Inhibitory activities of WQ-3810 and its analogs against DNA gyrase of *Mycobacterium leprae*

(らい菌のＤＮＡジャイレースに対するWQ-3810とその類縁体の阻害活性)

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LIST OF PAPERS

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CONTENTS

ABBREVIATION

ML	*Mycobacterium leprae*
WHO	World Health Organization
TB	Tuberculosis
MDT	Multidrug therapy
GyrA	DNA gyrase subunit A
GyrB	DNA gyrase subunit B
ATP	Adenosine triphosphate
QRDR	Quinolone resistance determining region
WT	Wild type
MT	Mutant type
G89C	An amino acid substitution from glycine to cysteine at position 89
A91V	An amino acid substitution from alanine to valine at position 91
D95G	An amino acid substitution from aspartic acid to glycine at position 95
D464N	An amino acid substitution from aspartic acid to asparagine at position 464
E502D	An amino acid substitution from glutamic acid to Aspartic acid at position 502
Asp464	An amino acid aspartic acid at position 95
$GyrA^{WT}$	DNA gyrase subunit A which has not containing amino acid substitutions
$GyrA^{G89C}$	DNA gyrase subunit A which is containing an amino

	acid substitution from glycine to cysteine at position 89
$GyrA^{A91V}$	DNA gyrase subunit A which is containing an amino acid substitution from alanine to valine at position 91
$GyrA^{D95G}$	DNA gyrase subunit A which is containing an amino acid substitution from aspartic acid to glycine at position 95
$GyrB^{WT}$	DNA gyrase subunit B which has not containing amino acid substitutions
$GyrB^{D464N}$	DNA gyrase subunit B which is containing an amino acid substitution from aspartic acid to asparagine at position 464
$GyrB^{E502V}$	DNA gyrase subunit B which is containing an amino acid substitution from glutamic acid to valine at position 502
OFX	Ofloxacin
MFX	Moxifloxacin
IPTG	Isopropyl-beta-D-thiogalactopyranoside
LB	Luria burtani
TBE	Tris-borate-EDTA
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
R	Relaxed pBR322 DNA
S	Supercoiled pBR322 DNA

IC_{50}	Concentration for 50% inhibitory activity

PREFACE

Leprosy is a chronic, infectious disease caused by *Mycobacterium leprae*. The spread of leprosy has been kept under control using an MDT recommended by WHO. However, in 2016 alone, more than 200,000 new cases were reported [1]. Drug resistance problem is one of the main burden of the bacterial pathogens control. Fluoroquinolones are considered to be an important bactericidal drug for treating leprosy because of the broad spectrum against bacterial pathogens. Thus, OFX which is the fluoroquinolone used for single skin lesion paucibacillary cases as part of the above-mentioned MDT [2]. However, the threat of drug resistance are start to be concerned even against ofloxacin.

Fluoroquinolones block the activity of the enzyme, DNA gyrase, which has a crucial role in DNA replication and transcription [3,4]. DNA gyrase is an isotetramer enzyme consisting of two subunits A (GyrA) and two subunits B (GyrB) [4]. Bacteria can develop fluoroquinolone resistance by substituting amino acids in quinolone resistance-determining regions (QRDR) in both of GyrA and B [5]. In case of clinical OFX-resistant *M. leprae*, it replaces glycine with cysteine at position 89 (G89C) and A91V in GyrA [2,6]. In addition, D95G in GyrA which is the most frequently amino acid substitution in quinolone-resistant *M. tuberculosis*, also contributes to quinolone resistance in *M. leprae* [7]. By contrast, D461N and N499D in GyrB are found less frequently in clinical strains of *M. tuberculosis* [8,9]. However, experimentally but not clinically, it was shown that D464N and N502D in GyrB of DNA gyrases in *M. leprae*, which is identical to D461N and N499D in *M. tuberculosis*, cause

quinolone resistance [10].

The current fluoroquinolone remedy, ofloxacin, can not strongly inhibit the enzymatic activity of DNA gyrase containing those amino acid substitutions [7, 10]. Further, recurrence is a major setback when trying to control leprosy because relapse cases have a higher possibility of being accompanied with resistance to anti-leprosy drugs and hence it limits the number of drugs available for leprosy MDT [2,11-14]. Thus, to make a more reliable therapeutic regimen against quinolone-resistant leprosy, screening for drug candidates and developing new drugs are needed.

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WQ-3810 inhibits DNA gyrase activity in ofloxacin-resistant *Mycobacterium leprae*

Introduction

Moxifloxacin (MFX) is known to be a more effective fluoroquinolone against leprosy than OFX [7,10,15,16], and its bactericidal activity is estimated to be equivalent to that of rifampicin, which is one of the first-line drugs in MDT [15]. In multibacillary leprosy cases, MFX was proved to kill leprosy bacilli within days or weeks after a single dose [16]. In contrast, WQ-3810 is a newly developed fluoroquinolone with an innovative NH_2-based molecular structure at the R1 group (Fig 1A) [17, 18]. Although it has been reported that WQ-3810 has a strong bactericidal effect on several pathogenic bacteria [19], WQ-3810 is yet to be tested against *M. leprae*.

In the present study, we aimed to test WQ-3810 as a new drug candidate for *M. leprae.* Fluoroquinolones OFX (Fig 1B) and MFX (Fig 1C) were used as control drugs. To assess the potency of WQ-3810 as a therapeutic drug to treat leprosy and to facilitate comparison between WQ-3810 and control drugs, *in vitro* assays were conducted using recombinant *M. leprae* DNA gyrases including wild type (ML-GyrA^{WT}) and mutants bearing amino acid substitutions in GyrA (ML GyrA^{D91V} and ML-GyrA^{D95G}) and GyrB (ML-GyrB^{D464N} and ML-GyrB^{N502D}) instead of measuring minimum inhibitory concentration as *M. leprae* can not be cultured on artificial medium. In addition, an *in-silico* study was carried out to understand the molecular interaction between WQ-3810 and DNA gyrases.

Materials and Methods

Antibacterial agents

WQ-3810 was kindly provided by Wakunaga pharmaceutical Co., Ltd. (Osaka, Japan). Moxifloxacin and ofloxacin were purchased from LKT Laboratories, Inc. (St. Paul, MN) and FUJIFILM Wako Pure Chemical Industries Corp. (Osaka, Japan), respectively.

Bacterial strains and expression plasmids

The Thai-53 strain of *M. leprae* [20], 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 (Thermos Fisher Scientific Inc.; Waltham, MA) was used for cloning. *E. coli* strains Rosetta-gami^{TM} 2(DE3)pLysS and BL21(DE3)pLysS (Merck KGaA, Darmstadt, Germany) were used for protein expression. The plasmid vector pET20b(+) (Merck KGaA) was used to construct expression plasmids. Relaxed pBR322 DNA (Inspiralis Ltd.; Norwich, UK) was used for the DNA supercoiling assay.

Preparation of recombinant DNA gyrase subunits

DNA gyrase expression plasmids encoding ML-GyrA^{WT}, ML-GyrA^{G89C}, ML-GyrA^{A91V} and ML-GyrA^{D95G} and ML-GyrB^{WT}, ML-GyrB^{D464N} and ML-GyrB^{N502D} were constructed as previously described [7,10,21].

Expression and purification of recombinant DNA gyrase subunits were conducted as previously reported [7,21-23]. Briefly, expression plasmids bearing either *gyrA* or *gyrB* of *M. leprae* were transformed in *E. coli* Rosetta-gami2(DE3)pLysS or BL21(DE3)pLysS, respectively. Transformants were cultured in Luria-Bertani (LB) broth up to the log phase, under ampicillin selection (100 μg/ml).

Expression of DNA gyrases was induced by adding 1 mM isopropyl-beta-D-

thiogalactopyranoside (FUJIFILM Wako Pure Chemical Industries Corp.), and further incubated for 16 to 24 h at 12 or 14 °C. Harvested *E. coli* were lysed by sonication (10 times for 40 s at output level 3 and 40% duty cycle with 40-s intervals) using Sonifier 250 (Branson, Danbury, CT). The supernatant was obtained by centrifugation (10,000× *g* for 30 min). Recombinant DNA gyrase subunits in the supernatant were purified by Ni-NTA Agarose column (Thermo Fisher Scientific Inc.) chromatography, as per the manufacturer's protocol. Next, the standard buffer was replaced by DNA gyrase dilution buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl, 2 mM DTT, 1 mM EDTA), and recombinant DNA gyrase subunits were analyzed by PD-10 chromatography.

Assessment of supercoiling activity

ATP-dependent DNA supercoiling assays were carried out as previously described [21]. Briefly, the DNA supercoiling activity of purified DNA gyrases was assessed using 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, 0.36 mg/mL of BSA, 6.5% glycerol (w/v) and 1 mM ATP], relaxed pBR322 DNA (4 nM), ATP (1 mM), and DNA gyrase subunits; of ML-GyrA^{WT}, ML-GyrA^{G89C}, ML-GyrA^{A91V} or ML-GyrA^{D95G} (40 nM each) and ML-GyrB^{WT}, ML-GyrB^{D464N} or ML-GyrB^{N502D} (40 nM each) in a total volume of 30 µl. For DNA gyrases with ML-GyrA^{G89C}, and ML-GyrB^{WT} (160 nM each) were also used. The mixtures were incubated for 90 min at 30 °C. The reaction was stopped by adding 7.5 µL of 5× dye mix (5% SDS, 25% glycerol and 0.25 mg/mL of bromophenol blue). Next, 10 µL from each reaction mixture was subjected to electrophoresis on 1% agarose gel in 1× Tris-borate-EDTA buffer (TBE; 89 mM Tris, 89 mM borate, 2 mM EDTA, 8.3 pH). The gel was then stained with ethidium bromide (0.7 µg/mL).

Fluoroquinolone-inhibited DNA supercoiling assay

The concentration of fluoroquinolone necessary to inhibit the enzymatic activity of DNA gyrase by 50% was calculated as one IC_{50} resulting from the fluoroquinolone-inhibited DNA supercoiling assay, based on the method described by Fisher and Pan [24]. The assay was conducted with 30 µL of DNA gyrase reaction buffer, 4 nM relaxed pBR322 DNA, 40 nM GyrA (ML-$GyrA^{WT}$, ML-$GyrA^{A91V}$ or ML-$GyrA^{D95G}$), 40 nM GyrB (ML-$GyrB^{WT}$, ML-$GyrB^{D464N}$ or ML-$GyrB^{N502D}$) and fluoroquinolones. WQ-3810 and MFX were used in concentrations from 0.13 to 64 µg/mL for subunit combinations ML-$GyrA^{WT}$, ML-$GyrA^{A91V}$, and ML-$GyrA^{D95G}$ with ML-$GyrB^{WT}$. Similarly, 0.13 to 320 µg/mL was used for subunit combinations ML-$GyrB^{D464N}$, and ML-$GyrB^{N502D}$ with ML-$GyrA^{WT}$. OFX was used in concentrations from 1 to 512 µg/mL for every combination of subunits. Reactions were conducted for 90 min at 30 °C and stopped by adding 7.5 µL of dye mix. Next, 10 µL from each mixture was subjected to electrophoresis on 1% agarose gel in 1× TBE and stained with 0.7 µg/mL of ethidium bromide. The intensity of supercoiled DNA bands for electrophoresis was estimated with ImageJ software.

Simulations for molecular interaction among DNA gyrase, DNA and fluoroquinolones.

Molecular docking and visualization studies were carried out using Molecular Operating Environment (MOE) (Chemical Computing Group ULC, Montreal, Quebec, Canada.) software and MolDesk Basic v1.1.54 (IMSBIO co., Ltd, Tokyo, Japan). The *M. leprae* DNA gyrase molecular structure had not been classified. Therefore, the coordinates of DNA gyrase for structure-based molecular modeling were retrieved from the Protein Data Bank (PDB) with the PDB ID: 5BTA (Crystal structure model of Mtb-gyrase complex) as the amino acid sequence of both GyrA and B in Mtb-gyrase were highly homologous to *M. leprae*. The artificial amino acid substitution in GyrB of Mtb-D461N (D464N in ML) was introduced by WinCoot release 0.8.9.2. Ligand location and pocket size were set using the MFX coordinates, which were retrieved from the 5BTA structural information.

Optional parameters in MOE software were used to create a topology file, which included the addition of hydrogen atoms, the calculation of a grid potential, and a docking simulation. The flexible docking method was used, and scores were calculated as the sum of five potentials: accessible surface area, coulomb potential, hydrogen bonds, hydrogen bond considering anisotropy, and van der Waals interactions. Protein-ligand binding free energy was estimated with MOE software using the Amber 10: EHT force-field which is containing the MOE software package and the default parameters of the MOE Dock application. The results of molecular docking were visualized with PyMOL v1.3. Distances between amino acids and the side chains of WQ-3810 were calculated using MOE software.

Results

ATP-dependent DNA supercoiling activity of DNA gyrases.

The enzymatic activity of purified DNA gyrase subunits was assessed with a DNA supercoiling assays using relaxed pBR322 DNA as the substrate. Relaxed DNA was supercoiled when GyrA, GyrB and ATP were all present and no DNA supercoiling activity was observed without any of these three (Fig 2). To detect the DNA supercoiling activity, 40 nM each was sufficient in DNA gyrases with ML-GyrA^{WT}, ML-GyrA^{A91V}, ML-GyrA^{D95G}, ML-GyrB^{WT}, ML-GyrB^{D464N} and ML-GyrB^{N502D}. In contrast, DNA gyrase with ML-GyrA^{G89C} needed 160 nM of both GyrA and GyrB to show sufficient activity.

Inhibitory activity of fluoroquinolones against *M. leprae* recombinant DNA gyrases.

The results of supercoiling inhibitory assay against WT and mutant DNA gyrases under various concentrations of WQ-3810, MFX and OFX were shown in Fig. 3 and IC_{50}s calculated from the results are shown in Table 1. All fluoroquinolones examined in this study exhibited DNA supercoiling inhibitory activity against all DNA gyrases in dose-dependent manners. WQ-3810 and MFX showed higher DNA supercoiling inhibitory activities against DNA gyrases containing amino acid substitutions in GyrA than OFX in the statistical analysis. Then, WQ-3810 also showed better inhibitory activity than MFX and OFX against the DNA gyrase harboring ML-GyrB^{N502D}. In comparison with ML-GyrB^{D464N}, the inhibitory activity of WQ-3810 was lower than that of MFX.

In silico study of the molecular interaction between DNA gyrases, the DNA molecular structure and fluoroquinolones.

The fluoroquinolone binding site consists of subunits GyrA, GyrB and the DNA

molecular structure. 5BTA, the *M. tuberculosis* DNA gyrase 3D structural model, has an intact fluoroquinolone binding site and the 3D structure has a ligand model of MFX positioned at the fluoroquinolone binding site. A computational simulation for WQ-3810 was carried out based on the binding site 5BTA in MFX. The docking simulation of WQ-3810 showed a docking score of -7.1960 (Fig. 4A) for the original model and -5.1241 (Fig. 4B) for the amino acid-substituted model of Mtb-GyrB -D461N (D464N in ML). In addition, the distances between the R1 group, R7 groups of each fluoroquinolone and the side chain of each amino acid were calculated. While the distance between the R1 group of WQ-3810 and the side chain of Mtb-GyrB-Asp461 was 1.96 Å, that of MFX was 9.36 Å (Fig. 5). While, the distance between the R7 group of WQ-3810 and the side chain of Mtb-GyrB-Asn499 (Asn502 in ML) was 7.21 Å, that of MFX was 6.90 Å (Fig 5). In the ligand interaction simulation, the NH_2 molecule of the R1 (2,4-difluoro-5-aminopyridine substituent) group in WQ-3810 was able to associate with Mtb-GyrB-Asp461, but this association was not observed when Asp was replaced by asparagine (Fig 5).

Discussion

The potential bactericidal effect of WQ-3810 on *Acinetobacter baumannii*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Neisseria gonorrhoeae* and *E. coli* has been proved [19]. Nonetheless, until now the effect of WQ-3810 on *M. leprae* was unknown. To elucidate this, instead of measuring minimum inhibitory concentration, we calculated the inhibitory activity of WQ-3810 against DNA gyrase of *M. leprae*, because *M. leprae* is yet to be cultured on an artificial medium. The result of DNA gyrase inhibitory assay, IC_{50}, have been used as a reliable criteria to analyze the potency of fluoroquinolone against *M. leprae* [4, 5, 8, 19, 20].

Recombinant DNA gyrases assessed in the current study showed a readily enzymatic activity, except for DNA gyrase with ML-GyrA^{G89C}, which needed a 4-fold concentration to express when compared with the other gyrases. Additionally, the amino acid substitution G89C in GyrA is rare when compared with A91V in the field [2,6]. Therefore, we decided to focus instead on the inhibitory effect of WQ-3810 on DNA gyrase with ML-GyrA^{A91V}, which is considered a more representative amino acid substitution of the OFX-resistant DNA gyrase in *M. leprae*. DNA gyrase with ML-GyrA^{D95G}, showing a stronger fluoroquinolone resistance than that of A91V [7, 21], was also assessed in the present work. ML-Asp95 in GyrA may provide the most crucial linkage between fluoroquinolones and DNA. A linkage constructed by aspartic acid at an equivalent position has been found in DNA gyrase *E. coli*, *S. aureus*, *S. pneumoniae* and *M. tuberculosis* [23, 24].

In the present study, IC_{50} of WQ-3810 against DNA gyrase with ML-GyrA^{A91V} was similar to that against ML-GyrA^{WT} wheres IC_{50}s of OFX was significantly higher (3.9-fold; $P < 0.01$) and that of MFX was slightly higher (1.5-fold; $P = 0.1061$) against DNA gyrase with ML-GyrA^{A91V} comparing to that with ML-GyrA^{WT}, respectively. On the contrary, IC_{50} of WQ-3810 showed 4.3-fold increase ($P < 0.01$) against DNA gyrase with ML-GyrA^{D95G}

comparing to that with ML-GyrA^{WT} while the increase of IC_{50}s of OFX and MFX were 12.8-fold ($P < 0.01$) and 9.9-fold ($P < 0.01$), respectively. Only OFX showed significantly increased IC_{50} against DNA gyrase with ML-GyrA^{A91V} and suggested the decrease of OFX binding affinity because of the amino acid substitution A91V [25]. Distinct IC_{50}s among examined fluoroquinolones suggested the importance of interaction between GyrB and R1, R7 and R8 groups. Additional linkages of WQ-3810 with GyrB might provide better inhibitory effects against DNA gyrases with amino acid substitutions in GyrA.

To understand the detailed molecular interaction between DNA gyrases and WQ-3810, an *in-silico* study was carried out. Due to the molecular structure of *M. leprae* DNA gyrase is not listed in the PDB yet, we selected the DNA gyrase molecular structure of *M. tuberculosis* instead. 5BTA is the 3D molecular structural model of the *M. tuberculosis* DNA gyrase listed in PDB. This structural model shows intact isotetramers (two GyrAs and two GyrBs) with cleaved DNA and MFX. In addition, the information regarding the coordinates of MFX positioned at the fluoroquinolone binding site of 5BTA, was used for the docking simulation of WQ-3810. The *M. tuberculosis* DNA gyrase amino acid sequence around QRDR is identical to that of *M. leprae*. And the homologous amino acid substitutions at QRDR of *M. leprae* DNA gyrases associated with quinolone resistance are shown a similar quinolone resistance effect as that of amino acid substitutions in *M. tuberculosis* DNA gyrases [7, 10, 21]. Thus, we speculated that an *in-silico* study using the 5BTA structure could provide a reliable simulation regarding the association of WQ-3810 with the QRDR of *M. leprae* DNA gyrases.

Upon completion of the *in-silico* study, an additional linkage and specific amino acids were observed (Fig 5). It was detected that the NH_2 molecule of the R1 group in WQ-3810 interacted with Mtb-GyrB-Asp461, but that this interaction disappeared when asparagine was substituted to aspartic acid (Fig 5). These results were in agreement with the decreased score of WQ-3810 with mutant DNA gyrase, when compared with that of WT (Fig 4). Thus, we

theorized that a specific R1 group in WQ-3810 was the source of the additional linkage that contributed to a stronger relationship with Mtb-GyrB-Asp461, which MFX did not have. To confirm this theory, additional DNA gyrase inhibitory assays using recombinant *M. leprae* DNA gyrase subunits ML-GyrA^{WT}, ML-GyrB^{WT}, ML-GyrB^{D464N} and ML-GyrB^{N502D} were conducted (Fig. 3B) [10]. WQ-3810 had an almost 2-fold higher IC_{50} ($P < 0.01$) for DNA gyrase with ML-GyrB^{D464N} than MFX (Table 1). This result seemed to explain that WQ-3810 have a stronger interaction with GyrB-Asp464 of *M. leprae* DNA gyrase than MFX. Amino acid substitutions in GyrB that cause quinolone resistance has not been reported in clinical *M. leprae* yet, although admittedly, the number of reports related to quinolone-resistant leprosy is still limited. Even as data of the association of *M. tuberculosis* with quinolone resistance caused by amino acid substitutions is vast, information of quinolone resistance in clinical isolates with mutation in GyrB is rare [8, 9]. Amino acid substitutions in QRDR of GyrB at position of 461 and 499 may cause significant changes in DNA gyrase activity. Moreover, interaction of WQ-3810 with the QRDR of GyrB can potentially inhibit the activity of DNA gyrase with amino acid substitutions in QRDR of GyrA. Therefore, WQ-3810 may be a good candidate compound for treating the quinolone-resistant leprosy.

In conclusion, WQ-3810 showed a better inhibitory effect on DNA gyrases of *M. leprae* than OFX did. WQ-3810 also showed a better effect on DNA gyrase with ML-GyrA^{D95G} than MFX. Upon an *in-silico* study, it was theorized that an interaction of the R1-group in WQ-3810 with aspartic acid at position of 464 existed and that it may have enhanced its inhibitory effect on DNA gyrase with ML-GyrA^{D95G}. A subsequent experiment using recombinant DNA gyrase subunits confirmed the inhibitory effect of WQ-3810 on DNA gyrase with ML-GyrA^{D95G}. A possible treatment application of WQ-3810 against OFX-resistant leprosy was demonstrated in the present work. The results deriving from the present study showing the interaction between the R1 group in WQ-3810 and gyrases may contribute to design innovative fluoroquinolones that could help lower the possibility of

further emergence of antibiotic-resistant *M. leprae* and other pathogens.

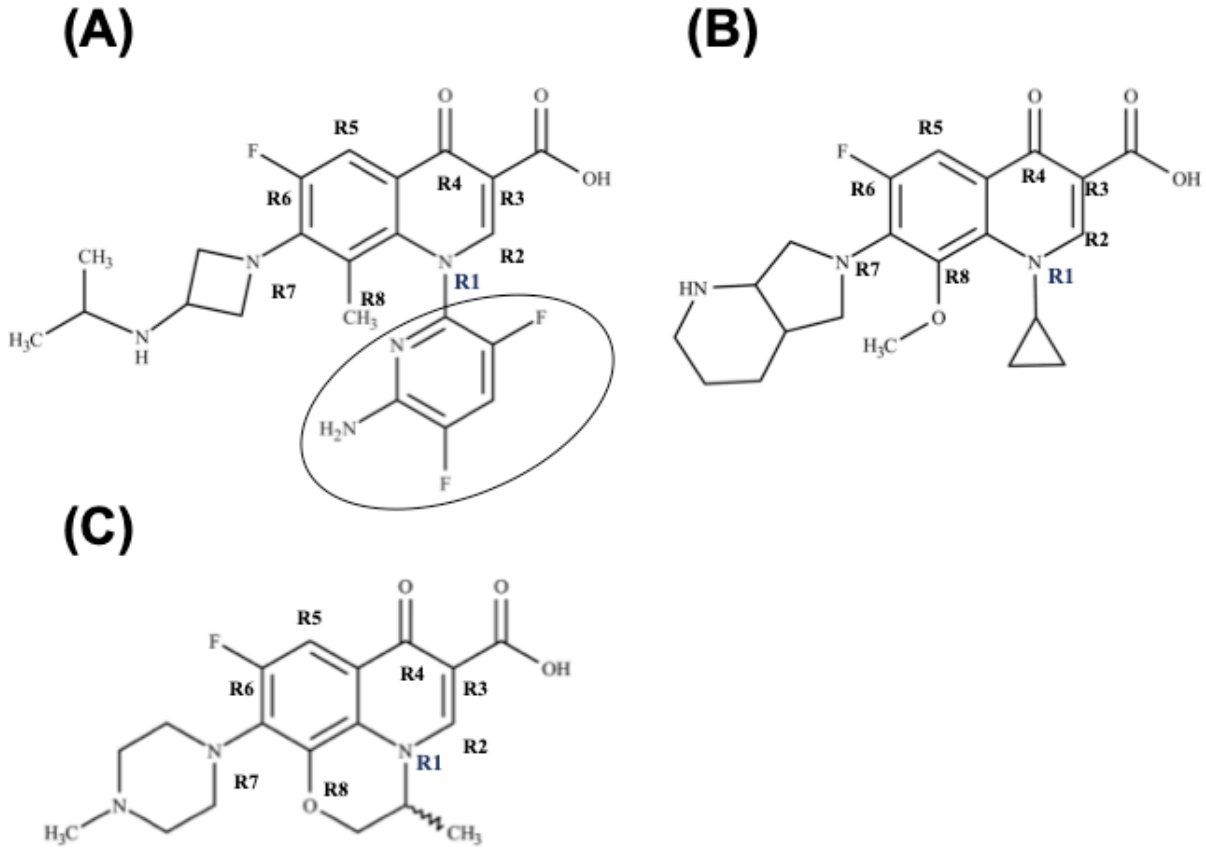


Fig 1. Structures of the quinolones tested in the present study

(A) WQ-3810 (B) Moxifloxacin (C) Ofloxacin

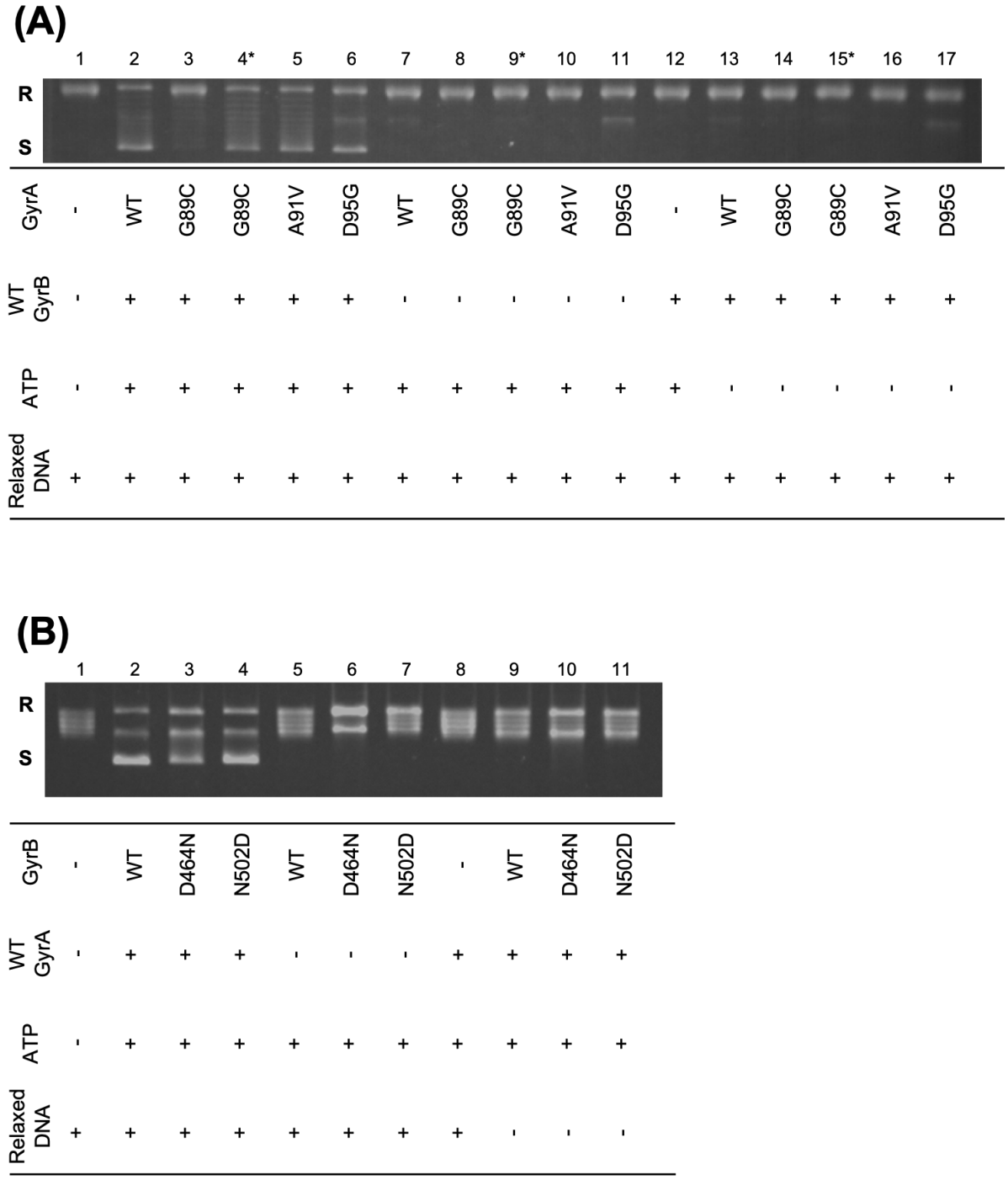


Fig 2. ATP-dependent DNA supercoiling assay

(A) The supercoiling activity of DNA gyrases consisting of ML-GyrA^{WT}, ML-GyrA^{G89C}, ML-GyrA^{A91V} or ML-GyrA^{D95G} and ML-GyrB^{WT} was confirmed. Relaxed DNA (pBR322) was incubated with GyrA, GyrB, or both, of the subunits in the presence or absence of ATP. Lane 1: relaxed pBR322 DNA only; lanes 2–6: relaxed pBR322 DNA, ATP, GyrA (40 nM), and GyrB (40 nM); lanes 7–11: relaxed pBR322 DNA, ATP, and GyrA (40 nM); lane 12: relaxed

pBR322 DNA, ATP, and GyrB (40 nM); lane 13–17: relaxed pBR322 DNA, GyrA (40 nM), and GyrB (40 nM). *Lane 4, 9 and 15: The amount of DNA gyrase subunit was 4-fold (160 nM). (B) The supercoiling activity of DNA gyrases consisting of ML-GyrB^{WT}, ML-GyrB^{D464N} or ML-GyrB^{N502D} and ML-GyrA^{WT} was 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 only; lanes 2–4: relaxed pBR322 DNA, ATP, GyrA (40 nM), and GyrB (40 nM); lanes 5–7: relaxed pBR322 DNA, ATP, and GyrB (40 nM); lane 8: relaxed pBR322 DNA, ATP, and GyrA (40 nM); lane 9–11: relaxed pBR322 DNA, GyrA (40 nM), and GyrB (40 nM). R and S at the left side of agarose gel indicates the position of relaxed DNA and supercoiled DNA, respectively.

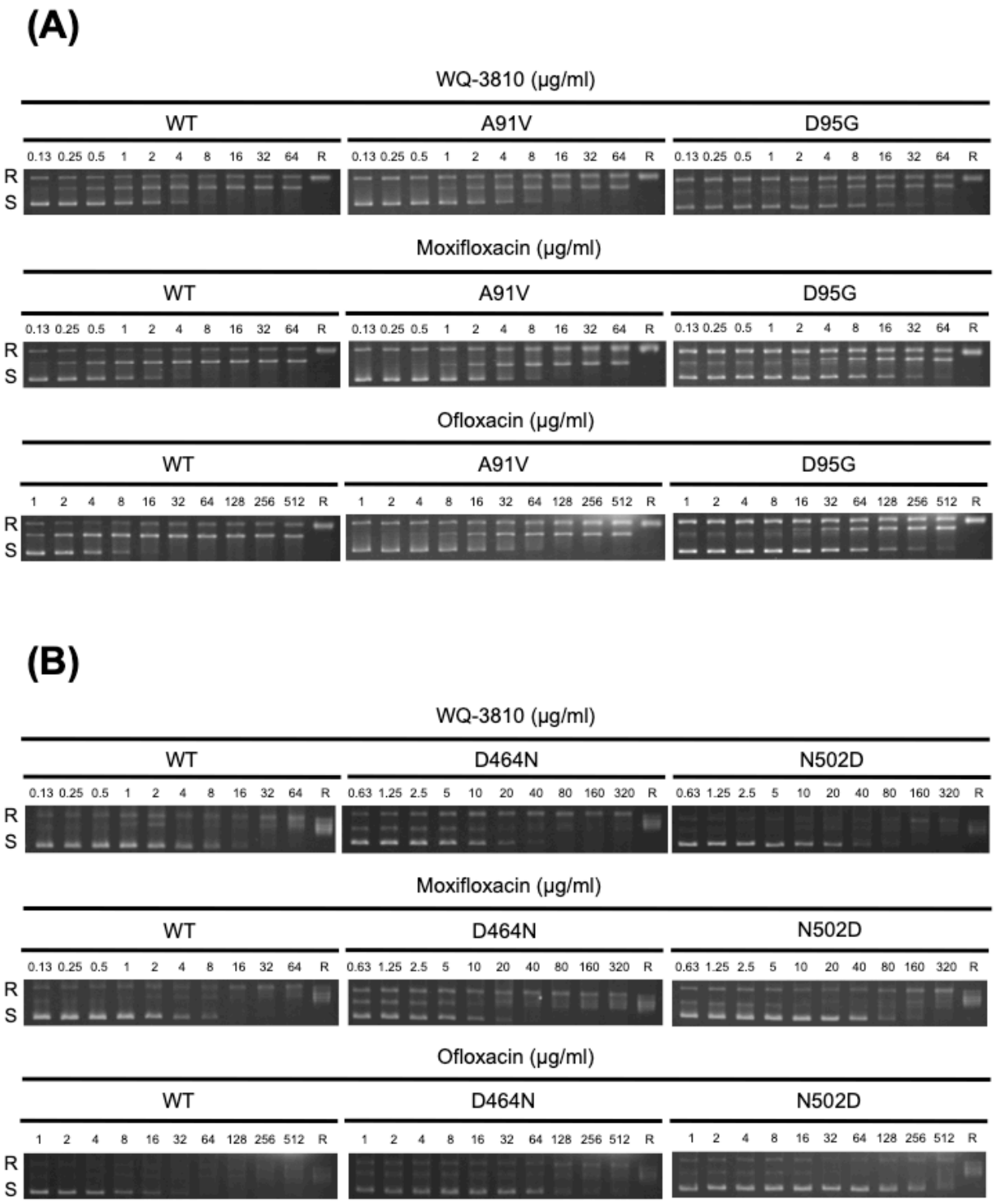


Fig 3. Fluoroquinolone-inhibited DNA supercoiling assay

(A) Relaxed DNA (pBR322) was mixed and incubated with GyrA, GyrB, ATP and quinolones at the indicated concentrations. Each quinolone was screened for its inhibitory effect on WT DNA gyrases and mutant DNA gyrases with ML-GyrA^{A91V} and ML-GyrA^{D95G} substitutions. Lanes labeled as R indicate relaxed pBR322 DNA. (B) Relaxed pBR322 DNA was mixed and

incubated with GyrA, GyrB, ATP and quinolones at the indicated concentrations. Each quinolone was screened for its inhibitory effect on WT DNA gyrases and mutant DNA gyrases with ML-GyrB^{D464N} and ML-GyrB^{N502D} substitutions. Lanes labeled as R indicate relaxed pBR322 DNA. R and S at the left side of agarose gel indicates the position of relaxed DNA and supercoiled DNA, respectively.

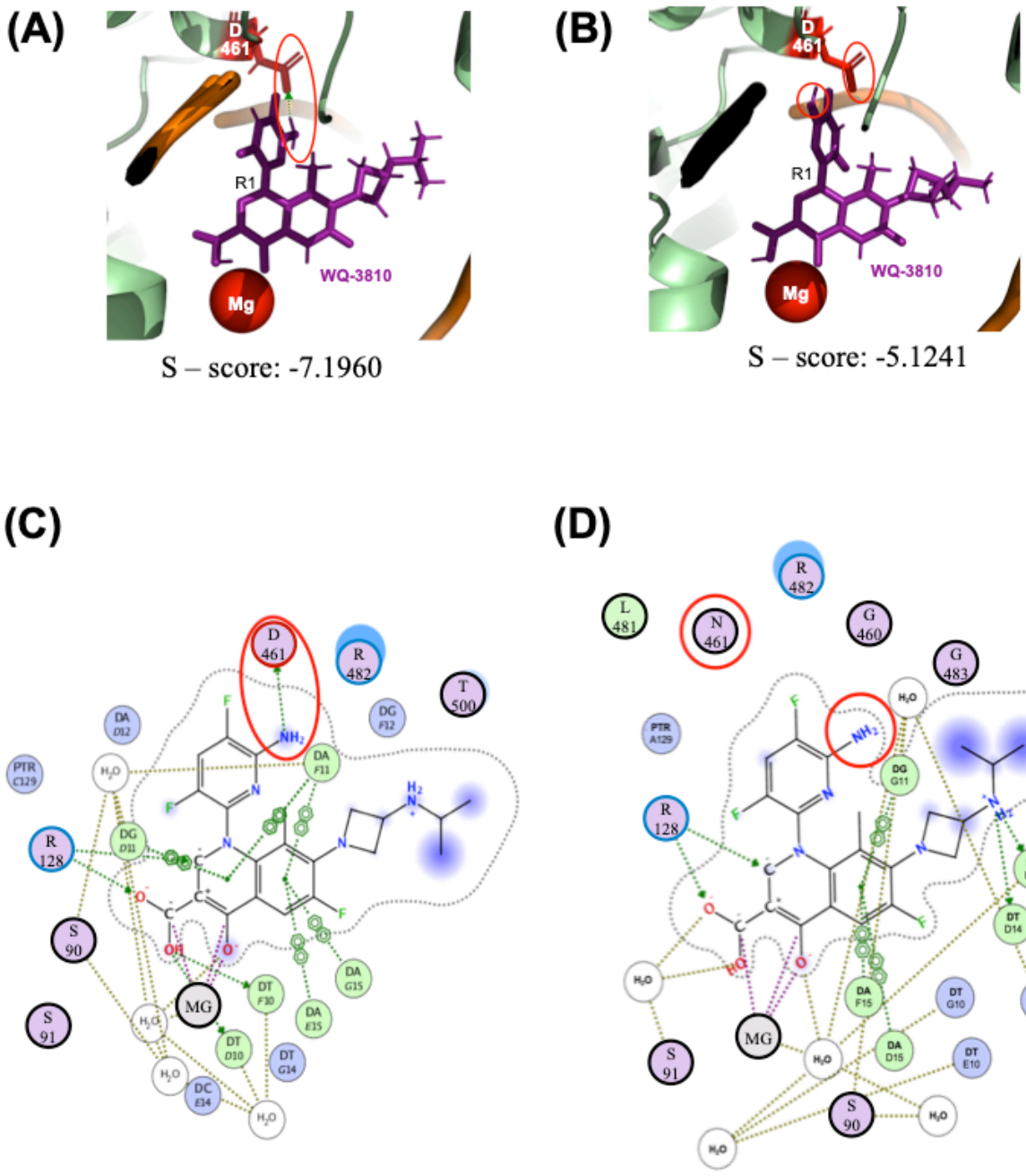


Fig 4. Molecular interaction of WQ-3810 with DNA gyrases.

(A) indicates the docking simulation result of WQ-3810 (shown in purple) and 5BTA without MFX. Aspartic acid at the amino acid position 461 of GyrB is circled in red and the Mg ion is shown as a blue circle. The specific linkage between NH_2 of the R1 group in WQ-3810 and aspartic acid at position 461 of GyrB are circled in red. (B) indicates the docking simulation result of WQ-3810 and the 5BTA without MFX, in which aspartic acid is replaced by asparagine at position 461 in GyrB. Asparagine at 461 and NH_2 of the R1 group in WQ-3810

are circled in red. (C) indicates the molecular interaction of (A). (D) indicates the molecular interaction of (B). WQ-3810 docking scores are shown in this figure.

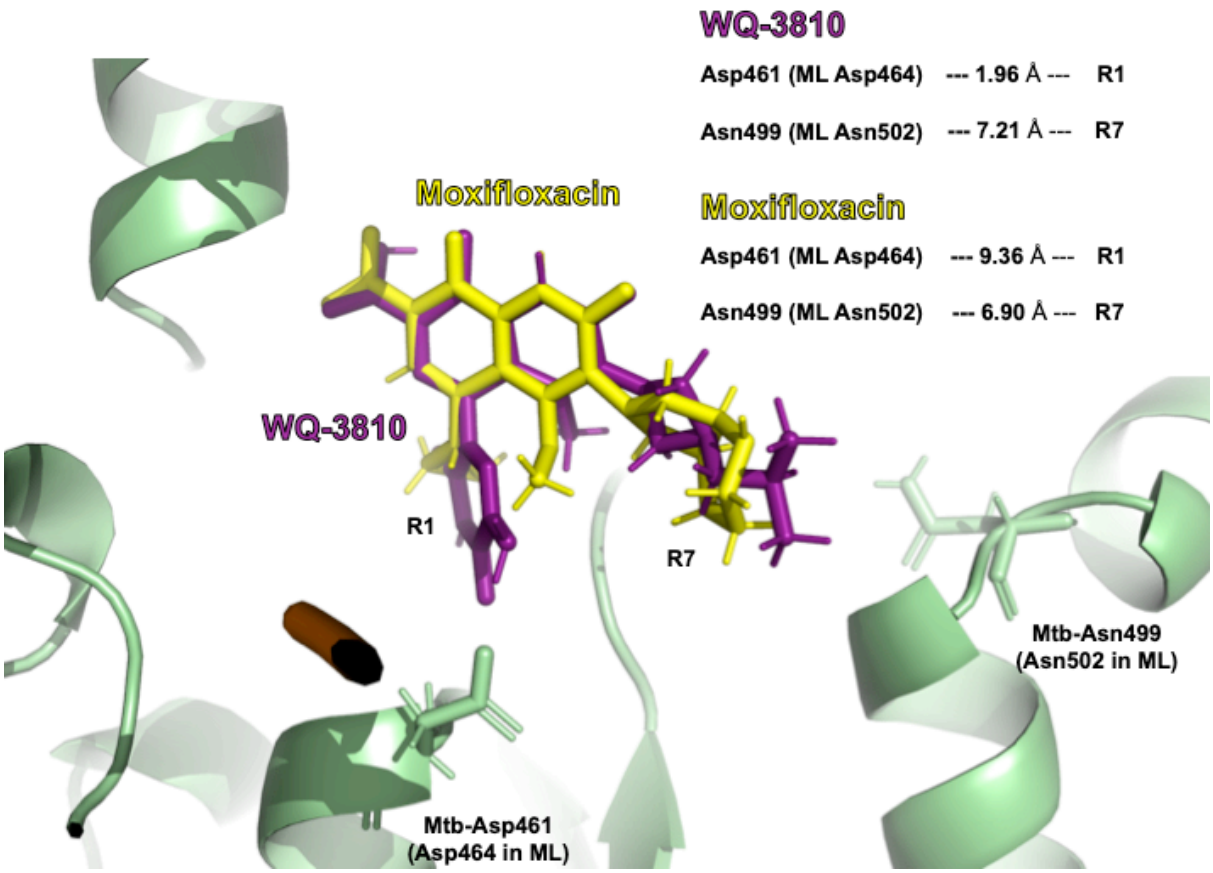


Fig 5. Molecular interaction between DNA gyrase, the DNA structure and WQ-3810.

MFX is shown in yellow and WQ-3810 in purple. The spatial distance information is shown in this figure.

Table 1. IC_{50}s of quinolones for ML DNA gyrases in WT and mutants

Drug	IC_{50} ± SD (μg/mL)				
	WT (n=6)	A91V (n=3)	D95G (n=3)	D464N (n=3)	N502D (n=3)
WQ-3810	1.6 ± 0.4	1.7 ± 0.5	6.9 ± 1.8	10.5 ± 0.4	22.0 ± 3.0
MFX	1.4 ± 0.5	2.1 ± 0.3	13.9 ± 1.9	5.1 ± 0.2	45.1 ± 10.3
OFX	3.7 ± 0.2	14.3 ± 0.4	47.3 ± 6.9	79.1 ± 9.1	124.2 ± 3.6

IC_{50}: Quinolone concentration for 50% inhibitory activity against DNA gyrase
WT: Wild type
SD: Standard deviation

Summary

Background

Mycobacterium leprae causes leprosy and ofloxacin is used to control this bacterium. However, specific amino acid substitutions in DNA gyrases of *M. leprae* interferes with the effect of ofloxacin.

Methodology/principal findings

Here we tested the inhibitory effect of WQ-3810 on DNA gyrases in *M. leprae*, using recombinant gyrases. We theorized that WQ-3810 and DNA gyrases interacted, which was tested *in silico*.

Compared with control drugs ofloxacin and moxifloxacin, WQ-3810 showed a better inhibitory effect on ofloxacin-resistant DNA gyrases of *M. leprae*. The *in-silico* study showed that, unlike control drugs, a specific linkage between a R1 group in WQ-3810 and aspartic acid at position 464 in the subunit B of DNA gyrases existed, which would enhance the inhibitory effect of WQ-3810. This linkage was confirmed in a further experiment, using recombinant DNA gyrases with amino acid substitutions in subunits B instead.

Conclusions/significance

The inhibitory effect of WQ-3810 was likely enhanced by the specific linkage between a R1 group residue in its structure and DNA gyrases. Using interactions like the one found in the present work may help design new fluoroquinolones that contribute to halt the emergence of antibiotic-resistant pathogens.

CHAPTER II

Interaction of quinolones carrying novel R1 group with *M. leprae* DNA gyrase

Introduction

WQ-3810 is newly developed fluoroquinolone with an innovative NH_2-based molecular structure, 6-amino-3,5-difluoropyridin-2-yl at the R1 group (Fig 6B)[18, 19]. This compound has been reported to have strong bactericidal effects on several pathogenic bacteria [18, 19, 21]. Further, it has shown that reliable inhibitory activity against *M. leprae* DNA gyrase baring a quinolone-resistant amino acid substitution in GyrA [29]. This superiority might be supported by the molecular structural characteristic of R1 group on WQ-3810 [29]. For clear understanding of how the novel molecular structure of R1 group contribute to the inhibitory activity, further experimental evidence was necessary.

WQ-3334, WQ-4064 and WQ-4065 possess similar molecular structure with WQ-3810 on R8 (WQ-3334) and R1(WQ-4064 and WQ-4065) group those of basic quinolone molecular structures, respectively. Though, those compounds are expected the reliable inhibitory activity against DNA gyrase of *M. leprae* as same of WQ-3810, there are yet to be tested. Therefore, those three candidates can be reliable comparative controls to analyze the reliability of novel R1 group in insight of molecular structure against *M. leprae* DNA gyrase.

In present study, WQ-3810, WQ-3334, WQ-4064 and WQ-4065 were used for test of R1 groups utility in DNA gyrase of *M. leprae*. To facilitate comparison between those WQ-compounds, *in vitro* assays were conducted using recombinant *M. leprae* DNA gyrases including wild type and mutants bearing amino acid substitutions in GyrA ML-GyrA^{D95G} and GyrB ML-GyrB^{D464N} instead of measuring minimum inhibitory concentration. Furthermore, an *in silico* study was carried out to understand the molecular interaction between WQ-

compounds and DNA gyrase.

Materials and Methods

Antibacterial agents

WQ-3810, WQ-3334, WQ-4064 and WQ-4065 were kindly provided by Wakunaga pharmaceutical Co., Ltd. (Osaka, Japan) (Fig 6).

Preparation of recombinant DNA gyrase subunits

DNA gyrase expression plasmids encoding ML-GyrA^{WT}, ML-GyrA^{D95G}, ML-GyrB^{WT} and ML-GyrB^{D464N} were constructed as previously described [10, 21, 23].

Expression and purification of recombinant DNA gyrase subunits were conducted as previously reported [10, 23-25]. Briefly, expression plasmids bearing either gyrA or gyrB of *M. leprae* DNA gyrase were transformed in *E. coli* Rosetta-gami2(DE3)pLysS or BL21(DE3)pLysS, respectively. Transformants were cultured in Luria-Bertani (LB) broth up to the log phase, under ampicillin selection (100 μg/ml).

Expression of DNA gyrases were induced by adding 1 mM isopropyl-beta-D-thiogalactopyranoside (FUJIFILM Wako Pure Chemical Industries Corp, Osaka, Japan), and further incubated for 16 to 24 h at 12 or 14 °C. Harvested *E. coli* were lysed by sonication (10 times for 40 s at output level 3 and 40% duty cycle with 40-s intervals) using Sonifier 250 (Branson, Danbury, CT). The supernatant was collected by centrifugation (10,000× g for 30 min). Recombinant DNA gyrase subunits in the supernatant were purified by Ni-NTA Agarose column (Thermo Fisher Scientific Inc.) chromatography, as per the manufacturer's protocol and dialyzed against DNA gyrase dilution buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl, 2 mM DTT, 1 mM EDTA), and recombinant DNA gyrase subunits were extracted by PD-10 chromatography.

Fluoroquinolone-inhibited DNA supercoiling assay

DNA supercoiling assay was performed based on the method described by Fisher and Pan [26]. Briefly, 30 µL of DNA gyrase reaction buffer, 4 nM relaxed pBR322 DNA, 40 nM of each subunit GyrA (ML-GyrA^{WT} or ML-GyrA^{D95G}), GyrB (ML-GyrB^{WT} or ML-GyrB^{D464N}) and fluoroquinolones. All of WQ-compounds were used in the concentration from 0.13 to 64 µg/mL for subunit combination ML-GyrA^{WT} and ML-GyrB^{WT}. WQ-3810 and WQ-3334 were used for further assay step in the concentration from 0.13 to 64 µg/ml for subunit combinations ML-GyrA^{D95G} with ML-GyrB^{WT} and ML-GyrA^{WT} with ML-GyrB^{D464N}, respectively. Reactions were conducted for 90 min at 30 °C and arrested by adding 7.5 µL of dye mix. Next, 10 µL from each mixture was subjected to electrophoresis on 1% agarose gel in 1× TBE and stained with 0.7 µg/mL of ethidium bromide. The intensity of supercoiled DNA bands for electrophoresis was estimated with ImageJ software and IC_{50}s were calculated by the AAT Bioquest web tool.

Simulations for molecular interaction among DNA gyrase, DNA and fluoroquinolones.

Molecular docking and visualization studies were carried out using MOE software (Chemical Computing Group ULC, Montreal, Quebec, Canada.) software and MolDesk Basic v1.1.54 (IMSBIO co., Ltd, Tokyo, Japan). The *M. leprae* DNA gyrase molecular structure has not been classified, yet. Therefore, the coordinates of DNA gyrase for structure-based molecular modeling were retrieved from the Protein Data Bank (PDB), with the PDB ID: 5BTA (Crystal structure model of Mtb-gyrase complex) which is *M. tuberculosis* DNA gyrase 3D structural model that is highly homologous to *M. leprae*.

Ligand location and pocket size were set using the MFX coordinates which is possessed in the 5BTA as a ligand component, which were retrieved from the 5BTA structural information. Optional parameters in MOE software were used to create a topology file, which included the addition of hydrogen atoms, the calculation of a grid potential, and a docking simulation. The flexible docking method was used, and scores were calculated as the

sum of five potentials: accessible surface area, coulomb potential, hydrogen bonds, hydrogen bond considering anisotropy, and van der Waals interactions. Protein-ligand binding free energy was estimated with MOE software using the Amber 10: EHT force-field and the default parameters of the MOE Dock application. The results of molecular docking were visualized with PyMOL v1.3. Distances between amino acids and the side chains of WQ-3810 and WQ-3334 were calculated using PyMOL v1.3.

Results

Inhibitory activity of fluoroquinolones against *M. leprae* recombinant DNA gyrases.

All fluoroquinolones inhibited DNA gyrases in a dose-dependent manner (Fig. 7 and 8). IC_{50}s calculated by the assays are shown in Table 2. As a result, WQ-3334 showed most reliable inhibitory activity against WT DNA gyrase. In contrast, WQ-4064 and WQ-4065 indicate weaker inhibitory activity than that of WQ-3334 and WQ-3810. Against quinolone-resistant DNA gyrases, WQ-3334 showed increased IC_{50}s in both of DNA gyrase with ML-$GyrA^{D95G}$ and ML-$GyrB^{D464N}$ as same as WQ-3810. Especially, those two compounds showed higher IC_{50}s against DNA gyrase with ML-$GyrB^{D464N}$ than DNA gyrase with ML-$GyrA^{D95G}$. In comparison with those two compounds, WQ-3334 showed lower IC_{50}s against both of quinolone-resistant DNA gyrases than those of WQ-3810.

In silico study of the molecular interaction between DNA gyrase, DNA and fluoroquinolones.

The fluoroquinolone binding site consists of subunits GyrA, GyrB and DNA molecule. PDB ID: 5BTA which is the *M. tuberculosis* DNA gyrase 3D structural model, possess the intact fluoroquinolone binding site and MFX positioned at the binding site as a ligand model. A computational simulation for WQ-3334 was carried out based on the binding site of MFX in 5BTA. The docking simulation of WQ-3334 showed a s-score as -28.4434 (Fig. 9). In addition, distance between the R1 group of WQ-3334 and the side chain of amino acid at position 461 (Asp461 in ML) was calculated to be 1.9 Å (Fig. 9). Though, the distance was close enough to make a specific association between R1 group and side chain of Mtb-Asp461 which have shown in Chapter I (Fig 4C), the NH_2 molecule of the R1 group in WQ-3334 was not shown the same association with Mtb-Asp461 (Asp464 in ML) in this Chapter II ligand interaction prediction using WQ-3334 (Fig 10A).

Discussion

The R1 group, 6-amino-3,5-difluoropyridin-2-yl is novel molecular characteristics of WQ-3810 comparing to other fluoroquinolones and the satisfactory bactericidal effect of WQ-3810 on *Acinetobacter baumannii*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *E. coli* and *Salmonella* Typhimurium has been reported [19, 28].

In previous study, the inhibitory activity of WQ-3810 against DNA gyrase of *M. leprae* was calculated [29] to elucidate the potential of the compound as a remedy for leprosy, instead of measuring minimum inhibitory concentration because *M. leprae* is yet to be cultured on artificial media. IC_{50}s calculated by supercoiling inhibitory assay have been used as an reliable criteria for the potency of fluoroquinolone against *M. leprae* [6, 7, 10, 21, 22]. From the previous study, WQ-3810 showed satisfactory inhibitory activity against quinolone-resistant DNA gyrases which are containing the amino acid substitutions on GyrA QRDR. By further *in silico* study, WQ-3810 was shown to have additional linkage with GyrB that might enhance the inhibitory activity against quinolone-resistant DNA gyrase bearing GyrA amino acid substitutions [29]. For clear understanding of this speculation, the additional comparison with WQ-3810 and other WQ-compounds which has similar molecular characteristics seemed to be necessary.

In this study, WQ-3334, WQ-4064 and WQ-4065 were chosen as a candidate group and compared with WQ-3810 as these compounds have similar molecular structure with WQ-3810. WQ-3334 has only the difference of brome atom at R8 group which might cause the change of the angle of R1 group (Fig 6C). WQ-4064 and 4065 has 6-methylamino-3,5-difluoropyridin-2yl and 6-ethlylamino-3,5-difluoropyridin-2yl at R1 group, respectively (Fig 6D and 6E).

WQ-4064 and WQ-4065 showed weaker inhibitory activity against DNA gyrase with ML-$GyrA^{WT}$ than WQ-3334 and WQ-3810. Especially, the IC_{50} was critically increased

when the 6-methylamino-3,5-difluoropyridin-2yl (WQ-4064) was changed to 6-ethlylamino-3,5-difluoropyridin-2yl (WQ-4065) (Fig 6D, 6E and 8A and Table 2). It is suspected that the R1 group, 6-amino-3,5-difluoropyridin-2-yl (WQ-3810 and WQ-3334) make better association with GyrB than those two types of R1 group positioned in WQ-4064 and WQ-4065. Practically, the IC_{50} of WQ-3334 against DNA gyrase with ML-$GyrA^{WT}$ was 4 times and 39 times lower than that of WQ-4064 (P<0.01) and WQ-4065 (P<0.01), respectively. Therefore, WQ-3810 and WQ-3334 was further compared using DNA gyrase bearing ML-$GyrA^{D95G}$ or ML-$GyrB^{D464N}$.

The aspartic acid at the position 95 in *M. leprae* DNA gyrase may provide the most crucial linkage between fluoroquinolones and DNA gyrase. The speculation can be supported by reports that substitutions from aspartic acid to another amino acids such as glycine at an equivalent position has been found in quinolone-resistant DNA gyrase in *E. coli*, *S. aureus*, *S. pneumoniae* and *M. tuberculosis* [25, 26]. In present study, increased IC_{50}s of WQ-3810 and WQ-3334 were observed with DNA gyrase bearing ML-$GyrA^{D95G}$ (Table 2). Interestingly, WQ-3334 showed better inhibitory activity than WQ-3810 (P < 0.01) against the DNA gyrase with ML-$GyrA^{D95G}$. It is indicated that the molecular structure of WQ-3334 has higher activity with this quinolone-resistant DNA gyrase than WQ-3810.

The 464^{th} amino acid in GyrB of *M. leprae* DNA gyrase was considered as an important factor for the inhibitory activity of WQ-3810 because of the speculated linkage between R1 group of WQ-3810 and GyrB [29]. This linkage might contribute to an enhanced inhibitory activity against quinolone-resistant DNA gyrase with GyrA amino acid substitutions like D95G [29]. In present study, dramatically increased IC_{50}s were found in both of WQ-3810 and WQ-3334 against DNA gyrase with ML-$GyrB^{D464N}$ and the rate of increase for those two compounds were almost same (WQ-3334: 7 times and WQ-3810: 6 times) (Table 2). it is indicate that the R1 group might support the binding affinity of WQ-3334 with similar way of WQ-3810, however, the IC_{50} of WQ-3334 is better than WQ-3810

($P < 0.01$) (Table 2).

To understand the detailed molecular interaction between receptor (DNA gyrases) and ligand (WQ-3810 or WQ-3334), an *in silico* study was carried out. Because the molecular structure of *M. leprae* DNA gyrase is not listed in the PDB yet, that of *M. tuberculosis* DNA gyrase was selected. PDB ID: 5BTA is the 3D molecular structural model of the *M. tuberculosis* DNA gyrase. This structural model show intact heterotetramers (two GyrAs and two GyrBs) with cleaved DNA and MFX. Hence, the information regarding the coordinates of MFX positioned at the fluoroquinolone binding site of 5BTA was used for the docking simulation of WQ-3334 following previous study [29]. The amino acid sequence around QRDR in *M. tuberculosis* DNA gyrase is identical to that of *M. leprae*, indeed, those homologous amino acid substitutions at QRDR of *M. leprae* DNA gyrase have been shown to confer similar resistance as in *M. tuberculosis* DNA gyrases [10, 21, 23]. Thus, an *in silico* study using the 5BTA structure could provide a reliable simulation regarding the association of WQ-compounds with the *M. leprae* DNA gyrase.

WQ-3334 showed satisfactory binding affinity against 5BTA in the computational simulation and this result is better than that of WQ-3810 reported in previous study (Fig 4) [29]. In structural insight, there are two different points between WQ-3334 and WQ-3810. One of those is the distance between NH_2 on R1 group of WQ-3334 and 461th aspartic acid in GyrB was slightly longer than that of WQ-3810. It might be related with the different size of R8 group. WQ-3334 has brome as a R8 group (Atomic size: 0.114 nm) and this molecule is significantly different with methyl group (Atomic size: carbon = 0.077 nm and hydrogen = 0.037) in WQ-3810. Those different molecule size might contribute to make different angle of R1 group. This theoretical approach is in good agreement with the report of Kuramoto et al. in 2003 [17]. Secondly, the trend of R7 group in WQ-3334 was different from that of WQ-3810. It is speculated that the slightly changed distance between NH_2 of R1 group of both compounds and 461th amino acid make lower association of R1 group, thus the R7

group of WQ-3334 might contribute to the new linkage with other amino acid in GyrB, instead of direct linkage between R1 group and 464th amino acid. This speculation supports the results that higher inhibitory activity of WQ-3334 against both of DNA gyrase with ML-GyrA^{D95G} (P<0.01) and ML-GyrB^{D464N} (P<0.01) than WQ-3810.

Amino acid substitutions in GyrB that cause quinolone resistance have not been reported in clinical *M. leprae* yet. Even in *M. tuberculosis* with vast amount of information on quinolone resistance associating amino acid substitutions in GyrA, reports on that in GyrB is rare [11, 12]. Amino acid substitutions in QRDR of GyrB may cause significant changes in DNA gyrase activity. Moreover, interaction of WQ-3810 and WQ-3334 with the QRDR of GyrB can potentially inhibit the activity of DNA gyrase with amino acid substitutions in QRDR of GyrA. Therefore, those molecular structural characteristics of WQ-3810 and WQ-3334 which have significant interaction with GyrB may lead designing strategic drug which is targeting the quinolone resistant leprosy.

In conclusion, WQ-3810 and WQ-3334 which have same R1 group (6-amino-3,5-difluoropyridin-2yl) showed a better inhibitory effect on DNA gyrase of *M. leprae* than those of WQ-4064 and WQ-4065 which have different R1 group (6-methylamino-3,5-difluoropyridin-2yl and 6-ethylamino-3,5-difluoropyridin-2yl, respectively). WQ-3334 also showed a better effect on DNA gyrase against both of DNA gyrases with ML-GyrA^{D95G} and ML-GyrB^{D464N} than that of WQ-3810. Upon an *in silico* study, it was theorized that the different R8 group may change the total affinity valance with DNA gyrase in comparison with WQ-3810. The results derived from the present study showing the interaction between the WQ-compound and DNA gyrase may contribute to design innovative fluoroquinolones that could help lower the possibility of further emergence of antibiotic-resistant *M. leprae* and other pathogens.

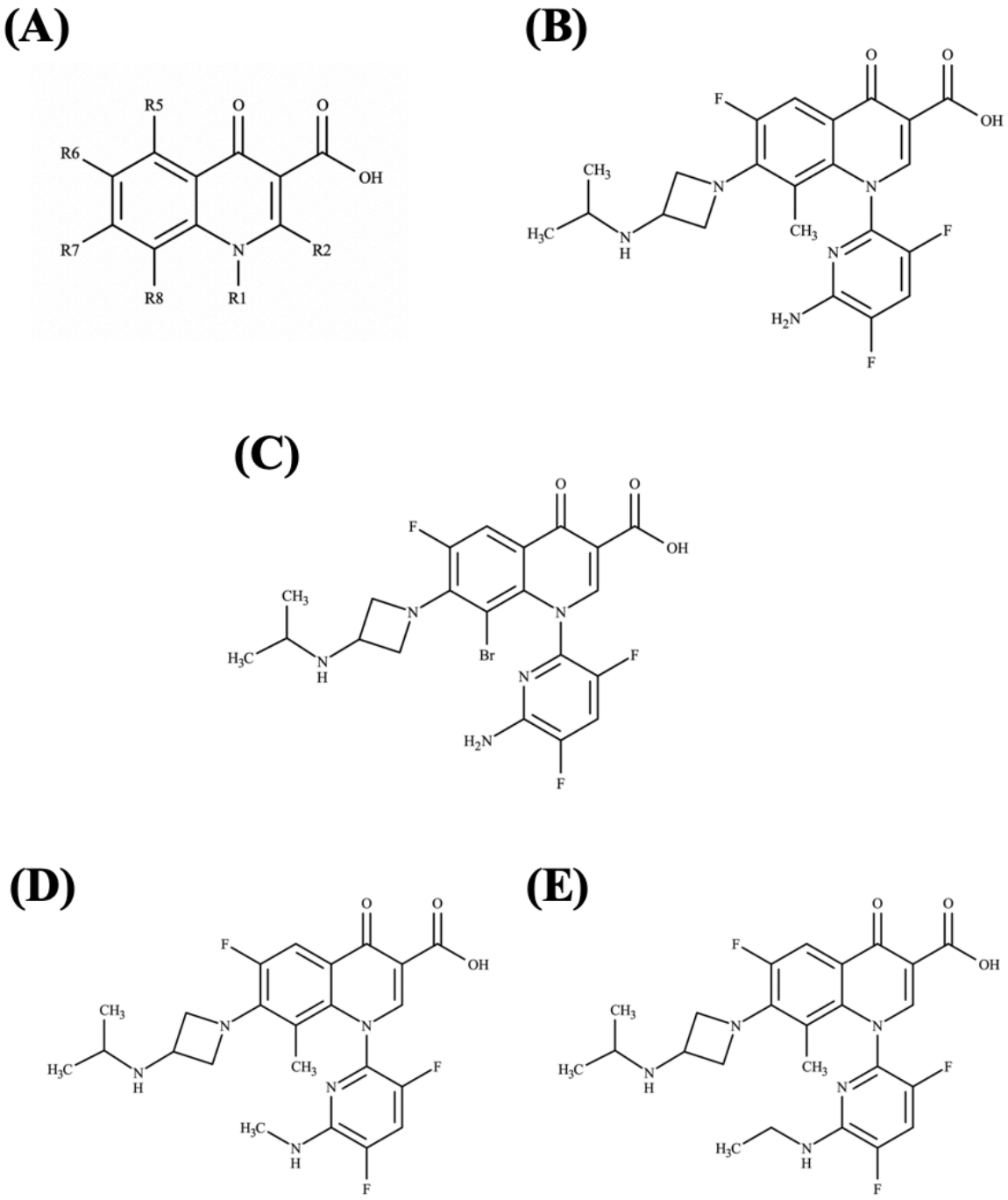


Fig 6. Structures of the quinolones tested in the present study

(A) Position of each R-group in the basic quinolone structure (B) WQ-3810 (C) WQ-3334 (D) WQ-4064 (E) WQ-4065

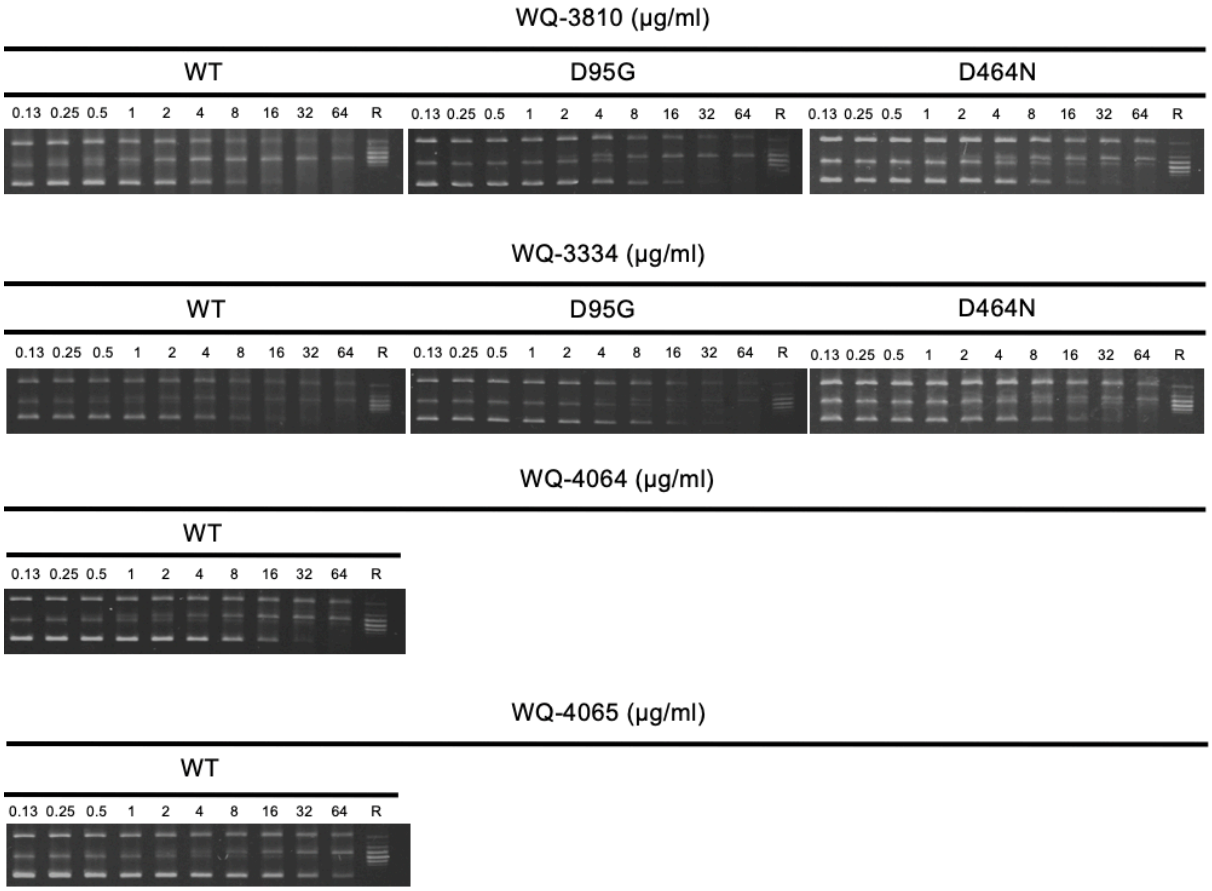


Fig 7. Fluoroquinolone-inhibited DNA supercoiling assay

Relaxed DNA (pBR322) was mixed and incubated with GyrA, GyrB, ATP and quinolones at the indicated concentrations. Each quinolone was screened for its inhibitory effect on WT DNA gyrases and mutant DNA gyrases with ML-GyrA^{D95G} and ML-GyrB^{D464N} substitutions. Lanes labeled as R indicate relaxed pBR322 DNA.

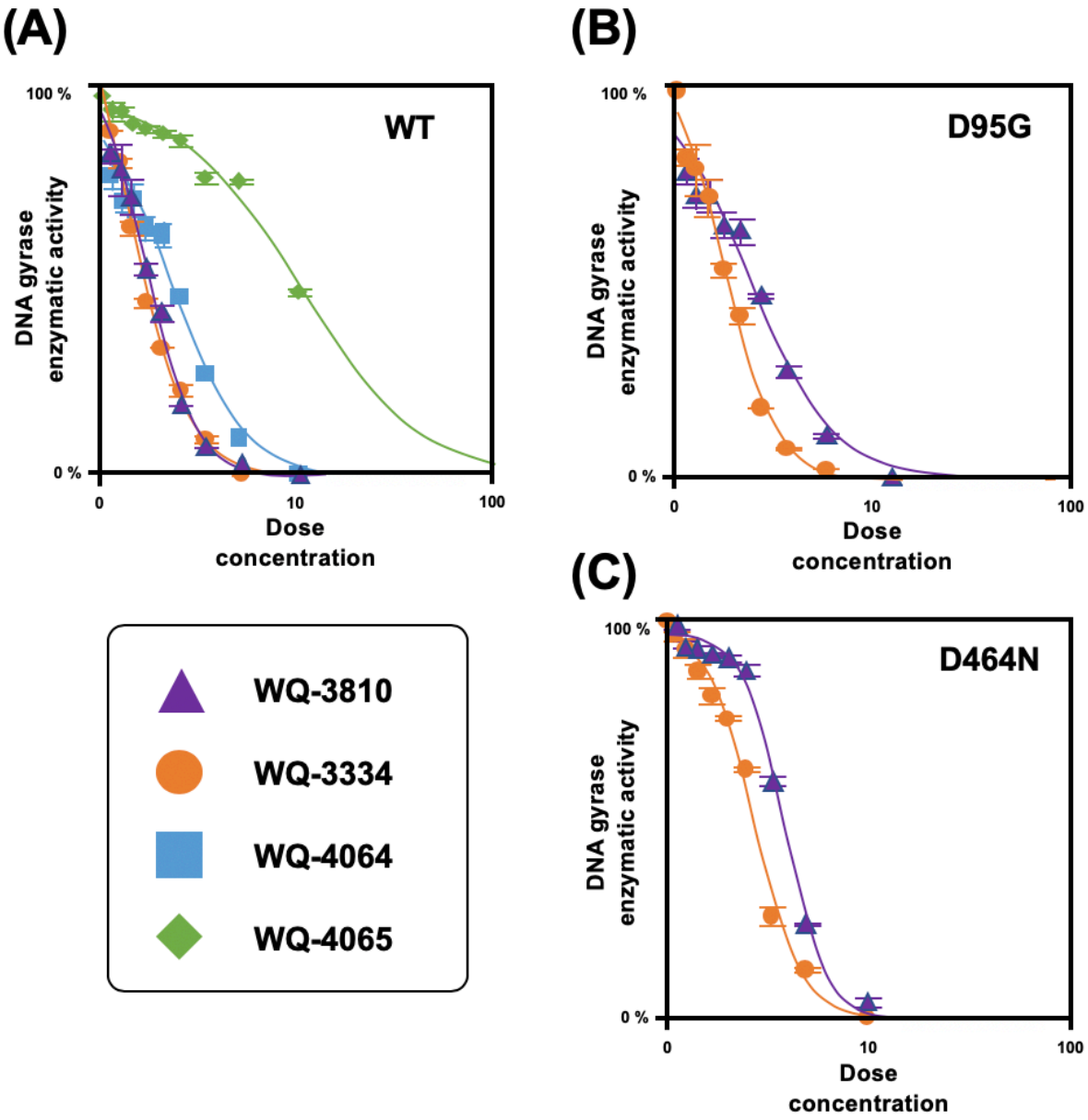


Fig 8. Sigmoidal graph for DNA gyrase activities in dose dependent of fluoroquinolones. (A) Inhibitory activities of those WQ-compounds (WQ-3810, WQ-3334, WQ-4064 and WQ-4065) against WT DNA gyrase were indicated in sigmodal graph, respectively. (B) Inhibitory activities of those WQ-3810 and WQ-3334 against mutant DNA gyrase with ML-GyrA^{D95G} were indicated in sigmoidal graph, respectively. (C) Inhibitory activities of those WQ-3810 and WQ-3334 against mutant DNA gyrase with ML-GyrB^{D464N} were indicated in sigmoidal graph, respectively.

Compound	S-score	Compound	S-score
WQ-3810	-7.1960	WQ-3334	-28.4434

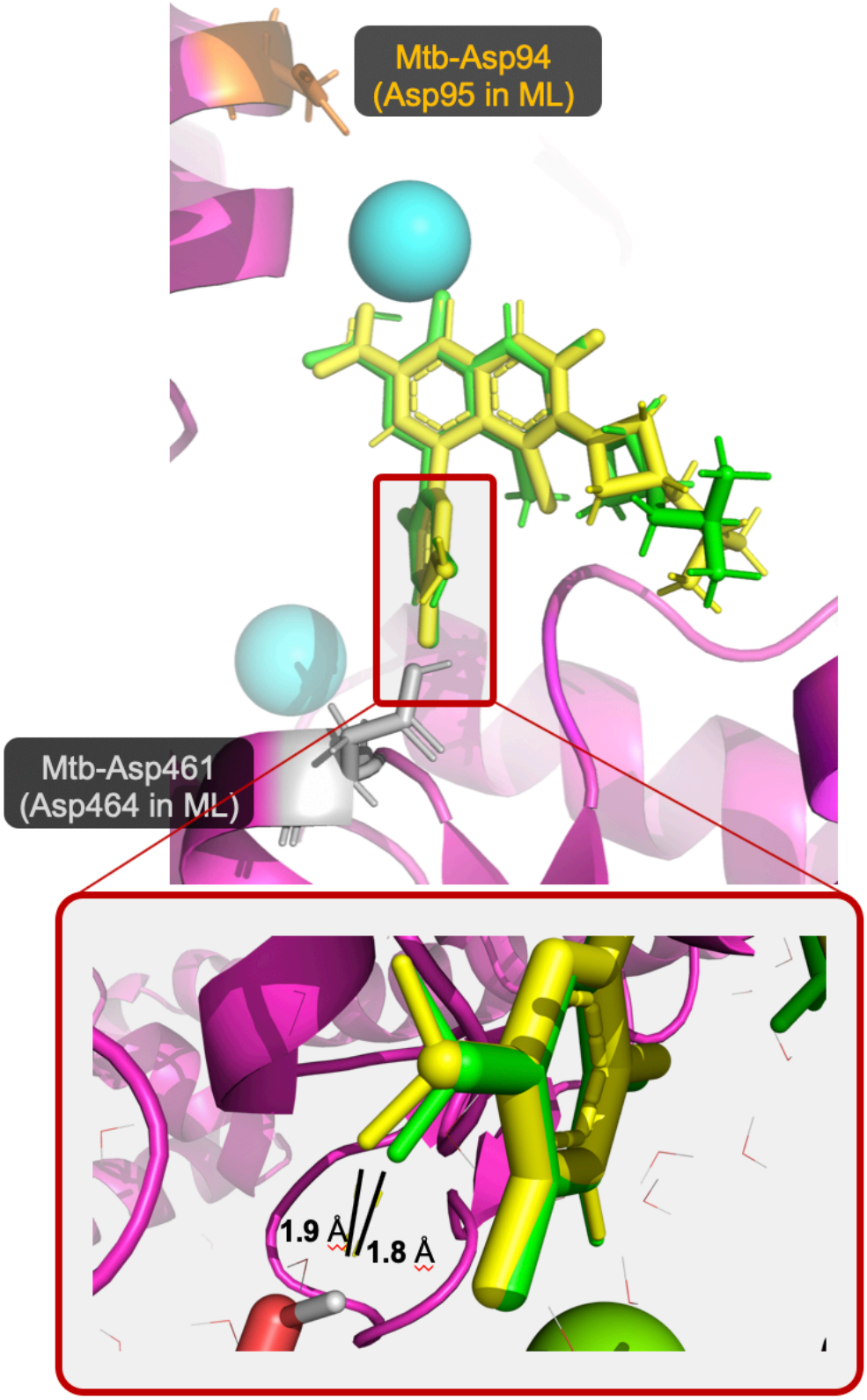
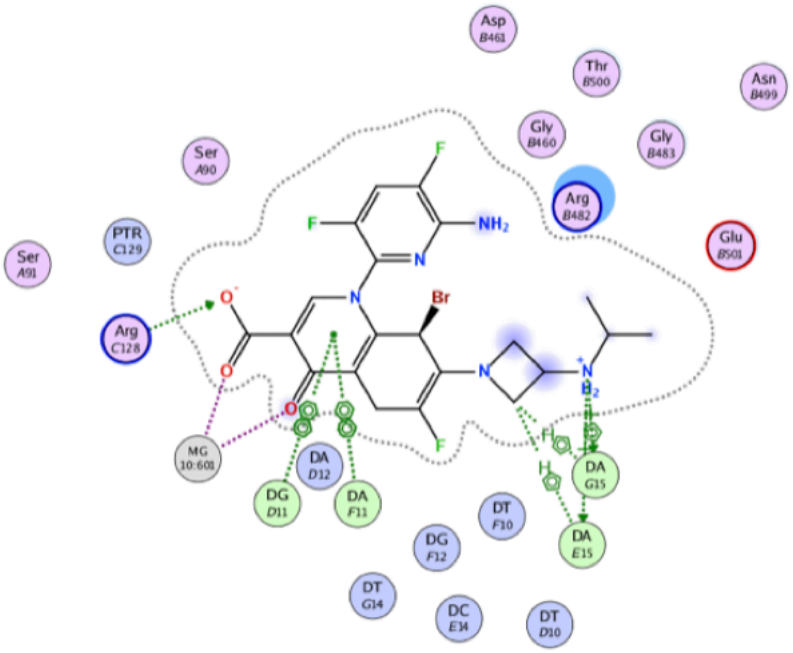


Fig 9. Molecular interaction of WQ-3334 with DNA gyrases.

Indicates the docking simulation result of WQ-3334 (shown in yellow quinolone compound) as an 3D coordinates in left side and s-score in right top side on this figure. Previously analyzed docking simulation result of WQ-3810 (shown in green quinolone compound) is overlapped and compared with those WQ-3334 results. Those calculated spatial distances between R1 group of each quinolone compound and 461th amino acid of 5BTA is displayed in right bottom side. DNA gyrase molecular structure is shown in purple and Mg ion shown in cyan ball. Those related amino acid positions with this study are marked as a orange for 94^{th} amino aicd and gray for 461^{th} amino acid, respectively.

(A)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Ser A91
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DT D10
DA G15
DA E15

(B)

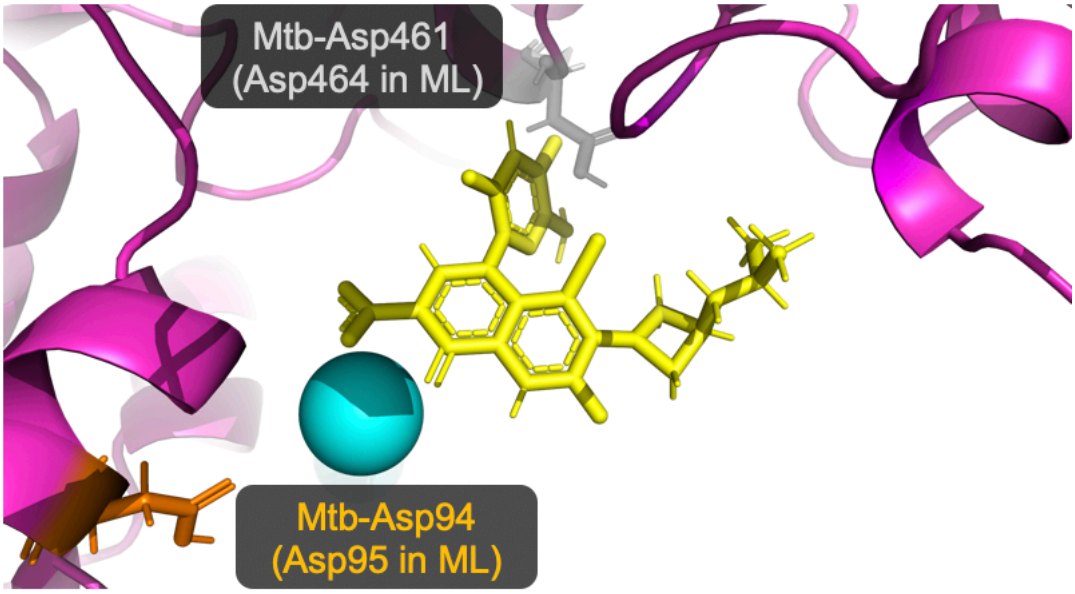
Mtb-Asp461
(Asp464 in ML)
Mtb-Asp94
(Asp95 in ML)

Fig 10. Molecular interaction between DNA gyrase, the DNA structure and WQ-3334. (A) Indicates the molecular interaction information of WQ-3334 with 5BTA. (B) Visualized the interaction information to 3D coordinates. Those related amino acid positions with this study are marked as a orange for 94^{th} amino aicd and gray for 461^{th} amino acid, respectively.

Table 2. IC_{50}s of quinolones for ML DNA gyrases in WT and mutants

Drug	IC_{50} ± SD (μg/mL)			
	WQ-3810	WQ-3334	WQ-4064	WQ-4065
WT (n=3)	1.4 ± 0.1	0.8 ± 0.0	4.4 ± 0.2	31.2 ± 1.0
D95G (n=3)	7.3 ± 0.7	3.5 ± 0.1	ND	ND
D464N (n=3)	9.9 ± 0.1	4.9 ± 0.1	ND	ND

IC_{50}: Quinolone concentration for 50% inhibitory activity against DNA gyrase
WT: Wild type
SD: Standard deviation

Summary

Background

In previous study, WQ-3810 with novel R1 group showed reliable inhibitory activity against ofloxacin resistant DNA gyrases of *M. leprae*. It is suspected that the molecular structural characteristics of R1 group might support the inhibitory activity. To understand the effectiveness of the R1 molecular structure against DNA gyrase of *M. leprae*, further analysis was necessary.

Methodology/principal finding

To understand the reliability of R1 group in quinolones, the inhibitory effect WQ-3810, WQ-3334, WQ-4064 and WQ-4065 against DNA gyrase of *M. leprae* was compared using recombinant DNA gyrases. In addition, *in silico* study was performed to understand the detail information of molecular interaction between DNA gyrase and WQ-compound.

Those WQ-3334 and WQ-3810 which has 6-amino-3,5-difluoropyridin-2-yl on R1 group showed better inhibitor effects on *M. leprae* DNA gyrase than WQ-4064 and WQ-4065 which has 6-methlylamino-3,5-difluoropyridin-2-yl and 6-ethlyamino-3,5-difluoropyridin-yl, respectively. In further investigation using quinolone-resistant DNA gyrase of *M. leprae*, WQ-3334 showed better activity than WQ-3810. R8 group is only different molecule between those two. The R8 group is suspected as a determination factor of the R1 group angle by *in silico* study. And this difference might cause the different binding affinity with DNA gyrase. This speculation is supported by the present *in silico* study results.

Conclusions/significance

The inhibitory effect of WQ-compound which has novel R1 group (6-amino-3,5-difluoropyridin-2-yl) against DNA gyrase can be enhanced by the improved bind affinity with changed R8 group molecule. This understanding may help to design the new fluoroquinolone that contribute to overcoming the ofloxacin resistant leprosy.

CONCLUSION

Leprosy is chronic infection disease caused by *Mycobacterium leprae* and fluoroquinolone is the main key to control this burden. In current reports, more than 200,000 new cases are found in a year. Quinolone-resistant *M. leprae* is one of the reason why the leprosy is still remaining as a human threat. Thus, new candidate for the quinolone-resistant *M. leprae* is urgently needed.

In this study, the fluoroquinolone compound which has a novel molecular structure was tested against DNA gyrase of *M, leprae* because the bacteria is hard to be cultured in artificial media. Further, *in silico* study was carried out to understand the molecular structural interaction between the compound and DNA gyrase.

CHAPTER I verified the superiority of WQ-3810 which has a novel molecular structure at the R1 group against ofloxacin-resistant DNA gyrase of *M. leprae*. Additionally, the specific molecular interaction between the R1 group of WQ-3810 and 464th amino acid in GyrB of DNA gyrase was speculated.

CHAPTER II verified the importance of R1 group, 6-amino-3,5-difluoropyridin-2-yl for the ofloxacin-resistant DNA gyrase of *M. leprae*. Additionally, R8-group was focused as an important factor for the inhibitory activity of fluoroquinolone which has the novel R1 group.

This theoretical approach may contribute to design innovative fluoroquinolones that could help lower the possibility of further emergence of antibiotic-resistant *M. leprae* and other pathogens.

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Softwares

1. ImageJ software - https://imagej.nih.gov/ij/download.html
2. Molecular Operating Environment (MOE) (Chemical Computing Group ULC, Montreal, Quebec, Canada - https://www.chemcomp.com/index.htm
3. MolDesk Basic v1.1.54 (IMSBIO co., Ltd, Tokyo, Japan)
4. Protein Data Bank (PDB) - http://www.rcsb.org/pdb/
5. WinCoot release 0.8.9.2 - http://bernhardcl.github.io/coot/
6. PyMOL v1.3 - http://www.pymol.org/
7. AAT Bioquest web tool - https://www.aatbio.com