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Factors Affecting *In Vitro* Activity of LB20304, a New Fluoroquinolone

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LB20304 is a novel fluoroquinolone that exhibits a potent broad spectrum antibacterial activity against both gram-positve and gram-negative bacteria. The MICs (Minimal Inhibitory Concentration) of LB20304 were determined against both gram-positve and gram-negative bacteria under various conditions including several media, pHs, and inoculum concentrations. The *in vitro* activity of LB20304 was not significantly affected by the changes in testing conditions such as components of media and inoculum concentrations, but it was slightly reduced by acid condition. The MICs and MBCs (Minimal Bactericidal Concentration) of LB20304 against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* were hardly affected by the presence of 50 % human serum, mouse serum, guinea pig serum or horse serum, and the MBCs were equal to or at most four-times higher than the MICs. The activities of LB20304 were decreased by the presence of high concentraion of Mg^{++} or human urine (pH, 5.5) in the test media. The frequencies of mutants resistant to LB20304 were similar to or lower than those found in ciprofloxacin and sparfloxacin

Key words : LB20304, Quinolone, MIC, MBC, Resistance

INTRODUCTION

The fluoroquinolone antimicrobial agents have been used in the therapy of many infections since they were introduced into the market (Neu, 1992, Wolfson *et al*, 1989). Recently, however, quinolone-resistant gram-positive bacteria, such as MRSA and Streptococci, have developed frequently due to their wide use for the treatment of various infections in human (Blumberg *et al*, 1991, Kaatz *et al*, 1991). Since the current fluoroquinolones, such as ciprofloxacin, lomefloxacin, ofloxacin and fleroxacin, lack a sufficient activity against gram-positive bacteria which are major pathogenic strains of respiratory tract infections, and anaerobic bacteria which cause intra-abdominal infections (Raviglione *et al*, 1990; Thys *et al*, 1989), there is strong interest in finding novel quinolone compounds that provide improved activity against gram-positive organism and anaerobes while retaining the potent activity of ciprofloxacin against gram-negative bacteria.

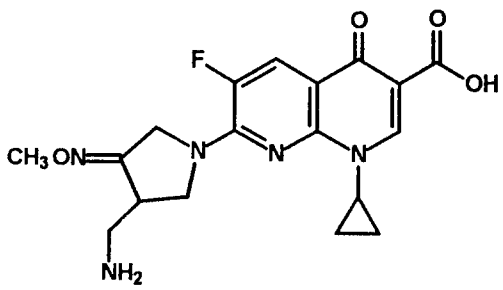

Fig. 1. Chemical structure of LB20304

LB20304, [7-(3-aminomethyl-4-methoxyimino-pyrrolidin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-[1.8]-naphthyridine-3-carboxylic acid], is a new quinolone antibacterial agent synthesized at LG Chemical Ltd. (Kim *et al*, 1995) (Fig. 1). This compound has shown a broad-spectrum antibacterial activity. Its *in vitro* activity was superior to those of the currently available quinolones against most bacterial strains including gram-positive bacteria and anaerobes (Oh *et al*, 1995).

In this paper, we examined the effects of various test conditions on the *in vitro* activity of LB20304. And the frequencies of mutants resistant to LB20304, ciprofloxacin and sparfloxacin were also evaluated.

MATERIALS AND METHODS

Antimicrobial agents

LB20304 was synthesized at the Biotech Research

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Institute, LG Chem Research Park, LG Chemical Ltd., Taejon, Korea. All comparative quinolone compounds were obtained directly from their manufacturers.

Bacterial strains

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

In vitro tests

The MICs were determined by either broth dilution or 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 (MHB), and then these overnight cultures were diluted with the same fresh medium to the density of approximately 10^7 CFU/ml and applied to Mueller-Hinton agar (MHA) plates or MHB, which have serially diluted antimicrobial agent, by use of an automatic MIC-2000 multipin inoculator (Dynatech Laboratories, Inc., Alexandria, VA.) to yield 10^4 CFU per spot. The MICs were determined after 18 h of incubation at 35°C. The concentrations of the bacterial suspensions were determined by measuring the optical density or the turbidity and were verified by determining standard colony counts on antibiotic-free agar plates. The MIC was considered to be the lowest concentration that completely inhibited bacterial growth on agar plates, disregarding a single colony or a faint haze caused by the inoculum. The MBC was defined as the lowest concentration which induced more than 99.9 % reduction in CFU after 18 h of incubation at 35°C (NCCLS M26-7, 1992).

The effects of inoculum concentrations (10^4, 10^5, or 10^6 CFU per spot) on the activity of LB20304 were examined by determining the MICs against laboratory standard strains by the methods described as above. Three different media were used for evaluating the effects on *in vitro* activity of LB20304. And pH effects were determined in media adjusted to the pHs indicated in Table III with NaOH or HCl. For examining the cation effects on the activity of LB20304, $MgCl_2$ was added to MHB at the concentrations of 4.5 mM and 9 mM, respectively, and the MICs and MBCs against *S. aureus, E. faecalis, E. coli* and *P. aeruginosa* were determined. Serum effects were also examined in media containing 50 % heat-inactivated serums as indicated in Table V.

Table I. Effect of inoculum concentrations on the activity of LB20304

Strains	Code	MIC (μg/ml)		
		10^4cfu	10^5cfu	10^6cfu
S. aureus	6538p	≤0.008	≤0.008	≤0.008
S. aureus	giorgio	≤0.008	≤0.008	0.031
S. aureus	77	0.016	0.031	0.031
S. aureus	241	4	4	4
S. epidermidis	887E	≤0.008	≤0.008	≤0.008
S. epidermidis	178	4	4	4
E. faecalis	29212A	0.031	0.063	0.063
B. subtilis	ATCC 6633	≤0.008	≤0.008	≤0.008
M. luteus	ATCC 9341	0.13	0.13	0.13
E. coli	10536	≤0.008	≤0.008	≤0.008
E. coli	3190Y	≤0.008	≤0.008	≤0.008
E. coli	851E	≤0.008	0.016	0.016
E. coli	3455E (TEM3)	0.13	0.13	0.13
E. coli	3739E (TEM5)	0.063	0.13	0.13
E. coli	2639E (TEM9)	0.031	0.031	0.031
P. aeruginosa	1912E	0.5	0.5	0.5
P. aeruginosa	10145	0.5	0.5	0.5
P. aeruginosa	6065Y	8	8	8
A. calcoaceticus	15473A	0.031	0.063	0.063
C. diversus	2046E	0.031	0.031	0.063
E. cloacae	1194E (IND+VE)	0.031	0.063	0.063
E. cloacae	P99	≤0.008	0.016	0.016
K. aerogenes	1976E (SHV-1)	0.063	0.13	0.13
K. aerogenes	1082E (K1+)	0.031	0.031	0.063
P. vulgaris	6059A	0.25	0.25	0.25
S. marcescens	1826E	0.25	0.5	0.5
S. typhimurium	14028A	0.031	0.031	0.031

Table II. Effect of media on the activity of LB20304

Strains	Code	MIC (μg/ml)		
		Mueller-Hinton Agar	Brain Heart Infusion Agar	Tryptic Soy Agar
S. aureus	6538p	≤0.008	≤0.008	0.031
S. aureus	giorgio	≤0.008	≤0.008	≤0.008
S. aureus	77	0.016	0.016	0.031
S. aureus	241	4	4	8
S. epidermidis	887E	≤0.008	≤0.008	0.016
S. epidermidis	178	4	8	8
E. faecalis	29212A	0.031	0.031	0.063
B. subtilis	ATCC 6633	≤0.008	≤0.008	≤0.008
M. luteus	ATCC 9341	0.13	0.13	0.25
E. coli	10536	≤0.008	≤0.008	≤0.008
E. coli	3190Y	≤0.008	≤0.008	≤0.008
E. coli	851E	≤0.008	≤0.008	0.016
E. coli	3455E (TEM3)	0.13	0.25	0.25
E. coli	3739E (TEM5)	0.063	0.13	0.13
E. coli	2639E (TEM9)	0.031	0.016	0.031
P. aeruginosa	1912E	0.5	0.25	0.5
P. aeruginosa	10145	0.5	0.5	0.5
P. aeruginosa	6065Y	8	8	8
A. calcoaceticus	15473A	0.031	0.063	0.063
C. diversus	2046E	0.031	0.016	0.063
E. cloacae	1194E (IND+VE)	0.031	0.016	0.031
E. cloacae	P99	≤0.008	≤0.008	≤0.008
K. aerogenes	1976E (SHV+1)	0.063	0.063	0.13
K. aergenes	1082E (K1+)	0.031	0.031	0.063
P. vulgaris	6059A	0.25	0.25	0.5
S. marcescens	1826E	0.25	0.25	0.5
S. typhimurium	14028A	0.031	0.031	0.031

Table III. Effect of pH on the activity of LB20304

Strains	Code	MIC (μg/ml)		
		pH6	pH7	pH8
S. aureus	giorgio	≤0.008	≤0.008	≤0.008
S. aureus	77	0.031	0.016	≤0.008
S. epidermidis	887E	0.016	≤0.008	≤0.008
B. subtilis	ATCC 6633	≤0.008	≤0.008	≤0.008
M. luteus	ATCC 9341	0.25	0.13	0.13
E. coli	10536	≤0.008	≤0.008	≤0.008
E. coli	851E	0.063	≤0.008	≤0.008
E. coli	3455E (TEM3)	0.5	0.13	0.063
E. coli	3739E (TEM5)	0.25	0.063	0.031
E. coli	2639E (TEM9)	0.13	0.031	≤0.008
P. aeruginosa	10145	1	0.5	0.25
A. calcoaceticus	15473A	0.25	0.031	0.031
C. diversus	2046E	0.13	0.031	≤0.008
E. cloacae	1194E (IND+VE)	0.13	0.031	≤0.008
E. cloacae	P99	0.016	≤0.008	≤0.008
K. aerogenes	1976E (SHV-1)	0.5	0.063	0.031
K. aerogenes	1082E (K1+)	0.13	0.031	≤0.008
P. vulgaris	6059A	1	0.25	0.13
S. marcescens	1826E	1	0.25	0.13
S. typhimurium	14028A	0.063	0.031	≤0.008

In vitro frequency of resistant cells

Test organisms were grown in MHB at 35°C with shaking until the mid-exponential growth phase was achieved. The bacteria were then concentrated by centrifugation, and approximately 10^9 to 10^{10} CFU of bacteria were smeared onto MHA plates containing each drug at the concentrations of four times the MIC. The numbers of colonies were counted after 48 h incubation at 35°C. The frequency of spontaneous mu-

Table IV. Effect of pH and Mg^{++} on the MICs of LB20304

Strains		MIC (μg/ml)					
		MHB		MHB+4.5 mM Mg^{++}		MHB+9 mM Mg^{++}	
		pH 7.2	pH 5.5	pH 7.2	pH 5.5	pH 7.2	pH 5.5
S. aureus	6538p	0.031	0.063	0.25	0.5	0.5	1
E. faecalis	29212A	0.13	0.25	1	2	2	4
E. coli	3190Y	0.016	0.13	0.25	0.5	1	2
P. aeruginosa	1912E	1	2	16	64	16	32

Table V. Effect of serum and urine on the activity of LB20304

Materials			*S. aureus* 77	*E. coli* 851E	*P. aeruginosa* 1912E
Mueller-Hinton broth		MIC	0.016	0.016	0.5
		MBC	0.031	0.016	0.5
MHB*+50% human serum		MIC	0.016	0.016	0.5
		MBC	0.031	0.031	1
MHB*+50% mouse serum		MIC	0.016	0.016	0.25
		MBC	0.063	0.031	0.5
MHB*+50% guinea pig serum		MIC	0.031	0.016	0.5
		MBC	0.063	0.031	1
MHB*+50% horse serum		MIC	0.016	0.016	0.5
		MBC	0.063	0.031	1
MHB*+50% urine	pH 5.5	MIC	0.13	1	8
		MBC	0.25	2	32
	pH 7.4	MIC	0.31	0.063	1
		MBC	0.063	0.063	2

MHB*, Mueller-Hinton broth

tations 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.

RESULTS

Factors affecting *in vitro* activity

The MICs of LB20304 against standard strains remained unchanged at an inoculum of 10^5 or 10^6 CFU per spot in Muller-Hinton agar compared with the preferred concentration of 10^4 CFU per spot as shown in Table I. And the activities of LB20304 against laboratory standard strains were nearly same in any of three different media; Muller-Hinton agar, brain heart infusion agar, tryptic soy agar (Table II). However, the activities of LB20304 were inhibited by acid condition and by the presence of high concentrations of Mg^{++} as shown in Table III and IV, respectively. The MICs of LB20304 in the presence of 9 mM Mg^{++} were 16- to 64-fold higher than those assayed in MHB against *S. aureus* 6538p, *E. faecalis* 29212A, *E. coli* 3190Y and *P. aeruginosa* 1912E. The activities of LB20304 were minimally affected by acid conditions, with a 2- to 4-fold increase in the MICs at pH 5.5. The effects of serum and urine on the MICs for representative *S. aureus, E. coli,* and *P. aeruginosa* are shown in Table V. The activities of LB20304 (MIC and MBC) were hardly affected by the presence of 50% human serum, mouse serum, guinea pig serum, or horse serum in Muller-Hinton broth. The activity of LB20304 was slightly affected by the presence of human urine at pH 7.4. However, there were 8- to 64-fold increases in the MICs and MBCs of LB20304 when human urine at pH 5.5 was added in broth.

Table VI. Frequency of mutants resistant to LB20304, ciprofloxacin and sparfloxacin

Strains	Drugs	MIC (μg/ml)	Mutation frequency*
E. coli 3190Y	LB20304	0.008	1.2×10^{-9}
	Ciprofloxacin	0.13	3.0×10^{-8}
	Sparfloxacin	0.063	1.2×10^{-9}
P. aeruginosa 1912E	LB20304	0.008	5.6×10^{-8}
	Ciprofloxacin	0.008	3.3×10^{-8}
	Sparfloxacin	0.016	2.5×10^{-8}
S. aureus 6538p	LB20304	0.25	1.5×10^{-10}
	Ciprofloxacin	0.13	2.1×10^{-10}
	Sparfloxacin	1	6.2×10^{-9}

*Mutants were selected at 4×MIC concentration

Mutation frequency of resistance

Table VI shows the frequency of resistant cells to LB 20304, ciprofloxacin and sparfloxacin. The frequencies of spontaneous mutants resistant to LB20304

in *E. coli*, *P. aeruginosa* and *S. aureus* were 1.2×10^{-9}, 5.6×10^{-8} and 1.5×10^{-10}, respectively. LB20304 induced mutant cells less than ciprofloxacin in *E. coli* and *S. aureus*. On the other hand, LB20304 induced more resistant cells than ciprofloxacin in *P. aeruginosa*.

DISCUSSION

LB20304 is a new fluoroquinolone which has shown a potent activity against gram-positive, gram-negative and anaerobic bacteria *in vitro* and *in vivo*, and improved pharmacokinetic profiles in animals (Oh *et al*, 1995).

This study showed that inoculum size and components of media had little effect on the activity of LB 20304 against both gram-positive and gram-negative bacteria. The acidification of the growth medium decreased the activity of LB20304, as the activities of many fluoroquinolones were influenced greatly by pH (Chin *et al*, 1994; Marshall *et al*, 1993). And the activity of LB20304 was greatly affected by high concentration of Mg^{++} in media. It has been reported that the decreased activity of quinolones in the presence of divalent cations might be accounted for by one of two mechanisms: (i) the quinolone forms a complex with the magnesium and is then too bulky to enter the cell via the porins; or (ii) the divalent cations bind to polyphosphates in the LPS, stabilizing the complex and preventing subsequent damage by the quinolone (Marshall *et al*, 1994). But the significience of the effect of Mg^{++} on the use of these drug in clinical practice is not clear. Although there were decreases in the activity of LB20304 in presence of acidic urine and high concentration of Mg^{++}, the MICs of LB20304 against most of the clinical isolates were within the range of concentrations attainable in urine because LB20304 was excreted through urine at high concentration. The frequencies of spontaneous mutants resistant to LB20304 in *S. aureus*, *E. coli* and *P. aeruginosa*, were similar to or slightly lower than those found in ciprofloxacin and sparfloxacin.

In view of its improved antibacterial activity compared with currently available quinolones, further pharmacological and clinical studies would be necessary to establish the clinical usefulness of this compound.

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