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Studies on DNA gyrase of quinolone-resistant *Mycobacterium leprae*

（キノロン耐性らい菌の **DNA** ジャイレースに関する研究）

Tomoyuki Yamaguchi

CONTENTS

ABBREVIATIONS..... 1

PREFACE 3

CHAPTER I:

DC-159a shows inhibitory activity against DNA gyrases of *Mycobacterium leprae*

Introduction 10

Materials and Methods 12

- Antibacterial agents

- Bacterial strains and expression plasmids

- Construction of expression plasmids

- Expression and purification of recombinant DNA gyrase subunits

- ATP-dependent DNA supercoiling assay

- Quinolone-inhibited DNA supercoiling assay

- Quinolone-mediated DNA cleavage assay

Results..... 17

- Construction and purification of recombinant WT and mutant DNA gyrase

subunits

- ATP-dependent DNA supercoiling activities of DNA gyrases

- Half maximal inhibitory concentrations of quinolones against WT and mutant DNA gyrases

- Concentrations of quinolones that convert 20% of input DNA to the linear form against WT and mutant DNA gyrases

Discussion 27

Summary 35

CHAPTER II:

Quinolone resistance-associated amino acid substitutions affect enzymatic activity of *Mycobacterium leprae* DNA gyrase

Introduction 36

Materials and Methods 37

- Construction of expression plasmids and purification of recombinant DNA gyrase subunits

- Time course DNA supercoiling assay

Results 39

- Alteration of DNA supercoiling activity by quinolone resistance-associated

amino acid substitutions

- Supplementation of reduced activity of Gly89Cys-substituted DNA gyrase by increasing the enzyme concentration

Discussion **46**

Summary **50**

CONCLUSION **52**

ACKNOWLEDGEMENTS **54**

和文要旨 **56**

REFERENCE **60**

ABBREVIATIONS

MDT	Multidrug therapy
WHO	World Health Organization
DNA	Deoxyribonucleic acid
GyrA	DNA gyrase subunit A
GyrB	DNA gyrase subunit B
QRDR	Quinolone resistance-determining region
WT	Wild type
Gly89Cys	Amino acid substitution from glycine at position 89 to cysteine
Ala91Val	Amino acid substitution from alanine at position 91 to valine
Asp95Gly	Amino acid substitution from aspartic acid at position 95 to valine
LB	Luria-Bertani
IPTG	Isopropyl-beta-D-thiogalactopyranoside
Tris	Trishydroxymethylaminomethane
EDTA	Ethylenediamine tetra-acetic acid

ATP	Adenosine triphosphate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBE	Tris-borate-EDTA
His-tag	Hexahistidine-tag
MIC	Minimum inhibitory concentration
IC_{50}	Drug concentration that inhibit DNA supercoiling by 50%, or half maximal inhibitory concentrations
CC_{20}	Drug concentration that converts 20% of input DNA to the linear form

PREFACE

Mycobacterium leprae is an uncultivable bacterium known as a causative agent of leprosy, also known as Hansen's disease, which mainly affects skin and peripheral nerves of humans and causes subsequent severe disabilities. The mode of transmission of leprosy has not been conclusively proven; however, nasal droplets are considered to be the main route of the person-to-person transmission[44, 46].

Humans being are the major reservoir, but not the only susceptible species. Besides human cases, natural infection of leprosy has been observed also in some animal species such as nine-banded armadillos, mangabey monkeys and chimpanzees[16, 46, 51]. In addition, recent studies have indicated possible transmission between nine-banded armadillos and humans[47, 52]. Thus, leprosy has come to be known also as a zoonosis.

Like the other infectious diseases, shortening the duration of infectivity by proper treatment is an effective strategy to reduce the risk of further transmission of leprosy to healthy individuals in the community[54]. Although this chronic infectious disease had been considered to be incurable for a long time, it is now well accepted that this disease can be treated by chemotherapy[39]. Owing to a MDT introduced by WHO in

the 1980s, world prevalence of leprosy has been dramatically reduced (Figure 1)[57–59]. Nonetheless, leprosy still remains a public health concern with more than 210,000 new cases every year mainly in Asian, Latin American and African countries[44, 57] (Figure 2).

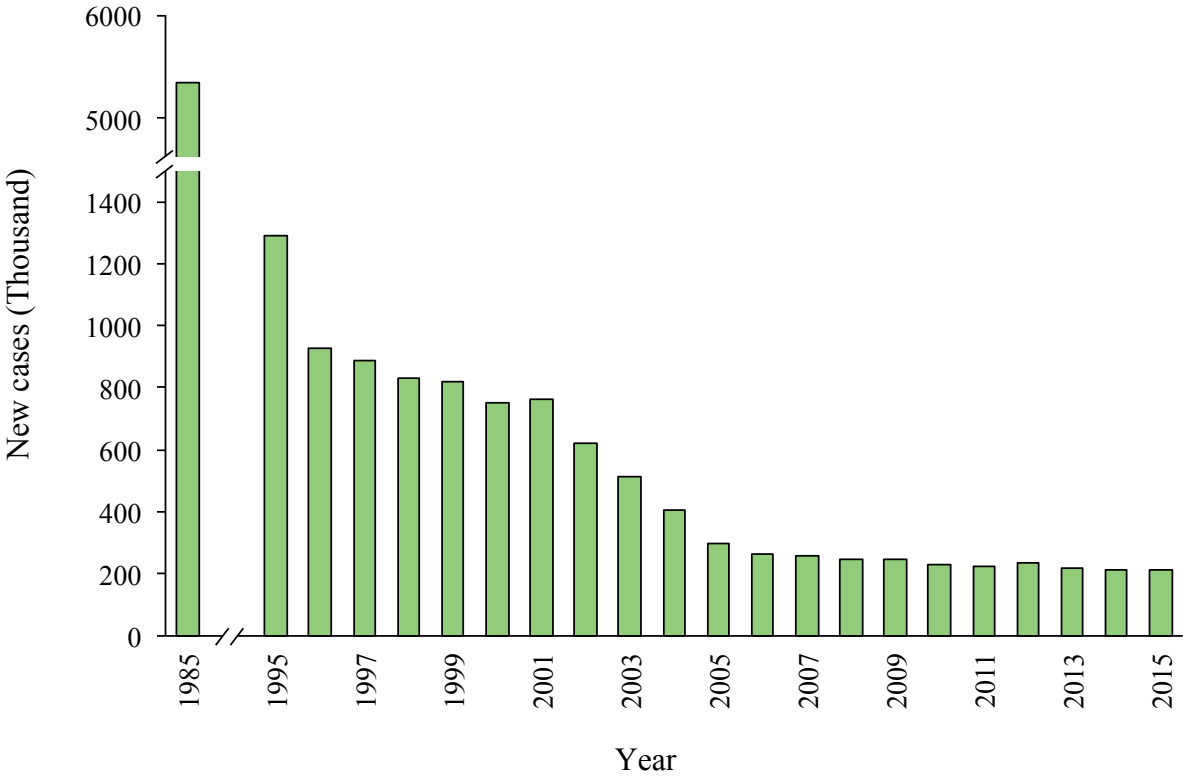


Figure 1. Trends in global new case detection of leprosy[57–59)]

The numbers of new cases in Europe are excluded until the region reported 18 new cases in 2015.

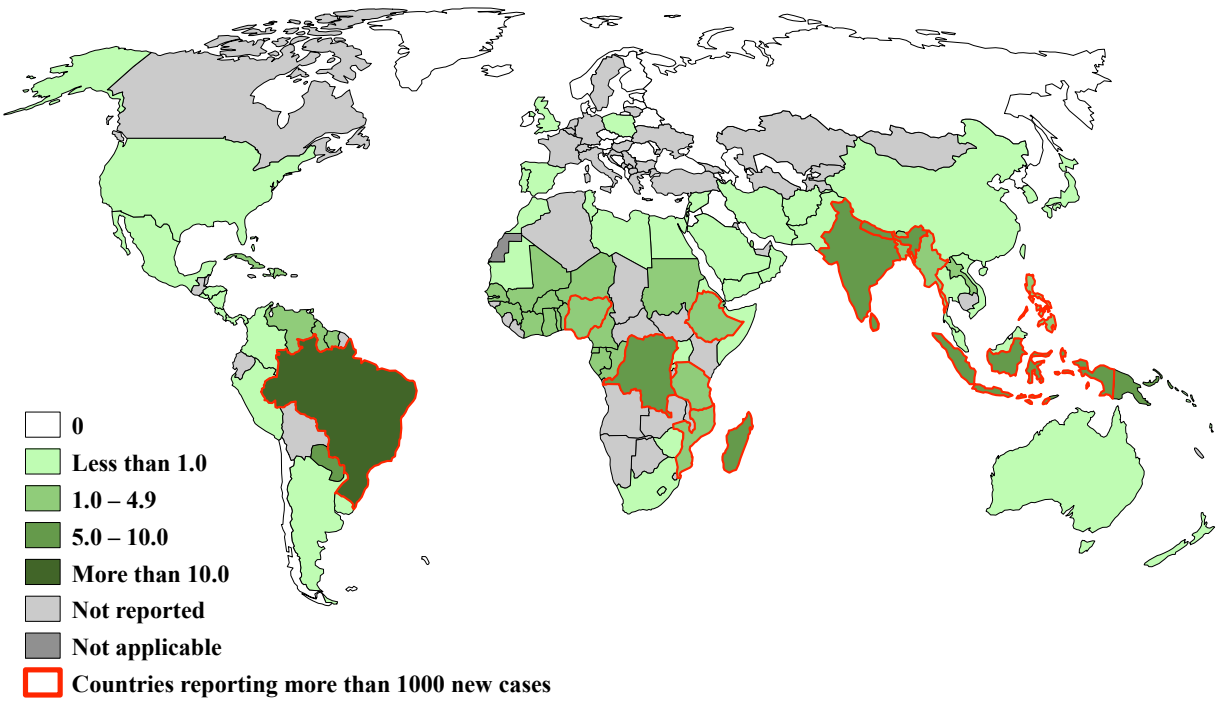


Figure 2. Detection rates of new leprosy cases in 2015[57)]

Darker green color indicates a higher burden of leprosy on the corresponding country. Countries highlighted with red borders reported more than 1000 new cases with red border and accounted for almost 95% of the global total.

Quinolones, including fluoroquinolones, are recognized as an effective antibacterial class widely used for treatment of various bacterial infections[20]. Also for treatment of leprosy, this drug class is considered to be important antibacterials. Quinolones are regularly used in a MDT regimen for treatment of single-skin lesion paucibacillary leprosy. In addition, some members of quinolones are also reportedly effective against multibacillary leprosy[25, 42].

Quinolones target two bacterial type II DNA topoisomerases that play an essential role in DNA replication and transcription[8, 19]. One of the bacterial Type II DNA topoisomerases, DNA gyrase alleviates positive supercoils of DNA accumulated during DNA replication and transcription by introducing negative supercoils while the other bacterial type II DNA topoisomerase, topoisomerase IV decatenates interlinked daughter chromosomes after DNA replication[43]. DNA gyrase introduces negative supercoils by a cycle of cleaving double stranded DNA, passing another part of the DNA molecule through the cleavage and resealing the cleavage[8, 43]. This enzyme has been focused on as a target of antibacterials because of the crucial roles during DNA replication and transcription, and also their uniqueness: Those are essential for all bacteria, but absent in eukaryotes such as human being and animals[9]. Unlike other bacteria, mycobacteria lack topoisomerase IV and possess only DNA gyrase as a type II

topoisomerase in their cells, which is thought to play also the role of decatenation[31]. Thus, for mycobacterial species, including *M. leprae*, DNA gyrase is the sole target of quinolones[7]. As Quinolones interfere with DNA gyrase supercoiling activity by binding to the site where DNA gyrase cleaves the double strands, quinolone resistance can arise as a result of substitutions in amino acids that are close to the quinolone-binding site[1, 45]. Therefore, the region in DNA gyrase consisting these amino acids is called QRDR. DNA gyrase is a tetrameric enzyme composed of two GyrA and two GyrB, and both GyrA and GyrB have QRDRs in their sequences[21]. In clinical strains of *M. leprae*, only two types of amino acid substitution in GyrA, Gly89Cys and Ala91Val, have been found to be responsible for acquisition of quinolone resistance. According to previous reports, the majority of quinolone-resistant *M. leprae* strains has Ala91Val, and that Gly89Cys has been found only in a very limited number of cases[17, 29, 33, 56]. In addition to these amino acid substitutions found in clinical strains, it was previously demonstrated that experimentally-induced Asp95Gly also hinders the inhibitory activity of quinolones[61]. Although Asp95Gly has not been found in clinical *M. leprae* strains, its equivalent substitution, aspartic acid at position 94 to glycine, is frequently reported in *M. tuberculosis* clinical isolates[6, 50, 55].

This thesis is composed of two chapters and the conclusion. First, in Chapter I,

a potency of a newly developed quinolone DC-159a against *M. leprae* DNA gyrase was examined as an antileprosy drug. Then in Chapter II, focusing on DNA gyrase as an indispensable enzyme for bacteria, effect on the enzymatic activity of *M. leprae* DNA gyrases by amino acid substitutions related to acquisition of quinolone resistance were investigated.

CHAPTER I:

DC-159a shows inhibitory activity against DNA gyrases of *Mycobacterium leprae*

Introduction

In current MDT regimens, ofloxacin is the quinolone used for single skin lesion paucibacillary cases[56]. Although ofloxacin is adopted in MDT, it is not the most potent quinolone. Bactericidal activity differs greatly among quinolones. For example, moxifloxacin is known to be a more effective quinolone against leprosy than ofloxacin[10, 42, 61, 62], and its bactericidal activity is estimated to be equivalent to that of rifampicin, one of the first line drugs in MDT[10]. A study on human multibacillary leprosy cases demonstrated that moxifloxacin can kill leprosy bacilli with a single dose within days or weeks[42]. Sitafloxacin, another quinolone, has also been found to be highly potent against *Mycobacterium leprae* in both *in vivo* and *in vitro* studies[12, 13]. Previous studies that assessed the inhibitory efficacies of quinolones, including ofloxacin, moxifloxacin and sitafloxacin found that sitafloxacin was more effective than either ofloxacin or moxifloxacin[61, 62]. Recently, DC-159a, a newly developed 8-methoxy quinolone, was reported to have high antibacterial efficacy against various bacterial species including *M. tuberculosis*[14, 22, 23]. Although many studies have shown its potential as a remedy for

bacterial infection, the efficacy of DC-159a against *M. leprae* has not been elucidated yet.

Recurrence of leprosy is a major obstacle for control of the disease because relapse cases are more likely to be accompanied with resistance to drugs used in MDT, which limits the choice of anti-leprosy drugs[37, 49, 56, 63]. Recurring cases are usually considered to result from therapeutic failure due to inadequate or incomplete treatment, and drug resistance can also be acquired at this time[24]. Thus, compliance with the planned course of medication is an important factor that can influence the treatment outcome because the recommended MDT can take as long as 12 months. To that end, introduction of quinolones to MDT regimens that are more potent than ofloxacin, owing to their ability to clear *M. leprae* bacilli rapidly, would be expected to improve patient compliance by shortening the medication period. In this study, I focused on three powerful quinolones, namely, moxifloxacin, sitafloxacin and DC-159a. In order to assess the potencies of these drugs as remedies for leprosy and to facilitate comparison between them, I conducted *in vitro* quinolone-mediated assays using recombinant *M. leprae* DNA gyrases including WT and mutants bearing amino acid substitutions Gly89Cys, Ala91Val and Asp95Gly.

Materials and Methods

Antibacterial agents

DC-159a and sitafloxacin were kindly provided by Daiichi-Sankyo Co., Ltd. (Tokyo, Japan). Moxifloxacin was purchased from LKT Laboratories, Inc. (St. Paul, MN). Ampicillin was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Bacterial strains and expression plasmids

The Thai-53 strain of *M. leprae*[36], maintained at the Leprosy Research Center, National Institute of Infectious Diseases (Tokyo, Japan), was used to prepare *M. leprae* DNA. *Escherichia coli* strain TOP-10 (Thermo Fisher Scientific Inc.; Waltham, MA) was used for cloning. *E. coli* strains Rosetta-gami 2(DE3)pLysS and BL21(DE3)pLysS (Merck KGaA, Darmstadt, Germany) were used for protein expression. The plasmid vector pET-20b(+) (Merck KGaA) was used for the construction of expression plasmids. Relaxed and supercoiled pBR322 DNA (John Innes Enterprises Ltd.; Norwich, United Kingdom) were used for the DNA supercoiling assay and DNA cleavage assay.

Construction of expression plasmids

DNA gyrase expression plasmids coding WT GyrA, GyrA with Ala91Val (Ala91Val-GyrA), GyrA with Asp95Gly (Asp95Gly-GyrA) and WT GyrB were constructed as described by Yokoyama et al.[61] The expression plasmid for GyrA with Gly89Cys (Gly89Cys-GyrA) was constructed in a similar way using primer pairs of k-45 (5′-GGCATATGACTGATATCACGCTGCCACCAG-3′) and k-58 (5′-CGATGCGTCGCAGTGCGGATGG-3′), and k-57 (5′-CCATCCGCACTGCGACGCATCG-3′) and k-46 (5′-ATAACGCATCGCCGCGGGTGGGTCATTACC-3′). The nucleotide sequence of GyrA gene with Gly89Cys in the plasmid was confirmed using a BigDye Terminator v3.1 cycle sequencing kit and an ABI Prism 3130xI genetic analyzer (Thermo Fisher Scientific Inc.).

Expression and purification of recombinant DNA gyrase subunits

Recombinant DNA gyrase subunits were expressed and purified as previously described[26, 34, 61]. Briefly, each expression plasmid bearing either gyrA or gyrB of *M. leprae* was transformed in *E. coli* Rosetta-gami 2(DE3)pLysS or BL21(DE3)pLysS. The transformants were cultured in LB broth under ampicillin selection (100 μg/mL) up

to the log phase. The expression of DNA gyrase was induced by the addition of 1 mM IPTG (Wako Pure Chemical Industries Ltd., Osaka, Japan) and further incubation at 12 or 14°C for 16 to 24 h. The harvested *E. coli* were lysed by sonication (10 times for 40 s at output level 3 and 40% duty cycle with 40-s intervals) (Sonifier 250; Branson, Danbury, CT) and centrifugation at 10,000 × *g* for 30 min. The recombinant DNA gyrase subunits in the supernatants were purified by Ni-NTA Agarose (Thermo Fisher Scientific Inc.) column chromatography and dialyzed against DNA gyrase dilution buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl, 2 mM DTT, and 1 mM EDTA). The purified proteins were examined by SDS-PAGE with Prestained Protein Marker, Broad Range (7-175 kDa) (New England Biolab; Hitchin, UK).

ATP-dependent DNA supercoiling assay

ATP-dependent DNA supercoiling assays were carried out as previously described[15, 26, 33, 34, 61]. Briefly, DNA supercoiling activities of the purified subunits were examined with a reaction mixture consisting of DNA gyrase reaction buffer (35 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 1.8 mM spermidine, 24 mM KCl, 5 mM DTT, 1mM ATP, 0.36 mg/mL BSA and 6.5% w/v glycerol), relaxed pBR322 DNA (180 ng), ATP (1 mM) and DNA gyrase subunits (30.0 ng of WT, Gly89Cys-, Ala91Val- or

Asp95Gly-GyrA and 24.4 ng of WT GyrB) in a total volume of 18 μL. For the assays of DNA gyrase with Gly89Cys, 60.0 ng Gly89Cys-GyrA and 48.8 ng WT GyrB were also examined. The mixtures were incubated at 30°C for 1.5 h and the reaction was stopped by adding 4.5 μL of 5× dye mix (5% SDS, 25% glycerol, 0.25 mg/mL bromophenol blue). Next, 10 μL of each reaction mixture was subjected to electrophoresis with a 1% agarose 1× TBE buffer gel. The agarose gel was stained with ethidium bromide (0.7 μg/mL).

Quinolone-inhibited DNA supercoiling assay

The quinolone-inhibited DNA supercoiling assay was based on the method described by Fisher and Pan[15]. Each assay was conducted in 18 μL of DNA gyrase reaction buffer, with relaxed pBR322 DNA (180 ng) and DNA gyrase subunits. For the assays of WT DNA gyrase and mutant DNA gyrases with Ala91Val and Asp95Gly, 30.0 ng GyrA and 24.4 ng WT GyrB were mixed. For the assays of DNA gyrase with Gly89Cys, 60.0 ng Gly89Cys-GyrA and 48.8 ng WT GyrB were used instead. The reactions were continued at 30°C for 1.5 h and stopped by the addition of 4.5 μL of 5× dye mix. Next, 10 μL of each mixture was subjected to electrophoresis on 1% agarose 1× TBE gels and stained with ethidium bromide. To assess the inhibitory effects of

quinolones on DNA gyrases, the amount of DNA supercoiled in the reactions was quantified with ImageJ (http://rsbweb.nih.gov/ij) by determining IC_{50}s, in the presence or absence of serial two-fold increases in the concentrations of DC-159a, sitafloxacin and moxifloxacin. Each assay was conducted three times to confirm its reproducibility.

Quinolone-mediated DNA cleavage assay

The DNA cleavage assay was also based on the method by Fisher and Pan[15]. Each assay was carried out in 18 μL of the DNA gyrase reaction buffer with supercoiled pBR322 DNA (180 ng), DNA gyrase subunits (the same concentrations as the quinolone-inhibited DNA supercoiling assays) and increasing concentrations of DC-159a, sitafloxacin and moxifloxacin. After 2-hour incubation at 30°C, the cleaving reactions were stopped by adding 2.7 μL of 2% SDS and 2.7 μL of proteinase K (1 mg/mL). Proteinase K reactions were continued for a further 30 min at 37°C, then stopped by the addition of 5.9 μL of 5× dye mix. Next, 10 μL of the reaction mixtures were electrophoresed on 0.8% agarose 1× TBE gels, and stained with ethidium bromide. To assess concentrations of quinolones that convert 20% of input DNA to the linear form (CC_{20}s), the amount of cleaved DNA was quantified with ImageJ. Each assay was conducted three times to confirm its reproducibility.

Results

Construction and purification of recombinant WT and mutant DNA gyrase subunits

The expression plasmids of WT GyrA, Ala91Val-GyrA, Asp95Gly-GyrA and WT GyrB previously constructed in this laboratory were used[61]. The DNA fragment including GyrA genes with Gly89Cys was amplified from the WT GyrA expression plasmid[61] and introduced into pET-20b(+). Recombinant subunits were expressed with C-terminal His-tags for purification by Ni-NTA Agarose resin, as the His-tag does not interfere with the catalytic functions of GyrA and GyrB[3, 4, 26, 33, 34, 61, 62]. Expressed recombinant DNA gyrase subunits were purified as soluble His-tagged 80 kDa proteins of GyrA and 75 kDa proteins of GyrB. The purity of the recombinant proteins was confirmed by SDS-PAGE (Figure 3).

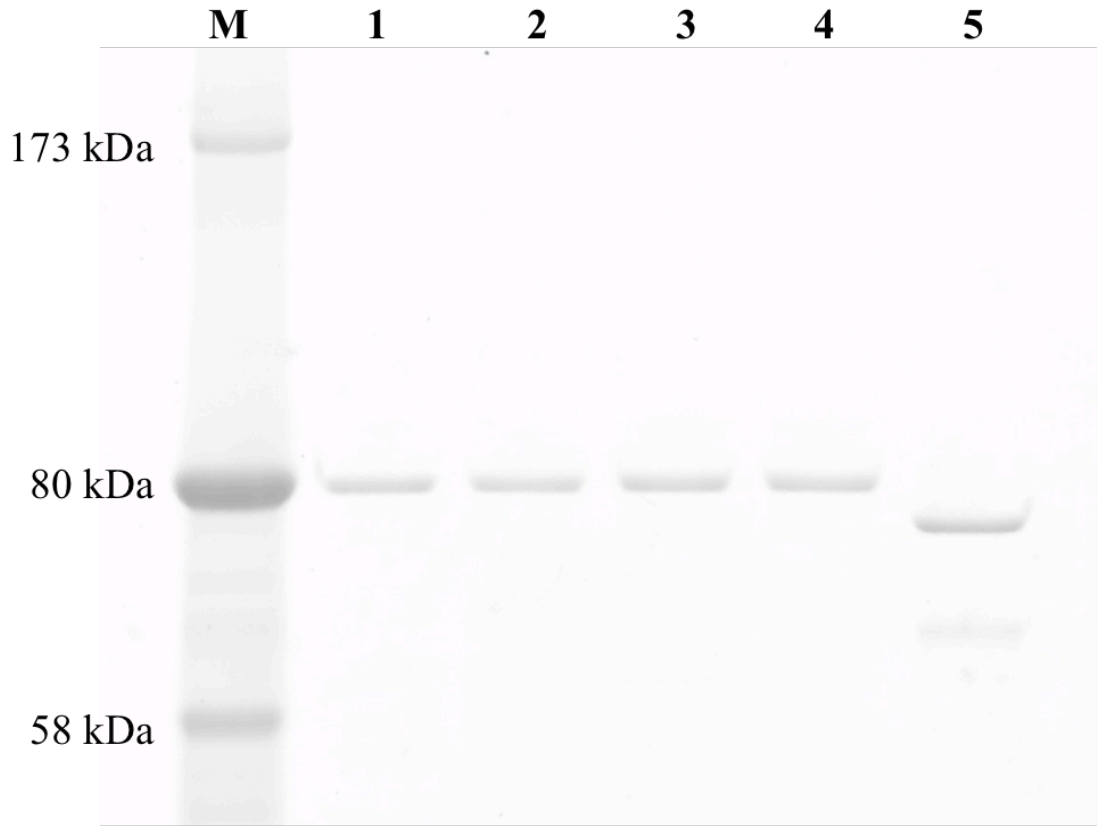
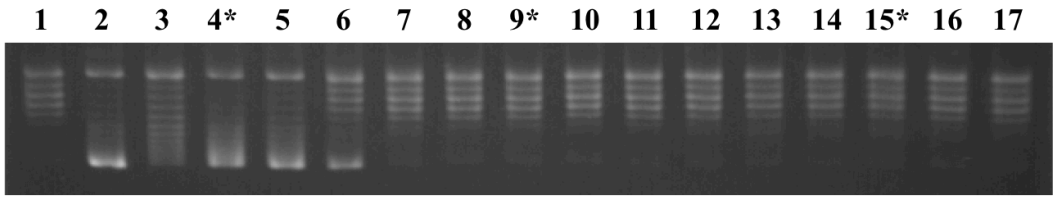


Figure 3. SDS-PAGE analysis of purified *M. leprae* DNA gyrase subunits.
The His-tagged recombinant DNA gyrase subunits were expressed in *E. coli* and purified by Ni affinity column chromatography. One hundred ng of each purified subunit was loaded on a 5-20% gradient polyacrylamide gel. Lane M: Protein marker, lane 1: WT-GyrA, lane 2: Gly89Cys-GyrA, lane 3: Ala91Val-GyrA, lane 4: Asp95Gly-GyrA, lane 5: WT-GyrB.

ATP-dependent DNA supercoiling activities of DNA gyrases

Combinations of GyrA (WT or mutant with Gly89Cys, Ala91Val, or Asp95Gly) and WT-GyrB were examined for DNA supercoiling activities using relaxed pBR322 DNA as a substrate in the presence or absence of ATP (Figure 4). Relaxed DNA was supercoiled when GyrA, GyrB and ATP were all present; no supercoiling activity was observed in conditions lacking any of them.



	1	2	3	4*	5	6	7	8	9*	10	11	12	13	14	15*	16	17
GyrA	–	WT	Gly89Cys	Gly89Cys	Ala91Val	Asp95Gly	WT	Gly89Cys	Gly89Cys	Ala91Val	Asp95Gly	–	WT	Gly89Cys	Gly89Cys	Ala91Val	Asp95Gly
WT GyrB	–	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+
ATP	–	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
Relaxed DNA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Figure 4. ATP-dependent DNA supercoiling assay.

Supercoiling activities of DNA gyrases composed of WT-GyrA, Gly89Cys-GyrA, Ala91Val-GyrA or Asp95Gly-GyrA and WT-GyrB were confirmed. Relaxed pBR322 DNA was incubated with GyrA, GyrB, or both, of the subunits in the presence or absence of ATP. Lane 1: relaxed pBR322 DNA alone; lanes 2-6: relaxed pBR322 DNA, ATP, GyrA and GyrB; lanes 7-11: relaxed pBR322 DNA, ATP and GyrA; lane 12: relaxed pBR322 DNA, ATP and GyrB; lane 13-17: relaxed pBR322 DNA, GyrA and GyrB.

* Twice the amount of DNA gyrase subunits was used in these assays.

Half maximal inhibitory concentrations of quinolones against WT and mutant DNA gyrases

The IC_{50}s were determined by the quinolone-inhibited DNA supercoiling assays. Dose-dependent inhibition was observed in each combination of quinolones and DNA gyrases (Figure 5). As shown in Table 1, the IC_{50}s widely varied among the tested quinolones. Both DC-159a and sitafloxacin showed much lower IC_{50}s against every DNA gyrase than moxifloxacin did. Respective IC_{50}s of DC-159a and sitafloxacin against WT DNA gyrase were 2.8- and 5.5-fold lower when compared with those of moxifloxacin, which were 9.8- and 11.9-fold lower against the DNA gyrase with Gly89Cys, 3.0-, 5.3-fold lower against the DNA gyrase with Ala91Val, and 4.4- and 6.4-fold lower against DNA gyrase with Asp95Gly. Fold changes of DC-159a and sitafloxacin between IC_{50}s against the WT and the mutant DNA gyrases were at most 7.0 and 9.5, respectively, whereas that of moxifloxacin reached up to 20.5.

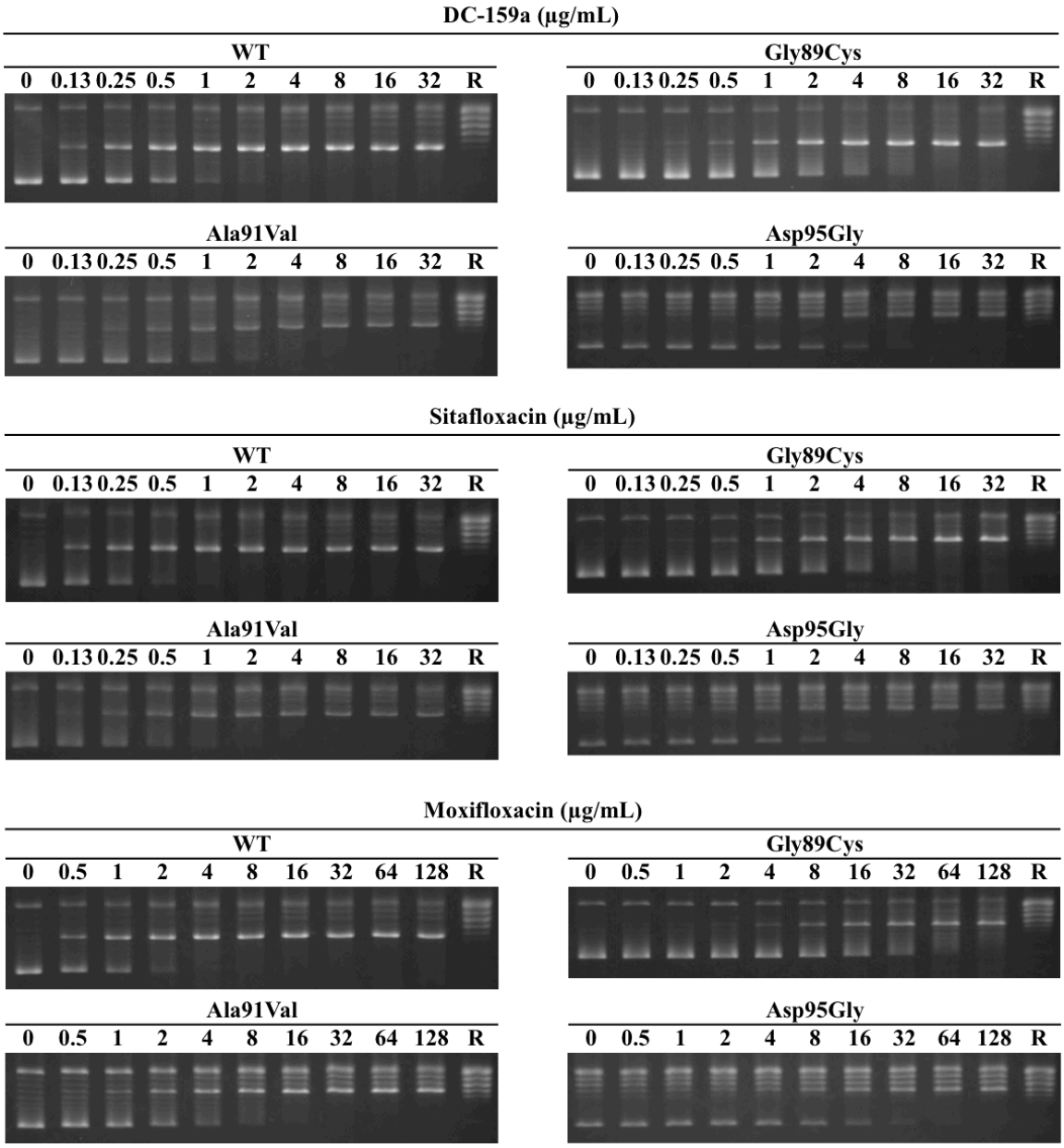


Figure 5. Quinolone-inhibited DNA supercoiling assay.

Relaxed pBR322 DNA was mixed and incubated with GyrA, GyrB, ATP and quinolones at the indicated concentrations. Each quinolone was examined for its inhibitory activity against WT DNA gyrase and mutant DNA gyrases with Gly89Cys-GyrA (indicated as Gly89Cys), Ala91Val-GyrA (Ala91Val) and Asp95Gly-GyrA substitution (Asp95Gly). The lanes labeled as R indicate relaxed pBR322 DNA.

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

Drug	IC_{50} (μg/mL)			
	WT	Gly89Cys	Ala91Val	Asp95Gly
DC-159a	0.4 ± 0.0	2.3 ± 0.3	0.7 ± 0.1	2.8 ± 0.1
Sitafloxacin	0.2 ± 0.0	1.9 ± 0.1	0.4 ± 0.1	1.9 ± 0.1
Moxifloxacin	1.1 ± 0.0	22.6 ± 2.9	2.1 ± 0.1	12.2 ± 1.3

Each value is expressed as the mean ± standard deviation (n = 3).

Concentrations of quinolones that convert 20% of input DNA to the linear form against WT and mutant DNA gyrases

The CC_{20}s of the three quinolones were determined by DNA cleavage assays. Dose-dependency of DNA cleavage is shown in Figure 6. The CC_{20}s of all tested conditions are summarized in Table 2. The CC_{20}s of DC-159a and sitafloxacin against all the tested DNA gyrases were lower than those of moxifloxacin. Respective CC_{20}s of DC-159a against the WT and the mutant DNA gyrases with Gly89Cys, Ala91Val and Asp95Gly were 4.0-, 13.5-, 5.5- and 8.3-fold lower, and respective CC_{20}s of sitafloxacin were 4.0-, 9.8-, 5.5- and 8.9-fold lower than those of moxifloxacin. Fold changes of DC-159a and sitafloxacin between CC_{20}s against the WT and the mutant DNA gyrases were no more than 14.0 and 13.0, respectively, whereas that of moxifloxacin reached up to 29.0.

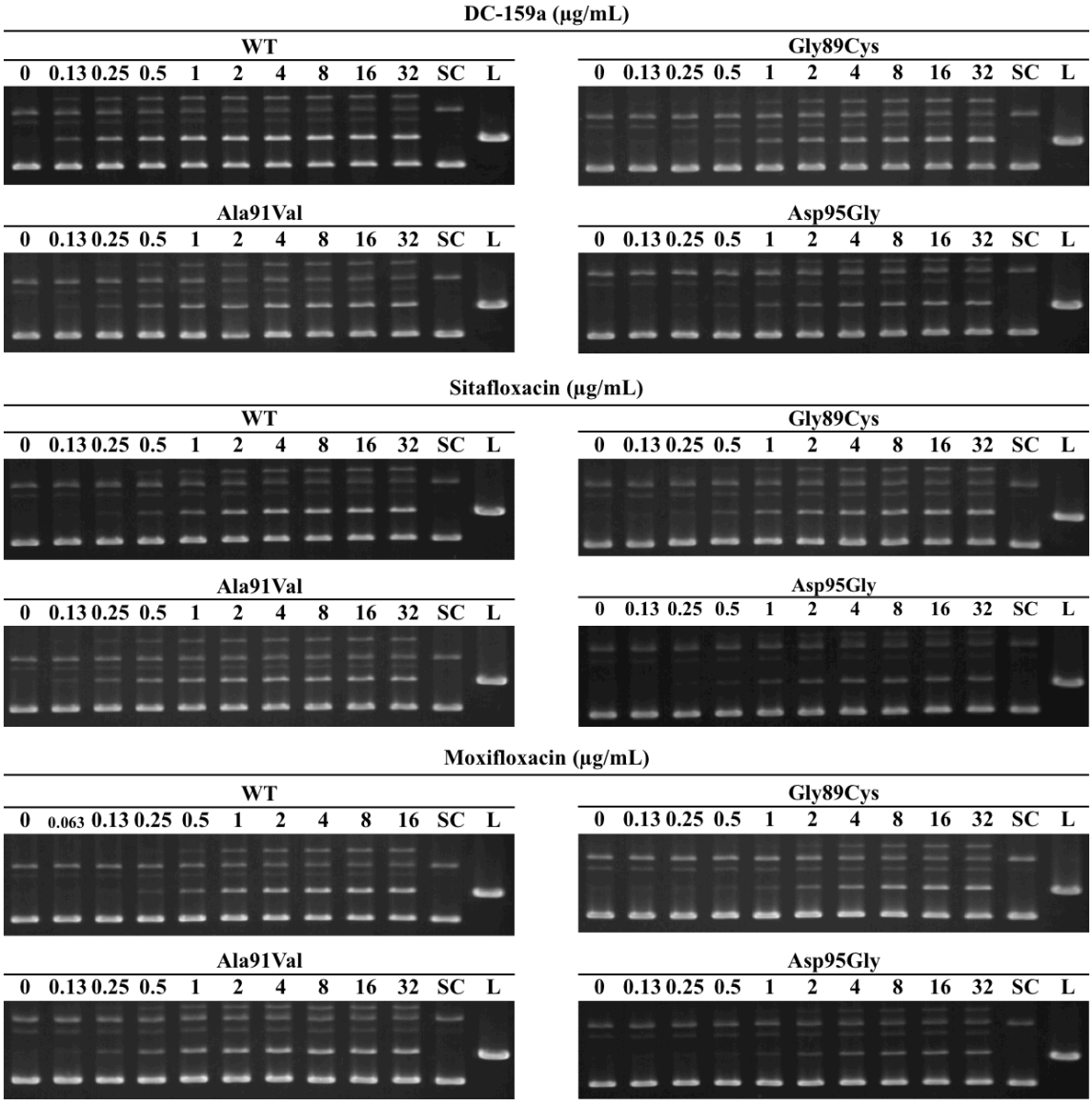


Figure 6. Quinolone-mediated DNA cleavage assay.

Supercoiled pBR322 DNA was mixed and incubated with GyrA, GyrB, ATP and quinolones at the indicated concentrations. Each quinolone was examined for its cleaving activity against WT DNA gyrase and mutant DNA gyrases with Gly89Cys-GyrA (indicated as Gly89Cys), Ala91Val-GyrA (Ala91Val) and Asp95Gly-GyrA (Asp95Gly). The lanes labeled as SC and L indicate supercoiled and linearized pBR322 DNA, respectively.

Table 2. CC_{20}s of quinolones against DNA gyrases of WT and mutant *M. leprae*.

Drug	CC_{20} (μg/mL)			
	WT	Gly89Cys	Ala91Val	Asp95Gly
DC-159a	0.1 ± 0.0	0.8 ± 0.1	0.4 ± 0.1	1.4 ± 0.3
Sitafloxacin	0.1 ± 0.0	1.1 ± 0.3	0.4 ± 0.1	1.3 ± 0.4
Moxifloxacin	0.4 ± 0.0	10.8 ± 2.2	2.2 ± 0.4	11.6 ± 1.9

Each value is expressed as the mean ± standard deviation (n = 3).

Discussion

In this study, we focused on quinolones expected to have high potency against the DNA gyrases of WT and ofloxacin-resistant *M. leprae*. We examined the inhibitory activity of moxifloxacin, sitafloxacin and DC-159a by measuring their IC_{50}s and CC_{20}s using the quinolone-inhibited DNA supercoiling assay and the quinolone-mediated DNA cleavage assay using recombinant WT and mutant DNA gyrases.

Usually, potencies of antibacterial agents against bacteria are evaluated and compared using MICs of the agents[2]. The MICs are defined as the lowest concentrations to inhibit visible growth of microorganisms, and are determined by conducting *in vitro* drug susceptibility tests, exposing target organisms directly to the agents. This parameter is also used to estimate clinical efficacies of antibacterial drugs. However, determination of the MICs is not always possible. In the case of *M. leprae*, as this bacterium is yet to be cultured on any artificial media, MICs are not currently available. Thus, for quinolone assessment, instead of MICs, ICs and CCs have been examined. In that regard, correlations between MICs and ICs or CCs have been reported in previous studies. For example, *M. tuberculosis,* which, similar to *M. leprae*, possesses DNA gyrase as a sole target of quinolones, has high positive correlations between MIC and ICs or CCs[3]. Hence, for the present work we considered that *M.*

leprae would also show these correlations and that IC_{50}s and CC_{20}s could be used for estimating bactericidal efficacies of quinolones against *M. leprae*.

In the quinolone-inhibited DNA supercoiling assay and the quinolone-mediated cleavage assay, IC_{50}s and CC_{20}s of all tested quinolones became lowest when they were examined for WT DNA gyrase. Interestingly, in both assays DC-159a and sitafloxacin always showed lower IC_{50}s and CC_{20}s than moxifloxacin. The lower level of these IC_{50}s and CC_{20}s indicated that DC-159a and sitafloxacin have higher potencies than moxifloxacin. In addition, IC_{50}s and CC_{20}s of DC-159a and sitafloxacin against the mutant DNA gyrases showed the lower fold changes from the values of the WT DNA gyrase when compared with that of moxifloxacin. That difference implied that DC-159a and sitafloxacin retained their inhibitory activities even against mutant DNA gyrases.

Compared with other types of DNA gyrases, twice the amounts of DNA gyrase subunits (60.0 ng of Gly89Cys-GyrA and 48.8 ng of WT GyrB) were used for the assays of the DNA gyrase with Gly89Cys because IC_{50}s and CC_{20}s could not be measured at the same concentrations of DNA subunits. The extent of the effect of this substitution on IC_{50}s of quinolones was previously estimated[33]. IC_{50}s of quinolones against the DNA gyrase with Gly89Cys were reported to be no lower than those against

the DNA gyrase with Ala91Val[33]. This previously reported outcome indicates that Gly89Cys in GyrA can confer equal or higher resistance to quinolones as Ala91Val. In the present study, we provide evidence that is in agreement with previous work. For example, we found IC_{50}s of moxifloxacin against DNA gyrase with Gly89Cys to be more than 10 times higher than IC_{50}s of moxifloxacin against DNA gyrase with Ala91Val. Even though it is likely that IC_{50}s increased in the assays for DNA gyrase with Gly89Cys because twice the amount of DNA gyrase subunits was used, the large gap observed in IC_{50}s between Gly89Cys and Ala91Val may not be solely due to differences in assay conditions.

Although moxifloxacin showed the highest IC_{50} and CC_{20} values for all types of DNA gyrase in the present work, it should be noted that moxifloxacin has been shown to be effective in leprosy treatment. For instance, in previous *in vitro* studies, moxifloxacin showed a much higher inhibitory effect on *M. leprae* DNA gyrase than ofloxacin did[33, 61, 62]. Moreover, a strong bactericidal activity of moxifloxacin was also reported in human cases of leprosy[41, 42]. Therefore, the fact that in the present study DC-159a and sitafloxacin were shown to be more potent than moxifloxacin suggests that there is a strong likelihood they will be far more successful for treating leprosy than ofloxacin.

So far, only Gly89Cys and Ala91Val in GyrA have been found in clinical strains as amino acid substitutions that can confer ofloxacin resistance to *M. lepra*e, and a majority of ofloxacin-resistant *M. leprae* strains bear the latter[56]. In the present study, it was observed that the IC_{50}s and CC_{20}s of DC-159a and sitafloxacin against DNA gyrase with Ala91Val were the same or lower than those values for moxifloxacin against WT DNA gyrase. Taken together with the higher activity shown by moxifloxacin in comparison with ofloxacin, this outcome implies that DC-159a and sitafloxacin could be effective even against the majority of ofloxacin-resistant cases if the drugs could attain the same concentration in leprosy lesions as does moxifloxacin.

Sitafloxacin is already commercially available in Japan and Thailand. Its recommended dosage for bacterial infections is no more than 100 mg twice a day, whereas the other quinolones are usually administered at a dosage of at least 200 mg once or twice a day[27, 53]. Even at the relatively low dose of 100 mg twice a day, the pharmacokinetic and pharmacodynamic properties of sitafloxacin indicate that its efficacy against gram-positive or -negative bacteria is the same or better than that exerted by moxifloxacin and ofloxacin at their usual dose[53]. In addition, *in vivo* and *in vitro* studies have reported a good synergistic effect of sitafloxacin with rifampicin against *M. leprae*[12, 13]. For these reasons, sitafloxacin seems to be a good option for the

treatment of leprosy. However, there is a concern about its safe use in living bodies due to the presence of a chlorine atom at R_8 in its structure and therefore, its administration remains controversial. For example, while some published articles reported no serious problems associated with sitafloxacin as long as it was administered at a clinically recommended dosage, others reported phototoxicity of this drug in *in vivo* experiments[5, 11, 27, 35, 40, 48].

In contrast, DC-159a is neither commercially available nor has it been assessed with *M. leprae* DNA gyrases yet. However, DC-159a has a similar structure to sitafloxacin (Figure 7). The noteworthy structural difference between these two drugs is at the R_8 substituent. Indeed, DC-159a has a methoxy group at this position, whereas, as described above, sitafloxacin has a chlorine atom as the corresponding substituent. The R_8 substituent has been considered to be responsible for adverse effects, especially phototoxicity and 8-methoxy quinolones have been reported to have less phototoxicity than 8-halogenated quinolones[28, 30, 40]. In fact, moxifloxacin, which has a methoxy group at the position, showed no phototoxicity in a mouse model[18, 32]. Because quinolones bring better therapeutic outcomes when they are administered at a higher dose and the adverse effects are mostly dose-dependent, this characteristic may be crucial[28, 38]. In this respect, DC-159a seems to have an advantage. It can then be

expected that this drug will have a lower frequency of adverse effects than 8-halogenated quinolones in patients with skin lesions when it is administered at high doses to achieve success in treatment of ofloxacin-resistant leprosy.

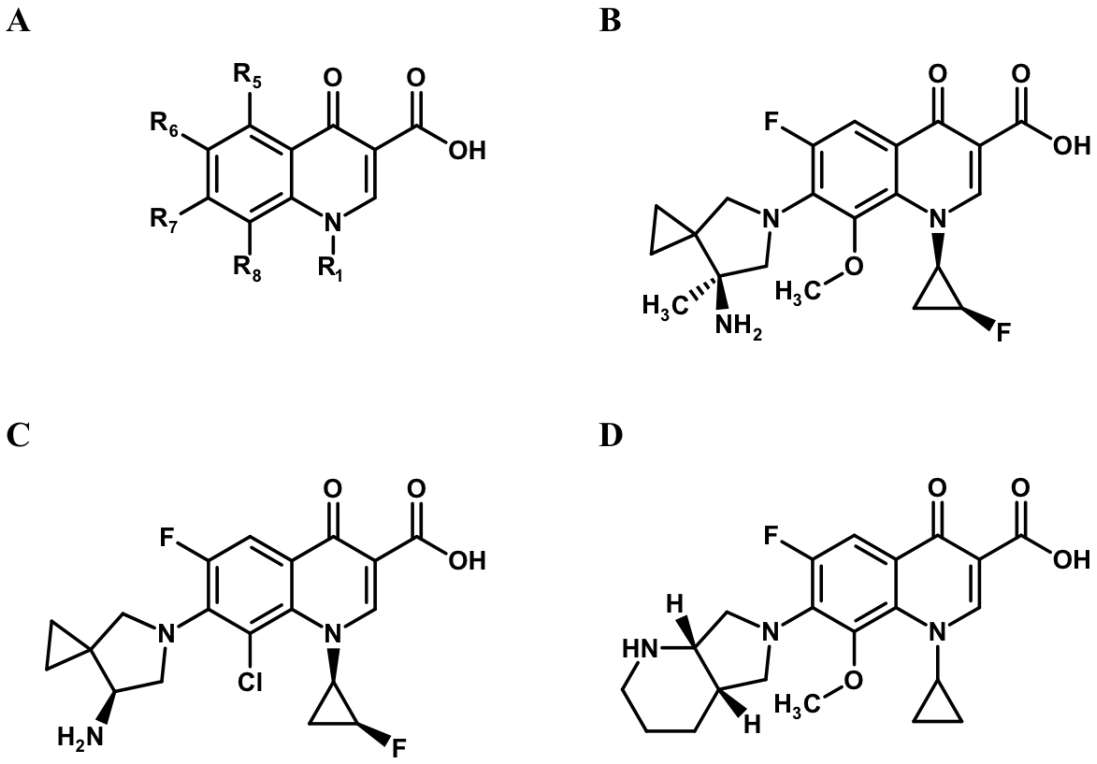


Figure 7. Structures of quinolones tested in this study.

(A) The basic structure of quinolones, (B) DC-159a, (C) Sitafloxacin and (D) Moxifloxacin.

In conclusion, we found that the inhibitory activities of DC-159a and sitafloxacin are sufficient and these drugs are much more effective against *M. leprae* DNA gyrases with any reported mutations related to quinolone resistance than moxifloxacin. Moreover, we showed that these drugs possess strong inhibitory effects even against the mutant DNA gyrases of most ofloxacin-resistant strains. DC-159a in particular seems to be a very promising candidate that may supersede the current quinolone remedies because its structural characteristics suggest a reduced likelihood of adverse effects. However, current *in vivo* data for DC-159a including its distribution to skin lesions and its adverse effects in humans are still scarce. These findings provide strong evidence to warrant further investigation to assess the effectiveness of DC-159a in clinical leprosy cases.

Summary

Some new quinolones have been attracting interest due to their remarkable potency that is reportedly better than that of ofloxacin, the fluoroquinolone currently recommended for treatment of leprosy. For example, DC-159a, a recently developed 8-methoxy fluoroquinolone, has been found to be highly potent against various bacterial species. Nonetheless, the efficacy of DC-159a against *M. leprae* is yet to be examined. To gather data that can support highly effective fluoroquinolones as candidates for new remedies for leprosy treatment, I conducted *in vitro* assays to assess and compare the inhibitory activities of DC-159a and two potent fluoroquinolones, sitafloxacin and moxifloxacin that are already known to be more effective against *M. leprae* than ofloxacin. The *in vitro* assays showed that high potencies of DC-159a and sitafloxacin excelled that of moxifloxacin. In addition, these two drugs retained their inhibitory activities even against DNA gyrases of ofloxacin-resistant *M. leprae*. The results suggested that these antibacterial drugs can be good candidates that may supersede current fluoroquinolone remedies. DC-159a in particular is very promising because it is expected to be less likely to cause adverse effects.

CHAPTER II:

Quinolone resistance-associated amino acid substitutions affect enzymatic activity of *Mycobacterium leprae* DNA gyrase

Introduction

It is already clarified and proven that all of the three amino acid substitutions, Gly89Cys, Ala91Val and Asp95Gly contribute to quinolone resistance. Nevertheless, the effect of these amino acid substitutions on *M. leprae* DNA gyrase activity itself, and the reason Asp95Gly has been clinically unreported until now, remain unclear. Hence, to further elucidate the enzymatic activity of WT and mutant *M. leprae* DNA gyrases with Gly89Cys, Ala91Val and Asp95Gly amino acid substitutions, I conducted *in vitro* assays using recombinant DNA gyrase subunits.

Materials and Methods

Construction of expression plasmids and purification of recombinant DNA gyrase subunits

DNA gyrase expression plasmids coding WT GyrA, Gly89Cys-GyrA, Ala91Val-GyrA, GyrA with Asp95Gly-GyrA and WT GyrB constructed in Chapter I were used. Recombinant DNA gyrase subunits were expressed, purified and examined in a similar way to Chapter I.

Time course DNA supercoiling assay

Time course DNA supercoiling assays were carried out under the following conditions. Each assay was carried out in 30 μL of DNA gyrase reaction buffer, with 2 nM relaxed pBR322 DNA, 8 nM GyrA (WT, Gly89Cys-, Ala91Val- or Asp95Gly-GyrA) and 8 nM WT GyrB. The reactions were continued at 30 °C and stopped at 0, 10, 20, 40, 60, 90, 120, 240, 360 and 600 min by addition of 7.5 μL of 5× dye mix (5% SDS, 25% glycerol, 0.25 mg/mL of bromophenol blue). Next, 10 μL of each mixture was subjected to electrophoresis with Lambda DNA-HindIII Digest (New England Biolabs) on 1% agarose 1× TBE gels and stained with 1 μg/mL of ethidium bromide. To assess the activity of DNA gyrases, the amount of DNA supercoiled in the

reactions was quantified with ImageJ (http://rsbweb.nih.gov/ij/) by comparing band brightness of the supercoiled DNA with that of the Lambda DNA-HindIII Digest. Each assay was conducted thrice to confirm its reproducibility.

For DNA gyrases with WT GyrA and Gly89Cys-GyrA, time course DNA supercoiling assay was also carried out using reaction mixtures of 4 nM relaxed PBR322 DNA, 8 nM GyrA and 8 nM WT GyrB (1:1 mixture of relaxed DNA and DNA gyrase), reaction mixtures of 4 nM DNA, 16 nM GyrA and 16 nM GyrB (1:2 mixture) and reaction mixtures of 4 nM DNA, 24 nM GyrA and 24 nM GyrB (1:3 mixture). The reactions were continued at 30 °C and stopped at 0, 10, 20, 30, 40, 50, 60, 90, 120, 240, 360 and 600 min by addition of 7.5 μL of 5× dye mix. Gel electrophoresis and image analysis were conducted in a similar manner as described above. Each assay was conducted thrice to confirm its reproducibility.

Results

Alteration of DNA supercoiling activity by quinolone resistance-associated amino acid substitutions

Recombinant *M. leprae* GyrAs and GyrB were purified and subjected to time course DNA supercoiling assay. Each GyrA (WT, Gly89Cys-, Ala91Val-, or Asp95Gly-GyrA) was mixed with WT GyrB to form DNA gyrase and its supercoiling activity was observed for 600 min. Obtained electrophoretic images showed sequential changes of supercoiling by WT and mutant DNA gyrases (Figure 8). Then, reaction curves of the DNA gyrases were obtained by analyzing the intensity of each band on the images at the position of the supercoiled form, (Figure 9). WT DNA gyrase yielded the highest amount of supercoiled DNA. In comparison, the amount of supercoiled DNA produced by DNA gyrases with Gly89Cys-, Ala91Val- and Asp95Gly-GyrA at 600 minutes was 22.0%, 68.9% and 30.7%, respectively, of the amount produced by the WT. Furthermore, in the assay using DNA gyrase with Asp95Gly-GyrA, a large part of the input relaxed DNA remained unsupercoiled at its initial position after observation ended (Figure 10).

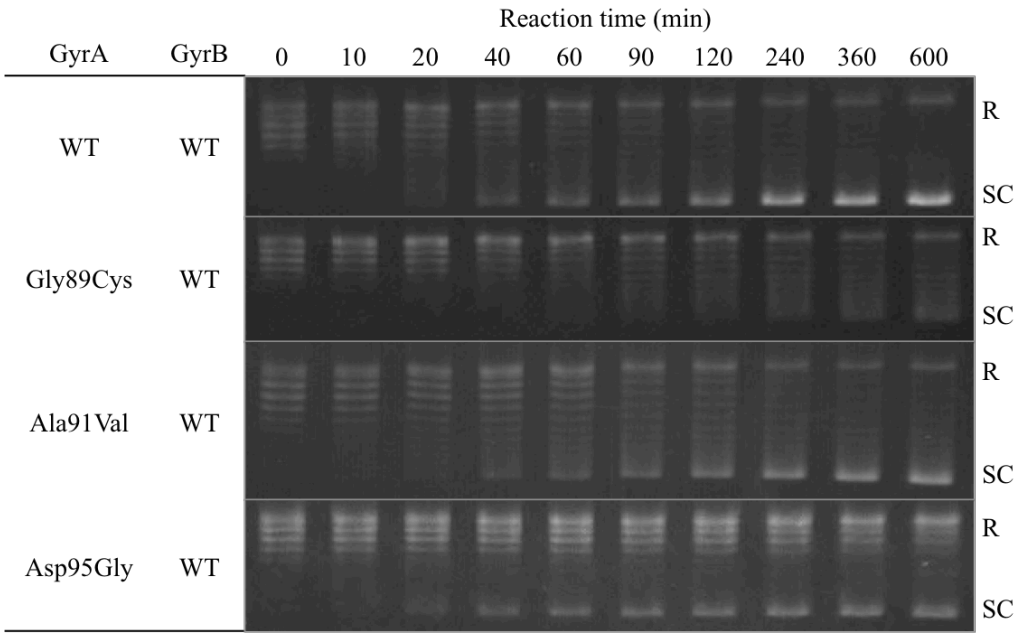


Figure 8. Time-dependent DNA supercoiling by wild and mutant types of *M. leprae* DNA gyrases.

Relaxed pBR322 DNA (2 nM) was mixed and incubated with GyrA (8 nM), GyrB (8 nM), and ATP (1 mM). The supercoiling reaction was then stopped at each time point (as indicated) and examined by gel electrophoresis. R and SC denote the positions of the relaxed and supercoiling forms, respectively.

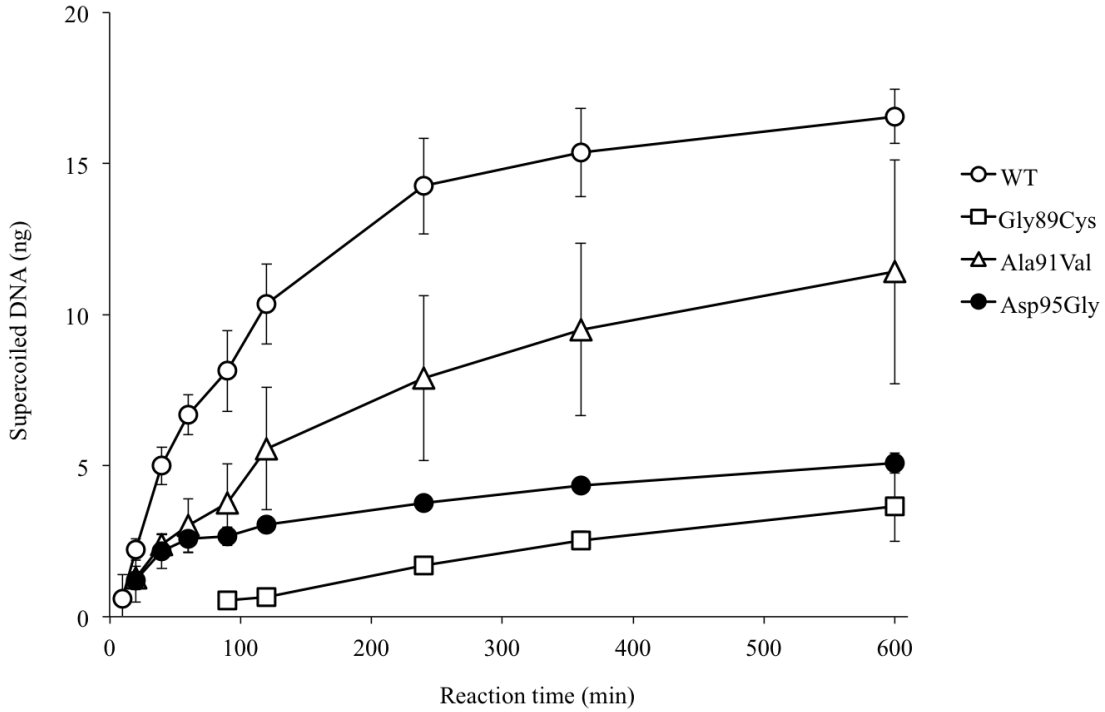
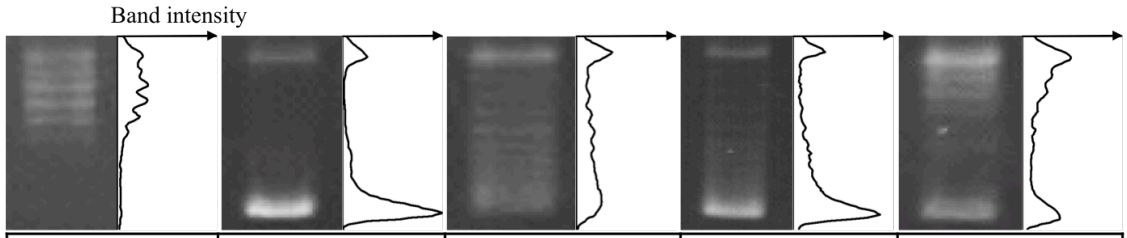


Figure 9. Time-dependent increase in the amount of DNA supercoiled by wild and mutant types of *M. leprae* DNA gyrases.

The amount of supercoiled DNA was calculated by analyzing the bands intensities measured at the position of the supercoiled form on electrophoresis images obtained from the time course DNA supercoiling assay. Data of DNA gyrases with Ala91Val and Asp95Gly at 10 min and of DNA gyrase with Gly89Cys until 90 min were not available because band intensities were not sufficient for measurement. Each symbol and bar indicate the mean ± standard deviation (n = 3).



GyrA	WT	WT	Gly89Cys	Ala91Val	Asp95Gly
GyrB	WT	WT	WT	WT	WT
Reaction time (min)	0	600	600	600	600

Figure 10. Band intensities of electrophoretic images at the initial and the final time points.

The graphs indicate band intensities of corresponding positions on electrophoretic images. The intensities directly correlate to the amount of DNA molecules at the corresponding positions.

Supplementation of reduced activity of Gly89Cys-substituted DNA gyrase by increasing the enzyme concentration

To further characterize the lowest level of supercoiling activity of DNA gyrase with Gly89Cys-GyrA, an additional time course assay was conducted with 1:1, 1:2 and 1:3 molecular ratio of relaxed DNA to DNA gyrase. Figure 11 and 12 show changes in the supercoiling reaction and the amount of supercoiled DNA when more DNA gyrase was introduced to the reaction mixture. WT DNA gyrase supercoiled relaxed DNA molecules more rapidly under a higher molecular ratio. DNA gyrase with Gly89Cys-GyrA also produced more supercoiled DNA when a larger amount of DNA gyrase subunits was applied. Compared with the amount of supercoiled DNA produced under the 1:2 ratio, the amount of supercoiled DNA at 600 min produced by DNA gyrase with Gly89Cys-GyrA under the 1:3 ratio was increased by 48.8% and the gap with DNA gyrase with WT GyrA became smaller. The curve for the assay using Gly89Cys-GyrA under the 1:1 ratio could not be obtained because calculation of the amount of supercoiled DNA via image analysis was not possible.

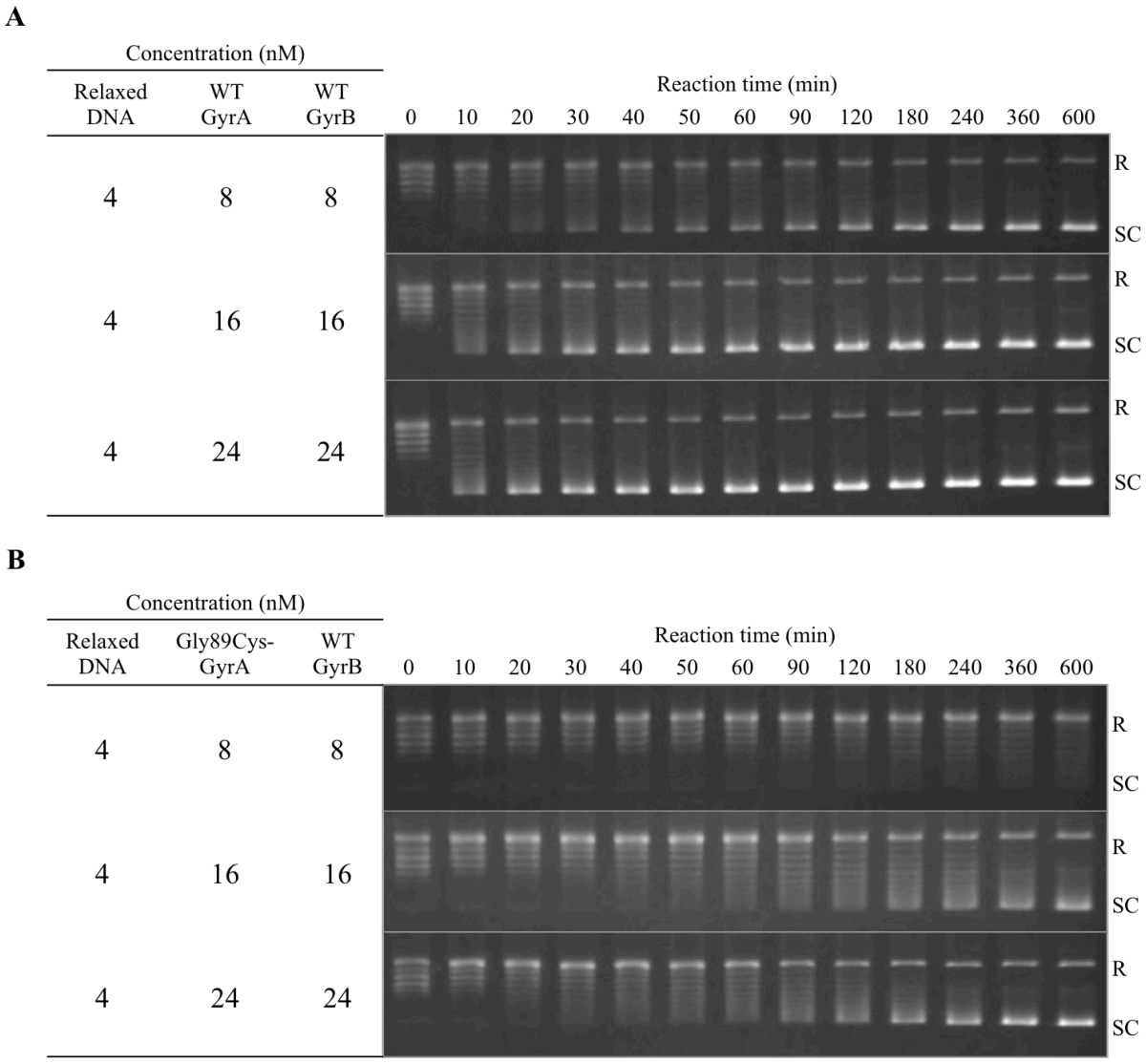


Figure 11. Supercoiling activity of WT and Gly89Cys *M. leprae* DNA gyrases at various molecular concentrations.

Supercoiling of (A) DNA gyrase with WT GyrA and (B) DNA gyrase with Gly89Cys-GyrA were examined under conditions of different subunit concentrations. Relaxed pBR322 DNA (4 nM), GyrA (8, 16 or 24 nM) and WT GyrB (8, 16 or 24 nM) were mixed with ATP (1 mM) and incubated at 30 °C. The reaction was stopped at each time point (as indicated) and then examined by gel electrophoresis. R and SC denote positions of the relaxed and supercoiling forms, respectively.

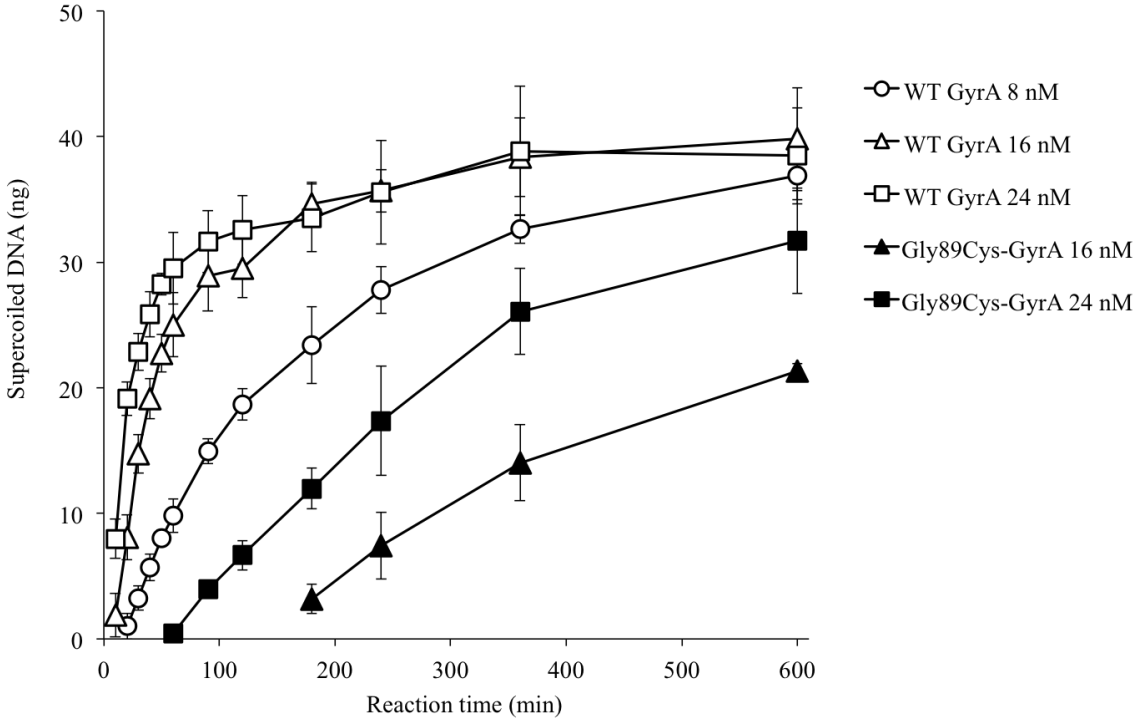


Figure 12. The amount of DNA Supercoiled by WT and Gly89Cys *M. leprae* DNA gyrase at various molecular concentrations.

Band intensities at the position of the supercoiled form on the electrophoresis images were measured to calculate the amount of supercoiled DNA at the indicated time points. Data of 8 nM WT GyrA at 10 min, of 16 nM Gly89Cys-GyrA until 60 min and of 24 nM Gly89Cys-GyrA until 180 min and a curve for 8 nM Gly89Cys-GyrA were not available because band intensities were not sufficient for measurement. Each symbol and bar indicates the mean ± standard deviation (n = 3).

Discussion

In this study, focusing on the effects caused by quinolone resistant-conferring amino acid substitutions on the enzymatic activity of *M. leprae* DNA gyrase, I conducted time course DNA supercoiling assays to examine the supercoiling activity of WT and three mutant types of DNA gyrases. The results clearly showed a particular effect of each amino acid substitution on the supercoiling activity of DNA gyrase (Figure 8). Using time course assays, I showed that amino acid substitution Ala91Val causes the least effect on the supercoiling activity of *M. leprae* DNA gyrase. Even though previous studies established that the level of quinolone-resistance conferred by this substitution was the lowest[60, 61], considering the highest supercoiling activity among the tested mutant DNA gyrases, it seems reasonable that this amino acid substitution is selected by the majority of quinolone-resistant *M. leprae* strains.

Unlike DNA gyrase with Ala91Val amino acid substitution found at clinical level, DNA gyrase with Gly89Cys-GyrA showed the lowest supercoiling activity. Nonetheless, the fact that this amino acid substitution was found in clinical *M. leprae* strains is a strong evidence that DNA gyrase with Gly89Cys-GyrA does not totally lose enzymatic activity[17, 56]. The electrophoretic image and its band intensities indicated that DNA gyrase with Gly89Cys-GyrA was gradually converting the entire set of input

relaxed DNA molecules to the supercoiled form in a similar manner as DNA gyrases with WT or Ala91Val-GyrA did, even though supercoiling was quite slow (Figure 9 and 10). Therefore, it seems that the amount of DNA gyrase is important for the rate by which DNA gyrase with Gly89Cys-GyrA induces supercoiling. To demonstrate that the DNA supercoiling rate can be supplemented by the molecular amount of DNA gyrase subunits, I conducted an additional assay of time course DNA supercoiling by changing the molecular ratio of circular DNA and DNA gyrase from 1:1 to 1:3. In this assay, to clearly identify the difference between assay conditions, the amount of relaxed DNA (4 nM) was applied twice to assist the low supercoiling activity of DNA gyrase with Gly89Cys-GyrA. The results obtained from this assay clearly showed that the amount of supercoiled DNA produced by DNA gyrase with Gly89Cys-GyrA can be increased when larger amounts of DNA gyrase subunits are applied. In addition, both the electrophoretic images and the reaction curves showed that the supercoiling activity of DNA gyrase with Gly89Cys became more similar to that of WT, as a larger amount of enzymes was introduced (Figure 11 and 12). These findings suggested that DNA gyrase with Gly89Cys can play a role similar to that of WT as long as subunits are sufficiently expressed in the bacterial cells. However, it should be noted that the time course assays in this study had a limitation since they were not conducted with the same DNA and

DNA gyrase concentrations as those found in actual bacterial cells. Therefore, to estimate and discuss the *in vivo* impact of Gly89Cys amino acid substitution on survival of *M. leprae*, further investigation to establish the number of molecules of DNA gyrase functioning inside individual bacilli of *M. leprae* is required.

No amino acid substitution at position 95 has been previously found in clinical strains of *M. leprae*, although its equivalent substitution, Asp94Gly, is frequently reported in *M. tuberculosis*. Previously conducted *in vitro* studies using recombinant DNA gyrase with Asp95Gly demonstrated that this substitution could also contribute to quinolone resistance[60, 61]. It was shown that the level of quinolone resistance brought about by Asp95Gly is higher than that by Ala91Val, and the same or higher than that by Gly89Cys. In this study, it was revealed that the supercoiling activity of *M. leprae* DNA gyrase was largely affected by with Asp95Gly, but was still higher than that of DNA gyrase with Gly89Cys-GyrA, which has been reported in clinical strains[56]. The electrophoretic images indicated that the supercoiling process between DNA gyrase with Asp95Gly-GyrA and the other DNA gyrases was clearly different (Figure 8). DNA gyrase with Asp95Gly-GyrA did not supercoil all the input relaxed DNA molecules, and part of the relaxed DNA remained at their initial positions, whereas DNA gyrases found in clinical strains, including WT, gradually supercoiled the

entire set of input relaxed DNA molecules (Figure 10). It seems that the change in the supercoiling process represents a critical defect in the function of *M. leprae* DNA gyrase caused by Asp95Gly, which may be a reason Asp95Gly has not been clinically reported, despite conferring a high level of quinolone resistance while keeping the supercoiling activity higher than Gly89Cys. I speculate that DNA gyrase with Asp95Gly less frequently move out from the bound DNA molecule to another after giving a supercoil than the other DNA gyrases, and that this excessive persistence on DNA might interrupt DNA replication and transcription and be fatal for the DNA gyrase function. To confirm this speculation, I plan further analysis to examine other DNA gyrase properties, including DNA-DNA gyrase binding affinity.

In summary, I showed that amino acid substitutions that confer quinolone resistance to *M. leprae* DNA gyrase simultaneously cause diminution of enzymatic activity. Furthermore, it was demonstrated that amino acid substitutions Gly89Cys and Ala91Val reduced the enzymatic activity of DNA gyrase, but did not alter the innate supercoiling process of DNA gyrase as Asp95Gly did. From the results of this study, I can conclude that changes in the supercoiling process of DNA gyrase caused by Asp95Gly may be a plausible explanation why this amino acid substitution has never been reported in clinical strains of quinolone-resistant *M. leprae*.

Summary

It is proven that amino acid substitutions, Gly89Cys, Ala91Val and Asp95Gly in QRDR of *M. leprae* DNA gyrase are responsible for quinolone resistance. However, the effect of those mutations on the enzymatic activity of DNA gyrase is yet to be studied in depth. Hence, to further elucidate the effect of mutations, I conducted *in vitro* assays using WT and mutant recombinant DNA gyrases to compare supercoiling reactions of them. The time course DNA supercoiling assays showed that all tested mutant DNA gyrases converted less DNA from relaxed to supercoiled form than the WT did, and that each amino acid substitution had its own unique effect on the enzymatic activity of DNA gyrase. DNA gyrase with Ala91Val was found to possess the highest activity among the mutant DNA gyrases. DNA gyrase with Asp95Gly, previously unreported in clinical *M. leprae* strains, supercoiled in a different manner than did the other tested DNA gyrases, including the WT. As DNA gyrase with Asp95Gly did not supercoil all the input DNA molecules, part of these molecules remained in the original relaxed state. Intriguingly, DNA gyrase with Gly89Cys turned out to possess the lowest level of activity despite being found in clinical strains. It was also suggested that DNA gyrase with Gly89Cys may play a role similar to that of wild type DNA gyrases, as long as a sufficient amount of subunits are expressed in the

bacterial cells. These results clearly reflect the fact that the majority of quinolone-resistant *M. leprae* strains have Ala91Val, and only a few cases have been found to have Gly89Cys. The results also suggest that Asp95Gly may cause changes in the supercoiling process of DNA gyrase, which may be a reason this substitution has not been found in clinical strains.

CONCLUSION

DNA gyrase is an indispensable enzyme for *M. leprae* and also a highly important target for antimicrobials. Quinolones are one of antibacterial classes that inhibit the DNA gyrase activity, and widely used for treatment of bacterial infection, including leprosy. Although ofloxacin is used in the current MDT, some other quinolones are expected to have high potency but have not been adapted to the standard regimen yet. Thus, there still remained necessity to investigate the efficacy of those quinolones as more optimized choices and the characteristics of DNA gyrase as the drug target.

First, in the Chapter I, I demonstrated that a newly developed quinolone, DC-159a can be a very promising candidate with sufficient potency even against DNA gyrase of the majority of quinolone-resistant *M. leprae* and expectation of less likelihood to cause adverse effects.

In the following Chapter II, from an aspect of the DNA gyrase activity, I described why the majority of quinolone-resistant *M. leprae* has Ala91Val, but not Gly89Cys. In addition, I also suggested a reason for no clinical report of Asp95Gly, which had been experimentally proved to have the enzymatic activity.

Through the results above, I consider that although *M leprae* can obtain resistance to quinolones, this antibacterial family still has potential enough to overcome this obstacle. The findings from the present study can be a foundation for deeper understanding of DNA gyrase that helps identify the possible types of *M. leprae* DNA gyrase as drug targets and assist development of the best quinolone that can contribute to leprosy control.

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和文要旨

らい菌（*Mycobacterium leprae*）はハンセン病の原因となる抗酸菌である。かつてハンセン病は治療が非常に困難な病と考えられていたが、現在は化学療法によって完治が可能な感染症として認識されている。ハンセン病の化学療法においては、複数系統の抗菌薬を同時に併用することが推奨されており、キノロン系と呼ばれる抗菌薬系統は病変が単一の少菌型症例に対する処方に含まれている。キノロン系抗菌薬は細菌 DNA の複製や転写に必須の酵素である II 型トポイソメラーゼを標的とすることが知られている。II 型トポイソメラーゼの一つである DNA ジャイレースは、DNA に負の超らせんを導入することで複製や転写の際に生じる正の超らせんを解消する役割を担うが、この負の超らせん化の際には DNA ジャイレースによって DNA の切断、ねじれの導入及び DNA の再結合が繰り返される。このときキノロン系抗菌薬は DNA の切断部分に入り込むことで再結合を妨げ DNA ジャイレースによる超らせん化を阻害する。そのため、キノロン系抗菌薬が結合する部位周辺の DNA ジャイレース上の領域にアミノ酸置換が起きるとキノロン系抗菌薬に対する耐性がもたらされる。現在、らい菌の DNA ジャイレースにキノロン耐性を与えるアミノ酸置換として、臨床株で確認されている DNA ジャイレース A サブユニット上の 89 位、91 位のアミノ酸置換と、実験的に示された 95 位のアミノ酸置換のみが報告されている。

本研究では DNA ジャイレースの持つキノロン系抗菌薬の標的としての側面と細菌の必須酵素としての側面に注目し、キノロン耐性らい菌の DNA ジャイレースに対する新規開発キノロン系抗菌薬の阻害活性と、キノロン耐性をもたらすアミノ酸置換がらい菌 DNA ジャイレースの酵素活性そのものに与える影響について明らかにした。

1. 新規開発キノロン系抗菌薬 DC-159a のらい菌 DNA ジャイレース阻害活性

キノロン系抗菌薬はその基本骨格に付属する側鎖によって II 型トポイソメラーゼの阻害活性が大きく異なる。ハンセン病に対する多剤併用療法ではオフロキサシンが現在用いられているが、より高いらい菌 DNA ジャイレースの阻害活性が認められるキノロンがいくつか報告されている。本研究では、キノロン耐性をもたらすアミノ酸置換を導入したらい菌 DNA ジャイレースを使用し、らい菌 DNA ジャイレースを強力に阻害することが既に認められているモキシフロキサシン及びシタフロキサシンと、高い活性が期待される新規開発キノロン系抗菌薬 DC-159a の阻害活性を試験した。その結果、シタフロキサシン及び DC-159a のらい菌 DNA ジャイレース阻害活性はモキシフロキサシンを大きく上回り、オフロキサシン耐性臨床株の大部分が持つ 91 位のアミノ酸置換を導入した DNA ジャイレースをも十分に阻害できることが見出された。特に DC-159a は、キノロン系抗菌薬の中でも副作用の報告が少ない誘導体に共通する特徴的な8位の側鎖構造を持つため、ハンセン病治療薬として期待されるべき抗菌薬であることが示

された。

2. キノロン耐性をもたらすアミノ酸置換が DNA ジャイレースの酵素活性に与える影響

DNA ジャイレース A サブユニットの 89 位、91 位及び 95 位のアミノ酸が置換されることによって、らい菌 DNA ジャイレースにキノロン系抗菌薬に対する耐性がもたらされることは既に明らかである。しかしながら、これらのアミノ酸置換が DNA ジャイレースの酵素活性そのものに与える影響については未だ解析されていない。本研究では、キノロン耐性を与えるアミノ酸置換を導入したらい菌 DNA ジャイレースによる DNA の超らせん化を観察することで、野生型及び変異型の酵素活性について比較検討した。試験の結果、キノロン耐性臨床株の大部分で確認される 91 位のアミノ酸変異を導入した DNA ジャイレースの超らせん化活性は変異型間で最も高く、一方 89 位のアミノ酸置換を持つ DNA ジャイレースでは超らせん化活性が最も低いことが示された。また 95 位のアミノ酸置換を導入した DNA ジャイレースでは他の DNA ジャイレースと異なる変化が超らせん化の過程にもたらされていることが確認された。らい菌臨床株において 95 位のアミノ酸置換は未だ報告はなく、今回の結果はその原因が反映されたものであると推察された。

以上の研究から、キノロン系抗菌薬にはその化学構造によってはキノロンに対する感受性を低下させた変異型らい菌に対しても十分な治療成果を期待できる派生体が存在すること、また DNA ジャイレースの酵素活性はらい菌が選択し得るアミノ酸置換と関係があることが示唆された。本研究により得られた知見は、ハンセン病治療におけるキノロン系抗菌薬の有用性を支持する重要な論拠を与えるとともに、キノロン耐性獲得の際にらい菌 DNA ジャイレースに起こり得る変異の特定へ繋がる基礎となると考えられ、ハンセン病の制御に向けた最適な化学療法指針の策定への貢献を期待し得るものである。

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