Postantibiotic and Sub-MIC Effects of Azithromycin and Isepamicin against Staphylococcus aureus and Escherichia coli

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Investigations of pharmacodynamic parameters (postantibiotic effect [PAE], sub-MIC effects [SMEs], etc.) have been progressively employed for the design of dosing schedules of antimicrobial agents. However, there are fewer in vivo than in vitro data, probably because of the simplicity of the in vitro procedures. In this study, we have investigated the in vitro PAE, SME, and previously treated (postantibiotic [PA]) SME (1/2 MIC, 1/4 MIC and 1/8 MIC) of azithromycin and isepamicin against standard strains of Staphylococcus aureus and Escherichia coli by using centrifugation to remove the antibiotics. In addition, the in vivo PAE and SME have been studied with the thigh infection model in neutropenic mice. Finally, in vivo killing curves with two dosing schedules were determined to examine whether the PAE can cover the time that antimicrobial agents are below the MIC. The two antimicrobial agents induced moderate-to-high in vitro PAEs, SMEs, and PA SMEs against S. aureus (>8 h) and E. coli (3.38 to >7.64 h). The in vivo PAEs were also high (from 3.0 to 3.6 h), despite the fact that isepamicin had lower times above the MIC in serum. Only azithromycin showed a high in vivo SME against the two strains (1.22 and 1.75 h), which indicated that the in vivo PAEs were possibly underestimated. In the killing kinetics, no great differences (<0.5 log10) were observed between the schedule that took the PAE into account and the continuous administration of doses. These results are comparable with those of other authors and suggest that these antimicrobial agents could be administered at longer intervals without losing effectiveness.

Over the last two decades, the number of studies of pharmacodynamic aspects of antimicrobial agents have progressively increased. The postantibiotic effect (PAE) is one of the parameters most extensively studied, and a great number of data have been obtained, especially in vitro. The PAE represents the suppression of growth of a microorganism after an exposure to an antimicrobial agent (16), and it is of important clinical interest, since longer dosing intervals could reduce toxicity and costs without a loss in effectiveness. To study this phenomenon in vivo, several models in animals have been used (12, 13, 20, 27, 29). The model of infection in the neutropenic mouse thigh (12) is one of the most employed models because it is faster and less laborious than others. We have previously observed long PAEs with quinolones (9) and aminoglycosides (18) by using this model, although meropenem did not induce significant values (8, 15). Other authors have also reported significant in vivo PAEs with macrolides (4) and aminoglycosides (4, 27) by using this model and others.

The effect of subinhibitory concentrations on microorganisms, previously treated (postantibiotic [PA] SME) or not (SME), is another important pharmacodynamic parameter (2). These sub-MIC concentrations may have a greater importance with some antimicrobial agents, such as the macrolides (with long half-lives) or the aminoglycosides (with high bactericidal activity). The SMEs have been studied in vitro with many antibiotics, including the groups studied here (19, 21–25). Azithromycin has been reported to exhibit in vitro long PA SMEs and SMEs against some respiratory tract pathogens (25), but isepamicin has not been investigated. Among the aminoglycoside antibiotics, only amikacin exhibited long PA SMEs and SMEs against some gram-negative microorganisms (24).

The study of the PA SME or SME in vivo has never been carried out according to the in vitro specifications or similar methods. However, complementary experiments to ensure that the suppression of the microorganism growth was a true PAE have generally been reported when the thigh infection model in mice had been performed (9, 18, 30). We have previously carried out these experiments and applied the formula of the in vitro SME to determine the analogous in vivo effect (9), but no significant values were obtained. In the case of macrolides, which have long half-lives, the importance of these assays may be greater.

The aim of the present study was to investigate the in vivo PAE, SME, and PA SME of azithromycin and isepamicin against standard strains of E. coli and S. aureus. Using the thigh infection model in neutropenic mice, we studied the in vivo PAE, as well as the possible in vivo SME (to ensure that the growth suppression was a true PAE). Finally, we applied this in vivo model to determine whether dosing schedules that include the PAE duration were as effective as continuous schedules that always maintained the levels of the antimicrobial agents in serum above the MIC.

MATERIALS AND METHODS

Microorganisms. S. aureus ATCC 25923 and E. coli ATCC 25922 were used in this study.

Antimicrobial agents. The antibiotics were obtained as reference powders with known potencies from the following pharmaceutical companies: isepamicin was obtained from Schering Plough S.A. (Madrid, Spain), and azithromycin was obtained from Pfizer S.A. (Madrid, Spain). Dilutions were made on the same day of the experiments. The MICs were determined by the microdilution standard method (14) in Mueller-Hinton (MH) broth.

Animals. Female BALB/c mice weighing 26 to 28 g were rendered neutropenic by intraperitoneal injection of cyclophosphamide (Laboratorios Fung, Madrid, Spain) at 150 and 100 mg/kg of body weight on days 0 and 3, respectively (12).

In vivo PAE. E. coli or S. aureus cells (10^7 CFU/ml in MH broth) in the logarithmic phase of growth were exposed to the drugs at 37°C in a shaking incubator for 0.5 to 1.5 h (depending on the activity against the strain) at the following therapeutic concentrations: E. coli, 80 mg/liter with azithromycin and
7 mg/liter with isepamicin; *S. aureus*, 20 mg/liter with azithromycin and 7 mg/liter with isepamicin.

One culture with the same microorganism was not exposed to the antimicrobial agent and remained as a nonexposed control culture.

The antimicrobial agent was removed by centrifugation (5 min at 1,200 × g). Nonexposed control cultures were also centrifuged. The treated and control cultures were then both divided into four tubes, with one of each used to determine the PAE by reincubation at 37°C for another 10 h. Samples were withdrawn, and viable bacteria were determined every hour. Each antibiotic–microorganism combination was studied at least two times on different occasions. The PAE was calculated by the formula (4)

\[
P_{\text{AE}} = \frac{T_{\text{PA}}}{C}
\]

where \(T_{\text{PA}}\) is the time required for the mean count of CFU in the thighs of pretreated and control mice, respectively, to increase by 1 log10 above its initial value. This formula is similar to that described by Odendahl et al. (23) to determine the in vitro SME.

In vivo killing curves. After the determination of the PAEs, their application in the dosing schedules was evaluated according to the in vivo killing kinetics. Neutropenic mice were divided into three different groups. All of them were inoculated i.m. into one thigh with 0.1 ml of a bacterial suspension (10^6 to 10^7 CFU/ml) in the log phase of growth. Two hours later (time zero), 0.2 ml of saline solution with the antimicrobial agent was injected subcutaneously (s.c.) into the treated mice, with saline solution administered alone to the other group (control group).

The second treated group was injected with the same doses approximately every time that drug levels in serum fell below the MIC for this antibiotic as follows: isepamicin, at time zero and every 1.1 h for *S. aureus* and *E. coli*; azithromycin, at time zero every 4.5 h for *S. aureus* and every 1.7 h for *E. coli*.

The first treated group was then injected s.c. with the antimicrobial agent every time that drug levels in serum fell below the MIC for this antibiotic as follows: isepamicin, at time zero and every 1.1 h for *S. aureus* and *E. coli*; azithromycin, at time zero every 4.5 h for *S. aureus* and every 1.7 h for *E. coli*.

Groups of three to four animals from the treated and control groups were then killed every hour for the 1st 4 h and every 2 h up to the 10th h after drug administration. At each sampling time, thigh muscles were removed and homogenized in 9 ml of ice-cold 0.85% NaCl, and viable counts on MH agar plates were determined. The in vivo PAE was calculated by the formula (27)

\[
P_{\text{AE}}(t) = C_{\text{pre}} \times e^{-k \cdot t}
\]

where \(C_{\text{pre}}\) and \(C_{\text{post}}\) are the concentrations of the antimicrobial agent in serum at time zero and at times zero, 4, and 8 h for *E. coli*; azithromycin, at times zero and 8 h for *S. aureus* and at times zero and 5 h for *E. coli*.

### RESULTS

**MICs.** The MICs of azithromycin and isepamicin for *S. aureus* and *E. coli* are shown in Table 1. Since macrolide antibiotics have no great activity against gram-negative strains, the MIC of azithromycin for *E. coli* was moderate (8 mg/liter).

<table>
<thead>
<tr>
<th>Antimicrobial agent and microorganism</th>
<th>MIC (mg/liter)</th>
<th>Concentration (mg/liter)</th>
<th>Time pre-exposure (h)</th>
<th>PAE (h)</th>
<th>SME (h) at indicated fraction of MIC</th>
<th>PA SME (h) at indicated fraction of MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/2</td>
<td>1/4</td>
</tr>
<tr>
<td><strong>Isepamicin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>2</td>
<td>7</td>
<td>1</td>
<td>2.30</td>
<td>6.11</td>
<td>&gt;9.28</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>2</td>
<td>7</td>
<td>0.5</td>
<td>1.50</td>
<td>1.97</td>
<td>1.72</td>
</tr>
<tr>
<td><strong>Azithromycin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>2</td>
<td>20</td>
<td>1</td>
<td>2.20</td>
<td>4.51</td>
<td>&gt;8.08</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>8</td>
<td>80</td>
<td>1.5</td>
<td>3.50</td>
<td>4.74</td>
<td>&gt;7.64</td>
</tr>
</tbody>
</table>

In vitro PAE, SME, and PA SME. The results of all experiments are shown in Table 1. All of the in vitro PAEs were moderate, those of azithromycin generally being longer than those of isepamicin (2 h more with *E. coli*).

Generally, the SME and PA SME values were also long, even if the PAE was subtracted from them. Isepamicin induced longer PAEs and SMEs on *S. aureus* than on *E. coli*, with PA SMEs greater than 8 h at all fractions of MICs. Nevertheless, the time of preexposure was also shorter (0.5 h), and it was reduced because it was too bactericidal and no counts were obtained in the PA SME assays. On the other hand, azithromycin induced longer PAEs and SMEs on *E. coli* (with 7 MIC longer than *S. aureus*), while the latter microorganism showed higher PA SMEs (greater than 8 h).

Some PA SMEs were not determined exactly, because at the
end of the experiment, the cultures did not increase 1 log_{10} CFU. The differences among the times of preexposure were due to previous results that indicated the exposures that were adequate (not excessively bactericidal).

In vivo PAE. Figure 1 shows the in vivo PAE curves of the two antimicrobial agents, and Table 2 shows the results of the in vivo delay of growth. The highest PAE was that of isepamicin against S. aureus (3.6 h), but it was very similar to those of azithromycin (3.5 h). Although all values are highly significant, the PAE/T>MIC ratio is very low for azithromycin against S. aureus in comparison with those of the other microorganisms-drug combinations (0.77 against 2.05 to 3.27).

The pharmacokinetic parameters are also shown in Table 2. The peak drug levels were rapidly reached (0.25 to 1 h), while the AUCs were similar for isepamicin and the 20-mg/kg dose of azithromycin.

In vivo determination of the SME. The in vivo SMEs are also shown in Table 2. The negative values can be explained by the different levels of growth of the microorganisms in the different mice, and only when isepamicin was assayed against S. aureus was a significant negative value (~0.5 h) found. On the other hand, azithromycin induced high SMEs against the two strains tested. The values of 1.75 and 1.22 h (with S. aureus and E. coli, respectively) could indicate that azithromycin is highly bacteriostatic in subinhibitory concentrations.

In vivo killing curves. Table 3 shows the lethal effects of azithromycin and isepamicin on S. aureus and E. coli with the two schedules of antimicrobial administration. There were not large differences between the two dosing schedules. The greatest difference was observed with isepamicin and S. aureus, but it was not greater than 0.5 log_{10} CFU. The results are also shown in Fig. 2, where the administrations in schedule B (which takes into account the PAE) are pointed out.

The total doses administered are also shown in Table 3. It can be observed that schedule B reduced the amount of antimicrobial agent administered by 33 to 80%, corresponding to the largest reductions with isepamicin.

### DISCUSSION

The PAE is one of the most important and best known pharmacodynamic parameters. This effect has been intensively studied since it was described in the late 1940s (1, 6). However, there are fewer data available in vivo than in vitro (4), probably because of the simplicity of the in vitro procedures. The study of the in vivo PAE began when this effect was described (6), but reliable and standardized methods have not been employed until the last 2 decades (12, 16).

The thigh infection model in neutropenic mice has been one of the most employed, and a great amount of data have been obtained (3). With aminoglycosides, the PAEs against most of the standard strains have been considerably long. Against S. aureus, PAEs of 3.4 to 6.7 h have been obtained with gentamicin by this model (4, 11, 18), while a PAE of 2.3 h has been reported with amikacin and other techniques (27). With E. coli, a wide range of PAEs have been reported: 1.4 to 4.5 h with gentamicin (4, 11, 18), 1.7 to 2.9 h with netilmicin, and 1.8 to 2.1 h with tobramycin (10). With amikacin and with another model, a PAE of 3.8 h has been determined (27). Isepamicin, as well as netilmicin, is a compound related to gentamicin, and the PAEs obtained in the present study are similar to those of these two antibiotics.

The in vivo PAE of azithromycin has not yet been studied. The in vitro results show a moderate PAE from 2.2 to 4.7 h against other strains than those studied here (5, 25). However, the in vivo PAEs of other macrolides have already been investigated. Erythromycin induced PAEs up to 6.8 h against S. aureus and other gram-positive microorganisms (3, 4), and clindamycin also showed high values (7.1 h) with this strain (4). Nevertheless, the high values obtained with these macrolides could be due in part to their long periods above the MIC in serum, since they have long half-lives. In our study, this interval was also long in the case of S. aureus, despite the fact that its

### TABLE 2. Doses, pharmacokinetic parameters, times that levels of antimicrobial agent in serum exceeded the MIC, in vivo PAEs, and in vivo SMEs of isepamicin and azithromycin against S. aureus and E. coli

<table>
<thead>
<tr>
<th>Antimicrobial agent and microorganism</th>
<th>Peak drug level (µg/ml)</th>
<th>Time for peak level (h)</th>
<th>AUC (mg/liter · h)</th>
<th>Dose (mg/kg)</th>
<th>T&gt;MIC (h)</th>
<th>In vivo PAE (h)*</th>
<th>PAE/T&gt;MIC (h)</th>
<th>In vivo SME (h)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isepamicin</td>
<td></td>
<td></td>
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<tr>
<td>S. aureus</td>
<td>37.47</td>
<td>0.5</td>
<td>22.75</td>
<td>7</td>
<td>1.1</td>
<td>3.6 ± 0.4</td>
<td>3.27</td>
<td>−0.8 ± 0.4</td>
</tr>
<tr>
<td>E. coli</td>
<td>37.47</td>
<td>0.5</td>
<td>22.75</td>
<td>7</td>
<td>1.1</td>
<td>3.0 ± 0.4</td>
<td>2.72</td>
<td>−0.1 ± 0.3</td>
</tr>
<tr>
<td>Azithromycin</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>7.89</td>
<td>0.25</td>
<td>19.34</td>
<td>20</td>
<td>4.5</td>
<td>3.5 ± 0.5</td>
<td>0.77</td>
<td>1.75 ± 0.2</td>
</tr>
<tr>
<td>E. coli</td>
<td>82.61</td>
<td>1.00</td>
<td>96.89</td>
<td>80</td>
<td>1.7</td>
<td>3.5 ± 0.5</td>
<td>2.05</td>
<td>1.22 ± 0.1</td>
</tr>
</tbody>
</table>

* Each value represents the mean of three experiments ± standard error.
PAE was very similar to that of isepamicin (3.5 and 3.6 h, respectively).

The effect of subinhibitory concentrations is another important pharmacodynamic parameter. By standardized in vitro techniques, significant values have been observed with many antimicrobial agents (2), even though this activity is not the same against bacteria in the PAE phase (PA SME) or SME phase. These effects have been studied with three macrolides (including azithromycin) against respiratory tract pathogens (25), with values higher than 12 h against *Streptococcus pneumoniae*. In this study, azithromycin induced long SMEs and PA SMEs against the two microorganisms, but if the in vitro PAE is subtracted, the SMEs are clearly lower than those of *S. aureus* (>8 h).

The SMEs of the aminoglycosides have not been examined in detail. Only the PA SMEs and SMEs of amikacin against *E. coli* are reported (24), in which the values were higher (>22.3 h with 0.3 × MIC) than those obtained in the present assay with isepamicin (although the preexposure period was 2 h). Our PA SMEs against *S. aureus* were longer than 9.28 h, and the SMEs were also long. Although the sub-MICs for *E. coli* were lower than those for *S. aureus*, the relative effect on this microorganism is greater, because the time of preexposure is only 0.5 h. This lower time was chosen because no CFU counts were detected with an assay of 1 h.

The in vivo investigation of the SMEs is more difficult than in vitro, and consequently the number of investigations reported are considerably lower. In one of the first in vivo experiments, Oshida et al. (26) observed that the inactivation of amoxicillin by an injection of penicillinase in mice shortened the duration of the PAE. On the other hand, almost all of the in vivo PAE experiments performed with the thigh infection model in mice have included killing curves to examine the possible SMEs (9, 18, 26, 30). Moreover, those killing curves have been determined by a method similar to that used for our in vivo SME lethality curves, although they have not employed the formula described above. With this assay, some authors have observed that the subinhibitory concentrations of some antibiotics were active and increased the PAE period (26, 30). We were interested in carrying out an in vivo experiment similar to that employed in the in vitro PAE determination. In the design of the technique, we included the inoculation of in vitro-pretreated microorganisms approximately at the time that the antimicrobial drug levels in serum fell below the MIC, since it was not possible to inactivate these compounds. Unfortunately, in the present study, we employed microorganisms in the log phase because reliable results were not obtained, since microorganisms in the PAE phase did not infect the thigh and were rapidly killed (data not shown). Despite the use of microorganisms in the log phase, azithromycin induced a short significant SME against the two strains tested (1.75 and 1.22 h), suggesting that the 3.5 h determined for the in vivo PAE really reflects the combined action of sub-MICs and PAE.

The importance of this subinhibitory effect of azithromycin could be greater considering the following circumstances. First, the microorganisms were in the log phase instead of the PAE phase, with the in vitro PAEs of this antibiotic being considerably high. Second, the elimination rate in small animals is approximately six times higher than that in humans (25, 26), and the exposure time could be increased notably. Finally, the high accumulation of azithromycin in tissues (including polymorphonuclear leukocytes and macrophages) and posterior release (7, 10, 17) also increase the exposure time in some of these tissues. All of these factors, together with the action of the defensive system, indicate that longer dosing intervals for

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### TABLE 3. Total doses administered and lethal effects of two different administrations of isepamicin and azithromycin against *S. aureus* and *E. coli* on in vivo killing kinetics

<table>
<thead>
<tr>
<th>Antimicrobial agent and microorganism</th>
<th>Schedule A</th>
<th>Schedule B</th>
<th>Difference between schedules A and B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lethal effect (log_{10} CFU)</td>
<td>Total dose (mg/kg)</td>
<td>Lethal effect (log_{10} CFU)</td>
</tr>
<tr>
<td>Isepamicin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>−4.13 ± 0.3</td>
<td>70</td>
<td>−3.73 ± 0.4</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>−4.68 ± 0.4</td>
<td>70</td>
<td>−4.58 ± 0.5</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>−2.81 ± 0.4</td>
<td>60</td>
<td>−2.44 ± 0.3</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>−2.41 ± 0.4</td>
<td>400</td>
<td>−2.09 ± 0.5</td>
</tr>
<tr>
<td><em>E. coli</em></td>
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</table>

* Difference in log_{10} CFU between control and treated growth curves at the end of the in vivo killing kinetics.

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**FIG. 2.** In vivo killing kinetics of isepamicin and azithromycin. Isepamicin: ■, control; ●, 7 mg/kg at zero hour and every hour; ●, 7 mg/kg at zero hour and at the end of PAE plus time that drug levels in serum exceeded the MIC. Azithromycin: ■, control; ●, 20 mg/kg at time zero and every 4.5 h for *S. aureus* and 80 mg/kg at zero hour and every 2 h for *E. coli*; ●, 20 and 80 mg/kg for *S. aureus* and *E. coli*, respectively, at zero hour and at the end of the PAE plus time that drug levels in serum exceeded the MIC. The arrows represent drug administrations in schedule B.
this antimicrobial agent could be allowed. With this objective, we examined the in vivo killing curves with two different dosing schedules. In terms of the lethal effect, the schedule B treatment with azithromycin (which included the PAE) was only 13\% less effective than schedule A against the two strains. With isepamicin, which did not show SMEs in vivo, the effectiveness was even higher (90 to 97\%), probably because of its greater bactericidal (lethal) effect (Table 3).

We conclude that the pharmacodynamic parameters of azithromycin and isepamicin are important enough to have an influence on the dosing designs of these antimicrobial agents. They showed long PAEs not only in vitro but also in vivo, and the in vitro SMEs or PA SMEs also seem to be high; the in vivo SMEs are also significant in the case of azithromycin. The effectiveness could then be maintained, and longer dosing intervals could also reduce costs and toxicity (which is important in the case of aminoglycosides). However, further experiments should be performed to confirm these findings and study new ones (e.g., predictive pharmacokinetic parameter for efficacy and emergence of resistance).

REFERENCES


