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Effectiveness of fluoroquinolones with difluoropyridine derivatives as R1 groups on the *Salmonella* DNA gyrase in the presence and absence of plasmid-encoded quinolone resistance protein QnrB19

Running title: Novel quinolones and *Salmonella* with QnrB19

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Abstract

Aims: WQ-3810 has strong inhibitory activity against *Salmonella* and other fluoroquinolone-resistant pathogens. The unique potentiality of this is attributed to 6-amino-3,5-difluoropyridine-2-yl at R1 group. The aim of this study was to examine WQ-3810 and its derivatives WQ-3334 and WQ-4065 as the new drugs candidate for wild type *Salmonella* and that carrying QnrB19.

Materials and methods: The half maximal inhibitory concentrations (IC₅₀s) of WQ-3810, WQ-3334 (Br atom in place of methyl group at R8) and WQ-4065 (6-ethylamino-3,5-difluoropyridine-2-yl in place of 6-amino-3,5-difluoropyridine-2-yl group at R1) in the presence or absence of QnrB19 were assessed by *in vitro* DNA supercoiling assay utilizing recombinant DNA gyrase and QnrB19.

Results: IC₅₀s of WQ-3810, WQ-3334 and WQ-4065 against *Salmonella* DNA gyrase were 0.031 ± 0.003, 0.068 ± 0.016 and 0.72 ± 0.39 µg/mL, respectively, while QnrB19 increased IC₅₀s of WQ-3810, WQ-3334 and WQ-4065 to 0.44 ± 0.05, 0.92 ± 0.34 and 9.16 ± 2.21 µg/mL, respectively.

Conclusion: WQ-3810 and WQ-3334 showed stronger inhibitory activity against *S. Typhimurium* DNA gyrases than WQ-4065 even in the presence of QnrB19. The results suggest that 6-amino-3,5-difluoropyridine-2-yl group at R1 is playing an important role and WQ-3810 and WQ-3334 to be good candidates for *Salmonella* carrying QnrB19.

Keywords: Difluoropyridine, QnrB19, *Salmonella*, DNA gyrase

Introduction

Substantial evidence states that non-typhoidal *Salmonella* (NTS) causes about 153 million gastroenteritis and 57,000 deaths in humans worldwide. (1). *S. enterica* subsp. *enterica* serovar Enteritidis and Typhimurium are the major serotypes that cause foodborne diseases (2). Even though NTS infections are self-limiting, in severe cases and immune compromised patients, the use of antimicrobials is advised (2–4). In such cases, traditional first-line antimicrobials for example, ampicillin, chloramphenicol and trimethoprim/sulfamethoxazole are used. Fluoroquinolones such as ciprofloxacin have also been drugs of choice for treating NTS infections in both humans and animals following resistance to first line drugs (5).

However, resistance to ciprofloxacin among many *Salmonella* serovars circulating in humans and animal-foods was reported. For instance, NTS were reported to be resistant to ciprofloxacin with the high prevalence in patients from Taiwan (48.1%), Thailand (46.2%), Korea (36.5%), Singapore (24.5%), Philippines (14.9%), Hong Kong (7.1%), and Sri Lanka (8.0%), China (5.5%) and the US (2.7%) (6–8). In addition, multidrug-resistant *S. Typhimurium* with definitive phage type 104 (DT104) was detected to have been contaminating meat and meat products (9). In Europe, *S. Enteritidis* has been seen resistant to ciprofloxacin in 13.3% from clinical isolates and poultry products (10–12).

In the wake of this, the World Health Organization (WHO) declared fluoroquinolone-resistant *Salmonella* spp. as a high priority bacterial infection (13). Hence, research and development of the effective antimicrobial agents to address the emerging microbial resistance to traditional antimicrobials is necessary (13).

The resistance mechanism of fluoroquinolones is often associated with chromosomal mutations conferring amino acids substitutions at the quinolone-resistant determining regions (QRDR) of DNA gyrase subunits (GyrA and GyrB) and the DNA topoisomerase IV subunits (ParC and ParE) in Gram-negative and Gram-positive bacteria respectively. Additionally,

quinolone resistance is also associated with plasmid-mediated quinolone resistance (PMQR) mechanism (14, 15). PMQR has emerged in Enterobacteriaceae, rapidly transmitted by horizontal gene transfer thereby posing a significant threat to use of fluoroquinolones (15). Plasmid-encoded quinolone resistance protein Qnr has been reported in PMQR and comprise of seven families, namely; QnrA, QnrB, QnrC, QnrD, QnrS, QnrE and QnrVC pentapeptide repeat proteins. Plasmid-encoded quinolone resistance protein Qnr bind to DNA gyrase and decrease susceptibility to quinolones (16). Furthermore, efflux pumps (OqxAB, QepA and QaqBIII) and Aac(6')-Ib-cr (an aminoglycoside-, ciprofloxacin- and norfloxacin-inactivating acetyl-transferase) have also been reported to mediate quinolone resistant (15, 16).

Recently, WQ-3810, a novel fluoroquinolone showed strong inhibitory activity against several pathogens. Kazamori and others (17) reported stronger inhibitory activity of WQ-3810 against fluoroquinolone-resistant clinical isolates of *Escherichia coli* and *Acinetobacter baumannii* than other fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin and gamifloxacin) by MIC and IC₅₀ measurement. Low toxicity of WQ-3810 was also mentioned in the same report. Koide and others (18) reported higher inhibitory activity of WQ-3810 than ciprofloxacin and nalidixic acid against *Salmonella* DNA gyrase with amino acid substitutions in GyrA. Similarly, WQ-3810 was shown to have higher inhibitory activity than levofloxacin and ofloxacin against *Mycobacterium tuberculosis* and *M. leprae* DNA gyrases with fluoroquinolones-resistance conferring amino acid substitutions (19, 20). These findings highlighted WQ-3810 as an effective novel antimicrobial agent against fluoroquinolone-resistant pathogens. The uniqueness is attributed to the R1 groups (6-amino-3,5-difluoropyridine-2-yl) and R7 groups (3-isopropylaminoazetizine-1-yl) enhancing the binding affinity to DNA gyrase (17, 21). Recently synthesized fluoroquinolones, WQ-3334 and WQ-4065, with similar molecular structure as WQ-3810 at R1 (WQ-3334) and R8 (WQ-4065) positions would also be expected to have strong inhibitory effect on fluoroquinolone resistant bacteria.

In our previous study, the presence of QnrB19 was demonstrated to add quinolone resistance to wild type *Salmonella* DNA gyrase (22). However, there is no information on the inhibitory effect of novel fluoroquinolones with 6-amino-3,5-difluoropyridine-2-yl as R1 groups against QnrB19 protected *Salmonella* DNA gyrase supercoiling activity. Hence, the present study aimed to examine WQ-3810, WQ-3334 and WQ-4065 as new drug candidates for wild type *Salmonella* and that carrying QnrB19.

Materials and Methods

Antibacterial agents

The antibacterial agents, WQ-3810, WQ-3334 and WQ-4065 (Figure 1) were provided by Wakunaga Pharmaceutical Co., Ltd. (Osaka, Japan).

Expression and purification of *Salmonella* DNA gyrase subunits and QnrB19

Salmonella Typhimurium DNA gyrase subunits and QnrB19 used in the present work were prepared as in our previous work (22). Briefly, *Salmonella* DNA gyrase subunits (GyrA and GyrB) were expressed and purified according to the procedures (23-26) using *E. coli* BL21 (DE3)/pLysS (Merck KGaA, Darmstadt, Germany) carrying *gyrA* or *gyrB* or *qnrB19* in pET-28b(+) plasmid vector (Merck KGaA). The soluble proteins were loaded onto the column chromatography with Ni-NTA agarose resin (Thermos Fisher Scientific Inc.; Waltham, MA) to snare the his-tagged protein. The quality and quantity of all purified proteins were analyzed by SDS-PAGE as shown in Figure 2.

Inhibition of *Salmonella* DNA gyrase by quinolones and protection by QnrB19

The DNA supercoiling activity of *Salmonella* DNA gyrase was evaluated as previously described (22-26). Briefly, reaction mixture (30 μ L) contained DNA gyrase assay buffer (35 mM

Tris-HCl pH 7.5; 24 mM KCl; 4 mM MgCl₂; 2 mM DTT; 1.8 mM spermidine; 6.5% glycerol; 0.1 mg/mL of BSA), 1 mM ATP, 0.3 µg relaxed pBR322 DNA (Inspiralis Ltd.; Norwich, UK), 18 nM DNA gyrase subunits (with/without fluoroquinolones), 0.16 µg/mL of WQ-3810, 0.64 µg/mL of WQ-3334 and 2.56 µg/mL of WQ-4065, and various concentrations of QnrB19 (6 to 4,374 nM; 3-fold serial increases). The reactions were incubated at 35°C for 60 min and stopped by adding 8 µL of 5X dye mix (5% SDS, 25% glycerol and 0.25 mg/mL of bromophenol blue). After incubation, the reaction mixtures were diluted 4 times with a 1× DNA sample buffer (0.1% SDS; 0.05% Bromo Phenol Blue; 10% glycerol; and 100 mM Tris-HCl, pH 8.0) and loaded onto 1% agarose gel in Tris-borate-EDTA (TBE) buffer for electrophoresis (96 min at 50 mA). The gel was stained with 0.5 µg/mL of GelRed (FUJIFILM Wako Pure Chemical Industries Ltd., Osaka, Japan). The amount of DNA supercoiling activity was measured by tracing the intensity of the bands with software ImageJ (<http://rsbweb.nih.gov/ij>). To confirm reproducibility, DNA supercoiling activity assay was carried out three times.

Impact of QnrB19 on IC₅₀ of fluoroquinolones

To examine the ability of QnrB19 to provide fluoroquinolone resistance to DNA gyrase, the DNA supercoiling activity of *Salmonella* DNA gyrase in the presence of QnrB19 and fluoroquinolones was investigated as previously described (22). The reaction mixture was prepared in a final volume of 30 µL consist of DNA gyrase assay buffer, 1 mM ATP, relaxed pBR322 DNA (0.3 µg) with 18 nM DNA gyrase subunit, fluoroquinolones (3-fold serial concentration increases); 0.01 - 3.2 µg/mL of WQ-3810, 0.032 - 10 µg/mL of WQ-3334, and 0.32 - 100 µg/mL of WQ-4065, in the absence or presence of 18 nM QnrB19. Reactions, gel electrophoresis and measuring DNA supercoiling activity were performed as explained in previous sections. Inhibitory effects of WQ-3810, WQ-3334 and WQ-4065 on DNA gyrase were assessed by determining the drug concentrations required to reduce 50% of supercoiling activity

139 (IC₅₀s), using the calculator at AAT Bioquest (<https://www.aatbio.com/tools/ic50-calculator>). To
140 confirm reproducibility, the DNA supercoiling activity assay was carried out in triplicate.
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Results

Inhibitory activities of WQ-3810, WQ-3334 and WQ-4065 against *Salmonella* DNA gyrase.

Table 1 lists IC₅₀s of WQ-3810, WQ-3334 and WQ-4065 as calculated by *in vitro* DNA gyrase assay with increasing concentration of each quinolone. Comparing to WQ-3810, WQ-3334 and WQ-4065 showed 2.2 and 23 times higher IC₅₀s, respectively.

Impact of QnrB19 on *Salmonella* DNA gyrase supercoiling activity in the presence or absence of fluoroquinolones

Figure 3 shows the interaction between QnrB19, *Salmonella* DNA gyrase and fluoroquinolones. At a concentration between 6 -18 nM, QnrB19 showed little or less impact on DNA supercoiling activity of DNA gyrase. QnrB19 exhibited a concentration-dependent inhibitory activity against DNA gyrase at concentrations higher than 54 nM, while it completely inhibited the DNA gyrase activity at the highest concentration of 4,374 nM (Figure 3A). WQ-3810 at the concentration of 0.16 µg/mL, WQ-3334 at 0.64 µg/mL and WQ-4065 at 2.56 µg/mL reduced the DNA gyrase activity to 24.2, 11.1 and 19.3 %, respectively (Figure 3B, C, D and E) where QnrB19 at the concentration of 6 - 162 nM restored the DNA gyrase activity in dose dependent manner to approximately 4.45-4.28 times (WQ-3810), 5.78-6.80 times (WQ-3334) and 4.89-5.18 times (WQ-4065) (Figure 3C).

Effect of QnrB19 on IC₅₀s of fluoroquinolones

In a concentration-dependent manner, WQ-3810, WQ-3334 and WQ-4065 inhibited *Salmonella* DNA gyrase activity. Addition of 18 nM QnrB19, same concentration as DNA gyrase, diminished the inhibitory activities of all compounds examined in this study (WQ-3810; Figure 4A and D, WQ-3334; Figure 4B and E and WQ-4065; Figure 4C and F). Table 1 shows the effect of QnrB19 on IC₅₀s of fluoroquinolones against *Salmonella* DNA gyrase. The IC₅₀ of WQ-3810,

WQ-3334 and WQ-4065 were increased by 14.2-, 13.5- and 12.7 -fold, respectively, with 18 nM QnrB19.

Discussion

Fluoroquinolones are drugs of choice for treating NTS infections, however, acquisition of resistance by *Salmonella* pathogens have limited the use of these drugs. WQ-3810 with unique substituents at R1 (6-amino-3,5-difluoropyridine-2-yl) and R7 (3-isopropylaminoazetizine-1-yl) has showed high activity against fluoroquinolone resistant pathogens. Two novel fluoroquinolones, WQ-3334 and WQ-4065 with similar structure to WQ-3810 has also been developed. However, there is limited information on the correlation between the structural features and inhibitory activities of these compounds against *Salmonella* DNA gyrase. In addition, there is no report on the impact of Qnr families against the activity of these compounds.

Park and others (20) showed that interaction of R1 group in WQ-3810 with aspartic acid at position 464 in GyrB could enhance the inhibitory effect of this compound against DNA gyrase of *M. leprae*. The R1 group on WQ-3810 and WQ-3334 may contribute to high inhibitory activity of these quinolones (Table 1). A distinction at R8 between WQ-3810 [CH₃] and WQ-3334 [Br] (Figure 1) might explain the cause of 2.2-fold higher IC₅₀ of the latter. In contrast, WQ-4065 with 6-ethylamino-3,5-difluoropyridine-2-yl at the R1 position showed 23.2-fold higher IC₅₀ (Figure 1). The addition of ethyl group at position 6 in R1 group strongly affected the DNA gyrase inhibitory activity. These explains the significance of the structure of R1 group in relation to the effectiveness of these novel fluoroquinolones.

At lower concentration (6 - 18 nM), QnrB19 had little effect on DNA supercoiling activity of both DNA gyrase GyrA and GyrB) (Figure 3C). However, increasing concentration of QnrB19 reduced the activity of DNA gyrase in concentration dependent manner evident by complete inhibition of DNA supercoiling activity at the highest concentration of 4,374 nM of QnrB19 (Fig

3A). In this study, QnrB19 showed the ability to protect DNA gyrase from the effect of WQ-3810, WQ-3334 and WQ-4065 in a concentration dependent manner (Figure 3). At the concentration of 18 - 162 nM, QnrB19 showed restoring ability on WQ-3810, WQ-3334 and WQ-4065 inhibited DNA supercoiling activity of DNA gyrase by approximately 4.3 to 4.5-, 5.8 to 6.8- and 4.9 to 5.2-folds, respectively (Figure 3C). This agreed with the results of our previous work, where, at the concentration of 54 nM, QnrB19 showed the maximum restoring ability on the nalidixic acid (50 µg/mL), norfloxacin (1 µg/mL) and ciprofloxacin (0.5 µg/mL) inhibited DNA supercoiling activity of DNA gyrase by approximately 1.8, 3.6 and 7.1-fold, respectively (15). Comparing the results in this study with our previous study, QnrB19 showed restoring ability on the novel fluoroquinolones (WQ-3810, WQ-3334 and WQ-4065) inhibited DNA gyrase activities similar to norfloxacin and ciprofloxacin, but higher than nalidixic acid. Thus, it is worth noting that QnrB19 protected DNA gyrase activity from old and new quinolone with distinct properties.

Under the existence of QnrB19 (18nM), the IC₅₀s of WQ-3810, WQ-3334 and WQ-4065 increased by 14.2-, 13.5- and 12.7-folds, respectively. In our previous work (22), the presence of same molar concentration of QnrB19 caused 3.0- and 2.7-fold increase of IC₅₀s of ciprofloxacin and norfloxacin, respectively, while there was no effect on the IC₅₀ of nalidixic acid. These results suggested the structural differences, especially at R7 group (3-isopropylaminoazetizine-1-yl in WQ-compounds, piperazine in ciprofloxacin and norfloxacin, and none in nalidixic acid) to be the cause of distinct increase of IC₅₀s by the addition of QnrB19. Conversely, better choice of R7 group may contribute to develop quinolones strong for the effect of Qnrs. This will be elucidated in our future study.

Elsewhere, IC₅₀s obtained by DNA supercoiling assay targeting wild type *Salmonella* DNA gyrase and MICs obtained by conventional culture-based drug susceptibility tests have been compared. Ciprofloxacin showed IC₅₀ of 0.25 ± 0.05 µg/mL and MIC of 0.0078 µg/mL (32-fold

lower than IC₅₀). On the other hand, nalidixic acid exhibited the IC₅₀ of 65.1 ± 19.08 µg/mL and MIC of 4 µg/mL (16-fold lower than IC₅₀) (23). In our previous study, MIC of WQ-3810 was 2.4-fold lower than IC₅₀ (18). These facts show that the correlation between IC₅₀s and MICs varies between quinolones. The Clinical and Laboratory Standards Institute (CLSI) 2019 defined MIC breakpoint for *Salmonella* spp. resistance to ciprofloxacin, norfloxacin and nalidixic acid to be ≥ 1, ≥ 16 and ≥ 32 µg/mL respectively (27). Predicted MICs of WQ-3810, 3334, 4065 against wild type *Salmonella* and those under the existence of QnrB19 were 0.013, 0.030, 0.30, 0.18, 0.38 and 3.8 µg/mL, respectively. WQ-3810 and 3334 would be good candidate drugs for the treatment of salmonellosis caused by either WT *Salmonella* or that carrying QnrB19.

In conclusion, novel fluoroquinolones, WQ-3810, WQ-3334 and WQ-4065 with unique difluoropyridine derivatives at R1 groups were assessed in this study to observe the inhibitory activity against wild type *Salmonella* DNA gyrase and interaction with plasmid-encoded quinolone resistance protein QnrB19. Our results show that WQ-3810 and WQ-3334 with 6-amino-3,5-difluoropyridine-2-yl at the R1 group showed stronger inhibitory activity against *Salmonella* DNA gyrases with a lower IC₅₀s than WQ-4065 which have a 6-ethylamino-3,5-difluoropyridine-2-yl group at the R1 position either in the absence or presence of QnrB19. The results suggested that WQ-3810 and WQ-3334 could be good candidate drug for wild type *Salmonella* and *Salmonella* carrying QnrB19.

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References

1. Jessica, M.H., and B.B. Beau. CDC. 2019. Salmonellosis (Nontyphoidal) - Chapter 4. [accessed 13 September 2020]. Available at <https://wwwnc.cdc.gov/travel/yellowbook/2020/travel-related-infectious-diseases/salmonellosis-nontyphoidal>.
2. WHO. 2019. *Salmonella* (non-typhoidal). [accessed 13 September 2020] Available at [https://www.who.int/news-room/fact-sheets/detail/salmonella-\(non-typhoidal\)](https://www.who.int/news-room/fact-sheets/detail/salmonella-(non-typhoidal)).
3. Bula-Rudas, F.J. , M.H. Rathore, and N.F. Maraqa. 2015. *Salmonella* Infections in Childhood. Adv in Pediatr. 62(1):29-58.
4. Garcia, C., N. Hinostroza, L. Astocondor, T. Ochoa, J. Jacobs, and For The Salmoiber Cytel Network. 2019. Characterization of ESBL-Producing *Salmonella* enterica Serovar Infantis Infection in Humans, Lima, Peru. Am J Trop Med Hyg. 101:746-748.
5. Crump, J.A., M. Sjölund-Karlsson, M.A. Gordon, and C.M. Parry. 2015. Epidemiology, clinical presentation, laboratory diagnosis, antimicrobial resistance, and antimicrobial management of invasive *Salmonella* infections. Clin Microbiol Rev. 28(4):901-937.
6. Li, Y., Xie, X., Xu, X., Wang, X., Chang, X., Wang, C., Wang, A., He, Y., Yu, H., Wang, X., and Zeng, M. 2014. Nontyphoidal *Salmonella* infection in children with acute gastroenteritis: Prevalence, serotypes, and antimicrobial resistance in Shanghai, China. Foodborne Pathog Dis. 11:200-206.
7. Crump, J.A., F.M. Medalla, K.W. Joyce, A.L. Krueger, R.M. Hoekstra, J.M. Whichard, E.J. Barzilay, and Emerging Infections Program NARMS Working Group. 2011. Antimicrobial resistance among invasive nontyphoidal *Salmonella* enterica isolates in the United States: National antimicrobial resistance monitoring system, 1996 to 2007. Antimicrob Agents Chemother. 55: 1148-1154.

8. Lee H.Y., L.H. Su, M.H. Tsai, S.W. Kim, H.H. Chang, S.I. Jung, K.H. Park, J. Perera, C. Carlos, B.H. Tan, G. Kumarasinghe, T. So, A. Chongthaleong, P.R. Hsueh, J.W. Lui, J.H. Song, and C.H. Chiu. 2009. High rate of reduced susceptibility to ciprofloxacin and ceftriaxone among nontyphoid *Salmonella* clinical isolates in Asia. *Antimicrob Agents Chemother.* 53(6):2696-2699.
9. [REDACTED] and the DT104 Study Group [REDACTED]
[REDACTED]
[REDACTED] European Food Safety Authority. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2015. *EFSA J.* 2017;15. [accessed 30 Nov 2018]. Available at <https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2017.4694>.
- [REDACTED] Threlfall, E.J., M. Day, E.de. Pinna, A. Charlett, and K.L. Goodyear. 2006. Assessment of factors contributing to changes in the incidence of antimicrobial drug resistance in *Salmonella* enterica serotypes Enteritidis and Typhimurium from humans in England and Wales in 2000, 2002 and 2004. *Int J Antimicrob Agents.* 28: 389-395.
- [REDACTED] Mølbak, K., P.G. Smidt, and H.C. Wegener. 2002. Increasing Quinolone Resistance in *Salmonella enterica* serotype Enteritidis. *Emerg Infect Dis.* 8: 514-515.
13. Cuypers, W.L., J. Jacobs, V. Wong, E.J. Klemm, S. Deborggraeve, and S. Van Puyvelde. 2018. Fluoroquinolone resistance in *Salmonella*: Insights by wholegenome sequencing. *Microb Genom.* 4(7).
14. Deweese, J.E., and N. Osheroff. 2010. The use of divalent metal ions by type II topoisomerases. *Metallomics.* 2(7):450-459.
15. Aldred, K.J., R.J. Kerns, and N. Osheroff. 2014. Mechanism of quinolone action and resistance. *Biochemistry.* 18;53(10):1565-1574.
16. Robicsek, A., G.A. Jacoby, and D.C. Hooper. 2006. The worldwide emergence of plasmid-

- mediated quinolone resistance. *Lancet Infect Dis.* 6(10):629-640.
17. Kazamori, D., H. Aoi, K. Sugimoto, T. Ueshima, H. Amano, K. Itoh, Y. Kuramoto, and A. Yazaki. 2014. In vitro activity of WQ-3810, a novel fluoroquinolone, against multidrug-resistant and fluoroquinolone-resistant pathogens. *Int J Antimicrob Agents.* 44:443-449.
18. Koide, K., S. Kongsoi, C. Nakajima, and Y. Suzuki. 2019. WQ-3810 exerts high inhibitory effect on quinolone-resistant DNA gyrase of *Salmonella* Typhimurium. *Biosci Biotechnol Biochem.* 12:2249-2256.
19. Ouchi, Y., T. Mukai, K. Koide, T. Yamaguchi, J.H. Park, H. Kim, K. Yokoyama, A. Tamaru, S.V. Gordon, C. Nakijima, and Y. Suzuki. 2020. WQ-3810: A new fluoroquinolone with a high potential against fluoroquinolone-resistant *Mycobacterium tuberculosis*. *Tuberculosis (Edinb).* 120:101891.
20. Park, J.H., T. Yamaguchi, Y. Ouchi, K. Kentaro, S. Mori, H. Kim, T. Mukai, C. Nakijima, and Y. Suzuki. 2020. WQ-3810 inhibits DNA gyrase activity in ofloxacin-resistant *Mycobacterium leprae*. *J Infect Chemother.* 26(4):335-342.
21. Itoh, K., Y. Kuramoto, H. Amano, D. Kazamori, and A. Yazaki. 2015. Discovery of WQ-3810: design, synthesis, and evaluation of 7-(3-alkylaminoazetidin-1-yl)fluoro-quinolones as orally active antibacterial agents. *Eur J Med Chem.* 103:354-360.
22. Pachanon, R., K. Koide, S. Kongsoi, C. Nakajima, T.F. Kapalamula, O. Suthienkul, and Y. Suzuki. 2020. Interaction of the plasmid-encoded quinolone resistance protein QnrB19 with *Salmonella* Typhimurium DNA gyrase. *J infect Chemother.* 26:1139-1145.
23. Kongsoi, S., K. Yokoyama, A. Suprasert, F. Utrarachkij, C. Nakajima, O. Suthienkul, and Y. Suzuki. 2015. Characterization of *Salmonella* Typhimurium DNA gyrase as a target of quinolones. *Drug Test Anal.* 7:714-720.
24. Matrat, S., S. Petrella, E. Cambau, W. Sougakoff, V. Jarlier, and A. Aubry. 2007. Expression and purification of an active form of the *Mycobacterium leprae* DNA gyrase and

its inhibition by quinolones. *Antimicrob Agents Chemother.* 51(5):1643-1648.

25. Kim, H., C. Nakajima, K. Yokoyama, Z. Rahim, Y.U. Kim, H. Oguri, and Y. Suzuki. 2011.

Impact of the E540V amino acid substitution in GyrB of *Mycobacterium tuberculosis* on quinolone resistance. *Antimicrob Agents Chemother.* 55(8):3661-3667.

26. Yokoyama, K., H. Kim, T. Mukai, M. Matsuoka, C. Nakajima, and Y. Suzuki. 2012. Amino

acid substitutions at position 95 in GyrA can add fluoroquinolone resistance to

Mycobacterium leprae. *Antimicrob Agents Chemother.* 56(2):697-702.

27. Clinical and Laboratory Standards Institute. 2019. Performance standards for antimicrobial

susceptibility testing. M100 standard. 29th ed. Clinical and Laboratory Standards Institute,

Wayne, PA.

Figure legends

Figure 1. Chemical structures of fluoroquinolones used in the present study

A; Basic structure of fluoroquinolone, B; WQ-3810, C; WQ-3334, D; WQ-4065.

Differences in WQ-3334 were WQ-4065 from WQ-3810 were shown by blue and red, respectively.

Figure 2. Purified recombinant DNA gyrase subunits of *Salmonella* and QnrB19 used in this study.

Recombinant DNA gyrase of *Salmonella* and QnrB19 purities were examined by SDS-PAGE. Each protein sample (500 ng) was loaded on a SuperSep™ Ace 5-20% gradient gel. lane M: protein size markers, lane 1: GyrA, lane 2: GyrB, lane 3: QnrB19. “†” denotes GyrA with the MW of ~97 kDa, “‡” denotes GyrB with the MW of ~89 kDa, “*” denotes recombinant QnrB19 with the MW of ~25 kDa.

Figure 3. Inhibition of wild type *Salmonella* DNA gyrase by fluoroquinolones and protection by QnrB19

Reaction mixtures were diluted 4 times with 1× DNA sample buffer and 10 µL of them were analyzed by electrophoresis in 1% agarose gels. Lanes 2 - 16: contained 0.3 µg relaxed plasmid pBR322, reaction buffer and 18 nM DNA gyrase subunit. (A) Lane 1 - 8: with the absence of fluoroquinolone; lane 9 - 16: with the presence of 0.16 µg/mL WQ-3810. (B) Lane 1 - 8: with the presence 0.64 µg/mL WQ-3334; lane 9 - 16: with the presence of 2.56 µg/mL WQ-4065. Lanes 2 and 10: with 6 nM QnrB19; lanes 3 and 11: with 18 nM QnrB19; lanes 4 and 12: with 54 nM QnrB19; lanes 5 and 13: with 162 nM QnrB19; lanes 6 and 14: with 486 nM QnrB19; lanes 7 and 15: with 1458 nM QnrB19; lanes 8 and 16: with 4374 nM QnrB19; lane 17: relaxed plasmid pBR322. R, SC, L and N denote relaxed, supercoiled, linear and nicked form of pBR322 DNA,

respectively. In panel C, dotted lines with thick circles denote the effect of QnrB19 on the DNA supercoiling activity of DNA gyrase (expressed as % activity comparing to that without fluoroquinolones). Dotted lines with filled triangles denote those with the presence of 2.56 $\mu\text{g/mL}$ WQ-4065. Solid lines with filled squares and filled circles denote those with the presence of 0.16 $\mu\text{g/mL}$ WQ-3810 and 0.64 $\mu\text{g/mL}$ WQ-3334, respectively.

Figure 4. Effect of QnrB19 on IC₅₀s of fluoroquinolones

Reaction mixtures were diluted 4 times with 1 $\times$ DNA sample buffer and 10 μL of them were analyzed by electrophoresis in 1% agarose gels. Lane 1 - 13: Reaction mixture contained 0.3 μg relaxed plasmid pBR322, reaction buffer and 18 nM DNA gyrase subunit; Lanes 2 - 7: with 0.01 - 3.2 $\mu\text{g/mL}$ WQ-3810 (A), with 0.032 - 10 $\mu\text{g/mL}$ WQ-3334 (B), with 0.32 - 100 $\mu\text{g/mL}$ WQ-4065 (C) in the absence of QnrB19; lanes 8 - 13: with 0.01 - 3.2 $\mu\text{g/mL}$ WQ-3810 (A), with 0.032 - 10 $\mu\text{g/mL}$ WQ-3334 (B), with 0.32 - 100 $\mu\text{g/mL}$ WQ-4065 (C) in the presence of 18 nM QnrB19; lane 14: relaxed plasmid pBR322. Solid lines with filled squares in panels D, E and F denote the impact of WQ-3810, WQ-3334 and WQ-4065 on the DNA supercoiling activity of DNA gyrase (expressed as % activity comparing to that without QnrB19), respectively. Dotted lines with filled triangles denote those in the presence of 18 nM QnrB19.

376 **Table 1. Impact of IC₅₀s of fluoroquinolones on WT *Salmonella* DNA gyrase with/without QnrB19**

Fluoroquinolones	R-1 ^a	R-6 ^a	R-7 ^a	R-8 ^a	IC ₅₀ ^b (µg/mL)		Ratio (Without: With QnrB19)
					Without QnrB19	With QnrB19	
WQ-3810	6-amino-3,5-difluoropyridine-2-yl	F	3-isopropylaminoazetizine-1-yl	CH ₃	0.031 ± 0.003	0.44 ± 0.05	1:14.2
WQ-3334	6-amino-3,5-difluoropyridine-2-yl	F	3-isopropylaminoazetizine-1-yl	Br	0.068 ± 0.016	0.92 ± 0.34	1:13.5
WQ-4065	6-ethylamino-3,5-difluoropyridine-2-yl	F	3-isopropylaminoazetizine-1-yl	CH ₃	0.72 ± 0.39	9.16 ± 2.21	1:12.7

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378 ^a Position of substituents in the structures of fluoroquinolones.

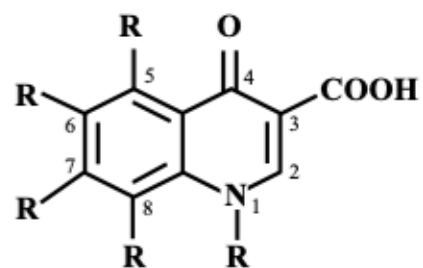
379 ^b IC₅₀: Concentrations of fluoroquinolones to inhibit the supercoiling activity of DNA gyrase by 50%.

380

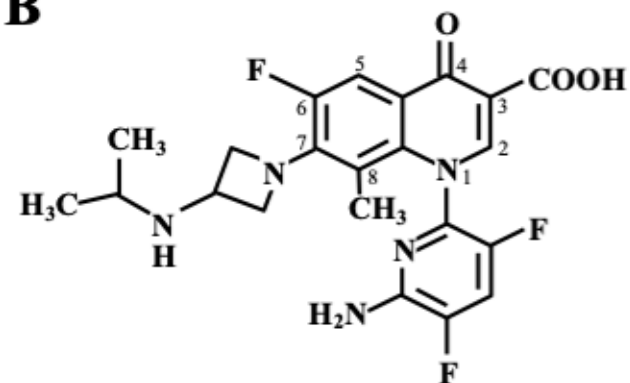
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Pachanon Figure 1

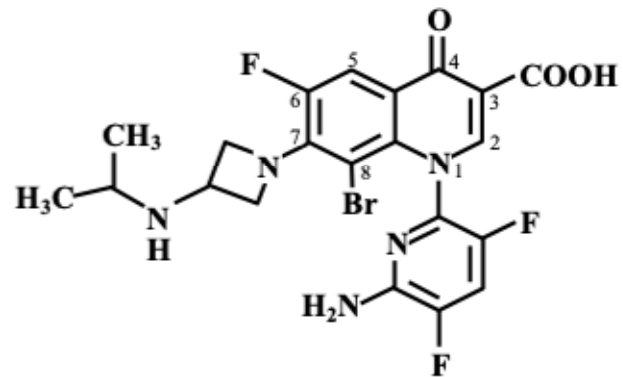
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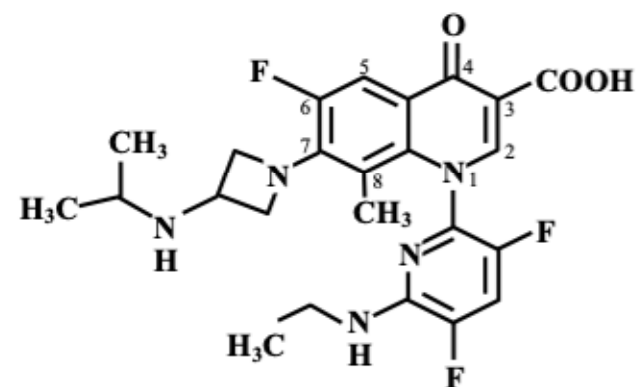
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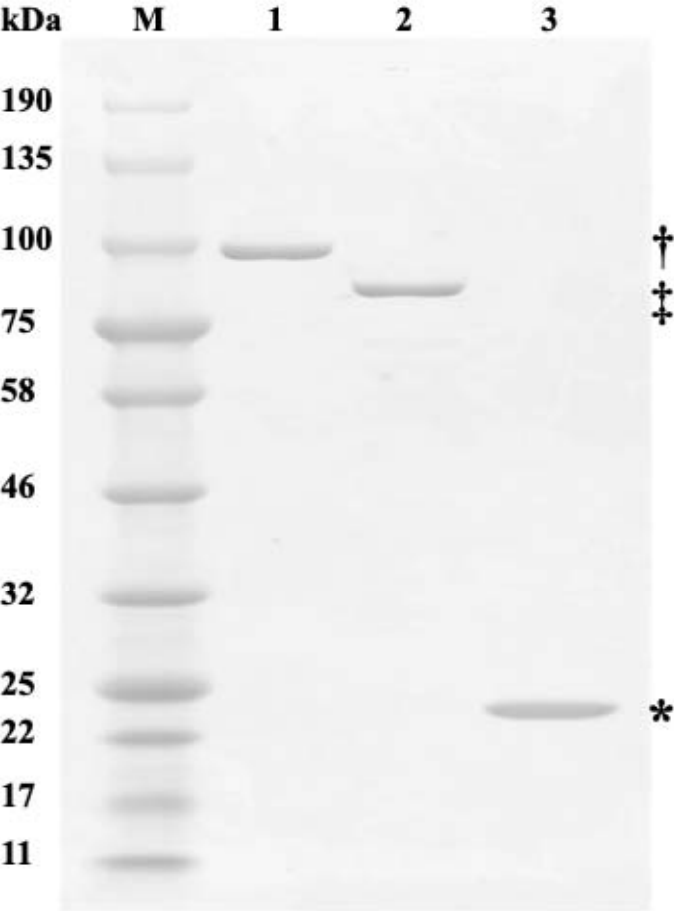
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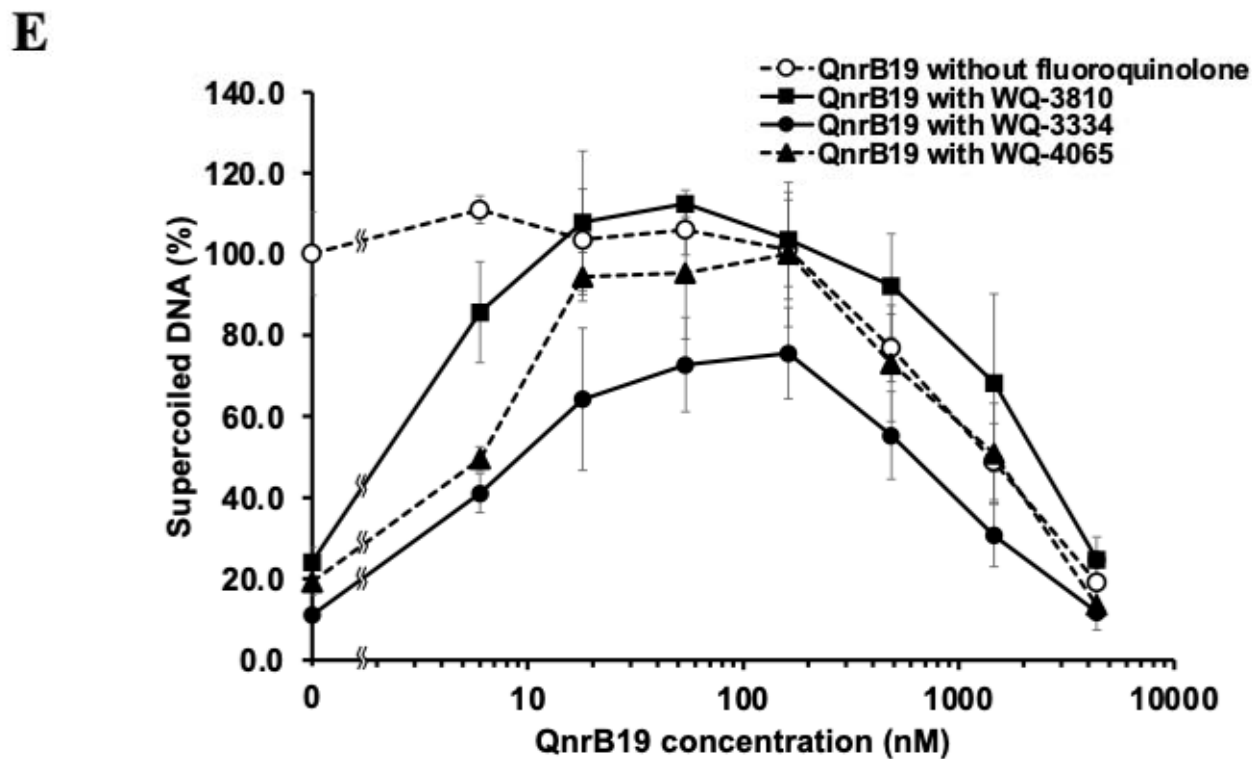
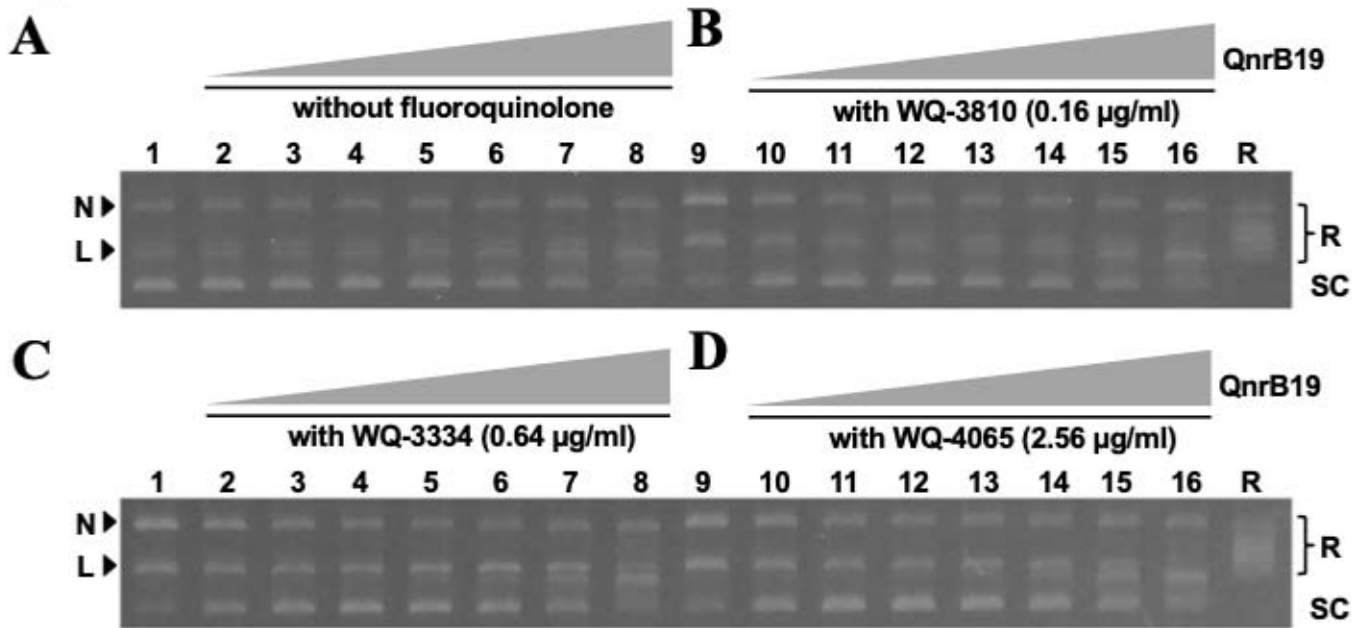
D



Pachanon Figure 2



Pachanon Figure 3



Pachanon Figure 4

