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The effects of increasing levels of quinolone resistance on in-vitro activity of four quinolones

Kenneth S. Thomson* and Christine C. Sanders

Center for Research in Anti-Infectives & Biotechnology, Department of Medical Microbiology and Immunology, Creighton University School of Medicine, Omaha, NB 68178, USA

A panel of 266 clinically isolated Gram-positive cocci and Gram-negative bacilli with varying levels of resistance to ciprofloxacin were analysed for susceptibility to Du-6859a, ciprofloxacin, ofloxacin, temafloxacin and nalidixic acid. Staphylococci were divided into ciprofloxacin-susceptible, moderately resistant and highly resistant subgroups. Du-6859a was the most potent quinolone against all taxa. As ciprofloxacin resistance increased to high levels, MICs of all quinolones increased but Du-6859a MICs increased least, and ciprofloxacin MICs increased most. Less susceptible single-step mutants were selected from 80% of 15 representative clinical isolates exposed to ciprofloxacin, 71% of isolates exposed to temafloxacin, 67% of isolates exposed to Du-6859a and 53% of isolates exposed to ofloxacin. Du-6859a inhibited more mutants (67%) at a concentration of 1 mg/L than did the other quinolones (26–43%) at their susceptible breakpoints. Du-6859a was the most rapidly bactericidal quinolone in time–kill studies with *Enterococcus faecalis* and *Enterococcus faecium*. This study indicated that Du-6859a is more potent than the comparator quinolones, is less affected by the mechanisms responsible for high-level quinolone resistance and may be less likely to select resistant mutants if it has a susceptible breakpoint of 1 mg/L.

Introduction

Increasing bacterial resistance to currently available quinolones has reduced their effectiveness and may compromise future use of this important class of antibacterial drugs. Of various proposals to limit the spread of quinolone resistance, the only factor that has been examined in detail is drug potency.[1] Spontaneous mutations that reduce quinolone susceptibility usually result in only modest increases in MIC (two- to eight-fold) and occur at low frequency.[2–4] Therefore, the bacteria that are most likely to develop quinolone resistance in a single mutational step are those for which fluoroquinolone MICs are high in the susceptible range, i.e. eight-fold or less below the susceptible breakpoint. This implies that the likelihood of resistance emerging can be reduced by use of fluoroquinolones of potency sufficient to inhibit both the pathogen infecting a patient and the less susceptible single-step mutant which may also be present or may emerge during therapy.[5,6] For this reason, a study was designed to compare the activities of four quinolones against clinical and laboratory isolates that possess varying levels of resistance to ciprofloxacin and also to investigate the potential of the quinolones to select resistant mutants.

Materials and methods

Strains

Two hundred and sixty-six strains of Gram-positive and Gram-negative bacteria with different levels of resistance to ciprofloxacin were recovered from patients throughout the USA, Spain, Sweden, the UK and Australia. The clinical strains were chosen for their different levels of resistance to ciprofloxacin. They were not random clinical isolates. An additional isogenic panel of strains of *Escherichia coli* was tested to determine the effects of specific mutations on the activity of the study quinolones.

* Tel: +1-402-280-1881; Fax: +1-402-280-1225.

Susceptibilities

Antibiotic susceptibilities were determined by agar dilution testing with Mueller–Hinton agar and an inoculum of 10^4 cfu per spot.[7] This medium was supplemented with 4% NaCl when staphylococci were tested against oxacillin, and with 5% defibrinated sheep blood when *Streptococcus pneumoniae* was tested. Tests with *S. pneumoniae* were incubated in 10% CO_2 in air. Antibiotic solutions were prepared on the day of use from the following reference powders: Du-6859a (Daiichi Pharmaceutical Co., Ltd, Tokyo, Japan), temafloxacin (Abbott Laboratories, Chicago, IL, USA), ofloxacin (R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ, USA), ciprofloxacin (Bayer, Inc., West Haven, CT, USA), and nalidixic acid (Sigma Chemical Co., St Louis, MO, USA).

The staphylococci were separated into three phenotypic groups on the basis of their susceptibility to ciprofloxacin: susceptible (MIC $\leqslant$ 0.25 mg/L), moderately resistant (MIC = 0.5–4 mg/L), and highly resistant (MIC $\geqslant$ 8 mg/L).

Mutational frequencies

Fifteen strains of *Staphylococcus aureus*, coagulase-negative staphylococci, *S. pneumoniae*, *Enterococcus faecalis*, *Enterococcus faecium*, *E. coli*, *Serratia marcescens*, *Acinetobacter* sp., *Stenotrophomonas* (previously *Xantho - monas*) *maltophilia*, *Burkholderia* (previously *Pseudo - monas*) *cepacia* and *Pseudomonas aeruginosa* were grown in Mueller–Hinton broth or Todd–Hewitt broth (*S. pneumoniae* only) until the mid-logarithmic phase. Inocula of 10^7–10^9 cfu were added to Mueller–Hinton agar or Todd–Hewitt agar (*S. pneumoniae*) containing super-inhibitory concentrations (2–16 times the MIC) of antibiotic. The actual inoculum used was determined by agar dilution plate counts. After 48–72 h of incubation at 35°C in air (with 10% CO_2 added for *S. pneumoniae*), mutational frequencies were calculated from the results obtained for plates containing the highest drug concentration on which colonies were obtained. The colonies selected by this procedure were tested for their antibiotic susceptibilities as described above.

Time–kill studies

The bactericidal activity of Du-6859a, ofloxacin and ciprofloxacin against *E. faecalis* 103 and *E. faecium* 129 was investigated in time–kill experiments. Stationary phase cultures were exposed to each quinolone at multiples of 0.5, 1 and 4 $\times$ the MIC, and subcultured for viable counts at 0, 2, 4, 6 and 24 h on to Mueller–Hinton agar containing 5 mM $FeCl_3$ to overcome the effect of drug carryover. The viable counts were compared with those of drug-free control cultures.

Results

In-vitro activity

Du-6859a was the most potent quinolone overall (Table I). It was at least four times more potent than each of the other quinolones against staphylococci, enterococci, pneumococci, members of the family Enterobacteriaceae, *Acinetobacter* spp., *S. maltophilia*, *Chrysobacterium* spp. and *B. cepacia*. Ciprofloxacin was similar to Du-6859a in potency against ciprofloxacin-susceptible and intermediate isolates of *P. aeruginosa* (ciprofloxacin MICs $\leqslant$ 2 mg/L), but four times less potent against the most resistant isolates. The numbers of isolates requiring >1 mg/L of Du-6859a or ciprofloxacin, or >2 mg/L of ofloxacin or temafloxacin for inhibition are shown in Table II. The percent of isolates inhibited by these concentrations was 91% for Du-6859a, 70% for temafloxacin, 63% for ofloxacin and 55% for ciprofloxacin. Resistance to 1 mg/L of Du-6859a occurred in *P. aeruginosa* (eight isolates), *S. aureus* (six isolates), coagulase-negative staphylococci (two isolates), *Acinetobacter* spp. (five isolates), *E. coli* (two isolates) and *E. faecium* (one isolate).

Studies with the isogenic panel of strains of *E. coli* (Table III) indicated that the activity of Du-6859a was not reduced by specific mutations in gyrase subunit A (*nalA*), gyrase subunit B (*nalC* and *nalD*), or a purported impermeability mutation (*nalB*). In contrast, four-fold or greater increases in MICs of the other quinolones were associated with *nalA* (all comparison drugs), *nalB* and *nalC* (nalidixic acid) and *nalD* (ofloxacin, ciprofloxacin and nalidixic acid).

Effect of ciprofloxacin resistance on potency

Changes in the potency of the quinolones relative to each other were detected by comparing differences between their MIC_{50}s and MIC_{90}s in Table I. In this analysis, the MIC_{90}s of Du-6859a increased least, indicating that it was the quinolone least affected by the mechanisms responsible for high-level resistance, while ciprofloxacin was the agent most affected. The relative potencies of the quinolones against organisms with varying levels of quinolone resistance were examined in more detail with staphylococci. Analysis of the cumulative susceptibilities of three groups of staphylococci, susceptible, moderately resistant and highly resistant strains, showed that against ciprofloxacin-susceptible (MIC $\leqslant$ 0.25 mg/L) isolates of *S. aureus* (Figure 1a), Du-6859a was four to eight times more potent than temafloxacin, and eight to 16 times more potent than ofloxacin or ciprofloxacin. However, against highly resistant isolates (ciprofloxacin MIC $\geqslant$ 8 mg/L) Du-6859a was 32 times more potent than temafloxacin, 32–64 times more potent than ofloxacin, and 64–128 times more potent than ciprofloxacin (Figure 1c). This indicated that resistance increased less to Du-6859a than to the

Table I. Comparative in-vitro activities of quinolones against 266 clinical isolates

Organism	No. of strains	Quinolone	MIC (mg/L) range	50%	90%
S. aureus	54	Du-6859a	0.007–8	0.03	2
		ofloxacin	0.12–64	0.5	32
		temafloxacin	0.06–64	0.25	16
		ciprofloxacin	0.12–>128	0.5	64
Coagulase-negative staphylococci	53	Du-6859a	0.007–2	0.06	1
		ofloxacin	0.06–128	1	64
		temafloxacin	0.06–128	1	64
		ciprofloxacin	0.03–>128	2	128
Enterococcus spp.	30	Du-6859a	0.03–4	0.25	0.25
		ofloxacin	1–128	4	8
		temafloxacin	0.5–128	2	8
		ciprofloxacin	0.25–>128	2	4
S. pneumoniae	21	Du-6859a	0.03–0.25	0.06	0.12
		ofloxacin	2–4	2	4
		temafloxacin	0.5–2	1	2
		ciprofloxacin	0.5–8	1	2
E. coli	18	Du-6859a	0.004–4	0.007	4
		ofloxacin	0.015–128	0.06	64
		temafloxacin	0.007–128	0.03	64
		ciprofloxacin	0.002–128	0.007	64
		nalidixic acid	2–>128	8	>128
Citrobacter freundii (9)	29	Du-6859a	0.007–1	0.03	0.5
Enterobacter cloacae (5)		ofloxacin	0.03–16	0.25	8
Klebsiella pneumoniae (9)		temafloxacin	0.015–32	0.5	4
S. marcescens (6)		ciprofloxacin	0.002–8	0.03	2
		nalidixic acid	2–>128	8	>128
Chrysobacterium spp. (6)	12	Du-6859a	0.03–0.25	0.06	0.12
B. cepacia (6)		ofloxacin	2–4	2	4
		temafloxacin	0.5–2	1	2
		ciprofloxacin	0.5–8	1	2
		nalidixic acid	8–128	16	64
P. aeruginosa	20	Du-6859a	0.03–8	0.5	4
		ofloxacin	0.5–>128	4	64
		temafloxacin	0.5–128	4	64
		ciprofloxacin	0.06–32	0.5	16
S. maltophilia	16	Du-6859a	0.015–0.5	0.12	0.25
		ofloxacin	0.25–8	1	4
		temafloxacin	0.25–8	1	4
		ciprofloxacin	0.25–16	2	16
Acinetobacter spp.	13	Du-6859a	0.004–2	0.5	2
		ofloxacin	0.03–32	4	16
		temafloxacin	0.03–64	2	64
		ciprofloxacin	0.015–128	8	128
		nalidixic acid	4–>128	128	>128

Table II. Numbers of clinical isolates requiring quinolone concentration of >1 mg/L of Du-6859a or the susceptible breakpoint[a] for inhibition

Organism	No. of strains tested	No. of isolates resistant to 1 mg/L of Du-6859a or the susceptible breakpoint concentration[a] of:			
		Du-6859a	ofloxacin	temafloxacin	ciprofloxacin
S. aureus	54	6	16	16	21
Coagulase-negative staphylococci	53	2	24	24	28
Enterococcus spp.	30	1	18	11	19
E. coli	21	0	9	0	9
E. cloacae	5	0	0	0	0
K. pneumoniae	9	0	1	0	1
C. freundii	9	9	2	2	1
S. marcescens	6	0	2	2	2
P. aeruginosa	20	7	1	11	8
Acinetobacter spp.	13	5	7	6	8
S. maltophilia	16	0	3	3	14
Chrysobacterium spp.	6	0	0	0	4
B. cepacia	6	0	1	1	2
Total	266	23	98	80	121

[a]2 mg/L of ofloxacin, temafloxacin; 1 mg/L of ciprofloxacin.

Table III. Influence of specific mutations on activity of DU-6859a and comparator quinolones

Strain	Genotype	MIC (mg/L)				
		DU-6859a	ofloxacin	temafloxacin	ciprofloxacin	nalidixic acid
AFE-1	wild type	0.015	0.06	0.03	0.007	4
AFE-7	*nalA* mutant	0.03	0.5	0.25	0.12	>128
AFE-12	*nalB* mutant	0.015	0.06	0.06	0.007	16
AFE-17	*nalC* mutant	⩽0.0005	0.007	0.004	0.007	64
AFE-22	*nalD* mutant	0.015	0.25	0.06	0.03	64

comparator quinolones. A similar trend was detected with coagulase-negative staphylococci (data not shown), with Du-6859a again being the most active quinolone and the least affected by increasing quinolone resistance.

Mutational frequencies

The ability of the quinolones to select less susceptible single-step mutants was determined by exposing 15 isolates to superinhibitory concentrations (two to eight times the MIC) of each quinolone. Forty-two of the mutants were Gram-positive and 26 were Gram-negative. Mutants at least four times less susceptible than the parental strain to at least one quinolone were selected from 80% of the strains exposed to ciprofloxacin, 71% of strains exposed to temafloxacin, 67% of strains exposed to Du-6859a, 53% of strains exposed to ofloxacin and each of the four strains exposed to nalidixic acid (Table IV). Mutational frequencies of 10^{-8}–10^{-6} were obtained for 57 (84%) of the 68 single-step mutants selected by these quinolones.

Of the 68 mutants, the percentages of mutants inhibited by 1 mg/L of Du-6859a or the susceptible breakpoint of the other quinolones were: Du-6859a, 67%; ofloxacin, 43%; temafloxacin, 35%; and ciprofloxacin, 26%. None of the mutants was inhibited by <16 mg/L of nalidixic acid.

Less susceptible mutants of *S. pneumoniae* 211 could only be selected with ciprofloxacin, and then only at 2 × MIC (frequency 10^{-6}). Exposure to superinhibitory concentrations of the other quinolones did not yield less susceptible mutants.

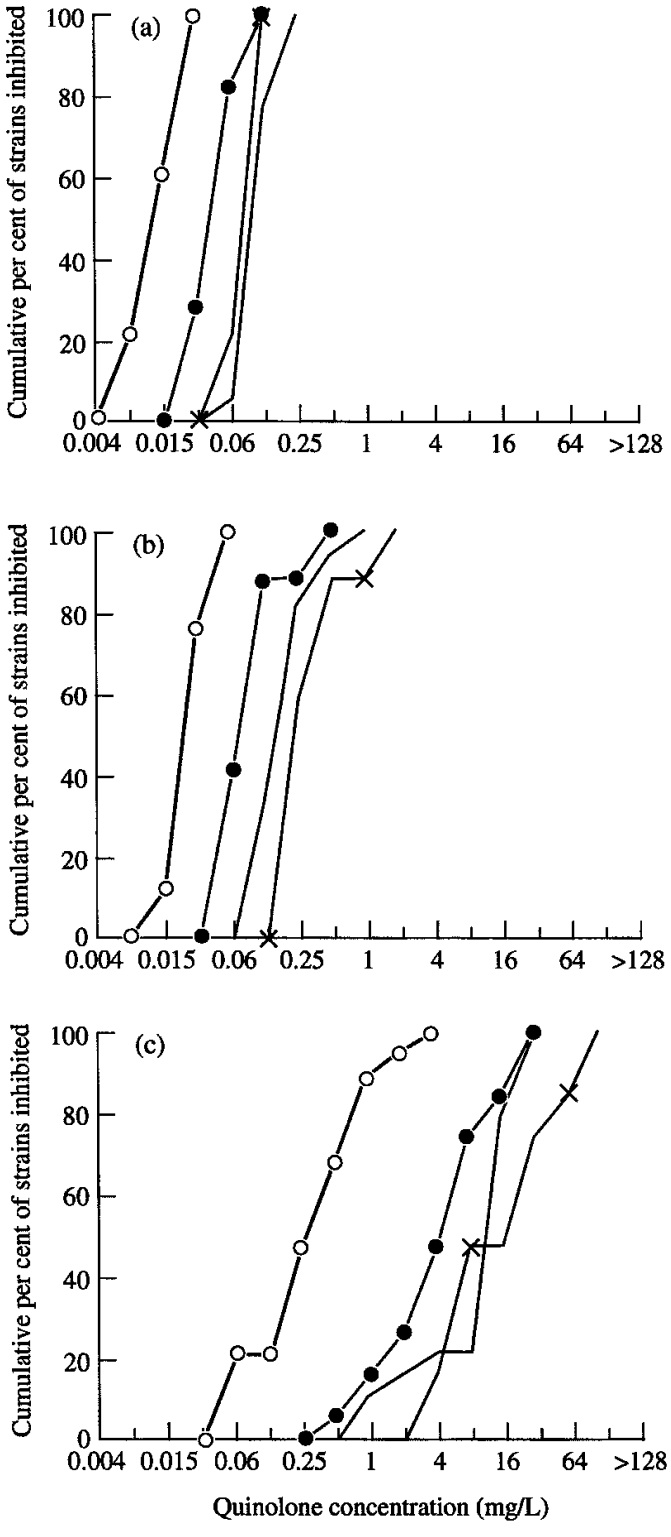

Figure 1. Comparative activity of DU-6859a (○), ofloxacin (—), temafloxacin (●) and ciprofloxacin (×) against 54 isolates of *S. aureus* grouped by their ciprofloxacin susceptibility. Ciprofloxacin susceptibilities and numbers of strains tested were as follows: (a) MIC ≤ 0.25 mg/L (n = 18); (b) MIC = 0.5–4 mg/L (n = 17); (c) MIC ≥ 8 mg/L (n = 19).

Mutational changes in antibiotic susceptibility

The majority of the 68 mutants exhibited only modest increases (up to eight-fold) in their quinolone MICs when compared with the parental strains (see below). However, larger increases in some quinolone MICs (≥16-fold) were detected in some of the mutants derived from parental strains of *S. aureus, Staphylococcus epidermidis, Staphylococcus lugdunensis, E. coli, S. marcescens, P. aeruginosa, Acinetobacter* sp. *B. cepacia* and *S. maltophilia*. The only species from which no mutants exhibited such high-level increases in MICs were *E. faecalis* and *S. pneumoniae.* These large increases in quinolone MIC occurred most frequently with nalidixic acid (52% of all mutants exhibited ≥16-fold increased in MIC) and less frequently with the other quinolones: ciprofloxacin (37%), temafloxacin (23%), ofloxacin (19%) and Du-6859a (16%). When the drugs that selected this subset of mutants were examined, nalidixic acid selected the highest percentage of these mutants (35%). The percentages for the other quinolones were ciprofloxacin, 22%; temafloxacin, 20%; Du-6859a, 16%; and ofloxacin, 6%. Representative parents and mutants exhibiting large increases in at least one quinolone MIC are shown in Table V.

Bactericidal activity against enterococci

The results of the time–kill experiments with *E. faecalis* 103 and *E. faecium* 129 are shown in Figures 2 and 3, respectively. Du-6859a, ofloxacin and ciprofloxacin were slowly bactericidal (≥3 $\log_{10}$ decrease in viable count) only at 4 × MIC. These concentrations were at or above resistant breakpoints of ofloxacin (8 mg/L) and ciprofloxacin (4 and 8 mg/L). For Du-6859a, 0.5 mg/L was bactericidal for both strains, with approximately 14 h exposure required for the detection of bactericidal activity against *E. faecalis* 103 (Figure 2) and 6 h exposure required for *E. faecium* 129 (Figure 3). These results indicated that Du-6859a was the most rapidly bactericidal quinolone.

At the MIC of each agent, less susceptible mutants were selected from *E. faecalis* 129. In tests with *E. faecium* at the MIC of each drug, only Du-6859a reduced the viable count during the first 6 h, but after this period outgrowth of a less susceptible mutant was detected. In contrast ofloxacin and ciprofloxacin were bacteriostatic for 6 h at the MIC, after which viable counts increased.

Discussion

Recent uncritical use of quinolones in the USA and other countries has resulted in the emergence of quinolone resistance at rates greater than originally anticipated.[6,8–11] Therefore, when new quinolones are evaluated it is important to take into account both activity against isolates that are resistant to currently available quino-

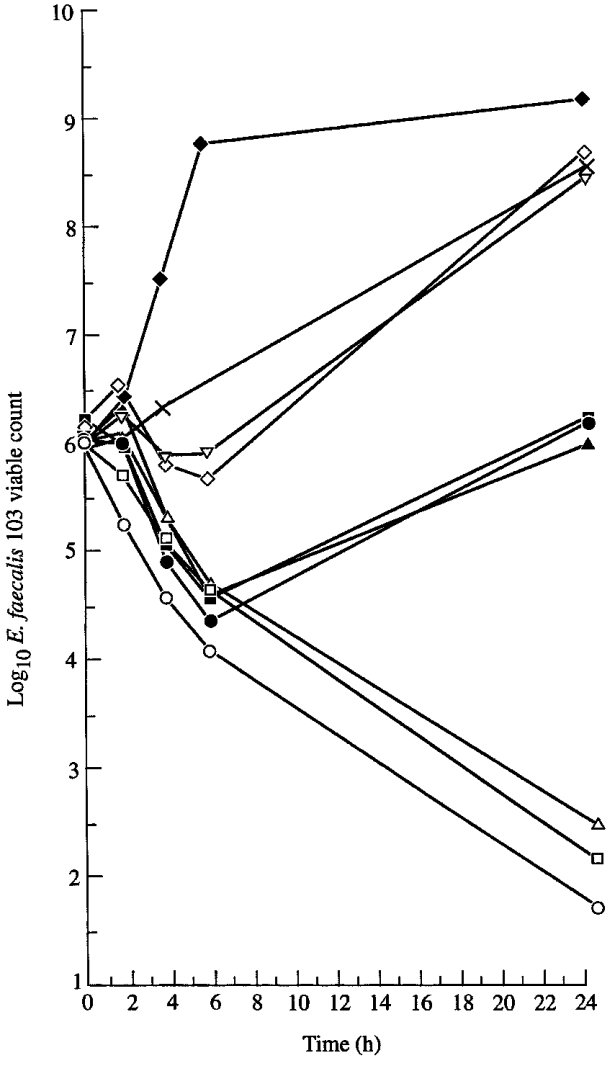

Figure 2. Time–kill study results for activities of Du-6859a, ciprofloxacin and ofloxacin against *E. faecalis* 103. ◆, control; ▽, Du-6859a at 0.5 × MIC; ●, Du-6859a at 1 × MIC; ○, Du-6859a at 4 × MIC; ◇, ciprofloxacin at 0.5 × MIC; ▲, ciprofloxacin at 1 × MIC; △, ciprofloxacin at 4 × MIC; ×, ofloxacin at 0.5 × MIC; ■, ofloxacin at MIC; □, ofloxacin at 4 × MIC.

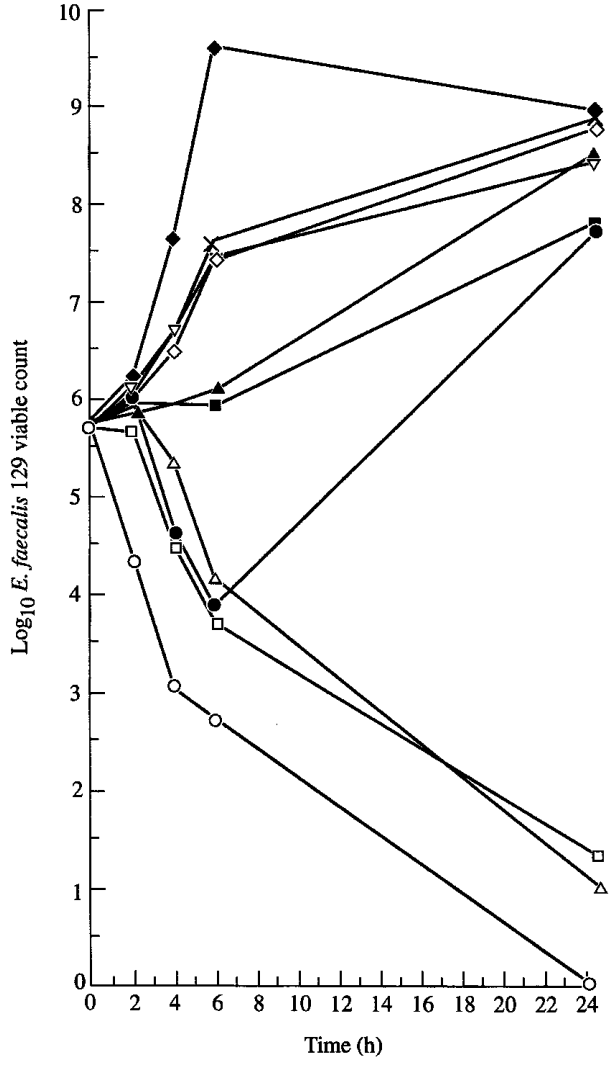

Figure 3. Time–kill study results for activities of Du-6859a, ciprofloxacin and ofloxacin against *E. faecium* 129 (symbols as in Figure 2).

lones, and the potential for susceptible isolates to develop resistance.

In this study Du-6859a was more potent than the comparator quinolones against all groups of bacteria tested, and was least affected by the mechanisms of quinolone resistance encountered. This finding was consistent with the data of Nakane *et al*.,[12] but contrasted with the report of Marshall & Jones,[13] who observed that Du-6859a and ciprofloxacin were comparable in activity against recent clinical isolates from the family Enterobacteriaceae. This discrepancy may have occurred because the current study, and that of Nakane *et al*., contained more isolates with high-level ciprofloxacin resistance, whereas the Marshall & Jones study contained only few isolates with low-level ciprofloxacin resistance, and none with high-level resistance.

In time–kill studies, Du-6859a was the most rapidly bactericidal quinolone against the two enterococcal strains, but none of the quinolones exhibited the rapidly lethal activity that is characteristic of Du-6859a and other quinolones against more susceptible bacteria.[14] None of the study quinolones may have been sufficiently bactericidal for monotherapy in serious infections caused by *E. faecalis* or *E. faecium*.

In the in-vitro mutational studies Du-6859a inhibited more mutants at a concentration of 1 mg/L than did the comparator quinolones at their susceptible breakpoints. Du-6859a was also the quinolone to which the lowest percentage of mutants exhibited large (≥16-fold) MIC increases.

These features suggest that if serum and body fluid concentrations >1 mg/L can be achieved safely, Du-6859a

Table IV. Single-step mutants selected by DU-6859a, ofloxacin, temafloxacin, ciprofloxacin and nalidixic acid

Mutational frequency[a]	Multiple of MIC	No of mutants selected by					
		DU-6859a	ofloxacin	temafloxacin	ciprofloxacin	nalidixic acid	total
10^{-5}	2	3	1	1	3		8
	4	1			1		2
	8						0
10^{-6}	2	2		2	2	2	8
	4		1	1	2		4
	8	1			1		2
10^{-7}	2	1	3	1	1	2	8
	4	1	1	1	4	1	8
	8	3	4	2	3	1	13
10^{-8}	2		1				1
	4	3	1	2	1	1	8
	8	2			3		5
10^{-9}	2						0
	4						0
	8					1	1
No. of mutants selected from:							
Gram-positive parents		10	9	10	13	0	42
Gram-negative parents		7	3	0	8	8	26
total[b]		17	12	10	21[c]	8	68
No. tested[d]		15	15	6	15	4	

[a]Mutational frequencies were calculated from the results obtained for plates containing the highest drug concentration on which colonies were obtained.
[b]Totals are the total numbers of mutants selected.
[c]*S. epidermidis* 98 yielded two distinctly different mutants when it was exposed to ciprofloxacin.
[d]Three parental strains of *S. aureus*, two parental strains of *S. epidermidis*, and single parental strains of *Acinetobacter* sp., *B. cepacia*, *E. coli*, *P. aeruginosa*, *S. marcescens*, *S. maltophilia*, *E. faecium*, *E. faecalis*, *S. lugdunensis* and *S. pneumoniae* were tested. Eleven strains with high-level nalidixic acid resistance were not tested with nalidixic acid and nine strains were not tested with temafloxacin after its withdrawal by the manufacturer.

may be more likely than currently available quinolones to retain activity against populations of bacteria that contain less susceptible strains due to previous exposure to quinolones.

Acknowledgements

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Table V. Representative parents and mutants showing high-level increases in quinolone MICs

Organism	Parent or mutant	Selecting drug multiples of MIC at which mutants were selected[a]	Mutational frequency	MIC (mg/L) (fold increase in MIC)				
				DU-6859a	ofloxacin	temafloxacin	ciprofloxacin	nalidixic acid
S. lugdunensis 103	parent	–[b]	–	0.015	0.25	0.25	0.12	–[c]
	mutant	ciprofloxacin 4×	10^{-7}	0.06 (8)	2 (8)	2 (8)	2 (16)	–
	mutant	ciprofloxacin 8×	10^{-7}	0.06 (8)	2 (8)	2 (8)	2 (16)	–
E. coli GB 13	parent	–	–	0.12	0.5	0.5	0.12	16
	mutant	ciprofloxacin 8×	10^{-8}	0.5 (4)	16 (32)	4 (8)	4 (32)	>512 (≥64)
	mutant	nalidixic acid 2×	10^{-7}	0.5 (4)	8 (16)	4 (8)	2 (16)	>512 (≥64)
S. marcescens 69	parent	–	–	0.12	0.5	0.5	0.25	4
	mutant	DU-6859a 8×	10^{-7}	1 (8)	4 (8)	16 (32)	4 (16)	64 (16)
	mutant	ciprofloxacin 4×	10^{-7}	0.5 (4)	4(8)	8 (16)	2 (8)	32 (8)
P. aeruginosa 142	parent	–	–	0.5	4	4	2	>512
	mutant	DU-6859a 2×	10^{-7}	2 (4)	>32 (≥16)	>32 (≥16)	16 (8)	>512
	mutant	ciprofloxacin 2×	10^{-6}	2 (4)	>32 (≥16)	>32 (≥16)	16 (8)	>512
S. maltophilia GM 79	parent	–	–	0.007	2	–[d]	2	16
	mutant	DU-6859a 4×	10^{-7}	0.25 (32)	16 (8)	–	32 (16)	512 (32)
	mutant	nalidixic acid 8×	10^{-7}	0.12 (16)	32 (32)	–	32 (16)	256 (16)

[a]Multiple at which mutants were selected. All parental strains were exposed to each quinolone at 2, 4 and 8 × the MIC.
[b]parental strains, i.e. history not relevant.
[c]nalidixic acid not tested because parental strain was resistant.
[d]temafloxacin not tested because drug was withdrawn by manufacturer.

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