ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1995, p. 1522–1525 Vol. 39, No. 7
0066-4804/95/$04.00+0

Improved Bactericidal Activity of Q-35 against Quinolone-Resistant Staphylococci

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Received 30 January 1995/Returned for modification 11 April 1995/Accepted 7 May 1995

The bactericidal effects of Q-35, sparfloxacin, tosufloxacin, and ofloxacin on 18 strains of methicillin-resistant *Staphylococcus aureus* (MRSA) and 3 strains of *Staphylococcus epidermidis* were studied by a viable-count method. Staphylococci as used in this study were clearly divided into two groups with respect to their susceptibilities to sparfloxacin. MICs of Q-35 and tosufloxacin were 0.05 to 0.78 μg/ml for sparfloxacin-susceptible strains (MICs, 0.05 to 0.2 μg/ml) and 1.56 to 12.5 μg/ml for sparfloxacin-resistant strains (6.25 to 25 μg/ml). All the sparfloxacin-resistant strains of MRSA tested contained the *gyrA* mutation at codon 84. Time-kill studies showed that Q-35 decreased the viable counts from ≈10^7 CFU/ml to 10^3 to 10^5 CFU/ml within 3 h at concentrations greater than the MICs against both sparfloxacin-susceptible and -resistant strains. In contrast, sparfloxacin, tosufloxacin, and ofloxacin produced bacteriostatic effects at 3 h after exposure against sparfloxacin-resistant strains at concentrations which were greater than the respective MICs, whereas these quinolones were bactericidal against sparfloxacin-susceptible strains. The rapid bactericidal activities of Q-35 against sparfloxacin-resistant MRSA were reduced when the methoxy group of Q-35 at the 8 position was substituted with fluorine or hydrogen. Thus, our data suggest that the introduction of a methoxy group into the 8 position of quinolones contributes to the bactericidal activities of fluoroquinolones against quinolone-resistant staphylococci.

Staphylococci have been recognized as important pathogens. These organisms can be resistant to multiple antimicrobial agents, which severely limits therapeutic options in selected instances. Development of the fluoroquinolone class of antimicrobial agents provided an effective option for the therapy of serious infections caused by multidrug-resistant staphylococci, such as methicillin-resistant *Staphylococcus aureus* (MRSA). However, subsequent to increased use of fluoroquinolones, the emergence of fluoroquinolone-resistant strains of *S. aureus* and *Staphylococcus epidermidis* has been reported with growing frequency (12, 13). Tosufloxacin (4) and sparfloxacin (8), which were originally designed to improve activity against gram-positive bacteria, also exhibited better antibacterial activity than some comparative agents against fluoroquinolone-resistant staphylococci.

Possible mechanisms of fluoroquinolone resistance in staphylococci have been elucidated (10, 11, 14, 16, 17). One of these mechanisms occurs via a mutational alteration of DNA gyrase (10, 11). DNA gyrase is a tetrameric protein consisting of two A and two B subunits, encoded by the *gyrA* and *gyrB* genes, respectively. High levels of quinolone resistance in *S. aureus* have therefore been attributed to a point mutation of *gyrA* (14). Alternatively, reduced drug accumulation has been associated with resistance and occurs via alterations in the *norA* gene which encodes an efflux pump (17). This mechanism, however, confers resistance lower than that observed with *gyrA* mutations (5). Furthermore, resistance to fluoroquinolones can be mediated by alterations to the *flq* locus (16). This locus alteration also confers low-level resistance, but the exact mechanism by which it does so has as yet not been fully evaluated. Recently, however, Ferrero et al. (3) reported the finding of the topoisomerase IV gene in *S. aureus* and implied that it is located at the *flq* locus.

We are currently developing a broad-spectrum antibacterial agent, namely, Q-35 (balofloxacin), which is highly active against gram-positive bacteria (7). Although tosufloxacin and sparfloxacin are more active than Q-35 against fluoroquinolone-susceptible staphylococci, the opposite is true for resistant strains, against which Q-35 has greater potency than these fluoroquinolones (7). Thus, Q-35 may exhibit good clinical effects against infectious diseases caused by fluoroquinolone-resistant staphylococci. In this study, we investigated the bactericidal activity of Q-35, ofloxacin, tosufloxacin, and sparfloxacin against staphylococci and we described the difference in bactericidal activity between Q-35 and other fluoroquinolones against fluoroquinolone-resistant staphylococci.

MATERIALS AND METHODS

Antimicrobial agents. Q-35, 8-H Q-35 (an analog with a change from the 8-methoxy group to hydrogen), 8-F Q-35 (8-methoxy group changed to fluorine), and 7-4 Q-35 [7-(3-methylaminopiperidine) changed to 7-(4-methylaminopiperidine)] were synthesized as a free base at the Research Foundation of Chugai Pharmaceutical Co. Ltd. (6). Ofloxacin (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan), ciprofloxacin chloride (Bayer Pharmaceutical Co. Ltd., Osaka, Japan), tosufloxacin (Toyama Chemistry Co., Ltd., Tokyo, Japan), and sparfloxacin (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) were used as reference quinolones.

Q-35, Q-35 analogs, and the reference quinolones except ciprofloxacin chloride were initially dissolved in 0.1 N NaOH, diluted with water, and neutralized with 0.1 N HCl. Ciprofloxacin chloride was dissolved in water.

Bacterial strains. Bacterial strains used in this study were recent clinical isolates collected from various hospitals in Japan between 1990 and 1993. All *S. aureus* strains were resistant to methicillin.

Determination of MICs. MICs were determined by the twofold agar dilution method recommended by the Japan Society of Chemotherapy (2), with Mueller-Hinton (MH) agar (Difco Laboratories, Detroit, Mich.). The overnight broth cultures of the bacterial strains were diluted with broth corresponding to a final concentration of about 10^6 CFU/ml. Five microliters of each bacterial suspension was applied with an inoculator (Microplanter; Sakura Seisakusho, Tokyo, Japan)

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TABLE 1. Mutations in the *gyrA* gene and antibacterial activities of Q-35 and reference quinolones against MRSA and *S. epidermidis*

Strain	*gyrA*[a] mutation	MIC (μg/ml)[b]				
		Q-35	OFLX	CPFX	TFLX	SPFX
MRSA ATJ-8	−	0.39	3.13	12.5	0.39	0.05
MRSA ATJ-17	−	0.39	3.13	12.5	0.78	0.05
MRSA ATJ-29	−	0.78	3.13	12.5	0.78	0.05
MRSA ATJ-34	−	0.05	0.39	0.39	0.05	0.05
MRSA QA 389	−	0.2	0.78	3.13	0.2	0.05
MRSA ATJ-2	−	0.78	3.13	12.5	0.78	0.2
MRSA ATJ-10	+	3.13	25	100	12.5	6.25
MRSA ATJ-18	+	6.25	25	100	6.25	6.25
MRSA ATJ-26	+	6.25	25	100	6.25	6.25
MRSA ATJ-27	+	1.56	6.25	12.5	6.25	6.25
MRSA ATJ-43	+	1.56	12.5	12.5	3.13	6.25
MRSA QA 266-1	+	6.25	25	100	12.5	6.25
MRSA ATJ-4	+	3.13	25	50	6.25	12.5
MRSA ATJ-22	+	6.25	50	>100	3.13	12.5
MRSA ATJ-38	+	6.25	25	>100	3.13	12.5
MRSA ATJ-42	+	6.25	50	100	3.13	12.5
MRSA QA 217	+	3.13	25	100	12.5	12.5
MRSA ATJ-49	+	12.5	50	>100	6.25	25
S. epidermidis QA 184-2	NT	0.2	0.78	0.39	0.2	0.1
S. epidermidis QA 129	NT	1.56	12.5	50	12.5	6.25
S. epidermidis QA 244-1	NT	6.25	100	12.5	12.5	12.5

[a] Agarose gel analysis of PCR-amplified DNA digested with *Hin*fI. −, *Hin*fI site present; +, *Hin*fI site absent; NT, not tested.

[b] OFLX, ofloxacin; CPFX, ciprofloxacin; TFLX, tosufloxacin; SPFX, sparfloxacin.

onto an agar plate containing twofold serial dilutions of quinolones, delivering a final inoculum of 5×10^3 CFU per spot.

Detection of *gyrA* mutation in *S. aureus*. Restriction fragment length polymorphism analysis of PCR-amplified DNA was performed by the method of Tokue et al. (15). In brief, the 124-bp DNA fragment of the *gyrA* sequence was amplified by PCR. Two oligonucleotide primers were synthesized for PCR: 5′-ATGAA CAAGGTATGACACCGG-3′ (complementary to positions 87 to 67 bp upstream of the *Hin*fI site [5]) and 5′-CGTTACCATGCATACCGAGT-3′ (complementary to positions 18 to 37 bp downstream of the *Hin*fI site). The PCR products were digested with *Hin*fI, and subsequent cleavage of the PCR product was evaluated by a combination of electrophoresis through a 3.5% agarose gel (NuSieve GTG; FMC BioProducts, Rockland, Maine) and ethidium bromide staining. The absence of the *Hin*fI site indicated the mutation TCA→TTA at codon 84. The *gyrA* of *S. epidermidis* was not amplified with primers used in this study.

Bactericidal activity. Each strain was grown in MH broth (Difco) for 20 h. The viable count of each individual strain was then adjusted to approximately 10^7 CFU/ml in MH broth. Nine milliliters of each of the bacterial suspensions was individually transferred into test tubes (diameter, 30 mm), and then the antibacterial agents at a selected concentration or sterile distilled water was added to give a final volume of 10 ml. The test tubes were then incubated aerobically at 37°C with constant agitation. One-hundred-microliter samples were removed at fixed intervals, and several dilutions of each sample were prepared in saline as required and subsequently plated onto MH agar. Drug carryover into agar was minimal at a 100-fold dilution of the sample with agar. The number of colonies was counted after 24 h of incubation at 37°C. The minimum countable number was 10^2 CFU/ml.

RESULTS

Antibacterial activity and *gyrA* mutation. The antibacterial activities of Q-35, ofloxacin, ciprofloxacin, tosufloxacin, and sparfloxacin were determined for 18 strains of MRSA and 3 strains of *S. epidermidis* clinical isolates (Table 1). The susceptibility of 21 strains of staphylococci to ciprofloxacin ranged between 0.39 and >100 μg/ml. The susceptibility to sparfloxacin clearly divided the strains into the two following groups: susceptible strains (0.05 to 0.20 μg/ml) and resistant strains (6.25 to 25 μg/ml). No intermediate strains were observed. The MICs of Q-35 against sparfloxacin-susceptible strains ranged between 0.05 and 0.78 μg/ml, and those for resistant strains ranged between 1.56 and 12.5 μg/ml.

The point mutation of *gyrA* at codon 84 (Ser), as detected by PCR-restriction fragment length polymorphism analysis, was present in all sparfloxacin-resistant strains. In sparfloxacin-susceptible strains, this mutation was absent.

Killing-curve studies. The bactericidal activity of Q-35 was compared with that of tosufloxacin and sparfloxacin with *S. aureus* ATJ-26 as represented in Fig. 1. Q-35 produced the greatest bactericidal response after 1 h of exposure compared with the other quinolones tested. After a 4-h exposure, Q-35 decreased the viable counts from 1.7×10^7 CFU/ml to approximately 10^4 CFU/ml at drug concentrations that were greater than the MIC. Tosufloxacin and sparfloxacin produced no bactericidal effect until after 2 h of exposure to quinolone at all of the concentrations tested. After 4 or 6 h, tosufloxacin and sparfloxacin exhibited weak bactericidal activity at values greater than the MIC.

Bactericidal activities at various concentrations of quinolones. The bactericidal activities of four quinolones at various concentrations against *S. aureus* ATJ-26 (sparfloxacin resis-

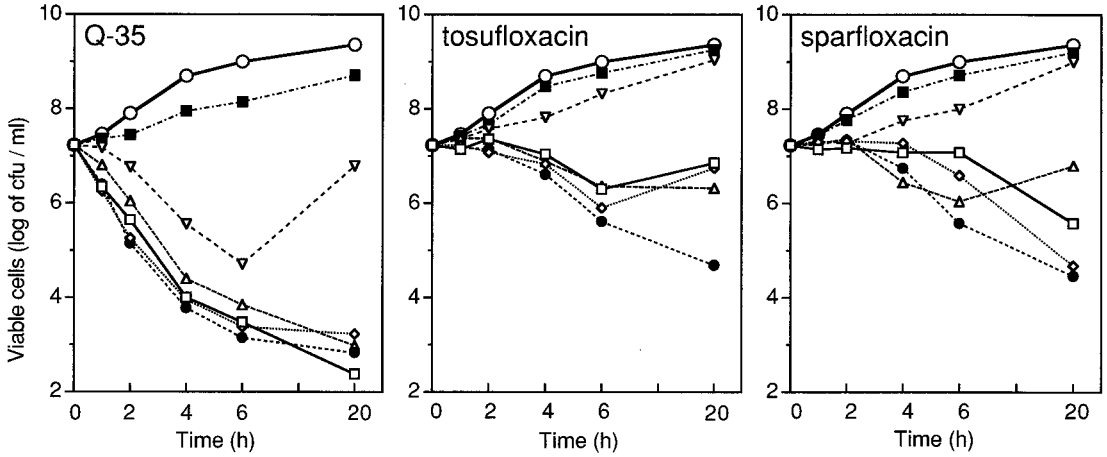

FIG. 1. Bacterial killing kinetic curves of Q-35 (MIC, 6.25 μg/ml), tosufloxacin (MIC, 6.25 μg/ml), and sparfloxacin (MIC, 6.25 μg/ml) against *S. aureus* ATJ-26. ○, control; ■, one-fourth of the MIC; ▽, one-half of the MIC; △, MIC; ●, twice the MIC; ◇, four times the MIC; □, eight times the MIC.

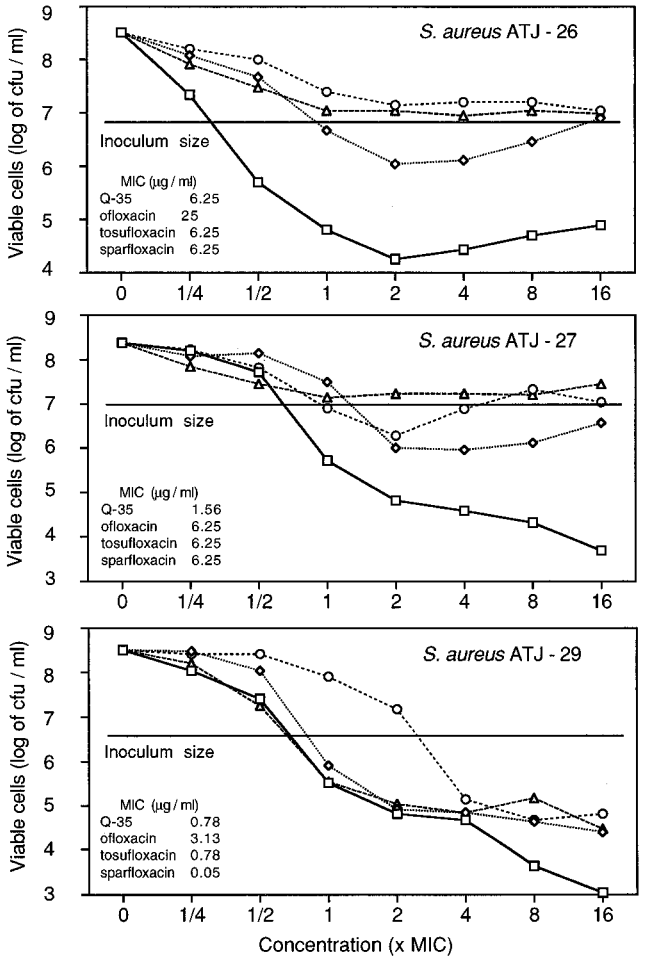

FIG. 2. Bactericidal effects of Q-35 (□), ofloxacin (◇), sparfloxacin (○), and tosufloxacin (△) against *S. aureus* at various concentrations.

tant), ATJ-27 (resistant), and ATJ-29 (susceptible) after 3 h of exposure were investigated.

Tosufloxacin and sparfloxacin were unable to significantly kill the resistant strains of ATJ-26 and ATJ-27 at any of the concentrations tested, while Q-35 exhibited bactericidal activity at values greater than the MIC (Fig. 2). In comparison, ofloxacin produced intermediate activity. Q-35 and ofloxacin produced a biphasic action with ATJ-26.

All quinolones exhibited bactericidal activities against the susceptible strain ATJ-29 at values greater than one to four times the MIC. The growth-inhibitory concentration of sparfloxacin in this condition was two- to fourfold higher than the MIC as tested by the agar dilution method.

Bactericidal activities against 23 strains of staphylococci. The bactericidal activities of Q-35, ofloxacin, tosufloxacin, and sparfloxacin were compared in both quinolone-susceptible and -resistant strains of *S. aureus* (20 strains) and *S. epidermidis* (3 strains) so as to characterize relative potencies against sparfloxacin-susceptible and -resistant strains. Each bacterial strain was tested at approximately 10^7 CFU/ml and exposed to Q-35, ofloxacin, tosufloxacin, and sparfloxacin at eight times the MIC. Bactericidal activity of each strain was calculated as the decrease in viable counts after 3 h of exposure to each of the quinolones. The relationship between the MIC and bactericidal activity for each compound tested is shown in Fig. 3.

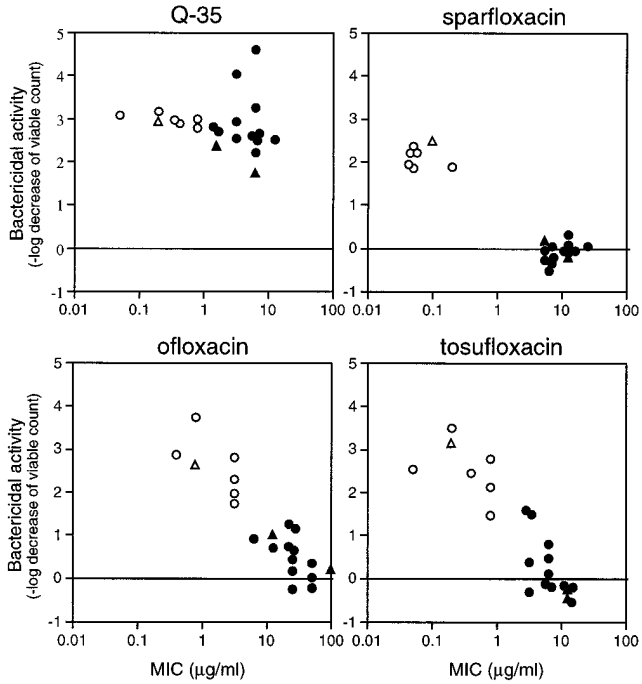

FIG. 3. Relationship between MIC and bactericidal activity for staphylococci. *S. aureus* (circle) and *S. epidermidis* (triangle) cells were exposed to eight times the MIC of each quinolone for 3 h. In each panel, sparfloxacin-susceptible and -resistant strains are represented by open and closed symbols, respectively.

Q-35 decreased the viable counts of both sparfloxacin-susceptible and -resistant strains, and most strains showed decreases on the order of 3 log CFU/ml (Fig. 3). Two sparfloxacin-resistant strains, ATJ-4 and ATJ-8, showed decreases on the order of 4 log CFU/ml.

Against sparfloxacin-susceptible strains, ofloxacin and tosufloxacin showed a bactericidal effect ranging between 1.5 and 4 log CFU/ml. However, the bactericidal activities of these quinolones were decreased with resistant strains. Highly resistant strains were not killed by these quinolones.

Sparfloxacin produced a bactericidal effect against sparfloxacin-susceptible strains on the order of 2 log CFU/ml (Fig. 3). However, sparfloxacin produced no bactericidal effect against resistant strains. In addition, bactericidal activity of sparfloxacin clearly divided strains into the two groups and was directly related to the MIC (Fig. 3).

Bactericidal activities of Q-35 analogs. The bactericidal activities of Q-35 against *S. aureus* ATJ-27 (sparfloxacin resistant) were compared with those of Q-35 analogs, namely, 8-H Q-35, 8-F Q-35, and 7-4 Q-35 (Fig. 4). At values greater than the MIC, both Q-35 and 7-4 Q-35 were profoundly bactericidal; 8-F Q-35 was mildly bactericidal whereas 8-H Q-35 exhibited only bacteriostatic activity. All of these analogs of Q-35 exhibited bactericidal activities against sparfloxacin-susceptible *S. aureus* (data not shown).

DISCUSSION

The bactericidal activity of fluoroquinolones against fluoroquinolone-susceptible staphylococci has been extensively investigated (7–9). However, relatively little attention has been given to the bactericidal activity against fluoroquinolone-resistant strains. In this study, we examined the differences in bactericidal activity between Q-35 and other quinolones. Q-35

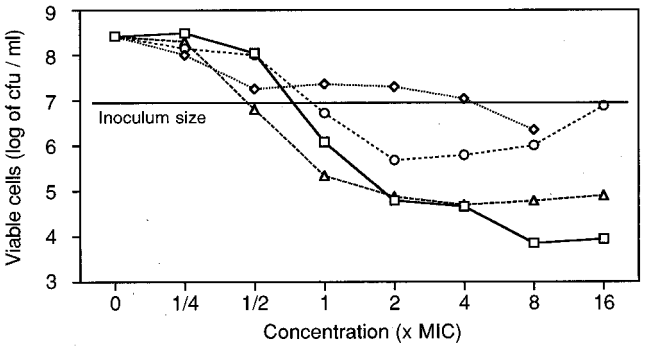

FIG. 4. Bactericidal effects of Q-35 (MIC, 1.56 μg/ml; □), 8-H Q-35 (MIC, 100 μg/ml; ◇), 8-F Q-35 (MIC, 6.25 μg/ml; ○), and 7-4 Q-35 (MIC, 1.56 μg/ml; △) against *S. aureus* ATJ-27 at various concentrations.

produced bactericidal activity against sparfloxacin-resistant staphylococci, whereas ofloxacin, tosufloxacin, and sparfloxacin did not show bactericidal activities until after 3 h of exposure to quinolones. We elucidated the characteristic activity of Q-35 by examining the effects of its analogs against sparfloxacin-resistant MRSA. Substitution of the 8-methoxy group with hydrogen or fluorine significantly decreased the bactericidal activity in resistant strains. Thus, it is implied that the 8-methoxy group is particularly important to the process of killing resistant strains of staphylococci. These analogs were all effective against susceptible strains.

In this study, the MICs of sparfloxacin against *S. aureus* clearly divided the organisms into susceptible and resistant strains, and all sparfloxacin-resistant strains contained the *gyrA* mutation at codon 84 (Ser). Because of increased hydrophobicity (1), sparfloxacin was reported not to be influenced by low-level resistance mechanisms, such as that of the efflux pump which is encoded by the *norA* gene (8, 17). Against the resistant strains, sparfloxacin was not bactericidal. These results suggest that the *gyrA* mutation not only caused the observed increase in the MIC but was also responsible for the associated resistance to bacterial killing by quinolones.

We have previously reported that Q-35 inhibited the gyrase purified from *S. aureus* FDA 209P at an approximately 10-fold-lower concentration than that of tosufloxacin or sparfloxacin (6, 7). It was thought that this unique bactericidal activity of Q-35 was related to this potent inhibition of gyrase by Q-35.

In conclusion, Q-35 produced improved bactericidal activities against quinolone-resistant staphylococci, and the introduction of a methoxy group into the 8 position of quinolones contributed to these activities. Further studies are important for elucidation of the exact mechanisms of bacterial killing.

ACKNOWLEDGMENTS

We especially thank Toshio Iwasawa for his assistance. We also thank Peter Kowalski-Saunders for helpful suggestions and discussions.

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