In vitro and in vivo antibacterial activity of modithromycin against streptococci and Haemophilus influenzae

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Objectives: In vitro and in vivo antibacterial activities of modithromycin against Streptococcus pneumoniae, Streptococcus pyogenes and Haemophilus influenzae were examined.

Methods: MICs were determined by the broth microdilution method. Experimental infection of epithelial cell line A549 was performed to compare the intracellular activity and lasting effects of the antimicrobial agents. To evaluate in vivo efficacy, the rat pulmonary infection model was used.

Results: Modithromycin had MICs of \( \leq 1 \) mg/L against all the clinical strains of both streptococci, including erythromycin-resistant strains. In particular, the MICs of modithromycin for \( \text{erm}(B)\)- or \( \text{mef}(A)\)-carrying \( S. \) pyogenes were 16–32 times or 2–4 times lower than those of telithromycin, respectively. The MIC90 of modithromycin for \( H. \) influenzae was 8 mg/L, which was 4 times higher than that of telithromycin. Modithromycin, as well as azithromycin, showed a lasting inhibitory effect on bacterial growth of cell-associated \( H. \) influenzae compared with telithromycin and levofloxacin after removal of the agents from the apical medium. In the pulmonary infection model, modithromycin showed greater or comparable efficacy against \( \text{erm}(B)\)-carrying \( S. \) pneumoniae and \( H. \) influenzae, respectively, than telithromycin, regardless of having an MIC that was 2 or 4 times higher for these strains.

Conclusions: Modithromycin has the most potent anti-\( S. \) pyogenes activity of the antimicrobial agents tested. Modithromycin also has the better in vivo efficacy against \( S. \) pneumoniae and \( H. \) influenzae, which might be due to its lasting intracellular activity.

Keywords: macrolides, \( \text{erm} \), efflux, lungs

Introduction

Macrolide antimicrobial agents, such as clarithromycin and azithromycin, have unique pharmacokinetic characteristics, e.g. efficient distribution into respiratory tract tissues, and broad-spectrum activity against Gram-positive and -negative respiratory pathogens. Therefore, they have been frequently used to treat respiratory tract infections, including community-acquired pneumonia, sinusitis and pharyngitis. However, macrolide resistance in Gram-positive cocci, particularly in Streptococcus pneumoniae, has been increasing throughout the world; therefore, continuous use of the current macrolide agents could lead to an increase in treatment failures.

To overcome the macrolide resistance of Gram-positive cocci, the novel bicyclolide agent modithromycin (EP-013420, EDP-420, S-013420) was discovered by Enanta Pharmaceuticals (Figure 1). Modithromycin possesses the unique structural feature of a C-6,11-bridged ether of a 14-membered macrolide ring containing a pyrazole–pyridine side chain at the bridged-linker and a ketone at the C-3 position. In the present study, we examined the in vitro and in vivo antibacterial activities of modithromycin against streptococcal respiratory pathogens, including erythromycin-resistant strains and Haemophilus influenzae.
were commercially obtained from US Pharmacopeia (Rockville, MD, USA). Levofloxacin was commercially obtained from LKT Laboratories, Inc. (St Paul, MN, USA).

Bacterial strains
A total of 298 clinical strains, including S. pneumoniae, Streptococcus pyogenes and H. influenzae, which had been collected at Japanese medical facilities from 2005 to 2006, were used in the MIC determination study. H. influenzae ATCC 10211 mutants lacking each component of the AcrA-AcrB-TolC efflux pump were constructed as follows: each component gene of acrA (HA0894), acrB (HA0895) and tolC (HA1462) was amplified by PCR and ligated in pUC18. Next, each of the three genes was fused and disrupted by a kanamycin-resistant cassette (kanR) from pUC4K. Transformation of ATCC 10211 was performed by homologous recombination with each of the kanR-inserted acrA, acrB and tolC fragments. For time–kill studies, S. pneumoniae ATCC 49619 and H. influenzae ATCC 51907 were used. For the intracellular antibacterial activity study, six clinical strains of non-typeable H. influenzae were used. For pulmonary infection, clinical strains of erm(B) gene-carrying erythromycin-resistant S. pneumoniae KP058 and ampicillin-resistant H. influenzae KP097 were used.

MIC determination
MICs were determined by the broth microdilution method recommended by the CLSI.11 Cation-adjusted Mueller–Hinton broth (CAMHB; Difco) supplemented with 5% lysed horse blood was used as the test medium for both streptococci. For H. influenzae, the Haemophilus test medium (HTM; Nissui Pharmaceutical Co., Ltd, Japan) was used. Bacterial colonies cultured overnight on agar plates were suspended and inoculated into the broth medium containing serial dilutions of the antimicrobial agents in a 96-well microplate to a final concentration of ~5×10^8 cfu/mL. The MIC was defined as the lowest concentration of the antimicrobial agent that inhibits visible growth after incubation for 20 h at 35°C in ambient air. The MIC interpretation of susceptible, intermediate resistant or resistant was determined based on the CLSI breakpoint.12

Detection of erythromycin resistance genes
The mef(A/E) (efflux pump), erm(B) (ribosome methylase) and erm(TR) (ribosome methylase), which is responsible for the resistance to erythromycin, in both streptococci were detected by a conventional PCR. PCR was performed according to the manufacturer’s instructions using two sets of primers for the mef(A/E) and erm(B) genes, which were commercially obtained from Wakunaga Pharmaceutical Co., Ltd (Osaka, Japan). For the erm(TR) genes, PCR was performed using the primers reported previously.13 As a result, at least one gene of mef(A/E), erm(B) or erm(TR) was detected in all the erythromycin-intermediate-resistant and -resistant strains of S. pneumoniae and S. pyogenes.

Time–kill study
Exponential growth phase bacteria were adjusted to ~10^6 cfu/mL in CAMHB supplemented with 5% lysed horse blood for S. pneumoniae or HTM for H. influenzae, and were then exposed to antimicrobial agents at concentrations of 0.5×, 1×, 2×, 4× and 8× MIC. After 1, 2.5, 4 and 6 h incubation at 37°C with constant shaking, viable bacterial counts were determined.

Experimental infection of epithelial cells
Human alveolar epithelial cell line A549 was obtained from ATCC. Aliquots of the cell suspension in the test medium (RPMI 1640 (Invitrogen Corporation) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Corporation)) were seeded in a 96-well tissue culture plate (1.6×10^5 cells/0.1 mL/well) and allowed to form confluent monolayer cells with 48 h incubation at 37°C in humidified air containing 5% CO₂. Then, the monolayer cells were infected with a bacterial suspension of each of six H. influenzae strains at densities of 2.8×10^8 to 1.3×10^9 cfu/0.1 mL/well. All six strains were exponentially grown and achieved a similar level of bacterial burden (~8.5 log cfu/mL) during 24 h incubation (they did not grow in the absence of the epithelial cells). Next, the extracellular non-adherent bacteria in the apical medium were removed and the cells were gently washed twice with fresh medium. Then, aliquots (0.1 mL) of the test medium containing antimicrobial agents were added at a concentration of 2× MIC to duplicate wells. After 12 h incubation at 37°C in humidified air containing 5% CO₂, determination of viable bacteria was performed in either one of the two wells. In the other well, the apical medium was removed by washing and replaced with fresh test medium, and the cells were then incubated again without the agents in the apical medium for 12 h. At each sampling timepoint (12 and 24 h after initiation of treatment), to determine the cell-associated bacteria, infected cells were gently washed twice after removal of the apical medium containing extracellular non-adherent bacteria and then lysed by vigorous pipetting with 0.1 mL of saline containing 1% saponin. This saponin concentration did not interfere with bacterial survival. The numbers of cell-associated bacteria (intra- and intercellular bacteria as well as cell-surface-adherent bacteria) in the suspension were counted on chocolate agar.

Pulmonary infection in rat
Specific-pathogen-free CD(SD) rats (male, 5 weeks old) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The bacterial suspension of the challenge organism was prepared with MHB containing 0.5% agar noble (Difco). Rats were anaesthetized by intraperitoneal injection of a ketamine and xylazine mixture, and then 0.1 mL of the bacterial suspension was inoculated intratracheally into the lung via a polyethylene catheter. The inoculum sizes of KP058 and KP097 were 3.8×10^8 to 1.3×10^9 cfu/rat, respectively. The antimicrobial agents prepared in 0.5% methylcellulose solutions were orally administered 4 h after infection for KP058, and 4, 24, 48 and 72 h after infection for KP097. The rats were sacrificed 24 h after infection for KP058 and 96 h after infection for KP097, and then viable bacterial counts in the lungs were determined. This experimental protocol was approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University.
Statistical analysis

Differences in the viable bacterial counts between study groups were analysed by Dunnett’s multiple comparison test or Welch’s t-test. Statistical significance was defined by a P value of <0.05.

Results

MICs of antimicrobial agents for clinical strains

Modithromycin showed MICs of ≤1 mg/L for all the clinical strains of both streptococci, including erythromycin-resistant strains, although some strains were highly resistant to clarithromycin for erythromycin-intermediate-resistant and -resistant strains of S. pneumoniae and S. pyogenes were 0.5 and 1 mg/L, respectively. It should be noted that the MIC90 of modithromycin for erythromycin-intermediate-resistant and -resistant S. pyogenes was 32 times lower than that of telithromycin. The MIC90 of modithromycin for H. influenzae was 8 mg/L, which was 4 times higher than that of telithromycin and azithromycin.

Comparison of the activities of modithromycin and telithromycin against erm and mef gene-carrying streptococci

Modithromycin and telithromycin showed similar MIC distributions for mef(E) and/or erm(B) gene-carrying S. pneumoniae (Figure 2). However, the MIC distributions for mef(A) or erm(B) gene-carrying S. pyogenes differed between these antimicrobial agents. Although all the erm(B) gene-carrying strains except one showed moderate to high MICs of telithromycin of 2–32 mg/L, modithromycin showed MICs of 0.125 or 1 mg/L for these strains. There were 16- to 32-fold differences in individual MICs between these antimicrobial agents. In addition, the MICs of modithromycin for mef(A) gene-carrying S. pyogenes were 2–4 times lower than those of telithromycin. On the other hand, all the erm(TR) gene-carrying S. pyogenes tested were susceptible to both antimicrobial agents, with MICs of ≤0.063 mg/L.

Influence of efflux pump on antibacterial activity against H. influenzae

The susceptibilities to modithromycin and other antimicrobial agents of H. influenzae ATCC 10211 and its mutant strains that lacked a component of the AcrA-AcrB-TolC efflux pump are shown in Table 2. The in vitro activity of modithromycin showed 8–16 times lower MICs for these mutants compared with their parental strain, indicating that this pump contributed to the reduced susceptibility to modithromycin of H. influenzae. These MIC changes occurred with other macrolide and ketolide agents tested, but not with ampicillin. The azithromycin MIC ratio between the parent and these mutant strains was slightly lower than that of the other macrolide and ketolide agents tested. However, all the macrolide and ketolide agents tested as well as the bicyclolide modithromycin showed similar MICs of 0.25 or 0.5 mg/L for these mutant strains.

*Table 1. Antibacterial activities of modithromycin and other antimicrobial agents against clinical strains*

<table>
<thead>
<tr>
<th>Organism (number of strains)</th>
<th>Erythromycin-susceptible S. pneumoniae&lt;sup&gt;a&lt;/sup&gt; (14)</th>
<th>Erythromycin-intermediate-resistant and erythromycin-resistant S. pneumoniae&lt;sup&gt;a&lt;/sup&gt; (86)</th>
<th>Erythromycin-susceptible S. pyogenes&lt;sup&gt;a&lt;/sup&gt; (78)</th>
<th>Erythromycin-intermediate-resistant and erythromycin-resistant S. pyogenes&lt;sup&gt;a&lt;/sup&gt; (21)</th>
<th>H. influenzae (99)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antimicrobial agent</td>
<td>MIC (mg/L)</td>
<td>Antimicrobial agent</td>
<td>MIC (mg/L)</td>
<td>Antimicrobial agent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>range</td>
<td></td>
<td>range</td>
<td></td>
</tr>
<tr>
<td>Erythromycin-susceptible</td>
<td>modithromycin</td>
<td>≤0.063–0.125</td>
<td>telithromycin</td>
<td>≤0.063</td>
<td>clarithromycin</td>
</tr>
<tr>
<td>S. pneumoniae&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(14)</td>
<td></td>
<td>telithromycin</td>
<td>≤0.063</td>
<td>clarithromycin</td>
</tr>
<tr>
<td>Erythromycin-intermediate-</td>
<td>modithromycin</td>
<td>≤0.063–1</td>
<td>telithromycin</td>
<td>≤0.063–1</td>
<td>clarithromycin</td>
</tr>
<tr>
<td>resistant and erythromycin-</td>
<td>(86)</td>
<td>0.5</td>
<td></td>
<td>≤0.063</td>
<td></td>
</tr>
<tr>
<td>resistant S. pneumoniae&lt;sup&gt;</td>
<td>modithromycin</td>
<td>≤0.063–0.125</td>
<td>telithromycin</td>
<td>≤0.063</td>
<td>clarithromycin</td>
</tr>
<tr>
<td>a&lt;/sup&gt;</td>
<td>(86)</td>
<td></td>
<td>telithromycin</td>
<td>≤0.063</td>
<td>clarithromycin</td>
</tr>
<tr>
<td>Erythromycin-susceptible</td>
<td>modithromycin</td>
<td>≤0.063–1</td>
<td>telithromycin</td>
<td>≤0.063–32</td>
<td>clarithromycin</td>
</tr>
<tr>
<td>S. pyogenes&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(78)</td>
<td>1.25</td>
<td>telithromycin</td>
<td>0.5</td>
<td>clarithromycin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>clarithromycin</td>
<td>2–16</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>CNS breakpoint MICs of erythromycin: susceptible, ≤0.25 mg/L; and intermediate resistant and resistant, ≥0.5 mg/L.
Time–kill study against S. pneumoniae and H. influenzae

Modithromycin showed concentration-dependent antibacterial activity against both S. pneumoniae ATCC 49619 and H. influenzae ATCC 51907, which was comparable to that of telithromycin (Figure 3). On the other hand, clarithromycin and azithromycin showed slightly weaker activity against ATCC 51907 and ATCC 49619, respectively, compared with modithromycin.

Antibacterial activity against epithelial cell-associated H. influenzae

Modithromycin killed \(~\sim 2.5\) log cfu/mL mean cell-associated bacteria during the 12 h treatment (from the initial bacterial counts), which was superior in bacterial reduction compared with clarithromycin and azithromycin (Figure 4). It was considered that some of the killed bacteria were localized intracellularly, because ampicillin, which has poor cell penetration, hardly killed the cell-associated bacteria, even though they were exposed to an extracellular concentration of 512× MIC of ampicillin (data not shown). To evaluate the effects of each antimicrobial agent accumulated in the cells on bacterial growth, an additional 12 h incubation was performed after removal of the antimicrobial agents from the apical medium. Both modithromycin and azithromycin inhibited the bacterial growth strongly, but telithromycin and levofloxacin did not. With respect to bacterial growth, removal of the antimicrobial agents influenced levofloxacin the most, with the mean increase in the bacterial counts of the six strains being 1.6 log cfu/mL.

In vivo efficacy against S. pneumoniae and H. influenzae

Modithromycin showed a dose-dependent in vivo efficacy against the pulmonary growth of erm(B) gene-carrying erythromycin-resistant S. pneumoniae KP058 (Table 3). A single oral administration of modithromycin at a dose of 30 mg/kg resulted in a significant reduction of viable bacteria in the lungs; the reduction was \(~\sim 5\) log cfu/g compared with the non-treated group. On the other hand, clarithromycin and azithromycin were inactive in this experimental model, reflecting their own higher MICs. At this dose, modithromycin was significantly better in bacterial reduction than telithromycin, as well as clarithromycin and azithromycin.

Modithromycin also showed in vivo efficacy against ampicillin-resistant H. influenzae KP097. Oral administration of modithromycin at a dose of 100 mg/kg/day for 4 days resulted in a significant reduction of viable bacteria in the lungs; the reduction was \(~\sim 4\) log cfu/g compared with the non-treated group. At this dose, modithromycin was significantly better in...
bacterial reduction than clarithromycin having the same MIC and had comparable efficacy to telithromycin, regardless of having a 4-fold higher MIC. Azithromycin showed the most potent efficacy among the antimicrobial agents tested.

**Discussion**

The percentage of streptococcal strains, such as *S. pneumoniae*, resistant to macrolide agents differs considerably from region to region. In this study, 86% and 21% of the test strains of *S. pneumoniae* and *S. pyogenes*, respectively, showed either intermediate resistance or resistance to erythromycin. These strains also showed cross-resistance to clarithromycin and azithromycin, with MICs of >64 mg/L. The percentages of resistant strains were approximately consistent with those of previous surveillance reports for Japan. Thus, the high resistance rate, particularly in *S. pneumoniae*, makes it difficult to use macrolide agents as a first-choice agent in Japan. On the other hand, the ketolide agent telithromycin has been developed to have potent activity against erythromycin-resistant streptococci. In this study, telithromycin showed excellent activity against erythromycin-intermediate-resistant and -resistant strains of *S. pneumoniae* (MIC of 0.25 mg/L), but not against erythromycin-intermediate-resistant and -resistant strains of *S. pyogenes* (MIC of 32 mg/L).

There are two predominant macrolide resistance mechanisms in Gram-positive organisms: target site modification of the 23S rRNA domain V caused by ribosome methylase; and active efflux pumps. The reason telithromycin retains antibacterial activity against erythromycin-resistant strains of *S. pneumoniae* has been shown to be caused by additive structural modifications of the alkyl-aryl side chain at the C-11 to C-12 cyclic carbamate and replacement of the cladinose sugar at C-3 with a ketone. This side chain enables additional binding interaction on domain II of 23S rRNA and sustains potent activity against erythromycin-resistant strains by binding to the...
methylated domain V. On the other hand, the replacement with a ketone prevents the acquisition of the resistance by overexpression of the efflux pump, which causes low- to middle-level resistance to 14- and 15-membered macrolide agents. The novel bicyclolide modithromycin also showed excellent antibacterial activity against the erythromycin-intermediate-resistant and -resistant streptococci, with MICs of \( \leq 1 \) mg/L. In particular, modithromycin was more active against \( \text{erm}(B) \) or \( \text{mef}(A) \) gene-carrying \( S. \text{pyogenes} \) than telithromycin. The improved activity of modithromycin against these resistant strains was considered to be due to its unique structural feature. Modithromycin, as well as telithromycin, contains the aromatic oxime side chain at the C-6 to C-11 bridged-linker and the ketone at the C-3 position, which would presumably contribute to the strong binding to the active sites and be little affected by the efflux pumps. The additional mechanisms of action for modithromycin against macrolide-resistant streptococci, including \( S. \text{pyogenes} \) which are less susceptible to telithromycin, need to be clarified by further evaluation.

\[ \text{H. influenzae} \] are also important pathogens that cause respiratory tract infections. Modithromycin showed potent in vitro activity against clinical strains of \( \text{H. influenzae} \), which was comparable to that of clarithromycin but inferior to those of telithromycin and azithromycin. In general, macrolide agents were not

![Figure 4. Antibacterial activity of modithromycin and other antimicrobial agents with an extracellular concentration of 2× MIC against epithelial cell-associated \( \text{H. influenzae} \) for 12 h of treatment (white bars), followed by removal of the agents from the apical medium and an additional 12 h of incubation (black bars). MIC ranges for the six strains were as follows: modithromycin (MOD), 4–8 mg/L; telithromycin (TEL), 1–2 mg/L; clarithromycin (CLR), 4–8 mg/L; azithromycin (AZM), 0.5–1 mg/L; and levofloxacin (LVX), 0.016 mg/L. #, significant difference between modithromycin- and other antimicrobial agent-treated groups after 12 h of treatment (\( P<0.05 \); Dunnett); *, significant difference between timepoints of 12 and 24 h in each antimicrobial agent-treated group (\( P<0.05 \); Welch).](image)

### Table 3. Therapeutic efficacy of modithromycin and other antimicrobial agents in the rat pulmonary infection model

<table>
<thead>
<tr>
<th>Organism (inoculum size)</th>
<th>Antimicrobial agent</th>
<th>MIC (mg/L)</th>
<th>Dose (mg/kg/dose)</th>
<th>Viable bacteria in the lungs (log cfu/g), mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Erythromycin-resistant} ) ( S. \text{pneumoniae} ) KP058 ( (3.8 \times 10^6 ) cfu/ra)a</td>
<td>modithromycin</td>
<td>0.125</td>
<td>30</td>
<td>4.77±0.72b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>6.65±0.96b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>8.36±0.76</td>
</tr>
<tr>
<td></td>
<td>telithromycin</td>
<td>0.063</td>
<td>30</td>
<td>6.03±0.70b,c</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>7.66±1.40b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>8.95±0.78</td>
</tr>
<tr>
<td></td>
<td>clarithromycin</td>
<td>( &gt;64 )</td>
<td>30</td>
<td>9.25±0.69b</td>
</tr>
<tr>
<td></td>
<td>azithromycin</td>
<td>( &gt;64 )</td>
<td>30</td>
<td>9.45±0.19b</td>
</tr>
<tr>
<td></td>
<td>non-treated</td>
<td>—</td>
<td>0</td>
<td>9.50±0.14</td>
</tr>
<tr>
<td>( \text{H. influenzae} ) KP097 ( (2.8 \times 10^6 ) cfu/ra)b</td>
<td>modithromycin</td>
<td>8</td>
<td>100</td>
<td>3.80±0.58b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30</td>
<td>6.96±0.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>6.99±0.57</td>
</tr>
<tr>
<td></td>
<td>telithromycin</td>
<td>2</td>
<td>100</td>
<td>3.63±0.32b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30</td>
<td>6.71±0.93</td>
</tr>
<tr>
<td></td>
<td>clarithromycin</td>
<td>8</td>
<td>100</td>
<td>6.31±1.28b,c</td>
</tr>
<tr>
<td></td>
<td>azithromycin</td>
<td>1</td>
<td>100</td>
<td>3.48±0.18b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30</td>
<td>4.75±1.12b,c</td>
</tr>
<tr>
<td></td>
<td>non-treated</td>
<td>—</td>
<td>0</td>
<td>5.86±1.52b</td>
</tr>
</tbody>
</table>

aAntimicrobial agents were orally administered 4 h after infection. The number of viable bacteria in the lungs was determined 24 h after infection (\( n=5 \)).
bSignificant difference between non-treated and antimicrobial agent-treated groups (\( P<0.05 \); Dunnett).
cSignificant difference between modithromycin- and other antimicrobial agent-treated groups at the same dose (\( P<0.05 \); Dunnett).
dAntimicrobial agents were orally administered 4, 24, 48 and 72 h after infection. The number of viable bacteria in the lungs was determined 96 h after infection (\( n=5 \)).
active enough against Gram-negative organisms compared with Gram-positive organisms. It is noted that one of the reasons for this difference in susceptibility is the presence of chromosomally encoded multicomponent type drug efflux pumps in Gram-negative organisms, which are engaged in intrinsic resistance to the macrolide agents.\textsuperscript{5} Sanchez et al.\textsuperscript{17} and Dean et al.\textsuperscript{18} have already reported that the chromosomally encoded AcrA-AcrB-ToIC efflux pump of \textit{H. influenzae} is closely linked to the intrinsic macrolide resistance, and the lack of these efflux pump components decreased the MIC of erythromycin $\sim$8–128 times in comparison with each parental strain, including clinical strains. Our results also showed that intrinsic resistance to not only macrolide agents but also bicyclolide modithromycin and ketolide telithromycin in \textit{H. influenzae} was conferred by this efflux pump, though the contribution to the resistance varied among these agents. Interestingly, as all macrolide agents tested showed similar MICs for the mutant strains, the ability to bind to the ribosome seemed to be similar among these antimicrobial agents against this strain.

In the rat pulmonary infection model, compared with telithromycin, modithromycin showed the greater or comparable efficacy against \textit{erm}(B)-carrying erythromycin-resistant \textit{S. pneumoniae} or ampicillin-resistant \textit{H. influenzae}, respectively, regardless of having an MIC that was 2 or 4 times higher for these strains. However, in the time–kill study, the antibacterial activities of modithromycin against both \textit{S. pneumoniae} and \textit{H. influenzae} were comparable to those of telithromycin at the same MIC concentration. To evaluate this discrepancy, we performed the experimental infection of epithelial cells with \textit{H. influenzae} strains. Van Schilfgaarde et al.\textsuperscript{19} have demonstrated that \textit{H. influenzae} invade the intra- and intercellular spaces of epithelial cells, followed by clustering, and they could be shielded from bactERICidal activity by antimicrobial agents having a poor cell penetration, such as for $\beta$-lactams and amino-glycosides. It means that the experimental cell infection model is beneficial for assessing the intracellular antibacterial activities and cellular pharmacokinetics of antimicrobial agents, which would better indicate the in vivo efficacies of the agents than in vitro time–kill studies. The present study has demonstrated that all the test agents except clarithromycin with an extracellular concentration of $2 \times \text{MIC}$ killed $\sim$2–2.5 log cfu/mL of cell-associated \textit{H. influenzae} for 12 h treatment. As for clarithromycin, the weakness of the antibacterial activity in the time–kill study might be reflected in the intracellular antibacterial activity against \textit{H. influenzae}. On the other hand, the removal experiment has shown that modithromycin as well as azithromycin inhibited the growth of cell-associated bacteria for an additional 12 h incubation, while telithromycin and levofloxacin did not. This lasting activity of azithromycin was considered to be caused by its cellular pharmacokinetic features. Kratzer et al.\textsuperscript{20} have demonstrated that the intracellular-to-extracellular concentration ratio of azithromycin is $\sim$4 and 10 times higher than that of telithromycin and quinolone agents (ciprofloxacin and moxifloxacin), respectively, after 8 h treatment using human bronchial epithelial cells. Similarly, by comparison of release from cells, Bosnar et al.\textsuperscript{21} have reported that azithromycin exhibits slower release from pre-loaded cells, such as phagocytic and epithelial cells, than telithromycin and clarithromycin. In addition, levofloxacin showed rapid cellular pharmacokinetics with respect to both influx into cells and efflux from cells compared with macrolide agents. These findings suggest that modithromycin would have the potential of high accumulation and retention in epithelial cells, similar to azithromycin. These considerations of cellular pharmacokinetics agree with the results of our in \textit{vivo} studies using the rat pulmonary infection model. In addition, it has been evaluated that the lung/plasma AUC ratio of modithromycin in rats was 68.7,\textsuperscript{22} which was significantly larger than that of telithromycin, which was reported to be 7.8 by Yamazaki et al.\textsuperscript{23} This high distribution rate of modithromycin into lung would also be partly due to the cellular pharmacokinetics noted above and might be correlated with the in \textit{vivo} efficacy. However, we must look more carefully into the cellular pharmacokinetics of other kinds of cells, such as phagocytic cells, which is known to be related to efficacious bacterial killings by macrolide agents at the site of infection.\textsuperscript{1,24} In addition, other possible reasons for lasting activity would be a post-antibiotic effect (PAE). Some researchers have demonstrated that the PAE of azithromycin is longer than that of clarithromycin against \textit{H. influenzae}, but no study has been conducted with modithromycin.\textsuperscript{25–27}

In summary, the novel bicyclolide modithromycin has better \textit{in vitro} activity against streptococci, particularly erythromycin-resistant \textit{S. pyogenes}, which are less susceptible to telithromycin. Moreover, modithromycin has better \textit{in vivo} efficacy against \textit{S. pneumoniae} and \textit{H. influenzae}, which might be due to its lasting intracellular activity. Modithromycin would be a useful antimicrobial agent for the treatment of respiratory infections caused by these pathogens.

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