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Studies on the antibacterial activity of novel fluoroquinolones against quinolone resistant *Salmonella* Typhimurium

(キノロン耐性 *Salmonella* Typhimurium に対する新規フルオロキノロンの抗菌作用に関する研究)

Kentaro Koide

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ABBREVIATIONS

95%CI	95% Confidence interval
Asp87Asn	Amino acid substitution from aspartic acid at position 87 to asparagine
Asp87Gly	Amino acid substitution from aspartic acid at position 87 to glycine
Asp87Tyr	Amino acid substitution from aspartic acid at position 87 to tyrosine
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CLSI	The Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
GyrA	DNA gyrase subunit A
GyrB	DNA gyrase subunit B
His-tag	Hexa-histidine tag
IC_{50}	Half maximal inhibitory concentration
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria-Bertani

MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant Staphylococcus aureus
MSSA	Methicillin susceptible Staphylococcus aureus
NCBI	National Center for Biotechnology Information
Ni-NTA	Nickel-nitrilotriacetic acid
OD	Optical density
QRDR	Quinolone resistance determining region
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser83Phe	Amino acid substitution from serine at position 83 to Phenylalanine
TBE	Tris-borate-EDTA
Tris	Tris hydroxy methyl aminomethane
WHO	World Health Organization
WT	Wildtype

PREFACE

Nontyphoidal *Salmonella* is an important causative pathogen of foodborne diseases. WHO estimated that diseases caused by 31 foodborne hazards and nontyphoidal *Salmonella* infection were among the greatest threats to human health [26]. *Salmonella enterica* is the main gastroenteritis- and invasive nontyphoidal *Salmonella* infection-causing bacterial species. In addition, this bacterial species has more than 2,500 serovars and, among them, *Salmonella enterica subsp. enterica* serovar Typhimurium and *Salmonella enterica subsp. enterica* serovar Enteritidis are two of the major foodborne infection- causing serovars [23]. In general, antibiotics do not expedite gastroenteritis resolution, prolong the excretion of the organism, and are not recommended in cases without complications. However, elders, infants and people with immunocompromised conditions require treatment with antibiotics to prevent increased mortality. Chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole were formerly used in the treatment of severe gastroenteric cases, particularly in treating susceptible patients such as the elderly and infants [7]. However, an increasing rate of resistance to these antibiotics in nontyphoidal *Salmonella* has been reported in many countries for the last two decades [16, 56]. The emergence of antimicrobial resistant bacteria has been attributed to the misuse and overuse of antibiotics [59]. In the veterinary field, a large amount of antibiotics is used in sub-therapeutic doses for the growth promotion during animal production along with timely exposure period, creating an ideal environment for selecting bacteria which have genes

that are prone to resistance [61]. The outright overuse of antibiotics may accelerate the emergence of antimicrobial resistant nontyphoidal *Salmonella* because the prevalent reservoir of nontyphoidal *Salmonella* is the intestinal tract of a wide range of animals, for instance cattle and poultry [25]. Therefore, the interdisciplinary research between veterinary and human medicine under the concept of "One Health" is essential to address the growing threat of food borne disease caused by antimicrobial resistant nontyphoidal *Salmonella*.

Quinolones are known to be effective against nontyphoidal *Salmonella* which are resistant to multiple drugs used in treatment currently [53]. Quinolones are synthetic antibacterial drugs used to treat bacterial infections, with nalidixic acid considered the first-generation synthetic quinolone[2]. Initially, nalidixic acid was only effective against Gram-negative bacteria and thus its antibacterial spectrum was narrow. However, the antibacterial activity of nalidixic acid was drastically improved by the addition of a fluorine atom at the position C6 of the quinolone ring [4, 15]. Quinolones fluorinated at the position C6 with an improved spectrum are especially known as fluoroquinolones [4]. Ciprofloxacin is a representative fluoroquinolone categorized as second-generation quinolone [48]. In general, ciprofloxacin is safe for eukaryotes, with high treatment efficacy and a wide antibacterial spectrum. Therefore, ciprofloxacin is a clinically useful antimicrobial drug that has been selected by WHO as an essential medicament [62]. Moreover, fourth-generation fluoroquinolones (e.g. sitafloxacin) was developed by substituting groups at N1, C7 and C8 positions of the basic structure

(Figure 1) [48]. They have broad-spectrum potent antibacterial activity. Consequently, fluoroquinolones are regarded as one of the most clinically important antibiotics.

However, clinical isolates of nontyphoidal *Salmonella* resistant to quinolones have emerged and spread rapidly [7, 22, 35, 44]. Hence, previously treatable infectious diseases caused by nontyphoidal *Salmonella* nowadays are becoming fatal to people with low immunity due to the unavailability of effective antibacterial drugs. Resistance to quinolones is typically caused by bacterial mutation due to amino acid substitutions in DNA gyrase, the target enzyme of quinolone [51]. DNA gyrase consists of two GyrA and two GyrB subunits [51]. The function of DNA gyrase is to introduce negative supercoils into the DNA to relieve the accumulation of twisted bacterial DNA prior to enzymic translocation [8]. Therefore, this enzyme is essential for DNA replication and transcription. Quinolones bind to DNA gyrases and inhibit enzymic activity, resulting in a bactericidal effect. However, quinolones cannot bind to DNA gyrases when a specific mutation has occurred on the binding site of DNA gyrases because this mutation first alters the structure of the target enzyme and subsequently the binding affinity of fluoroquinolones to this enzyme, resulting in quinolone resistance [51]. These mutations have been clustered in a region of the gene product between amino acids 67 and 122, so this region is called QRDR [2]. In particular, amino acid substitutions at position 83 and 87 in GyrA has been frequently detected in quinolone resistant nontyphoidal *Salmonella* [47, 58]. Amino acids at these positions are considered to be strongly

involved in the binding to quinolones [3), 54)]. Therefore, it is critical to find quinolones showing high affinity to DNA gyrases with amino acid substitutions at positions 83 and 87.

In my PhD study, inhibitory effects of two newly developed fluoroquinolones, DC-159a and WQ-3810, on DNA gyrase were evaluated. There was no report regarding the antibacterial activity of these fluoroquinolones against quinolone resistant nontyphoidal *Salmonella*. This thesis is composed of the two chapters and conclusion. In Chapter I, the inhibitory effect of DC-159a against the activity of *S.* Typhimurium was evaluated. In Chapter II, the inhibitory effect of another novel fluoroquinolone, WQ-3810, on DNA gyrase was investigated. By summarizing these two results, this study will contribute to investigation of the promising therapeutic agents against quinolone resistant nontyphoidal *Salmonella*.

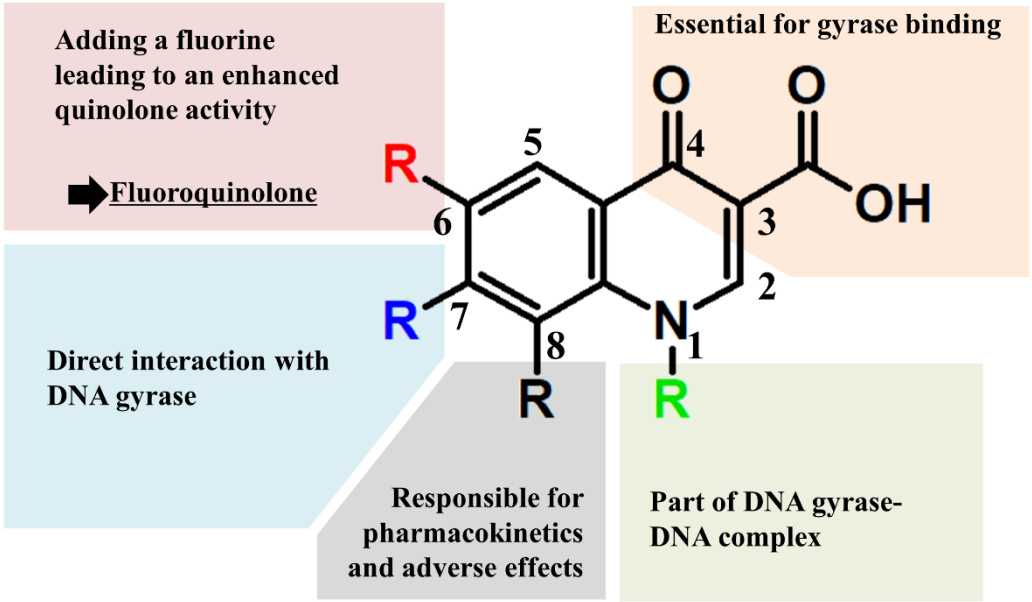

Figure 1. Basic structures of quinolone (modified *M. Andersson et al.,* 2003. and *R. Schaumann, et al.,* 2007.)

CHAPTER I

Inhibitory effect of DC-159a on DNA gyrase

Introduction

DC-159a is a newly developed fluoroquinolone. Many studies have reported its high antibacterial activity. The first report which was published in 2008 by Hoshino et al. showed MIC of DC-159a was lower against MSSA and quinolone susceptible MRSA than ciprofloxacin [24]. In particular, DC-159a exerted the highest antibacterial activity against quinolone resistant MRSA compared with ciprofloxacin and the other clinically available quinolones, which DC-159a can be an effective agent against quinolones resistant bacteria. However, for *Escherichia coli* and *Salmonella spp.*, MIC of DC-159a was higher than ciprofloxacin. DC-159a showed very different antibacterial activity for many pathogens. In another study published in the same year by Clark et al., DC-159a yielded lower MIC against quinolone susceptible and resistant *Streptococcus pneumoniae* than the other quinolone [10]. In the study. MIC of DC-159a against quinolone resistant strains did not depend on the number or type of mutations in QRDR. Thus, DC-159a might have a strong inhibitory activity on mutant DNA gyrase in quinolone resistant bacteria. Direct inhibitory effect of DC-159a on DNA gyrase was investigated by two groups, Okumura et al and Yamaguchi et al [39, 63]. They evaluated direct inhibitory effects of DC-159a on DNA gyrase of *Streptococcus pneumoniae* and *Mycobacterium*

leprae. In the results, DC-159a inhibited DNA gyrase activity as much as or more than clinically available quinolones. Moreover, DC-159a showed a strong inhibitory effect on mutant DNA gyrase containing amino acid substitution on GyrA in the study by Yamaguchi et al. Therefore, DC-159a can be expected to have a potent inhibitory effect regardless of amino acid substitutions in DNA gyrase of *S*. Typhimurium.

No study of DC-159a has yet been made on quinolone resistant *Salmonella*. Given that high inhibitory effect on DNA gyrase with amino acid substitutions on GyrA is demonstrated, the high antibacterial activity of DC-159a is expected as a therapeutic agent for foodborne disease caused by quinolone resistant nontyphoidal *Salmonella*. In this chapter, the inhibitory effect of DC-159a against quinolone resistant DNA gyrase were evaluated and also the antibacterial activity of DC-159a against quinolone resistant nontyphoidal *Salmonella* was estimated.

Materials and methods

Quinolone used in this chapter

DC-159a was kindly provided by Daiichi-Sankyo Co., Ltd. (Tokyo, Japan). Ciprofloxacin and nalidixic acid were used as comparators in this study. Those were purchased form LKT Laboratories, Inc. (St Paul, MN) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. Chemical structures of each quinolone were shown in Figure 2.

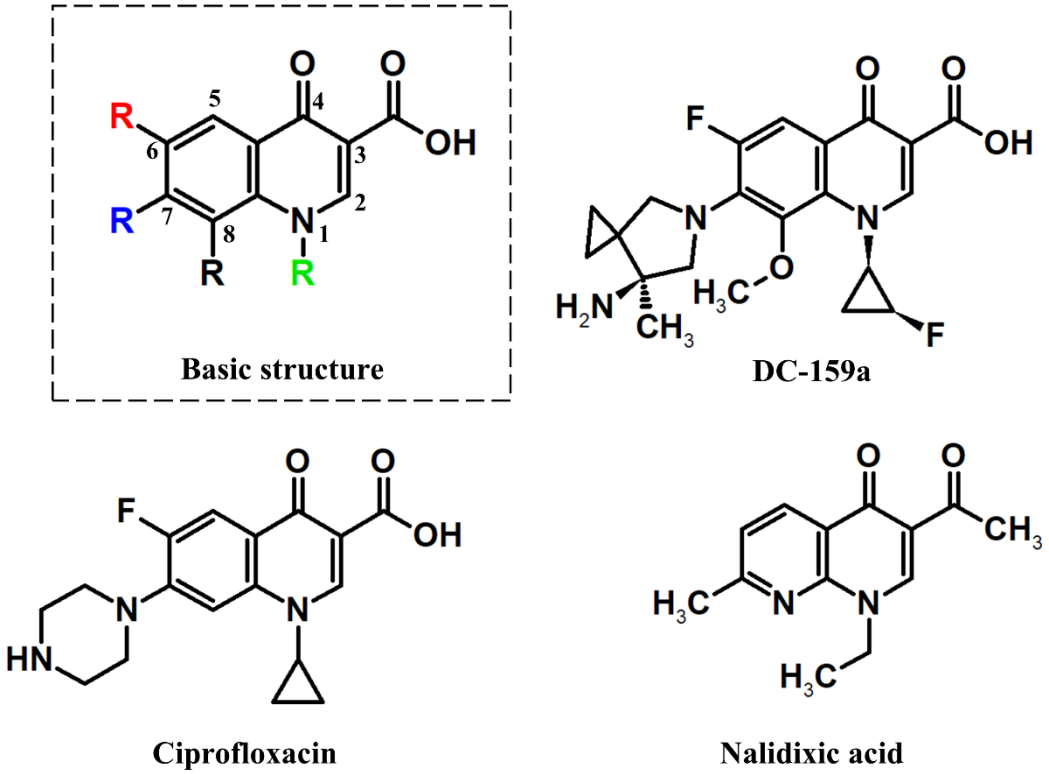

Figure 2. Chemical structures of quinolones used in this chapter

Recombinant DNA gyrases

Recombinant DNA gyrase was obtained as separate subunits, GyrA and GyrB. Seven subunits in total were produced in this study. Expression plasmids, including the coding regions of GyrA and GyrB of *S*. Typhimurium, were previously constructed [30]. Mutations Ser83Phe, Asp87Asn, Asp87Gly, Asp87Tyr, and Ser83Phe-Asp87Asn were introduced into QRDR. The plasmids were then introduced into *Escherichia coli* BL21(DE3) (Merck KGaA, Darmstadt, Germany), and protein expression was induced in the bacterial cells. A colony of transformed *E. coli* was inoculated into Luria-Bertani broth with ampicillin (1 μg/ml) and incubated at 37 □C until the optical density value reached 0.60. Protein expression was induced by adding 1 mM IPTG, purchased from Wako Pure Chemical Industries, Ltd. The incubation period of recombinant *E. coli* carrying the GyrA expression plasmid was 40 hr, and the temperature after adding IPTG was 16 ºC. Separately, recombinant *E. coli* carrying GyrB was incubated for 13 hr at 18 ºC. Harvested *E. coli* was centrifuged, and then sonicated at a 30% duty cycle with 10 cycles of 40 sec on and 40 sec off (Sonifier 250; Branson, Danbury, CT) to release the expressed protein. The recombinant DNA gyrase subunits were then purified by Ni-NTA agarose column chromatography and dialyzed against DNA gyrase dilution buffer (50 mM Tris-HCl, pH 7.5; 100 mM KCl, 2 mM DTT, 1 mM EDTA). To avoid denaturation, glycerol was added to the obtained protein. Finally, the mixture was stored in small aliquots at -80 ºC until further use. SDS-PAGE was used to assess the purity of proteins.

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Evaluation of quinolone concentration to inhibit the enzyme activity of DNA gyrase

DNA gyrase introduces negative supercoils into bacterial DNA as described in Preface. The supercoiling activity of the purified DNA gyrases was detected using agarose gel electrophoresis. Relaxed pBR322 (302.4 ng) and DNA gyrases (GyrA: 30.6 ng, GyrB: 28.3 ng) were incubated at 35 ^{o}C in a gyrase assay solution [35 mM Tris-HCl, 6 mM $MgCl_2$, 1.8 mM spermidine, 24 mM KCl, 5 mM DTT, 0.36 mg/ml of BSA and 6.5% glycerol (w/v)]. This reaction mixture contained serially diluted concentrations of quinolones. After 40-min incubation, the reaction was stopped by adding 8 μl of a stop solution (5% SDS, 25% glycerol and 0.25 mg/ml of bromophenol blue) [18]. Next, 10 μl of the reaction mixture was loaded onto 1% agarose gel in 0.5xTBE buffer for electrophoresis for two hours at 40 mA. The supercoiling activity of DNA gyrases can be easily distinguished and separated by gel electrophoresis because gyrases introduce negative supercoils into relaxed DNA, and supercoiled DNA is more compact than relaxed DNA [28), 31)]. After electrophoresis, the agarose gel was stained with 0.5 μg/ml of ethidium bromide. The presence of supercoiled DNA was confirmed under UV light. The amount of supercoiled DNA was measured by the intensity of its band using digital image processing software ImageJ (http://rsbweb.nih.gov/ij). All assays were run in triplicate to eliminate experimental bias and to confirm reproducibility.

The concentration of each quinolone required to reduce by 50% DNA gyrase activity (IC_{50})

was used as the baseline to assess their inhibitory effect. Intensity of the supercoiled DNA band of a quinolone-free sample was defined as 100% control. Data of the decreasing DNA band intensity caused by incremental quinolone concentration was fitted to a four-parameter log-logistic model. IC_{50} was estimated using a model for dose-response[34), 52)]. In addition to the calculation of the parameters of the model and the estimation of IC_{50}, the 95% confidence interval of IC_{50} was also estimated. All analyses were conducted using statistical software R version 3.2.5 and the add-on package "drc" version 3.0.

Antimicrobial susceptibility testing

MIC was measured by broth microdilution method. Antibacterial activity of DC-159a was tested using two strains of nontyphoidal *Salmonella*, *S*. Typhimurium NBRC 13245 and *S*. Enteritidis NBRC 3313, according to CLSI recommended procedures [11)]. Bacterial suspension of each strain was transferred to a 96-well tray containing serially diluted quinolones and incubated overnight at 37 ºC. MIC was defined as the lowest concentration of quinolone that completely inhibits bacterial growth in the well. This examination was run in triplicate for each strain.

Estimation of MICs against *S*. Typhimurium and *S*. Enteritidis carrying mutant DNA gyrase

Based on the data from a previous study of *S*. Typhimurium DNA gyrase, it was estimated

that there was a correlation between the different quinolones [20), 31)]. Moreover, as DC-159a showed the same logarithmic correlation between the IC_{50} and the corresponding MIC, it was possible to estimate the MICs of DC-159a against mutant strains with single- or double-mutant DNA gyrases from the results of the supercoiling inhibitory assay. Regression lines were calculated based on log(IC_{50}) and log(MIC) of the three quinolones against WTs using *S*. Typhimurium and *S*. Enteritidis in a separate manner. Next, log(MIC) was calculated from the regression coefficient and log(IC_{50}), and the estimated MIC was determined by converting the variable log(MIC) from logarithm to integer.

Results

IC_{50}s of DC-159a and other quinolones

Inhibitory effects of DC-159a and the other quinolones were evaluated using purified DNA gyrases. The recombinant subunits were successfully prepared with high purity, which was confirmed by SDS-PAGE. (Figure 2) Electrophoretic patterns of supercoiling activity attenuated by IC_{50}s of quinolones are shown in Figure 3. Inhibition activity of DC-159a against WT and mutant DNA gyrases of *S*. Typhimurium was estimated by the IC_{50}, and compared with that of ciprofloxacin and nalidixic acid. Dose-dependent inhibition activity of every quinolone within the 0.25 – 600 μg/ml range was confirmed. The inhibitory effects of tested quinolones are shown in Figure 4. The IC_{50}s of each quinolone are summarized in Table 1. The IC_{50}s of DC-159a and ciprofloxacin against WT DNA gyrases were 0.32 μg/ml (95% CI: 0.25 – 0.40 μg/ml) and 0.25 mg/mL (95% CI: 0.19 – 0.32 μg/ml), respectively. There was no significant difference between them. However, DC-159a showed more potent activity against mutant DNA gyrases than did ciprofloxacin. Furthermore, the IC_{50}s of DC-159a against mutant DNA gyrases were significantly different from those of ciprofloxacin. The biggest differences between the IC_{50}s of DC-159a and ciprofloxacin were observed in double mutant DNA gyrases. For example, the IC_{50} of DC-159a against the DNA gyrase bearing double mutation Ser83Phe-Asp87Asn was less than 1/55 that of ciprofloxacin. Moreover, while the IC_{50} of DC-159a against double mutant DNA gyrases showed a 27.5-fold increase in comparison with that against WT DNA gyrase, the IC_{50} of ciprofloxacin against double-mutant DNA gyrase showed a 1,920-fold

increase when compared with that against WT. In contrast, the concentration of nalidixic acid required to achieve supercoiling inhibition activity against every WT and mutant DNA gyrase was much higher than those of DC-159a and ciprofloxacin.

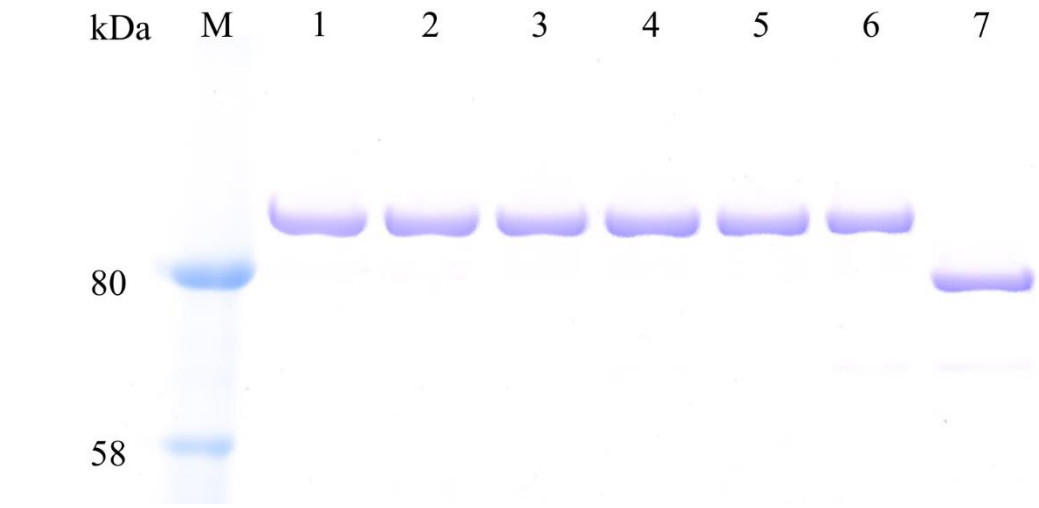

Figure 2. SDS-PAGE analysis of purified DNA gyrase subunits.

Table 1. IC_{50}s of quinolones against WT and mutant DNA gyrases.

Quinolone	IC_{50} (μg/ml)					
	WT	Ser83Phe	Asp87Asn	Asp87Gly	Asp87Tyr	Ser83Phe-Asp87Asn
DC-159a	0.32 (0.25 – 0.40)	1.0 (0.94 – 1.1)	1.4 (1.2 – 1.6)	1.2 (1.0 – 1.4)	1.2 (0.92 – 1.4)	8.8 (7.4 – 10)
Ciprofloxacin	0.25 (0.19 – 0.32)	3.6 (3.3 – 4.0)	3.9 (3.1 – 4.7)	2.6 (2.2 – 2.9)	3.6 (2.8 – 4.5)	480 (420 – 530)
Nalidixic acid	28 (14 – 42)	450 (370 – 540)	440 (330 – 540)	370 (270 – 460)	530 (410 – 650)	600 (450 – 750)

Note: The range in brackets is the 95% confidence interval.

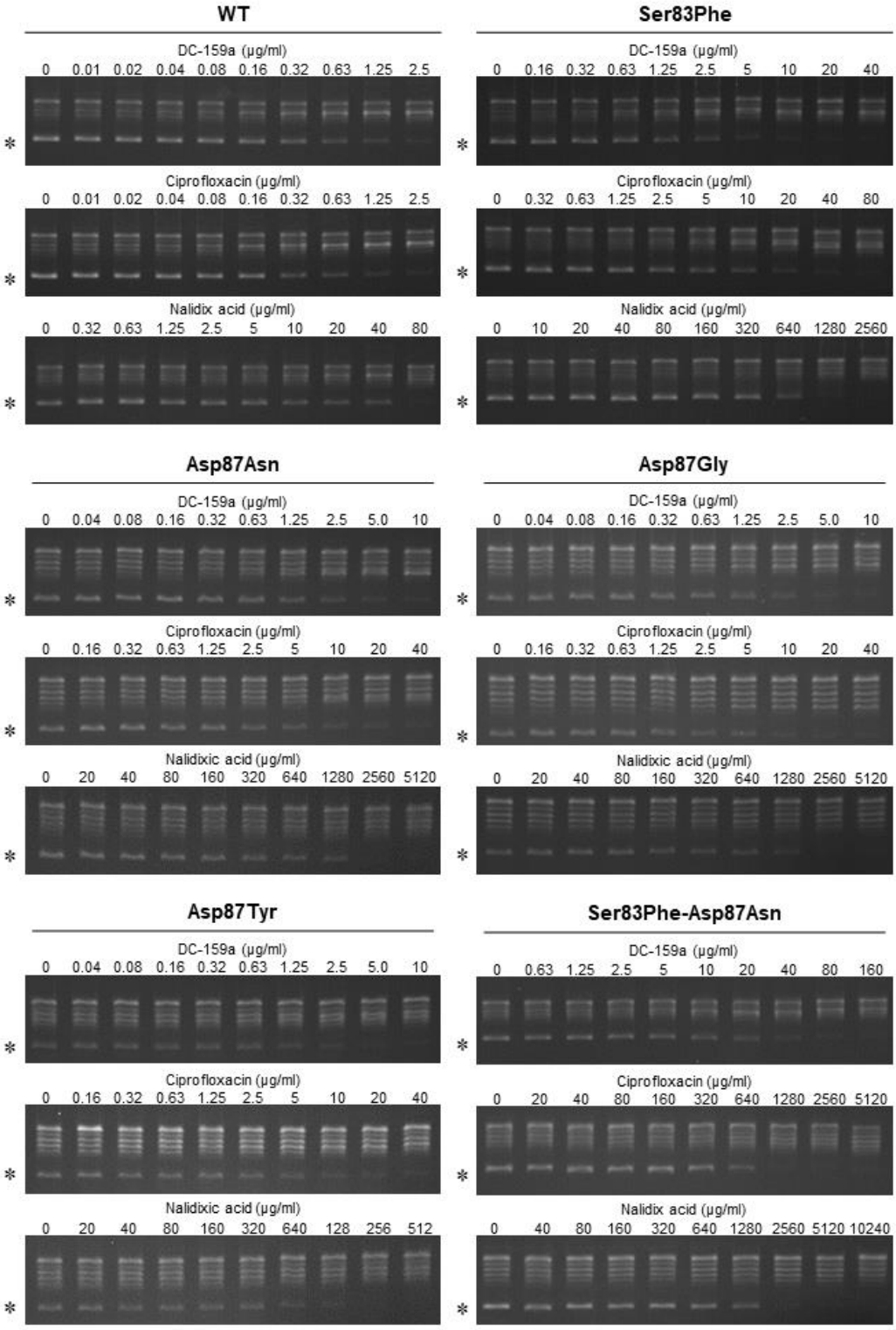

Figure 3. Supercoiling inhibitory activity of quinolones. Asterisks (*) indicate the band of supercoiled DNA.

MICs of DC-159a and other quinolones

To examine and compare the bactericidal effect of DC-159a with that of ciprofloxacin and nalidixic acid, MICs against *S.* Typhimurium and *S.* Enteritidis standard strains were measured. The results of antimicrobial susceptibility testing are shown in Table 2. MICs of DC-159a against these two strains were different, with the growth inhibitory activity of DC-159a being higher than that of nalidixic acid but lower than that of ciprofloxacin.

Estimated MICs of quinolones

Based on the above results of IC_{50} and MIC, MICs of DC-159a, ciprofloxacin, and nalidixic acid against *Salmonella* carrying mutant DNA gyrase were estimated. The estimates were summarized in Table 3. The increase of estimated MIC was confirmed from the MIC for type strains of *S.* Typhimurium and *S.* Enteritidis. According to the CLSI standard, breakpoints of ciprofloxacin for *Salmonella spp.* are classified as: ≤ 0.06 μg/ml, susceptible; 0.12 – 0.5 μg/ml, intermediate; ≥ 1.0 μg/ml, resistant[11]. For strains with single mutant DNA gyrases, the MIC of ciprofloxacin was estimated to be in the range of 0.33 to 0.60 μg/ml in this study, which all strains were classified as an intermediate. The estimated MIC against strains carrying double mutant DNA gyrase were 70.0 and 85.4 μg/ml. These are much higher than the breakpoint of resistance. In nalidixic acid, all estimates are more than 50 μg/ml. On the other hand, the MIC of DC-159a for single mutant strains was less

than half that of ciprofloxacin in spite of higher observed MIC for type stains than ciprofloxacin.

Table 2. MICs for *S*. Typhimurium and *S*. Enteritidis

Quinolones	MIC (μg/ml)	
	S. Typhimurium	*S*. Enteritidis
DC-159a	0.13	0.06
Ciprofloxacin	0.016	0.016
Nalidixic acid	4.0	4.0

Table 3. Estimated MICs for *S*. Typhimurium and *S*. Enteritidis

Quinolone	Serovar	Estimated MIC (μg/ml)				
		Ser83Phe	Asp87Asn	Asp87Gly	Asp87Tyr	Ser83Phe-Asp87Asn
DC-159a	*S*. Typhimurium	0.16	0.22	0.19	0.19	1.34
	S. Enteritidis	0.12	0.17	0.14	0.14	1.20
Ciprofloxacin	*S*. Typhimurium	0.55	0.60	0.40	0.55	70.02
	S. Enteritidis	0.46	0.50	0.33	0.46	85.4
Nalidixic acid	*S*. Typhimurium	65.7	64.2	54.1	77.2	87.3
	S. Enteritidis	79.7	77.8	64.7	94.9	108

Discussion

The emergence of nontyphoidal *Salmonella* infections caused by quinolone resistant pathogens has become one of the most significant public health concerns worldwide. DC-159a is a newer fluoroquinolone that has already been shown to have potent activity against various microorganisms including some with amino acid substitutions in DNA gyrases [13), 24), 63)]. In this chapter, I evaluated the potential bactericidal effect of DC-159a against *S.* Typhimurium by (1) measuring its IC_{50} and MIC, and (2) comparing its antibacterial activity with that of ciprofloxacin and nalidixic acid.

IC_{50}s of every investigated quinolone against mutant DNA gyrases were higher than those against WT DNA gyrases. Consequently, observed IC_{50}s against double-mutant DNA gyrases were higher than those against single-mutant DNA gyrases. Amino acids at position 83 and 87 have previously been described as being strongly related to the binding of quinolones to DNA gyrase [42), 54)]. Therefore, the binding affinities of ciprofloxacin and nalidixic acid were expected to drastically decrease due to IC_{50} differences between WT and mutant DNA gyrases. Nonetheless, the binding affinity of DC-159a was maintained even when amino acids were substituted. Thus, of all quinolones tested by the supercoiling inhibitory assay, DC-159a showed the most potent inhibitory activity against mutant DNA gyrases of *S.* Typhimurium, especially double-mutant DNA gyrases.

As described above, it is well known that fluorination at C6 of the quinolone ring improves the antibacterial activity. In addition to fluorination at C6, the structure of ciprofloxacin at positions

N1 and C7 is different from that of nalidixic acid. N1 belongs to the enzyme–DNA complex, and a cyclopropyl at position N1 in ciprofloxacin is currently considered the most optimal substituent for the formation of an enzyme–DNA binding complex [43]. Position C7 is believed to directly interact with the DNA gyrase, and a bulky side chain at C7 plays an important role in the prolongation of half-lives and the direct interaction with DNA gyrase [57]. Likely due to these mechanisms, the inhibitory activity of ciprofloxacin against DNA gyrases was greater than that of nalidixic acid, and its observed IC_{50} lower than that of nalidixic acid. In contrast, DC-159a possesses a 3-aminopyrrolidine substituent at C7 [24]. Apart from DC-159a, sitafloxacin is the only quinolone that presents a similar structure at C7 [6, 30]. A previous study reported that the inhibitory activity against mutant DNA gyrases of *S.* Typhimurium showed by sitafloxacin was relatively stronger than that of ciprofloxacin. Since the IC_{50} values showed by DC-159a against *Mycobacterium leprae* DNA gyrases are similar to those shown by sitafloxacin, a substituent at C7 in DC-159a can be considered a key factor for maintaining a strong affinity to mutant DNA gyrases [63].

Our results from the supercoiling inhibitory assay were in agreement with previous antimicrobial susceptibility testing against clinical isolates [12, 40, 60]. For example, in our supercoiling inhibitory assay, the IC_{50} of ciprofloxacin against double-mutant DNA gyrases was greater than that against WT DNA gyrases. This result is in concordance with previous work reporting an association between double mutation in GyrA and a high level of resistance in clinical

isolates. In addition, regardless of the mutation in GyrA, a concentration of nalidixic acid higher than that of ciprofloxacin was required to inhibit the activity of DNA gyrases. Nonetheless, the detected IC_{50}s of ciprofloxacin were significantly lower than those of nalidixic acid. These results were also in agreement with previous studies, as it was reported that the MIC of nalidixic acid against clinical isolates of *S*. Typhimurium was higher than that of ciprofloxacin [12), 40)]. MIC values are considered to be strongly related to IC_{50}s [20), 31)]. The IC_{50}s of DC-159a were lower than those of ciprofloxacin; hence, low MICs of DC-159a against ciprofloxacin resistant *Salmonella* strains were also expected.

In the antimicrobial susceptibility testing, MICs of DC-159a against *S.* Typhimurium and *S.* Enteritidis were 0.13 and 0.06, respectively (Table 2). According to NCBI databases, *S.* Typhimurium and *S*. Enteritidis have the same amino acid sequences in GyrA, and this sequence was determined to be a WT. Thus, the susceptibility of their DNA gyrase against quinolones was presumed to be the same. These results suggested that the rate of DC-159a accumulation was different between *S*. Typhimurium and *S*. Enteritidis. In the data from MIC estimation, although it was considered that DC-159a permeability and/or accumulation were lower than those of ciprofloxacin, the MIC of DC-159a was lower than that of ciprofloxacin. Interestingly, the MIC of DC-159a against strains with double-mutant DNA gyrases was less than 1/50 that of ciprofloxacin. *Salmonella* isolates carrying double-mutant DNA gyrases showed a higher level of quinolone resistance than those with a single mutant. It was also estimated that MICs of DC-159a against each

mutant strain were much lower than those of nalidixic acid. Therefore, it was clear that DC-159a showed potential to be used as an effective therapeutic agent against salmonellosis caused by quinolone resistant bacteria.

Quinolones exhibit dose-dependent bactericidal effects [37]. Thus, the maximum concentration of a drug that a living organism can tolerate (C_{max}) must be considered. The C_{max} of DC-159a in murine models has previously been reported [1, 19]. Based on those findings, the C_{max} values of DC-159a were similar to those of ofloxacin in serum of male juvenile rats receiving a single oral administration. In a separate study [55], the C_{max} of ofloxacin in a healthy human male was examined. The recommended oral dosage of ofloxacin is 200 – 400mg twice daily [29]. When a 200 mg tablet of ofloxacin was administered, the C_{max} was 1.74 mg/ml [55]. It is therefore expected that if a similar amount of DC-159a (200 mg) was administered to human, the C_{max} would be close to 1.74 mg/ml. This DC-159a concentration would suffice to exert a bactericidal effect because the MIC of DC- 159a was estimated to be 1.34 mg/ml at most (Table 3). Moreover, since fluoroquinolones are absorbed from the intestine [5], the concentration of fluoroquinolone in the intestinal tract would be higher than it would be in serum. Based on this evidence, it is believed that DC-159a could exert a potent bactericidal effect against nontyphoidal *Salmonella* in the intestinal tract even at a lower dose.

Since quinolones are dose-dependent antibiotics, usually high doses of quinolones exert excellent therapeutic effects [38]. However, as the adverse effects are also dose dependent [41], a

quinolone with fewer adverse effects was required. It is believed that the bactericidal activity and the adverse effects are strongly related to the structure of quinolones as well. In addition to the aforementioned structure–activity relationship of quinolones, a substituent at position C8 has been found to be responsible for adverse effects [36), 41)]. Nonetheless, the substituent at C8 in DC-159a is a methoxy moiety, and C8-methoxy quinolones have been reported to have less adverse effects such as low phototoxicity [33), 49)]. Therefore, a high dose of DC-159a should be readily available, and the antimicrobial activity against quinolone resistant *S.* Typhimurium and *S*. Enteritidis sufficient.

Conclusion

This study demonstrated that DC-159a has two advantages over ciprofloxacin and nalidixic acid: (1) it is more effective at inhibiting the activity of DNA gyrases with reported mutations in QRDR, especially double mutant DNA gyrases, and (2) it shows a potent antimicrobial activity at lower doses against quinolone resistant *S*. Typhimurium and *S*. Enteritidis. In addition, it will cause less adverse effects even if administered at high doses. It can be concluded that DC-159a is a promising antibiotic candidate that is safe and with potent in vitro activity for treating infectious human diseases such as those caused by quinolone resistant nontyphoidal *Salmonella*.

Chapter II

Inhibitory effect of WQ-3810 on DNA gyrase

Introduction

WQ-3810 is a newer fluoroquinolone than DC-159a, so a few studies have reported the antibacterial activity of WQ-3810. However, its potent antimicrobial activity has been shown against some microorganisms. In the previous study [27], WQ-3810 exerted higher antibacterial activity against clinical isolates of *E. coli* and *Acinetobacter baumannii* containing amino acid substitutions on GyrA than other fluoroquinolones including ciprofloxacin. WQ-3810 is characterized by a very different structure from DC-159a and ciprofloxacin. In particular, WQ-3810 has unique substituents at the N1 and C7 positions. N1 and C7 substituents of quinolone are thought to be involved in binding affinity to DNA gyrase. Thus, it was expected that WQ-3810 would exert different inhibitory effects on DNA gyrases with amino acid substitution at position 83 and 87 in GyrA from DC-159a and other quinolones. Therefore, the objective of the present study was to assess the inhibitory effect of WQ-3810 on *S*. Typhimurium DNA gyrase with amino acid substitutions that putatively cause quinolone resistance. In addition, the antibacterial activity of WQ-3810 against nontyphoidal *Salmonella* was estimated.

Materials and methods

Quinolones

WQ-3810 was kindly provided by Wakunaga Pharmaceutical Co., Ltd. The chemical structure of WQ-3810 was shown in Figure 4. Other comparators were same with Chapter I.

Recombinant DNA gyrases

The inhibitory effect of WQ-3810 was evaluated against recombinant WT and mutant DNA gyrases used in Chapter I.

Supercoiling inhibitory assay

Inhibitory effect of WQ-3810 was evaluated by IC_{50}.

Antimicrobial susceptibility testing for nontyphoidal *Salmonella*

MICs of WQ-3810 for *S.* Typhimurium and *S.* Enteritidis were measured by same procedure with Chapter I. All experiments were performed in triplicate. the MICs of WQ-3810 against the strains carrying mutant DNA gyrases were also estimated and compared with the results in Chapter I.

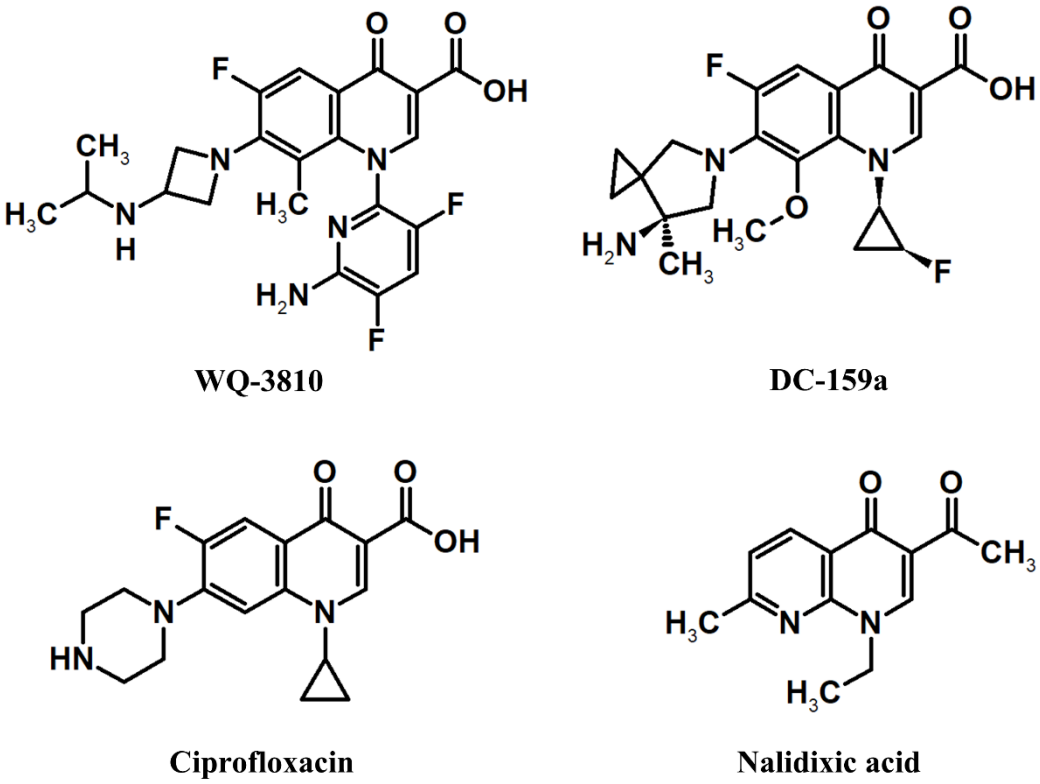

Figure 4. Structure of WQ-3810 and other quinolones used in this chapter

Results

IC_{50}s of WQ-3810

IC_{50}s of WQ-3810 were estimated using the purified DNA gyrases. Electrophoretic patterns of enzymic activity attenuated by IC_{50}s of WQ-3810 are shown in Figure 5 with the results of ciprofloxacin and nalidixic acid in Chapter I. IC_{50}s are summarized in Table 4. Although no significant differences were found between the IC_{50}s of WQ-3810 [0.31 μg/ml; 95% CI: 0.24 – 0.37 μg/ml] and ciprofloxacin (0.25 μg/ml; 95%CI: 0.19 – 0.32 μg/mL) against WT DNA gyrases, they were markedly lower than that of nalidixic acid (28 μg/ml; 95%CI: 14 – 42 μg/ml). Moreover, IC_{50}s of WQ-3810 against every single mutant DNA gyrase were lower than those of ciprofloxacin and nalidixic acid. IC_{50} of WQ-3810 against double mutant DNA gyrase was significantly greater than that against WT and single mutant DNA gyrases as same with ciprofloxacin and nalidixic acid. Nonetheless, compared with those of ciprofloxacin and nalidixic acid, IC_{50}s of WQ-3810 against double mutant DNA gyrases were considerably lower. The IC_{50} of WQ- 3810 against double mutant DNA gyrase was 45-fold greater that that against WT DNA gyrases, it was less than 1/45 that of ciprofloxacin. In the present study, when taking into consideration the position of the amino acid substitution, IC_{50}s of WQ-3810 against mutant DNA gyrases with an amino acid substitution at position 87 were lower than those against DNA gyrases with an amino acid substitution at position 83. Moreover, while the IC_{50} of WQ-3810 against DNA gyrase with Ser83Phe was 1.6 μg/ml (95%CI: 1.4 – 1.9 μg/ml), those against DNA gyrases with amino acid substitutions at position 87

were no greater than 0.92 μg/ml (95%CI: 0.72 – 1.1 μg/ml).

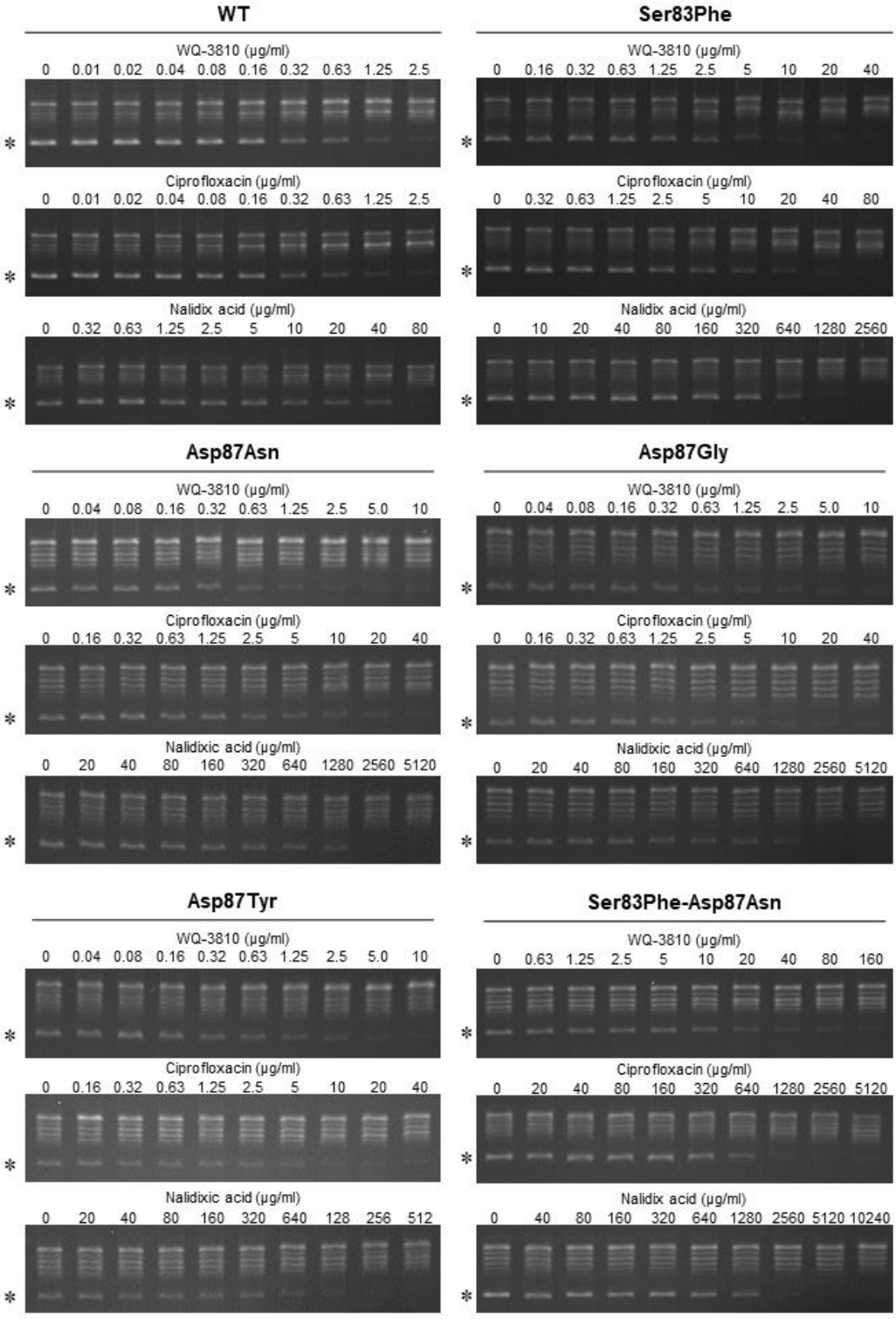

Figure 5. Inhibitory activity of WQ-3810 compared with that of ciprofloxacin and nalidixic acid.

Asterisks (*) indicate the band of supercoiled DNA

Table 4. IC_{50}s of WQ-3810 against WT and mutant DNA gyrases

Quinolones	IC_{50} (μg/ml)					
	WT	Ser83Phe	Asp87Asn	Asp87Gly	Asp87Tyr	Ser83Phe-Asp87Asn
WQ-3810	0.31 (0.24 – 0.37)	1.6 (1.4 – 1.9)	0.67 (0.52 – 0.82)	0.65 (0.56 – 0.73)	0.92 (0.72 – 1.1)	14 (10 – 18)
Ciprofloxacin	0.25 (0.19 – 0.32)	3.6 (3.3 – 4.0)	3.9 (3.1 – 4.7)	2.6 (2.2 – 2.9)	3.6 (2.8 – 4.5)	480 (420 – 530)
Nalidixic acid	28 (14 – 42)	450 (370 – 540)	440 (330 – 540)	370 (270 – 460)	530 (410 – 650)	600 (450 – 750)

Note: The range in brackets is the 95% confidence interval.

MICs of WQ-3810

MICs measured by the antimicrobial susceptibility testing are summarized in Table 5. WQ-3810 had different MICs against *S*. Typhimurium and *S*. Enteritidis. While the antibacterial activity of WQ-3810 was higher than that of nalidixic acid, it was lower than that of ciprofloxacin. Estimated MICs are summarized in Table 6.

Table 5. MICs of WQ-3810 against *S.* Typhimurium and *S.* Enteritidis

Quinolones	MIC (μg/ml)	
	S. Typhimurium	*S*. Enteritidis
WQ-3810	0.13	0.06
Ciprofloxacin	0.016	0.016
Nalidixic acid	4.0	4.0

Table 6. Estimated MICs of WQ-3810.

Quinolone	Serovar	Estimated MIC (μg/ml)				
		Ser83Phe	Asp87Asn	Asp87Gly	Asp87Tyr	Ser83Phe-Asp87Asn
WQ-3810	*S*. Typhimurium	0.25	0.11	0.10	0.14	2.11
	S. Enteritidis	0.20	0.08	0.08	0.11	1.96
Ciprofloxacin	*S*. Typhimurium	0.55	0.60	0.40	0.55	70.02
	S. Enteritidis	0.46	0.50	0.33	0.46	85.4
Nalidixic acid	*S*. Typhimurium	65.7	64.2	54.1	77.2	87.3
	S. Enteritidis	79.7	77.8	64.7	94.9	108

Note: Estimates of ciprofloxacin and nalidixic acid are same in Chapter I

Discussion

In the supercoiling activity assay, WQ-3810 exerted a higher inhibitory effect on mutant DNA gyrases with quinolone resistance-conferring amino acid substitutions than did ciprofloxacin and nalidixic acid. It is worth noting that the inhibitory effect of WQ-3810 differed depending on the position of the amino acid substitution in DNA gyrases. Indeed, WQ-3810 showed a high affinity to DNA gyrases with the amino acid at position 87 regardless of the occurrence of an amino acid substitution. This finding seems to indicate that binding of WQ-3810 to DNA gyrases is more dependent on the amino acid at position 83 than at position 87. WQ-3810 possesses unique substituents in its chemical structure at N1 and C7, and these substituents have been proposed to contribute to the affinity to DNA gyrases [32), 50)]. Previous work proposed a model of a quinolone-binding pocket in DNA gyrases [21)]. In that model, ciprofloxacin is positioned in the binding pocket between GyrA and GyrB. The substituent at N1 interacts with residues in GyrA and the substituent at C7 interacts with residues in GyrB, including serine at position 83 and aspartic acid at position 87. In the model, a change of substituent at N1 of the quinolone ring causes different binding affinity to amino acids at positions 83 and 87 in GyrA. In the case of WQ-3810, it possesses a 2-amino-6-tert-butylamino-3,5-difluoropyridin moiety at position N1, whereas ciprofloxacin has a cyclopropyl moiety at the same position. Hence, the substituent of WQ-3810 at N1 is structurally larger than that of ciprofloxacin. In addition, it is likely that the substituent at N1 in WQ-3810 is closer to the amino acid at position 83 than the substituent of ciprofloxacin. These unique characteristics likely help

WQ-3810 develop stronger interaction and affinity to DNA gyrases.

The substituent at C7 of the quinolone ring is thought to be critical for potent antimicrobial activity [9), 21)]. In the present work, when compared with ciprofloxacin and nalidixic acid, WQ-3810 showed lower IC_{50}s. In the aforementioned model, C7 is bound to GyrB and thus, it can be inferred that IC_{50} s are likely affected by C7. Based on this, IC_{50}s of quinolones against double mutant DNA gyrases estimated in the present study were likely influenced by the binding force to substituents at C7 in GyrB. Quinolone resistant nontyphoidal *Salmonella* with amino acid substitutions in GyrB is much rarer than those with amino acid substitutions in GyrA[14), 45), 46)]. This might be because of the fatal effect of amino acid substitutions in GyrB on bacterial survival. Assuming that the proportion of GyrB in binding of WQ-3810 to DNA gyrase is large, it can be expected that WQ-3810 is less likely to produce quinolone resistant *Salmonella* than other quinolones. Although no universally accepted explanation for this phenomenon is available to date, the results of the present study will likely contribute to the under- standing of the relationship between the structure and the antibacterial activity of quinolones.

Comparing to the IC_{50} of DC-159a in Chapter I, it is obvious that WQ-3810 exhibit a higher inhibitory effect on DNA gyrase with amino acid substitutions at position 87 than with that at position 83 in GyrA. Though there was no difference between the IC_{50} of WQ-3810 and DC-159a against WT DNA gyrase, the IC_{50} for mutant DNA gyrase was different between WQ-3810 and DC-

159a. While DC-159a showed high inhibitory effect on regardless of amino acid substitution on DNA gyrase, WQ-3810 showed higher inhibitory effect on DNA gyrase with amino acid substitution at position 87 than DC-159a. The substituent at N1 in WQ-3810 might be playing an important role in this phenomenon. Detailed analysis of structure-activity relationship in our future study may lead to a practical idea of designing novel fluoroquinolones effective for quinolone resistant *Salmonella* due to occurrence of mutations in *gyrA*.

While its inhibiting concentration depended on the amino acid substitution, it was demonstrated that WQ-3810 could inhibit the activity of DNA gyrases at a lower concentration than the other quinolones. However, to exert a bactericidal effect, a quinolone has to be taken up by the bacterial cell and accumulate intracellularly until reaching the concentration required to inhibit DNA gyrase activity. This is observed when uptake is suppressed, and bacteria readily show quinolone resistance afterward [17]. Considering that there was no difference between the IC_{50}s of WQ-3810 and ciprofloxacin against WT DNA gyrases, the potential reason for the MIC of WQ-3810 being higher than that of ciprofloxacin is that WQ-3810 is less permeable and easier to be excreted than ciprofloxacin. Thus, it can be argued that in terms of permeability and accumulation, WQ-3810 is an inferior drug compared with ciprofloxacin. Nonetheless, since its IC_{50}s were very low, it would be expected that MICs of WQ-3810 against strains with mutant DNA gyrases would be lower than those of ciprofloxacin. In particular, a strong antimicrobial effect would be expected against strains

with amino acid substitutions at position 87. Therefore, permeability and accumulation rate should be also important factors to consider when evaluating the antibacterial activity of WQ-3810.

While the estimated range of MICs of WQ-3810 against strains having DNA gyrases with Ser83Phe was 0.20 – 0.25 μg/ml, its range of MICs for strains having amino acid mutations at position 87 in GyrA was 0.08 – 0.14 μg/ml, which was very low. In a past epidemiological study, the number of nontyphoidal *Salmonella* strains containing amino acid substitutions at position 87 was greater than that with amino acid substitutions at position 83. In light of this, WQ-3810, with a higher antibacterial activity than ciprofloxacin and a higher antimicrobial activity against strains with amino acid substitutions at position 87, will be an effective quinolone antibacterial drug.

Conclusion

To summarize Chapter II, it was demonstrated that WQ-3810, which has a unique structure at positions N1 and C7, exerted a higher inhibitory effect on DNA gyrases with amino acid substitutions at position 87 in GyrA than did ciprofloxacin and nalidixic acid. It was shown that WQ-3810 was expected to exhibit effective antibacterial activity against nontyphoidal *Salmonella* even if they are resistant to quinolone. Furthermore, although further studies are required for the structure activity relationship of quinolones, the results obtained in this study were important findings for development of new quinolones which are particularly effective for quinolone resistant bacteria.

CONCLUSION

There is an urgent need to find a new treatment for food poisoning caused by quinolone resistant nontyphoidal *Salmonella*. Quinolone is the exceptional antibiotic which has an excellent oral bioavailability and broad-spectrum with superior safety profiles. Thus, quinolone resistance causes significant clinical impact. Although there are multiple mechanisms of quinolone resistance, amino acid substitution which occur rapidly in GyrA during the quinolone treatment is the most significant factor for causing quinolone resistance. A new quinolone that effectively inhibits DNA gyrase with amino acid substitutions is needed. In this study, inhibitory effects of two newly developed quinolones were evaluated against DNA gyrase of quinolone resistant *S*. Typhimurium.

First, the inhibitory effect of DC-159a on DNA gyrase of *S.* Typhimurium was demonstrated in Chapter I. Comparing to ciprofloxacin and nalidixic acid, DC-159a showed high inhibition activity on the mutant DNA gyrases. The antibacterial activity was expected to be outstanding against quinolone resistant nontyphoidal *Salmonella* possessing frequently reported mutations on DNA gyrase. Therefore, DC-159a can be expected to be effective even against high-level quinolone resistant *S.* Typhimurium which have a double mutant DNA gyrase.

Next, the inhibitory effect of WQ-3810 was evaluated in Chapter II. The results showed WQ-3810 had a higher inhibitory effect on mutant DNA gyrase than ciprofloxacin and also the unique residues at position N1 and C7 seems to have greatly increased the inhibitory effect on the

activity of DNA gyrase of quinolone resistant *S.* Typhimurium. In particular, WQ-3810 showed stronger inhibitory activity against DNA gyrase with amino acid substitution at position 87, which was an important finding for the structure-activity relationship of quinolone and a new therapy procedure proposed to select more effective quinolone antibiotics depending on the amino acid substitution on GyrA.

Both DC-159a and WQ-3810 showed excellent inhibitory activities on mutant DNA gyrase, and the promising antibacterial activities for quinolone resistant nontyphoidal *Salmonella* were proved in this study. Although the relationship between chemical structure of quinolone and inhibitory effect is not fully understood, the outcomes of this study can contribute the development of new quinolone and countermeasure for quinolone resistant nontyphoidal *Salmonella*.

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和文要旨

非チフス性サルモネラ属菌は家畜や家禽の腸管に保菌されており、腸内容物によって汚染された食品を介してヒトに感染する。年間の感染者数は約 9,400 万人、死亡者数は約 15.5 万人と推定されており、公衆衛生上非常に大きな問題である。非チフス性サルモネラ属菌は 2,500 種類以上の血清型に細分されており、その中でも *Salmonella* Typhimurium は食中毒の原因菌として世界各国で頻繁に報告されている。これまで治療薬としてアンピシリンやクロラムフェニコールなどが使用されてきたが、これらの抗生物質に対して耐性を示すサルモネラ菌が多く見つかっている。キノロンはこれらの薬剤耐性サルモネラ菌に対しても効果的である為重宝されてきた。しかし、近年ではキノロン耐性サルモネラ菌が世界的に増加傾向にあり対策が急がれている。

キノロンは細菌の増殖に関わる酵素を阻害することで殺菌的な作用を示す抗菌薬である。これまでに開発されたキノロンは共通の構造であるキノロン環を持ち、その側鎖構造を置き換えることで抗菌スペクトラムおよび殺菌効果を発展させてきた。キノロンの標的酵素である DNA ジャイレースは、DNA 複製開始時に DNA の二本鎖が解かれたことによって細菌の環状 DNA に生じた捻れを解消する働きをする為、細菌の DNA 複製に欠かせない酵素の 1 つである。DNA ジャイレースは GyrA および GyrB の 2 種のサブユニット 2 組からなる四量体を形成しており、キノロンはこれらのサブユニットに囲まれた部位に結合することで DNA ジャイレースの酵素活性を阻害する。そのため、キノロンが結合する部位周辺の DNA ジャイレース上にアミノ酸置換が生じることでキノロン耐性がもたらされる。特に GyrA を構成する 83 番目のセリンおよび 87 番目のアスパラギン酸が他のアミ

ノ酸に置換されたDNAジャイレースをもつキノロン耐性サルモネラ菌が多く分離されており、これらのアミノ酸がDNAジャイレースとキノロンの結合に直接的にかかわると考えられている。アミノ酸置換を含むDNAジャイレースに対しても高い酵素活性阻害作用をもつキノロンがキノロン耐性菌の制御に必要とされている。

本研究では新規キノロンであるDC-159aならびにWQ-3810の*S.* Typhimuriumの DNAジャイレースに対する直接的な阻害活性を明らかにし、キノロン耐性非チフス性サルモネラ属菌による感染症の治療薬としての可能性を評価した。

第一章では、第一三共株式会社によって開発されたDC-159aの酵素活性阻害作用および抗菌活性の試験を行った。DC-159aはこれまでに他の菌種に対して既存のキノロンよりも高い抗菌作用が確認されていることからキノロン耐性非チフス性サルモネラ属菌に対しても効果的であることが期待されるものであった。大腸菌を用いた組み換えタンパク質発現系によって*S.* Typhimuriumが保有するDNAジャイレースのGyrAおよびGyrBを作出した。GyrAはアミノ酸置換を含まない野生型に加えて、83番目もしくは87番目のアミノ酸置換を含む単変異型GyrA、およびその両方の位置にアミノ酸置換を含む二重変異型GyrAを作出した。組み換えDNAジャイレースに対するDC-159aの阻害活性をシプロフロキサシンおよびナリジクス酸と比較した結果、アミノ酸置換を含む変異型DNAジャイレースに対してDC-159aは最も高い阻害活性を示した。また、*S.* Typhimurium標準株に対する最小発育阻止濃度はシプロフロキサシンとほぼ等しい値を示し、変異型DNAジャイ

レースに対する阻害活性の差から DC-159a はシプロフロキサシンよりもキノロン耐性非チフス性サルモネラ属菌に効果的である可能性が明らかになった。

第二章では、湧永製薬株式会社によって開発された WQ-3810 を上述と同様の方法で評価した。WQ-3810 は前述の DC-159a およびその他のキノロンとはキノロン環の 1 位および 7 位の位置の側鎖構造が大きく異なる構造を持つ。組み換え DNA ジャイレースを使用した酵素活性阻害試験によって、WQ-3810 は変異型 DNA ジャイレースに対してシプロフロキサシンおよびナリジクス酸よりも高い阻害活性を示すことが明らかになった。さらに DC-159a との比較によって、GyrA の 87 番目の位置にアミノ酸置換を含む変異型 DNA ジャイレースに対してより高い阻害活性を示すことが確認された。このことから、キノロン耐性菌が持つアミノ酸置換の位置によってより効果的なキノロン系抗菌薬を治療薬として選択できる可能性に加えて、キノロンの構造と阻害活性の相関に寄与する新たな知見を得た。

DC-159a および WQ-3810 はキノロン耐性 *S.* Typhimurium の DNA ジャイレースに対して既存のキノロンよりも高い酵素活性阻害活性を示し、キノロン耐性非チフス性サルモネラ属菌による感染症に対して有効な治療薬となる可能性が高いことが本研究によって明らかとなった。さらに、それらの新規キノロンがもつ側鎖構造と本研究で測定された酵素活性阻害作用から、新たなキノロンの開発ならびにキノロン耐性非チフス性サルモネラ属菌による感染症の効果的な治療法の確立に貢

献することが期待された。

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