Bactericidal activity of amoxicillin against non-susceptible *Streptococcus pneumoniae* in an *in vitro* pharmacodynamic model simulating the concentrations obtained with the 2000/125 mg sustained-release co-amoxiclav formulation

David Sevillano¹, Almudena Calvo¹, María-José Giménez¹, Luis Alou¹, Lorenzo Aguilar¹, Eva Valero¹, Antonio Carcas² and José Prieto¹*

¹Microbiology Department, School of Medicine, Universidad Complutense, Madrid; ²Pharmacology Unit, Universidad Autónoma, Madrid, Spain

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**Objectives**: To investigate the bactericidal activity against *Streptococcus pneumoniae* of simulated amoxicillin serum concentrations obtained in humans after 2000/125 mg sustained-release (SR) and 875/125 mg co-amoxiclav administered twice and three times a day, respectively.

**Methods**: An *in vitro* computerized pharmacodynamic simulation was carried out and colony counts were determined over 24 h. Ten strains non-susceptible to amoxicillin (four of them exhibiting an MIC of 4 mg/L, five strains with an MIC of 8 mg/L and one strain with an MIC of 16 mg/L) were used.

**Results**: With amoxicillin 2000 mg, an initial inoculum reduction >99.99% was obtained for strains with an MIC of 4 mg/L, >99% for strains with an MIC of 8 mg/L and 70.6% for the strain with an MIC of 16 mg/L at 24 h sampling time. At this sampling time, no reduction of initial inocula was obtained with amoxicillin 875 mg/8 h for two of the four strains with an MIC of 4 mg/L, three of the five strains with an MIC of 8 mg/L, three of the five strains with an MIC of 8 mg/L or for the strain with an MIC of 16 mg/L.

**Conclusions**: The new co-amoxiclav 2000/125 mg SR formulation appears to offer advantages versus previous formulations with respect to bactericidal activity against current amoxicillin non-susceptible strains.

Keywords: β-lactams, pneumococcal isolates, inocula decrease

**Introduction**

A pharmacokinetically enhanced formulation of co-amoxiclav (16:1) that gives a time over MIC of at least 50% for strains with an amoxicillin MIC of 4 mg/L, with a twice daily administration for adults, has been developed.¹

Because the bactericidal activity of an antibiotic is important for pathogen eradication, and the more potent an antimicrobial agent the less likely is resistance selection,² the aim of this study was to investigate the bactericidal activity of this new formulation. In this study, we report the bactericidal activity of this new amoxicillin formulation in an *in vitro* model, as compared with the activity obtained with the previous 875/125 mg formulation, by simulating serum concentrations obtained in humans subsequent to those formulations.

**Materials and methods**

**Bacterial strains**

Ten strains of *Streptococcus pneumoniae* [four of them with an amoxicillin MIC of 4 mg/L (two serotype 9, one serotype 14 and one serotype 23), five with an MIC of 8 mg/L (one serotype 6, two serotype 14, one serotype 9 and one serotype 23) and one strain with an amoxicillin MIC of 16 mg/L (serotype 6)] were used. All strains were clinical isolates from community-acquired respiratory tract infections.

**Antibiotic**

Amoxicillin trihydrate laboratory reference standard was supplied by GlaxoSmithKline (Worthing, England, UK).

*Corresponding author. Tel: +34-91-3941508; Fax: +34-91-3941511; E-mail: jprieto@med.ucm.es
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MIC determination

MICs and MBCs were determined by a microdilution method following NCCLS methodology in Mueller–Hinton broth (Difco Laboratories, Detroit, MI, USA) supplemented with calcium, magnesium and 5% lysed horse blood. Additionally, MICs (prior to and after the simulation process) were determined in Todd–Hewitt broth (Difco Laboratories) supplemented with 0.5% yeast extract, using dilution steps of 1 mg/L. All determinations were performed five times and modal values are reported.

In vitro kinetic model

The model derives from the two-compartment kinetic model with artificial capillary units proposed by Blaser et al. and was designed to expose bacteria to changing antibiotic concentrations, without dilution of the bacterial inoculum. In brief, the model consists of a sterile central compartment (spinner flask, tubing and lumina of capillary bundles) representing the systemic circulation and a peripheral compartment representing the infection site (the intra-capsular circulat- ing tubing and the extra-capillary space between the two connected hollow fibre capsule filters of 0.2 μm pore size and 550 cm² filtering surface; FiberFlo, Minntech Corp, Minneapolis, MN, USA). The selectively permeable capillary walls (capsule filters of 0.2 μm pore size) allow the bi-directional diffusion of antibiotics and nutrients between the central and peripheral compartments, but prevent bacterial penetration into the central compartment. Capsules are placed in a 37°C incubator. Computerized peristaltic pumps (Maxterflex; Cole-Parmer Instrument Co., Chicago, IL, USA) draw the Todd–Hewitt broth medium, at a programmed rate, from the reservoir (placed in a 37°C water bath [HB 4 basic; IKA, Staufen, Germany]) to the central compartment for antibiotic dilution. A computer-controlled syringe pump (402 Dilutor Dispenser; Gilson SA, Villiers-le-Bel, France) allows the simulation of concentrations in serum, by infusion of the antibiotic into the central compartment until the C\text{max} is reached. The antibiotic-containing medium is pumped at 6 mL/min to the peripheral compartment, where it passes through the capillary walls and is distributed in the extra-capillary space (where the antibiotic interacts with bacteria) by unidirectional pressure diffusion. Additional pumps circulate the antibiotic–medium mixture at 25 mL/min within the extra-capillary space, through the intracapsular circulating tubing. Afterwards the mixture is re-circulated back to the central compartment via a second hollow fibre capsule, in the opposite direction. The elimination of the medium is at the same rate as the replacement of fresh medium in the central compartment, which maintains a constant volume in the system (400 mL), allowing the simulation of the antibiotic half-life (t\text{1/2}).

Kinetic simulations

Pharmacokinetic profiles of amoxicillin corresponding to the new co-amoxiclav 2000/125 mg sustained-release (SR) formulation (2000 mg of amoxicillin/12h) and to the conventional 875/125 mg formulation (875 mg of amoxicillin/8h) were simulated over 24 h. The flow rates of the peristaltic pumps were programmed to obtain a clearance of 1.38 mL/min in the first phase (0–5h) of the amoxicillin 2000 mg simulation and a clearance of 4.2 mL/min for the elimination phase (5–12h) and at 4.2 mL/min for amoxicillin 875 mg. These two flows in the peristaltic pumps were synchronized over 24h using Win Lin software (Cole-Parmer Instrument Co., Chicago, IL, USA).

Experiments

Prior to each experiment, 1–2 colonies from a fresh passage on Mueller–Hinton agar supplemented with cations and 5% lysed sheep blood, were inoculated in 20 mL of Todd–Hewitt broth supplemented with 0.5% yeast extract. The resulting suspension was allowed to grow to a density of 10^5 cfu/mL, as measured by a UV spectrophotometer (Hitachi U-1100); 15 mL of this inoculum was introduced into the peripheral compartment of the in vitro model 1 h prior to each simulation process to allow the microorganism to adapt to the medium. All initial inocula were in the range 2.5 × 10^6–2.5 × 10^7 cfu/mL.

Samples (0.5 mL) from the peripheral compartment were collected at 0, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 12.5, 13, 13.5, 14, 16, 18, 20, 22 and 24 h. Each sample was serially diluted 10-fold in 0.9% sodium chloride for bacterial counting in supplemented Mueller–Hinton agar with 5% sheep blood, which was incubated at 37°C, 5% CO2 for 24 h. At least five dilutions for each sample (including the non-diluted sample) were plated in triplicate. The limit of detection was 5 × 10^5 cfu/mL, and each experiment was performed in triplicate.

Pharmacokinetic analysis

Pharmacokinetic analysis was performed in triplicate, in bacteria-free simulations under the same experimental conditions. Samples (0.5 mL) from the peripheral compartment were obtained at 0, 0.5, 1, 1.5, 3, 4, 6, 8, 11, 12 and 12 h for amoxicillin SR/12 h and at 0, 0.5, 1, 1.5, 3, 4, 5 and 8 h for amoxicillin 875 mg/8 h. Concentrations were determined by bioassay using Micrococcus luteus ATCC 9341 as an indicator organism. All standards and samples were assayed in triplicate. The lower limit of detection was 0.06 mg/L, and the coefficient of variation between assays was 6.72% for the amoxicillin 2000 mg experiments and 7.61% for the amoxicillin 875 mg experiments.

Amoxicillin concentrations were analysed by a non-compartmental approach using the WinNonlin Professional program (Pharsight, Mountainview, CA, USA). Briefly, C\text{max} and T\text{max} were obtained directly from the observed data; the apparent elimination rate constant (k\text{el}) was calculated as the best-fit slope obtained from linear regression using the last measurements in the terminal phase of the curve (at least three time-concentration pairs). The area under the concentration-time curve from 0 to last (C\text{last}) measured concentration (AUC\text{last}) was calculated by the trapezoidal rule. The area under the concentration-time curve (AUC) was calculated as AUC\text{last} + C\text{last}/k\text{el}. The time that concentrations exceeded the 1 mg/L step MIC (t > MIC) for each strain was calculated graphically by plotting mean concentrations at each time point versus time.

Statistical analysis

Mean cfu/mL were calculated from the three values of colony counts at each time during the 24 h simulation. Initial inoculum reduction (IIR) at a determined time point was calculated as a percentage reduction in bacterial count compared with the initial inoculum.

Differences in %IIR between treatments at each time point were determined by the Mann–Whitney U-test. A P < 0.05 was considered statistically significant.

Results

The mean serum concentrations simulated over time were 9.65, 16.09, 13.34, 11.49, 7.89, 2.20, 0.40 and 0.12 mg/L at
0.5, 1.5, 3, 4, 6, 8, 11 and 12 h, respectively, for the 2000 mg amoxicillin formulation and 5.04, 12.19, 6.43, 3.83, 1.01 and 0.06 mg/L at 0.5, 1.5, 3, 4, 5 and 8 h, respectively, for the 875 mg amoxicillin formulation. Maximum serum concentrations (C<sub>max</sub>) were 16.1 ± 0.3 mg/L and 12.2 ± 2.0 mg/L and areas under the serum concentration-time curve (AUC) were 84.0 ± 5.1 mg·h/L and 33.0 ± 4.9 mg·h/L for amoxicillin 2000 and 875 mg, respectively. These values were within the range obtained in humans after administration of the doses simulated in this study, and in other pharmacodynamic simulations.<sup>7</sup>

When MICs/MBCs were determined using dilution steps of 1 mg/L, values obtained were (mg/L): 4/4 (two strains); 4/6 and 4/7 for strains with a standard MIC of 4 mg/L; 6/7, 6/8, 7/9, 8/8 and 8/9 for strains with a standard MIC of 8 mg/L; 9/9 for the strain with a standard MIC of 16 mg/L. Final isolates recovered at the end of the study showed the same (or one-step higher) MIC values as the initial isolates.

The time (% dosing interval) that serum levels exceeded the 1 mg/L step MIC (t > MIC) were in the range 19.16%–44.89% for amoxicillin 875 mg and 41.38%–59.3% for amoxicillin 2000 mg, for strains exhibiting MICs of 9–4 mg/L, respectively.

Mean reductions (%) in initial inocula during the 24 h study period are shown in Table 1, for the four strains with a standard MIC of 4 mg/L and the six strains with standard MIC >8 mg/L. In the absence of amoxicillin, the initial inocula increased (considering 0% was at 0 h) to 15194 ± 14968% at 8 h and to −10296 ± 6938% at 12 h for strains with an MIC of 4 mg/L, and to −17094 ± 15038% at 8 h and to −3846 ± 4059% at 12 h for strains with an MIC ≥8 mg/L.

Significant differences between both regimens (Table 1) for the strains with a standard MIC of 4 mg/L were found at the time points 20–24 h. The same differences were observed for strains with an MIC ≥8 mg/L from 6–12 h and from 16–24 h, with no reduction in colony counts in the latest periods for amoxicillin 875 mg/8 h.

By 24 h, amoxicillin 2000 mg produced an inoculum decrease ≥99.99% for the four strains with an MIC of 4 mg/L, 98.95%–99.94% for the five strains with an MIC of 8 mg/L and 70.64% for the strain with an MIC of 16 mg/L. With amoxicillin 875 mg/8 h, regrowth is shown by negative values.

<sup>a</sup>P = 0.028 versus 875 mg at the indicated times.

<sup>b</sup>P ≤ 0.005 versus 875 mg at the indicated times.

Table 1. Mean reductions of initial inocula over 24 h against the four strains with MIC 4 mg/L and the six strains with standard MIC ≥8 mg/L.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Amoxicillin SR 2000 mg</th>
<th>Amoxicillin 875/125 mg</th>
<th>Amoxicillin SR 2000 mg</th>
<th>Amoxicillin 875/125 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>1</td>
<td>95.00 ± 3.23</td>
<td>92.85 ± 5.89</td>
<td>89.57 ± 7.27</td>
<td>79.06 ± 36.64</td>
</tr>
<tr>
<td>3</td>
<td>99.63 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.53 ± 2.67</td>
<td>96.38 ± 4.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>99.06 ± 1.02</td>
</tr>
<tr>
<td>8</td>
<td>≥99.99 ± 0.01</td>
<td>99.37 ± 0.65</td>
<td>99.99 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>98.31 ± 2.76</td>
</tr>
<tr>
<td>12</td>
<td>99.97 ± 0.06</td>
<td>99.85 ± 0.16</td>
<td>99.50 ± 0.67</td>
<td>94.84 ± 8.78</td>
</tr>
<tr>
<td>16</td>
<td>99.98 ± 0.05</td>
<td>88.62 ± 22.41</td>
<td>97.04 ± 0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38.65 ± 79.37</td>
</tr>
<tr>
<td>20</td>
<td>≥99.99 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.37 ± 142.33</td>
<td>99.92 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34.93 ± 98.75</td>
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<tr>
<td>22</td>
<td>≥99.99 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.58 ± 65.72</td>
<td>98.71 ± 2.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−300.17 ± 546.35</td>
</tr>
<tr>
<td>24</td>
<td>≥99.99 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>−1310.55 ± 1741.21</td>
<td>94.78 ± 11.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−2482.24 ± 3446.52</td>
</tr>
</tbody>
</table>

Regrowth is shown by negative values.

<sup>a</sup>P = 0.028 versus 875 mg at the indicated times.

<sup>b</sup>P ≤ 0.005 versus 875 mg at the indicated times.

Figure 1. Mean values and standard deviations of reductions in cfu/mL (log<sub>10</sub>) at 24 h obtained with the amoxicillin 875 mg/8 h (black bars) and amoxicillin SR 2000 mg/12 h (diagonally striped bars) formulations against strains grouped by 1 mg/L step MIC.
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no reduction in counts at 24 h was obtained in two out of four strains with an amoxicillin MIC of 4 mg/L, in three out of five strains with an MIC of 8 mg/L or in the strain with an MIC of 16 mg/L.

Figure 1 shows mean values and standard deviations of reductions in cfu/mL (log_{10}) at 24 h obtained with both formulations against strains as grouped by 1 mg/L step MIC.

Discussion

Although co-amoxiclav 2000 mg SR has shown a good clinical efficacy in community-acquired pneumonia, in such studies there would be only a small number of patients infected with pneumococci that have a high amoxicillin MIC. Since it is important to investigate situations for which clinical trials cannot provide an adequate response, in this case, because the probability of pathogens with a reduced susceptibility profile is low, the pharmacodynamic simulation reported here was carried out.

We chose a pharmacokinetic–pharmacodynamic approach based on killing curves as this is considered a more rational approach to describe drug–bacteria interactions than the classical MIC or 24 h MBC approach.

Five of the 10 strains exhibited no reduction in initial inocula at 24 h with the amoxicillin 875 mg regimen. The surviving subpopulation exhibited an MIC similar to the prior simulation and can be considered tolerant to the amoxicillin 875 mg concentration profile after this third dose. Seventy percent of clinical isolates of penicillin-resistant pneumococci exhibit defective lysis, the prevalence of tolerance being much higher than in isolates of penicillin-resistant pneumococci exhibit defective lysis, the prevalence of tolerance being much higher than in

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References