Inhibitory activity of garenoxacin against DNA gyrase of *Mycoplasma pneumoniae*

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**Objectives:** Garenoxacin, a des-fluoro(6)-quinolone, exhibits potent activity against *Mycoplasma pneumoniae*, including macrolide-resistant strains. There has been no report on the inhibitory activity of garenoxacin against the target enzyme of *M. pneumoniae*.

**Methods:** Subunits of DNA gyrase (GyrA and GyrB) proteins of *M. pneumoniae* FH were separately expressed as His-tagged proteins in *Escherichia coli* Chaperone Competent Cell BL21 by IPTG induction of plasmids containing the respective *gyrA* and *gyrB* genes. The inhibitory activities of garenoxacin, moxifloxacin, gatifloxacin and levofloxacin against DNA gyrase were evaluated by the inhibition of supercoiling activity (*n* = 3).

**Results:** Against *M. pneumoniae* FH, garenoxacin showed 2- to 16-fold more potent activity than the other quinolones. The mean IC$_{50}$ of garenoxacin for DNA gyrase of *M. pneumoniae* was 2.5 mg/L. Garenoxacin showed the most potent inhibitory activity against *M. pneumoniae* DNA gyrase among the quinolones tested. The IC$_{50}$ values of the quinolones for DNA gyrase roughly correlated with each MIC value.

**Conclusions:** The antimycoplasmal activity of the quinolones was almost certainly due to inhibition of the supercoiling activity of DNA gyrase. Garenoxacin was considered a valuable quinolone in the treatment of infectious diseases caused by *M. pneumoniae*.

**Keywords:** recombinant proteins, purification, supercoiling activity, quinolones

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**Introduction**

*Mycoplasma pneumoniae* is a common pathogen causing community-acquired respiratory tract infections, mainly in children and young adults. In some cases, this organism can cause severe, systemic disease, especially in the setting of a debilitated or immunocompromised host. Although macrolides are usually considered the first-choice agents for chemotherapy for *M. pneumoniae* infection in children, it was reported that 30.6% of the strains isolated from patients in Japan in 2006 were macrolide resistant. Also, in Shanghai, China, 90 of 100 (90%) *M. pneumoniae* strains isolated from paediatric patients from March 2008 to July 2009 were resistant to erythromycin. Further, during an outbreak of *M. pneumoniae* infection in Italy in 2010, *M. pneumoniae* strains from 11 (25.6%) of the 43 paediatric patients hospitalized were macrolide resistant. Given this background, it was considered that an alternative to macrolides is needed in the treatment of respiratory tract infections, such as pneumonia, caused by *M. pneumoniae*.

Garenoxacin is a des-fluoro(6)-quinolone that exhibits excellent in vitro activity against *M. pneumoniae* and is expected to be active in infectious diseases caused by this organism.

The target enzymes of quinolones are considered to be DNA gyrase and topoisomerase IV (TopoIV), which are essential enzymes for controlling the topological state of DNA in DNA replication and transcription. DNA gyrase and TopoIV have been purified from many bacterial species, and the inhibitory activities of quinolones against these enzymes have been measured; however, to our knowledge, there is no report on the inhibitory activity of quinolone against mycoplasmal DNA gyrase.

In this study, the MIC of garenoxacin was determined for *M. pneumoniae* FH (ATCC 15531), and compared with those of moxifloxacin, gatifloxacin and levofloxacin. In addition, recombinant DNA gyrase of *M. pneumoniae* was purified using competent cells of *Escherichia coli*, and then the inhibitory activity of garenoxacin against this target enzyme was evaluated and compared with those of the above-mentioned quinolones.

**Methods**

The bacterial strain used in this study was *M. pneumoniae* FH (ATCC 15531), purchased from the ATCC.

The quinolones employed were garenoxacin, moxifloxacin, gatifloxacin and levofloxacin. Garenoxacin was synthesized at the Research Laboratory.
Laboratories, Toyama Chemical Co., Ltd (Toyama, Japan). Moxifloxacin and gatifloxacin were extracted and purified from commercially available tablets (moxifloxacin: Avelox, Bayer Yokuhin, Ltd, Osaka, Japan; and gatifloxacin: Gatilfo, Kyorin Pharmaceutical Co., Tokyo, Japan). The purities of these two agents were >93% and >98%, respectively, as determined by HPLC assay. Levofloxacin was purchased from LKT Laboratories, Inc. (Saint Paul, MN, USA).

A broth microdilution method was performed to determine the MICs. Serial 2-fold dilutions of antibacterial agents prepared in PPL0 broth (Difco Inc., Detroit, MI, USA) containing 10^7 – 10^8 cfu/mL. M. pneumoniae were placed in 96-well microplates. The microplates were sealed with adhesive sheets and incubated at 37°C for 5 days. The MIC was defined as the lowest concentration of a drug at which metabolism of the organism was inhibited, as evidenced by a lack of colour change in the medium at the time when the drug-free control first showed a colour change.

DNA gyrase genes (gyrA and gyrB) were derived from purified DNA (ATCC 15531D) of M. pneumoniae FH. Oligonucleotide primers were designed for the amplification of gyrA and gyrB genes, and their subsequent insertion into SacI and PstI sites, respectively, in the pTrChis vector (Life Technologies Inc., Carlsbad, CA, USA). PCR was performed with primer sets of mpgA11 (5'-ATGCCGAGCTGATGGCAAAGCAACAAGATC-3' ; SacI site underlined) and mpgA21 (5'-AACCTGCAAGTGCAGTAATTGGTTCG-3' ; PstI site underlined) and mpgB11 (5'-ATGCCGAGCTGATGGCAAAGCAACAAGATC-3' ; SacI site underlined) and mpgB21 (5'-AACCTGCAAGTGCAGTAATTGGTTCG-3' ; PstI site underlined), respectively.

Each of the genes amplified by PCR was cloned into the pTrChis vector. All PCRs were performed under the following conditions: DNA was amplified for 30 cycles, for 10 s at 98°C, 10 s at 55°C and 150 s (for gyrA) or 120 s (for gyrB) at 72°C. DNA amplification was performed in a final volume of 50 μL with 1.25 U of PrimeSTAR HS DNA polymerase (TakaRa Bio Inc., Shiga, Japan). Amplified PCR products of gyrA and gyrB were ~2.5 and ~2.0 kb, respectively, and their DNA sequences were confirmed.

The subunits of DNA gyrase protein (GyrA and GyrB) from M. pneumoniae FH were separately expressed as His-tagged proteins in E. coli Chaperone Competent Cell BL21 (TakaRa Bio Inc., Shiga, Japan) by IPTG induction. IPTG was added to a final concentration of 1 mM and induction was continued overnight at 15°C. The enzymes were purified using a modification of the Pan and Fisher method. Overproduced proteins were purified using Ni-NTA affinity resin and dialysed with Amicon Ultra-15 (Merck Millipore, Darmstadt, Germany). The overproduced proteins were stored at ≏85% glycerol in storage buffer [50 mM Tris-HCl (pH 8.0), 100 mM KCl, 1 mM EDTA, 2 mM DTT and 20% glycerol] until use. The molecular masses of GyrA and GyrB, as determined by SDS–PAGE, were ~93.4 and ~73.8 kDa, respectively (data not shown).

The supercoiling activity of DNA gyrase was measured, as previously described. A 0.5 U of supercoiling activity was defined as the amount of GyrA and GyrB proteins required to supercoil 50% of 0.1 μg of relaxed pBR322 DNA (Wako Pure Chemical Industries, Ltd, Osaka, Japan). The specific activities of His-tagged GyrA and GyrB were determined as 2.1×10^6 and 5.3×10^5 U/mg, respectively. IC50 values were defined as the drug concentration that reduced the enzymatic activity observed with drug-free controls by 50%. The inhibitory activities of the antibacterial agents against DNA gyrase were evaluated by the inhibition of supercoiling activity (n = 3).

For the supercoiling inhibition test, the reaction buffer was composed of 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM MgCl2, 75 μg/mL BSA, 100 μg/mL RNA and 2 mM ATP, 0.1 μg of relaxed pBR322 DNA, and 1 U of GyrA and 1 U of GyrB proteins. After incubation at 37°C for 1 h, 2 μg of Proteinase K (Wako Pure Chemical Industries, Ltd) was added to the reaction mixture. Each quinolone was added to the reaction mixture after serial dilution from its maximum concentration in five steps. The concentrations of quinolones in the reaction mixtures were as follows: garenoxacin, 6.25–0.39 mg/L; moxifloxacin and gatifloxacin, 12.5–0.78 mg/L; and levofloxacin, 100–6.25 mg/L. After incubation at 37°C for 30 min, the reaction mixtures were subjected to 0.8% agarose gel electrophoresis.

### Results

The MIC of garenoxacin for M. pneumoniae FH was 0.0313 mg/L, 2- to 16-fold lower than that of moxifloxacin, gatifloxacin and levofloxacin, respectively (Table 1). The IC50 of garenoxacin for DNA gyrase of M. pneumoniae FH was 2.50 mg/L; those of moxifloxacin, gatifloxacin and levofloxacin were 7.44, 5.71 and 47.5 mg/L, respectively. The MICs also roughly correlated with the IC50s (r² = 0.98). The MIC result favoured the DNA gyrase as the principal target. Among the quinolones tested, garenoxacin showed the most potent inhibitory activity against DNA gyrase.

### Discussion

Garenoxacin had potent inhibitory activity against M. pneumoniae DNA gyrase, suggesting that it is a valuable quinolone in the treatment of infectious diseases caused by M. pneumoniae.

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### Transparency declarations

All authors are employees of Toyama Chemical Co., Ltd.

### References


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**Table 1. Inhibition of M. pneumoniae FH DNA gyrase by quinolones**

<table>
<thead>
<tr>
<th>Quinolone</th>
<th>MIC (mg/L)</th>
<th>IC50 for DNA gyrase (mg/L)</th>
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<tbody>
<tr>
<td>Garenoxacin</td>
<td>0.0313</td>
<td>2.50 (2.01–3.20)*</td>
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<tr>
<td>Moxifloxacin</td>
<td>0.0625</td>
<td>7.44 (6.10–9.55)</td>
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<tr>
<td>Gatifloxacin</td>
<td>0.125</td>
<td>5.71 (4.24–8.45)</td>
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<tr>
<td>Levofloxacin</td>
<td>0.5</td>
<td>47.5 (40.8–56.7)</td>
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*95% confidence limits.


