Title: **Interaction of quinolones carrying new R1 group with *Mycobacterium leprae* DNA gyrase**

Short title: Novel quinolones and *M. leprae* DNA gyrase

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Abstract

Background

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* and the treatment of choice is ofloxacin. And specific amino acid substitutions in DNA gyrase of *M. leprae* have been reported leading to resistance against the drug. In our previous study, WQ-3810, a fluoroquinolone with a new R1 group (6-amino-3,5-difluoropyridin-2-yl) was shown to have a strong inhibitory activity on ofloxacin-resistant DNA gyrases of *M. leprae*, and the structural characteristics of its R1 group was predicted to enhance the inhibitory activity.

Methodology/principal finding

To further understand the contribution of the R1 group, WQ-3334 with the same R1 group as WQ-3810, WQ-4064, and WQ-4065, but with slightly modified R1 group, were assessed on their activities against recombinant DNA gyrase of *M. leprae*. An *in-silico* study was conducted to understand the molecular interactions between DNA gyrase and WQ-compounds. WQ-3334 and WQ-3810 were shown to have greater inhibitory activity against *M. leprae* DNA gyrase than others. Further, analysis using quinolone-resistant *M. leprae* DNA gyrases, showed that WQ-3334 had greater inhibitory activity than WQ-3810. The R8 group was shown to be a factor for the linkage of the R1 groups with GyrB by an *in-silico* study.

Conclusions/significance

The inhibitory effect of WQ compounds that have a new R1 group against *M. leprae* DNA gyrase, can be enhanced by improving the binding affinity with different R8 group molecules. The information obtained by this work could be applied to design new fluoroquinolones effective for quinolone-resistant *M. leprae* and other bacterial pathogens.

Keywords: *Mycobacterium leprae*, DNA gyrase, GyrB, WQ-3810, WQ-3334

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Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*. The spread of this disease has been controlled by a multidrug therapy (MDT) recommended by the World Health Organization (WHO). Nonetheless, more than 200,000 new cases were still reported in 2019 [1]. Relapse cases especially, are a serious concern because of a higher possibility of being accompanied with resistance to anti-leprosy drugs, and hence, limiting the number of drugs available for the treatment of leprosy [11-16]. To better treat MDT-resistant leprosy, alternative drugs are needed. A fluoroquinolone, ofloxacin (OFX), is currently being used for the treatment of MDT-resistant leprosy [2].

Fluoroquinolones bind to DNA gyrases and inhibit their enzymatic activity crucial for DNA transcription and replication of bacteria [3, 4]. DNA gyrase consists of two subunits, A (GyrA) and subunits B (GyrB) [4]. The quinolone resistance of DNA gyrase is developed by substituting amino acids around quinolone binding sites, so-called quinolone resistance-determining regions (QRDR), in either GyrA or GyrB [5]. In particular, amino acid substitution from aspartic acid to glycine at the position of 94 (D94G) in GyrA is the most frequently found substitution in quinolone-resistant *M. tuberculosis*. Homologous amino acid substitution was experimentally confirmed to contribute to quinolone resistance in *M. leprae* [2, 6]. By contrast, amino acid substitutions in QRDR of GyrB are less frequently found in clinical strains; nonetheless, it is believed that mutations in GyrB may be significantly related to DNA gyrase enzymatic activity. Therefore, D461N in GyrB of *M. tuberculosis* and homologous amino acid substitution~~s~~ in *M. leprae* have been proved to confer quinolone resistance [8, 9].

To develop an efficient therapeutic regimen against OFX-resistant leprosy, there is an urgent need for screening of new drugs and analyzing their individual structural characteristics.

WQ-3810 is a quinolone compound which has an innovative NH_2-based molecular structure, 6-amino-3,5-difluoropyridin-2-yl, at the R1 group (Fig 1B) [18, 19]. This compound has been reported to have a strong bactericidal effect on several pathogenic bacteria [18, 19, 21]. Furthermore, it was shown to have strong inhibitory activity against *M. leprae* DNA gyrase with quinolone-resistant amino acid substitutions in GyrA [29]. This superior inhibitory property may be enhanced by the structural characteristic of the R1 group [29]. Thus, understanding the contribution of the R1 group to the inhibitory activity against *M. leprae* DNA gyrase, seemed to be necessary.

WQ-3810 shares structural characteristics with the compounds WQ-3334, WQ-4064 and WQ-4065 though with differences at R8 to WQ-3334, and R1 to WQ-4064 and WQ-4065. Due to the distinct molecular structures, these compounds seem to exert different inhibitory activities against wild-type (WT) and mutant DNA gyrases of *M. leprae*. In light of this, the interaction of the molecular structures of the WQ compounds with DNA gyrases remains unknown.

In the present study, WQ-3810, WQ-3334, WQ-4064, and WQ-4065 were assessed on the ability of the R1 group to inhibit *M. leprae* DNA gyrase. To compare activities of these compounds, *in vitro* assays were conducted using recombinant DNA gyrases, including WT and mutants bearing amino acid substitutions, D95G and D464N in GyrA and GyrB, respectively. In addition, an *in-silico* study was carried out to understand the molecular interaction between WQ-compounds and DNA gyrases.

Materials and Methods

Antibacterial agents

Quinolones WQ-3810, WQ-3334, WQ-4064, and WQ-4065 were provided as 100 % pure compounds by Wakunaga Pharmaceutical Co., Ltd. (Osaka, Japan).

Bacterial strains and expression plasmids

Thai-53 strain of *M. leprae* [20], maintained at the Leprosy Research Center, National Institute of Infectious Diseases (Tokyo, Japan), was used for the preparation of *M. leprae* DNA. *Escherichia coli* strain TOP-10 (Thermos Fisher Scientific, Waltham, MA) was used for cloning. While *E. coli* strains Rosetta-gami^{TM} 2(DE3)pLysS and BL21(DE3)pLysS (Merck KGaA, Darmstadt, Germany) were used for protein expression. Further, plasmid vector pET20b(+) (Merck KGaA) was used to construct expression plasmids, and relaxed pBR322 plasmid DNA (Inspiralis Ltd., Norwich, UK) was used for the assessment of supercoiling activity.

Preparation of recombinant DNA gyrase subunits

DNA gyrase expression plasmids encoding ML-GyrA^{WT} and ML-GyrA^{D95G} and 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 carrying either *gyrA* or *gyrB* of *M. leprae* were introduced into *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, Osaka, Japan) to the culture, and further incubated for 16 to 24 h at 12 or 14 °C. After

incubation, *E. coli* cells were harvested and lysed by sonication (10 times for 40 s at output level 3 and 40% duty cycle with 40-s intervals) using a Sonifier 250 (Branson, Danbury, CT). The supernatant was collected by centrifugation (10,000× *g* for 30 min) and recombinant DNA gyrase subunits were purified by Ni-NTA Agarose (Thermo Fisher Scientific) column chromatography, as per 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).

Fluoroquinolone-inhibited DNA supercoiling assay

A DNA supercoiling assay was carried out in 30 µL reaction mixture consisting of 1 x 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 [26]. All WQ compounds were used at concentrations 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 assays in concentrations 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}. Reactions were conducted for 90 min at 30 °C and stopped by adding 7.5 µL of dye mix. Then, 10 µL from each reaction mixture was analyzed by 1% agarose gel electrophoresis in 1× TBE buffer (Nacalai Tesque, Inc., Osaka, Japan) stained with 0.7 µg/mL of ethidium bromide. The intensity of the supercoiled DNA bands in agarose gel was calculated by the software, ImageJ (https://imagej.nih.gov/ij/download.html) and the IC_{50}s were calculated with the AAT Bioquest web tool (https://www.aatbio.com).

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.

https://www.chemcomp.com/index.htm) and MolDesk Basic v1.1.54 (IMSBIO co., Ltd, Tokyo, Japan). As the *M. leprae* DNA gyrase molecular structure is yet to be elucidated, the coordinates of the DNA gyrase were retrieved from the Protein Data Bank (PDB, http://www.rcsb.org/pdb/) for structure-based molecular modeling and the PDB ID #5BTA (https://www.rcsb.org/structure/5BTA; Crystal structure model of Mtb-gyrase complex), the *M. tuberculosis* DNA gyrase 3D structural model, highly homologous to *M. leprae*, was used.

Ligand location and pocket-size were set using the coordinates of moxifloxacin (MFX), which is found in the PDB ID #5BTA model as a ligand component. Optional parameters in software MOE 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 by MOE 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 (http://www.pymol.org/). Distances between amino acids and the side chains of WQ-3810 and WQ-3334 were calculated using PyMOL v1.3.

Results

Inhibitory effect of fluoroquinolones on *M. leprae* recombinant DNA gyrases.

All fluoroquinolones inhibited DNA gyrases depending on the doses (Fig. 2). The trend of inhibitory activity of each quinolone against WT and mutant DNA gyrase was as shown in Fig 3. When compared against WT DNA gyrase, WQ-3334 showed the highest inhibitory activity than compounds, WQ-3810, WQ-4064, and WQ-4065 as shown in Fig 3A. Further, using mutant DNA gyrases, WQ-3334 showed better inhibitory activity than that of WQ-

3810 against both quinolone-resistant DNA gyrase with ML-GyrA^{D95G} and ML-GyrB^{D464N} (Fig 3B and C). The IC_{50}s calculated by the assays are shown in Table 1. WQ-3334 showed the highest inhibitory effect on WT DNA gyrase. In addition, the IC_{50}s of WQ-3810 and WQ-3334 against quinolone-resistant DNA gyrases with ML-GyrA^{D95G} and ML-GyrB^{D464N} were increased with the increase in IC_{50}s against DNA gyrase with ML-GyrB^{D464N} greater than that against DNA gyrase with ML-GyrA^{D95G} (Table 1). Moreover, the increases in the IC_{50}s of WQ-3334 against both mutant DNA gyrases were lower than those of WQ-3810.

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

Fluoroquinolone binding site consists of subunits GyrA, GyrB, and the DNA molecule. PDB ID #5BTA, which is the *M. tuberculosis* DNA gyrase 3D structural model, possesses ~~the~~ an intact fluoroquinolone binding site and MFX positioned at the binding site as a ligand model. The docking simulation of WQ-3334 showed an s-score of -28.4434 (Fig. 4). In addition, the distance between the R1 group of WQ-3334 and the side chain of an amino acid at position 461 (Asp461 in ML) was found to be 1.9 Å (Fig. 4.). In the simulation, the NH_2 molecule in the R1 group (2,4-difluoro-5-aminopyridine substituent) of WQ-3334 seemed to polarly associate with Mtb-Asp461 (Asp464 in ML) (Fig 5).

Discussion

The R1 group, 6-amino-3,5-difluoropyridin-2-yl, is a new molecular structure for fluoroquinolones. WQ-3810 with this R1 group showed a strong bactericidal effect on *Acinetobacter baumannii*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *E. coli*, and *Salmonella* Typhimurium [19, 28].

In the previous study, to elucidate the potential of WQ-3810 as a drug for leprosy, its inhibitory effect against DNA gyrases of *M. leprae* was assessed instead of measuring the minimum inhibitory concentration [29]. This approach was used because *M. leprae* is yet to be cultured in artificial media. The IC_{50}, calculated using a supercoiling inhibitory assay, was used as a reliable criterion for the therapeutic potency of fluoroquinolones against *M. leprae* [6, 7, 10, 21, 22]. Thus, WQ-3810 showed strong inhibitory activity against quinolone-resistant DNA gyrases, which have amino acid substitutions in GyrA QRDR. In a subsequent *in silico* study, WQ-3810 showed an additional association with GyrB that may enhance its inhibitory effect on quinolone-resistant DNA gyrases bearing GyrA amino acid substitutions [29]. For a better understanding of this assumption, an additional comparison with WQ-compounds having different R1 and R8 groups was deemed necessary.

In the present study, compounds WQ-3810, WQ-3334, WQ-4064, and WQ-4065 were compared with each other. When compared with WQ-3810 (Fig 1B), WQ-3334 had only one different atom of bromine at the R8 group (Fig 1C), which may have caused a change in the angle of the R1 group. Furthermore, unlike WQ-3810, WQ-4064 and 4065 have 6-methylamino-3,5-difluoropyridin-2yl and 6-ethlylamino-3,5-difluoropyridin-2yl, respectively, at the R1 group (Fig 1D and 1E). In an inhibitory assay, WQ-4064 and WQ-4065 showed weaker inhibitory effects on DNA gyrase with ML-$GyrA^{WT}$ than WQ-3334 and WQ-3810 (Fig 3A). The IC_{50} notably increased by substituting 6-amino-3,5-difluoropyridin-2yl group in WQ-3810 to 6-methylamino-3,5-difluoropyridin-2yl or 6-ethlylamino-3,5-difluoropyridin-2yl at R1 group in WQ-4064 and 4065, respectively (Table 1). It seemed that at the R1 group, 6-amino-3,5-difluoropyridin-2-yl (WQ-3810 and WQ-3334) associated with GyrB better than those in WQ-4064 and WQ-4065. The IC_{50} of WQ-3334 against DNA gyrase with ML-$GyrA^{WT}$ was almost 4-fold and 39-fold lower than those of WQ-4064

(P<0.005) and WQ-4065 (P<0.005), respectively. In light of this, WQ-3810 and WQ-3334 were further compared using DNA gyrases bearing ML-GyrA^{D95G} and ML-GyrB^{D464N}.

Aspartic acid at position 95 in GyrA of *M. leprae* DNA gyrase may provide a metal ion bridge effect, which seems to be a crucial linkage between fluoroquinolones and DNA gyrases. An equivalent effect has been found at the same position in quinolone-resistant DNA gyrase in *E. coli*, *S. aureus*, *S. pneumoniae*, and *M. tuberculosis* [25, 26]. In the present study, an increase in the IC_{50}s of WQ-3810 and WQ-3334 was observed with DNA gyrases bearing ML-GyrA^{D95G} (Table 1). Interestingly, WQ-3334 showed a greater inhibitory effect on DNA gyrase with ML-GyrA^{D95G} than did WQ-3810 (P < 0.005) (Fig 3B and Table 1).

Aspartic acid at-position 464 in GyrB of *M. leprae* DNA gyrase has been considered an important amino acid for the inhibitory effect of WQ-3810, probably due to a greater association of the R1 group in WQ-3810 with GyrB [29]. This association may contribute to enhanced ~~the~~ inhibitory effect on quinolone-resistant DNA gyrase with GyrA amino acid substitutions such as D95G [29]. In this study, substantial increases in IC_{50}s against DNA gyrase with ML-GyrB^{D464N} were found in both WQ-3810 and WQ-3334, and the rates of increase for these two quinolones were almost the same (WQ-3810: 7-fold; WQ-3334: 6-fold) (Table 1). These results may indicate that the R1 group enhances the binding affinity of WQ-3334 in a similar manner to that of WQ-3810. However, the trend of inhibitory activity (Fig 3C) and IC_{50} of WQ-3334 was better than that of WQ-3810 (P < 0.005) (Table 1). This may be due to differences between the R8 groups, which may affect the R1 group and hence, enhance the binding affinity of WQ-3334 to wild and mutant type DNA gyrases.

To further understand the molecular interaction between DNA gyrases with WQ-3810 and WQ-3334, an *in-silico* study was carried out. The molecular structure of *M. leprae* DNA gyrase is not yet listed in the protein databank (PDB), hence, the DNA gyrase of *M. tuberculosis* was used. The amino acid sequence of QRDR and the surrounding region of

DNA gyrase in *M. leprae* is identical to that of *M. tuberculosis.* Besides, several amino acid substitutions at QRDR in both GyrA and GyrB of *M. leprae* have been shown to confer resistance similar to those of *M. tuberculosis* [10, 21, 23]. Thus, it was theorized that an *in-silico* study using the 5BTA model could provide a reliable simulation to elucidate the association of target compounds with *M. leprae* DNA gyrases. PDB ID: 5BTA is the 3D molecular structural model of the intact heterotetramers consisting of two GyrAs and GyrBs of *M. tuberculosis*, cleaved DNA, and MFX. Hence, the information regarding the coordinates of MFX positioned at the quinolone binding site of 5BTA was used for the docking simulation of WQ-3334 following a previous study conducted at these premises [29].

A better binding affinity to DNA gyrase of WQ-3334 than that of WQ-3810 was estimated by the computational simulation (Fig 4). The distance between R1 group of WQ-3334 and 461st Asp in GyrB was slightly longer than that of WQ-3810 and the angle between R7 and R1 group in WQ-3334 was shown to be distinct from that in WQ-3810 (Fig 4). This might cause the different binding affinity (S-score) of WQ-3334 to DNA gyrase from WQ-3810 and associate with the distinct inhibitory activities of these compounds against *M. leprae* DNA gyrase. This was supported by the predicted molecular interaction shown in Fig 5A and B as 2D and 3D graphics, respectively. This theoretical approach was in good agreement with the report by Kuramoto et al. [17].

Amino acid substitutions in GyrB associated with quinolone resistance have not yet been reported in clinical *M. leprae*, perhaps due to the limited number of reports related to quinolone-resistant leprosy. Though numerous reports on quinolone-resistance associated amino acid substitutions in GyrA have been published, information on clinical isolates showing quinolone resistance due to amino acid substitutions in GyrB is rare [11, 12]. This fact may indicate that amino acid substitutions in QRDR of GyrB associate with a significant reduction of DNA gyrase activity. Moreover, the 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, acquiring more information on the molecular structural characteristics of WQ-3810 and WQ-3334 may be the first steps to designing better drugs that target quinolone-resistant leprosy.

In conclusion, WQ-3810 and WQ-3334 with 6-amino-3,5-difluoropyridin-2yl at the R1 group showed better inhibitory activity against DNA gyrase of *M. leprae* than WQ-4064 and WQ-4065 with 6-methylamino-3,5-difluoropyridin-2yl and 6-ethylamino-3,5-difluoropyridin-2yl, respectively, at the R1 group. WQ-3334 showed greater inhibitory activity against both DNA gyrase with ML-GyrA^{D95G} and ML-GyrB^{D464N} than WQ-3810. Additionally, the *in-silico* study suggested the impact of R8 group on the affinity of WQ-3810 and WQ-3334 to DNA gyrases. The information obtained by the present work could be applied to design new fluoroquinolones effective for-several quinolone-resistant *M. leprae* and other bacterial pathogens.

Acknowledgments

This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and the Joint Research Program of the Research Center for Zoonosis Control, Hokkaido University to YS, and in part by Japan Agency for Medical Research and Development (AMED) under Grant Number JP19fm0108008, JP19fk0108042, JP19jm0510001, and JP18jk0210005 to YS. We are grateful to Wakunaga Pharmaceutical Co., Ltd. for providing WQ-3810, WQ-3334, WQ-4064, and WQ-4065.

ICMJE Statement: All authors meet the ICMJE authorship criteria.

COI statement: There is no conflict of interest.

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Table. 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

ND: Not determined

Figure legends

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

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

Fig 2. 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 3. Sigmoidal graph for DNA gyrase activity of fluoroquinolones in a dose-dependent manner

(A) The inhibitory activity of compounds WQ-3810, WQ-3334, WQ-4064, and WQ-4065 against WT DNA gyrase is shown as sigmodal graphs. (B) The inhibitory activity of WQ-3810 and WQ-3334 against mutant DNA gyrase with ML-GyrA^{D95G} is shown as sigmoidal graphs. (C) The inhibitory activity of WQ-3810 and WQ-3334 against mutant DNA gyrase with ML-GyrB^{D464N} is shown as sigmoidal graphs.

Fig 4. Molecular interaction of WQ-3334 with DNA gyrases

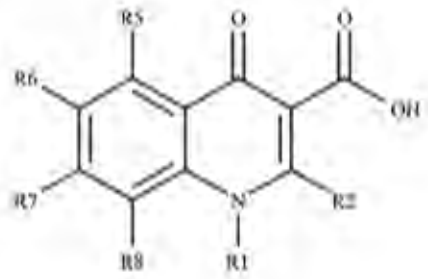
The docking simulation result of quinolone WQ-3334 is shown in yellow. The 3D coordinates are on the left and the s-score on the right top. The docking simulation result (analysis from a previous study) of quinolone WQ-3810 is shown in green. For comparison, the results are overlapped. The calculated spatial distances between the R1 group of each quinolone and the 461$^{st\ th}$ amino acid of 5BTA are shown on the right bottom. The DNA

gyrase molecular structure is shown in purple and the Mg ion is shown as a cyan-colored sphere. Amino acid positions related to the present study are shown in orange (94th amino acid) and gray (461$^{st\ th}$ amino acid).

Fig 5. Molecular interaction between DNA gyrase, the DNA structure, and WQ-3334

(A) Molecular interaction of WQ-3334 with 5BTA. (B) Visualization of the interaction as 3D coordinates. Amino acid positions related to the present study are shown in orange (94th amino acid) and gray (461$^{st\ th}$ amino acid).

(A)



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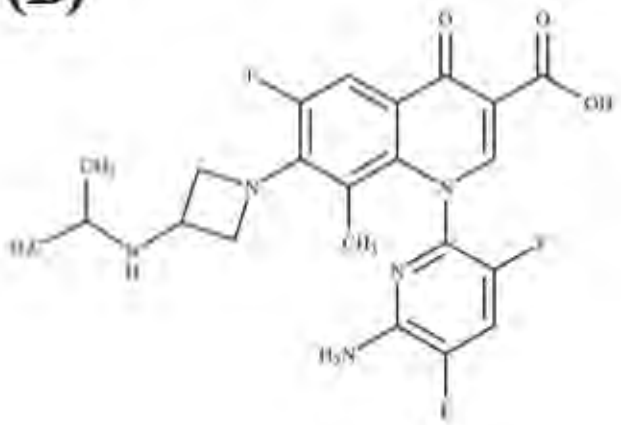

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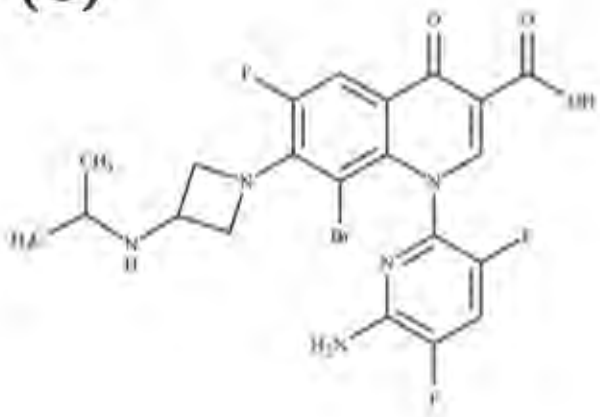

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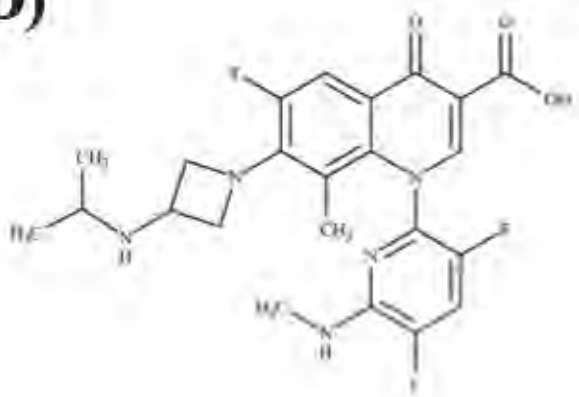

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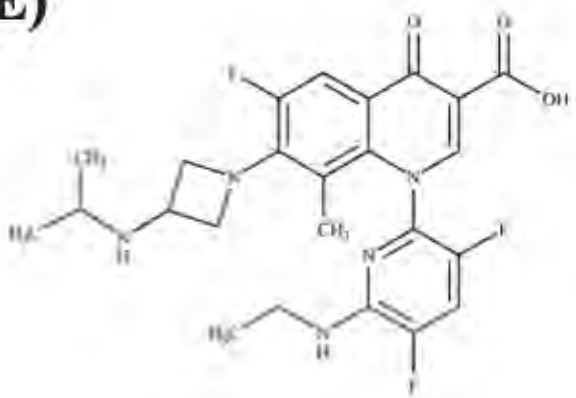
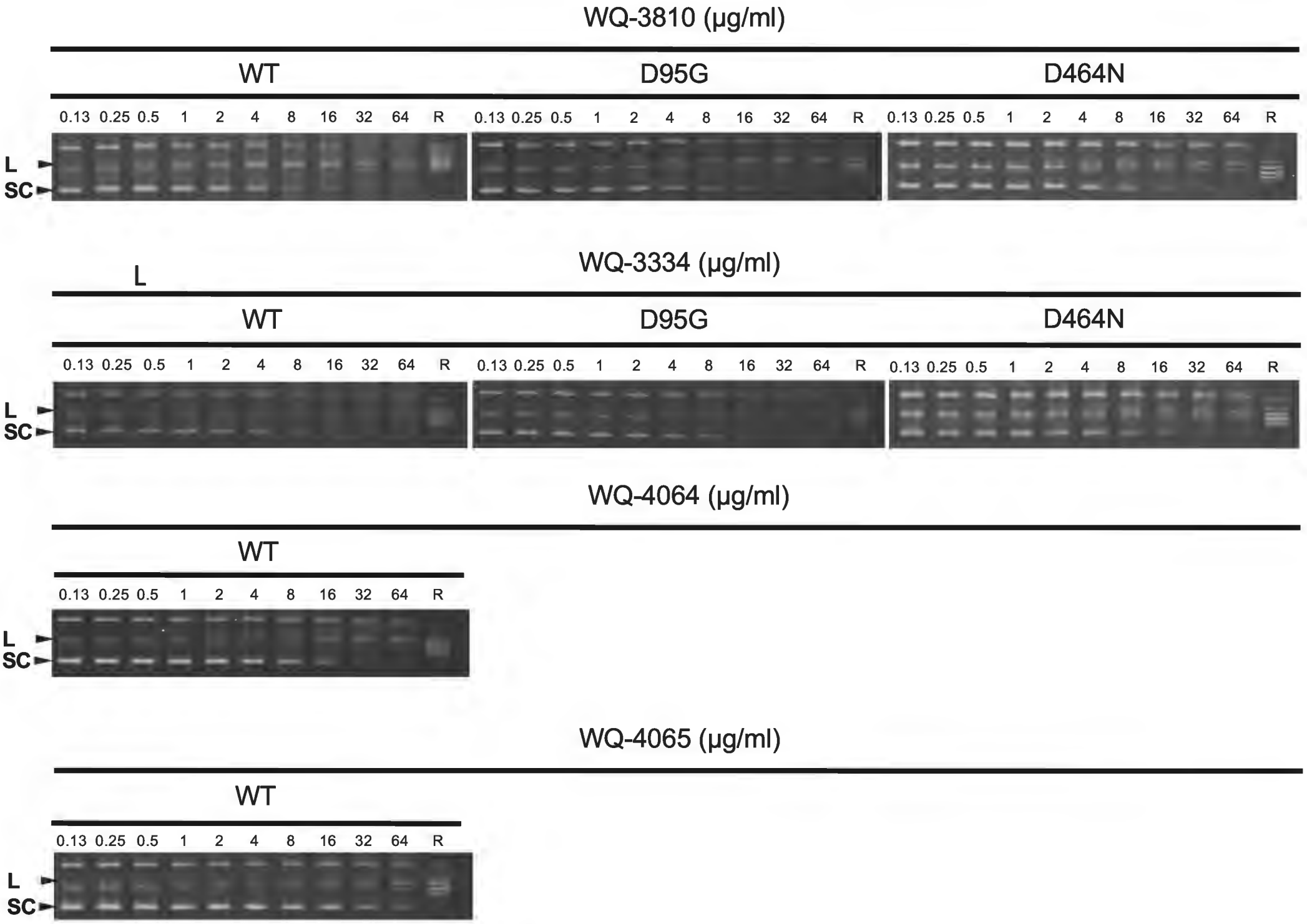
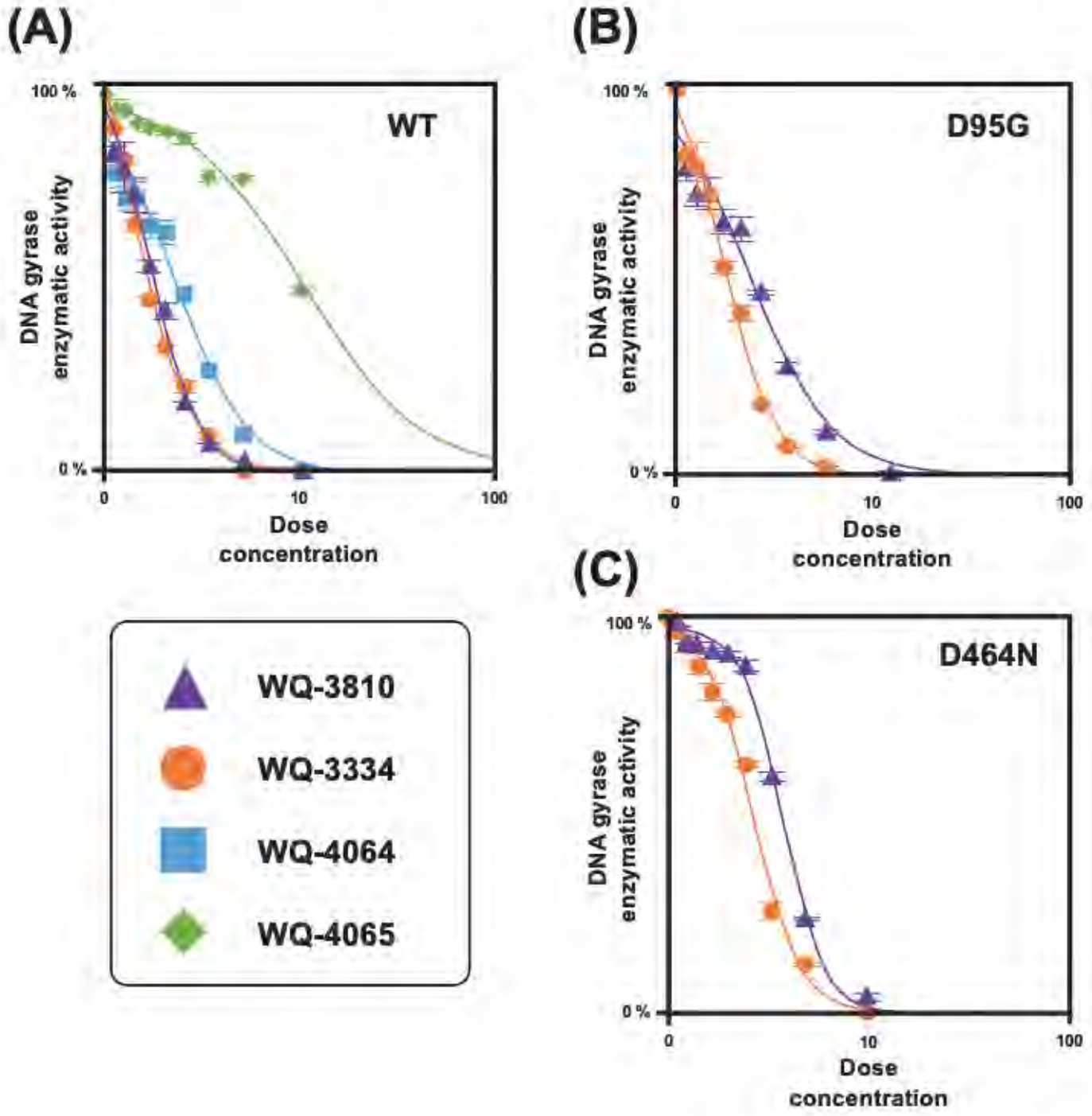


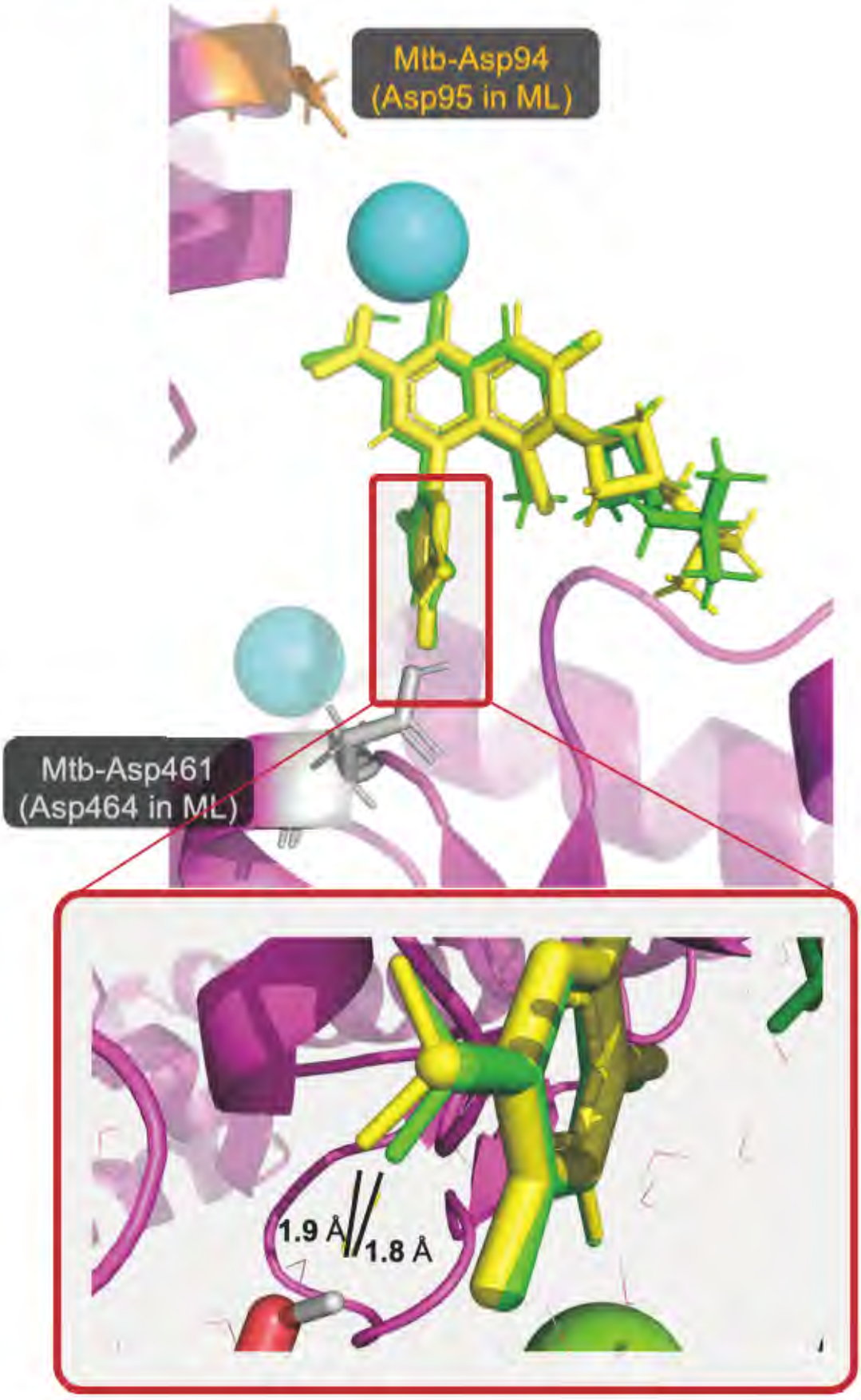
Park et al. Fig 1


WQ-3810 (μg/ml)
WT
D95G
D464N
0.13 0.25 0.5 1 2 4 8 16 32 64 R
L
SC
WQ-3334 (μg/ml)
WQ-4064 (μg/ml)
WQ-4065 (μg/ml)



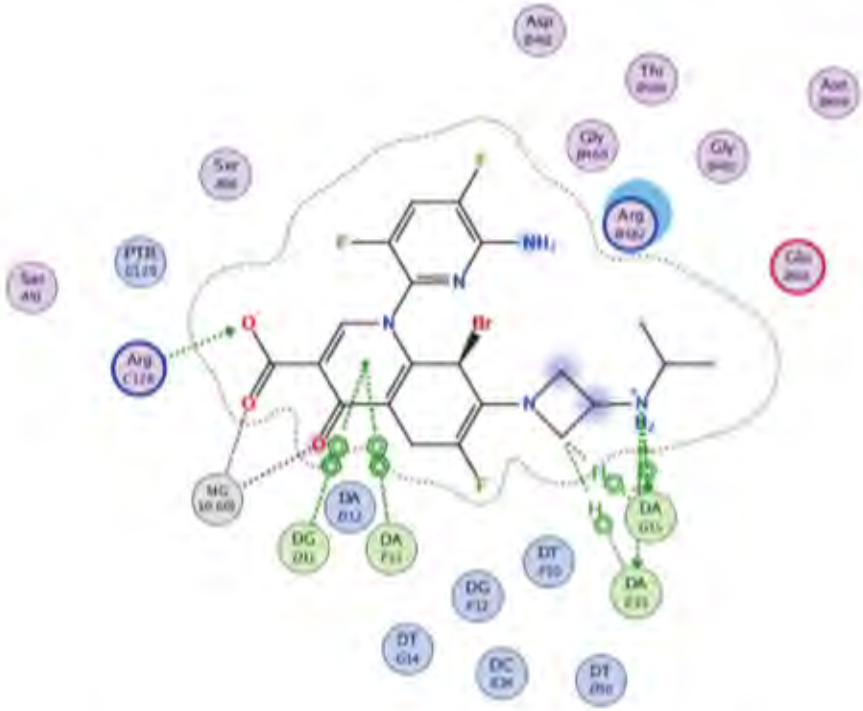
Park et al. Fig 3

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



Park et al. Fig 4

(A)



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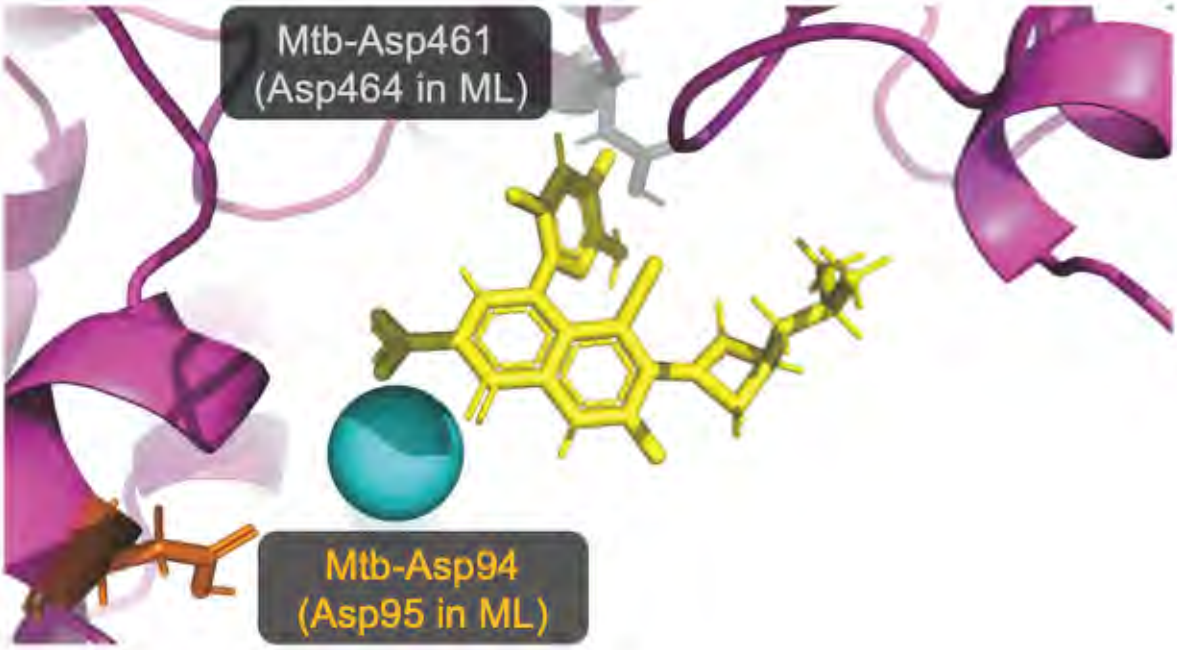

Park et al. Fig 5