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In Vitro Activity of Amifloxacin against Outer Membrane Mutants of the Family *Enterobacteriaceae* and Frequency of Spontaneous Resistance

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Amifloxacin showed potent inhibitory activity against DNA gyrase of *Escherichia coli*. The difference in the susceptibilities of lipopolysaccharide-deficient *Salmonella typhimurium* mutants and their parent strain was less than twofold, and the difference in the susceptibilities of porin-deficient *E. coli* mutants and their parent strain was less than twofold. There was cross resistance among the quinolone group of agents; however, the decrease in MIC for *norB* mutants was slightly lower than that of other fluoroquinolones. Cell lysis was induced with combined treatment of amifloxacin and sodium dodecyl sulfate in *E. coli*. The frequency of mutants spontaneously resistant to amifloxacin was extremely low in all species tested.

Fluoroquinolone antibacterial agents have broad and potent antibacterial activity. The excellent antibacterial activity of fluoroquinolones may be due to their strong inhibitory action against DNA gyrase, a target enzyme of quinolones, and their efficient penetration of the outer membrane (10, 18). Genetic and biochemical analyses of *Escherichia coli* mutants resistant to fluoroquinolone have indicated that the resistance is associated with decreased outer membrane permeability and altered DNA gyrase (8, 9, 13).

To investigate the mode of action of the fluoroquinolone amifloxacin (1, 14), inhibitory activity against *E. coli* DNA gyrase, activity against the outer membrane mutants of *E. coli* and *Salmonella typhimurium*, and frequency of spontaneous resistance were determined in this study.

MATERIALS AND METHODS

Drugs. The following antimicrobial agents were provided by the indicated manufacturers: amifloxacin, Sterling-Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan; norfloxacin, Kyorin Seiyaku Co., Ltd., Tokyo, Japan; ofloxacin and nalidixic acid, Daiichi Seiyaku Co., Ltd., Tokyo, Japan; ciprofloxacin, Bayer Yakuhin Co., Ltd., Osaka, Japan; and cefoxitin, Banyu Yakuhin Co., Ltd., Tokyo, Japan.

Bacterial strains. Nalidixic acid-resistant and norfloxacin-resistant mutants of *E. coli* MH5 (*gyrA*) and KEA13 (*norB*) were originated from *E. coli* K-12 strain KL16 (Hfr *thi relA*) (11). Lipopolysaccharide (LPS)-deficient (*rfa*) mutants of *S. typhimurium* LT2 (supplied by B. A. D. Stocker) (10) and porin-deficient mutants of *E. coli* K-12 were used to investigate the effect of the outer membrane on the activity of quinolones. *E. coli* MC4100 [F$^-$ *araD139* Δ*(argF-lac)U169 rpsL150 relA1 fibB25 ptsF25 deo-1*], MH1160 (MC4100 *ompR1*), MH760 (MC4100 *ompR2*), and MH1461 (MC4100 *envZ11*) were donated by S. Mizushima (19). The parent strain, MC4100, shows osmoregulated production of OmpF and OmpC proteins (7, 8). MH1160 lacks OmpF and OmpC proteins. MH760 lacks OmpC protein and produces OmpF protein constitutively, while MH1461 lacks OmpF protein and produces OmpC protein constitutively. All strains used in this study were maintained in the Laboratory of Drug Resistance in Bacteria, School of Medicine, Gunma University, Gunma, Japan, and the Episome Institute, Gunma, Japan.

Susceptibility testing. The susceptibilities of the strains to the compounds were studied by an agar dilution method. The inocula were prepared as follows. For all strains, the organisms were grown overnight in sensitivity test broth (Nissui Seiyaku) to yield viable counts of about 10^9 CFU/ml. By using an inoculating device (Sakuma Seisakusyo), approximately 10^4 CFU of bacterial culture was inoculated onto agar plates containing serial twofold dilutions of the agents. The medium used for the agar dilution procedure was sensitivity disk agar (Nissui Seiyaku), i.e., modified Mueller-Hinton agar. The MIC was determined after incubation at 37°C for 18 h.

Determination of frequency of spontaneous mutants. The spontaneous mutants resistant to quinolones were selected by plating a 0.1-ml sample of an overnight culture (final inoculum, approximately 10^9 CFU/ml) onto plates containing concentrations of four or eight times the MIC of each drug. After 48 h of incubation at 37°C, the number of viable cells was counted. The frequency of spontaneous mutants resistant to each drug was calculated by dividing the number of resistant cells by the number of viable cells in the sample.

Inhibitory activity against *E. coli* DNA gyrase. *E. coli* KL16 (9, 10) was used as the source of DNA gyrase. Crude enzyme was prepared by treatment with lysozyme (Sigma Chemical Co., St. Louis, Mo.) and ammonium sulfate, as described by Sugino et al. (23). The crude enzyme was loaded on novobiocin-Sepharose and heparin–Sepharose CL-6B columns (22) (Pharmacia, Tokyo, Japan) and was eluted by the method of Sato et al. (21). The reaction conditions for DNA supercoiling activity and enzyme activity were based on a method of Gellert et al. (5, 6). One unit of enzyme is the amount that brings 50% of relaxed pBR322 DNA to the supercoiling form in agarose gel electrophoresis. The reaction mixture, containing subunit A and B proteins, drug solution, and pBR322 relaxed by topoisomerase I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), was incubated at 37°C for 2 h. The reaction was stopped by the addition of proteinase K (Sigma) at a final concentration of 1% and was subjected to agarose gel electrophoresis. The gel

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TABLE 1. Comparison of antibacterial activity of amifloxacin against *S. typhimurium* LPS-deficient mutants and *E. coli* porin-deficient mutants

Species and strain	Phenotype or genotype	Efficacy ratio[a]					
		AMFX	NFLX	OFLX	CPFX	NA	CFX
S. typhimurium							
SL3770	Wild type	1 (0.20)	1 (0.10)	1 (0.20)	1 (0.025)	1 (6.25)	1 (3.13)
SL3749	*rfaL*	1	1	1	1	1	1
SL3750	*rfaJ*	1	1	1	1	0.5	1
SL3769	*rfaG*	0.5	1	1	1	0.5	1
SL3789	*rfaF*	0.5	1	0.5	1	0.125	1
SL1102	*rfaE*	0.5	1	0.5	1	0.125	1
E. coli							
MC4100	Wild type	1 (0.10)	1 (0.05)	1 (0.05)	1 (0.013)	1 (3.13)	1 (1.56)
MH1461	$OmpF^-$ $OmpC^+$	2	4	2	4	1	8
MH760	$OmpF^+$ $OmpC^-$	0.5	1	1	1	1	1
MH1160	$OmpF^-$ $OmpC^-$	1	2	2	2	1	16

[a] Given as the ratio of the MICs for the LPS-deficient and the porin-deficient mutants to those for the parent strains. Numbers in parentheses are MICs (in micrograms per milliliter). Abbreviations: AMFX, amifloxacin; NFLX, norfloxacin; OFLX, ofloxacin; CPFX, ciprofloxacin; NA, nalidixic acid; CFX, cefoxitin.

was stained with ethidium bromide (0.5 μg/ml) and photographed with a UV transilluminator. The negatives were traced with a densitometer (Joko, Tokyo, Japan).

Effect on the outer membrane. The effect of amifloxacin on the outer membrane was determined by the lysis of growing cells induced with sodium dodecyl sulfate (SDS) as described by Chapman et al. (3). Amifloxacin was added to logarithmically growing cells of *E. coli* MC4100 in antibiotic medium 3 (Difco Laboratories, Detroit, Mich.) containing SDS, and the lysis was monitored photometrically at 600 nm by using BIOSCREEN C (Labsystems, Helsinki, Finland).

RESULTS

Inhibitory activity of *E. coli* DNA gyrase. The 50% inhibitory concentrations for supercoiling activity of *E. coli* KL16 DNA gyrase of amifloxacin (MIC, 0.10 μg/ml), norfloxacin (MIC, 0.05 μg/ml), ofloxacin (MIC, 0.05 μg/ml), and ciprofloxacin (MIC, 0.013 μg/ml) were 2.47, 1.16, 0.86, and 0.62 μg/ml, respectively. The inhibitory activity of amifloxacin was four times less than that of ciprofloxacin.

Susceptibility of the outer membrane mutants. The antibacterial activity of amifloxacin against LPS-deficient mutants of *S. typhimurium* and porin-deficient mutants of *E. coli* is shown in Table 1.

The MICs for LPS-deficient mutants (SL3769, SL3789, and SL1102) of amifloxacin were twofold lower than the MIC for the parent strain, and the relative activity of amifloxacin against LPS-deficient mutants was comparable to that of ofloxacin.

The OmpF protein-deficient mutant MH1461 was less susceptible to amifloxacin than the parent strain, MC4100, but MH760, which lacks the OmpC protein and constitutively produces OmpF protein, was twice as susceptible as MC4100. The OmpF and OmpC protein-deficient mutant MH1160 showed susceptibility equal to that of MC4100. Strains MH1461 and MH1160 were two- to fourfold less susceptible to norfloxacin, ofloxacin, and ciprofloxacin than MC4100 was, and MH760 showed susceptibility equal to that of MC4100. All porin-deficient mutants showed susceptibility to nalidixic acid equal to that of MC4100. The susceptibility pattern of porin-deficient mutants for amifloxacin was slightly different from those for other quinolones tested.

Susceptibility of quinolone-resistant mutants. The antibacterial activity of amifloxacin against *E. coli gyrA* mutant MH5 and *norB* mutant KEA13 was determined (Table 2). Both strains showed cross resistance to amifloxacin; however, the decrease in susceptibility of KEA13 to amifloxacin was slightly lower than that of other fluoroquinolones.

Effect on the outer membrane. The effect of amifloxacin on the outer membrane was determined (Fig. 1). Cell lysis was observed in combined treatment with amifloxacin and SDS in *E. coli*.

Frequency of spontaneous resistance. Table 3 shows the frequencies of spontaneous mutants resistant to amifloxacin, norfloxacin, ofloxacin, and ciprofloxacin. The frequency of spontaneous mutants resistant to amifloxacin at concentrations of four to eight times the MICs was about 10^{-8} to $<10^{-9}$/CFU. In *Serratia marcescens*, resistance to norfloxacin, ofloxacin, and ciprofloxacin at a concentration of four times the MIC occurred at frequencies of about 10^{-5} to 10^{-6}/CFU. On the other hand, the frequency of spontaneous mutants resistant to amifloxacin was about 10^{-8}/CFU.

TABLE 2. Comparison of antibacterial activity of amifloxacin against *E. coli* quinolone-resistant mutants

E. coli strain	Phenotype or genotype	Efficacy ratio[a]					
		AMFX	NFLX	OFLX	CPFX	NA	CFX
KL16	Wild type	1 (0.10)	1 (0.05)	1 (0.05)	1 (0.013)	1 (3.13)	1 (3.13)
MH5	*gyrA*	8	8	8	4	32	1
KEA13	*norB*	2	4	4	4	4	4

[a] Given as the ratio of the MIC for the quinolone-resistant mutant to that for the parent strain. Numbers in parentheses are MICs (in micrograms per milliliter). Abbreviations: AMFX, amifloxacin; NFLX, norfloxacin; OFLX, ofloxacin; CPFX, ciprofloxacin; NA, nalidixic acid; CFX, cefoxitin.

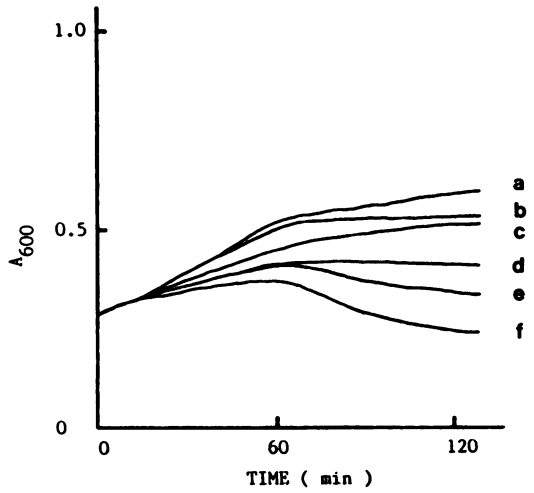

FIG. 1. Effect of amifloxacin on SDS-induced lysis of *E. coli* MC4100. a, No addition; b, 0.05% SDS; c, 0.39 μg of amifloxacin per ml; d, 0.78 μg of amifloxacin per ml; e, 0.39 μg of amifloxacin per ml and 0.05% SDS; f, 0.78 μg of amifloxacin per ml and 0.05% SDS.

DISCUSSION

Fluoroquinolones have expanded antibacterial spectra and enhanced antibacterial activities in comparison with the older, nonfluorinated quinolones, such as nalidixic acid. The potent inhibitory action of fluoroquinolones against DNA gyrase and their efficient permeation of the outer membrane may contribute to their antibacterial activities (10, 18). Amifloxacin is a fluoroquinolone and has a broad spectrum and potent activities that are almost comparable to those of norfloxacin and ofloxacin (1, 4, 14–16).

Amifloxacin and other fluoroquinolones inhibited DNA gyrase activity, and this inhibitory action may explain the antibacterial activities of the agents.

Furthermore, we determined the activity of amifloxacin against the outer membrane mutants. The differences in the susceptibilities of LPS-deficient mutants and smooth strains to amifloxacin were less than twofold. Hirai et al. (10) noted a good correlation in the decrease in MICs for rough mutants compared with the MICs for the wild-type strain; the more hydrophobic the compound, the larger the decrease in the MIC. According to the data of Chapman et al. (3), amifloxacin is more hydrophobic than norfloxacin, ofloxacin, and nalidixic acid. Despite the high hydrophobicity of the drug, the activity of amifloxacin was little affected by alterations in LPS structure. These results suggest that amifloxacin may be one of the exceptions to the correlation and that the aminoethyl group at position 1, an amifloxacin-specific moiety, and the methylpiperazine group at position 7 of the quinolone nucleus may play an important role in penetrating the permeability barrier of LPS.

The OmpF-deficient mutant was less susceptible to amifloxacin than the parent strain was, and OmpF-deficient–OmpC-constitutive mutants showed increased susceptibility. These results suggest that amifloxacin may penetrate the OmpF porin and that the OmpF porin dependency of amifloxacin may be slightly lower than those of norfloxacin and ciprofloxacin for penetration through the outer membrane. Furthermore, the OmpF- and OmpC-deficient mutant MH1160 showed susceptibility to amifloxacin equal to that

TABLE 3. Frequency of spontaneous resistance to amifloxacin

Organism and strain	Drug	MIC (μg/ml)	Frequency of spontaneous resistance with:	
			4 × MIC	8 × MIC
Staphylococcus aureus Smith	Amifloxacin	0.78	$<1.0 \times 10^{-8}$	$<1.0 \times 10^{-8}$
	Norfloxacin	0.39	3.0×10^{-8}	$<1.0 \times 10^{-8}$
	Ofloxacin	0.20	$<1.0 \times 10^{-8}$	$<1.0 \times 10^{-8}$
	Ciprofloxacin	0.20	1.0×10^{-8}	$<1.0 \times 10^{-8}$
Escherichia coli ML4707	Amifloxacin	0.05	$<9.1 \times 10^{-9}$	$<9.1 \times 10^{-9}$
	Norfloxacin	0.05	$<9.1 \times 10^{-9}$	$<9.1 \times 10^{-9}$
	Ofloxacin	0.05	$<9.1 \times 10^{-9}$	$<9.1 \times 10^{-9}$
	Ciprofloxacin	0.025	$<9.1 \times 10^{-9}$	$<9.1 \times 10^{-9}$
Proteus mirabilis IF03849	Amifloxacin	1.56	$<4.2 \times 10^{-9}$	$<4.2 \times 10^{-9}$
	Norfloxacin	0.39	$<4.2 \times 10^{-9}$	$<4.2 \times 10^{-9}$
	Ofloxacin	0.78	$<4.2 \times 10^{-9}$	$<4.2 \times 10^{-9}$
	Ciprofloxacin	0.20	$<4.2 \times 10^{-9}$	$<4.2 \times 10^{-9}$
Enterobacter cloacae 963	Amifloxacin	0.39	9.8×10^{-8}	7.1×10^{-8}
	Norfloxacin	0.39	1.4×10^{-8}	5.4×10^{-9}
	Ofloxacin	0.39	9.8×10^{-8}	5.0×10^{-8}
	Ciprofloxacin	0.20	4.5×10^{-8}	7.1×10^{-8}
Serratia marcescens GN7577	Amifloxacin	3.13	9.0×10^{-8}	$<4.8 \times 10^{-9}$
	Norfloxacin	3.13	3.2×10^{-6}	$<4.8 \times 10^{-9}$
	Ofloxacin	3.13	1.5×10^{-6}	$<4.8 \times 10^{-9}$
	Ciprofloxacin	1.56	1.3×10^{-5}	1.0×10^{-7}
Pseudomonas aeruginosa GN11189	Amifloxacin	1.56	$<4.8 \times 10^{-9}$	$<4.8 \times 10^{-9}$
	Norfloxacin	1.56	$<4.8 \times 10^{-9}$	$<4.8 \times 10^{-9}$
	Ofloxacin	1.56	$<4.8 \times 10^{-9}$	$<4.8 \times 10^{-9}$
	Ciprofloxacin	0.39	$<4.8 \times 10^{-9}$	$<4.8 \times 10^{-9}$

of MC4100. We also found that amifloxacin in combination with SDS induced cell lysis (Fig. 1). These results may be interpreted to mean that amifloxacin penetrates the outer membrane through OmpF porin pores and phospholipid bilayers (nonporin pathway), like fleroxacin (2, 3).

The *gyrA* mutant showed cross resistance to amifloxacin, just as it did to other quinolones tested. However, the difference in susceptibility of *norB* mutant KEA13 and the parent strain to amifloxacin was slightly less than differences in response to the other quinolones. The *norB*-determined resistance to norfloxacin with a decrease in OmpF protein (11) and the smaller difference in susceptibilities to amifloxacin may be explained by the balance of its permeation route dependency as described above.

Amifloxacin elicited a low frequency of spontaneous resistance in all species tested; however, other quinolones elicited relatively higher frequencies, especially in *Serratia marcescens*. This property of amifloxacin was considered interesting from the point of view of resistance selection. The OmpF and OmpC mutations would not be sufficient to cause resistance to amifloxacin, since the decrease in MIC for porin-deficient mutants of *E. coli* was less than twofold. These factors might contribute to the lower mutation frequencies elicited by amifloxacin in gram-negative bacteria.

Outer membrane mutations have been found to contribute to resistance to various antibiotics (12, 20). Amifloxacin would be a useful agent for such isolates that showed resistance to antibiotics by outer membrane mutations.

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