ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1995, p. 1467–1471 Vol. 39, No. 7
0066-4804/95/$04.00+0
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Contribution of the C-8 Substituent of DU-6859a, a New Potent Fluoroquinolone, to Its Activity against DNA Gyrase Mutants of *Pseudomonas aeruginosa*

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Received 15 December 1994/Returned for modification 7 March 1995/Accepted 4 May 1995

Inhibitory effects of five quinolones against DNA gyrases purified from four quinolone-resistant clinical isolates of *Pseudomonas aeruginosa* and the quinolone-susceptible strain PAO1 were examined. All of the quinolone-resistant strains tested were found to be DNA gyrase mutants. The 50% inhibitory concentrations (IC_{50}s) of the quinolones for these DNA gyrases roughly correlated with their MICs. Interestingly, gyrase inhibition by DU-6859a was found to be significantly less affected by these mutations than inhibition by other currently available quinolones. To assess the enhanced activity shown by DU-6859a, the effects of quinolones with altered substituents at the N-1, C-7, and C-8 positions of the quinolone ring of DU-6859a were tested. Measurement of MICs for four DNA gyrase mutants and IC_{50}s for their purified DNA gyrases showed that removal of the C-8 chlorine of DU-6859a significantly increased MICs and IC_{50}s for DNA gyrase mutants. However, no deleterious effects were observed when either the fluorine on the cyclopropyl substituent at the N-1 position or the cyclopropyl ring at the C-7 substituent was removed. Moreover, removal of the C-8 chlorine also increased the MIC for 19 of 20 quinolone-resistant clinical isolates. Our results led to the conclusion that DU-6859a is much more active against quinolone-resistant clinical isolates of *P. aeruginosa* than other currently available quinolones, probably because of its strong inhibitory effects against mutant quinolone-resistant DNA gyrases, and that the C-8 chlorine is necessary for these potent effects.

DU-6859a (the sesquihydrate of DU-6859), (−)-7-[(7S)-amino-5-azaspiro[2,4]heptan-5-yl]-8-chloro-6-fluoro-1-[(1*R*,2*S*)-*cis*-2-fluoro-1-cyclopropyl]-1,4-dihydro-4-oxoquinolone-3-carboxylic acid, is a new fluoroquinolone with antibacterial activity significantly better than that of any currently available quinolone (13, 21). One of its unique characteristics is its activity against quinolone-resistant clinical isolates of *Pseudomonas aeruginosa* (21). Sato et al. reported that the activity of DU-6859a was roughly comparable to that of ciprofloxacin (CPFX) at the level of the MIC at which 50% of the isolates were inhibited, but its activity against ofloxacin-resistant strains was more than four times greater than that of CPFX (21). Since strains of *P. aeruginosa* are often isolated from clinical specimens and since the frequency of quinolone-resistant *P. aeruginosa* strains has increased (7), this high level of potency of DU-6859a has attracted considerable interest. It has been reported that the quinolone resistance mechanisms in *P. aeruginosa* involve chromosomal mutations, affecting drug permeability (3, 6, 11, 18–20) or the drug sensitivity of DNA gyrase (4), the main target of the quinolones (10). DNA gyrase is thought to be a tetramer composed of two subunits, A2 and B2 (10). Inoue et al. reported that alterations in the A subunit render *P. aeruginosa* resistant to quinolones (10). Several studies have indicated that the majority of quinolone-resistant clinical isolates of *P. aeruginosa* alter DNA gyrase (30, 31). In light of the predominant gyrase mutations found in quinolone-resistant clinical isolates, we postulated that the potent activity of DU-6859a against clinical isolates might be due to its improved inhibitory activity against DNA gyrase mutants. In order to confirm this hypothesis, we purified DNA gyrases from four quinolone-resistant clinical isolates of *P. aeruginosa* and compared the inhibitory effects of five quinolones against the supercoiling activities of these gyrases. Moreover, the effects of three derivatives of DU-6859a were also compared to assess the contribution of each substituent of DU-6859a to the potency of its effect against quinolone-resistant *P. aeruginosa*. To our knowledge, this is the first report to examine the relationship between the structure of quinolones and their activity against DNA gyrase with or without quinolone resistance alterations.

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MATERIALS AND METHODS

Strains. Twenty quinolone-resistant strains (DNS 5001, DNS 5002, DNS 5003, DNS 5004, DNS 5005, DNS 5006, DNS 5007, DNS 5008, DNS 5009, DNS 5010, DNS 5011, DNS 5012, DNS 5013, DNS 5014, DNS 5015, DNS 5016, DNS 5017, DNS 5018, DNS 5019, and DNS 5020) were selected for study on the basis of their high-level resistance to ofloxacin (MICs, >25 μg/ml) from a total of 103 clinical isolates of *P. aeruginosa* collected in Japan in 1990. DNS 5001, DNS 5002, DNS 5003, and DNS 5004 were chosen at random from those 20 strains. *P. aeruginosa* PAO1 was used as a reference strain having wild-type DNA gyrase.

Chemicals. Levofloxacin (LVFX), CPFX, tosufloxacin, sparfloxacin (SPFX), DU-6859a, DU-6611 (a 7-[3-(s)-amino-1-pyrrolidinyl] derivative of DU-6859), DU-6668 (a 1-cyclopropyl derivative of DU-6859), and DW-8186 (an 8-hydrogen derivative of DU-6859) were synthesized at the Exploratory Research Laboratories I, Daiichi Pharmaceutical Co., Ltd. The chemical structures of these compounds are shown in Fig. 1. The eight quinolone compounds were dissolved in 0.1 N NaOH and diluted with distilled water. All other chemicals were purchased from their respective manufacturers and were at least of analytical grade.

Purification of DNA gyrase and measurement of inhibition by quinolones. DNA gyrase was purified from DNS 5001, DNS 5002, DNS 5003, DNS 5004, and PAO1 by the procedure of Inoue et al., using a novobiocin-Sepharose column (10). The effects of quinolones on the supercoiling activity of DNA gyrase were determined by measuring 50% inhibitory concentrations (IC_{50}s) by the procedure of Inoue et al. (10), using 0.5 U of DNA gyrase and 100 ng of pBR322

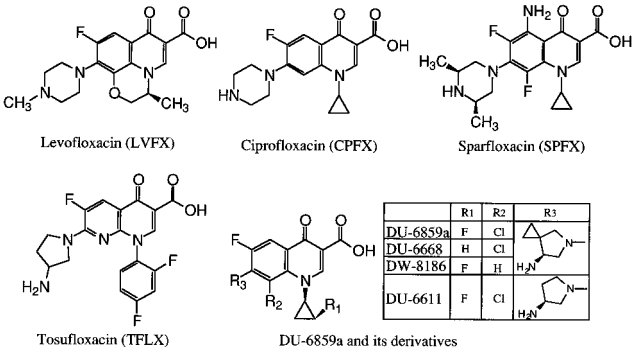

FIG. 1. Chemical structures of quinolone compounds.

plasmid DNA (Boehringer Mannheim GmbH, Mannheim, Germany) previously relaxed with calf thymus topoisomerase I (Bethesda Research Laboratories, Ltd., Cergy Pontoise, France). One unit of gyrase activity was defined as the amount of gyrase required to fully supercoil 100 ng of relaxed DNA in 90 min at 37°C. The specific activities of the purified DNA gyrases were 52.9 to 108 U/mg of protein.

Determination of MICs. MICs were determined by the standard broth dilution method with Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) (16). The MIC was defined as the lowest drug concentration that prevented visible bacterial growth.

RESULTS

Comparison of antigyrase and antibacterial activities of DU-6859a and other quinolones. The IC_{50}s of five quinolones for DNA gyrases isolated from five *P. aeruginosa* strains and their respective MICs for intact bacteria are summarized in Table 1. The MICs of CPFX, LVFX, SPFX, and tosufloxacin for all of the clinical isolates were from 32 to over 2,000 times those for strain PAO1, indicating quinolone resistance in those isolates. The IC_{50}s of LVFX for DNA gyrases from these resistant strains were 8 to over 400 times higher than the IC_{50}s for PAO1, which suggested that all of the clinical isolates were DNA gyrase mutants. A good correlation ($r = 0.83$) between IC_{50}s and MICs for all strains tested was observed, with the exception of DNS 5002 (Fig. 2). The MIC of DU-6859a or CPFX against PAO1 was 0.1 μg/ml, which was between one-half and one-quarter of the MICs of the other quinolones. Therefore, DU-6859a was one of the most potent agents against the quinolone-susceptible strain PAO1. Of the clinical isolates tested, DNS 5002 was the most highly quinolone resistant with respect to both MIC and IC_{50}. The MICs of CPFX, LVFX, or SPFX for DNS 5002 were 1,000 to 2,000 times those for PAO1. The MIC of DU-6859a, on the other hand, increased by only 250 times. IC_{50}s of CPFX, LVFX, or SPFX for this strain's gyrase were from 439 to over 2,000 times as high as the IC_{50}s for PAO1 gyrase, whereas against DNS 5002 gyrase, DU-6859a had less than 1/18 of its activity against PAO1 gyrase. Thus, DU-6859a was considerably more active than other quinolones against quinolone-resistant DNA gyrase from DNS 5002. Although the differences were not as large as those with DNS 5002, similar effects were seen with DNS 5001, DNS 5003, and DNS 5004 also. These differences in IC_{50} and MIC indicate that DU-6859a is much more active than other quinolones against these DNA gyrase mutants because of its stronger inhibition of mutant gyrase.

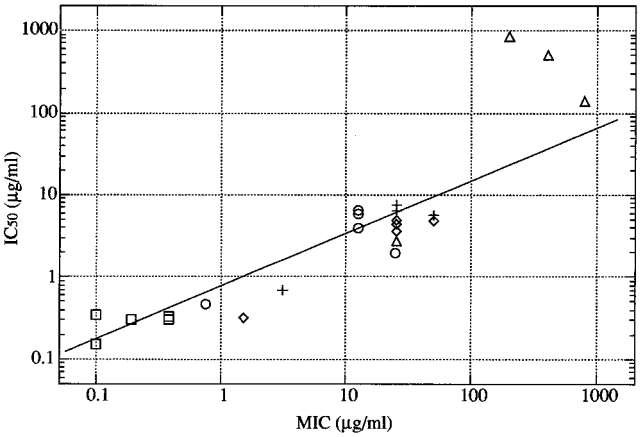

FIG. 2. Correlation between MIC and IC_{50}. Correlation coefficient, 0.83. Symbols: ○, DNS 5001; △, DNS 5002; +, DNS 5003; ◇, DNS 5004; □, PAO1.

Contribution of the C-8 substituent to the potent activity of DU-6859a against DNA gyrase mutants. DU-6859a has several structural features that distinguish it from other fluoroquinolones, such as the fluorine at the N-1 position on its cyclopropane ring, the spirocyclopropane on the C-7 aminopyrrolidine ring, and the chlorine at the C-8 position. Since potent activity against DNA gyrase mutants is unique to DU-6859a, we speculated that all or some of these substituents might be responsible for this potent activity. In order to assess their contribution, the MICs and levels of gyrase inhibition of three derivatives of DU-6859a, namely, DU-6668, DU-6611, and DW-8186 (Fig. 1), for DNA gyrases from DNS 5001, DNS 5002, DNS 5003, DNS 5004, and PAO1 were measured. The MICs and IC_{50}s of the four quinolones are listed in Table 2. DU-6611 (the 7-[3-(s)-amino-1-pyrrolidinyl] derivative of DU-6859) had MICs and IC_{50}s that were approximately one-half of or equal to those of DU-6859a for all strains tested. The MICs and IC_{50}s of DU-6668 (the 1-cyclopropyl derivative of DU-6859) and DU-6859a were comparable for all the strains tested. In contrast, the IC_{50}s of DW-8186 for DNA gyrase

TABLE 1. Antigyrase and antibacterial activities[a] of quinolones against *P. aeruginosa* strains

Drug	Concn (μg/ml) for strain:									
	DNS 5001		DNS 5002		DNS 5003		DNS 5004		PAO1	
	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC
DU-6859a	0.46	0.78	2.79	25	0.70	3.13	0.32	1.56	0.15	0.10
CPFX	4.54	12.5	831	200	5.14	25	4.64	25	0.34	0.10
LVFX	2.49	25	136	800	5.05	25	4.34	25	0.31	0.39
SPFX	6.49	12.5	509	400	5.60	50	4.86	50	0.33	0.39
TFLX[b]	5.50	12.5	>50	>50	6.32	>50	3.43	25	0.30	0.19

[a] Assays were performed as described in Materials and Methods.
[b] TFLX, tosufloxacin.

TABLE 2. Antigyrase and antibacterial activities[a] of DU-6859a and its derivatives

Drug[b]	Concn (μg/ml) for strain:									
	DNS 5001		DNS 5002		DNS 5003		DNS 5004		PAO1	
	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC
DU-6859a	0.46	0.78	2.79	25	0.70	3.13	0.32	1.56	0.15	0.10
DU-6611	0.24	0.39	3.76	12.5	0.36	3.13	0.33	0.78	0.083	0.05
DU-6668	0.42	1.56	1.60	25	0.64	3.13	0.54	1.56	0.16	0.10
DW-8186	1.12	6.25	16.9	>100	1.58	12.5	1.40	6.25	0.086	0.10

[a] Assays were performed as described in Materials and Methods.
[b] Chemical structures of the compounds are shown in Fig. 1.

mutants were two to six times greater than those of DU-6859a, while the IC_{50}s of DW-8186 for PAO1 were approximately half that of DU-6859a. Similar differences were observed with MICs. These results indicated that the potent activity of DU-6859a against DNA gyrase mutants is retained or even improved upon removal of the fluorine on the cyclopropyl substituent at the N-1 position or the cyclopropyl ring at the C-7 substituent; however, removal of the C-8 chlorine significantly increased the IC_{50}s as well as the MICs for the DNA gyrase mutants. On the basis of these results, we concluded that the unique activity of DU-6859a against DNA gyrase mutants of *P. aeruginosa* was mainly attributable to the C-8 chlorine substituent.

In order to obtain further information about the effect of the C-8 chlorine substituent against quinolone-resistant *P. aeruginosa*, the activities of DU-6859a, DU-6611, DU-6668, DW-8186, and CPFX against 20 quinolone-resistant clinical isolates of *P. aeruginosa* were compared (Table 3). The MICs of DU-6859a, DU-6668, DW-8186, and CPFX for PAO1 were 0.1 μg/ml, which was twice that of DU-6611. Against most of the quinolone-resistant strains, DU-6668 showed activity comparable to that of DU-6859a, but this activity was half that of DU-6611. In contrast, the MICs of DW-8186 and CPFX for all strains except DNS 5019 were more than twice that of DU-6859a. These results indicated that the effects of C-8 chlorine against four DNA gyrase mutants were similar in most of the other quinolone-resistant strains as well.

TABLE 3. Antibacterial activities of quinolones against 20 quinolone-resistant clinical isolates and PAO1

Strain	MIC (μg/ml) of:				
	DU-6859a	DU-6611	DU-6668	DW-8186	CPFX
DNS 5001	0.78	0.39	1.56	6.25	12.5
DNS 5002	25	12.5	25	>100	200
DNS 5003	3.13	3.13	3.13	12.5	25
DNS 5004	1.56	0.78	1.56	6.25	25
DNS 5005	1.56	0.78	1.56	6.25	6.25
DNS 5006	1.56	0.78	1.56	6.25	6.25
DNS 5007	6.25	3.13	12.5	25	25
DNS 5008	3.13	1.56	3.13	12.5	25
DNS 5009	3.13	1.56	3.13	6.25	12.5
DNS 5010	0.78	0.39	1.56	1.56	6.25
DNS 5011	3.13	1.56	3.13	12.5	25
DNS 5012	3.13	1.56	6.25	25	25
DNS 5013	3.13	1.56	1.56	6.25	12.5
DNS 5014	1.56	0.78	3.13	6.25	25
DNS 5015	3.13	1.56	3.13	12.5	12.5
DNS 5016	3.13	1.56	6.25	12.5	25
DNS 5017	25	12.5	25	100	>100
DNS 5018	1.56	0.78	1.56	1.56	1.56
DNS 5019	3.13	1.56	3.13	12.5	25
DNS 5020	50	25	25	>100	>100
PAO1	0.1	0.05	0.1	0.1	0.1

Contribution of subunits A and B of DNS 5001 and DNS 5002 gyrases to resistance. The IC_{50}s of LVFX for DNA gyrases from three clinical isolates (DNS 5001, DNS 5003, and DNS 5004) were comparable and were about eight times higher than those for PAO1 gyrase. However, the IC_{50}s of LVFX for DNS 5002 gyrase were more than 400 times that for PAO1 gyrase, which indicated that DNS 5002 may have mutations in the A or B subunit of DNA gyrase that are different from or additional to those in the other three clinical isolates. To clarify the resistance mechanism of these strains, the IC_{50}s of LVFX for DNA gyrases reconstituted by combination of A and B subunits from DNS 5001 (A_m and B_m, where m means moderately resistant), DNS 5002 (A_h and B_h, where h means highly resistant), and PAO1 (A_s and B_s, where s means susceptible) were assessed. The IC_{50}s of LVFX for A_s plus B_s, A_m plus B_s, A_s plus B_m, A_h plus B_s, A_s plus B_h, A_m plus B_m, and A_h plus B_h are shown in Table 4. Combination of any gyrase B subunit with the A subunit from PAO1 (wild type) altered the IC_{50} of LVFX, increasing or decreasing it by no more than a factor of 2. Furthermore, combination of wild-type subunit B with gyrase A from either quinolone-resistant strain did not significantly alter the IC_{50} from that seen with the resistant A and resistant B subunits. These results suggest that the resistance mechanism of DNS 5001 or DNS 5002 against quinolones is mainly due to one or more alterations in the subunit A protein. However, DNS 5002 may have different or additional mutations in the subunit A protein to give it greater quinolone resistance than DNS 5001 has.

TABLE 4. Inhibitory activities of LVFX against reconstituted DNA gyrase

Strain from which subunit derived		IC_{50} (μg/ml)[a]
Subunit A[b]	Subunit B[c]	
PAO1	PAO1	0.31
PAO1	DNS 5001	0.52
PAO1	DNS 5002	0.60
DNS 5001	PAO1	3.43
DNS 5001	DNS 5001	2.49
DNS 5002	PAO1	162
DNS 5002	DNS 5002	136

[a] Assays were performed as described in Materials and Methods.
[b] Subunits A from PAO1, DNS 5001, and DNS 5002 were designated A_s, A_m, and A_h, respectively.
[c] Subunits B from PAO1, DNS 5001, and DNS 5002 were designated B_s, B_m, and B_h, respectively.

DISCUSSION

Our data show that DU-6859a has considerably lower MICs and IC_{50}s for four DNA gyrase mutants of *P. aeruginosa* than other quinolones. Although we studied only 4 isolates from a total of 20 quinolone-resistant clinical isolates of *P. aeruginosa*, larger differences in MICs between DU-6859a and CPFX were also observed with all the other 16 isolates (Table 3). Moreover, our finding that all of four randomly selected strains possessed altered DNA gyrase supported the predominant quinolone-resistant phenotype for *P. aeruginosa* (30, 31). From these results, we concluded that DU-6859a shows much better activity against quinolone-resistant clinical isolates of *P. aeruginosa* than other currently available quinolones (21), probably because of its much better inhibitory effects against mutant DNA gyrases.

Good correlation ($r = 0.83$) between MICs and gyrase IC_{50}s of quinolones for clinical isolates DNS 5001, DNS 5003, DNS 5004 was observed, and this confirms that the main target of the quinolones is DNA gyrase (10). However, the correlation between MIC and IC_{50} for DNS 5002 was less than those observed for the other clinical strains (Fig. 2). For example, the MIC of LVFX for DNS 5002 was four times that of CPFX, while the IC_{50} of CPFX for DNS 5002 was more than four times that of LVFX. Since DNS 5002 is a clinical isolate, this strain may have other mechanisms of quinolone resistance in addition to DNA gyrase alterations. The differences between the MICs and the IC_{50}s might be due to different permeability profiles for the two compounds. On the other hand, several studies concerning the abilities of quinolones to inhibit other topoisomerases, such as topoisomerase I (26) and topoisomerase III (24), have been reported (15, 24). Moreover, recent reports suggest the possibility of topoisomerase IV (12) as the primary or secondary target of quinolones against *Staphylococcus aureus* and *Escherichia coli*, especially for DNA gyrase mutants (1, 8). It may follow, then, that these quinolones inhibit topoisomerases other than DNA gyrase in the highly resistant DNA gyrase mutant DNS 5002, preventing a direct correlation between MICs and IC_{50}s.

Quinolone resistance mutations were reported to be present in both the *gyrA* and *gyrB* genes, which produce the A and B subunits of DNA gyrase, respectively (14, 30). Yoshida et al. indicated that most of the quinolone-resistant clinical isolates of *P. aeruginosa* were not *gyrB* but *gyrA* mutants (30). Although *gyrA* mutations are thought to be the major mechanism conferring quinolone resistance, little is known about how resistance is caused by *gyrA* mutations. Our findings that DNS 5001 and DNS 5002 have a quinolone resistance alteration(s) in the A subunit of DNA gyrase suggested that DU-6859a showed potent activity against at least two *gyrA* mutants. Since DU-6859a is unique among fluoroquinolones in that it appears to be less affected by such alterations, further study of this drug might lead to a better understanding of the mechanism of resistance caused by *gyrA* mutations and of the interaction between subunit A of DNA gyrase and quinolones. Several studies have examined the interaction between DNA gyrase and quinolones (22, 28). Shen et al. suggested the possibility of an interaction between DNA gyrase and the quinolone C-7 substituent (22), which was proposed to interact with subunit B of DNA gyrase (28). Yoshida et al. proposed a quinolone pocket model, in which the quinolone binds to a gyrase-DNA complex with affinities determined by both A and B subunits in concert (27–29). Our study indicated that the C-8 substituent is important for activity against *gyrA* mutants of *P. aeruginosa*, which suggests the possibility of an interaction between the C-8 substituent and subunit A of DNA gyrase.

Since quinolone-resistant *P. aeruginosa* DNA gyrase was reported to have mutations in the so-called quinolone resistance-determining region that are similar to those of *E. coli* and *S. aureus* (5, 14, 17, 23, 27), it is possible that DU-6859a has potent activity against *gyrA* mutants of *E. coli* and *S. aureus* as well. Moreover, it was reported that another C-8 chloro quinolone, CI960, showed potent activity against ciprofloxacin-resistant *S. aureus* as well as *P. aeruginosa* in a way similar to that of DU-6859a (2, 25). However, Hoshino et al. suggested that such potent activity against mutant DNA gyrase was not clear in the case of *E. coli* or *S. aureus* (9). These data suggested that DU-6859a may be active against only some kinds of mutants with alterations in *gyrA*. Further studies concerning the structure-activity relationship of quinolones and genetic research on DNA gyrase mutants of *P. aeruginosa* are in progress.

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