In vitro activity of sitafloxacin against Mycobacterium tuberculosis with gyrA/B mutations isolated in Japan

Lina Yi,1,2,3*, Akio Aono,2 Kinuyo Chikamatsu,2 Yuriko Igarashi,2 Hiroyuki Yamada,2 Akiko Takaki2 and Satoshi Mitarai2,3

Abstract

Purpose. Sitafloxacin (SFX) is a new fluoroquinolone (FQ) that has shown a strong bactericidal effect against Mycobacterium tuberculosis (Mtb) in vitro. However, data on SFX efficacy against Mtb with gyrA/B mutations and its epidemiological cut-off (ECOFF) value remain limited. Therefore, we evaluated and compared the in vitro activity of SFX against gyrA/B-mutant Mtb to that of moxifloxacin (MFX), levofloxacin (LFX) and ciprofloxacin (CFX), and determined the ECOFF for SFX.

Methodology. A total of 109 clinical Mtb isolates, including 73 multidrug-resistant (MDR) isolates, were subjected to minimum inhibitory concentration (MIC) analysis in oleic-albumin-dextrose-catalase (OADC)-supplemented Middlebrook 7H9 medium. Our results showed that SFX had lower cumulative MIC than MFX, LFX and CFX. Furthermore, we performed direct DNA sequencing of the quinolone-resistance-determining regions (QRDRs).

Results. We identified the following mutations: D94G, D94A, A90V, D94H, D94N and G88A in gyrA; and A543V, A543T, E540D, R485C, D500A, I552S and D577A in gyrB. Based on our results, an ECOFF of 0.125 µg ml⁻¹ was proposed for SFX. With this ECOFF, 15 % of LFX-resistant isolates with MIC ≥2 µg ml⁻¹ were susceptible to SFX.

Conclusion. SFX had the lowest cumulative MIC and a relatively low ECOFF value against Mtb, indicating that SFX was not only more effective against gyrA-mutant isolates, but also MDR isolates in Japan.

INTRODUCTION

The World Health Organization (WHO) reported that approximately 500,000 people worldwide developed multidrug-resistant tuberculosis (MDR-TB) in 2015, while an additional 100,000 developed rifampicin-resistant tuberculosis (RR-TB) [1]. Better chemotherapeutic interventions are needed to prevent the transmission of drug-resistant TB. Therefore, the WHO has updated the guidelines for the treatment of drug-resistant tuberculosis and currently recommends fluoroquinolones (FQs) as group A drugs to treat MDR-TB and RR-TB [2]. However, FQ-resistant Mycobacterium tuberculosis (Mtb) strains and the incidence of FQ-resistant TB have increased [3, 4].

Among the group A drugs, levofloxacin (LFX) is the most frequently used drug to treat MDR-TB in Japan [5]. LFX resistance was reported in 3.2 and 6.1 % of isolates from frequently used drug to treat MDR-TB in Japan [5]. LFX resistance was reported in 3.2 and 6.1 % of isolates from among MDR isolates in Japan. Moreover, a previous study reported that half of MDR isolates in Japan were resistant to FQs [such as LFX, sparfloxacin and ciprofloxacin (CFX)] [6]. Thus it is crucial to determine the most efficacious FQ to treat MDR-TB.

Sitafloxacin (SFX) is a synthetic broad-spectrum 8-chloro-fluoroquinolone approved for use in Japan [7]. Studies conducted in Japan and Thailand showed good activity for SFX against Mtb in vitro [8, 9]. A previous study showed that CFX-resistant clinical isolates were more susceptible to SFX and gatifloxacin than to other FQs [10]. However, studies that assess the MICs for SFX and the significance of gyrA/B mutations with a larger number of isolates in Japan are lacking. Therefore, we investigated the correlations between the MICs of SFX and other FQs, including moxifloxacin (MFX), LFX and CFX, and mutations in gyrA/B as
assessed by direct DNA sequencing of the quinolone resistance-determining regions (QRDRs). We also determined the tentative epidemiological cut-off (ECOFF) value of SFX against Mtb. To provide a visual comparison of the MICs of different FQs, cumulative percentage distribution was used to compare the MICs of SFX with those of MFX, LFX and CFX.

METHODS

Bacterial isolates

A total of 109 M. tuberculosis clinical isolates were randomly selected from a collection maintained and preserved by the Tuberculosis Research Committee (Ryoken, Tokyo, Japan). Among the 109 isolates, 73 (67 %) were MDR and 36 (33 %) were non-MDR isolates. Two of the 36 non-MDR isolates were resistant to isoniazid (INH) (Table S1). These isolates were collected from TB patients with Mtb-positive culture results throughout Japan in 2002 and 2007, and each isolate was given a unique identification number.

All isolates were confirmed by conventional biochemical and/or immuno-chromatography methods (Capilia TB; TAUNS, Numazu, Japan) [11] and tested for drug susceptibility to INH and rifampicin by the conventional proportion method [5]. Furthermore, the susceptibility to LFX was also evaluated using the proportion method on 1 % Ogawa medium [equivalent to the Löwenstein-Jensen (LJ) method] [5]. Furthermore, the susceptibility to LFX was also evaluated using the proportion method mentioned above. M. tuberculosis H37Rv (ATCC 27294; ATCC, Manassas, VA, USA) was used as a control.

Determination of the minimum inhibitory concentrations

The MICs of SFX (Lot #023WCG; Daiichi Sankyo, Tokyo, Japan), MFX (#32477; Sigma-Aldrich, St Louis, MO, USA), LFX (#28266; Sigma-Aldrich) and CFX (#17850; Sigma-Aldrich) were determined in Middlebrook 7H9 broth supplemented with 10 % oleic albumin dextrose catalase (OADC). A broth microdilution method using 7H9 broth has been described previously [12]. Each drug was dissolved in 0.1 NaOH and a serial two-fold broth microdilution was performed for this study. SFX, MFX, LFX and CFX were suspended in 7H9 broth and their final concentrations ranged from 0.008–8, 0.016–16, 0.03–32 and 0.03–32 µg ml⁻¹ for SFX, MFX, LFX and CFX, respectively. Bacterial growth was assessed after 1 week of incubation at 37 °C with 5 % CO₂. Bacterial suspensions were prepared by adding 5 ml of 7H9 broth medium into 0.05 ml of the original bacterial suspension until an optical density (OD) of 0.15–0.24 was reached. The MIC was defined as the lowest concentration of drug that inhibited visible growth of the bacteria. The cumulative percentage of MICs was used to compare the MICs for different FQs.

DNA extraction and sequencing

Genomic DNA of the Mtb isolates was extracted according to a previously described method [13], and 5 µg ml⁻¹ of DNA was used in the PCR mixtures. The primers for gyrA were 5’-GAT GAC AGA CAC GAC GTT GC-3’ (forward) and 5’-GGG CTT CGG TGT ACC TCA T-3’ (reverse) [14]. The primers for gyrB were 5’-GAG TTG GTG CGG CGT AAG AGC-3’ (forward) and 5’-CAA GAT CGT GCT GAT GGC CG-3’ (reverse) [15]. AmpliTaq Gold (Roche, Pleasanton, CA, USA) was used to amplify the DNA. After amplification, direct sequencing of the QRDRs was performed. The sequencing primers were 5’-GAT AGA CAC GAC GTT GC-3’ for gyrA and 5’-GAG TTG GTG CGG CGT AAG AGC-3’ for gyrB. The QRDR in gyrA ranged from codon 74 to 113, and the QRDR in gyrB ranged from codon 500 to 540 [16, 17].

Statistical analysis

The chi-square test was performed to compare the proportions of susceptible and resistant isolates for each FQ. The Mann–Whitney U test was performed to compare MIC values as continuous variables for each FQ. P<0.05 was considered statistically significant. Statistical analyses were performed using SPSS for Windows, version 22.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Minimum inhibitory concentrations of fluoroquinolones

Fig. 1 shows the cumulative percentage of MICs for SFX, MFX, LFX and CFX. The approximated cumulative % (50) values were 0.06, 0.25, 0.5 and 0.5 µg ml⁻¹ for SFX, MFX, LFX and CFX, respectively. The approximated cumulative % (90) values were 1, 4, 8 and 16 µg ml⁻¹ for SFX, MFX, LFX and CFX, respectively. The median [interquartile range (IQR)] MIC of SFX, MFX, LFX and CFX against 37 gyrA mutants was 0.5 (0.25–1.0 µg ml⁻¹), 2 (1.0–4.0 µg ml⁻¹), 8 (4–8 µg ml⁻¹) and 8 µg ml⁻¹ (4–16 µg ml⁻¹), respectively. Four gyrB-mutant isolates showed relatively low MICs; against these four isolates, the highest MICs were 0.125, 0.5, 1 and 1 µg ml⁻¹ for SFX, MFX, LFX and CFX, respectively. The MICs of the reference strain (H37Rv) were 0.03, 0.125, 0.5 and 0.5 µg ml⁻¹ for the four FQs, respectively. The MICs for all the isolates evaluated in this study are shown in Table S1 (available in the online Supplementary Material).

Correlations between mutations in gyrA and gyrB and the MIC values

Among the 109 isolates, 37 (34 %) isolates had mutations in gyrA with mutation patterns of D94G, D94A, A90V, D94H, D94N and G88A. Among the 73 MDR and 36 non-MDR isolates, 30 (41.1 %) and 7 (19.4 %) were gyrA mutants, respectively. Most mutations (75.7 %) in the 37 gyrA-mutant isolates were found in codon 94 (Table 1).

Among the 109 isolates, 8 (7 %) isolates had mutations in gyrB with mutation patterns of A543V, A543T, E540D, R485C, D500A, I552S and D577A. Half of the gyrB-mutant isolates also had mutations in gyrA and all eight were MDR isolates. Among the four isolates that only had mutations in gyrB, two had double gyrB mutations, while the other two had either the R485C or the A543T mutation (Table 2); these four isolates did not greatly increase the MICs of the FQs.
The MICs were significantly higher against the gyrA mutants than the wild-type isolates ($P<0.001$). Two isolates showed higher MICs for FQs despite the absence of gyrA mutations in the QRDR. The MICs against these two isolates were 0.25, 0.125, 4 and 8 $\mu$g ml$^{-1}$, and 0.25, 2, 4 and 8 $\mu$g ml$^{-1}$ for SFX, MFX, LFX and CFX, respectively.

**Correlations between different mutation patterns and the MICs**

One isolate showed amino-acid substitutions in three different gyrA codons (G88A+A90V+D94G). The MICs of SFX, MFX, LFX and CFX against this isolate were 0.5, 4, 16 and 32 $\mu$g ml$^{-1}$, respectively (Table 1). Two isolates showed substitutions in two different gyrB codons (E540D+A543V and I552S+D577A). The MICs of SFX, MFX, LFX and CFX against these two isolates were 0.125, 0.5, 1 and 1 $\mu$g ml$^{-1}$, and 0.125, 0.25, 0.5 and 0.5 $\mu$g ml$^{-1}$, respectively. Isolates with double gyrB mutations in the vicinity of the QRDR had higher MICs (Table 2).

**Determination of ECOFF value for fluoroquinolones**

The distributions of MICs for the four FQs are shown in Fig. 2. MFX, LFX and CFX, but not SFX, showed bimodal distribution of MICs, which clearly segregated the phenotypically wild-type isolates from the mutants. As previously described, the tentative ECOFF value can be determined by identifying the end of the phenotypically wild-type distribution to distinguish the resistant and susceptible isolates [18]. Therefore, we determined the ECOFF values as 0.125, 0.5, 1 and 1 $\mu$g ml$^{-1}$ for SFX, MFX, LFX and CFX, respectively. We utilized these ECOFFs to screen for LFX-resistant isolates showing MIC $\geq$2 $\mu$g ml$^{-1}$, which is the cut-off value for LFX against Mtb, as described in previous studies [19, 20]. A total of 39 isolates were LFX-resistant, showing an MIC of $\geq$2 $\mu$g ml$^{-1}$. Furthermore, gyrA mutations were absent in only two of the isolates. Using these ECOFFs, 15% (6/39), 13% (5/39) and 0% of isolates were susceptible to SFX, MFX and CFX, respectively. All of the six and five SFX- and MFX-susceptible isolates above were MDR isolates. Except for one MFX-susceptible isolate with no gyrA mutation, all six and four isolates were gyrA mutants.

Among the 73 MDR isolates, 26 (35.6%), 28 (38.4%) and 29 (39.7%) were resistant to SFX, MFX and LFX, respectively. Furthermore, gyrA mutations were found in 24/26 (92.3%) isolates resistant to SFX and 6/47 (12.8%) isolates susceptible to SFX ($P<0.001$); 26/27 (92.9%) isolates resistant to MFX and 4/46 (8.7%) isolates susceptible to MFX ($P<0.001$); and 30/32 (93.8%) isolates resistant to LFX and 0/41 (0%) isolates susceptible to LFX ($P<0.001$). There were no significant differences in susceptibility or resistance to SFX, MFX and LFX in isolates with gyrB mutations only.

**DISCUSSION**

LFX, MFX and ofloxacin are currently recommended by the WHO for the treatment of MDR-TB [21]. However, data supporting the efficacy of SFX against TB are limited because SFX has not been used worldwide. In this study, we determined the MICs of LFX, MFX, SFX and CFX against multiple clinical isolates of Mtb, and performed direct DNA sequencing of the QRDRs in these isolates. We evaluated the activity of SFX in vitro against gyrA/B mutants as well as MDR isolates obtained from Japan. We found that SFX had the lowest MIC values when compared to other FQs such as
LFX, MFX and CFX. SFX showed the lowest cumulative MIC and had a relatively low ECOFF value against Mtb in our study, indicating that SFX is potentially a more effective agent against gyrA-mutant isolates as well as MDR isolates in Japan.

Using an agar dilution method in 7H11 medium, a previous study showed that SFX had lower MIC<sub>50</sub> and MIC<sub>90</sub> than LFX against non-MDR and MDR isolates [22]. As noted above, that study utilized solid medium and the proportion of MDR isolates was different from that in our study, which may explain the fact that they observed higher MIC values than were observed in our study. A study conducted in Thailand also showed that SFX had lower MICs than other FQs against FQ-susceptible Mtb and isolates with gyrA/B mutations in 7H10 medium [9], and thus SFX was suggested to be more efficacious for Mtb treatment. In agreement with these findings, our results showed that SFX had lower cumulative MICs than MFX, indicating that SFX may be superior to MFX for Mtb treatment. To confirm these findings, additional studies using 7H9 medium are needed.

The WHO does not currently recommend the use of CFX to treat MDR-TB [21]. In our study, CFX showed the highest MICs among the FQs evaluated; this finding lent further support to the WHO recommendation. However, a recent study showed that the use of LFX or MFX did not significantly influence the final treatment outcome in patients with FQ-susceptible MDR-TB [23]. This finding suggested that MIC was not the sole indicator of treatment efficacy. Based on this thought, SFX may not yield a better treatment outcome when compared to MFX or LFX, despite displaying the lowest MIC values.

A previous study using 7H10 agar medium suggested tentative ECOFF values of 1.0 µg ml<sup>-1</sup> for CFX and 0.5 µg ml<sup>-1</sup> for LFX and MFX [24]. On the basis of the findings of the present study, we suggested ECOFF values of 0.125, 0.5 and 1 µg ml<sup>-1</sup> for SFX, MFX and CFX, respectively. These values were based on a breakpoint at 2 µg ml<sup>-1</sup> for LFX in 7H9 medium. The proposed breakpoint for MFX agrees with that recommended by the WHO, which was determined using the mycobacteria growth indicator tube (MGIT).

### Table 1. The MICs of fluoroquinolones against gyrA-mutant isolates

<table>
<thead>
<tr>
<th>Mutation patterns</th>
<th>Isolates, n (%)</th>
<th>MIC of LFX (range), µg ml&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>MIC of MFX (range), µg ml&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>MIC of SFX (range), µg ml&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>MIC of CFX (range), µg ml&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Number of MDR and (non-MDR) isolates, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>A90V (GCG→GTG)</td>
<td>7 (18.9)</td>
<td>(2–4)</td>
<td>(0.5–4)</td>
<td>(0.125–0.5)</td>
<td>(2–16)</td>
<td>3 (4)</td>
</tr>
<tr>
<td>D94G (GAC→GGC)</td>
<td>13 (35.1)</td>
<td>(4–16)</td>
<td>(2–8)</td>
<td>(0.25–4)</td>
<td>(8–32)</td>
<td>12 (1)</td>
</tr>
<tr>
<td>D94H (GAC→CAC)</td>
<td>2 (5.4)</td>
<td>8</td>
<td>(4–8)</td>
<td>1</td>
<td>8</td>
<td>1 (1)</td>
</tr>
<tr>
<td>D94N (GAC→GGC)</td>
<td>2 (5.4)</td>
<td>8</td>
<td>(2–8)</td>
<td>(0.25–1)</td>
<td>(4–32)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>D94A (GAC→GCC)</td>
<td>8 (21.6)</td>
<td>(2–8)</td>
<td>(0.25–2)</td>
<td>(0.06–1)</td>
<td>(2–16)</td>
<td>8 (0)</td>
</tr>
<tr>
<td>A90V (GCG→GTG) and D500A (GAC→GCC)</td>
<td>1 (2.7)</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>1 (0)</td>
</tr>
<tr>
<td>A90V (GCG→GTG) and A543V (GCG→GTG)</td>
<td>1 (2.7)</td>
<td>8</td>
<td>2</td>
<td>0.125</td>
<td>8</td>
<td>1 (0)</td>
</tr>
<tr>
<td>D94A (GAC→GCC) and A543V (GCG→GTG)</td>
<td>2 (5.4)</td>
<td>8</td>
<td>(2–4)</td>
<td>0.25</td>
<td>8</td>
<td>2 (0)</td>
</tr>
<tr>
<td>G88A (GAC→GCC) and A90V (GCG→GTG) and D94G (GAC→GCC)</td>
<td>1 (2.7)</td>
<td>16</td>
<td>4</td>
<td>0.5</td>
<td>32</td>
<td>1 (0)</td>
</tr>
</tbody>
</table>

*Percentage of total gyrA mutants.

LFX, levofloxacin; MFX, moxifloxacin; SFX, sitafloxacin; CFX, ciprofloxacin; MDR, multidrug-resistant.

### Table 2. MICs of fluoroquinolones against gyrB-mutant isolates without gyrA mutations

<table>
<thead>
<tr>
<th>Mutation patterns</th>
<th>Isolates, n (%)</th>
<th>MIC of LFX, µg ml&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>MIC of MFX, µg ml&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>MIC of SFX, µg ml&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>MIC of CFX, µg ml&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Number of MDR isolates, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>R485C (CGT→GT)</td>
<td>1 (12.5)</td>
<td>1</td>
<td>0.5</td>
<td>0.125</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>A543T (GCG→AAG)</td>
<td>1 (12.5)</td>
<td>0.5</td>
<td>0.25</td>
<td>0.06</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>E546D (GAA→GAC) and A543V (GCG→GT)</td>
<td>1 (12.5)</td>
<td>1</td>
<td>0.5</td>
<td>0.125</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>IS552S (ATC→AGC) and D577A (GAT→GCT)</td>
<td>1 (12.5)</td>
<td>0.5</td>
<td>0.25</td>
<td>0.125</td>
<td>0.5</td>
<td>1</td>
</tr>
</tbody>
</table>

*Percentage of total gyrB mutants.

LFX, levofloxacin; MFX, moxifloxacin; SFX, sitafloxacin; CFX, ciprofloxacin; MDR, multidrug-resistant.
medium [25]. Although the breakpoints for CFX and MFX determined in a previous study were comparable to ours [24], additional studies using 7H9 medium should be performed to confirm these breakpoints. Since data on the breakpoint of SFX are limited, we determined the ECOFF of SFX in this study. The appropriate breakpoint setting can influence the choice of drug for the most effective treatment; thus, additional information on the breakpoint of FQs, including that of SFX, is needed. In our study, 15 and 13% of the LFX-resistant isolates were susceptible to SFX and MFX, respectively, indicating that SFX is a more effective agent against MDR isolates with \textit{gyrA} mutations.

\textit{Mtb} resistance to FQs can be mainly attributed to mutations in QRDRs [26], specifically that in the \textit{gyrA} gene [27]. The presence of \textit{gyrA} mutation has also been associated with high-level resistance to FQs [28]. The relationship between mutations in \textit{gyrA/B} and the MICs of FQs has been reported in various settings [19, 20, 29]. Furthermore, double mutations in \textit{gyrA} and \textit{gyrA/B} combined mutations were shown to positively correlate with resistance to FQs [30]. Mutations in certain \textit{gyrB} codons were also associated with a significant increase in the MICs of FQs [31], albeit to a lower extent when compared to those observed with \textit{gyrA} mutant isolates [32]. Isolates with \textit{gyrA} mutations showed higher MICs for the four FQs evaluated in our study. Mutations in codons 90 and 94 of \textit{gyrA} were the most frequently observed mutations (34%), with D94G being the most frequently observed mutation pattern. This finding was in agreement with those of previous reports [19, 31, 33]. In particular, mutations in \textit{gyrA} showed a strong correlation with resistance to FQs among the MDR isolates. In the non-MDR isolates in our study, nearly 20% of isolates were \textit{gyrA} mutants; these isolates showed high MICs and resistance to FQs. This finding suggested that the use of FQs in Japan carries a risk of inducing resistance, even when used to treat non-MDR-TB. Since FQs are widely used to treat bacterial infections, the rate of FQ resistance may increase. Therefore, as indicated in a previous study, a susceptibility test for FQs should be considered [6].

Similarly, the association between \textit{gyrB} mutations and FQ resistance has been reported in several studies. These reports showed that E540V and E540D mutations were
involved in resistance to FQs [30, 31]. Additionally, G512R mutation was reported to correlate with resistance to MFX and ofloxacin [34]. In our study, E540D and A577V, which were found in the same isolate, and R485C, which was found in a different isolate, were associated with resistance to MFX and SFX. Moreover, the 1552S and 577A mutations, found in one isolate, raised the MIC of SFX to its breakpoint of 0.125 μg ml⁻¹. However, the A543T mutation in gyrB appeared to be less relevant for the efficacy of FQs in our study. Since mutations in gyrB are found frequently, further studies are needed to confirm these findings. Isolates with only gyrB mutation did not increase the MICs, indicating that there were no significant differences in susceptibility or resistance to different FQs for the gyrB mutants in our study. To our knowledge, the D577A mutation of gyrB has not been reported elsewhere.

In conclusion, our study confirmed the importance of mutations in gyrA for conferring resistance to SFX, MFX, LFX and CFX. Furthermore, despite showing fewer effects than the gyrA mutations, several mutations in gyrB were associated with increased MICs for the four FQs evaluated. In our study, SFX displayed the lowest MIC values and had a relatively low ECOFF against Mtb, suggesting its superior efficacy against not only gyrA-mutant Mtb isolates, but also MDR isolates in Japan.

Funding information
The authors received no specific grant from any funding agency.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The Ryoken General Assembly approved the collection and use of sputum samples from TB patients. All patients provided written informed consent.

References


