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In vitro development of resistance to a novel fluoroquinolone, DW286, in methicillin-resistant *Staphylococcus aureus* clinical isolates

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***In vitro* development of resistance to a novel fluoroquinolone, DW286, as well as to ciprofloxacin, gemifloxacin, sparfloxacin and trovafloxacin, was investigated in eight methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates. The strains were subcultured in subinhibitory concentrations of each agent during a 50 day period. Subculturing in most agents led to the selection of 37 mutants with increased MICs. The DW286 MICs were increased from 0.004–0.031 to 0.125–0.5 mg/L in five strains after 13–47 passages, and were not increased in three strains. The ciprofloxacin, gemifloxacin, sparfloxacin and trovafloxacin-selected mutants showed relatively weak cross-resistance to DW286. DNA sequencing analyses of all of the selected mutants revealed a few point mutations responsible for the high level of resistance, but actually these variations did not confer high resistance to fluoroquinolones. In the presence of reserpine, an inhibitor of the Gram-positive efflux pump, of 36 mutants 22 had two- to 16-fold lower ciprofloxacin MICs, and 20 had two- to 16-fold lower gemifloxacin MICs. However, sparfloxacin, trovafloxacin and DW286 were not good substrates for efflux pumps.**

Keywords: DW286, fluoroquinolone, MRSA, antibiotic resistance

Introduction

DW286 is a novel fluoroquinolone, 7-[3-(aminomethyl)-4-(methoxyimino)-3-methyltetrahydro-1H-1-pyrrolyl]-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro[1,8] naphthyridine-3-carboxylic acid hydrochloric acid salt. DW286 showed potent *in vitro* antibacterial activity against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and quinolone-resistant *S. aureus* (QRSA), and the most potent efficacy in experimental systemic mouse models of MRSA and QRSA infections.[1]

Fluoroquinolones with superior antimicrobial activities have been developed to treat infections caused by mainly Gram-positive pathogens, including MRSA. However, the extensive use of quinolones brought about the rapid emergence of resistant MRSA strains.[2]

Quinolones directly inhibit DNA synthesis by interacting with DNA gyrase (the two subunits encoded by the *gyrA* and *gyrB* genes) and DNA topoisomerase IV (the two subunits encoded by the *grlA* and *grlB* genes for *S. aureus*, and *parC* and *parE* for other organisms). The mechanism of quinolone resistance falls into three categories: (i) the nucleotide changes at *gyrA* and *gyrB*; (ii) the nucleotide changes at *grlA* and *grlB*; and (iii) the alteration in drug permeation to the target enzymes, mediated by efflux pumps. Most of the quinolone-resistant strains from laboratory or clinical sources have point mutations clustered in a narrow region, called the quinolone-resistance determining region (QRDR), near the enzyme active sites.[3,4] Also, several studies have demonstrated that subculturing staphylococci and pneumococci in subinhibitory concentrations of quinolones can lead to the *in vitro* selection of resistant mutants.[5,6]

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Table 1. Resistance selection results

	Initial MIC (mg/L)[b]					Selected resistance			MIC after 10 antibiotic-free subcultures					Alterations[c] on enzymes of:			
Strain[a]	DW286	CIP	GEM	SPX	TVA	agent[b]	MIC	no. of passages	DW286	CIP	GEM	SPX	TVA	GrlA	GrlB	GyrA	GyrB
1	0.008	0.063	0.016	0.063	0.063	DW286	NR[d]							ND[e]	ND	ND	ND
						CIP	4	26	0.5	4	0.5*[f]	0.25	0.25	E84K	R390P D395Y		
						GEM	0.25	32	0.031	2	0.25	0.25	0.25			S84L	
						SPX	0.25	45	0.008	1*	0.063*	0.25	0.063			S84L	
						TVA	2	15	0.016	32**	0.5*	2	2		R390P D395Y		
2	0.008	0.063	0.016	0.031	0.063	DW286	NR							ND	ND	ND	ND
						CIP	8	19	0.063*	8*	0.25*	0.125	0.25		R390P D395Y		
						GEM	0.5	23	0.063	2*	0.5**	8*	0.25*			S84L	
						SPX	0.5	43	0.031	1	0.125*	0.5	0.5		E559Q	S84L	
						TVA	0.5	23	0.031	4	0.25*	0.5	0.5	S80F			
3	0.016	0.031	0.031	0.125	0.063	DW286	0.5	45	0.5	0.5	0.5	1*	2			E88K	
						CIP	32	10	0.25	32*	0.5	2	2	E84K	R390P D395Y		
						GEM	1	29	0.25*	8*	1	4	1		R390P D395Y	S84L	
						SPX	0.5	29	0.5	1*	0.25	0.5	0.5		E559Q	S84L	
						TVA	0.125	3	revertant								
4	0.031	0.031	0.016	0.031	0.016	DW286	0.25	13	0.25	0.063*	0.125*	0.25	1				
						CIP	4	5	0.125	4*	0.125	0.125	0.5	E84K			
						GEM	0.5	8	0.063	1*	0.5*	0.25	1				
						SPX	0.25	9	0.063	1	0.25	0.25	1			S84L	
						TVA	0.25	15	0.063	0.5	0.016	0.031	0.25				
5	0.008	0.063	0.031	0.25	0.031	DW286	0.5	26	0.5*	0.5	0.5	0.5	1				
						CIP	8	12	0.125	8*	0.25	0.5*	0.5	E84K	R390P D395Y		
						GEM	0.5	34	0.031*	16**	0.5**	0.5	0.5			E88K	
						SPX	2	15	0.008	0.5	0.031	2	0.063			E88K	
						TVA	0.5	15	0.125	1	0.25*	1	0.5	S80F	E559Q		
6	0.004	0.016	0.016	0.125	0.016	DW286	0.125	47	0.125*	32**	2**	2*	0.5		R390P	E88K	
						CIP	64	15	0.25	64*	0.5	2	2	E84K			
						GEM	0.25	23	0.031	4	0.25	1	0.25		E559Q		
						SPX	1	45	0.063	32**	2**	1*	0.5		R390P D395V	E88K	
						TVA	0.25	26	0.125	16*	0.5*	0.5	0.25	S80F			
7	0.008	0.031	0.031	0.125	0.063	DW286	NR							ND[d]	ND	ND	ND
						CIP	2	10	0.016	2*	0.031*	0.25	0.125	E84K			

	GEM	0.125		30		0.031	2		0.125	0.5	0.25		E559Q	S84L					
	SPX	2		3		0.008	0.5*		0.031*	2	0.063		R390P						
													D395Y						
													E422D						
	TVA	0.25		35		0.016	2*		0.063*	0.25	0.25		E559Q						
8	DW286	0.125		19		0.125	4*		0.125*	0.5	0.25								
	CIP	8		9		0.125	8*		0.125*	2	2	E84K							
	GEM	0.063		12		0.063	2*		0.063*	1	0.25		E559Q	S84L					
	SPX	0.125		14		0.016	0.25		0.016	0.125	0.125			S84L					
	TVA	0.5		7		0.063	4		0.5	0.5	0.5	S80F							
0.004		0.016	0.004	0.031	0.016														

[a]Strain, 1; *S. aureus* 722, 2; *S. aureus* 726, 3; *S. aureus* 3583, 4; *S. aureus* 5298; 5, *S. aureus* TMS33; 6, *S. aureus* TMS64; 7, *S. aureus* U31; 8, *S. aureus* R572.
[b]CIP, ciprofloxacin; GEM, gemifloxacin; SPX, sparfloxacin; TVA, trovafloxacin.
[c]Alterations refer to changes between the parent strain and the selected mutant strain. All parent strains had wild-type GrlA, GrlB, GyrA and GyrB.
[d]NR, no increase in MIC detected.
[e]ND, not determined.
[f]Selected mutants had fold decrease in MICs in the presence of reserpine. *Two- to four-fold decrease; **eight- to 16-fold decrease.

In order to evaluate the potential of using DW286 for the treatment of infections, we need to determine whether it selects increased MICs and resistance development for staphylococci. Therefore, we sequentially exposed eight clinical isolates of MRSA to subinhibitory concentrations of DW286 and comparative agents. Genotypic analyses were carried out using DNA sequence analyses, and the involvement of efflux pumps in the resistance development was determined.

Materials and methods

Bacterial strains

S. aureus clinical isolates were collected and identified from the intensive care units (ICUs) of hospitals in Seoul, South Korea, during 1999–2000. Among them, six isolates of methicillin-resistant (MIC ≥ 16 mg/L) and ciprofloxacin-susceptible (≤2 mg/L) *S. aureus* (MRSA and QSSA) were selected: *S. aureus* 722, *S. aureus* 726, *S. aureus* 3583, *S. aureus* 5298, *S. aureus* R572 and *S. aureus* U31. Two clinical strains (MRSA and QSSA) were kindly provided by Professor Goto of Toho University, Japan: *S. aureus* TMS 33 and *S. aureus* TMS 64.

Antimicrobial agents

The antimicrobial agents used in this study were as follows: trovafloxacin was provided by Pfizer, Inc., New York, NY, USA; and DW286, ciprofloxacin, gemifloxacin and sparfloxacin were synthesized at the R & D Centre, Dong Wha Pharmaceutical Ind. Co., Ltd, An-Yang, South Korea.

MIC determination

MICs were determined by the microdilution method with Mueller–Hinton broth (Difco Laboratories, Detroit, MI, USA), according to the recommendation by the NCCLS.[7] Susceptibility breakpoints for each agent are as follows: ciprofloxacin ≤ 2 mg/L; gemifloxacin ≤ 1 mg/L; sparfloxacin ≤ 0.5 mg/L; and trovafloxacin ≤ 1 mg/L.

Serial passage

Parent strains were fully grown in antibiotic-free Mueller–Hinton broth (Difco) at 37°C for 18 h, and ~5×10^7 cfu/mL of each strain were inoculated into each of a series of tubes, containing 2 mL of an appropriate broth with antibiotic concentrations consisting of doubling dilutions below and above the MIC of each agent for each strain. After an incubation at 37°C for 24 h, an inoculum from the tube nearest the MIC, which had the same turbidity as the antibiotic-free control, was taken, diluted 1:100 and then transferred to a new tube containing the appropriate broth with antibiotics. These

passages were carried out for 50 days. During this time period, when the MIC of any agent for any strain increased four-fold, the passaging was stopped. The selected mutant strain was subcultured in antibiotic-free Mueller–Hinton agar (Difco) for another 10 serial passages. Additionally, the selected mutant for a certain agent was tested with the other agents, to see whether the mutant acquired cross-resistance.

Analysis of total DNA by pulsed-field gel electrophoresis (PFGE)

To determine whether the selected mutants were derived from those tested at the beginning of the study, the parent strains and the strains with increased MICs obtained after the last passage were tested by PFGE. Genomic DNAs were digested by the *Sma*I restriction endonuclease and electrophoresed using a CHEF-DR II system (Bio-Rad Laboratories, Hercules, CA, USA). All of the mutants had PFGE patterns identical to those of the parent strains.

Amplification of QRDRs and DNA sequence analysis

To determine whether the mutants selected during serial passages had alterations in the QRDRs of topoisomerase VI or DNA gyrase in comparison with their parent strains, the fragments of *grlA*, *grlB*, *gyrA* and *gyrB* containing the QRDRs were amplified by PCR. Genomic DNAs were used as templates for the amplification of *grlA*, *grlB*, *gyrA* and *gyrB* by the PCR method, using the primers and cycling conditions described by Schmitz *et al*.[8] The sequences of *grlA* and *grlB* are adjacent to each other, and so are those of *gyrA* and *gyrB*. On the basis of this continuity, we amplified *gyrB–gyrA* and *grlB–grlA*, respectively. The oligonucleotides used for PCR were as follows: for *gyrB–gyrA*, 5′ primer 1400-CAGCGTTAGATGTAGCAAGC-1419 and 3′ primer 2781-CAGGACCTTCAATATCCTCC-2800; for *grlB–grlA*, 5′ primer 1520-CGATTAAAGCACAACAAGCAAG-1541 and 3′ primer 2829-CTTGATGGCAATACCATTGGTTC-2851. PCR conditions were 30 s at 95°C for denaturation; 30 s at 50°C for *gyrB–gyrA* and 30 s at 57°C for *grlB–grlA* for annealing; and 1 min at 72°C for primer extension. Elongation was carried out for 35 cycles. The sizes of the PCR products obtained in these reactions were 1401 bp for *gyrB–gyrA* and 1332 bp for *grlB–grlA*.

We sequenced the DNAs of all the mutants selected by each of the five agents. The PCR products were purified using a Qiagen PCR purification kit, as recommended by the manufacturer (Qiagen, Valencia, CA, USA). PCR-amplified DNA fragments were sequenced by the dye terminator method in both the forward and reverse directions, using an automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Efflux system

The MICs of the agents for the selected mutants and parent strains were determined in the presence and absence of 20 mg/L reserpine (Sigma, St Louis, MO, USA), as described by Boos *et al*.[5]

Results and discussion

MICs for mutants

The increments of the MICs resulting from subculturing in subinhibitory concentrations of agents for strains are summarized in Table 1.

Of 37 mutant strains, 36 showed stable phenotypes in antibiotic-free media for another 10 serial passages.

Subculturing in ciprofloxacin gave increased MICs, rising from 0.016–0.063 to 2–64 mg/L after 5–26 serial passages. Subculturing in the presence of gemifloxacin gave increased MICs, rising from 0.004–0.031 to 0.063–1 mg/L after 8–34 serial passages. Subculturing in sparfloxacin gave increased MICs, rising from 0.031–0.25 to 0.125–2 mg/L after 3–45 serial passages. Subculturing in trovafloxacin gave increased MICs, rising from 0.016–0.063 to 0.125–2 mg/L after 3–35 serial passages.

In contrast to the other four fluoroquinolones, which gave increased MICs in all eight strains, subculturing in the presence of DW286 gave increased MICs for five of the eight strains, with MICs rising from 0.004–0.031 to 0.125–0.5 mg/L after 13–47 serial passages. The minimum number of subcultures for the selection of mutants by DW286 was 13, which was much longer than those of the other fluoroquinolones. In the present study, DW286 was the least likely to select mutants with highly increased MICs during the 50 day period.

Cross-resistance among selected mutants

The MICs for 36 selected mutants were determined to identify whether they acquired cross-resistance to other fluoroquinolones as well. Table 2 shows the distribution of each mutant with respect to the MIC of each agent.

Based on the susceptible breakpoints, 17 mutants were resistant to ciprofloxacin (range 4–64 mg/L), two mutants to gemifloxacin (2 mg/L), 14 mutants to sparfloxacin (range 1–8 mg/L) and five mutants to trovafloxacin (2 mg/L).

The DW286 MICs for all of the selected mutants were distributed in the range from 0.008 to 0.5 mg/L. Even though 25 of the 36 mutants showed at least four-fold increases in their DW286 MICs, the highest MIC was no more than 0.5 mg/L. The susceptible breakpoint of DW286 has yet to be determined, but from this result the ability of DW286 to cause cross-resistance is relatively weak compared with the other fluoroquinolones.

Table 2. Distribution of mutants with respect to quinolone MICs

Agent	No. of mutants for which the MIC (mg/L) was[a]													
	0.008	0.016	0.031	0.063	0.125	0.25	0.5	1	2	4	8	16	32	64
DW286	3	4	6	8	7	4	4	–[b]	–	–	–	–	–	
CIP	–	–	–	1	–	1	5	6	6	**6**	**4**	**2**	**4**	**1**
GEM	–	2	3	3	6	8	11	1	**2**	–	–	–	–	
SPX	–	–	1	–	3	8	10	**5**	**7**	**1**	**1**	–	–	
TVA	–	–	–	3	2	11	10	5	**5**	–	–	–	–	

[a]Bold type represents resistance to this quinolone.
[b]–, none.

Mutations in DNA topoisomerase IV

Most of the DW286 selected mutants did not have point mutations in the topoisomerase IV subunits, but strain 6, exposed to DW286, had Arg-390→Pro in GrlB (Table 1). The amino acid change of Glu-84→Lys was present in seven mutant strains selected by ciprofloxacin. Within the seven mutant strains, four mutants with the single mutation of Glu-84→Lys in GrlA were susceptible to all of the fluoroquinolones, except ciprofloxacin. The other three mutants, with triple mutations in GrlA (Glu-84→Lys) and GrlB (Arg-390→Pro and Asp-395→Tyr), were resistant to two or three of the fluoroquinolones tested, but were still susceptible to DW286 and gemifloxacin. Even though the mutant strains with the Ser-80→Phe mutation in GrlA showed a low level of resistance to ciprofloxacin or sparfloxacin, they were all susceptible to the other fluoroquinolones.

The mutations detected in GrlA and GrlB in this study do not seem to be directly involved in the development of a high level of resistance.

Mutations in DNA gyrase

Ten mutant strains had Ser-84→Leu, and five had Glu-88→Lys in GyrA, but there was no amino acid change in GyrB. Even though they showed resistance to one or two fluoroquinolones, they were still susceptible to DW286 (its MIC was the lowest) and other fluoroquinolones. Therefore, we could confirm that the Ser-84 mutation in GyrA alone does not confer a high level of resistance. All of the mutant strains with Ser-84 or Glu-88→Lys in GyrA lacked Ser-80→Phe or Glu-84→Lys in GrlA, either of which is required for the development of high-level resistance in *S. aureus*, so they were not highly resistant compared with the strains used in other studies.

Except for two of the DW286 selected mutants, most lacked point mutations at the hot spots of the GyrA or GyrB subunit. This result indicates that DW286 does not seem to induce point mutations at the hot spots in gyrase easily.

GyrB does not seem to play an important role in the resistance mechanism,[9,10] and our study also shows that GyrB is not involved in the resistance development.

Efflux system

In the presence of reserpine, 28 of 36 mutants had lower DW286, ciprofloxacin, gemifloxacin, sparfloxacin and trovafloxacin MICs (data not shown). Of these, 22 mutants had two- to 16-fold lower ciprofloxacin, and 20 mutants had two- to 16-fold lower gemifloxacin MICs. Our study shows that the selected mutant strains were more susceptible to ciprofloxacin and gemifloxacin in the presence of reserpine. The sparfloxacin, trovafloxacin and DW286 MICs were slightly affected by reserpine, but the fold decrease in the MICs was lower compared with those of ciprofloxacin and gemifloxacin. Sparfloxacin, trovafloxacin and DW286 resistances are not greatly affected by reserpine.

In summary, our results show that the novel fluoroquinolone DW286 has good *in vitro* and *in vivo* activity against wild-type MRSA isolates and is still more potent than the other fluoroquinolones against mutant strains selected via serial passages. In addition, it does not cause strong cross-resistance to other fluoroquinolones and the effect of reserpine on this novel fluoroquinolone is negligible. The results indicate that DW286 could be used in clinical trials for a long treatment period without causing high levels of resistance. However, the clinical importance of DW286 resistance needs to be confirmed in further studies, because a small number of clinical isolates was used in this study and DW286 does not show a high activity against highly quinolone-resistant *S. aureus*.[1]

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