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Structure-Activity Relationship of Fluoroquinolone in *Escherichia coli*

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Structure-activity relationship of 20 fluoroquinolones was studied using the susceptible and 4 resistant *Escherichia coli* which were developed against 4 fluoroquinolones [ciprofloxacin (1), KR-10755 (6), norfloxacin (2), and ofloxacin (3)] in our laboratory. The C-7 and C-8 substituents of fluoroquinolone were important in various functions such as the inhibitory activity on DNA gyrase, permeability, and efflux. Among 20 fluoroquinolones, compounds with a 3-methyl-3,7-diazabicyclo[3.3.0]octan-1(5)-ene-7-yl substituent at the C-7 position or a chlorine substituent at the C-8 position showed a good inhibitory activity on DNA gyrase (especially a mutated DNA gyrase). Compounds with a 3,7-diazabicyclo [3.3.0]octan-1(5)-ene-7-yl substituent at the C-7 position showed good permeability in the susceptible and resistant strains, while compounds with a fluorine substituent at the C-8 position were less effluxed from cells.

Key words : Fluoroquinolone, *Escherichia coli*, Structure-Activity-Relationship

INTRODUCTION

During the past decade, many efforts were focused on the synthesis of new fluoroquinolones and the evaluation of their antibacterial activities. As a result of these, extensive studies of the structure activity relationship (SAR) of fluoroquinolones have been published (Chu and Fernandes, 1989; Coll *et al.*, 1996; Gootz *et al.*, 1994; Kitamura *et al.*, 1995; Klopman *et al.*, 1996; Wolfson and Hooper, 1985). In most of the previous studies, however, the activity of fluoroquinolones was determined with MIC (Minimum Inhibitory Concentration) (Chu and Fernandes, 1989; Coll *et al.*, 1996; Klopman *et al.*, 1996; Wolfson and Hooper, 1985), and only a small number of studies have been reported about the inhibitory activity on DNA gyrase as the target site of fluoroquinolone (Gootz *et al.*, 1994; Kitamura *et al.*, 1995). There are two factors in determining MIC of fluoroquinolones: one is the inhibitory activity on the target site and the other is the concentration inside cells. To predict and design a new compound with an improved inhibitory activity, the contribution of each substituent to the inhibitory activity on DNA gyrase or permeability should be individually evaluated, and then an assembly of each substituent or a study on the cooperativity between each substituent should be followed. Recently, an energy dependent efflux system was reported as one of the resistant mechanism, so the structural characteristic that influence an efflux needs to be studied, too. Since the occurrence of resistant strains has been increasing as the use of fluoroquinolones increased, it is important to search for a better antibacterial agent capable of killing the resistant strain. Also, the emergence of resistance can shorten significantly the useful lifetime of an antibacterial agent.

In this work, we tried to find out the structure important for the inhibition of DNA gyrase, permeability, and efflux in the fluoroquinolone resistant strains as well as the susceptible strain.

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MATERIALS AND METHODS

Bacterial strains

The fluoroquinolone resistant strains used in this study were laboratory developed strains which were obtained by a serial passage of the susceptible strain *E. coli* 078 in nutrient broth (NB) containing gradually increasing concentrations of fluoroquinolones [ciprofloxacin (1), KR-10755 (6), norfloxacin (2), and ofloxacin (3)] (Lee *et al.*, 1992).

Antimicrobial agents and other reagents

KR series fluoroquinolones (Fig. 1) were synthesized at Korea Research Institute of Chemical Technology,

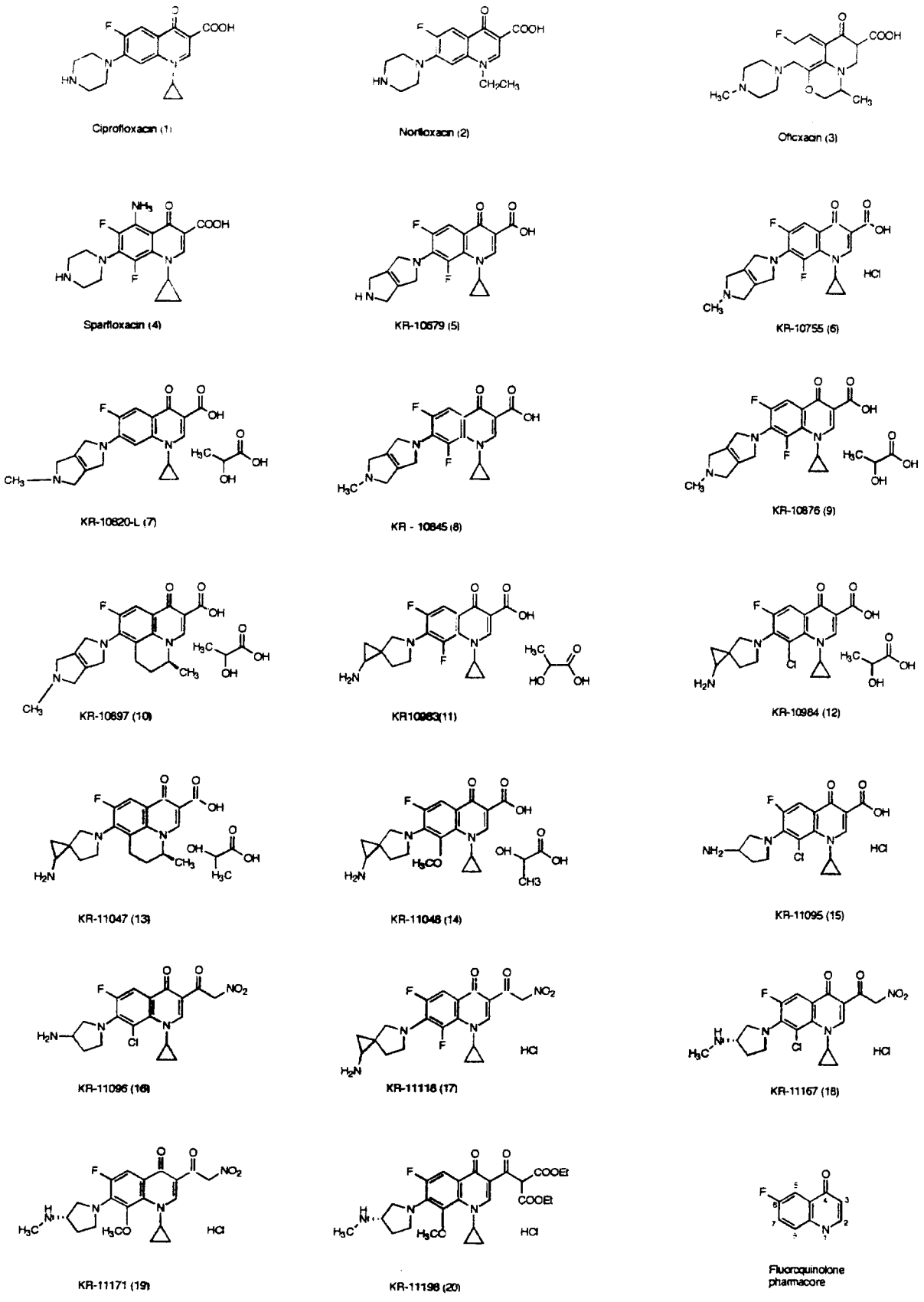

Fig. 1. The structures of twenty fluoroquinolones.

and the other fluoroquinolones were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]TTP (specific activity: 30 Ci/mmol) and [^{3}H]thymidine (specific activity: 50 Ci/mmol) were purchased from Amersham (Buckinghamshire, England). dNTP mixture was a product of Pharmacia Biotech (Uppsala, Sweden).

Effect of fluoroquinolone on *in vivo* DNA synthesis

The effect of fluoroquinolone on *in vivo* DNA synthesis was assayed as described by Park *et al.* (1996).

Effect of fluoroquinolone on *in vitro* DNA synthesis in permeabilized cells

In vitro DNA synthesis in permeabilized cells was assayed as described by Park *et al.* (1996).

Assay of fluoroquinolone inside cells

The concentration of fluoroquinolone inside cells was assayed as described by Kim *et al.* (1996). To examine the efflux effect, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to the cell suspension.

RESULTS

Effect of fluoroquinolone on *in vivo* DNA synthesis

Considering *in vivo* DNA synthesis in the absence of fluroquinolone as 100%, DNA synthesis in the susceptible strain was less than 40% of the control in the presence of each fluoroquinolone (Table I). In the 4 resistant strains, however, DNA synthesis was not decreased but rather enhanced by the addition of most fluoroquinolones except KR-10679(5). This fluoroquinolone showed potent inhibitory activity in the susceptible strain. There was no significant difference between the inhibitory activities of developed and KR series fluoroquinolones on the susceptible and the resistant strains.

Effect of fluoroquinolone on *in vitro* DNA synthesis

To observe the inhibitory activity of fluoroquinolone on DNA gyrase avoiding effects from an uptake barrier or an efflux system, DNA synthesis in the presence of fluoroquinolone was assayed using permeabilized cells. KR series fluoroquinolones had little inhibitory activity on *in vitro* DNA synthesis in the susceptible strain (Table II), while developed fluoroquinolones showed potent inhibitory activities. However, there was no difference between the inhibitory actvities of developed and most of KR series fluoroquinolones on the 4 resistant strains except KR10845(8) and KR11047(13) which showed better activities than developed fluoroquinolones. Interestingly, KR10845(8) with a 3-methyl-3,7-diazabicyclo[3.3.0]octan-1(5)-ene-7-yl substituent at the C-7 position showed a good activity on the resistant strains than on the susceptible strain. Also this compound showed better inhibitory activity than developed fluoroquinolones in the resistant strains. Fluoroquinolones with the same structure except a substituent at the C-7 position and in salt form were evaluated to characterize the structural importance of KR10845(8): first, KR10679(5), which does

Table I. Effect of fluoroquinolones on *in vivo* DNA synthesis

antimicrobial agents	DNA synthesis (%)				
	susceptible strain	ciprofloxacin resistant strain	KR-10755 resistant strain	norfloxacin resistant strain	ofloxacin resistant strain
ciprofloxacin (1)	18.9	244.2	235.1	211.3	250.7
norfloxacin (2)	18.0	190.5	320.2	276.9	273.7
ofloxacin (3)	13.2	380.3	288.9	287.6	328.9
sparfloxacin (4)	23.1	420.8	338.0	316.9	348.3
KR-10679 (5)	7.7	89.8	102.3	93.5	100.0
KR-10755 (6)	12.4	370.6	342.4	303.3	274.1
KR-10820-L (7)	20.6	527.2	273.7	503.5	321.0
KR-10845 (8)	10.5	395.6	354.4	260.4	331.4
KR-10876 (9)	12.8	289.8	336.7	278.2	223.0
KR-10897 (10)	13.9	456.9	343.0	258.7	301.5
KR-10983 (11)	10.8	300.8	362.1	232.2	283.2
KR-10984 (12)	11.4	320.8	294.1	198.0	210.3
KR-11047 (13)	12.7	402.9	255.6	366.2	317.3
KR-11048 (14)	14.6	471.4	283.9	340.4	302.7
KR-11095 (15)	10.8	115.6	156.4	71.9	338.5
KR-11096 (16)	15.7	420.7	325.1	380.6	270.6
KR-11118 (17)	18.1	416.5	368.6	269.3	263.6
KR-11167 (18)	15.0	356.5	273.3	341.3	362.1
KR-11171 (19)	33.6	329.5	362.0	339.4	375.0
KR-11198 (20)	23.7	287.2	278.6	290.6	129.3

Table II. Effect of fluoroquinolones on *in vitro* DNA synthesis

antimicrobial agents	DNA synthesis (%)				
	susceptible strain	ciprofloxacin resistant strain	KR-10755 resistant strain	norfloxacin resistant strain	ofloxacin resistant strain
ciprofloxacin (1)	23.8	158.7	87.7	65.7	51.9
norfloxacin (2)	18.1	161.0	98.9	72.2	55.9
ofloxacin (3)	19.4	98.7	102.3	72.0	65.4
sparfloxacin (4)	21.1	88.3	76.8	74.8	43.0
KR-10679 (5)	85.4	105.6	79.2	97.2	72.3
KR-10755 (6)	58.1	110.7	80.6	48.7	45.9
KR-10820-L (7)	76.2	97.4	65.2	66.7	48.1
KR-10845 (8)	80.8	76.9	69.5	55.0	41.3
KR-10876 (9)	67.1	94.2	70.1	63.5	45.7
KR-10897 (10)	78.0	110.6	73.4	68.3	62.2
KR-10983 (11)	52.4	99.2	75.2	77.2	52.8
KR-10984 (12)	67.5	82.3	78.4	49.6	48.8
KR-11047 (13)	85.6	85.7	73.2	62.4	48.0
KR-11048 (14)	64.8	108.1	76.4	56.1	51.2
KR-11095 (15)	64.5	101.9	75.7	62.4	48.2
KR-11096 (16)	72.9	127.5	70.9	63.3	59.9
KR-11118 (17)	83.0	91.7	80.3	71.1	55.5
KR-11167 (18)	84.1	86.7	88.6	65.1	68.3
KR-11171 (19)	80.8	116.1	94.7	97.0	54.7
KR-11198 (20)	82.8	130.4	72.2	67.3	63.7

not have a N-3-methyl group at the C-7 substituent, showed a weak inhibitory activity; second, the inhibitory activities of KR10755(6) with a hydrogen chloride and KR10876(5) with a lactic acid were better than that of KR10679(9) and less than that of KR 10845(8) in the resistant strains; third, KR10876(9) and KR10820-L(7) were characterized to determine the contribution of the C-8 substituent to the inhibition of *in vitro* DNA synthesis. Removal of the C-8 fluorine had a little negative effect on the inhibitory activity.

Compounds with chloro substituent at the C-8 position [KR10984(12), KR11095(15), KR11096(16), and KR11167(18) generally showed a good inhibitory activity on the mutated DNA gyrase. When the C-8 chlorine was replaced with other substituent such as methoxy group [KR11048(14) or KR11171(19)], the inhibitory activity was decreased.

Permeability and efflux of fluoroquinolones

KR10679(5) showed the best permeability in all strains (Table III) and this was the only one with better permeability than ciprofloxacin. In order to find out which substituent was responsible for the high permeability, KR10679(5) was compared with KR10845(8). The addition of a N-3-methyl group on a 3,7-diazabicyclo[3.3.0]octan-1(5)-ene-7-yl substituent at the C-7 position significantly decreased permeability, showing the importance of the C-7 substituent in the permeability.

Based on the difference of permeabilities between in the susceptible and the resistant strains, 20 fluoroquinolones could be grouped into three categories: first, compounds with higher permeabilities in the susceptible strain than the resistant ones [e.g., norfloxacin(2), sparfloxacin(4), KR10679(5), KR10983(11), and KR11048 (14)]; second, compounds with higher permeabilities in the resistant strains than the susceptible one [e.g., ofloxacin(3) and KR10755(6); third, compounds with high permeability in a certain resistant strain [e.g., KR 11047(13) with high permeability only in HK3140 resistant strain].

Sparfloxacin(4), KR10679(5), KR10876(9), and KR 10983(11) were less effluxed than ciprofloxacin in most of the strains (Table VI). The common structural charactristic of these was a fluorine at the C-8 position. On the other hand, compounds with a methoxy group at the C-8 position were better effluxed than ciprofloxacin. Fluoroquinolones with a 1-amino-5-azaspiro[2,4]heptane-5-yl substituent with lactic acid at the C-7 position [e.g., KR10984(12), KR11047(13), and KR11048(14) were less effluxed in the resistant strains than the susceptible one.

DISCUSSION

Because of the importance of fluoroquinolones as antimicrobial agents, the relationship between drug structure and its activity has been studied extensively.

Up to now, N-1, C-6, C-7, and C-8 positions were proven to be more malleable than other positions in quinolone moiety (Mitscher *et al.*, 1989; Mitscher *et al.*, 1993; Tillotson, 1996; Wentland, 1990). A small, lipophilic, and aliphatic substituent attached to N-1

Table III. Accumulated amounts of fluoroquinolone inside cells

antimicrobial agents	concentration of fluoroquinolone (μg/ml)									
	susceptible strain		ciprofloxacin resistant strain		KR-10755 resistant strain		norfloxacin resistant strain		ofloxacin resistant strain	
	-CCCP	+CCCP	-CCCP	+CCCP	-CCCP	+CCCP	-CCCP	+CCCP	-CCCP	+CCCP
ciprofloxacin (1)	1.49	1.65	2.47	2.77	0.81	1.11	1.19	1.11	1.78	1.86
norfloxacin (2)	0.82	1.79	1.39	1.07	1.32	0.94	0.98	0.95	0.99	1.49
ofloxacin (3)	0.84	0.70	1.26	2.15	0.88	0.78	0.86	0.81	1.15	2.08
sparfloxacin (4)	1.27	1.42	0.93	0.94	0.82	0.64	1.01	0.98	0.60	0.79
KR-10679 (5)	6.49	8.37	5.32	4.46	7.83	7.00	6.11	7.40	6.15	6.90
KR-10755 (6)	0.69	0.51	0.97	1.37	0.84	0.62	0.63	0.99	0.67	1.19
KR-10820-L (7)	0.92	1.16	1.23	2.33	0.46	0.79	0.80	0.82	0.76	0.98
KR-10845 (8)	0.91	1.44	1.74	2.08	0.99	1.10	0.88	0.69	1.09	0.77
KR-10876 (9)	0.97	1.00	0.74	0.87	0.83	0.91	0.90	0.85	0.72	0.92
KR-10897 (10)	0.58	1.33	0.98	1.28	0.89	0.64	0.67	0.70	0.64	0.73
KR-10983 (11)	1.32	1.45	0.67	0.65	0.53	0.43	0.88	2.22	1.10	0.74
KR-10984 (12)	0.50	0.78	1.13	1.71	1.05	0.61	1.10	0.76	0.92	0.74
KR-11047 (13)	1.05	1.69	0.53	0.56	1.43	1.71	0.98	1.00	0.93	1.27
KR-11048 (14)	0.64	1.49	1.34	0.76	0.65	1.29	0.84	0.83	0.73	0.70
KR-11095 (15)	1.01	1.00	1.00	1.15	0.48	0.98	0.77	0.73	0.69	0.63
KR-11096 (16)	0.97	0.62	1.25	0.94	0.43	0.55	1.03	0.73	0.57	1.04
KR-11118 (17)	1.19	1.20	1.46	2.01	1.14	1.92	1.07	1.14	1.45	0.83
KR-11167 (18)	0.76	1.29	0.71	1.99	0.70	1.19	0.98	0.64	1.08	0.70
KR-11171 (19)	1.00	0.98	1.61	2.11	0.58	1.06	0.79	0.96	0.77	1.21
KR-11198 (20)	0.71	1.00	0.75	0.81	0.90	0.78	0.76	0.99	0.63	1.07

Table IV. Efflux effects

antimicrobial agents	efflux effect (%)*				
	susceptible strain	ciprofloxacin resistant strain	KR-10755 resistant strain	norfloxacin resistant strain	ofloxacin resistant strain
ciprofloxacin (1)	9.3	10.8	27.3	-6.8	4.7
norfloxacin (2)	54.1	-30.3	-40.4	-2.6	33.6
ofloxacin (3)	-20.6	41.4	-12.8	-6.1	44.9
sparfloxacin (4)	10.3	1.2	-27.3	-2.9	24.5
KR-10679 (5)	22.5	-19.2	-11.8	17.3	11.0
KR-10755 (6)	-33.3	29.0	-36.8	36.0	43.6
KR-10820-L (7)	20.9	47.1	41.4	2.4	22.2
KR-10845 (8)	37.1	16.3	10.2	-27.0	-40.4
KR-10876 (9)	3.1	15.2	8.8	-6.5	21.0
KR-10897 (10)	56.1	23.4	-39.1	4.8	11.5
KR-10983 (11)	9.0	-3.1	-22.0	27.5	-48.7
KR-10984 (12)	35.2	34.2	-72.2	-44.3	-24.2
KR-11047 (13)	38.0	5.1	16.5	1.8	27.1
KR-11048 (14)	56.9	-75.5	50.0	-1.1	-5.1
KR-11095 (15)	-0.9	13.4	50.6	-5.3	-10.7
KR-11096 (16)	-57.0	-33.0	21.3	-40.2	45.0
KR-11118 (17)	1.0	27.6	40.9	6.4	-75.0
KR-11167 (18)	40.7	64.5	41.1	-53.6	-54.3
KR-11171 (19)	-1.8	23.8	45.5	18.2	36.2
KR-11198 (20)	29.2	7.4	-16.2	22.6	40.8

*efflux effect (%)=(b-a)/b × 100 (a, accumulated fluoroquinolone inside cells in the absence of CCCP; b, accumulated fluoroquinolone inside cells in the presence of CCCP).

led to the best inhibitory activity (Mitscher *et al.*, 1993) and C-6 fluorine substituent was superior in MIC to all others so far (Chu and Fernandes, 1989; Mitscher *et al.*, 1993). The possession of a five- or six-membered nitrogen heterocycle at the C-7 position improved an inhibitory activity (Tillotson, 1996). The most useful groups on the C-8 position were small and hydrophobic like fluorine, chlorine, and methyl (Mitscher *et al.*, 1993). Twenty compounds used in this work had a big variation at the C-7 and C-8 position than other positions.

In *in vivo* DNA synthesis, KR10679(5) and KR11095 (15) showed potent activities in the resistant strains. It was proved that the potent activity of KR10679(5) was resulted not from a good inhibitory activity on DNA gyrase but from a good permeability. However, the factor for a potent inhibitory activity of KR11095 (15) could not be determined.

One interesting thing was that *in vivo* DNA synthesis in the resistant strains was increased above the control (DNA synthesis in the absence of fluoroquinolone) in the presence of fluoroquinolone except KR-10679(5). It was not clear why the addition of fluoroquinolone increased DNA synthesis. We assumed that quinolone induced DNA damage and increased DNA synthesis to repair.

In *in vitro* DNA synthesis, developed fluoroquinolones lost their potent activities on the resistant strains. This must have been caused by the mutations of target site-DNA gyrase or topoisomerase IV. When quinolone resistance determining regions of DNA gyrase and topoisomerase IV of 4 resistant strains were sequenced, mutations were found (data not shown). The common structural characteristic of developed fluroquinolones was a (4-methyl)piperazinyl group at the C-7 position which gave a strong inhibition on the DNA gyrase of the susceptible strain but not on the mutated DNA gyrase.

A previous study by Gootz *et al.* (1994) suggested that a methyl group on a piperazinyl substituent was not an important factor on the inhibitory activity on the DNA gyrase in contrast to mammalian topoisomerase II. Ofloxacin which has a methyl group on a piperazinyl substituent showed no difference in activity compared with other developed ones. On the other hand, a methyl group on a 3,7-diazabicyclo[3.3.0]octan-1(5)-ene-7-yl substituent showed a good inhibitory activity on the mutated DNA gyrases, which was shown by the comparison of KR10845(8) with KR10679(15). However, the addition of other group such as lactic acid or hydrogen chloride on this substituent decreased the inhibitory activity, indicating that the characteristic of a C-7 substituent was an important factor on the mutated DNA gyrase.

Kitamura *et al.* (1995) suggested that the addition of chlorine at the C-8 position had an increased inhibitory effect on the mutated DNA gyrase. In our study, the compounds with chlorine at the C-8 showed a good inhibitory activities on the mutated DNA gyrases, and substitution of chlorine with other group (such as methoxy group) decreased the inhibitory activity. Fluorine as well as chlorine at the C-8 position showed positive inhibitory effects. From these, it could be said that the C-8 position was important on the inhibitory activity on the mutated DNA gyrase.

Shen *et al.* (1989) suggested that a C-7 substituent is a critical factor in the interaction with DNA gyrase. This suggestion was also agreed with our results. However, according to the results using the resistant strains, it seemed that both C-7 and C-8 were involved in the interaction with the mutated DNA gyrase. The interaction mechhanism of DNA gyrase, fluoroquinolone, and DNA has not been known yet, but presumed only by yeast topoisomerase II (Pan *et al.*, 1996). So it is essential to define SAR of fluoroquinolone and the interaction mechanism with DNA gyrase for developing a better compound.

There are two factors in determining the fluoroquinolone concentration inside cells: one is the permeability of compounds and the other is the efflux of compounds. Structural characteristics that promote permeability or induce lower efflux of compounds have not been well characterized. In this work, KR-10679(5) with a 3,7-diazabicyclo[3.3.0]octan-1(5)-ene-7-yl substituent at the C-7 position showed about six times higher permeability compared with the other compounds. As mentioned above, methyl group at this position had a good activity on the mutated DNA gyrase. Considering the activity of KR10679(5) on the permeability and DNA gyrase together, the potent inhibitory activity of KR-10679(5) on *in vivo* DNA synthesis was due to the good permeability rather than the inhibitory activity on DNA gyrase. However, since MIC of fluoroquinolone in the resistant strain was known to be more affected by the mutation of DNA gyrase than that of entry routes (Heisig *et al.*, 1993; Hirai *et al.*, 1986), we suggest a structure with more potent activity on the mutated DNA gyrase is better than the one with good permeability.

It was very difficult to find out a specific structural charateristic for the less efflux. Only the C-7 and C-8 substituents affected efflux. There might be two possibilities for the poor relationship between the structure and efflux: first, genes for an efflux pump could be mutated, so the kinds of well or poorly effluxed compounds could be quite different in every strains; second, several substituents could be involved in the affinity for an efflux pump. The whole size or charge of a quinolone might determine the affinity for the efflux pump.

Some compounds showed negative efflux, in other words, fluoroquinolone was less accumulated inside

cells in the presence of CCCP than in the absence of CCCP. It seemed that cells died at a high concentration of CCCP, as we reported that CCCP had a different inhibitory effect on each strain in a previous paper (Park *et al.,* 1996).

Conclusively, we suggest that the C-7 and C-8 substituent were important factors on various functions such as inhibitory activity on DNA gyrase, permeability, and efflux of fluoroquinolone. Among these 20 fluoroquinolones, compounds with a 3-methyl-3,7-diazabicyclo[3.3.0]octan-1(5)-ene-7-yl substituent at the C-7 position or a chlorine substituent at the C-8 position showed better inhibitory activity on DNA gyrase, especially on the mutated DNA gyrase, compounds with 3,7-diazabicyclo[3.3.0]octan-1(5)-ene-7-yl substituent at the C-7 position showed good permeability in both of the susceptible and resistant strains, and finally compounds with a fluorine substituent at the C-8 position were less effluxed from cells.

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