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Bacterial Resistance to LB20304, a New Fluoroquinolone Antibiotic

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In vitro studies were conducted to dertermine the frequency rate of spontaneous resistance to LB20304 and to dertermine whether cross-resistance to other antimicrobial agents develops. In eight strains of bacteria, the frequency of mutation to LB20304 at the concentrations of four and eight times the minimal inhibitory concentration (MIC) ranged from less than 4.0×10^{-10} to 2.2×10^{-8}. These results were similar to those found for other new fluoroquinolones. The development of stepwise resistance was determined by repeated subculture in broth in the presence of increasing concentration of the compounds. Exposure of bacteria to increasing concentrations of LB20304 resulted in the selection of organisms with higher MICs. There were 4- to 128-fold increases in the MIC of LB20304 for bacterial strains of *Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli* and *Pseudomonas aeruginosa*. However, those strains selected after repeated exposure were well within the susceptibility range for LB20304 except for *Pseudomonas aeruginosa*. The resistant isolates selected with LB20304 showed cross-resistance when tested against ciprofloxacin and *vice versa*.

Key words : LB20304, Fluoroquinolone, Frequency of resistance, Cross-resistance

INTRODUCTION

The new fluoroquinolones have been used for nearly 10 years for the treatment of community-acquired and hospital-acquired infections. The intensive use of fluoroquinolones raised concerns about the development of resistant strains reported shortly after these antibacterial agents were introduced for clinical use (Acar and Francoual, 1990; Goldstein and Acar, 1995). Two principal mechanisms of resistance to the fluoroquinolones have been described. First, alteration of the DNA gyrase (*gyrA* and *gyrB*), which is the target site of the quinolones (Sato *et al.*, 1986; Yamagishi *et al.*, 1986). Second, diminished accumulation of quinolones inside the cell as a result of either decreased uptake or increased efflux (Hooper *et al.*, 1986; Li *et al.*, 1994). Generally, alteration of DNA gyrase results in higher levels of quinolone resistance than decreased permeability or enhanced efflux. To date, most clinical fluoroquinolone resistance has been due to mutations in *gyrA* (Piddock, 1995). Resistant strains derived from alteration of DNA gyrase exhibited cross-resistance to other fluoroquinolones but not to unrelated antibiotics. On the other hand, mutants with an alteration of the bacterial outer membranes or permeability factors, which are usually found in gram-negetive bacteria such as Enterobacteriaceae and *P. aeruginosa*, were also cross-resistant to unrelated antibiotics, such as β-lactams, chloramphenicol, trimethoprim and tetracyclines. The incidence of resistance to fluoroquinolones varies depending on bacterial species, compounds and concentration of drugs. Therefore, the resistance study is very important for the evaluation of new quinolone compounds. Indeed, nalidixic acid, the prototype of quinolones, is hardly used these days because of rapid development of resistance during therapy.

LB20304 is a new quinolone antibacterial agent synthesized at LG Chemical Ltd. (Kim *et al.*, 1995) (Fig. 1). LB20304 has shown potent activities against gram-positive, gram-negative and anaerobic bacteria *in vitro* and *in vivo*, and improved pharmacokinetic profiles in animals (Oh *et al.*, 1996; Kim *et al.*, 1996; Paek *et al.*, 1996; Oh *et al.*, 1995). This compound has many advantages over the currently available quinolone antibiotics in terms of antibacterial activities and pharmacokinetic profiles.

In this study, we examined the frequency of mutants resistant to LB20304 and the development of stepwise resistance by repeated subculture. The cross-

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resistance between LB20304 and other antibiotics was also studied.

MATERIALS AND METHODS

Test compounds

LB20304 was synthesized at the Biotech Research Institute, LG Chem Research Park, LG Chemical Ltd., Taejon, Korea. All comparative quinolone compounds were obtained directly from their manufacturers.

Test organisms

The bacterial strains used in this study were clinical isolates from human clinical specimens or laboratory standard strains obtained from American Type Culture Collection (ATCC) and Glaxo Group Research Ltd. All isolates were stored frozen at -70°C.

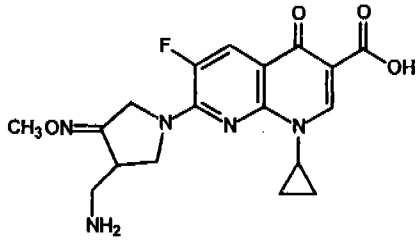

Fig. 1. Chemical structure of LB20304

In vitro MIC determination

The MICs were determined by the agar dilution methods as described by the National Committee for Clinical Laboratory Standards M7-A3 (NCCLS, 1993). Test strains were grown for 18 h in Mueller-Hinton broth (Difco Laboratories, Detroit, Michigan) and then diluted with the same fresh medium to the density of approximately 10^7 CFU/ml. These strains were applied to Mueller-Hinton agar plates containing a serially diluted antimicrobial agent, by using an automatic MIC-2000 multipin inoculator (Dynatech Laboratories, Inc., Alexandria, VA.) to yield 10^4 CFU per spot. The MIC was considered to be the lowest concentration that completely inhibited bacterial growth on agar plates after 18 h of incubation at 35°C, disregarding a single colony or a faint haze caused by the inoculum.

In vitro frequency of resistant cells

Test organisms were grown in Mueller-Hinton broth at 35°C with shaking until the midexponential growth phase was achieved. The bacteria were then concentrated by centrifugation, and approximately 10^9 to 10^{10} CFU of bacteria were smeared onto Mueller-Hinton agar plates containing each drug at the concentrations of four times or eigth times the MIC. The

Table I. Frequency of spontaneous mutants resistant to LB20304 and other quinolones

Strain		Frequency of resistance to indicated agent			
		LB20304	ciprofloxacin	sparfloxacin	lomefloxacin
S. aureus giorgio	4×MIC[a]	1.1×10^{-9}	8.3×10^{-9}	$<5.6\times10^{-10}$	2.8×10^{-9}
	8×MIC[b]	5.6×10^{-10}	$<5.6\times10^{-10}$	$<5.6\times10^{-10}$	$<5.6\times10^{-10}$
S. epidermidis 887E	4×MIC	4.0×10^{-10}	4.0×10^{-10}	4.0×10^{-10}	1.2×10^{-9}
	8×MIC	$<4.0\times10^{-10}$	$<4.0\times10^{-10}$	$<4.0\times10^{-10}$	$<4.0\times10^{-10}$
E. faecalis 29212A	4×MIC	$<2.4\times10^{-9}$	$<2.4\times10^{-9}$	$<2.4\times10^{-9}$	$<2.4\times10^{-9}$
	8×MIC	$<2.4\times10^{-9}$	$<2.4\times10^{-9}$	$<2.4\times10^{-9}$	$<2.4\times10^{-9}$
S. pyogenes PY009	4×MIC	$<4.8\times10^{-10}$	$<4.8\times10^{-10}$	$<4.8\times10^{-10}$	$<4.8\times10^{-10}$
	8×MIC	$<4.8\times10^{-10}$	$<4.8\times10^{-10}$	$<4.8\times10^{-10}$	$<4.8\times10^{-10}$
E. coli 10536	4×MIC	1.3×10^{-9}	2.2×10^{-9}	1.3×10^{-9}	3.9×10^{-9}
	8×MIC	4.3×10^{-10}	$<4.3\times10^{-10}$	$<4.3\times10^{-10}$	8.7×10^{-10}
E. cloacae 1194E	4×MIC	2.2×10^{-8}	2.8×10^{-8}	1.5×10^{-8}	6.7×10^{-9}
	8×MIC	1.2×10^{-9}	1.4×10^{-9}	1.6×10^{-10}	1.2×10^{-10}
S. marcescens 1826E	4×MIC	2.0×10^{-8}	3.3×10^{-10}	2.5×10^{-10}	2.8×10^{-9}
	8×MIC	1.7×10^{-10}	1.7×10^{-10}	$<8.3\times10^{-11}$	1.7×10^{-10}
P. aeruginosa 10145	4×MIC	8.5×10^{-9}	3.7×10^{-10}	1.5×10^{-9}	2.4×10^{-9}
	8×MIC	7.0×10^{-9}	$<1.9\times10^{-10}$	$<1.9\times10^{-10}$	$<1.9\times10^{-10}$

[a]Mutants were selected at 4 times the original MIC.
[b]Mutants were selected at 8 times the original MIC.

numbers of colonies were counted after 48 h incubation at 35°C. The frequency of spontaneous mutations selected by each compound was calculated as the ratio of the number of cells growing on drug-containing agar plates to the number of inoculated cells.

Stepwise resistance by serial passage

The development of stepwise resistance was determined by repeated exposure of bacteria to increasing concentrations of the compounds. Test organisms were grown in Mueller-Hinton broth at 35°C with shaking and then inoculated to fresh Mueller-Hinton broth containing each drug at twofold incremental concentrations. From the highest concentration showing visible growth, 1.5×10^5 CFU of test organisms were reexposed to twofold incremental concentrations until the concentration above which further growth did not occur was reached.

Cross-resistance of LB20304 with other antibiotics

The spontaneous mutants resistant to LB20304 or ciprofloxacin were produced in the presence of each drug (at a concentration of four times the MIC) as selecting agent. To check the cross-resistance, the MICs of LB20304, ciprofloxacin and cefpirome against the mutants selected with LB20304 or ciprofloxacin were determined as described above.

RESULTS

Frequency of mutations resistant to test compounds

The appearance of spontaneous resistance was determined for eight different strains. Table I shows the frequency of resistant cells to LB20304, ciprofloxacin, sparfloxacin and lomefloxacin. In general, spontaneous mutations rendering strains resistant to four times the MIC of LB20304 occurred at a low frequency ($<10^{-9}$) except for *Enterobacter cloacae* 1194E and *Serratia marcescens* 1826E. The frequency of single-step mutations of *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Streptpcoccus pyogenes, Escherichia coli, E. cloacae, S. marcescens* and *Pseudomonas aeruginosa* to LB

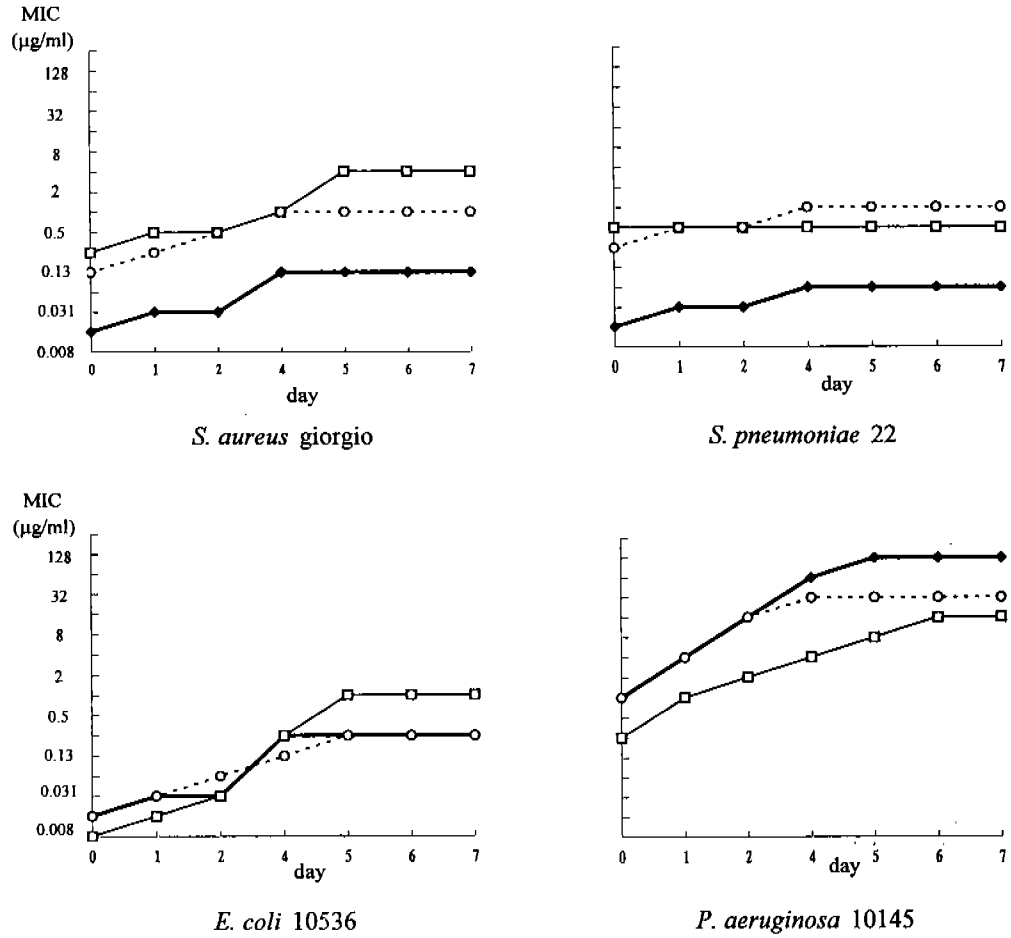

Fig. 2. Development of stepwise resistance to LB20304, sparfloxacin and cipofloxacin (—◆—, LB20304; ···○···, sparfloxacin; —□—, ciprofloxacin).

20304 at a concentration of eight times the MIC was 5.6×10^{-10}, $< 4.0\times10^{-10}$, $< 2.4\times10^{-9}$, $< 4.8\times10^{-10}$, 4.3×10^{-10}, 1.2×10^{-9}, 1.7×10^{-10} and 7.0×10^{-9}, respectively. These results were similar to those found for ciprofloxacin, sparfloxacin and lomefloxacin.

Development of stepwise resistance by serial passage

Exposure of bacteria to increasing concentrations of LB20304 or ciprofloxacin resulted in the selection of

Table II. Cross-resistance of LB20304 with ciprofloxacin and cefpirome

Strain			MIC (μg/ml)		
			LB20304	Ciprofloxacin	Cefpirome
S. aureus 6538p	wild-type		≤0.008	0.13	1
	resistance to LB20304[a]	# 1	0.13	0.5	1
		# 2	0.13	0.5	1
	resistance to ciprofloxacin[b]	# 1	0.063	0.25	1
		# 2	0.063	0.25	1
E. coli 3190Y	wild-type		≤0.008	≤0.008	0.031
	resistance to LB20304	# 1	0.13	0.13	0.031
		# 2	0.13	0.13	0.031
		# 3	0.13	0.13	0.031
		# 4	0.13	0.13	0.031
		# 5	0.063	0.13	0.031
		# 6	0.063	0.13	0.031
		# 7	0.063	0.13	0.031
		# 8	0.063	0.13	0.031
		# 9	0.13	0.13	0.031
	resistance to ciprofloxacin	# 1	0.13	0.25	0.063
		# 2	0.13	0.13	0.031
		# 3	0.13	0.13	0.031
		# 4	0.13	0.13	0.031
		# 5	0.13	0.13	0.031
		# 6	0.13	0.13	0.031
		# 7	0.13	0.13	0.031
P. aeruginosa 1912E	wild-type		0.5	0.25	4
	resistance to LB20304	# 1	8	2	16
		# 2	4	2	16
		# 3	8	2	16
		# 4	8	2	16
		# 5	8	2	16
		# 6	8	2	16
		# 7	8	2	16
		# 8	8	2	16
		# 9	16	2	16
		# 10	8	4	16
		# 11	8	2	16
		# 12	16	2	16
		# 13	8	4	16
	resistance to ciprofloxacin	# 1	16	4	16
		# 2	4	2	4
		# 3	2	1	2
		# 4	8	2	16
		# 5	8	2	16
		# 6	8	2	16
		# 7	8	4	16
		# 8	8	4	16
		# 9	8	2	16
		# 10	8	2	16

[a]Strains were selected with LB20304 as a selecting agent.
[b]Strains were selected with ciprofloxacin as a selecting agent.

organisms with higher MICs (Fig. 2). Prior to exposure to drug, the MIC of LB20304 for both *S. aureus* giorgio and *Streptococcus pneumoniae* 22 was 0.016 μg/ml. After 7 transfers, the MICs of LB20304 were 0.13 and 0.063 μg/ml, respectively. There was an eight-fold increase in the MIC of LB20304 for *S. aureus* and a four-fold increase in the MIC for *S. pneumoniae*, but these strains selected after repeated exposure were still highly susceptible to LB20304. In contrast, the MICs of sparfloxacin increased from 0.13 to 4 μg/ml for *S. aureus* and from 0.25 to 1 μg/ml for *S. pneumoniae* after 7 transfers. Resistance to LB 20304 in these two gram-positive strains developed more slowly than did that to sparfloxacin. For *E. coli*, there was a 16-fold increase (from 0.016 to 0.25 μg/ml) in the MIC of LB20304 but these strains were also well within the susceptibility range for LB20304. The development of resistance to LB20304 in *E. coli* was similar to that to sparfloxacin. On the other hand, there was a 128-fold increase (from 1 to 128 μg/ml) in the MIC of LB20304 for *P. aeruginosa*. LB20304 induced resistance more rapidly than sparfloxacin and ciprofloxacin. The strains selected after consecutive exposure of *P. aeruginosa* to LB20304 and other quinolones were highly resistant to all quinolones tested. The maximum increase in the MIC of LB20304 for all strains in a single subculture was eight-fold.

Cross resistance between LB20304 and other antibiotics

Strains selected for resistance exhibited cross-resistance between LB20304 and ciprofloxacin as shown in Table II. As the MICs of LB20304 against strains selected for resistance increased, so did the MICs of ciprofloxacin against these strains increase. For resistant isolates of *S. aureus* and *E. coli* selected with either LB20304 or ciprofloxacin, there was complete cross-resistance between LB20304 and ciprofloxacin, but no cross-resistance between LB20304 and β-lactam antibiotic, such as cefpirome. However, the resistant isolates of *P. aeruginosa* selected with either LB 20304 or ciprofloxacin showed cross-resistance to cefpirome as well as to each other.

DISCUSSION

Resistance to new fluoroquinolones as a consequence of single-step mutation occurs at a low frequency, and the frequency of mutation by new fluoroquinolones is usually several hundreds times lower than that induced by nalidixic acid (Neu, 1988). In the previous paper, the frequency of spontaneous resistance to LB20304 in three strains was reported (Paek *et al.*, 1996). The frequency of resistant strains to LB20304 in *S. aureus, E. coli* and *P. aeruginosa* was similar to or slightly lower than that observed for ciprofloxacin and sparfloxacin. In this study, the bacterial resistance to LB20304 was investigated intensively. The frequency of spontaneous resistance to LB20304 in new eight strains was also low like new fluoroquinolones and similar to that of ciprofloxacin, sparfloxacin and lomefloxacin. Furthermore, the development of resistance after consecutive exposure of two gram-positive strains to drugs demonstrated that the emergence of resistance to LB20304 was slower than that to sparfloxacin and ciprofloxacin. Although the MICs of LB20304 against the strains selected after consecutive exposure of *S. aureus, S. pneumoniae, E. coli* and *P. aeruginosa* to LB20304 increased, these strains were still well within the susceptibility range to LB20304 except for *P. aeruginosa*, considering the concentration of LB20304 that could achieve in blood, urine and various tissues after oral administration.

There was no cross-resistance between quinolones and other classes of drugs, with the exception of drug resistance related to changes in the bacterial outer membrane proteins (Neu, 1988). This study exhibited that there was complete cross-resistance in *S. aureus* and *E. coli* between LB20304 and ciprofloxacin, but no cross-resistance between LB20304 and cefpirome, a fourth-generation parenteral cephalosporin. Therefore, these mutant strains of *S. aureus* and *E. coli* seemed to be drived from an alteration of DNA gyrase. On the other hand, resistant strains of *P. aeruginosa* selected with either LB20304 or ciprofloxacin showed cross-resistance to cefpirome as well as to each other. The changes in the bacterial outer membrane proteins (porin proteins) of the resistant strains of *P. aeruginosa* seemed to reduce the permeability of both quinolones and cephalosporins (Sanders *et al.*, 1984; Piddock, 1991). Although LB20304 shared cross-resistance with other fluoroquinolones, it had potent activity against β-lactam-resistant strains which produce β-lactamase (Kim *et al.*, 1996).

Further studies on the mechanism of bacterial resistance to LB20304 would be necessary to establish the clinical usefulness of this compound.

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