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In Vitro Activities of Garenoxacin (BMS-284756) against *Haemophilus influenzae* Isolates with Different Fluoroquinolone Susceptibilities

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The in vitro activity of garenoxacin (BMS-284756) against 62 clinical *Haemophilus influenzae* isolates with different fluoroquinolone susceptibilities was determined by the microdilution susceptibility testing method and compared with the activities of other oral quinolones and nonquinolone oral antimicrobial agents. Cefixime presented the highest intrinsic activity (MIC at which 50% of the isolates tested were inhibited [MIC_{50}], 0.01 μg/ml), followed by garenoxacin, moxifloxacin, and ciprofloxacin (MIC_{50}, 0.06 μg/ml), levofloxacin (MIC_{50}, 0.12 μg/ml), cefuroxime (MIC_{50}, 1.0 μg/ml), and amoxicillin-clavulanate (MIC_{50}, 1.0/0.5 μg/ml), amoxicillin (MIC_{50}, 2 μg/ml), azithromycin (MIC_{50}, 4 μg/ml), and erythromycin (MIC_{50}, 8 μg/ml). In strains with ciprofloxacin MICs of ≤0.06 μg/ml, ciprofloxacin and garenoxacin displayed similar MIC_{50}s and MIC_{90}s, one dilution lower than those of moxifloxacin and levofloxacin. For strains for which ciprofloxacin MICs were ≥0.12 μg/ml, MIC_{50}s were similar for the four quinolones tested, although garenoxacin presented the widest activity range (0.03 to 32 μg/ml) and the highest MIC at which 90% of the isolates tested were inhibited (16.0 μg/ml). For strains without amino acid changes in the quinolone resistance determining region (QRDR) of GyrA and ParC, garenoxacin MICs were ≤0.03 μg/ml; with a single amino acid change in GyrA, garenoxacin MICs were 0.06 to 0.12 μg/ml; with one amino acid change each in GyrA and ParC, garenoxacin MICs were 0.5 to 2.0 μg/ml; one amino acid change in ParC combined with two amino acid changes in GyrA increased the MICs to ≥4 μg/ml for all assayed quinolones. We conclude that garenoxacin has excellent activity against *H. influenzae*, although progressive acquired resistance was observed by step-by-step mutation in the QRDR of *gyrA* and *parC*.

Garenoxacin is a novel des-fluoro(6)-quinolone that differs from earlier quinolones in its lack of a fluorine atom at the C-6 position and an isoindolin-5-yl substitution at the 7 position (17). It has reportedly shown increased activity relative to the other quinolones against gram-positive organisms (3, 15), including methicillin (oxacilin)-resistant staphylococci and some *Enterococcus* spp. (5). Broad antianaerobic coverage and superior activity against fastidious organisms has also been described (9). The difluoromethoxy substituent at position 8, instead of a methoxy group, has been shown to improve bacteriostatic and bactericidal activity and decrease the selection of resistant mutants (10). Garenoxacin has good oral bioavailability (11), and toxicological findings indicate low chondrotoxicity in juvenile rats, making it a potentially suitable therapy for children and adolescents (12).

Little is known about the activity of this compound against *Haemophilus influenzae* strains whether or not they have reduced susceptibility to other fluoroquinolones. Although fluoroquinolones remain among the most powerful in vitro antimicrobial agents against *H. influenzae* and are also highly effective as oral treatments of respiratory tract infections (6), resistance to them has been recognized (4, 7, 16). Therapeutic failure in community-acquired pneumonia associated with levofloxacin resistance has recently been described for *H. influenzae* (2). We have previously shown that strains with ciprofloxacin MICs of ≤0.06 μg/ml remain free of quinolone resistance determining region (QRDR) mutations in both *gyrA* and *parC*, while the first mutation in *gyrA* appears in strains with ciprofloxacin MICs of ≥0.12 μg/ml (M. Pérez-Vázquez et al., submitted for publication).

On the other hand, it is of interest to study the activities of new compounds that are to be introduced onto the market against well-characterized collections of resistant organisms (8). These studies are useful for defining the intrinsic activities of new drugs, even when resistance mechanisms may compromise these compounds.

Our study was designed to determine the comparative activity of garenoxacin (BMS-284756) and those of other orally administered quinolones and of other nonquinolone antimicrobial agents tested against *H. influenzae* isolates displaying different fluoroquinolone susceptibility values. It also aimed to ascertain the influence of GyrA and/or ParC QRDR amino acid changes on the activity of garenoxacin.

MATERIALS AND METHODS

Test isolates. A total of 62 clinical *H. influenzae* isolates and two reference strains from the American Type Culture Collection (ATCC 49247 and ATCC 51907) were studied. This batch of strains was distributed into two different groups: (i) group I included two American clinical strains and 30 Spanish clinical isolates from the collection of the *Haemophilus* Reference Laboratory. They were selected on the basis of their reduced susceptibility to ciprofloxacin (MIC, ≥0.12 μg/ml). This group comprised respiratory specimens from patients with cystic fibrosis or chronic respiratory infections, obtained as part of antibiotic resistance surveillance in Spanish clinical isolates. (ii) Group II, which included a fully susceptible control group of 30 strains with ciprofloxacin MICs of ≤0.06 μg/ml, was selected and matched according to the following criteria: similar date of isolation, geographical area, clinical diagnosis, anatomical source, capsulation

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TABLE 1. In vitro activities of four quinolones and different nonquinolone antimicrobials against *H. influenzae* isolates with different levels of susceptibility to ciprofloxacin

Drug[a]	MIC (μg/ml)					
	Group I[b]			Group II[c]		
	50%	90%	Range	50%	90%	Range
Fluoroquinolones						
CIP	0.01	0.03	0.007–0.06	2	4	0.12–32
LEV	0.03	0.03	0.01–0.06	1	2	0.12–32
MOX	0.03	0.06	0.01–0.12	2	4	0.06–32
GAR	0.01	0.03	0.001–0.06	1	16	0.03–32
β-Lactams						
AMOX	2	128	0.12–128	4	128	0.06–128
AMX/CLV	0.5	4	0.12–8.0	2	8	0.06–8.0
CXM	1	8	0.12–16	2	16	0.25–64
CFX	0.01	0.12	0.01–0.25	0.06	0.5	0.01–0.5
Macrolides						
ERY	8	16	2.0–16	16	64	0.25–256
AZT	4	8	1.0–8.0	4	32	0.06–128

[a] CIP, ciprofloxacin; LEV, levofloxacin; MOX, moxifloxacin; GAR, garenoxacin; AMOX, amoxicillin, AMX/CLV, amoxicillin-clavulanate, CXM, cefuroxime CFX, cefixime; ERY, erythromycin; AZT, azithromycin.
[b] CIP MIC, ≤ 0.06 μg/ml.
[c] CIP MIC, ≥ 0.12 μg/ml.

status, and biotype. The majority of strains were collected between 1994 and 2002 from patients living in the central area of Spain.

Susceptibility testing. Reference broth microdilution method was performed according to the NCCLS guidelines (13, 14). Haemophilus test medium was prepared with Mueller-Hinton broth (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) supplemented with HTM supplement (Oxoid) and yeast extract (5‰) (Difco, Detroit, Mich.). Microtiter plates were inoculated to produce a final inoculum density of approximately 5×10^5 CFU/ml. This density was monitored at regular intervals by making colony counts. The inoculated plates were incubated at 35°C for 20 to 24 h in ambient air before results were interpreted. The MIC was defined as the lowest concentration of antibiotic that inhibited growth.

Antimicrobial agents. Garenoxacin (BMS-284756) was supplied by Bristol-Myers Squibb laboratories (Madrid, Spain). The other antimicrobial agents, including a β-lactamase inhibitor compound (amoxicillin, azithromycin, cefixime, cefuroxime, ciprofloxacin, erythromycin, levofloxacin, moxifloxacin, nalidixic acid, and clavulanate), were provided by their respective manufacturers or purchased from Sigma (Madrid, Spain).

Amplification and sequence analysis of the QRDR regions of *gyrA* and *parC* genes. One isolate was randomly selected for QRDR analysis from each group of strains with identical garenoxacin MICs (MIC range, 0.007 to 32 μg/ml). Amplification was performed in a 50-μl final volume: 5 μl of DNA template, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 1 μM (each) primer (Pharmacia), 200 μM (each) deoxynucleoside triphosphate, and 2.5 U of *Taq* polymerase (Roche). The PCR program applied was as follows: denaturation at 94°C for 5 min; 30 amplification cycles of 94°C for 1 min, annealing at 54°C for 1 min, and polymerization at 72°C for 1 min; and a final 10-min cycle at 72°C to extend amplicons fully.

A 400-bp fragment including the QRDR of *gyrA* and *ParC* was amplified. Specific primers used were the following: GYRA-F (5′-CCGCCGCGTAC TATTCTCAAT-3′), GYRA-R (5′-GTTGCCATCCCCACCGCAATACCA-3′), PARC-F (5′-TCTGAACTTGGCTTAATTGCC-3′), and PARC-R (5′-GC CACGACCTTGCTCATAAAT-3′). PCR products were purified with a PCR purification kit (Qiagen, Hilden, Germany). Sequencing of fragments was done on both DNA strands with the Big DyeTM terminator cycle sequencing kit (Perkin-Elmer) according to the manufacturer's instructions. The products were resolved and analyzed with an ABI PRISMR 377 DNA sequencer. Nucleotide sequences were analyzed using the DNAstar (Madison, Wis.) program.

RESULTS AND DISCUSSION

When we compare in vitro activities of different antimicrobial agents against the 62 selected *H. influenzae* isolates in terms of the MICs at which 50% of the isolates tested were inhibited (MIC_{50}s), which normally represent modal MICs, cefixime had the highest intrinsic activity (0.01 μg/ml). This value was similar to that obtained with ciprofloxacin, moxifloxacin, and garenoxacin (0.06 μg/ml). Amoxicillin, amoxicillin-clavulanate, and cefuroxime presented similar MIC_{50}s of 1 to 2 μg/ml, and erythromycin and azitromycin were the less active agents, with MIC_{50}s of 8 and 4 μg/ml, respectively. However, a substantial difference was observed in the MICs at which 90% of the isolates tested were inhibited (MIC_{90}s): 0.25 μg/ml for cefixime and 2, 2, and 4 μg/ml for ciprofloxacin, moxifloxacin, and garenoxacin, respectively. For amoxicillin, the MIC_{90} was 128 μg/ml; for amoxicillin-clavulanate, 8 μg/ml; for cefuroxime, 16 μg/ml; for erythromycin, 32 μg/ml; and for azithromycin, 8 μg/ml.

Table 1 shows the activity of the assayed antimicrobial agents against *H. influenzae* isolates of groups I and II. Levofloxacin, moxifloxacin, and garenoxacin displayed similar MIC_{50}s and MIC_{90}s in group I of *H. influenzae*. For the group II strains, levofloxacin, moxifloxacin, and garenoxacin had markedly decreased activities. Nevertheless, for four isolates belonging to group II, MICs of garenoxacin were lower than those for ciprofloxacin; the MIC for one of them was 0.03 μg/ml for garenoxacin and 0.12 μg/ml for ciprofloxacin, while the MICs for the three additional isolates were 0.06 μg/ml for garenoxacin and 0.12 μg/ml (two strains) and 0.25 μg/ml (one strain) for ciprofloxacin.

Table 2 shows the *gyrA* and *parC* QRDR mutations leading to amino acid substitutions in selected *H. influenzae* isolates of the study collection. These isolates are representative of different garenoxacin MICs from group I and group II. For comparative purposes, other quinolone MICs are also included in this table.

Garenoxacin displayed high intrinsic activity against isolates without *gyrA* or *parC* mutations, with MICs ranging from 0.007 to 0.03 μg/ml, similar to ciprofloxacin, while the moxifloxacin and levofloxacin MIC range was 0.03 to 0.12 μg/ml (Table 2).

TABLE 2. Quinolone susceptibility and amino acid changes in GyrA and ParC QRDR fragments from representative *H. influenzae* isolates[a]

Strain no.	MIC (μg/ml)				Amino acid at position:					
					GyrA		ParC			
	CIP	LEV	MOX	GAR	84	88	82	83	84	88
ATCC 51907	0.007	0.03	0.03	0.007	Ser	Asp	Gly	Asp	Ser	Glu
1	0.01	0.01	0.03	0.001						
2	0.01	0.03	0.03	0.007						
3	0.01	0.03	0.03	0.01						
4	0.03	0.06	0.06	0.03						
5	0.12	0.12	0.12	0.06		Tyr				
6	0.25	0.12	0.12	0.12		Asn				
7	0.5	0.5	0.5	0.5		Asn				Lys
8	4	2	2	1		Asn			lle	
9	2	2	1	2		Tyr	Asp			
10	8	4	4	4	lle	Ala		Gly		
11	4	2	2	8	Leu	Asn			lle	
12	2	2	1	16	Leu	Asn		Asn		
13	32	32	32	32	Leu	Tyr	Asp			

[a] CIP, ciprofloxacin; LEV, levofloxacin; MOX, moxifloxacin; GAR, garenoxacin.

Garenoxacin displayed MICs of 0.06 to 0.12 μg/ml for isolates with a first step in the resistance mechanisms for quinolones (one amino acid change in GyrA). This value was slightly lower than that obtained with ciprofloxacin, moxifloxacin, and levofloxacin (0.12 μg/ml). However, the existence of one amino acid change in ParC combined with two amino acid changes in GyrA drastically diminished the in vitro activity of the four assayed quinolones (Table 2).

Garenoxacin is a desfluoroquinolone with a broad spectrum of activity against both gram-positive and -negative pathogens, including fastidious strains that commonly cause community-acquired respiratory tract infections (5). The SENTRY antimicrobial surveillance program (2000) demonstrated that the activity of garenoxacin against *H. influenzae* was similar to that of other quinolones, such as ciprofloxacin, gatifloxacin, levofloxacin, and moxifloxacin (MIC_{90}, ≤0.016 to 0.03 μg/ml) (1), consistent with data obtained in the present study for strains without amino acid changes in the QRDRs of *gyrA* and/or *parC*.

The *H. influenzae* population tested in our study includes both strains with and without resistance mechanisms for quinolones. The overall activity of garenoxacin was similar to that of ciprofloxacin (MIC_{90}, 4.0 μg/ml), but it was slightly lower than those of moxifloxacin and levofloxacin. To gain insight into garenoxacin activity, we separated our *H. influenzae* collection according to its susceptibility to ciprofloxacin; for fully ciprofloxacin-susceptible isolates (group I), ciprofloxacin, levofloxacin, and garenoxacin MIC_{90}s were similar (0.03 μg/ml), and for moxifloxacin the MIC_{90} was one $\log_2$ dilution higher. In group II, garenoxacin was the quinolone with the lowest activity, since its MIC_{90} was at least two $\log_2$ dilutions higher than that of the other tested quinolones, although in this group, the garenoxacin MIC range included lower values (0.03 μg/ml for garenoxacin, 0.06 μg/ml for moxifloxacin, and 0.12 μg/ml for ciprofloxacin and levofloxacin). This result is concordant with the fact that the older generation of quinolones (ciprofloxacin and ofloxacin) have relatively low gram-positive activity, and other new generation quinolones (including desquinolone) were modified to enhance mainly gram-positive activity.

We have shown that the primary target of garenoxacin in *H. influenzae* is DNA gyrase, the same as that of ciprofloxacin (7). A first amino acid modification in the QRDR of GyrA increased the garenoxacin MIC to 0.06 to 0.12 μg/ml; a second amino acid change in the QRDR of ParC enhanced its MIC to 0.5 to 2 μg/ml; and a double amino acid change in GyrA yielded the highest MICs for the strains in this collection (4.0 to 32 μg/ml). This increase in MICs was similar for ciprofloxacin, moxifloxacin, and levofloxacin, suggesting a step-by-step resistance mechanism common to the four quinolones tested.

In summary, we conclude that there was excellent garenoxacin activity against *H. influenzae* strains lacking quinolone resistance mechanisms. However, some amino acid changes in the QRDR region of GyrA and ParC clearly decreased the susceptibility of *H. influenzae* to garenoxacin, ciprofloxacin, levofloxacin, and moxifloxacin, reaching a MIC of 32 μg/ml for strains with two modifications in the QRDR region of GyrA and one modification in the QRDR of ParC.

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