ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2010, p. 143–148 Vol. 54, No. 1
0066-4804/10/$12.00 doi:10.1128/AAC.00248-09

In Vitro Pharmacodynamics of Levofloxacin and Other Aerosolized Antibiotics under Multiple Conditions Relevant to Chronic Pulmonary Infection in Cystic Fibrosis▽

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Received 20 February 2009/Returned for modification 9 July 2009/Accepted 30 September 2009

The inhalational administration of antibiotics can provide high concentrations locally in the lungs of cystic fibrosis patients and, thus, can be useful for the treatment of chronic bacterial infections. The present study evaluated the in vitro activities of levofloxacin, ciprofloxacin, tobramycin, amikacin, and aztreonam against clinical isolates of *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia*, *Alcaligenes xylosoxidans*, and *Staphylococcus aureus* from cystic fibrosis patients. Levofloxacin was the most potent antibiotic against all cystic fibrosis isolates tested, with MIC_{90}s ranging from 8 to 32 μg/ml. Levofloxacin was more potent than the aminoglycosides and aztreonam against *P. aeruginosa* biofilms. Time-kill assays with drug concentrations achievable in sputum following aerosol administration showed that levofloxacin had the most rapid rate of killing among mucoid and nonmucoid isolates of *P. aeruginosa*. In contrast to tobramycin, the bactericidal activity of levofloxacin was not affected by sputum from cystic fibrosis patients. The results of the study show that the high concentrations of levofloxacin readily achievable in the lung following aerosol delivery may be useful for the management of pulmonary infections in patients with cystic fibrosis.

Chronic bacterial infections of the airway are common in cystic fibrosis (CF) patients. It is the burden of these infections, particularly infections with *Pseudomonas aeruginosa*, that eventually lead to the accelerated morbidity and mortality of CF patients (10, 24). *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Alcaligenes xylosoxidans*, *Burkholderia cepacia* complex, and *Staphylococcus aureus* are among the pathogens that are the most frequently isolated from the sputum of CF patients (2, 30). Bacterial isolates from CF patients can be more resistant to antibiotics and tend to mutate more readily than isolates from non-CF patients (4, 33, 35).

The use of aerosolized antibiotics has emerged as an important strategy for the management of chronic lung infections in CF patients. Aerosol administration provides higher concentrations of drug to the site of infection than parenteral or oral administration. Studies of the pharmacokinetics (PKs) of antibiotics in CF patients demonstrate that aerosol doses achieve greater maximum concentrations (C_{max}) and areas under the concentration-time curve (AUCs) in sputum than systemic doses, which increase the PK-pharmacodynamic (PD) indices relative to the MIC (13, 14, 17).

Over a decade ago, tobramycin solution for inhalation became the first antibiotic approved by the FDA for the management of CF patients. Aerosolized tobramycin reaches an average concentration of 1,240 μg/g in sputum (13); however, antimicrobial potency is reduced in sputum from CF patients (20, 28). The twice-daily administration of aerosolized tobramycin for 28 days in three on-off cycles over 6 months showed a reduction in the *P. aeruginosa* bacterial counts in sputum and an improvement in the lung function of CF patients, although these effects tended to decline with the use of repeated courses (36). The use of aerosolized tobramycin for the treatment of pulmonary infections has been associated with the selection of multiple-antibiotic-resistant strains of *P. aeruginosa* (29).

In view of the reduction in the potency of tobramycin in sputum from CF patients and the limitation that tobramycin may be administered only every other month, additional antibiotics have been investigated for aerosol administration in CF patients. Levofloxacin, a fluoroquinolone with broad-spectrum activity, is bactericidal against *P. aeruginosa* (16) and is reported to have activity against *P. aeruginosa* isolates growing in biofilms (22, 31). Levofloxacin inhalation solution (MP-376) is a novel formulation of levofloxacin that is currently being evaluated in clinical trials and that may be safely and rapidly administered by the aerosol route. Following MP-376 administration, high levofloxacin concentrations are achieved in the sputum of CF patients, resulting in the high PK-PD exposures associated with bactericidal activity and a reduced possibility for the selection of resistance (8, 12, 23).

The studies described here were designed to determine the in vitro activities of levofloxacin and other antibiotics currently under evaluation for aerosol administration in patients with CF. The in vitro activities of levofloxacin, ciprofloxacin, tobramycin, amikacin, and aztreonam against CF isolates, including *P. aeruginosa* isolates growing in biofilms, were determined. The bactericidal activities of levofloxacin, tobramycin, and aztreonam against mucoid and nonmucoid *P. aeruginosa* isolates from patients with CF were compared. Finally, the effect of sputum on the bactericidal activity of levofloxacin and tobramycin was evaluated.

MATERIALS AND METHODS

Antibiotics. The antibiotics used for these studies included those currently administered to and in development as therapies for use in CF patients. Levofloxacin hydrochloride, ciprofloxacin hydrochloride, tobramycin sulfate, and ami-

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▽ Published ahead of print on 5 October 2009.

TABLE 1. In vitro antibiotic susceptibilities of isolates from CF patients[a]

Organism	Antibiotic	MIC (μg/ml)		
		50%	90%	Range
P. aeruginosa (n = 282)	Levofloxacin	2	8	≤0.03–32
	Ciprofloxacin	1	8	≤0.03–32
	Tobramycin	2	>32	≤0.03–>32
	Amikacin	16	>128	≤0.125–>128
	Aztreonam	4	128	≤0.125–>128
B. cepacia complex[b] (n = 49)	Levofloxacin	4	32	0.25–>32
	Ciprofloxacin	4	32	0.25–>32
	Tobramycin	>32	>32	4–>32
	Amikacin	128	>128	8–>128
	Aztreonam	32	>128	1–>128
S. maltophilia (n = 51)	Levofloxacin	2	8	0.25–8
	Ciprofloxacin	4	16	0.5–16
	Tobramycin	>32	>32	0.5–>32
	Amikacin	128	>128	1–>128
	Aztreonam	>128	>128	2–>128
A. xylosoxidans (n = 44)	Levofloxacin	4	16	1–16
	Ciprofloxacin	4	16	1–32
	Tobramycin	>32	>32	8–>32
	Amikacin	>128	>128	16–>128
	Aztreonam	64	>128	32–>128
S. aureus, methicillin resistant (n = 36)	Levofloxacin	4	32	0.125–32
	Ciprofloxacin	16	>32	0.125–>32
	Tobramycin	128	>128	0.125–>128
	Amikacin	8	16	0.5–32
	Aztreonam	>512	>512	>512
S. aureus, methicillin sensitive (n = 24)	Levofloxacin	0.125	0.25	0.06–0.5
	Ciprofloxacin	0.125	0.5	0.06–1
	Tobramycin	0.125	32	0.06–>32
	Amikacin	1	4	0.5–8
	Aztreonam	>128	>128	>128

[a] Isolates were recovered from CF patients between 1980 and 2007.
[b] The species distribution of the *B. cepacia* complex isolates was as follows: unclassified (n = 34), *B. dolosa* (n = 8), and *B. multivorans* (n = 7).

kacin disulfate were purchased from LKT Laboratories (St. Paul, MN). Aztreonam base was purchased from MP Biomedicals (Solon, OH).

CF isolates. *P. aeruginosa* (n = 282), *B. cepacia* complex (n = 49), *S. maltophilia* (n = 51), *A. xylosoxidans* (n = 44), methicillin (meticillin)-resistant *S. aureus* (MRSA) (n = 36), and methicillin-sensitive *S. aureus* (MSSA) isolates (n = 24) from the sputum from CF patients were obtained from three sites in the United States and also from the Laboratoire de Bactériologie, Hôpital Jean Minjoz (Besançon, France). The U.S. sites included the CF Referral Center for Susceptibility & Synergy Studies at Columbia University (New York, NY), the CF Foundation Therapeutics Development Network Resource Center for Microbiology at Seattle Children's Hospital (Seattle, WA), and the CF microbiology laboratory at the University of North Carolina at Chapel Hill (Chapel Hill, NC). The Seattle and New York laboratories receive isolates from throughout the U.S., and the majority of isolates from the Chapel Hill laboratory originated from the southeastern states. The *P. aeruginosa* isolates were collected from 1980 through 2007, and 47% were recent isolates (collected from 2001 through 2007). Isolates of the other organisms were collected from 2001 through 2007.

In vitro antimicrobial susceptibility testing. Antibiotic MICs were determined by the broth microdilution method, according to Clinical and Laboratory Standards Institute (CLSI) reference methods (5). The antibiotics were dissolved as specified, serially diluted twofold in cation-adjusted Mueller-Hinton broth (CAMHB), and tested at the following concentrations: for levofloxacin, ciprofloxacin, and tobramycin, from 0.03 to 32 μg/ml; for amikacin and aztreonam, from 0.125 to 128 μg/ml. The MIC endpoints were determined by reading the optical density of the plate wells at 600 nm with a SpectraMax Plus384 plate reader (Molecular Devices, Sunnyvale, CA) and were confirmed by visual inspection.

***P. aeruginosa* biofilm susceptibility.** Biofilm formation and susceptibility testing were performed as described previously (31). *P. aeruginosa* CF isolates with a range of MICs for levofloxacin, tobramycin, amikacin, and aztreonam were selected for use in the biofilm susceptibility assays. The concentrations of levofloxacin, tobramycin, amikacin, and aztreonam required to prevent biofilm cell growth, referred to as the biofilm inhibitory concentration, were compared to the MIC results for planktonic cells.

Bactericidal activity against *P. aeruginosa*. Time-kill studies were conducted to evaluate the bactericidal activity of levofloxacin compared to the activities of tobramycin and aztreonam against the same *P. aeruginosa* CF isolates used in the biofilm susceptibility assays. Overnight cultures were diluted and incubated at 37°C until they reached an optical density at 600 nm of about 0.3. The average inoculum at the start of the experiment was adjusted to between 5×10^5 and 1×10^6 CFU/ml. The culture volume was 10 ml in CAMHB. To simulate the range of antibiotic concentrations that are found in sputum after aerosol administration, the bactericidal effects of levofloxacin, tobramycin, and aztreonam were evaluated at concentrations of 0.2 mg/ml, 0.6 mg/ml, and 2.0 mg/ml (12–14). All cultures were incubated at 37°C with shaking. At 0, 0.5, 1, 2, 4, 8, and 24 h after inoculation, 0.5-ml samples were washed twice with fresh CAMHB to prevent drug carryover effects and serially diluted in 96-well plates with physiologic saline, and 0.01 ml was plated on Mueller-Hinton agar. The agar plates were incubated for up to 48 h at 37°C, and the bactericidal activity was assessed. The limit of detection was 2 log CFU/ml.

Sputum time-kill curves. Time-kill studies were performed with and without sputum from CF patients to assess the effect of sputum on the activities of levofloxacin and tobramycin. Sputum was collected from CF patients who had not received any antibiotic therapy for at least 48 h under an institutional review board-approved protocol. The sputum specimens were pooled and exposed to UV light to eliminate endogenous bacteria. Sterilization of the pooled sputum

TABLE 2. In vitro susceptibilities of biofilm and planktonic cells of *P. aeruginosa*[a]

Isolate	Mucoid	BIC (μg/ml)				MIC (μg/ml)			
		LVX	TOB	AMK	ATM	LVX	TOB	AMK	ATM
PA 1054	Y	1	1	4	>64	0.5	0.25	1	8
PA 1042	N	1	1	8	>8	2	0.5	4	0.5
PA 5063	N	1	2	16	>256	0.5	0.25	2	8
PA 5043	N	1	8	64	>64	2	8	64	1
ATCC 27853	N	2	1	8	>256	1	0.25	2	4

[a] Abbreviations: Y, yes; N, no; BIC, biofilm inhibitory concentration; LVX, levofloxacin; TOB, tobramycin; AMK, amikacin; ATM, aztreonam.

was confirmed by direct plating of sputum samples on Mueller-Hinton agar and overnight culture of 0.1 ml in 10 ml CAMHB.

P. aeruginosa PAM1032 (a strain overexpressing the multidrug efflux pump MexAB-OprM) was used as the test strain (25). The MICs of levofloxacin and tobramycin for PAM1032 are 2 and 0.25 μg/ml, respectively. The inoculum was prepared in sterile 10% sputum or CAMHB alone to approximately 1×10^6 CFU/ml, and then the antibiotics were added. Levofloxacin and tobramycin were tested at final concentrations of 1×, 2×, 4×, and 8× MIC. The cultures were incubated at 37°C with shaking and sampled for up to 4 h. At each sampling, 0.1 ml was removed from each culture and serially diluted in 96-well plates with physiologic saline, and 0.01 ml was plated on Mueller-Hinton agar for quantitation. The agar plates were incubated for up to 48 h at 37°C, and bactericidal activity was assessed. The limit of detection was 2 log CFU/ml.

RESULTS

The in vitro activities of the antibiotics are shown in Table 1. The fluoroquinolones levofloxacin and ciprofloxacin were overall the most potent agents tested and had similar levels of activity against gram-negative CF pathogens, while levofloxacin was more potent against MSSA and MRSA strains. Aztreonam was not active against MSSA or MRSA strains.

Table 2 compares the susceptibilities of the *P. aeruginosa* isolates by the standard MIC and biofilm susceptibility testing methods. Levofloxacin was the most potent agent tested in the biofilm assays and tended to be the agent least affected by biofilms; the increases in the MICs for the biofilms were 0.5- to 2-fold for levofloxacin, 1- to 8-fold for tobramycin and amikacin, and >8-fold for aztreonam.

The results of the time-kill assays with representative *P. aeruginosa* isolates by the use of concentrations reported to be

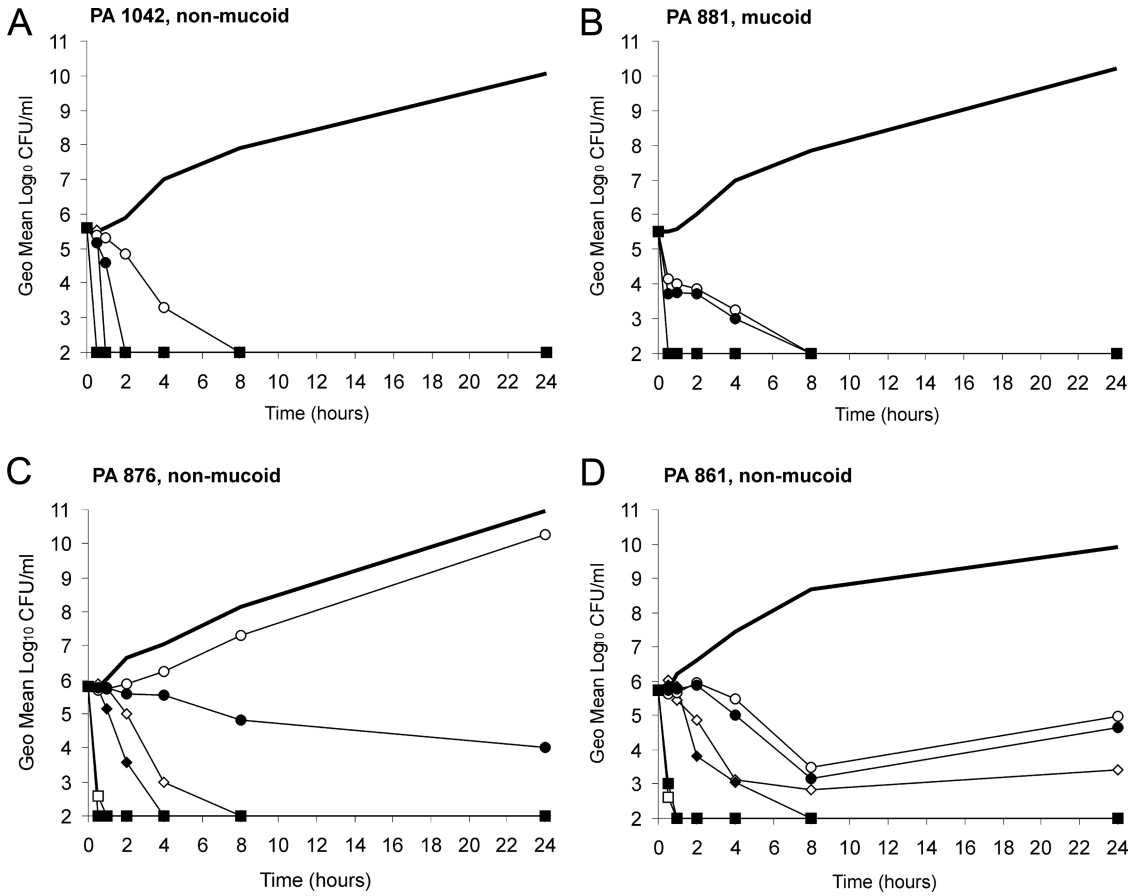

FIG. 1. Geometric mean log CFU/ml versus time for *P. aeruginosa* isolates from patients with CF. (A) Nonmucoid strain PA 1042 with MICs in the susceptible range for all antibiotics (levofloxacin MIC, 2 μg/ml; tobramycin MIC, 0.5 μg/ml; aztreonam MIC, 0.5 μg/ml); (B) mucoid strain PA 881 with MICs representing the MIC_{50}s for all antibiotics (levofloxacin MIC_{50}, 4 μg/ml; tobramycin MIC_{50}, 2 μg/ml; aztreonam MIC_{50}, 2 μg/ml); (C) nonmucoid strain PA 876 with MICs representing the MIC_{90}s for all antibiotics (levofloxacin MIC_{90}, 8 μg/ml; tobramycin MIC_{90}, 32 μg/ml; aztreonam MIC_{90}, >128 μg/ml); (D) nonmucoid strain PA 861 with MICs also in the resistant range for all antibiotics (levofloxacin MIC, 32 μg/ml; tobramycin MIC, 16 μg/ml; aztreonam MIC, 128 μg/ml). Thick lines, growth controls; squares, levofloxacin (□, 0.2 mg/ml; ■, 2.0 mg/ml); diamonds, tobramycin (◇, 0.2 mg/ml; ◆, 2.0 mg/ml); circles, aztreonam (○, 0.2 mg/ml; ●, 2.0 mg/ml). The limit of detection was 2 log CFU/ml.

TABLE 3. Bactericidal activities of high concentrations of levofloxacin, tobramycin, and aztreonam against *P. aeruginosa* isolates from CF patients[a]

Isolate	Mucoid	MIC (μg/ml)			Time to 3-log killing (h)								
					Levofloxacin			Tobramycin			Aztreonam		
		LVX	TOB	ATM	0.2 mg/ml	0.6 mg/ml	2.0 mg/ml	0.2 mg/ml	0.6 mg/ml	2.0 mg/ml	0.2 mg/ml	0.6 mg/ml	2.0 mg/ml
PA 1054	Y	0.5	0.25	8	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	24	24	24
PA 899	N	0.5	0.25	8	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	>24	24	24
PA 1022	N	1	>32	16	≤0.5	≤0.5	≤0.5	4	4	4	>24	>24	24
PA 1042	N	2	0.5	0.5	≤0.5	≤0.5	≤0.5	1	1	≤0.5	8	4	2
PA 5043	N	2	8	1	≤0.5	≤0.5	≤0.5	2	1	1	8	2	2
PA 880	N	4	1	0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	4	4	4
PA 881	Y	4	2	2	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	8	8	8
PA 1036	Y	8	1	32	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	>24	24	24
PA 5026	N	8	32	>128	≤0.5	≤0.5	≤0.5	2	1	≤0.5	>24	>24	>24
PA 876	N	8	32	>128	≤0.5	≤0.5	≤0.5	4	4	4	>24	>24	>24
PA 861	N	32	16	128	≤0.5	≤0.5	≤0.5	8	8	4	>24	>24	24
PA 5020	N	16	8	64	4	4	2	4	2	2	>24	>24	24
ATCC 27853	N	1	0.25	4	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	24	24	24

[a] Abbreviations: Y, yes; N, no; LVX, levofloxacin; TOB, tobramycin; ATM, aztreonam.

achievable in the sputum of CF patients demonstrated that the bactericidal activity of levofloxacin was more rapid and complete than the bactericidal activities of tobramycin and aztreonam (Fig. 1). Rapid and sustained killing of 11 of the 12 isolates tested by all concentrations of levofloxacin were observed within the first 30 min (Table 3). The same concentrations of tobramycin killed up to 58% of the isolates in 30 min. In contrast, the bactericidal activity of aztreonam was considerably slower, and the extent of bacterial killing by aztreonam was generally lower compared to that achieved with levofloxacin or tobramycin. The bactericidal activity of levofloxacin against nonmucoid and mucoid *P. aeruginosa* isolates was similar.

The bactericidal activities of levofloxacin and tobramycin against *P. aeruginosa* in the presence or absence of sputum from CF patients are shown in Table 4. While there was no change in the bactericidal activity of levofloxacin in sputum from CF patients, the activity of tobramycin was reduced at least eightfold.

DISCUSSION

While the aerosol administration of antibiotics to the lungs provides high concentrations of drug locally, the potencies and PDs of the drugs are still important to ensure that PK-PD indices are optimized to provide bacterial killing and reduce the possibility for the selection of resistance. The results of the multiple assays relevant to the pulmonary infections of CF patients performed in this study demonstrated that levofloxacin has good activity against *P. aeruginosa* at concentrations achievable in the lung following aerosol administration. Levofloxacin also had the lowest MICs against other pathogens found in the lungs of CF patients, including isolates of the *B. cepacia* complex, *S. maltophilia*, *A. xylosoxidans*, MSSA, and MRSA. The MICs of levofloxacin for *P. aeruginosa* and *B. cepacia* were comparable to those reported by Traczewski and Brown (38); however, it was not confirmed that the *B. cepacia* isolates were collected from CF patients. Similar levofloxacin MICs for *A. xylosoxidans* blood and respiratory isolates from the Latin American SENTRY Program (1997 to 2002) were reported by Gales et al. (11). Noviello et al. assayed MSSA and MRSA respiratory isolates for their susceptibilities to levofloxacin and reported that the potency of levofloxacin against MSSA was comparable to that achieved in the present study. Against MRSA, the levofloxacin MIC_{90} was fourfold lower than that reported in our study, which could be because they did not include CF isolates in their study (32). Weiss and colleagues also reported similar MICs for levofloxacin against predominantly respiratory tract specimens of *S. maltophilia* (41).

TABLE 4. Reduction in log CFU/ml for *P. aeruginosa* strain PAM1032 with levofloxacin or tobramycin in CAMHB with or without 10% sputum from CF patients[a]

Antibiotic concn (multiple of MIC)	Levofloxacin (MIC = 2 μg/ml)		Tobramycin (MIC = 0.25 μg/ml)	
	Without sputum	With sputum	Without sputum	With Sputum
1	−1.0	−1.3	−3.7	−2.0
2	−3.5	−3.2	−4.0	−2.2
4	−4.0	−3.7	−4.0	−2.7
8	−4.0	−4.0	−4.0	−3.0

[a] Starting inoculum, 10^6 CFU/ml.

The MICs for ciprofloxacin and levofloxacin against the CF isolates tested in this study are consistent with those found in other studies, which reported that the activity of levofloxacin was superior or equivalent to that of ciprofloxacin against isolates of *P. aeruginosa*, *B. cepacia* complex, *S. maltophilia*, *A. xylosoxidans*, MSSA, and MRSA from CF and non-CF patients (6, 16, 18, 21, 27, 40). Although levofloxacin and ciprofloxacin had similar MICs against *P. aeruginosa* (MIC_{90} = 8 μg/ml for both drugs), it was previously shown that resistant mutants of nonmucoid *P. aeruginosa* CF isolates could be more readily selected with ciprofloxacin in vitro (15).

Recent studies have linked *S. aureus* pulmonary infections with increased pulmonary inflammation (37) and poor outcomes in CF patients with MRSA infections (7). In addition to its potency against gram-negative pathogens from CF patients,

levofloxacin showed potency equivalent or superior to the potencies of other drugs against MSSA and MRSA strains.

Although most isolates were resistant to all antibiotics tested according to the current CLSI susceptibility breakpoints, it should be noted that these breakpoints apply only to the systemic routes of administration and may not be applicable to intrapulmonary doses (3, 9). Aerosol dosing of levofloxacin achieves much greater C_{max}/MIC and AUC/MIC ratios than intravenous or oral delivery methods. For example, the reported serum levofloxacin C_{max} and AUC for a single 750-mg intravenous dose of levofloxacin are 11.5 μg/ml and 110 μg · h/ml, respectively (Levaquin package insert). In contrast, aerosolized levofloxacin (administered as MP-376) can achieve C_{max} and AUC values in the sputum of CF patients more than 100 times higher than those achieved with levofloxacin delivered by the intravenous or oral route (12). Such high concentrations of levofloxacin in sputum have the potential to reduce the development of bacterial resistance (9, 17, 34).

The biofilms formed by adherent *P. aeruginosa* cells in the lungs present a challenge for antimicrobial therapy. The resistance of biofilm cells to antibiotics can be attributed to a lower rate of antibiotic diffusion into cells by the surrounding extracellular glucans matrix (26). The fluoroquinolone antibiotics tend to be among the agents that are the most active against *P. aeruginosa* cells growing in biofilms (31). While some studies show that aminoglycosides also have activity against *P. aeruginosa* biofilms, a recent study showed that the aminoglycosides may also induce biofilm formation at low, subinhibitory concentrations (19). In the current study, the levofloxacin MICs against biofilms formed by *P. aeruginosa* isolates from CF patients were minimally changed, with the increases being twofold or less compared to the MICs obtained by reference broth microdilution methods. The tobramycin and amikacin MICs increased up to eightfold and the aztreonam MICs increased more than eightfold when these drugs were tested in the presence of biofilms. Aztreonam and other beta-lactams may show decreased activity against biofilm cells since they are inherently slowly growing, which may reduce the activities of cell wall inhibitors, such as aztreonam.

The bactericidal activity of levofloxacin against *P. aeruginosa* isolates from CF patients, including multidrug-resistant and mucoid isolates, was greater than the activities of tobramycin and aztreonam at clinically relevant lung concentrations. Of note, the rate of levofloxacin bactericidal activity was rapid, with most of the bacterial killing being observed in the first 30 min after exposure to high concentrations. Thus, high peak levels of levofloxacin following aerosol administration are expected to result in rapid bactericidal killing and suppress the emergence of resistant mutants (1, 39). While high concentrations of aminoglycosides can result in rapid bacterial killing, our results show that higher concentration/MIC ratios are needed for rapid killing by tobramycin than by levofloxacin. In contrast to levofloxacin and tobramycin, the slower, time-dependent bactericidal activity of aztreonam was readily apparent.

The activity of an antibiotic in the sputum matrix present in the lungs of CF patients is an important factor to be considered in the development of an aerosolized antibiotic. The antibiotic must penetrate and diffuse through sputum from CF patients, but not bind to the complex matrix, to act on bacterial cells embedded in sputum. Levofloxacin retained bactericidal activity in vitro in the presence of sputum from CF patients. In contrast, reduced killing by tobramycin was observed in sputum, similar to results reported previously (14).

The results of this study demonstrate that levofloxacin is an attractive agent for aerosol administration for the management of chronic lung infections in patients with CF. Clinical studies with MP-376 are under way to evaluate its safety and efficacy in CF patients.

ACKNOWLEDGMENTS

We extend our gratitude to Lisa Saiman, Peter H. Gilligan, and Patrick Plesiat for providing many of the CF isolates used in this study. We also thank Douglas Conrad for providing sputum specimens for the sputum time-kill assays.

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