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Activities of New Fluoroquinolones against Fluoroquinolone-Resistant Pathogens of the Lower Respiratory Tract

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The activities of six new fluoroquinolones (moxifloxacin, grepafloxacin, gatifloxacin, trovafloxacin, clinafloxacin, and levofloxacin) compared with those of sparfloxacin and ciprofloxacin with or without reserpine (20 μg/ml) were determined for 19 *Streptococcus pneumoniae* isolates, 5 *Haemophilus* sp. isolates, and 10 *Pseudomonas aeruginosa* isolates with decreased susceptibility to ciprofloxacin from patients with clinically confirmed lower respiratory tract infections. Based upon the MICs at which 50% of isolates were inhibited (MIC_{50}s) and MIC_{90}s, the most active agent was clinafloxacin, followed by (in order of decreasing activity) trovafloxacin, moxifloxacin, gatifloxacin, sparfloxacin, and grepafloxacin. Except for clinafloxacin (and gatifloxacin and trovafloxacin for *H. influenzae*), none of the new agents had improved activities compared with that of ciprofloxacin for *P. aeruginosa* and *H. influenzae*. A variable reserpine effect was observed for ciprofloxacin and *S. pneumoniae*; however, for 9 of 19 (47%) isolates the MIC of ciprofloxacin was decreased by at least fourfold, suggesting the presence of an efflux pump contributing to the resistance phenotype. The laboratory *parC* (Ser79) mutant strain of *S. pneumoniae* required eightfold more ciprofloxacin for inhibition than the wild-type strain, but there was no change in the MIC of sparfloxacin and only a 1-dilution increase in the MICs of the other agents. For efflux pump mutant *S. pneumoniae* the activities of all the newer agents, except for levofloxacin, were reduced. Except for clinafloxacin, all second-step laboratory mutants required at least 2 μg of all fluoroquinolones per ml for inhibition.

Infections of the lower respiratory tract continue to be a significant cause of morbidity and mortality, particularly in patients with underlying lung disease (24). Increasingly, fluoroquinolones are seen as drugs of choice for such infections, and there has been rapid development of new agents with improved in vitro activities against gram-positive bacteria, including pneumococci, compared to older agents such as ciprofloxacin. Clinafloxacin (CI-960, AM-1091, PD127391), gatifloxacin (AM-1155), grepafloxacin (OPC 17116), moxifloxacin (BAY 12-8039), and trovafloxacin (CP 99,219) all have excellent in vitro activities for pathogens that cause community-acquired respiratory tract infections, including activity for *Streptococcus pneumoniae* resistant to erythromycin and β-lactams (5, 8, 9, 11–13, 16, 17, 27, 29, 35, 37, 38, 41).

The pharmacokinetic properties of many of these new agents, in particular the concentration of fluoroquinolones within the bronchial mucosa, suggest that once-daily dosing will be sufficient to allow concentrations of the agent to be in excess of the MICs for the majority of pathogens causing respiratory tract infection (Table 1). Although some new fluoroquinolones, such as levofloxacin (HR355) (*L*-ofloxacin), have in vitro activities similar to those of older agents, it has been proposed that their pharmacokinetic properties give rise to greater concentration within the bronchial mucosa such that these agents would be effective in the treatment of respiratory tract infections (2).

Clinical failure of older fluoroquinolones such as ciprofloxacin and ofloxacin has already been reported for pneumococci (4, 5, 19, 22), *Pseudomonas aeruginosa* (20, 25, 34, 42), and *Haemophilus influenzae* (14). In addition, there have also been several reports of the selection and characterization of fluoroquinolone-resistant *S. pneumoniae* (15, 18, 23, 28, 30, 31, 36, 43).

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It is likely that the population of respiratory tract pathogens that these new fluoroquinolones will encounter will include those already exposed, and possibly resistant, to older agents such as ciprofloxacin and ofloxacin. Therefore, in this investigation we sought to compare the activities of six new fluoroquinolones (clinafloxacin, gatifloxacin, grepafloxacin, levofloxacin, moxifloxacin, and trovafloxacin) with those of sparfloxacin and ciprofloxacin in the presence and absence of the gram-positive bacterial efflux inhibitor reserpine. In vitro susceptibilities to new antibiotics are typically determined with a wide range of clinical isolates from culture collections; many of these isolates are historical and were isolated prior to the widespread clinical use of fluoroquinolones and so do not reflect the current bacterial population infecting community and hospital patients. The isolates in the present study were from patients with clinically confirmed lower respiratory tract infection within the last two years.

MATERIALS AND METHODS

Bacteria and growth conditions. From our culture collection 19 *S. pneumoniae* isolates, 3 *H. influenzae* isolates, six *Haemophilus parainfluenzae* isolates, and 10 *P. aeruginosa* isolates with decreased susceptibilities to ciprofloxacin (cutoff for *Haemophilus* spp., ≥0.03 μg/ml; cutoff for *S. pneumoniae* and *P. aeruginosa*, ≥0.5 μg/ml) from patients with clinically confirmed lower respiratory tract infections were selected. Five clinical isolates of ciprofloxacin-resistant *H. influenzae* were also obtained from two other hospitals, in Aberdeen (22) and London (33), United Kingdom. Control strains were *S. pneumoniae* M3 (NCTC 7466; capsulated) and M4 (NCTC 7465; no capsule), *H. influenzae* N4 (NCTC 8466), and *P. aeruginosa* G1 (NCTC 10662). Mutants of M3 and M4 were selected and characterized in a parallel study (Table 2) (32). The mutant pneumococci were stably resistant without antibiotic pressure and were selected, after a single exposure to fluoroquinolone, with mutation frequencies suggestive of a mutation in a single gene. The quinolone resistance-determining region of *gyrA*, *gyrB*, *parC*, and *parE* of each mutant was analyzed by PCR and DNA sequencing. The concentration of ciprofloxacin with or without reserpine accumulated was also determined for each mutant. Second-step mutants were obtained by exposing the first-step mutant to fluoroquinolone. All mutants and clinical isolates were maintained at −70°C on Protect beads without antibiotic and grown overnight in in brain heart infusion broth supplemented with NAD (10 μg/ml; Sigma) and hemin (10 μg/ml)

TABLE 1. Pharmacokinetic properties of fluoroquinolones[a]

Agent	Dosage	C_{max} (μg/ml)	$t_{1/2}$ (h)	Concn in:			Reference
				Bronchial mucosa (mg/kg of body weight)	Epithelial lining fluid (μg/ml)	Alveolar macrophage (μg/ml)	
Ciprofloxacin	500 mg, twice daily	3.01	5–7	4.4	ND	ND	39
Sparfloxacin	200 mg, single dose	1.4	ND	3.3	11.9	41.3	40
Clinafloxacin	400 mg, single dose	4.9	6.1	ND	ND	ND	Parke-Davis file
Gatifloxacin	400 mg, single dose	3.4	8–10	ND	ND	ND	26
Grepafloxacin	400 mg, for 4 days	1.2	12	1.4	5.04	87.3	10
Levofloxacin	500 mg, single dose	7.19	6	4	10.1	38.4	2
Moxifloxacin	400 mg, single dose	3.1	12–14	1.56	5.9	36.8	Bayer file
Trovafloxacin	200 mg, multiple dose	3.1	11	1.1	5.8	24.1	Pfizer file

[a] Abbreviations: C_{max}, maximum concentration of drug in serum; $t_{1/2}$, half-life; ND, not determined.

for the *Haemophilus* spp. and incubated at 37°C in 5% CO_2. The solid medium was Isosensitest agar supplemented with 5% defibrinated horse blood.

Antibiotics, and susceptibility determination. The MIC of each antibiotic for each strain was determined by the agar doubling-dilution method. All of the following antibiotics were gifts and were made up and used according to the manufacturers' instructions: ciprofloxacin and moxifloxacin (Bayer AG) sparfloxacin (Rhone DPC Europe, Paris, France), grepafloxacin (Glaxo Wellcome), gatifloxacin (Gruenthal GmbH) trovafloxacin (Pfizer, New York, N.Y.), clinafloxacin (Parke-Davis Warner Lambert, Ann Arbor, Mich.), and levofloxacin (Hoechst Marion Roussel). Plates containing doubling dilutions of antibiotic were inoculated by transferring 1 μl of the undiluted overnight culture to the surface of the agar with a multipoint inoculator (DenleyTech, Billingshurst, United Kingdom) to give a final inoculum size of 10^6 CFU. All plates were incubated in 5% CO_2 at 37°C overnight. The MIC of the antibiotic was defined as the lowest concentration of antibiotic (in micrograms per milliliter of agar) at which no more than 10 colonies were detected; a slight haze of growth was ignored. Reserpine (Sigma) was added to ciprofloxacin to a final concentration of 20 μg/ml. For the laboratory mutants, decimal dilutions of ciprofloxacin were used as previously described (31), to reflect the small but reproducible differences in susceptibility. All determinations were performed for all strains and agents in parallel on at least three separate occasions, until three identical values for each strain and agent were obtained. This allowed discrimination between MICs of different agents to within 1 dilution, typically the normal error of MIC determination experiments.

RESULTS

Activities for characterized mutant *S. pneumoniae*. Table 2 shows the susceptibilities of selected laboratory mutants. *S. pneumoniae* M26 had a mutation in *parC*, and this conferred an eightfold increase in the MIC of ciprofloxacin and a fourfold increase in the MIC of levofloxacin but no change in the MIC of sparfloxacin and only a 1-dilution increase in the MICs of the other agents tested.

Reserpine had no effect upon the MIC of ciprofloxacin for the wild-type quinolone-susceptible *S. pneumoniae* M3 and M4, and for the *parC* mutant of M3 (M26), the MIC of ciprofloxacin was only slightly reduced when reserpine was added. All mutants of M4 were efflux mutants (cross-resistant to unrelated agents such as erythromycin and ethidium bromide) and accumulated lower concentrations of ciprofloxacin than did M26 and M3 (wild-type concentrations were accumulated in the presence of reserpine [32]); consequently, the MIC of ciprofloxacin was reduced by reserpine. Except for levofloxacin, the activities of all the newer agents were reduced for these mutants, with a two- to fourfold decrease in activity for the most ciprofloxacin-resistant mutant (M22). However, clinafloxacin, moxifloxacin, and trovafloxacin were least affected.

All second-step mutants derived from the *parC* mutant M26 were also efflux mutants but varied in susceptibility to reserpine, suggesting variations in expression of the efflux gene(s). Clinafloxacin was most active against the second-step mutants and retained good activity. For the remainder of the agents, all second-step mutants required at least a 2-μg/ml concentration of all newer fluoroquinolones for inhibition. The differences in the MICs of each agent were only 1 to 2 doubling dilutions, with the order of activity (from greatest to least) being clinafloxacin, sparfloxacin, trovafloxacin, moxifloxacin, levofloxacin, gatifloxacin, and grepafloxacin.

Activities for clinical isolates from the respiratory tract with decreased susceptibility to ciprofloxacin. Table 3 shows the susceptibilities of the clinical isolates. All new agents, except for levofloxacin, had improved activities for all *S. pneumoniae* isolates compared with those of ciprofloxacin. The most active agent was clinafloxacin, followed by (in order of decreasing activity) trovafloxacin, moxifloxacin, gatifloxacin, sparfloxacin, and grepafloxacin. Three of the 19 clinical isolates of *S. pneumoniae* required 32 μg of ciprofloxacin per ml for inhibition and showed phenotypes resembling those of the second-step

TABLE 2. Susceptibilities of selected *S. pneumoniae* mutants to fluoroquinolones[a]

Strain	Generation	Description	MIC (μg/ml) of:								
			CIP	CIP-RES	SPAR	CLIN	GAT	GREP	LEV	MOX	TROV
M3	Parent	Wild type	0.5	0.5	0.25	0.06	0.5	0.5	0.5	0.25	0.12
M26	First step	ParC Ser79→Ala[b] mutant	4	2.5	0.25	0.12	1	1	2	0.5	0.25
M27	Second step	ParC Ser79→Ala efflux mutant	64	32	4	0.5	8	16	16	0.5	4
M30	Second step	ParC Ser79→Ala efflux mutant	64	16	2	0.5	8	16	4	8	4
M31	Second step	ParC Ser79→Ala efflux mutant	16	16	4	0.5	8	16	8	4	4
M4	Parent	Wild type[c]	1	1	0.12	0.12	0.5	0.5	2	0.25	0.12
M16	First step	Efflux mutant	6	2.5	0.5	0.12	1	1	2	0.25	0.25
M22	First step	Efflux mutant	12	1	0.5	0.25	1	1	2	0.5	0.25

[a] Abbreviations: CIP, ciprofloxacin; RES, reserpine; SPAR, sparfloxacin; CLIN, clinafloxacin; GAT, gatifloxacin; GREP, grepafloxacin; LEV, levofloxacin; MOX, moxifloxacin; and TROV, trovafloxacin.
[b] Ser-to-Ala mutation at position 79.
[c] Silent mutations at Val157 and Asp159 in *gyrB*.

TABLE 3. Susceptibility (μg/ml) of clinical isolates of *S. pneumoniae*, *Haemophilus* spp., and *P. aeruginosa*

Species and agent	MIC (μg/ml) of NCTC control strain	MIC (μg/ml)[a]		
		50%	90%	Range
S. pneumoniae (n = 19)				
Ciprofloxacin	0.5	1	32	2–32
Ciprofloxacin + reserpine	0.5	0.5	8	0.5–8
Sparfloxacin	0.25	0.5	16	0.03–32
Clinafloxacin	0.06	0.12	1	0.06–1
Gatifloxacin	0.5	0.25	4	0.03–8
Grepafloxacin	0.5	0.5	16	0.06–64
Levofloxacin	0.5	1	32	0.06–32
Moxifloxacin	0.5	0.25	4	0.06–4
Trovafloxacin	0.25	0.12	4	0.06–4
Haemophilus spp. (n = 5)				
Ciprofloxacin	0.008			0.5–8
Sparfloxacin	0.008			0.5–8
Clinafloxacin	0.004			0.12–1
Gatifloxacin	0.06			0.25–2
Grepafloxacin	0.12			1–8
Levofloxacin	0.06			1–16
Moxifloxacin	0.06			1–4
Trovafloxacin	0.06			0.25–1
P. aeruginosa (n = 10)				
Ciprofloxacin	1	2	8	0.25–8
Sparfloxacin	4	8	32	8–32
Clinafloxacin	1	1	4	0.06–4
Gatifloxacin	4	4	16	0.5–16
Grepafloxacin	8	8	32	0.5–32
Levofloxacin	1	4	16	0.5–16
Moxifloxacin	16	16	32	16–32
Trovafloxacin	0.5	4	32	0.5–32

[a] 50% and 90%, MICs at which 50 and 90% of the isolates are inhibited, respectively.

mutants, suggesting that these strains possessed mutations in more than one gene.

Except for clinafloxacin, gatifloxacin, and trovafloxacin, none of the new agents had improved activities compared with that of ciprofloxacin for the *H. influenzae* isolates.

For the *P. aeruginosa* isolates, except for clinafloxacin none of the new agents had improved activities compared with that of ciprofloxacin. The order of activity (from greatest to least) was levofloxacin, gatifloxacin, trovafloxacin, sparfloxacin, grepafloxacin, and moxifloxacin.

Affect of putative efflux pump on activity of clinical isolates of ciprofloxacin-resistant *S. pneumoniae*. A variable reserpine effect was observed for ciprofloxacin and the clinical isolates of *S. pneumoniae*: for 1 of 19 clinical isolates reserpine had no effect, for 9 of 19 clinical isolates there was a 1-dilution difference (twofold decrease in the MIC), for 6 of 19 clinical isolates there was a 2-dilution difference (fourfold decrease in the MIC), and for 3 of 19 clinical isolates there was a 3-dilution difference (eightfold decrease in the MIC). It is of interest that the three clinical isolates for which the MIC of ciprofloxacin was most affected by reserpine were not the most resistant to ciprofloxacin (data not shown), and there was no clear correlation between the MIC of any agent and the effect of reserpine. However, for the most-resistant clinical isolates, reserpine reduced the MIC of ciprofloxacin from 32 to 4 to 8 μg/ml.

DISCUSSION

Clinical failures have occurred when ciprofloxacin or ofloxacin has been used to treat pneumococcal respiratory tract infections (4, 19, 21), and so the purpose of the present study was to determine whether such resistant clinical isolates would be susceptible to the newer fluoroquinolones. These new agents have been shown in vitro to be so active against quinolone-susceptible *S. pneumoniae* that it has been suggested that older agents no longer be used (1). In addition, with the new fluoroquinolones it is far more difficult to select resistant strains from wild-type quinolone-susceptible bacteria than from ciprofloxacin- or ofloxacin-resistant bacteria (32). Therefore, it is possible that replacing older agents with newer agents will slow down the selection of fluoroquinolone-resistant mutants in the clinical setting and retain a predominantly sensitive, antibiotic-treatable population.

In many respects the clinical isolates described in the present study are the worst of the worst and present a challenge to any new fluoroquinolone. It should also be noted that such isolates are rare in our hospital unit; indeed, from the ~350 patients seen by physicians in specialist respiratory clinics per year (yielding approximately 1,500 specimens per year) about 90 *S. pneumoniae* isolates are obtained. Only 19 fluoroquinolone-resistant *S. pneumoniae* isolates (MIC of ciprofloxacin > 0.5 μg/ml) have been obtained since 1990, and for only two patients was treatment seriously affected.

In the present study, for comparison with the clinical isolates, mutant *S. pneumoniae* strains were also investigated, having been characterized in a parallel study (32). The first-step mutant strains had MICs greater than the typical serum and bronchial levels of ciprofloxacin attained in humans (Table 1). While the mutant strains were also less susceptible to newer fluoroquinolones, the increases in the MICs were small. It has been proposed that topoisomerase IV of *S. pneumoniae* is the primary target of fluoroquinolones; however, it is clear that the *parC* mutation of *S. pneumoniae* (M26) confers very little resistance to sparfloxacin, clinafloxacin, gatifloxacin, grepafloxacin, moxifloxacin, and trovafloxacin. These data suggest that these agents have similar activities for topoisomerase IV and DNA gyrase, so only a mutation in both genes would confer high-level resistance, whereas ciprofloxacin has preferential activity for topoisomerase IV and a lesser affinity for DNA gyrase, such that mutation in *parC* is sufficient to render a strain resistant. The second-step mutants were highly resistant to ciprofloxacin, and the decrease in susceptibility conferred to the other fluoroquinolones would probably define these mutants as clinically resistant to all of them except clinafloxacin.

Reserpine is an inhibitor of several gram-positive bacterial efflux pumps, including the putative efflux pump of *S. pneumoniae*, and in the presence of reserpine the MICs of norfloxacin and ciprofloxacin were decreased (3, 6). Efflux of fluoroquinolones by *S. pneumoniae* has been demonstrated by Zeller et al. (43), although the precise identification of the gene(s) and protein(s) involved has yet to be described. Brenwald et al. (7) showed that of 273 clinical isolates of *S. pneumoniae* with MICs of ciprofloxacin of >1 μg/ml, in the presence of 10-μg/ml reserpine 124 isolates had an increase in susceptibility to norfloxacin or ciprofloxacin of fourfold or more.

In the present study 20-μg/ml reserpine was used for comparison with data from accumulation experiments by this laboratory and others. A variable reserpine effect was observed for ciprofloxacin and the clinical isolates of *S. pneumoniae*; however, for 47% of the isolates the MIC of ciprofloxacin was decreased by at least fourfold, suggesting the presence of an efflux pump contributing to the resistance phenotype. However, there was no clear correlation between the MIC of any agent and the reserpine effect. It is probable that several efflux pumps exist in *S. pneumoniae*, and experiments with genetically defined mutants are required to determine precisely which fluoroquinolones are most affected.

If the recommended breakpoint concentrations for these newer fluoroquinolones are set at concentrations similar to those for other quinolones, at 1 to 8 μg/ml, then only clinafloxacin would be defined as clinically active against all the mutants and clinical isolates described in this study. However, at 1 μg/ml all of the new fluoroquinolones inhibited at least 50% of the clinical isolates of *S. pneumoniae*. Some of the ciprofloxacin-resistant *S. pneumoniae* isolates would also be resistant to some of the new agents, but based purely upon in vitro data the most likely to be affected are grepafloxacin and levofloxacin. With the active search for fluoroquinolones with improved activities against gram-positive bacteria, especially *S. pneumoniae*, there has been some loss of activity for gram-negative bacteria, and this is clearly shown for the ciprofloxacin-resistant *P. aeruginosa* isolates, for which all of the agents investigated in this study had poor activity. Two of the *H. influenzae* isolates studied would be considered to be clinically resistant to all of the fluoroquinolones studied.

It may well be that the recommended breakpoint concentrations for these new agents are higher than 4 μg/ml, especially as the calculation of these values in some countries, such as the United Kingdom, takes into account the pharmacokinetic properties of an antibiotic. All the new agents had substantially improved pharmacokinetics and concentrated to greater levels in the lungs compared with ciprofloxacin (Table 1). For those agents that accumulate to high concentrations and can be given in doses higher than 800 mg/day, the activity for ciprofloxacin-resistant *S. pneumoniae* may well be improved.

It is clear that the clinical judgement as to which new fluoroquinolone to use in the treatment of respiratory tract infections will be based upon several factors, including the prevalence of fluoroquinolone-resistant bacteria, pharmacokinetics (frequency of dosing and concentration within the lungs), toxicity, adverse reactions, and patient type. In general, the newer fluoroquinolones had enhanced activity for ciprofloxacin-resistant *S. pneumoniae*, but for the ciprofloxacin-resistant *P. aeruginosa* and *H. influenzae* isolates with decreased susceptibility to ciprofloxacin there was little improvement. Therefore, it is important to establish the bacterial etiology of lower respiratory tract infection and/or the patient population treated before using empiric fluoroquinolone treatment. For patients in the community, this information may not be readily available, and so knowledge of the prevalence of antibiotic-resistant bacteria in the community would be useful before treatment.

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