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1 **WQ-3810: a new fluoroquinolone with a high potential against fluoroquinolone-resistant**
2 ***Mycobacterium tuberculosis***

3

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25 **Abstract**

26 Fluoroquinolone (FQ) resistance in *Mycobacterium tuberculosis* (*Mtb*), caused by amino
27 acid substitutions in DNA gyrase, has been increasingly reported worldwide. WQ-3810 is a newly
28 developed FQ that is highly active against FQ-resistant pathogens; however, its activity against *Mtb*
29 has not been evaluated. Herein we examined the efficacy of WQ-3810 against *Mtb* through the use
30 of recombinant *Mtb* DNA gyrases. In addition, in vitro antimycobacterial activity of WQ-3810 was
31 evaluated against recombinant *Mtb* var. *bovis* Bacille Calmette–Guérin strains in which
32 gyrase-coding genes were replaced with *Mtb* variants containing resistance-conferring mutations.
33 WQ-3810 showed a higher inhibitory activity than levofloxacin against most recombinant DNA
34 gyrases with FQ-resistance mutations. Furthermore, WQ-3810 showed inhibition even against a
35 DNA gyrase variant harboring a G88C mutation which is thought to confer the highest resistance
36 against FQs in clinical *Mtb* isolates. In contrast, the FQ susceptibility test showed that WQ-3810
37 had relatively weak mycobactericidal activity compared with moxifloxacin. However, the
38 combination of WQ-3810 and ethambutol showed the greatest degree of synergistic activity against
39 recombinant strains. Since FQs and ethambutol have been used in multi-drug therapy for
40 tuberculosis, WQ-3810 might represent a new, potent anti-tuberculosis drug that can be effective
41 even against FQ-resistant *Mtb* strains.

42 **Keywords**

43 WQ-3810, fluoroquinolone resistance, *gyrA* mutations, *Mycobacterium tuberculosis*,
44 ethambutol, synergistic effect

45 1. Introduction

46 Tuberculosis (TB) is a global disease caused by infection with *Mycobacterium tuberculosis*
47 (*Mtb*) and with an estimated 10 million new cases and 1.6 million deaths in 2017 [1]. For the
48 treatment of TB, multidrug therapy using rifampicin, isoniazid, pyrazinamide and ethambutol is
49 necessary. These drugs target essential bacterial enzymes to inhibit transcription, fatty acid
50 synthesis and cell wall synthesis. However, multidrug-resistant TB (MDR-TB), which is resistant to
51 at least two drugs including rifampicin and isoniazid, has increased significantly and become a
52 persistent threat with 558,000 cases in 2017 [1]. MDR-TB requires treatment with second-line
53 drugs such as fluoroquinolones (FQs) and injectable agents.

54 FQs have been used as broad-spectrum antibacterial agents that inhibit DNA gyrase activity.
55 DNA gyrase is a type II DNA topoisomerase that catalyzes DNA supercoiling at the expense of ATP
56 hydrolysis, and has an essential role for DNA replication and transcription in bacteria. This enzyme
57 is composed of subunit A (GyrA) and B (GyrB) encoded by *gyrA* and *gyrB*, respectively. According
58 to epidemiological studies [2–4], amino acid substitutions at positions 88, 90, 94 and elsewhere in
59 the quinolone resistance-determining region of GyrA cause FQ resistance in *Mtb*. These
60 substitutions are thought to change the formation of FQ binding pocket of the enzyme, leading to
61 FQ resistance [5,6].

62 WQ-3810 is a FQ developed by Wakunaga Pharmaceutical Co., Ltd. with increased
63 lipophilicity and membrane permeability [7]. This novel drug showed high DNA gyrase inhibitory
64 activity and antibacterial activity [8] against multidrug-resistant and even FQ-resistant
65 gram-negative pathogens such as *Escherichia coli* and *Acinetobacter baumannii*. WQ-3810 is also
66 active against FQ-resistant clinical isolates of gram-positive bacteria such as *Streptococcus*
67 *pneumoniae* and methicillin-resistant *Staphylococcus aureus*. WQ-3810 has a high oral absorption
68 rate with a low potential for side effects. However, the enzyme inhibitory activity and
69 antimycobacterial activity of this drug has not been evaluated against *Mycobacterium* species.

70 To evaluate the inhibitory activity of WQ-3810 against *Mtb*, we devised a two pronged

71 approach that utilized (1) recombinant DNA gyrases derived from *Mtb* Beijing lineage, which are
72 most prevalent in Asian countries [9]; (2) recombinant *Mtb* var. *bovis* Bacille Calmette–Guérin
73 (BCG) strains in which the gyrase-coding genes were replaced with *Mtb* Beijing lineage variants
74 (rBCG), and used for in vitro antibacterial activity of WQ-3810. Our results suggest that WQ-3810
75 shows promise as a new anti-TB drug.

76

77 **2. Materials and Methods**

78 **2.1. Reagents and Kits**

79 FQs, levofloxacin (LVX) and moxifloxacin (MXF) were purchased from LKT Laboratories,
80 Inc. (St Paul, MN, USA) and WQ-3810 was a gift from Wakunaga Pharmaceutical Co., Ltd. (Tokyo,
81 Japan) (Fig. 1). Ampicillin, kanamycin, hygromycin and isoniazid (INH) were purchased from
82 FUJIFILM Wako Pure Chemical Co., Ltd. (Osaka, Japan), ethambutol (EMB) was purchased from
83 MP Biomedicals, LLC (Solon, OH, USA). DNA Ligation Kit, Mighty Mix, and In-Fusion® HD
84 Cloning Kit were obtained from Takara Bio Inc. (Shiga, Japan). Ni-nitrilotriacetic acid (Ni-NTA)
85 Agarose was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Restriction
86 enzymes were purchased from New England Biolabs, Inc. (Ipswich, MA, USA). Relaxed pBR322
87 DNA was purchased from John Innes Enterprises Ltd (Norwich, UK). BD Difco™ Middlebrook
88 7H9 broth, BD Difco™ Middlebrook 7H10 agar and BD BBL™ Middlebrook OADC Enrichment
89 were obtained from Becton, Dickinson and Co. (MD, USA), and MycoBroth was purchased from
90 Kyokuto Pharmaceutical Industrial Co., Ltd. (Tokyo, Japan). Oligonucleotide primers used for
91 cloning, mutagenesis and sequencing are listed in Supplementary Table S1.

92

93 **2.2. Bacterial strains and plasmids**

94 *Mtb* var. *bovis* BCG Tokyo 172 strains were provided from the Japan BCG Laboratory
95 (Tokyo, Japan). FQ-susceptible or -resistant *Mtb* Beijing lineage strains listed in Supplementary
96 Table S2 were obtained from Osaka Institute of Public Health (Osaka, Japan). *E. coli* strain DH5α

97 (Takara Bio Inc.) was used for DNA cloning. *E. coli* strains Rosetta-gami 2 (DE3) and BL21 (DE3)
98 pLysS (Merck KGaA, Darmstadt, Germany) were used for protein expression. Vector plasmids
99 pET-20b (+) and pET-19b (Merck KGaA) were used to construct plasmids for the expression of
100 *Mtb* GyrA and GyrB, respectively. A *Mtb* GyrB expression plasmid, pTB-B, was constructed in our
101 previous study [10]. A parental plasmid for allelic exchange, pΔAHm31, was constructed, as shown
102 in Supplementary Fig. S1. Recombinase expression plasmid, pJV53, and resolvase expression
103 plasmid, pYUB870 were gifts from Dr. Hatfull and Dr. Jacobs, respectively [11,12].
104

105 **2.3. Construction of DNA gyrase subunit expression plasmid**

106 A range of *Mtb* DNA gyrase expression plasmids were constructed: wild type (WT)-GyrA;
107 GyrA with amino acid substitution glycine to cysteine at position 88 (G88C-GyrA); A90V-GyrA;
108 D94A-GyrA; D94G-GyrA; D94H-GyrA; D94N-GyrA and D94Y-GyrA (Supplementary Table S3)
109 [10]. Briefly, gene fragments were amplified by polymerase chain reaction (PCR) in the reaction
110 mixture consisted of 1x PrimeSTAR MAX DNA Polymerase (Takara Bio Inc.), 0.3 μM of each
111 primer pair and 0.02 ng/μl of bacterial DNA from Osaka MDR-12 (Beijing lineage). Gene cassettes
112 encoding N-terminal and C-terminal of WT-GyrA were amplified using each primer pair
113 ON-873/ON-874 and ON-875/ON-876. Both of gene cassettes were used as templates for the
114 amplification of complete *gyrA* cassette with a primer pair, ON-873/ON-876. Using NdeI and XhoI,
115 the amplified products were transferred into pET20b(+) to construct 6x His-tagged WT-GyrA
116 expression plasmid (termed pET/*gyrA*/WT). Mutations were introduced into the WT-*gyrA* in the
117 plasmid by PCR with pairs of complementary primers containing the mutation of interest and
118 ON-873 or ON-874. The *gyrA* amplicon with each mutation was digested with NdeI and HpaI and
119 ligated into pET/*gyrA*/WT digested with the same restriction enzymes. A *gyrB* cassette was
120 amplified with ON-1518/ON-886 using pTB-B as a template. The PCR product was digested by
121 NdeI and XhoI, and transferred into pET19b to construct 6x His-tagged GyrB expression plasmid
122 (termed pET/*gyrB*/WT). The nucleotide sequences of the DNA gyrase gene in the plasmids were

123 confirmed using a BigDye Terminator (version 3.1) cycle sequencing kit and an ABI Prism 3130xl
124 genetic analyzer (Thermo Fisher Scientific Inc.), according to the manufacturer's protocol.

125

126 **2.4. Recombinant expression and purification of DNA gyrase subunits**

127 Recombinant DNA gyrase subunits were expressed and purified using a slightly modified
128 method described by Kim et al [10]. Expression of GyrA and GyrB in *E. coli* Rosetta-gami 2 (DE3)
129 or BL21 (DE3) pLysS, respectively, was induced by addition of 1 mM isopropyl
130 beta-D-thiogalactopyranoside and incubation at 18°C for 40 h or at 23°C for 5 h. After harvesting
131 the culture, *E. coli* was sonicated and the recombinant DNA gyrase in the supernatant was purified
132 by affinity chromatography using Ni-NTA Agarose. The purified protein fractions were analyzed by
133 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) stained with coomassie
134 brilliant blue (CBB). The proteins were quantified by Bradford Protein Assay Kit (Takara Bio Inc.).

135

136 **2.5. DNA supercoiling assay and inhibitory assay**

137 The assay monitoring ATP-dependent supercoiling activity of recombinant DNA gyrases
138 was performed as previously described [10]. Briefly, the activity was examined in 30 µl reaction
139 mixture consisting of 1x buffer (35 mM Tris-HCl pH 7.5, 6 mM MgCl₂, 1.8 mM spermidine, 24
140 mM KCl, 5 mM DTT, 0.36 mg/ml BSA, 6.5% w/v glycerol), 1 mM ATP, 2 nM relaxed pBR322
141 DNA, and 32 nM GyrA and GyrB subunits. After the mixture was incubated at 37°C for 60 min,
142 reactions were stopped by the addition of 7.5 µl of 5x dye mix (5% SDS, 25% glycerol, 0.25 mg/ml
143 bromophenol blue). The relaxed DNA and the supercoiled DNA were separated by agarose gel
144 electrophoresis. The inhibitory activities of FQs were similarly tested using FQ-mediated DNA
145 supercoiling activity inhibition. Various amounts of LVX, MXF or WQ-3810 were used as
146 inhibitors. The reaction mixture contained 1x buffer, 1 mM ATP, 2 nM relaxed pBR322 DNA, 4 nM
147 GyrA and GyrB subunits and the indicated concentration of FQs in a total volume of 30 µl. As
148 described in the methodology for the DNA supercoiling assay, the relaxed DNA and the supercoiled

149 DNA were separated by agarose gel electrophoresis after the reactions. To assess the inhibitory
150 activities of FQs on each DNA gyrase, the amount of supercoiled DNA in the reactions was
151 quantified with ImageJ software (<https://imagej.nih.gov/ij/>) and the FQ concentrations required to
152 inhibit the supercoiling activity by 50% (IC₅₀s) were calculated.

153

154 **2.6. Construction of plasmids containing allelic exchange substrates**

155 Plasmids containing allelic exchange substrates to allow homologous recombination
156 between plasmid-borne *Mtb* DNA gyrase genes and BCG chromosomal orthologues were
157 constructed (Supplementary Table S3) following previously described recombineering protocols
158 [13]. The genomic DNAs were extracted from BCG Tokyo 172 strain and *Mtb* Beijing lineage
159 strains with or without FQ-resistance mutations (Osaka MDR-12 (WT), Osaka MDR-23 (G88C),
160 Osaka MDR-14 (A90V), Osaka MDR-5 (D94A), Osaka MDR-13 (D94G) and Osaka MDR-79
161 (D94H)). An approximately 4.8 kb fragment containing *gyrA* and *gyrB* was amplified using
162 ON-1547/ON-1548. Similarly, a 0.7 kb fragment with Rv0007, a gene located downstream of
163 *gyrAB*, was amplified using ON-1558/ON-1559. The former *gyrAB* PCR product was digested with
164 HindIII and XbaI, and cloned upstream of the hygromycin resistance cassette (*hyg*) of a parental
165 pΔAHm31 plasmid. The latter Rv0007 product was directly cloned downstream of *hyg* in the
166 KpnI-digested plasmid using In-Fusion® HD Cloning Kit. To construct the substrates containing
167 novel FQ mutations, D94N or D94Y, the pΔAHm/*gyrAB*/WT plasmid containing WT allelic
168 exchange substrate was used as a PCR template. A 2.5 kb fragment and a 2.3 kb fragment were
169 amplified using ON-1566/ON-1568 and ON-1567/ON-1569 (D94N) or ON-1566/ON-1570 and
170 ON-1567/ON-1571 (D94Y). The amplified products were ligated to the pΔAHm/*gyrAB*/WT
171 plasmid digested with HindIII and XbaI, using In-Fusion® HD Cloning Kit. The nucleotide
172 sequence of the plasmid was confirmed by standard sequencing.

173

174 **2.7. Preparation of recombinant BCG**

175 The rBCG strains in which the DNA gyrase genes were replaced with *Mtb* DNA gyrase
176 orthologues (rBCG/*Mtb*) were established by a modified recombineering protocol [13]. All strains
177 were cultivated in Middlebrook 7H9 broth containing 10% OADC enrichment, 0.2% glycerol and
178 0.05% tyloxapol or on Middlebrook 7H10 agar supplemented with 10% OADC enrichment, 0.2%
179 glycerol and 0.05% tyloxapol unless otherwise indicated. The pJV53, encoding a recombinase, was
180 transformed into electrocompetent BCG cells, the recombinase expression was induced by the
181 addition of 0.2% acetamide, and induced-BCG cells were again prepared as electrocompetent cells.
182 The plasmids containing allelic exchange substrates were linearized by HindIII and transformed
183 into the recombinase-expressing cells. After plating the transformed cells on Middlebrook 7H10
184 agar with 20 µg/ml hygromycin and incubating to select transformants, colonies were screened by
185 colony PCR using ON-1564/ON-1565, which were designed to amplify the whole exchanged
186 sequence from outside the region. Then, homologous recombination was confirmed by sequencing
187 using primers listed in Supplementary Table S1. The colonies were incubated in Middlebrook 7H9
188 broth for 2 weeks and plated on Middlebrook 7H10 agar without drugs. After the loss of pJV53
189 from the BCG was confirmed by PCR using ON-1562/ON-1563, the BCG cells were again
190 prepared as electrocompetent cells. pYUB870, encoding a res-site-specific resolvase ($\gamma\delta$ -tnpR), was
191 transformed into the recombinants to remove the res sites flanking the *hyg* cassette. After selection
192 using Middlebrook 7H10 agar with 20 µg/ml kanamycin, the absence of the *hyg* cassette was
193 confirmed by PCR using ON-1564/ON-1565. The recombinants were cultured in Middlebrook 7H9
194 broth without antibiotics, and the loss of pYUB870 after continuous incubation and confirmed by
195 PCR using ON-1562/ON-1563.

196

197 **2.8. Drug susceptibility test**

198 Parental BCG and rBCG strains were analyzed to determine the minimum inhibitory
199 concentrations (MICs) of selected drugs. The broth microdilution method was carried out in
200 duplicate according to the manufacturer's instructions. A bacterial culture ($OD_{600nm} = 0.15$) was

201 diluted 40-fold with MycoBroth and 100 µl of the dilution was added to each well of a sterile round
202 bottom microtitre plate containing the 2-fold serially diluted drugs (100 µl/well). After the test plate
203 was incubated for 14 days at 37°C, the MIC was defined as the lowest concentrations of antibiotics
204 that inhibited visible bacterial growth. The panel of drugs was LVX, MXF, WQ-3810, EMB and
205 INH.

206

207 **2.9. Checkerboard assay**

208 Recombinant BCG strains, which have WT or G88C DNA gyrases, were used to assess the
209 interaction between FQs and cell wall synthesis inhibitors. Using a checkerboard assay [14],
210 fractional inhibitory concentration index (FICI) of each agent was determined in the presence of
211 sub-inhibitory concentrations of another. A bacterial culture ($OD_{600nm} = 0.15$) was diluted 40-fold
212 with MycoBroth and 100 µl of the dilution was added to each well of a sterile round bottom
213 microtitre plate containing the serially diluted concentrations of FQs (50 µl/well) and INH or EMB
214 (50 µl/well). After the test plate was incubated for 14 days at 37°C, the FICI of each combination
215 between agents was calculated using the following equation: $FICI = (MIC \text{ of drug A in the presence}$
216 $\text{of drug B} / MIC \text{ of drug A alone}) + (MIC \text{ of drug B in the presence of drug A} / MIC \text{ of drug B}$
217 $\text{alone})$. Minimum FICIs ($FICI_{ms}$) in the tested combinations are shown and $FICI_m \leq 0.5$ is regarded
218 as synergistic.

219

220 **3. Results**

221 **3.1. Expression and purification of recombinant DNA gyrase subunits**

222 Using a Bradford Protein Assay to calculate protein concentration, 1.0 to 3.5 mg of purified
223 DNA gyrase subunits were harvested from 500 ml cultures of the *E. coli*. The purity of the
224 recombinant proteins was confirmed by SDS-PAGE (Fig. 2). All the recombinant proteins were
225 obtained at high purity (>95%) with molecular weights of 93 kDa and 74 kDa for GyrA and GyrB,
226 respectively.

227

228 **3.2. DNA supercoiling activity of *Mtb* DNA gyrases**

229 DNA supercoiling activities using combinations of WT or each mutant GyrA and GyrB
230 were examined using relaxed pBR322 DNA as a substrate in the presence or absence of ATP (Fig.
231 3). GyrAB enzyme activities were only observed when ATP and recombinant DNA gyrase subunits
232 were all present. In this assay, we confirmed the enzyme activities of purified recombinant gyrases
233 using sufficient concentrations (32 nM). In our preliminary experiment using various concentrations
234 (2 to 64 nM) of enzymes, mutants had slightly weak activities compared with WT enzyme (data not
235 shown). Because excessive amount of enzyme might interfere the exact results of inhibitory assay,
236 we used lower concentrations (4 nM) than sufficient concentrations, for subsequent assays.

237

238 **3.3. Inhibition of DNA gyrases by FQs**

239 Inhibition of DNA supercoiling activities by FQs were observed in agarose gel
240 electrophoresis (Supplementary Fig. S2) and IC₅₀s of each FQ were determined to estimate the
241 inhibitory activity of FQs (Fig. 4). This revealed that the inhibitory activity of WQ-3810 against
242 WT DNA gyrase (IC₅₀s: 3.04 µg/ml) had no significant difference with that of MXF (IC₅₀s: 2.01
243 µg/ml) but was higher than that of LVX (IC₅₀s: 5.84 µg/ml). Similarly, WQ-3810 as well as MXF
244 showed lower IC₅₀s against mutant DNA gyrases than LVX. Hence, against mutants with A90V and
245 D94A substitutions, lower concentrations of WQ-3810 were required to inhibit GyrAB enzyme
246 activity (IC₅₀s: 15.8 and 11.2 µg/ml, respectively), while IC₅₀s of LVX were 96.7 and 91.9 µg/ml.
247 WQ-3810 IC₅₀s results were comparable to those of MXF (IC₅₀s of 7.50 and 10.0 µg/ml,
248 respectively). Similarly, WQ-3810 and MXF showed lower IC₅₀s against other mutations at codon
249 94, in the range from 39.5 to 118 µg/ml and from 33.5 to 74.5 µg/ml, respectively, compared to
250 those of LVX which ranged from 315 to 589 µg/ml. Interestingly, even against the G88C mutant,
251 which has been known to be one of the highest resistance-conferring mutations in both clinical
252 isolates [3,4] and experimentally-derived strains [15,16], WQ-3810 showed a considerably lower

253 IC₅₀s of 50.0 µg/ml when compared with LVX and MXF (IC₅₀s: 518 and 296 µg/ml, respectively).

254

255 **3.4. Antibacterial activity of FQs against rBCG strains**

256 Since genetic backgrounds and phenotypic characteristics among clinical strains are
257 diverse and often cryptic, the rBCG strains whose DNA gyrase genes were replaced with *Mtb*
258 orthologues were used to estimate the direct relationships between FQs and FQ
259 resistance-associated mutations in GyrA. As shown in Table 1, MICs of each FQ against WT and
260 mutant rBCG strains were determined to evaluate the antibacterial activity. LVX and WQ-3810
261 exhibited relatively high MICs against rBCG strains, ranging from 0.25 to 8 µg/ml and from 0.5 to
262 8 µg/ml, compared with MICs of MXF that were in a range from 0.0625 to 2 µg/ml. However, the
263 ratios of the MIC_{mutant} / MIC_{WT} for WQ-3810 were lower than for the other tested FQs, ranging
264 from 4- to 16-fold. In particular, the presence of the G88C mutation did not increase the MIC for
265 WQ-3810 (16-fold) as much it increased the MICs for LVX and MXF (64- and 128-fold,
266 respectively).

267

268 **3.5. Effect of cell wall synthesis inhibitors on the MIC of WQ-3810 against rBCG strains**

269 Interaction between FQs and cell wall synthesis inhibitors such as EMB and INH was
270 analyzed using a checkerboard assay. As shown in Table 2, only the combination of WQ-3810 and
271 EMB against both WT and G88C rBCG strains exhibited a synergistic relation, with an FICI_m of
272 0.5, while WQ-3810 also showed a lower FICI_m than MXF in combination with INH. Additionally,
273 the MICs of FQs against rBCG strains were examined with a sub-lethal concentration of EMB (1
274 µg/ml) to see any synergistic relations (Table 1). The MICs of WQ-3810 against rBGG strains were
275 determined to be between 0.0625 and 1 µg/ml, while those of MXF ranged from 0.0625 to 4 µg/ml.
276 As shown in Fig. 5, the addition of a sub-lethal concentration of EMB to the drug susceptibility test
277 increased the correlation between IC₅₀s and MICs of WQ-3810 (correlation coefficient value, R =
278 0.982), and caused an approximately 8-fold decrease in the MIC of WQ-3810.

280 **4. Discussion**

281 In this study, we evaluated the enzyme inhibitory activity of WQ-3810 using recombinant
282 *Mtb* DNA gyrases. For in vitro evaluation of the FQs, recombinant DNA gyrases derived from the
283 *Mtb* H37Rv strain, a type strain, have been used generally. On the other hand, in this study, the
284 recombinant DNA gyrases were derived from the Beijing lineage of *Mtb* that harbors
285 polymorphisms at positions Q21, T95 and D668 in the GyrA subunit of DNA gyrase. These are
286 different amino acids from these in the *Mtb* H37Rv subunit which instead possesses E21, S95 and
287 G668, respectively. Even though these positions have not been thought to confer FQ resistance
288 [2,17], position 95 is located in the quinolone resistance-determining region. Moreover, the DNA
289 gyrase polymorphisms in the *Mtb* Beijing lineage are highly conserved among the globally
290 dominant *Mtb* strains [17–19]. Therefore, in our study the *Mtb* Beijing lineage DNA gyrase was
291 used to evaluate the inhibitory activity of FQs. Comparing the results of IC₅₀s of conventional FQs
292 against Beijing lineage and H37Rv DNA gyrases from this study with the published data
293 [5,16,20,21], most of the mutations conferred equivalent FQ resistance to both Beijing and H37Rv
294 DNA gyrases. However, the G88C mutation conferred much higher resistance, with 89-fold and
295 147-fold increases in IC₅₀s of LXV and MXF in this study, while other reports showed 13- or
296 20-fold and 8.8-fold increases in IC₅₀s of LVX and MXF, respectively, against *Mtb* H37Rv DNA
297 gyrases [5,16]. This agrees with reports that clinical *Mtb* isolates harboring the G88C mutation
298 showed considerably higher MIC for FQs [3,4]. In addition, the G88C mutation has been observed
299 so far only in clinical strains with amino acids Q21, T95 and D668 in the GyrA subunit of DNA
300 gyrase as per data in the NCBI database (www.ncbi.nlm.nih.gov). Considering that the
301 polymorphisms in the *Mtb* H37Rv DNA gyrase are found infrequently in epidemiological studies
302 [18,19] and that FQ resistance has been more frequently reported in Beijing lineage isolates [22,23],
303 greater studies of the *Mtb* Beijing lineage DNA gyrase are strongly warranted so as to evaluate the
304 exact functioning of this enzyme.

305 Some researchers have previously published on the application of recombinant
306 mycobacteria to FQ evaluation. For example, Malik et al. produced A90V and D94G *Mtb* strains
307 using representative laboratory strains such as H37Rv, Erdman and CDC1551, but the number of
308 analyzed *gyrA* mutations was limited [24]. Moreover, they used temperature sensitive bacteriophage
309 and cosmids for homologous recombination and didn't remove the hygromycin resistance gene
310 from the recombinants, which might have had subtle influences on susceptibility tests. In a similar
311 vein, Yoshida et al. produced recombinant *M. smegmatis* and BCG strains with an expression
312 plasmid carrying *Mtb* H37Rv DNA gyrase genes [15]. Since the *Mtb* DNA gyrase was expressed on
313 the plasmid ectopically, it might have affected the expression level of the enzyme and interfere with
314 the results of drug susceptibility tests. In this study, we produced rBCG strains in which their own
315 DNA gyrase-coding genes were replaced with *Mtb* Beijing lineage variants by electroporation of a
316 recombinase-expressing plasmid and allelic exchange substrates. Since the hygromycin selection
317 marker was removed from the chromosomal DNA, it would not have any off target effects on gene
318 transcription. The *Mtb* DNA gyrase genes were inserted via homologous recombination into the
319 identical chromosomal locus in the BCG strain ensuring the genes were in the correct genomic
320 contact and under the same transcriptional controls as in *Mtb*. Of course BCG also belongs to the
321 same complex as *Mtb* with an identical 16S rRNA sequence and 99.9% similarity at the nucleotide
322 level [17,25]. Hence, the applications of rBCG strains produced in our study were useful to
323 precisely clarify the intracellular relationship between FQs and mutations in DNA gyrase.

324 According to the results of previous studies [26, 27], the combination of a
325 5-amino-2,4-difluorophenyl group at N-1 position, azetidiny group at C-7 position, and Cl or Br or
326 Me group at C-8 position of the quinolone ring gives potent antibacterial activity against diverse
327 bacterial species. In addition, the presence of a 2,4-difluorophenyl group at the N-1 position further
328 increased the in vitro potency as well as in vivo efficacy in mice model compared to the
329 cyclopropyl group at the same position in MXF and ciprofloxacin [26]. Additionally, the *m*-amino
330 group on the N1 phenyl ring enhanced the bactericidal activity, especially against FQ-resistant

331 strains [27]. The *m*-amino group might be effective as an electron acceptor in the FQ binding pocket.
332 Therefore, WQ-3810, developed from these analogues, also had good antibacterial activity against
333 FQ-resistant pathogens. Kazamori et al reported the potent antibacterial activity of WQ-3810
334 against FQ-resistant clinical isolates of *E. coli*, *A. baumannii*, *S. pneumoniae* and *S. aureus* [8].
335 They also analyzed the inhibitory activity of WQ-3810 against *E. coli* DNA gyrase using
336 recombinant proteins [8]. While WQ-3810 and LVX exhibited comparable inhibitory activity
337 against WT DNA gyrase, the inhibitory activity of WQ-3810 was nine-fold more potent against
338 DNA gyrase with substitutions of S83L and D87N (corresponding to the substitutions at the
339 positions 90 and 94 in *Mtb*) in GyrA than that of LVX. The inhibitory activity of WQ-3810 was also
340 analyzed against the *Mtb* Beijing lineage DNA gyrase in our study. WQ-3810 showed potency even
341 against the LVX-resistant enzymes and inhibited DNA gyrase carrying the G88C substitution more
342 strongly than MXF. These findings might be because of the unique conformational features at the
343 N1, C7 and C8 substituents of the quinolone ring in WQ-3810.

344 Both enzyme assay and bacterial assay showed that WQ-3810 has potent antimycobacterial
345 activities, in particular against variants carrying the G88C mutation which is responsible for high
346 level FQ resistance in clinical *Mtb* isolates [3,4]. According to the crystal structure of DNA gyrase,
347 the G88C amino acid substitution is located at the beginning of α -helix 4 of the breakage-reunion
348 domain of GyrA [28], which is assumed to play an important role in making the FQ binding pocket,
349 while amino acids 90 and 94 make a water-metal ion interaction with the C3/C4 keto acid of FQ
350 [6,29]. Therefore, the G88C substitution can change the conformation of the loop connecting
351 α -helix 3 and 4 and affect the depth of the FQ binding pocket, leading to FQ resistance [30]. In
352 addition, some groups have reported another FQ binding mode in DNA gyrase where position 88 of
353 GyrA directly binds the C7 substituent of FQs through GyrA-based cross-linking [31,32]. Hence,
354 substitutions at position 88 can disrupt the interaction between DNA gyrase and FQs. Considering
355 the potent activity of WQ-3810 against G88C mutants shown in our current study,
356 C7-azetidiny-substituted-FQs might bind the FQ-binding pocket of DNA gyrase without a strong

357 interaction at position 88. In epidemiological studies of *Mtb* clinical isolates the G88C substitution
358 has been identified at a low frequency, but it is easily produced under the pressure of novel potent
359 C8-methoxy FQs such as PD161144, PD161148 and DC-159a [16,31]. This indicates that the newly
360 developed FQs might increase the occurrence of the G88C mutation in *Mtb* clinical isolates.
361 Conversely, WQ-3810 is still efficacious against variants carrying the G88C mutation, as well and
362 other mutations, compared with conventional FQs.

363 Contrary to the potency of WQ-3810 against DNA gyrase, the FQ susceptibility test with
364 rBCG strains showed that WQ-3810 had a relatively weak antimycobacterial activity compared
365 with MXF. The lower efficacy of WQ-3810 against mycobacteria indicates that permeability of
366 WQ-3810 may be less for mycobacteria than other bacterial species because mycobacteria have a
367 thick lipid-rich cell wall composed of mycolic acids, which form a primary hydrophobic barrier
368 [33]. Many researchers have tried to develop lipophilic FQs to enhance their penetration into the
369 bacterial cytoplasm and oral absorption properties [34]. Similarly, Itoh et al designed and
370 synthesized WQ-3810 by the introduction of an alkyl group into 7-(3-aminoazetidin-1-yl)
371 fluoroquinolone so as to increase its lipophilicity [7]. Some reports show that such FQs have high
372 potency in general against bacterial species, but other reports indicate lipophilicity of FQs has little
373 importance for activity against mycobacteria [35]. To clarify the efficacy of WQ-3810 in
374 mycobacteria, a checkerboard assay with cell wall synthesis inhibitors was performed so as to
375 obstruct the formation of lipid-rich cell wall and hence potentially increase permeability. WQ-3810
376 showed lower FICI_{ms} than MXF in combination with INH or EMB. In particular, the combination
377 of WQ-3810 and EMB showed synergistic activity against FQ susceptible and resistant rBCG
378 strains. In addition, sub-lethal concentrations of EMB enhanced bactericidal activity of WQ-3810
379 but not MXF. Those results showed that WQ-3810 has antimycobacterial potency equal to or even
380 greater than MXF in combination with cell wall synthesis inhibitors. While INH inhibits cell wall
381 biosynthesis by blocking the biosynthesis of mycolic acids, EMB inhibits the
382 arabinosyl-transferases, leading disruption of arabinogalactan in the cell wall and liberation of

383 mycolic acid residues as well [36]. Therefore, EMB might make the mycobacterial membrane more
384 permeable to compound, especially lipophilic antibiotics [37]. In current situation, utilities of other
385 cell wall synthesis inhibitors such as delamanid and prothionamide could also be recommended as
386 second-line anti-tuberculosis drugs for synergizing WQ-3810 potency.

387 In conclusion, our study evaluated the efficacy of WQ-3810 against *Mtb* using recombinant
388 *Mtb* DNA gyrases and rBCG strains. The DNA gyrase inhibitory assay revealed that WQ-3810
389 showed inhibition against LVX-resistant DNA gyrases. In addition, WQ-3810 was efficacious even
390 against a DNA gyrase variant harboring a G88C mutation which conferred the highest resistance
391 against MXF. Furthermore, the combination of WQ-3810 and EMB exerted antimicrobial potency
392 equal to or even greater than MXF. In high TB burden countries, FQs can be easily obtained at
393 pharmacies and readily prescribed by medical doctors. In such countries, the ease of FQ availability
394 not only drives a high rate of FQ resistance acquisition in MDR-TB [38] but also non-MDR-TB
395 shows FQ mono-resistance [39,40]. FQ resistant TB is difficult to treat, but multi-drug therapy that
396 includes WQ-3810 and EMB might have potential as a new, potent anti-TB treatment regimen that
397 can be effective even against FQ-resistant *Mtb* in clinical settings.

398

399 **Conflicts of interest**

400 None to declare.

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414 quinolone resistance Cloning and Nucleotide Sequence of *Mycobacterium tuberculosis* gyrA
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535 **Tables**

536

537 **Table 1. Drug susceptibility of rBCG strains.**

Strain	MIC (µg/ml)					MIC (µg/ml) with 1 µg/ml EMB	
	Levofloxacin	Moxifloxacin	WQ-3810	Ethambutol	Isoniazid	Moxifloxacin	WQ-3810
BCG Tokyo 172	0.25	0.0625	0.5	2	0.0625	0.0625	0.125 – 0.0625
rBCG/BCG	0.25	0.0625	0.5	ND	ND	0.0625	0.0625
rBCG/Mtb/WT	0.25 (1x)	0.0625 (1x)	0.5 (1x)	2 (1x)	0.03125 (1x)	0.0625 (1x)	0.0625 (1x)
rBCG/Mtb/G88C	16 (64x)	8 (128x)	8 (16x)	2 (1x)	0.03125 (1x)	4 (64x)	0.5 (8x)
rBCG/Mtb/A90V	2 (8x)	0.5 (8x)	2 (4x)	ND	ND	0.25 (4x)	0.25 (4x)
rBCG/Mtb/D94A	2 (8x)	0.5 (8x)	2 (4x)	ND	ND	0.25 (4x)	0.25 (4x)
rBCG/Mtb/D94G	8 (32x)	2 (32x)	4 – 8 (8x – 16x)	ND	ND	1 (16x)	0.5 (8x)
rBCG/Mtb/D94H	8 (32x)	1 – 2 (16x – 32x)	4 (8x)	ND	ND	1 (16x)	0.5 (8x)
rBCG/Mtb/D94N	8 (32x)	2 (32x)	8 (16x)	ND	ND	2 (32x)	1 (16x)
rBCG/Mtb/D94Y	8 (32x)	1 – 2 (16x – 32x)	2 – 4 (4x – 8x)	ND	ND	1 (16x)	0.5 (8x)

538 Concentration ranges were as follows: levofloxacin (0.03125-32 µg/ml), moxifloxacin (0.01563-32 µg/ml), WQ-3810 (0.00781-32 µg/ml), ethambutol

539 (0.25-8 µg/ml), isoniazid (0.00781-4 µg/ml). Numbers in brackets: ratio of the MIC_{mutant} / MIC_{WT}. ND: not determined.

540 **Table 2. Interaction between FQs and cell wall synthesis inhibitors.**

Strain / drug combinations	MIC of FQ (µg/ml)	MIC of cell wall synthesis inhibitor (µg/ml)	FIC of FQ	FIC of cell wall synthesis inhibitor	FICI	FICI _m
rBCG/Mtb/WT						
Moxifloxacin + Ethambutol	0.0625	0	1	0	1	1
	0	2	0	1	1	
WQ-3810 + Ethambutol	0.5	0	1	0	1	0.5
	0.25	0.125	0.5	0.0625	0.5625	
	0.125	0.5	0.25	0.25	0.5	
	0.0625	1	0.125	0.5	0.625	
	0	2	0	1	1	
Moxifloxacin + Isoniazid	0.0625	0	1	0	1	1
	0	0.0625	0	1	1	
WQ-3810 + Isoniazid	0.5	0	1	0	1	0.75
	0.25	0.015625	0.5	0.25	0.75	
	0.125	0.03125	0.25	0.5	0.75	
	0	0.0625	0	1	1	
rBCG/Mtb/G88C						
Moxifloxacin + Ethambutol	8	0	1	0	1	0.75
	4	0.5	0.5	0.25	0.75	
	0	2	0	1	1	
WQ-3810 + Ethambutol	8	0	1	0	1	0.5
	2	0.5	0.25	0.25	0.5	

	0.5	1	0.0625	0.5	0.5625	
	0	2	0	1	1	
Moxifloxacin + Isoniazid	8	0	1	0	1	0.75
	2	0.03125	0.25	0.5	0.75	
	0	0.0625	0	1	1	
WQ-3810 + Isoniazid	8	0	1	0	1	0.625
	4	0.015625	0.5	0.25	0.75	
	1	0.03125	0.125	0.5	0.625	
	0	0.0625	0	1	1	

541 Concentration ranges were as follows: moxifloxacin (0.0625-32 µg/ml), WQ-3810 (0.0625-32 µg/ml), ethambutol (0.0625-4 µg/ml), isoniazid
542 (0.00195-0.125 µg/ml). Synergistic activity was evaluated by calculating fractional inhibitory concentration index (FICI). Minimum FICIs ($FICI_m$) $\leq$
543 0.5 is regarded as synergistic.

544 **Captions to illustrations**

545

546 **Fig. 1.** Structure of FQs used in this study. (A) The basic structure of quinolones. (B) Levofloxacin.
547 (C) Moxifloxacin. (D) WQ-3810.

548

549 **Fig. 2.** Purification of recombinant *Mtb* GyrA and GyrB. The His-tagged proteins were expressed
550 by *E. coli* and purified by Ni-NTA Agarose. Approximately 300 ng of each protein was loaded on
551 SDS-PAGE and stained with CBB. Lanes: M, protein size marker; 1, WT-GyrA; 2, G88C-GyrA; 3,
552 A90V-GyrA; 4, D94A-GyrA; 5, D94G-GyrA; 6, D94H-GyrA; 7 D94N-GyrA; 8, D94Y-GyrA; 9,
553 GyrB.

554

555 **Fig. 3.** ATP-dependent supercoiling activity of DNA gyrases. The reaction mixture (30 μ l)
556 containing 32 nM GyrA (WT or each mutant), 32 nM GyrB and 2 nM relaxed DNA and 1 mM ATP
557 was incubated at 37°C. After 60 min, the reactions were terminated by SDS and samples were
558 electrophoresed through a 1% agarose gel.

559

560 **Fig. 4.** FQ activities against recombinant *Mtb* DNA gyrase. Amount of DNA supercoiled by DNA
561 gyrase was quantified from agarose gel electrophoresis in triplicate. From the proportion of
562 supercoiled DNA, the IC₅₀s of three FQs were calculated. After assumptions of normality and
563 homogeneity of variances were tested using Shapiro-Wilk test and Bartlett's test respectively,
564 statistically significant differences in IC₅₀ from WQ-3810 were described using Dunnett's test (*,
565 p<0.05; **, p<0.01).

566

567 **Fig. 5.** Correlation between IC₅₀s and MICs of FQs. Log₂(IC₅₀)s of MXF or WQ-3810 against each
568 recombinant DNA gyrase were plotted on x axis. Log₂(MIC)s of corresponding FQs alone or with
569 EMB (1 μ g/ml) against each rBCG strain were plotted on y axis. R indicates correlation coefficient

570 value.

A **B** **C** **D**

Table 1 Drug susceptibility of rBCG strains

Strain	MIC ($\mu\text{g/ml}$)		
	Levofloxacin	Moxifloxacin	WQ-3810
BCG Tokyo 172	0.25	0.0625	0.5
rBCG/BCG	0.25	0.0625	0.5
rBCG/Mtb/WT	0.25 (1x)	0.0625 (1x)	0.5 (1x)
rBCG/Mtb/G88C	16 (64x)	8 (128x)	8 (16x)
rBCG/Mtb/A90V	2 (8x)	0.5 (8x)	2 (4x)
rBCG/Mtb/D94A	2 (8x)	0.5 (8x)	2 (4x)
rBCG/Mtb/D94G	8 (32x)	2 (32x)	4 – 8 (8x – 16x)
rBCG/Mtb/D94H	8 (32x)	1 – 2 (16x – 32x)	4 (8x)
rBCG/Mtb/D94N	8 (32x)	2 (32x)	8 (16x)
rBCG/Mtb/D94Y	8 (32x)	1 – 2 (16x – 32x)	2 – 4 (4x – 8x)

Concentration ranges were as follows: levofloxacin (0.03125-32 $\mu\text{g/ml}$), moxifloxacin (0.00781-4 $\mu\text{g/ml}$), isoniazid (0.00781-4 $\mu\text{g/ml}$). Numbers in brackets: ratio of the $\text{MIC}_{\text{mutant}} / \text{MIC}_{\text{WT}}$. NT

MIC (µg/ml) with 1 µg/ml EMB			
Ethambutol	Isoniazid	Moxifloxacin	WQ-3810
2	0.0625	0.0625	0.125 – 0.0625
ND	ND	0.0625	0.0625
2 (1x)	0.03125 (1x)	0.0625 (1x)	0.0625 (1x)
2 (1x)	0.03125 (1x)	4 (64x)	0.5 (8x)
ND	ND	0.25 (4x)	0.25 (4x)
ND	ND	0.25 (4x)	0.25 (4x)
ND	ND	1 (16x)	0.5 (8x)
ND	ND	1 (16x)	0.5 (8x)
ND	ND	2 (32x)	1 (16x)
ND	ND	1 (16x)	0.5 (8x)

1 (0.01563-32 µg/ml), WQ-3810 (0.00781-32 µg/ml), ethambutol (0.25-8 µg/ml),
): not determined.

Table 2 Interaction between FQs and cell wall synthesis inhibitors

Strain / drug combinations	MIC of FQ (μ g/ml)	MIC of cell wall synthesis inhibitor (μ g/ml)	FIC of FQ
rBCG/Mtb/WT			
Moxifloxacin + Ethambutol	0.0625	0	1
	0	2	0
WQ-3810 + Ethambutol	0.5	0	1
	0.25	0.125	0.5
	0.125	0.5	0.25
	0.0625	1	0.125
	0	2	0
Moxifloxacin + Isoniazid	0.0625	0	1
	0	0.0625	0
WQ-3810 + Isoniazid	0.5	0	1
	0.25	0.015625	0.5
	0.125	0.03125	0.25
	0	0.0625	0
rBCG/Mtb/G88C			
Moxifloxacin + Ethambutol	8	0	1
	4	0.5	0.5
	0	2	0
WQ-3810 + Ethambutol	8	0	1
	2	0.5	0.25
	0.5	1	0.0625
	0	2	0
Moxifloxacin + Isoniazid	8	0	1
	2	0.03125	0.25
	0	0.0625	0
WQ-3810 + Isoniazid	8	0	1
	4	0.015625	0.5
	1	0.03125	0.125
	0	0.0625	0

Concentration ranges were as follows: moxifloxacin (0.0625-32 μ g/ml), WQ-3810 (0.0625-32 μ g/ml).

Synergistic activity was evaluated by calculating fractional inhibitory concentration (FIC) index.

as synergistic.

FIC of cell wall synthesis inhibitor	FICI	FICI _m
0	1	1
1	1	
0	1	0.5
0.0625	0.5625	
0.25	0.5	
0.5	0.625	
1	1	
0	1	1
1	1	
0	1	0.75
0.25	0.75	
0.5	0.75	
1	1	
0	1	0.75
0.25	0.75	
1	1	
0	1	0.5
0.25	0.5	
0.5	0.5625	
1	1	
0	1	0.75
0.5	0.75	
1	1	
0	1	0.625
0.25	0.75	
0.5	0.625	
1	1	

μg/ml), ethambutol (0.0625-4 μg/ml), isoniazid (0.00195-

≡

on index (FICI). Minimum FICIs (FICI_m) 0.5 is regarded

Supplementary Table S1

Primer names

gyrA and *gyrB* cloning pr

ON-873

ON-874

ON-875

ON-876

ON-1518

ON-886

Complementary primers for

k-4

k-3

k-10

k-9

ON-1527

ON-1528

k-85

k-84

ON-1529

ON-1530

ON-1531

ON-1532

ON-1533

ON-1534

Cloning primers for plasmid

ON-1547

ON-1548

ON-1558

ON-1559

Mutagenesis primers for p

ON-1566

ON-1567

ON-1568

ON-1569

ON-1570

ON-1571

gyrA and gyrB sequencing

ON-841

ON-897

ON-888

ON-1100

ON-512

ON-877

ON-1166

ON-878

ON-879

ON-880

Screening primers

ON-1562

ON-1563

ON-1564

ON-1565

Mutated codons are shown

1. Oligonucleotide sequences of primers used in this study

Sequence (Nucleotide position)

primers for expression plasmids

CCCATATGACAGACACGACGTTGCCGCC (1-23 in gyrA), NdeI site
GTAAACCGGGCTTCGGTGTACCTCATCG (377-404 in gyrA), HpaI site
GGTTAACCCCGTTGGCGATGGAGATGC (398-424 in gyrA), HpaI site
GGCTCGAGTTAATGATGATGATGATGATTGCCCGTCTGGTCTGCGCCG
(2493-2517 in gyrA), XhoI site and 6-His tag
CCCATATGGCTGCCCAGAAAAAGAAGGCC (118-142 in gyrB), NdeI site
GGCTCGAGTTAGACATCCAGGAACCGAACATCC (2121-2145 in gyrB), XhoI site

or site-directed mutagenesis of expression plasmids

TCGACGCGTCGCAGTGCGGGTGG (252-274 in gyrA)
CCACCCGCACTGCGACGCGTCGA (252-274 in gyrA)
CGTAGATCGACACGTCGCCGTGC (258-280 in gyrA)
GCACGGCGACGTGTCGATCTACG (258-280 in gyrA)
CGCACCAGGGTGCGTAGATCGACG (269-293 in gyrA)
CGCGTCGATCTACGCCACCCTGGTGCG (267-293 in gyrA)
TGCGCACCAGGGTGCCGTAGATC (273-295 in gyrA)
GATCTACGGCACCCCTGGTGCGCA (273-295 in gyrA)
TGCGCACCAGGGTGTTGGTAGATCGACGC (268-295 in gyrA)
ACGCGTCGATCTACCAACACCCTGGTGCG (266-293 in gyrA)
TGCGCACCAGGGTGTTGTAGATCGACGC (268-295 in gyrA)
ACGCGTCGATCTACAACACCCTGGTGCG (266-293 in gyrA)
TGCGCACCAGGGTGTAGTAGATCGACGC (268-295 in gyrA)
ACGCGTCGATCTACTAACACCCTGGTGCG (266-293 in gyrA)

ids containing allelic exchange substrate

TCTAAGCTTCGGGACGCACCAGGAAGAAAGATG HindIII site
TATTCTAGATAGCTGCCCGATTCCTCCTCAGATCG XbaI site
GGTTGCTAGAGGATCCCCGGAGGAGGAATCGGGCAGCTAGGC
identical region to end of pΔAHm31 digested with KpnI
CATGATTACGAATTCGAGCTCGCGACCGTGATCATCCAGACGAAG
identical region to end of pΔAHm31 digested with KpnI

plasmids containing allelic exchange substrate

CGGCCAGTGCCAAGCTTCGG
identical region to end of pΔAHm31 digested with HindIII
CGATATTTGATTTAGGATACACCGTTCTAGATAGCTGCC
identical region to end of pΔAHm31 digested with XbaI
CAGGGTGTTGTAGATCGACGCGTCGCC
identical region to end of another mutated fragment

GCGTCGATCTACA**A**CACCCTGGTGCGCATG
identical region to end of another mutated fragment
CAGGGT**G**TAGTAGATCGACGCGTCGCC
identical region to end of another mutated fragment
GCGTCGATCTACT**A**CACCCTGGTGCGCATG
identical region to end of another mutated fragment

3 primers

TCGGACGCGTATGCGATATC (448-467 in gyrB)
CCTGAGACCACTCGTACCCGTCGCG (538-562 in gyrB)
ACATCAACCGCACCAAGAACGC (905-926 in gyrB)
CGGCACGTAAGGCACGAGAG (1373-1392 in gyrB)
GGCTTCGGTGTACCTCATCG (377-396 in gyrA)
TGCCGTATCAGGTCAACCACGAC (821-843 in gyrA)
CGATGACGATGCGTAAACCGACC (927-949 in gyrA)
GATCCGGGCGTCGGAGACCGTC (1221-1242 in gyrA)
GGCAAGGGCGTGCAGGGTGCGG (1621-1642 in gyrA)
TCTCGGCCAACGGGCAGTCCATC (2021-2043 in gyrA)

TGTCGGGCAATCAGGTGCGACAATC (96-120 in KanR2)
TGGCAAGATCCTGGTATCGGTCTGCG (666-691 in KanR2)
CACGTCGATCGGCCCAGAACAAGG ((-125)-(-102) in gyrB)
GCGTTGTTCAACAGGTCACCGACG (717-740 in Rv0007)

n in bold letters.

Comment

WT-GyrA F

WT-GyrA R

WT-GyrA F

WT-GyrA R

GyrB F

GyrB R

G88C-GyrA R

G88C-GyrA F

A90V-GyrA R

A90V-GyrA F

D94A-GyrA R

D94A-GyrA F

D94G-GyrA R

D94G-GyrA F

D94H-GyrA R

D94H-GyrA F

D94N-GyrA R

D94N-GyrA F

D94Y-GyrA R

D94Y-GyrA F

upstream F

upstream R

downstream, in fusion F

downstream, in fusion R

upstream, in fusion F

upstream, in fusion R

D94N, in fusion R

D94N, in fusion F

D94Y, in fusion R

D94Y, in fusion F

PCR to check the presence of pJV53 or pYUB870

PCR to check the presence of pJV53 or pYUB870

PCR to check sequence and Hyg^r cassette deletion

PCR to check sequence and Hyg^r cassette deletion

Supplementary Table S2. *Mtb* and rBCG strains used in this study.

Strain	Mutations based on genome sequence of NCBI (RefSeq)
	<i>gyrB</i>
<i>Mtb</i> Beijing lineage strains	
Osaka MDR-12	-
Osaka MDR-23	-
Osaka MDR-14	-
Osaka MDR-5	-
Osaka MDR-13	-
Osaka MDR-79	-
Parental BCG and rBCG strains	
BCG Tokyo 172*	G513A, C1167T, G1207T(A403S)
rBCG/BCG*	G513A, C1167T, G1207T(A403S)
rBCG/ <i>Mtb</i> /WT	-
rBCG/ <i>Mtb</i> /G88C	-
rBCG/ <i>Mtb</i> /A90V	-
rBCG/ <i>Mtb</i> /D94A	-
rBCG/ <i>Mtb</i> /D94G	-
rBCG/ <i>Mtb</i> /D94H	-
rBCG/ <i>Mtb</i> /D94N	-
rBCG/ <i>Mtb</i> /D94Y	-

*BCG specific mutations in gyrase coding genes

res: remaining res sequence downstream of gyrase coding genes

of *Mtb* str. Beijing/NITR203 registered at
 NC_021054.1)

gyrA

Characteristics

Source

-	RIF ^r , INH ^r	Osaka Institute of Public Health
G262T(G88C)	RIF ^r , FQ ^r , INH ^r	Osaka Institute of Public Health
C269T(A90V)	RIF ^r , FQ ^r , INH ^r	Osaka Institute of Public Health
A281C(D94A)	RIF ^r , FQ ^r , INH ^r	Osaka Institute of Public Health
A281G(D94G)	RIF ^r , FQ ^r , INH ^r	Osaka Institute of Public Health
G280C(D94H)	RIF ^r , FQ ^r , INH ^r	Osaka Institute of Public Health

C984T, G1323T, C1440T, T1842C		Japan BCG Laboratory
C984T, G1323T, C1440T, T1842C	res	This study
-	res	This study
G262T(G88C)	res, FQ ^r	This study
C269T(A90V)	res, FQ ^r	This study
A281C(D94A)	res, FQ ^r	This study
A281G(D94G)	res, FQ ^r	This study
G280C(D94H)	res, FQ ^r	This study
G280A(D94N)	res, FQ ^r	This study
G280T(D94Y)	res, FQ ^r	This study

Supplementary Table S3. P**Names***Mtb* DNA gyrase subunit exl

pET20b(+)

pET/gyrA/WT

pET/gyrA/G88C

pET/gyrA/A90V

pET/gyrA/D94A

pET/gyrA/D94G

pET/gyrA/D94H

pET/gyrA/D94N

pET/gyrA/D94Y

pET19b

pTB-B

pET/gyrB/WT

Plasmids containing allelic exl

pUC18

pΔAHm31

pΔAHm/gyrAB/BCG

pΔAHm/gyrAB/WT

pΔAHm/gyrAB/G88C

pΔAHm/gyrAB/A90V

pΔAHm/gyrAB/D94A

pΔAHm/gyrAB/D94G

pΔAHm/gyrAB/D94H

pΔAHm/gyrAB/D94N

pΔAHm/gyrAB/D94Y

Plasmids used for allelic exl

pJV53

pYUB854

pYUB870

Plasmids used in this study

Characteristics

Expression plasmids

Vector for protein expression, Amp^r

6x His-tagged WT-GyrA coding sequence in pET20b(+)

6x His-tagged G88C-GyrA coding sequence in pET20b(+)

6x His-tagged A90V-GyrA coding sequence in pET20b(+)

6x His-tagged D94A-GyrA coding sequence in pET20b(+)

6x His-tagged D94G-GyrA coding sequence in pET20b(+)

6x His-tagged D94H-GyrA coding sequence in pET20b(+)

6x His-tagged D94N-GyrA coding sequence in pET20b(+)

6x His-tagged D94Y-GyrA coding sequence in pET20b(+)

Vector for protein expression, Amp^r

6x His-tagged GyrB coding sequence in pET19b

6x His-tagged GyrB coding sequence (5'- 117 bp was deleted) in pET19b

Allelic exchange substrates

Vector for cloning allelic exchange substrate, Amp^r

hyg cassette with res sites in pUC18; excludes Amp^r sequence, Hyg^r

BCG-*gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31

WT-Mtb-*gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31

G88C-Mtb-*gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31

A90V-Mtb-*gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31

D94A-Mtb-*gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31

D94G-Mtb-*gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31

D94H-Mtb-*gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31

D94N-Mtb-*gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31

D94Y-Mtb-*gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31

Range

Shuttle plasmid expressing recombinase under the control of acetamide, Kan^r

hyg cassette with res sites was used to construct pΔAHm31

Shuttle plasmid expressing resolvase, Kan^r

Source/reference

Merck

This study

This study

This study

This study

This study

This study

This study

This study

Merck

Antimicrob agen chemothera, 2011, 55:3661–3667

This study

TaKaRa

This study

This study

This study

This study

This study

This study

This study

This study

This study

This study

Nature Method. 2007, 4:147-152

Microbiology, 2002, 148:3007-3017

Microbiology, 2002, 148:3007-3017

Supplementary Figure 1. Construction of pΔAHm31 plasmid.

A parental plasmid for the allelic exchange, pΔAHm31, was constructed as follows. At first, a *hyg* cassette with *res* sites was digested from pYUB854 using XbaI and HindIII. The product was ligated into a pUC18 vector digested by same enzymes. The constructed plasmid was termed p18HygX-Hi (A). To utilize single restriction enzyme sites of EcoRI and Sall in multi-cloning site, silent mutations were introduced into the *hyg* cassette. Using primers F15Hyg61-Eco (CTGCGGAACGACCAGGAGTTCTGGGAGCCGCTG) and R15Hyg-744 Sal (ATAGACGTCGGTGAAGTCCACGATCCCGGTGAC), a 684 bp fragment of *hyg* was amplified from pYUB854. Using Sall and EcoRI, a 3929 bp fragment was digested from p18HygX-Hi. Both fragments were ligated using In-Fusion® HD Cloning Kit. From the produced plasmid (p18HmXb-Hi), the mutated *hyg* cassette was digested with XbaI and NheI, and ligated into pUC18 treated with XbaI and Calf intestine Alkaline Phosphatase. The ligated plasmid was termed pAHm31. To shorten the plasmid size, an AmpR gene was removed. Using primers FpUC18 2668-Sca (TTAAAGTACTATCACGAGGCCCTTTCGTC) and RpUC18-1677 Sca (GCATAGTACTAGACAGATCGCTGAGATAGG), pAHm31 was amplified. The product was digested by ScaI and self-ligated at the ScaI site. The constructed plasmid was termed pΔAHm31 (B).

Levofloxacin (μg/ml)

Moxifloxacin (μg/ml)

Supplementary Figure 2. FQ activities against recombinant *Mtb* DNA gyrase.

The reaction (30 μl) containing 4 nM GyrA (WT or each mutant), 4 nM GyrB and 2 nM relaxed DNA, 1 mM ATP and indicated concentrations of FQ was incubated at 37°C. After 60 min, the reaction was terminated by SDS and samples were electrophoresed through a 1% agarose gel.

Supplementary Table 1. Oligonucleotide sequences of primers used in this study

Primer names	Sequence (Nucleotide position)	Comment
<i>gyrA</i> and <i>gyrB</i> cloning primers for expression plasmids		
ON-873	<u>CCCATATG</u> ACAGACACGACGTTGCCGCC (1-23 in <i>gyrA</i>), <u>NdeI site</u>	WT-GyrA F
ON-874	<u>GTTAACCGGGCTTCGGTGTACCTCATCG</u> (377-404 in <i>gyrA</i>), <u>HpaI site</u>	WT-GyrA R
ON-875	<u>GGTTAACCCCGTTGGCGATGGAGATGC</u> (398-424 in <i>gyrA</i>), <u>HpaI site</u>	WT-GyrA F
ON-876	<u>GGCTCGAGTTAATGATGATGATGATGATGATTGCCCGTCTGGTCTGCGCCG</u> (2493-2517 in <i>gyrA</i>), <u>XhoI site</u> and 6-His tag	WT-GyrA R
ON-1518	<u>CCCATATGGCTGCCCAGAAAAAGAAGGCC</u> (118-142 in <i>gyrB</i>), <u>NdeI site</u>	GyrB F
ON-886	<u>GGCTCGAGTTAGACATCCAGGAACCGAACATCC</u> (2121-2145 in <i>gyrB</i>), <u>XhoI site</u>	GyrB R
Complementary primers for site-directed mutagenesis of expression plasmids		
k-4	TCGACGCGTC G CAGTGCGGGTGG (252-274 in <i>gyrA</i>)	G88C-GyrA R
k-3	CCACCCGCACT G CGACGCGTCGA (252-274 in <i>gyrA</i>)	G88C-GyrA F
k-10	CGTAGATCGAC A CGTCGCCGTGC (258-280 in <i>gyrA</i>)	A90V-GyrA R
k-9	GCACGGCGAC G TGTCGATCTACG (258-280 in <i>gyrA</i>)	A90V-GyrA F
ON-1527	CGCACCAGGGT G GCGTAGATCGACG (269-293 in <i>gyrA</i>)	D94A-GyrA R
ON-1528	CGCGTCGATCTAC G CCACCCTGGTGCG (267-293 in <i>gyrA</i>)	D94A-GyrA F
k-85	TGCGCACCAGGGT G CCGTAGATC (273-295 in <i>gyrA</i>)	D94G-GyrA R
k-84	GATCTAC G GCAACCCTGGTGCGCA (273-295 in <i>gyrA</i>)	D94G-GyrA F
ON-1529	TGCGCACCAGGGT G TGGTAGATCGACGC (268-295 in <i>gyrA</i>)	D94H-GyrA R
ON-1530	ACGCGTCGATCTACC A CACCCTGGTGCG (266-293 in <i>gyrA</i>)	D94H-GyrA F
ON-1531	TGCGCACCAGGGT G TTGTAGATCGACGC (268-295 in <i>gyrA</i>)	D94N-GyrA R
ON-1532	ACGCGTCGATCTACA A CACCCTGGTGCG (266-293 in <i>gyrA</i>)	D94N-GyrA F
ON-1533	TGCGCACCAGGGT G TAGTAGATCGACGC (268-295 in <i>gyrA</i>)	D94Y-GyrA R

ON-1534	ACGCGTCGATCTACT A CACCCTGGTGCG (266-293 in gyrA)	D94Y-GyrA F
Cloning primers for plasmids containing allelic exchange substrate		
ON-1547	TCTAAGCTTCGGGACGCACCAGGAAGAAAGATG <u>HindIII site</u>	upstream F
ON-1548	TATTCTAGATAGCTGCCCCGATTCCTCCTCAGATCG <u>XbaI site</u>	upstream R
ON-1558	GGTTGCTAGAGGATCCCCGGAGGAGGAATCGGGCAGCTAGGC <u>identical region to end of pΔAHm31 digested with KpnI</u>	downstream, in fusion F
ON-1559	CATGATTACGAATTCGAGCTCGCGACCGTGATCATCCAGACGAAG <u>identical region to end of pΔAHm31 digested with KpnI</u>	downstream, in fusion R
Mutagenesis primers for plasmids containing allelic exchange substrate		
ON-1566	CGGCCAGTGCCAAGCTTCGG <u>identical region to end of pΔAHm31 digested with HindIII</u>	upstream, in fusion F
ON-1567	CGATATTTGATTTAGGATACACCGGTCTAGATAGCTGCC <u>identical region to end of pΔAHm31 digested with XbaI</u>	upstream, in fusion R
ON-1568	CAGGGTGTGTAGATCGACGCGTCGCC <u>identical region to end of another mutated fragment</u>	D94N, in fusion R
ON-1569	GCGTCGATCTACA A CACCCTGGTGCGCATG <u>identical region to end of another mutated fragment</u>	D94N, in fusion F
ON-1570	CAGGGTGTAGTAGATCGACGCGTCGCC <u>identical region to end of another mutated fragment</u>	D94Y, in fusion R
ON-1571	GCGTCGATCTACT A CACCCTGGTGCGCATG <u>identical region to end of another mutated fragment</u>	D94Y, in fusion F

gyrA and gyrB sequencing primers

ON-841	TCGGACGCGTATGCGATATC (448-467 in gyrB)
ON-897	CCTGAGACCACTCGTACCCGTCGCG (538-562 in gyrB)
ON-888	ACATCAACCGCACCAAGAACGC (905-926 in gyrB)
ON-1100	CGGCACGTAAGGCACGAGAG (1373-1392 in gyrB)
ON-512	GGCTTCGGTGTACCTCATCG (377-396 in gyrA)
ON-877	TGCCGTATCAGGTCAACCACGAC (821-843 in gyrA)
ON-1166	CGATGACGATGCGTAAACCGACC (927-949 in gyrA)
ON-878	GATCCGGGCGTCGGAGACCGTC (1221-1242 in gyrA)
ON-879	GGCAAGGGCGTGCAGGGTGCGG (1621-1642 in gyrA)
ON-880	TCTCGGCCAACGGGCAGTCCATC (2021-2043 in gyrA)

Screening primers

ON-1562	TGTCGGGCAATCAGGTGCGACAATC (96-120 in KanR2)	PCR to check the presence of pJV53 or pYUB870
ON-1563	TGGCAAGATCCTGGTATCGGTCTGCG (666-691 in KanR2)	PCR to check the presence of pJV53 or pYUB870
ON-1564	CACGTCGATCGGCCAGACAAGG ((-125)-(-102) in gyrB)	PCR to check sequence and Hyg ^r cassette deletion
ON-1565	GCGTTGTTCAACAGGTCACCGACG (717-740 in Rv0007)	PCR to check sequence and Hyg ^r cassette deletion

Mutated codons are shown in bold letters.

Supplementary Table 2. Mtb and rBCG strains used in this study.

Strain	Mutations based on genome sequence of <i>Mtb</i> str. Beijing/NITR203 registered at NCBI (RefSeq: NC_021054.1)		Characteristics	Source
	gyrB	gyrA		
<i>Mtb</i> Beijing lineage strains				
Osaka MDR-12	-	-	RIF ^r , INH ^r	Osaka Institute of Public Health
Osaka MDR-23	-	G262T(G88C)	RIF ^r , FQ ^r , INH ^r	Osaka Institute of Public Health
Osaka MDR-14	-	C269T(A90V)	RIF ^r , FQ ^r , INH ^r	Osaka Institute of Public Health
Osaka MDR-5	-	A281C(D94A)	RIF ^r , FQ ^r , INH ^r	Osaka Institute of Public Health
Osaka MDR-13	-	A281G(D94G)	RIF ^r , FQ ^r , INH ^r	Osaka Institute of Public Health
Osaka MDR-79	-	G280C(D94H)	RIF ^r , FQ ^r , INH ^r	Osaka Institute of Public Health
Parental BCG and rBCG strains				
BCG Tokyo 172*	G513A, C1167T, G1207T(A403S)	C984T, G1323T, C1440T, T1842C		Japan BCG Laboratory
rBCG/BCG*	G513A, C1167T, G1207T(A403S)	C984T, G1323T, C1440T, T1842C	res	This study
rBCG/ <i>Mtb</i> /WT	-	-	res	This study
rBCG/ <i>Mtb</i> /G88C	-	G262T(G88C)	res, FQ ^r	This study
rBCG/ <i>Mtb</i> /A90V	-	C269T(A90V)	res, FQ ^r	This study
rBCG/ <i>Mtb</i> /D94A	-	A281C(D94A)	res, FQ ^r	This study
rBCG/ <i>Mtb</i> /D94G	-	A281G(D94G)	res, FQ ^r	This study
rBCG/ <i>Mtb</i> /D94H	-	G280C(D94H)	res, FQ ^r	This study
rBCG/ <i>Mtb</i> /D94N	-	G280A(D94N)	res, FQ ^r	This study
rBCG/ <i>Mtb</i> /D94Y	-	G280T(D94Y)	res, FQ ^r	This study

*BCG specific mutations in gyrase coding genes

res: remaining res sequence downstream of gyrase coding genes

Supplementary Table 3. Plasmids used in this study

Names	Characteristics	Source/reference
<i>Mtb</i> DNA gyrase subunit expression plasmids		
pET20b(+)	Vector for protein expression, Amp ^r	Merck
pET/gyrA/WT	6x His-tagged WT-GyrA coding sequence in pET20b(+)	This study
pET/gyrA/G88C	6x His-tagged G88C-GyrA coding sequence in pET20b(+)	This study
pET/gyrA/A90V	6x His-tagged A90V-GyrA coding sequence in pET20b(+)	This study
pET/gyrA/D94A	6x His-tagged D94A-GyrA coding sequence in pET20b(+)	This study
pET/gyrA/D94G	6x His-tagged D94G-GyrA coding sequence in pET20b(+)	This study
pET/gyrA/D94H	6x His-tagged D94H-GyrA coding sequence in pET20b(+)	This study
pET/gyrA/D94N	6x His-tagged D94N-GyrA coding sequence in pET20b(+)	This study
pET/gyrA/D94Y	6x His-tagged D94Y-GyrA coding sequence in pET20b(+)	This study
pET19b	Vector for protein expression, Amp ^r	Merck
pTB-B	6x His-tagged GyrB coding sequence in pET19b	Antimicrob agen chemothera, 2011, 55:3661–3667
pET/gyrB/WT	6x His-tagged GyrB coding sequence (5'- 117 bp was deleted) in pET19b	This study
Plasmids containing allelic exchange substrates		
pUC18	Vector for cloning allelic exchange substrate, Amp ^r	TaKaRa
pΔAHm31	<i>hyg</i> cassette with res sites in pUC18; excludes Amp ^r sequence, Hyg ^r	This study
pΔAHm/gyrAB/BCG	BCG-gyrAB and Rv0007 fragments flanking <i>hyg</i> cassette in pΔAHm31	This study
pΔAHm/gyrAB/WT	WT-Mtb-gyrAB and Rv0007 fragments flanking <i>hyg</i> cassette in pΔAHm31	This study
pΔAHm/gyrAB/G88C	G88C-Mtb-gyrAB and Rv0007 fragments flanking <i>hyg</i> cassette in pΔAHm31	This study
pΔAHm/gyrAB/A90V	A90V-Mtb-gyrAB and Rv0007 fragments flanking <i>hyg</i> cassette in pΔAHm31	This study

pΔAHm/gyrAB/D94A	D94A-Mtb-gyrAB and Rv0007 fragments flanking <i>hyg</i> cassette in pΔAHm31	This study
pΔAHm/gyrAB/D94G	D94G-Mtb-gyrAB and Rv0007 fragments flanking <i>hyg</i> cassette in pΔAHm31	This study
pΔAHm/gyrAB/D94H	D94H-Mtb-gyrAB and Rv0007 fragments flanking <i>hyg</i> cassette in pΔAHm31	This study
pΔAHm/gyrAB/D94N	D94N-Mtb-gyrAB and Rv0007 fragments flanking <i>hyg</i> cassette in pΔAHm31	This study
pΔAHm/gyrAB/D94Y	D94Y-Mtb-gyrAB and Rv0007 fragments flanking <i>hyg</i> cassette in pΔAHm31	This study

Plasmids used for allelic exchange

pJV53	Shuttle plasmid expressing recombinase under the control of acetamide, Kan ^r	Nature Method. 2007, 4:147-152
pYUB854	<i>hyg</i> cassette with res sites was used to construct pΔAHm31	Microbiology, 2002, 148:3007-3017
pYUB870	Shuttle plasmid expressing resolvase, Kan ^r	Microbiology, 2002, 148:3007-3017