Rapid bactericidal activity of sitafloxacin against *Streptococcus pneumoniae*

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The initial bactericidal activity of quinolones against *Streptococcus pneumoniae* at the concentration equivalent to their respective peak serum concentration (C_max) and free drug fraction of C_max (fC_max) were investigated. The bactericidal activity of sitafloxacin (STFX), levofoxacin (LVFX), moxifloxacin (MFLX), and garenoxacin (GRNX) were compared by determining the actual killing of bacteria at C_max and fC_max for 1 and 2 hours based on the Japanese maximum dose per administration (100, 500, 400, and 400 mg, respectively). Against 4 quinolone-susceptible clinical isolates (wild-type), STFX with C_max and fC_max exhibited the most rapid bactericidal activity resulting in an average reduction of $\geq 3.0 \log_{10}$ colony forming units (CFU)/mL in 1 hour. STFX with C_max and fC_max also showed the most rapid and potent bactericidal activity against 9 clinical isolates with single parC (C/E) mutation, resulting in $\geq 3.0 \log_{10}$ CFU/mL average reduction in viable cells in 1 hour. STFX showed a statistically significant advantage in initial bactericidal activity over other quinolones for single mutants ($P<0.001$). The propensity that the difference in the initial bactericidal activity between STFX and other quinolones was higher in single mutants than wild-type strains, was confirmed using *S. pneumoniae* ATCC49619 (wild-type) and its laboratory single parC mutant. As a result, STFX showed a similar rapid and potent initial bactericidal activity against both strains, while initial bactericidal activity for other quinolones was significantly reduced in the single mutant ($P<0.05$). In conclusion, STFX has the most rapid and potent initial bactericidal activity against wild-type and single mutants of *S. pneumoniae* and its bactericidal activity is not affected by the presence of a single par mutation compared to LVFX, MFLX, and GRNX.
Introduction

*Streptococcus pneumoniae* is one of the most significant pathogens of community-acquired respiratory tract infections and accounts for two-thirds of mortality of those patients requiring hospital treatment\(^1\). Due to increasing levels of resistance in *S. pneumoniae* to commonly used antimicrobials, such as penicillins and macrolides, fluoroquinolone with improved antibacterial activity against *S. pneumoniae* are being recommended and used for the treatment of patients with community acquired pneumonia (CAP) caused by multidrug resistant strains\(^2\). Emerging quinolone-resistant *S. pneumoniae* is also a growing concern because high rates of levofloxacin (LVFX) used by geographic region were reported to be associated with increased quinolone-resistant pneumococci\(^3\), however, current global resistance rates remain low\(^4\)\(^-\)\(^6\).

In pneumococci, resistance to quinolones is primarily mediated via mutations in the quinolone resistance-determining region (QRDR) of target enzymes\(^7\). Even in the LVFX-susceptible clinical isolates, 6–25% of strains with LVFX MIC at 1\(\mu g/mL\) and 30–71% of strains with LVFX MIC at 2\(\mu g/mL\) were reported to possess a single mutation in QRDR\(^8\)\(^-\)\(^10\). The frequency of selection of resistant mutants were higher in single QRDR mutants than wild-type strains\(^11\)\(^-\)\(^13\). It is necessary to use an appropriate quinolone and regimen to eradicate bacteria and to minimise the development of resistance.

Sitafloxicin (STFX) is a fluoroquinolone with enhanced broad-spectrum antibacterial activity against Gram-positive and Gram-negative aerobes and anaerobes\(^14\). STFX was confirmed to possess a potent antimicrobial activity against *S. pneumoniae*, including quinolone-resistant isolates, compared with other quinolones\(^15\).

For the evaluation of the bactericidal activities of quinolones, many studies were conducted using the concentration based on the minimum inhibitory concentration (MIC). In the case of STFX, the MIC based study showed potent bactericidal activity of STFX against *S. pneumoniae*\(^16\), however, the concentration should be examined considering the clinical setting.

Since the pharmacodynamics of quinolones are concentration-dependent\(^17\)\(^-\)\(^18\), some investigators reported the antibactericidal activity using pharmacokinetics/pharmacodynamics parameters of the therapeutic efficacy of quinolones, AUC/MIC, peak serum concentration (C\(_{\text{max}}\))/MIC and C\(_{\text{max}}\)\(^\text{free}\) (fC\(_{\text{max}}\)), which might have an effect on the initial bactericidal activities of quinolones in the clinical setting.
Materials and Methods

Antimicrobial agents

STFX and LVFX were synthesized at Daiichi Sankyo Propharma Co., Ltd., Tokyo, Japan. Moxifloxacin (MFLX) was purchased from Sequoia Research Products Ltd., Pangbourne, UK and LKT Laboratories, Inc., Minnesota, USA. Garenoxacin mesilate (GRNX) was synthesized at Daiichi Sankyo Co., Ltd., Tokyo, Japan. The determination of MICs was performed by the broth dilution method according to the Clinical and Laboratory Standards Institute guidelines\(^2\).

Microorganisms

The bacterial strains used in this study are shown in Table 1. Four quinolone-susceptible clinical isolates of *S. pneumoniae* (GE00175, GE01045, GE01085, and 05712), which did not harbour mutations in the QRDR of the *gyrA*, *gyrB*, *parC* and *parE* genes, and 9 clinical isolates (GE00215, GE01064, 05101, 05667, 05696, 05298, 05674, 05706, and 1026523) harbouring a single mutation relating to quinolone-resistance in the QRDR of *parC* or *parE* of topoisomerase

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Amino acid substitution</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ParC</td>
<td>ParE</td>
</tr>
<tr>
<td>GE00175</td>
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<tr>
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<tr>
<td>GE00215</td>
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<tr>
<td>GE01064</td>
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<tr>
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<tr>
<td>05696</td>
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<tr>
<td>05298</td>
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</tr>
<tr>
<td>60</td>
<td>S79Y</td>
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</tbody>
</table>

a: no amino acid substitution
IV\textsuperscript{24,25} isolated from Japan or UK were used in this study. Strains ATCC49619 and strain 60, which is a mutant of ATCC49619 harbouring a single QRDR mutation in \textit{parC} (Ser\textsuperscript{79}Tyr\textsuperscript{13}), were also used in this study. The bacterium were stored at \( -80^\circ \text{C} \) before experiments.

**Time-kill study**

The bacterial strains were inoculated on heart infusion agar (HIA; Eiken Chemical Co., Ltd., Tokyo, Japan) containing 5\% sheep-defibrinated blood and incubated overnight at 35\(^\circ\)C. Several fresh colonies on the plate were suspended in brain heart infusion broth (BHI; Becton, Dickinson and Company (BD), NJ, USA) supplemented with 0.5\% yeast extract (BD) (BHIY) and incubated at 37\(^\circ\)C with shaking, to obtain the culture containing approximately 10\(^6\) colony forming units (CFU)/mL of bacteria. The initial bactericidal activities of STFX, LVFX, MFLX, and GRNX were examined with exposure of the culture to the concentration equivalent to their respective \( C_{\text{max}} \) at the Japanese maximum dose per administration (100, 500, 400, and 400 mg, respectively) and their \( fC_{\text{max}} \) at 37\(^\circ\)C for 2 hours. \( C_{\text{max}} \) of the quinolones was as follows: STFX 1.0\( \mu \)g/mL\textsuperscript{26}, LVFX 8.0\( \mu \)g/mL\textsuperscript{27}, MFLX 4.1\( \mu \)g/mL\textsuperscript{28}, and GRNX 7.2\( \mu \)g/mL\textsuperscript{29}. \( fC_{\text{max}} \) of the quinolones which was calculated using their respective protein binding ratio by the ultrafiltration method\textsuperscript{26-29} was as follows: STFX 0.5\( \mu \)g/mL, LVFX 5.6\( \mu \)g/mL, MFLX 2.05\( \mu \)g/mL, and GRNX 1.44\( \mu \)g/mL. Samples were taken for viable count determinations at 0, 1, and 2 hours after addition of the drug. Each sample was then diluted appropriately and spread on HIA plates. Antibacterial agents carryover was minimized by serial saline dilution. Saline dilution reduced antibacterial agents concentrations around the MIC for study organisms. The viable cell counts (log\(_{10}\) CFU/mL) were determined based on the numbers of colonies grown on the plates after incubation at 35\(^\circ\)C overnight combined with dilution factors. The detection limit was \( 1 \times 10^2 \) CFU/mL.

**Analysis of time-kill data**

Initial bactericidal activities were represented as the change in viable cell counts before and after treatment with quinolones. This was shown as the mean±SD of the results of quinolone-susceptible isolates or mutant strains, calculated by Microsoft Excel 2003.

**Statistical analysis**

The statistical significance between the STFX-treated group and other quinolone-treated groups was evaluated using Tukey’s comparison test. The statistical significance between \textit{S. pneumoniae} ATCC49619 and 60 treated with the same quinolone was evaluated using Student’s \( t \)-test. These analyses were performed by SAS System Release 8.2 (SAS Institute Inc., NC, USA).
Results

Antimicrobial activity

The antimicrobial activity of STFX and other quinolones to *S. pneumoniae* strains used are shown in Table 1. The MICs of STFX, LVFX, MFLX, and GRNX against wild-type strains of *S. pneumoniae* were 0.03–0.06, 1, 0.12–0.25, and 0.03–0.06 $\mu$g/mL, respectively. The MICs of STFX, LVFX, MFLX, and GRNX against single mutants of *S. pneumoniae* were 0.03–0.06, 1–2, 0.12–0.5, and 0.03–0.12 $\mu$g/mL, respectively.

**Bactericidal activity against wild-type and single QRDR mutant of S. pneumoniae**

The changes in mean viable cell counts of 4 wild-type strains of *S. pneumoniae* after treatment with quinolones at $C_{\text{max}}$ and $fC_{\text{max}}$ for 1 and 2 hours are shown in Fig. 1 and Fig. 2, respectively. STFX at $C_{\text{max}}$ and $fC_{\text{max}}$ exhibited the most potent initial bactericidal activity resulting in 3.75 and 3.77 log$_{10}$ CFU/mL reduction in viable counts at 1 hour and 4.46 and 4.13 log$_{10}$ CFU/mL reduction in viable counts at 2 hours, respectively. Especially, STFX at $fC_{\text{max}}$ exhibited significantly rapid bactericidal activity compared to other quinolones tested (1 hour: $P<0.001$ vs. LVFX and GRNX, $P<0.01$ vs. MFLX, 2 hours: $P<0.05$ vs. MFLX and GRNX).

The changes in mean viable cell counts of 9 single mutants of *S. pneumoniae* after treatment with quinolones at $C_{\text{max}}$ or $fC_{\text{max}}$ for 1 and 2 hours are shown in Fig. 3 or Fig. 4, respectively. STFX at $C_{\text{max}}$ and $fC_{\text{max}}$ exhibited the most potent initial bactericidal activity resulting in 3.75 and 3.26 log$_{10}$ CFU/mL reduction in viable counts at 1 hour and 3.96 and 3.85 log$_{10}$ CFU/mL reduction in viable counts at 2 hours, respectively. STFX showed a statistically significant advantage in initial bactericidal activity over other quinolones tested for single mutants at any condition ($P<0.001$).

The changes in the mean viable cell counts after treatment with STFX at $C_{\text{max}}$ for 1 and 2 hours were 0.87–1.53 and 0.75–1.27 log$_{10}$ CFU/mL, respectively in the wild-type strains and 2.14–3.05 and 1.27–2.37 log$_{10}$ CFU/mL, respectively in the single mutants. The changes in the mean viable cell counts after treatment with STFX at $fC_{\text{max}}$ for 1 and 2 hours were 1.35–2.22 and 0.97–1.25 log$_{10}$ CFU/mL, respectively in the wild-type strains and 1.98–2.56 and 1.73–2.39 log$_{10}$ CFU/mL, respectively in the single mutants. This means that the STFX showed a more rapid bactericidal activity in the single mutants than wild type strains.

**Bactericidal activity against wild-type and its laboratory single parC mutant of S. pneumoniae**

The initial bactericidal activity of wild-type *S. pneumoniae* ATCC49619 (parent strain) and strain 60, which is a single *parC* mutant of ATCC49619, was investigated. The mean changes in viable cell counts with 3 times of individual experiments after treatment of two strains with quinolones at $C_{\text{max}}$ or $fC_{\text{max}}$ for 1 and 2 hours are shown in Fig. 5 or Fig. 6, respectively. STFX at
C<sub>max</sub> exhibited the most potent early bactericidal activity against wild-type and a single parC mutant, resulting in 2.92 and 3.00 log<sub>10</sub> CFU/mL reduction in viable counts at 1 hour, respectively, and 3.70 and 3.71 log<sub>10</sub> CFU/mL reduction in viable counts at 2 hours, respectively. No significant effect on the initial bactericidal activity of STFX by a single parC mutation was confirmed.
On the contrary, in the case of other quinolones tested, significant reduction of bactericidal activity was confirmed in a single parC mutant compared to wild-type \((P<0.01\) vs. LVFX at 1 and 2 hours, \(P<0.05\) vs. MFLX and GRNX at 1 and 2 hours). STFX at \(C_{\text{max}}\) exhibited the most potent early bactericidal activity against wild-type and a single parC mutant resulting in 2.80 and 2.41 \(\log_{10}\) CFU/mL reduction in viable counts at 1 hour, respectively and 3.55 and 3.24 \(\log_{10}\) CFU/mL reduction in viable counts at 2 hours, respectively. No significant effect on the initial
bactericidal activity of STFX by the substitution of single amino acid was confirmed. In contrast, in the case of other quinolones tested, significant reductions of bactericidal activities were confirmed in the single mutant compared to wild-type strain ($P<0.01$ vs. LVFX at 1 and 2 hours, MFLX at 2 hours, GRNX at 1 hour, $P<0.05$ vs. MFLX at 1 hour and GRNX at 2 hours).
**Discussion**

The *in vitro* study described here was performed with the aim of determining the initial bactericidal activities of STFX, LVFX, MFLX, and GRNX against wild-type strains and single
QRDR mutants of *S. pneumoniae* at the concentration equivalent to their $C_{\text{max}}$ and $fC_{\text{max}}$ in 2 hours to predict the initial bactericidal activities of quinolones in the clinical setting. We revealed that STFX not only at $C_{\text{max}}$ but also $fC_{\text{max}}$ exhibited distinguished potent and rapid bactericidal activity against *S. pneumoniae*, compared to LVFX, MFLX, and GRNX. The advantage of the
The bactericidal activity of STFX over other quinolones tested stood out in single QRDR mutants. Among the other quinolones tested, GRNX was confirmed to possess an almost equivalent antibacterial activity to STFX against *S. pneumoniae* and the C<sub>max</sub> and fC<sub>max</sub> of GRNX were about 7 times and 3 times higher than those of STFX, respectively. However, the initial antibactericidal activity of STFX over other quinolones tested stood out in single QRDR mutants. Among the other quinolones tested, GRNX was confirmed to possess an almost equivalent antibacterial activity to STFX against *S. pneumoniae* and the C<sub>max</sub> and fC<sub>max</sub> of GRNX were about 7 times and 3 times higher than those of STFX, respectively. However, the initial antibactericidal activity of STFX over other quinolones tested stood out in single QRDR mutants. Among the other quinolones tested, GRNX was confirmed to possess an almost equivalent antibacterial activity to STFX against *S. pneumoniae* and the C<sub>max</sub> and fC<sub>max</sub> of GRNX were about 7 times and 3 times higher than those of STFX, respectively. However, the initial antibactericidal activity of STFX over other quinolones tested stood out in single QRDR mutants. Among the other quinolones tested, GRNX was confirmed to possess an almost equivalent antibacterial activity to STFX against *S. pneumoniae* and the C<sub>max</sub> and fC<sub>max</sub> of GRNX were about 7 times and 3 times higher than those of STFX, respectively.

Each bar represents the mean±S.D. of the change in viable cell count of 3 times of individual experiments. The statistical significance between ATCC49619 and 60 treated with the same quinolone was evaluated using Student’s t-test.

** : P<0.01, * : P<0.05 compared to ATCC49619 (Student’s t-test)
activity of GRNX at $C_{\text{max}}$ and $fC_{\text{max}}$ were lower than those of STFX at $C_{\text{max}}$ and $fC_{\text{max}}$. YOKOTA et al. demonstrated a similar experiment except for the concentration of STFX. They used $C_{\text{max}}$ and $fC_{\text{max}}$ for the dosage of STFX 50 mg, which was half of our study and reported a different result that the antibactericidal activity of STFX 50 mg at $C_{\text{max}}$ and $fC_{\text{max}}$ was lower than GRNX. Since the protein binding ratio of STFX is 50%, $fC_{\text{max}}$ of STFX 100 mg is almost equal to $C_{\text{max}}$ of STFX 50 mg. However, the initial bactericidal activity of $fC_{\text{max}}$ of STFX 100 mg was confirmed to be higher than that of $C_{\text{max}}$ of GRNX 400 mg in our study. This difference is considered to be attributable to the difference of bacterial strains, medium, and bacterial counts at the beginning of drug treatment between the two studies. On the contrary, in terms of LVFX and MFLX, SCHAFER, J. et al. reported that MFLX initially displayed faster killing than LVFX against a parC mutant with a Ser-79Phe mutation. It was confirmed in the report by ANDEREGG, T. R. et al. that GRNX and LVFX showed slow initial bactericidal activity against wild-type and QRDR mutant strains of S. pneumoniae at clinically achievable serum concentration, where these data support our study.

STFX was confirmed to possess a more potent and balanced dual-target inhibition against wild-type and single mutated DNA gyrase and topoisomerase IV of S. pneumoniae than other quinolones. Moreover, OKUMURA et al. reported that the rapid bactericidal activity of STFX was due to its ability to induce autolysis mediated by LytA, which is responsible for autolysis in S. pneumoniae. We speculate that these features are part of the reasons why STFX showed rapid and potent bactericidal activity against wild-type and single QRDR mutant strains of S. pneumoniae.

In conclusion, STFX at around the $C_{\text{max}}$ of the clinical dose (100 mg) showed the most potent and rapid antibactericidal activity against S. pneumoniae regardless of existence or non-existence of single QRDR mutation. These results suggest that STFX may show potent and rapid bactericidal activity in the treatment of infectious disease caused by S. pneumoniae.

Acknowledgment

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Sitafl oxacinのStreptococcus pneumoniaeに対する迅速な殺菌効果

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キノロン系抗菌薬のStreptococcus pneumoniaeに対する初期殺菌効果を、最高血漿中濃度（C_{max}）及びフリーボディC_{max}（fC_{max}）で検討した。すなわち、Sitafl oxacin（STFX）、Levofoxacin（LVFX）、Moxifloxacin（MFLX）、及びGarenoxacin（GRNX）を健康成人に国内における1回あたり最高用量（それぞれ100mg, 500mg, 400mg, 及び400mg）を投与した際のC_{max}及びfC_{max}を作用させ、薬剤作用1時間後及び2時間後における殺菌効果を比較検討した。その結果、STFXは野生型の臨床分離S. pneumoniae菌株、計4株に対し、C_{max}, fC_{max}いずれの濃度を作用した場合も、薬剤作用1時間以内に平均生菌数を3 log colony forming units (CFU)/mL以上減少させ、最も高い初期殺菌効果を示した。STFXは、par（C/E）に1ヶ所変異を有するS. pneumoniae（一変異型S. pneumoniae）、計9株に対しても、最も高い初期殺菌効果を示し、薬剤作用1時間以内に平均生菌数を3 log CFU/mL以上減少させた。STFXの一変異型S. pneumoniaeに対する初期殺菌効果は、対照キノロン系抗菌薬と比較して有意に高いものであった（P<0.001）。STFXと对照キノロン系抗菌薬との殺菌効果の差が、野生型より一変異型S. pneumoniaeで大きくなる傾向を確認するために、S. pneumoniae ATCC49619（野生型）と本株から実験的に作製されたparC一変異株を用いて同様の検討を実施した。その結果、STFXは2株に対して同等の高い初期殺菌効果を示したが、対照キノロン系抗菌薬では、一変異型S. pneumoniaeに対する初期殺菌効果が顕著に減少した（P<0.05）。

以上の結果より、STFXは野生型及び一変異型S. pneumoniaeに対して最も高い初期殺菌効果を示すことが明らかとなった。さらに、STFXの殺菌効果はLVFX、MFLX、及びGRNXと比較して、標的酵素の一変異の影響を受けにくいことが明らかになった。