Evaluation of Antimicrobial Activities of Clarithromycin and 14-Hydroxyclarithromycin against Three Strains of *Haemophilus influenzae* by Using an In Vitro Pharmacodynamic Model

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Received 25 October 1993/Returned for modification 17 January 1994/Accepted 26 June 1994

An in vitro pharmacodynamic model was used to simulate the in vivo pharmacokinetics of clarithromycin and 14-hydroxyclarithromycin in order to generate time-kill curves for three clinical isolates of *Haemophilus influenzae* (isolates 2019, 91-183, and 1746). Representative concentrations in serum or lung tissue and the pharmacokinetic parameters of clarithromycin and the 14-hydroxy metabolite, separately and in combination, were simulated for the time-kill studies. Amoxicillin-clavulanic acid was used as a control drug. The simulation of typical concentrations of the macrolides in serum in time-kill studies resulted in magnitudes of bacterial killing that were less than (for strains 2019 and 91-183, MICs = 4 mg/liter for clarithromycin and 14-hydroxyclarithromycin) or equal to (for strain 1746, MIC = 1 mg/liter for clarithromycin and 14-hydroxyclarithromycin) those observed in amoxicillin-clavulanic acid studies. When typical concentrations in lung tissue were simulated, total log decreases in bacterial counts were greater than those achieved with typical concentrations in serum and, in the case of strain 1746, exceeded the magnitude observed with the control drug. In each case, the time to 3-log-unit killing was longer for the macrolides than for amoxicillin-clavulanic acid. Time-kill curve analyses demonstrated the presence of synergy (defined as a 2-log-unit decrease in the CFU per milliliter between the combination and the most active constituent at any time point) for the combination of clarithromycin and 14-hydroxyclarithromycin at simulated concentrations in serum for one strain of *H. influenzae* (isolate 91-183). Synergism is likely bacterial strain specific, and the presence of synergy may be dependent on the antibiotic concentrations that are tested. Evaluation of the kill curve kinetics in terms of bactericidal rate for the various starting concentrations of clarithromycin did not result in a clear demonstration of either concentration-dependent or concentration-independent bactericidal activity.

Erythromycin has been used clinically since 1952 and has well-established efficacy in the treatment of many respiratory and cutaneous infections. However, gastrointestinal intolerance of the drug and variable activity against *Haemophilus influenzae* limit the usefulness of erythromycin. Clarithromycin, a new macrolide antibiotic, has been shown to be well tolerated and effective in the treatment of acute and mild to moderate upper and lower respiratory tract infections, including those caused by *H. influenzae* (7, 8, 11, 13). The active metabolite, 14-hydroxyclarithromycin, has been shown to inhibit strains of *H. influenzae* in an additive or synergistic fashion when tested in combination with the parent compound (4, 9, 12). Additionally, pharmacokinetic studies have shown that clarithromycin and 14-hydroxyclarithromycin achieve high therapeutic concentrations in lung and other respiratory tract tissues (5). As such, clarithromycin shows promise in the treatment of respiratory tract infections.

The purpose of the investigation described here was to perform pharmacodynamic time-kill studies of clarithromycin and 14-hydroxyclarithromycin, alone and in combination, against three different strains of *H. influenzae* in order to evaluate the issues of synergy and killing kinetics at clinically relevant antimicrobial concentrations.

**MATERIALS AND METHODS**

In vitro pharmacodynamic model. The in vitro pharmacodynamic model (Fig. 1) consists of a sealed glass vessel (volume, 1,020 ml) that is filled with an appropriate bacterial growth medium and fitted with input and output tubing (Masterflex LS thin wall tubing; Cole-Parmer Instrument Co., Chicago, Ill.) (6). The model is placed in a 37°C water bath positioned over a stir-hot plate (Nuovo II; Barnstead-Thermolyne Corp., Dubuque, Iowa); constant mixing of the medium is accomplished by placing a magnetic stir bar in the model. The desired peak concentrations of antibiotic are produced by bolus injection of a known quantity of drug into the model. First-order, one-compartment elimination kinetics are simulated by pumping antibiotic-free medium into the chamber via a peristaltic pump (Masterflex; Cole-Parmer Instrument Co.), thereby displacing an equal volume of antibiotic-containing medium. The pump rate necessary to simulate the proper drug elimination kinetics is calculated by using the following equation: clearance (CL) = k × Vd (Equation 1), where k is the elimination rate constant and Vd is the volume of distribution. The pump rate (in milliliters per minute) is equal to the product of the elimination rate constant (in minute⁻¹; k = 0.693/t1/2, where t1/2 is the half-life) and the volume of the model (in milliliters).

The elimination of two compounds with different half-lives can be simulated by using a dynamic reservoir of medium that contains the compound with the longer elimination half-life (Fig. 1) (3). Clearances are calculated for compounds A (the
TABLE 1. Characteristics of *H. influenzae* isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Clinical source</th>
<th>Type</th>
<th>Ampicillin susceptibilitya</th>
<th>β-Lactamase productionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>2019</td>
<td>Respiratory</td>
<td>Non-b</td>
<td>Susceptible</td>
<td>Negative</td>
</tr>
<tr>
<td>91-183</td>
<td>Respiratory</td>
<td>Non-b</td>
<td>Resistant</td>
<td>Positive</td>
</tr>
<tr>
<td>1746</td>
<td>Middle ear</td>
<td>b</td>
<td>Resistant</td>
<td>Positive</td>
</tr>
</tbody>
</table>

* a Performed by standard Kirby-Bauer disk diffusion method.
  b Performed by the cefinase assay.

TABLE 2. Time-kill kinetic studies performed for each bacterial strain

<table>
<thead>
<tr>
<th>Study</th>
<th>Antibiotic</th>
<th>Cmax (mg/liter)</th>
<th>Half-life (h)</th>
<th>Simulated kinetics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth control</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14</td>
<td>C</td>
<td>14</td>
<td>9</td>
<td>Lung</td>
</tr>
<tr>
<td>C3</td>
<td>C</td>
<td>3</td>
<td>3</td>
<td>Serum</td>
</tr>
<tr>
<td>HC7</td>
<td>HC</td>
<td>7</td>
<td>9</td>
<td>Lung</td>
</tr>
<tr>
<td>HC1</td>
<td>HC</td>
<td>1</td>
<td>6</td>
<td>Serum</td>
</tr>
<tr>
<td>C14 + HC7</td>
<td>C, HC</td>
<td>14, 7</td>
<td>9, 9</td>
<td>Lung</td>
</tr>
<tr>
<td>C3 + HC1</td>
<td>C, HC</td>
<td>3, 1</td>
<td>3, 6</td>
<td>Serum</td>
</tr>
<tr>
<td>Amox + CA</td>
<td>Amox, CA</td>
<td>8, 3</td>
<td>1, 1</td>
<td>Serum</td>
</tr>
</tbody>
</table>

* C, clarithromycin; HC, 14-hydroxyclarithromycin; numbers indicate concentrations (in milligrams per liter) Amox, amoxicillin; CA, clavulanic acid; Cmax, maximum (initial) antibiotic concentration.

solutions were bolus injected into each model in order to obtain the desired initial antibiotic concentrations.

**Bacterial strains.** Three clinical isolates of *H. influenzae* (isolates 2019, 91-183, and 1746; Table 1) were studied. *H. influenzae* isolates were nontypeable respiratory tract isolates that were susceptible and resistant to ampicillin, respectively. *H. influenzae* isolate was a type b, ampicillin-resistant strain isolated from a patient with a middle ear infection.

Bacteria were reconstituted from frozen stocks (−80°C) and were subcultured onto solid medium at least twice before use. Standardized cultures were prepared by inoculating 30 ml of broth medium with two to five colonies of *H. influenzae* that were grown on chocolate agar plates (Dimed, St. Paul, Minn.). This bacterial suspension was grown overnight at 37°C in a CO2-enriched environment (5 to 10%) and was then diluted 1:20 and grown to the turbidity of a McFarland 0.5 standard. This standardized suspension was diluted 1:10 in the in vitro model so that the initial inoculum in each experiment was 1 × 10⁷ to 2 × 10⁸ CFU/ml of exponentially growing bacteria.

**Growth medium.** Haemophilus Test Medium was used as the growth medium in each experiment (10). This was prepared from Mueller-Hinton broth (Difco, Detroit, Mich.), yeast extract (Difco), hematin (Sigma Chemical Co., St. Louis, Mo.), and β-NAD (Sigma). Magnesium and calcium were supplemented according to the guidelines of the National Committee for Clinical Laboratory Standards (10). Because of the decreased level of activity of clarithromycin at acidic pH, a 0.1 M phosphate buffer system (pH 7.2) was incorporated into the medium to prevent acidification upon bacterial growth (10).

**Quantification of viable bacteria.** Viable bacterial counts were determined by standard plate dilution techniques. Each broth sample was serially diluted (10-fold), and 100 μl of each dilution was plated on a chocolate agar plate (Dimed); all

TABLE 3. Summary of MIC and MBC data

<table>
<thead>
<tr>
<th>H. influenzae strain</th>
<th>Clarithromycin</th>
<th>14-Hydroxyclarithromycin</th>
<th>Clarithromycin and 14-hydroxyclarithromycin (2:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>isolate (mg/liter)</td>
<td>MBC (mg/liter)</td>
<td>MBC (mg/liter)</td>
<td>MBC (mg/liter)</td>
</tr>
<tr>
<td>2019</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>91-183</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>1746</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Results are for inocula of 10⁵ CFU/ml. Results of MICs and MBCs performed with inocula of 10⁶ CFU/ml were within 1 dilution tube of reported results.
plates were incubated overnight at 37°C in a CO₂-enriched (5 to 10%) environment. The colonies on each plate were counted; the plated dilution that yielded a colony count of between 30 and 300 was used to construct the time-kill curves. Inoculation of a plate with 100 μl of undiluted sample resulted in a lower limit of bacterial quantitation of 3 × 10⁵ CFU/ml.

**MIC and MBC determinations.** MICs and MBCs were determined by standard microtiter dilution techniques according to the current procedural recommendations of the National Committee for Clinical Laboratory Standards (10). Clarithromycin and 14-hydroxylclarithromycin were tested independently and in combination in a fixed 2:1 ratio. The medium used for MIC testing was the buffered Haemophilus Test Medium that was used in the in vitro model experiments. MICs and MBCs were determined in triplicate in inocula of both 2 × 10⁵ to 5 × 10⁵ and 1 × 10⁵ for all three strains of H. influenzae. Checkerboard MICs and MBCs for combinations of clarithromycin and 14-hydroxyclarithromycin (0.03 to 16 mg/liter for both compounds) were also determined in order to test for synergy (2).

**Time-kill kinetic studies.** Time-kill kinetic studies and growth control experiments were performed in the in vitro pharmacodynamic model. The pharmacokinetics of clarithromycin and 14-hydroxyclarithromycin, alone and in combination, in serum and lung tissue were simulated for each bacterial strain (Table 2) (5, 7). The pharmacokinetics of amoxicillin-clavulanic acid in serum were simulated as a reference. Eight different studies were initially performed for each bacterial strain; the antimicrobial concentrations used for these studies are given in Table 2 (5, 7). Experiments with initial clarithromycin concentrations of 8 and 50 mg/liter and simulated half-lives of 9 h were added in order to evaluate the dependency of killing rates on concentration.

Each study was a single-dose, 12-h, duplicate experiment with a starting bacterial inoculum of approximately 10⁷ CFU/ml. One-milliliter samples were drawn from the models at time zero; 30 s; 30 min; and 1, 2, 3, 4, 6, 9, and 12 h. Each sample was quantitatively analyzed for its bacterial count. Temperature and pH were recorded at each time point.

**Analysis.** Time-kill curve data were plotted as log CFU per milliliter versus time and were evaluated for total logarithmic decline and time to a 3-log-unit decline in bacterial numbers. Time-kill curves were also evaluated for the presence of a synergistic relationship between clarithromycin and the 14-hydroxy metabolite; synergy was defined as a 2-log₁₀-unit decrease in the number of CFU per milliliter for the combination in comparison with that for the most active constituent in the combination at any time point. Checkerboard MICs were evaluated for synergism on the basis of the sum of fractional inhibitory concentrations of clarithromycin and 14-hydroxyclarithromycin; a synergistic relationship was reported if the sum of the fractional inhibitory concentrations was less than 1 (2). The kill curves generated at different initial clarithromycin concentrations were analyzed by linear regression between 1 and 6 h in order to evaluate the dependency of bacterial killing on concentration.

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**FIG. 2.** Time-kill curves for *H. influenzae* 91-183 as simulated concentrations of clarithromycin (C) and 14-hydroxylclarithromycin (HC) in serum (A) and lung tissue (B). Amox/CA, amoxicillin-clavulanic acid.

**FIG. 3.** Time-kill curves for *H. influenzae* 2019 as simulated concentrations of clarithromycin (C) and 14-hydroxylclarithromycin (HC) in serum (A) and lung tissue (B). Amox/CA, amoxicillin-clavulanic acid.
RESULTS

Susceptibility testing. The MICs and MBCs of clarithromycin and 14-hydroxyclarithromycin for the study strains are given in Table 3. The MICs were within the ranges that have previously been reported for *H. influenzae* (7). Clarithromycin and 14-hydroxyclarithromycin were both bactericidal against all three strains of *H. influenzae*; MBCs were within 1 tube dilution of the MICs in all cases. No inoculum effect was noted; MICs and MBCs for high-inoculum (1 × 10^7 CFU/ml) tests were within 1 tube dilution of those for standard-inoculum (2 × 10^5 to 5 × 10^5 CFU/ml) tests.

Time-kill kinetic studies. Growth control curves performed for each bacterial strain demonstrated logarithmic growth to approximately 10^8 CFU/ml. The addition of the 0.1 M phosphate buffer to the medium did not affect the growth control curves. Time-kill curves for clarithromycin, 14-hydroxyclarithromycin, and amoxicillin-clavulanic acid against *H. influenzae* 2019, 91-183, and 1746 are illustrated in Fig. 2, 3, and 4, respectively. Results of the time-kill analyses are summarized in Table 4. Clarithromycin and 14-hydroxyclarithromycin produced consistent 3-log_{10} unit declines in bacterial counts only for *H. influenzae* 1746 (MIC, 1 mg/liter for both clarithromycin and 14-hydroxyclarithromycin). For the two bacterial strains for which the MICs were higher (4 mg/liter for both clarithromycin and the metabolite), clarithromycin and the 14-hydroxy metabolite were not consistently bactericidal (i.e., did not produce a 3-log_{10} unit decline in bacterial count). Amoxicillin-clavulanic acid produced rapid 3-log_{10} unit declines in bacterial counts for all three strains of *H. influenzae*; regrowth occurred after 6 h. In all cases, amoxicillin-clavulanic acid produced a faster bactericidal rate than clarithromycin or the 14-hydroxy metabolite.

Synergy studies. Synergy was defined as a 2-log_{10} unit decrease in the number of CFU per milliliter after treatment with the combination in comparison with that after treatment with the most active constituent in the combination at any time point in the time-kill study. These criteria were met for the simulated pharmacokinetics of clarithromycin in serum (maximum concentration of drug, 3 mg/liter; half-life, 3 h) and 14-hydroxyclarithromycin (maximum concentration of drug, 1 mg/liter; half-life, 6 h) in studies with *H. influenzae* 91-183. No evidence of synergism was noted upon analysis of time-kill studies of *H. influenzae* 2019 and 1746. Synergy was also noted against *H. influenzae* 91-183 in checkerboard MIC testing (the sum of the fractional inhibitory concentrations was less than one). Synergy was not noted against *H. influenzae* 2019 and 1746 by this method.

Concentration dependency studies. Experiments with initial clarithromycin concentrations of 3, 8, 14, and 50 mg/liter were evaluated in order to determine if clarithromycin killed in a concentration-dependent or a concentration-independent manner. The results of this analysis are given in Table 5. Bactericidal rate (defined as the slope of the linear regression line of the portion of the kill curve between 1 and 6 h) was highly correlated with the initial clarithromycin concentrations that were greater than the MIC for *H. influenzae* 91-183 (R^2 = 0.999). This relationship could not be demonstrated for the other *Haemophilus* strains.

**DISCUSSION**

Clarithromycin is a unique macrolide antibiotic in that it has a metabolite which has activity that is equal to or greater than that of the parent drug (7). The 14-hydroxy metabolite is of further interest because of the reported additive or synergistic relationships between the parent and the metabolite against *H. influenzae* (4, 9, 12). Synergism was noted by both time-kill and checkerboard MIC methods against one of the three *H. influenzae* strains tested in the present study. In the time-kill studies, the synergistic relationship of the drugs against this strain was present only for the simulated concentrations of clarithromycin and 14-hydroxyclarithromycin in serum. Simulation of higher concentrations in lung tissue resulted in time-kill curves that were almost identical for clarithromycin.

![Time-kill curves for *H. influenzae* 1746 as simulated concentrations of clarithromycin (C) and 14-hydroxyclarithromycin (HC) in serum (A) and lung tissue (B). Amox/CA, amoxicillin-clavulanic acid.](image-url)
14-hydroxyclarithromycin, and both compounds in combination. This suggests that the synergistic relationship does not improve a maximal or near-maximal effect of the individual components and may not be a clinically relevant consideration in the treatment of tissue infections when antibiotic concentrations are high.

Antibiotics are often classified as either concentration dependent or concentration independent (14). At antibiotic concentrations that are many times the MIC for bacteria, concentration-dependent antibiotics display an increased rate and extent of bacterial killing in comparison with those at concentrations near the MIC. In contrast, concentration-independent antibiotics have similar rates and extents of bacterial killing at concentrations near the MIC and at concentrations many times the MIC. Macrolide antibiotics have not been firmly established as either concentration dependent or concentration independent. Clarithromycin did not clearly exhibit either behavior in the present study. In most cases, the slope of the kill curve became more negative as the initial concentrations of clarithromycin were increased; however, this trend was variable. It is likely that concentration-independent behavior represents a saturation of concentration-dependent processes. This property may be variable within the range of the attainable concentrations of clarithromycin in serum and tissue.

The simulated concentrations of clarithromycin in lung tissue compared more favorably with those of amoxicillin-clavulanic acid than the simulated concentrations in serum in terms of the extent of bacterial killing. Bacterial killing was more rapid for amoxicillin-clavulanic acid than for clarithromycin and 14-hydroxyclarithromycin; this is likely an intrinsic difference between these two classes of antibiotics that is related to the different mechanisms of action. Regrowth was dramatic in the amoxicillin-clavulanic acid studies; however, regrowth occurred after 6 h, when amoxicillin and clavulanic acid concentrations had dropped to less than 0.125 and 0.05 mg/liter, respectively. Amoxicillin-clavulanate is typically administered at least every 6 h, and the regrowth phase of the kill curve after this time cannot be considered clinically relevant.

The limitations of the present study include the short duration and the one-dose nature of the time-kill curves as well as the limited number of strains studied. In general, the time-kill curves generated in the present study demonstrate that many strains of *H. influenzae* might effectively be treated with clarithromycin if antibiotic and metabolite concentrations at the site of infection are adequate.

**ACKNOWLEDGMENTS**

This study was supported by a grant from Abbott Pharmaceuticals.

**REFERENCES**