"Pharmacodynamic evaluation of the intracellular activities of antibiotics against Staphylococcus aureus in a model of THP-1 macrophages"

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Abstract
The pharmacodynamic properties governing the activities of antibiotics against intracellular Staphylococcus aureus are still largely undetermined. Sixteen antibiotics of seven different pharmacological classes (azithromycin and telithromycin [macrolides]; gentamicin [an aminoglycoside]; linezolid [an oxazolidinone]; penicillin V, nafcillin, ampicillin, and oxacillin [beta-lactams]; teicoplanin, vancomycin, and oritavancin [glycopeptides]; rifampin [an ansamycin]; and ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin [quinolones]) have been examined for their activities against S. aureus (ATCC 25923) in human THP-1 macrophages (intracellular) versus that in culture medium (extracellular) by using a 0- to 24-h exposure time and a wide range of extracellular concentrations (including the range of the MIC to the maximum concentration in serum [C(max); total drug] of humans). All molecules except the macrolides caused a net reduction in bacterial counts that was time and concentr...
Pharmacodynamic Evaluation of the Intracellular Activities of Antibiotics against *Staphylococcus aureus* in a Model of THP-1 Macrophages

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The pharmacodynamic properties governing the activities of antibiotics against intracellular *Staphylococcus aureus* are still largely undefined. Sixteen antibiotics of seven different pharmacological classes (azithromycin and telithromycin [macrolides]; gentamicin [an aminoglycoside]; linezolid [an oxazolidinone]; penicillin V, nafcillin, ampicillin, and oxacillin [β-lactams]; teicoplanin, vancomycin, and oritavancin [glycopeptides]; rifampin [an ansamycin]; and ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin [quinolones]) have been examined for their activities against *S. aureus* (ATCC 25923) in human THP-1 macrophages (intracellular) versus that in culture medium (extracellular) by using a 0- to 24-h exposure time and a wide range of extracellular concentrations (including the range of the MIC to the maximum concentration in serum [C\(_{\text{max}}\); total drug] of humans). All molecules except the macrolides caused a net reduction in bacterial counts that was time and concentration/MIC ratio dependent (four molecules tested in detail [gentamicin, oxacillin, moxifloxacin, and oritavancin] showed typical sigmoidal dose-response curves at 24 h). Maximal intracellular activities remained consistently lower than extracellular activities, irrespective of the level of drug accumulation and of the pharmacological class. Relative potencies (50% effective concentration or at a fixed extracellular concentration/MIC ratio) were also decreased, but to different extents. At an extracellular concentration corresponding to their C\(_{\text{max}}\)s (total drug) in humans, only oxacillin, levofloxacin, garenoxacin, moxifloxacin, and oritavancin had truly intracellular bactericidal effects (2-log decrease or more, as defined by the Clinical and Laboratory Standards Institute guidelines). The intracellular activities of antibiotics against *S. aureus* (i) are critically dependent upon their extracellular concentrations and the duration of cell exposure (within the 0- to 24-h time frame) to antibiotics and (ii) are always lower than those that can be observed extracellularly. This model may help in rationalizing the choice of antibiotic for the treatment of *S. aureus* intracellular infections.

*Staphylococcus aureus*, which often causes chronic or relapsing diseases (68), is reported to persist as an opportunistic intracellular organism both in vitro and in vivo (8, 10, 18, 30, 31, 34, 39). Antibiotic treatments should therefore be optimized not only toward the extracellular forms of *S. aureus* but also toward the intracellular forms of *S. aureus* to avoid creating a niche where bacteria may persist, cause cell alterations, and possibly, be selected for resistance if they are exposed to subtherapeutic concentrations (1). A large body of literature on the activities of antibiotics against intracellular *S. aureus* in various cellular models is available (see references 54, 66, 67, and 70 for reviews). Yet, many of these studies yield contradictory results, and we still lack a clear understanding of which parameters are truly critical for the expression of antibiotic activity in the intracellular milieu (11). In a previous study, we measured the activities of selected antibiotics characterized by a fair to high level of cellular accumulation against intracellular *S. aureus* in a model of unstimulated murine J774 macrophages (57). We observed that cellular accumulation was only partially and nonconsistently predictive of activity. In a subsequent pilot study, performed with human THP-1 macrophages, we also noted that β-lactams, which notoriously do not accumulate in cells, actually showed significant activity against intracellular *S. aureus* when their extracellular concentration was brought to a sufficiently high but still clinically meaningful level (36). This triggered us to broaden and systematize our approach. For this purpose, we selected typical representatives of seven classes of antibiotics with known activities against *S. aureus* and included in commonly used guidelines for the handling of staphylococcal infections. We concentrated our effort on THP-1 macrophages because these cells present many of the characteristics of human monocytes while forming a homogeneous and reproducible population (6). THP-1 macrophages have been successfully used in various studies aimed at characterizing the interactions between *S. aureus* and macrophages in a clinical context (19, 28, 49) and to analyze the potential relationship between the accumulation of antibiotics in cells and intracellular activity (48). In contrast to many other models, however, we explored a large array of extracellular concentrations (including the range observed in the serum of patients receiving conventional doses) and used incubation times up to 24 h. Our purpose, indeed, was to analyze the pharmacodynamic parameters governing the activities of these antibiotics against intra-
cellular forms of *S. aureus* in terms of both the concentration/ 
MRC ratio and the time of exposure. This approach was 
thought to be necessary to enable us to draw pharmacologically 
as well as clinically meaningful conclusions. 

(Parts of this study were presented at the 43rd Interscience 
Conference on Antimicrobial Agents and Chemotherapy, 
Chicago, Ill., 14 to 17 September 2003 [M. Barcia-Macay, C. 
Seral, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van 
Bambeke, Abstr. 43rd Intersci. Conf. Antimicrob Agents 
Chemother., abstr. A-1174, 2003], and at the 44th Interscience 
Conference on Antimicrobial Agents and Chemotherapy, Washington 
D.C., 30 October to 2 November 2004 [M. Barcia-Macay, C. 
Seral, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van 
Bambeke, Abstr. 44th Intersci. Conf. Antimicrob Agents 
Chemother., abstr. A1488, 2004].)

**MATERIALS AND METHODS**

**Bacterial strain, susceptibility testing, and time and dose-kil" curve studies in 
extracellular medium.** *S. aureus* (strain ATCC 25923, fully susceptible) was used 
for all experiments. All conditions for measurement of the MICs (at pH 7.3 
and 5.0) and the minimal bactericidal concentrations (MBCs) were exactly 
the same as those described earlier (57). Dose-kil" curve studies were performed as 
described previously (57), with the following modifications: (i) RPMI 1640 medium 
supplemented with 10% fetal calf serum rather than broth was systematically 
used to measure the extracellular activities to better mimic a true extracellular 
environment, and (ii) enumeration of colonies (for determination of CFU) was 
performed with an automated detector (14). All samples (diluted as needed) 
were prepared in a final volume of 1 ml, of which 50 

**Antimicrob. Agents Chemother.**
Corresponding linezolid had higher MBCs (equal to or higher than their olones (except ciprofloxacin). In contrast, the macrolides and
volones (except ciprofloxacin). In contrast, the macrolides and
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lar concentration was set at the
reference 11 for a review). Whenever possible, the extracellu-
consistent with their known behavior in other cell types (see
whether the accumulation of antibiotics in THP-1 cells was
Validation of the intracellular model. We first examined
whether the accumulation of antibiotics in THP-1 cells was consistent with their known behavior in other cell types (see reference 11 for a review). Whenever possible, the extracellular concentration was set at the $C_{\text{max}}$ of the drug (as defined in Table 1), but poor assay sensitivity forced us to use higher concentrations for a number of molecules. The 24-h time point was selected since this corresponded to the maximal duration of our studies with infected cells. Data are presented in Table 2. Linezolid, $\beta$-lactams, and gentamicin showed no or only modest accumulation (from 0.5- to 4.4-fold). The quinolones, vancomycin, and teicoplanin reached slightly higher levels (5- to 10-fold). Rifampin, azithromycin, and telithromycin achieved higher levels (17- to 38-fold); and oritavancin accumulated up to almost 150-fold.

We then characterized the course of the infection of THP-1 cells by the strain of S. aureus that we used (Fig. 1). In the absence of antibiotic, the number of bacteria collected from cells (after the washing procedure) increased almost at the same rate as that for bacteria incubated in complete culture medium in the absence of cells (extracellular infection). The medium of the infected cells, however, showed visible acidification at 24 h (compared to the medium of the uninfected cells), with the number of viable bacteria in low speed super-

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (mg/liter) pH 7.3</th>
<th>MBC (mg/liter)</th>
<th>Dosage and route</th>
<th>Human $C_{\text{max}}$ (mg/liter) (Total drug)</th>
<th>% Free drug</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azithromycin</td>
<td>0.5</td>
<td>512</td>
<td>8</td>
<td>500 mg p.o.</td>
<td>0.5</td>
<td>88</td>
</tr>
<tr>
<td>Telithromycin</td>
<td>0.06</td>
<td>4</td>
<td>2</td>
<td>800 mg p.o.</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.5</td>
<td>16</td>
<td>2</td>
<td>6 mg/kg i.v.</td>
<td>18</td>
<td>80</td>
</tr>
<tr>
<td>Linezolid</td>
<td>2</td>
<td>4</td>
<td>32</td>
<td>600 mg i.v.</td>
<td>21</td>
<td>69</td>
</tr>
<tr>
<td>Penicillin V</td>
<td>0.015</td>
<td>&lt;0.015</td>
<td>0.06</td>
<td>500 mg p.o.</td>
<td>6.3</td>
<td>20</td>
</tr>
<tr>
<td>Nafcillin</td>
<td>0.25</td>
<td>0.06</td>
<td>1</td>
<td>1,000 mg i.v.</td>
<td>40</td>
<td>3</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0.06</td>
<td>0.03</td>
<td>0.25</td>
<td>1,000 mg i.v.</td>
<td>47.6</td>
<td>85</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>0.125</td>
<td>0.06</td>
<td>0.25</td>
<td>500 mg i.v.</td>
<td>63</td>
<td>10</td>
</tr>
<tr>
<td>Teicoplanin</td>
<td>0.25</td>
<td>0.5</td>
<td>64</td>
<td>12 mg/kg i.v.</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1</td>
<td>1</td>
<td>16</td>
<td>15 mg/kg i.v.</td>
<td>50</td>
<td>45–90</td>
</tr>
<tr>
<td>Oritavancin</td>
<td>0.25</td>
<td>0.25</td>
<td>1</td>
<td>3 mg/kg i.v.</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0.0075</td>
<td>0.002</td>
<td>0.03</td>
<td>600 mg i.v.</td>
<td>18</td>
<td>10–20</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.125</td>
<td>1</td>
<td>1</td>
<td>750 mg p.o.</td>
<td>4.3</td>
<td>63</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>0.125</td>
<td>1</td>
<td>0.125</td>
<td>400 mg p.o.</td>
<td>4</td>
<td>62–76</td>
</tr>
<tr>
<td>Garenoxacin</td>
<td>&lt;0.03</td>
<td>0.125</td>
<td>0.03</td>
<td>400 mg p.o.</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>0.06</td>
<td>0.25</td>
<td>0.06</td>
<td>400 mg p.o.</td>
<td>4</td>
<td>52</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Cellular accumulation</th>
<th>Extracellular concn (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azithromycin</td>
<td>37.8 ± 1.3</td>
<td>5e</td>
</tr>
<tr>
<td>Telithromycin</td>
<td>27.9 ± 1.3</td>
<td>2d</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>4.4 ± 0.1</td>
<td>25f</td>
</tr>
<tr>
<td>Linezolid</td>
<td>0.5 ± 0.0</td>
<td>25f</td>
</tr>
<tr>
<td>Penicillin V</td>
<td>1.2 ± 0.1</td>
<td>15f</td>
</tr>
<tr>
<td>Nafcillin</td>
<td>2.6 ± 0.1</td>
<td>400f</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>1.0 ± 0.1</td>
<td>15f</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>4.0 ± 0.1</td>
<td>25f</td>
</tr>
<tr>
<td>Teicoplanin</td>
<td>7.4 ± 0.2</td>
<td>15f</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>6.3 ± 0.1</td>
<td>100f</td>
</tr>
<tr>
<td>Oritavancin</td>
<td>148.0 ± 12.0</td>
<td>25d</td>
</tr>
<tr>
<td>Rifampin</td>
<td>17.6 ± 0.9</td>
<td>50f</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>5.1 ± 0.1</td>
<td>4.3d</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>7.0 ± 0.6</td>
<td>4d</td>
</tr>
<tr>
<td>Garenoxacin</td>
<td>9.1 ± 0.3</td>
<td>4d</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>7.6 ± 0.3</td>
<td>4d</td>
</tr>
</tbody>
</table>

The molecules are ranked by pharmacological classes, with each class appearing by order of its mean level of intracellular activity (as shown in Fig. 5).

$^a$ Commonly observed maximal concentration in serum (and estimated percentage of free drug) after intravenous or oral administration of conventional doses to humans, based on the references indicated.

$^b$ The molecules are ranked by pharmacological classes, with each class appearing by order of its mean level of intracellular activity (as shown in Fig. 5).

$^c$ p.o., oral; i.v., intravenous.


$^e$ A concentration larger than the $C_{\text{max}}$ was used because of a lack of sensitivity of the microbiological assay.

$^f$ Concentration corresponding to the $C_{\text{max}}$ (as defined in Table 1).
natants and washing media amounting to approximately 17% of the total sample content (medium plus cells). Based on previous experience with J774 macrophages (57), gentamicin was added to the culture medium to prevent this contamination. As shown in Fig. 1, a gentamicin concentration as low as 0.01 its MIC reduced the extracellular contamination to an almost negligible level, while it still allowed a marked increase in the number of cell-associated CFU (to about 65% of what was seen without antibiotic). A further increase in the extracellular concentration of gentamicin to its MIC allowed extracellular contamination to go to undetectable levels, but with a further decrease in the cell-associated CFU, demonstrating interference with the intracellular multiplication of the bacteria. Yet, optical and electron microscopy of cells incubated for 24 h with gentamicin at the MIC still revealed the presence of actively multiplying bacteria within membrane-bound structures, consistent with intraphagolysosomal localization (see Fig. 6). Because of all those uncertainties in the true level of intracellular growth of \textit{S. aureus} and the potential impact of even low concentrations of gentamicin, intracellular activities were therefore examined and expressed not as the difference from the controls but in terms of variations of the cell-associated CFU from the original, postphagocytosis inoculum. Finally, careful examination of the bacterial cultures obtained from cell samples exposed to gentamicin or to other antibiotics (see below) failed to identify so-called small-colony variants.

### Kinetics of antibacterial effects at a fixed, large concentration (C\textsubscript{max})
Time-kill curves were obtained for eight molecules selected on the basis of (i) their increasing MBC/MIC ratios (from 1 [moxifloxacin] to 33 [telithromycin]; see Table 1) when they were tested in broth and (ii) their increasing levels of cellular accumulation (apparent cellular-concentration-to-extracellular-concentration ratio from less than 1 [linezolid] to about 150 [oritavancin] in uninfected cells; see Table 2). The results are shown in Fig. 2. By first considering the extracellular activities, it appears that gentamicin, rifampin, and oritavancin acted very fast, with bacterial counts reaching the limit of detection within 6 h or less, whereas linezolid and telithromycin, although they were tested at concentrations equal or close to their MBCs, were only slowly and poorly bactericidal. Oxacillin, vancomycin, and moxifloxacin reached the limit of detection upon prolonged incubation. There was thus only a poor correlation between the rates and extents of killing and the MBC/MIC ratios. By next considering the intracellular bacteria, overall decreases in the rates and extents of killing of
intracellular bacteria compared to those of extracellular bacteria were observed, but some antibiotics were more affected than others. Only oritavancin, moxifloxacin, and oxacillin achieved bactericidal effects (as defined by a 2-log decrease from the original inoculum) at 24 h. Rifampin and gentamicin, which were highly bactericidal toward extracellular bacteria, did not reach this limit (and their intracellular activities were actually close to those of vancomycin and linezolid). Telithromycin was essentially bacteriostatic. There was no correlation between intracellular activity and cellular accumulation among the eight drugs tested.

**Kinetics and influence of concentration on antibacterial effects in the MIC-C<sub>max</sub> range.** Six molecules were then selected from among the bactericidal drugs to examine the influence of concentration on the rate and extent of killing (Fig. 3). By first considering extracellular activities, the extent of killing was significantly concentration dependent for all drugs over the range of concentrations investigated. The rate of killing also increased with concentration for all drugs except rifampin, for which a low concentration (but still above the MIC) caused the antibiotic activity to plateau after 6 h. By next considering the intracellular activities, both the rate and the extent of killing of intracellular bacteria were considerably reduced compared to those of extracellular bacteria; but significant concentration-dependent effects were still observed with respect to both of these parameters for vancomycin, oxacillin, and oritavancin and with respect to the extent of killing for moxifloxacin and rifampin. For gentamicin, an increase in the extracellular concentration from 5 to 18 mg/liter (10- to 36-fold the MIC) was without significant effect at 6 h but caused a modest, albeit statistically significant, increase in activity at 24 h, the extent of which remained, however, very limited.

These experiments were then repeated with all drugs included in this study but were limited to the examination of the 24-h time point and to three critical concentrations (the MIC, 10 times the MIC, and C<sub>max</sub>, except for rifampin, in view of its very low MIC [see the Fig. 4 legend for the concentrations of rifampin used]). The results are shown in Fig. 4 in a synoptic fashion for ease of direct comparison of the results between molecules and, for each molecule, between its extracellular and intracellular levels of activity. The data show that (i) the macrolides were always bacteriostatic toward both extracellular and intracellular bacteria, whichever concentration was tested; (ii) the largest discrepancy between extracellular and intracellular activities occurred for gentamicin; and (iii) oxacillin (among the four penicillins tested), levofloxacin, garenoxacin, and moxifloxacin (among the four quinolones tested) and oritavancin were bactericidal toward intracellular bacteria (and the level of activity was in that order) but had to be used at concentrations close to or equal to their C<sub>max</sub>s to achieve such an effect. There was, again, no simple correlation between intracellular bactericidal effects and the MBC/MIC ratios or the levels of cellular accumulation (as measured in uninfected cells).

**Wide range of concentration-effect relationships (pharmacological comparisons).** Four molecules (oxacillin, gentamicin, moxifloxacin, and oritavancin) were selected to obtain full pharmacological dose-response curves based on (i) their demonstrated dose-effect relationships in the MIC-C<sub>max</sub> range and (ii) their contrasting behaviors with respect to their intracellular activity/extracellular activity ratios. Figure 5 shows the results, with the regression parameters and a detailed statistical analysis presented in Table 3. Against extracellular bacteria, all four drugs displayed similar relative potencies (50% effective concentrations [EC<sub>50</sub>s]) (33) and static concentrations at about their MICs and 0.3 their MICs, respectively. Their relative efficacies (maximum effects [E<sub>max</sub>s]) were significantly different (oxacillin < moxifloxacin < oritavancin ≈ gentamicin). Against intracellular bacteria, all four drugs had significant decreases in their relative efficacies (E<sub>max</sub>s), but these decreases were roughly similar (E<sub>max</sub> against intracellular bacteria/E<sub>max</sub> against extracellular bacteria ratios, 0.42 [minimum] to 0.64 [maximum]; because we could not reliably assess inoculum decreases larger than 4.2 log and arbitrarily set all larger values to 5, these ratios may actually be overestimated for highly bactericidal antibiotics such as oritavancin and gentamicin). In contrast, the relative potencies (EC<sub>50</sub>s) were very differently affected, with oxacillin and moxifloxacin showing no significant change compared to their corresponding potencies against extracellular bacteria, whereas marked decreases (9- to 14-fold) in potency (indicated by an increase in EC<sub>50</sub>) were noted for gentamicin and oritavancin. This partially translated into an increase in the static concentrations of about 2-, 4-, 7-, and 17-fold for moxifloxacin, oxacillin, gentamicin, and oritavancin, respectively.

**Morphological studies.** Electron microscopy (Fig. 6) was used to examine the morphological changes of phagocytosed *S. aureus* after exposure to the antibiotics in order to ascertain that the decreases in the numbers of CFU seen in our experiments were associated with visible changes in the number and/or morphology of the bacteria. Oxacillin and oritavancin were selected for use in this study, since both are reported to act on cell wall biosynthesis and/or the cell wall structure, making their potential action on the bacteria more easily recognizable. In the absence of these antibiotics (but in the presence of gentamicin at the MIC, to fully avoid extracellular contamination), phagocytosed bacteria were darkly stained (Fig. 6A), often actively multiplying, and surrounded by a thick cell wall (Fig. B and C). In cells incubated with oxacillin, a large number of intracellular bacteria appeared as ghosts with a rarefied cytoplasmic material (Fig. 6D and E) or with large, electron-lucent vacuoles (Fig. 6F). Ghosts were also commonly observed in infected cells incubated with oritavancin (Fig. 6G), with profiles often showing granular material sometimes opposed on the periphery of the bacterial body (Fig. 6H).

**DISCUSSION**

The data presented in this paper underline three main properties of antibiotics in relation to their intracellular activities that may not have been sufficiently detected in previous studies because of an insufficient duration of exposure and the investigation of a limited range of concentrations. The model used here has specifically tried to address these issues and has been validated to exclude the significant contribution of extracellular growth within the limits of the experimental setup.

A first and unanticipated property is that all classes of antibiotics tested, with the exception of the macrolides, showed significant intracellular killing when their extracellular concentration was brought to a sufficiently high level and the time of...
exposure was prolonged to 24 h. For two molecules at least (oxacillin and oritavancin), we could show that the decreases in cell-associated CFU are accompanied by evidence of severe morphological alterations of the intracellular bacteria, consistent with their known modes of action (3, 25), indicating true intracellular expression of drug-related activity. This property is actually the direct consequence of two factors. The first is that all antibiotics studied here, with the exception of the macrolides, show concentration-dependent effects (for the four molecules tested in detail, we even observed typical pharmacological dose-response curves with the classical basic properties of threshold, slope, and maximal effects upon increasing concentration [53], irrespective of their specific modes of action). This definitely helps to provide an understanding of why contradictory results are reported when only narrow ranges of extracellular concentrations are explored. The second factor, which is perhaps as critical as the first one, is that all drugs, with the exception of the macrolides and, surprisingly, rifampin, showed time-dependent effects when they were tested at low multiples of their MICs. Both concentration and time therefore appear to modulate the final response and need to be taken into account when results from different models are compared. We know that this first part of our conclusion may appear to be at variance with what has been drawn from previous studies of the pharmacodynamics of antibiotics, namely, that the activities of some drugs (most notably, the β-lactams) are predominantly time dependent, whereas the activities of others (most notably, the aminoglycosides and the fluoroquinolones) are mainly concentration dependent (15, 16). Our observations being what they are, we suggest that the way that the drugs appear and can be differentiated from one another in most models essentially depends on two factors, namely, (i) the value of the $E_{\text{max}}$ parameter of the pharmacological response (maximal activity) and the concentrations at which effects approaching $E_{\text{max}}$ are obtained and (ii) the size (how large) of the concentration range examined. (The $E_{\text{max}}$ values shown in Table 3 are negative numbers, since they pertain to decreases in bacterial counts. Greater activity is, therefore, strictly speaking, associated with a smaller $E_{\text{max}}$. Since this is rather counterintuitive, we use the term “maximal activity” throughout this

FIG. 4. Influence of concentration on the extent of antibiotic activity against extracellular and intracellular S. aureus. The graphs show the change in the number of CFU (Δ log CFU; means ± SDs; n = 3; most SD bars are smaller than the symbols) per ml of culture medium (extracellular) or in THP-1 macrophages (intracellular) per mg of cell protein. Each antibiotic was added at concentrations corresponding to its MIC (circles; 4 times the MIC for rifampin), 10 times its MIC (triangles; 530 times the MIC for rifampin), or its $C_{\text{max}}$ in humans (squares; total drug) [Table 1]. Thick dotted line, static effect; thin dotted line, −2-log change (bactericidal effect, as defined by the Clinical and Laboratory Standards Institute for bacteria growing in broth). AZM, azithromycin; TEL, telithromycin; GEN, gentamicin; LNZ, linezolid; PEN V, penicillin V; VAN, vancomycin; TEC, teicoplanin; NAF, nafcillin; AMP, ampicillin; RIF, rifampin; CIP, ciprofloxacin; OXA, oxacillin; LVX, levofloxacin; GRN, garenoxacin; MXF, moxifloxacin; ORI, oritavancin.

FIG. 3. Influence of concentration on the rate and the extent of the activities of antibiotics against extracellular and intracellular S. aureus. The graphs show the change in the number of CFU (Δ log CFU; means ± SDs; n = 3; most SD bars are smaller than the symbols) per ml of culture medium (extracellular) or in THP-1 macrophages per mg of cell protein (intracellular). Molecules are ordered by increasing bactericidal potential, as determined by their MIC/MBC ratios in broth (Table 1). Except for rifampin, each antibiotic was tested at three increasing concentrations corresponding to its MIC (circles), 10 times the MIC (triangles), and the $C_{\text{max}}$ in humans (squares; total drug); rifampin was used at 4 (circles) and 530 (triangles) times the MIC and at the $C_{\text{max}}$ in humans (squares; total drug). Dotted line, static effect. For analysis of variance (ANOVA), the same letter indicates no statistically significant difference between values; different letters indicate a P value <0.05. n.a., not applicable (below the detection level); extrac., extracellular; intrac., intracellular; VAN, vancomycin; OXA, oxacillin; MXF, moxifloxacin; RIF, rifampin; GEN, gentamicin; ORI, oritavancin.
This will increase the impact of the time during which the bacteria are exposed to the antibiotic and suggests that the activities of all these antibiotics actually appear to be mainly time dependent. Extrapolation of our data for the categorization of the activities of the drugs as concentration or time dependent in vivo cannot, however, be done without caution. A first uncertainty relates to the effective availability of the antibiotics in blood and extracellular fluids, which can be severely impaired by binding to proteins or other biological constituents. As a help to the reader, however, we have provided in Table 1 an estimation of the percentage of free drug in human serum for each $C_{\text{max}}$ used in our study. If it is assumed that it is only the free drug that drives activity, one could surmise that the clinically meaningful concentration range of antibiotics that are highly protein bound will shift toward lower values, making the activities of most of them more and more concentration dependent as their effective concentrations approach the EC$_{50}$s. Unfortunately, the model used here does not easily lend itself to a pertinent evaluation of even this simple effect of serum protein binding, because (i) the serum concentration is low, resulting in only weak and limited binding of antibiotics that are usually reported to be highly protein bound (36); (ii) this concentration cannot be markedly changed without causing cell death, thereby preventing most concentration-effect studies; and (iii) the serum is of bovine and not human origin. A second uncertainty is whether the results obtained with a constant concentration over a 24-h period are predictive of what may be observed in vivo with fluctuating concentrations, as will be the case unless drugs are administered by continuous infusion. This will need to be specifically addressed in future studies. However, recent data from a study examining the pharmacodynamics of erythromycin against intracellular Legionella pneumophila by the use of both static and kinetic models failed to reveal significant differences in behavior related to the type of exposure (60).

A second property that appears from the comparative analysis of the dose-effect is that intracellular activities consistently remain lower than the extracellular ones, whether one considers what can be obtained at any given extracellular concentration or the maximal achievable effects (the $E_{\text{max}}$ parameter; because we could not reliably assess inoculum decreases greater than 4.2 log, the intracellular $E_{\text{max}}/\text{extracellular } E_{\text{max}}$ ratios observed for drugs highly bactericidal toward extracellular bacteria may actually be underestimated). This property was seen for all molecules studied and is probably more related to bacterial or cellular parameters than to drug pharmacodynamic or pharmacokinetic ones. The present study offers no insight into the underlying mechanism. However, we know that S. aureus cells phagocytosed by macrophages sojourn and thrive in phagolysosomes (35, 52). We may reasonably suggest that the metabolic changes triggered by the exposure of bacteria to this specific environment and to an acid pH in particular (46) could play a critical role (69). Alternatively, it is possible that those bacteria that apparently remain insensitive to antibiotics are physically protected from direct contact with the drugs. These hypotheses need to be addressed in future work but may face the difficulty of the specific analysis of what may concern only a small, albeit significant, part of the original inoculum. Thus, we could not directly examine the role of the so-called small-colony variants, which have been linked to per-

![Graph showing dose-response curves of four selected antibiotics against extracellular (extra) and intracellular (intra) S. aureus. The graphs show the change in the number of CFU (Δ log CFU; means ± SD; n = 3; most SD bars are smaller than the symbols) per ml of culture medium (extracellular; closed gray symbols) or in THP-1 macrophages (intracellular; closed black symbols) per mg of cell protein. Dotted line, static effect. The sigmoidal function was used (Hill coefficient = 1 [42]; goodness of fit and regression parameters are shown in Table 3). Vertical arrow, $C_{\text{max}}$ in humans (total drug; Table 1).

![Graph showing dose-response curves of four selected antibiotics against extracellular (extra) and intracellular (intra) S. aureus. The graphs show the change in the number of CFU (Δ log CFU; means ± SD; n = 3; most SD bars are smaller than the symbols) per ml of culture medium (extracellular; closed gray symbols) or in THP-1 macrophages (intracellular; closed black symbols) per mg of cell protein. Dotted line, static effect. The sigmoidal function was used (Hill coefficient = 1 [42]; goodness of fit and regression parameters are shown in Table 3). Vertical arrow, $C_{\text{max}}$ in humans (total drug; Table 1).](image-url)
sistent and relapsing infections (51), since we failed to detect them in significant numbers in our experimental conditions. Likewise, it may prove difficult to determine to what extent a small subpopulation of all intracellular bacteria are sojourning in poorly accessible compartments.

A third property, and probably the most critical one, to be considered in drug selection is the fact that the relative potencies (as measured by the EC$_{50}$ parameter) of some molecules are markedly decreased against intracellular bacteria compared with those against extracellular bacteria. Gentamicin and oritavancin appear to be the most affected, even though both drugs primarily concentrate in lysosomes and related vacuoles (62, 64), where S. aureus is thought to localize. These vacuoles are acidic, which will markedly decrease the activities of aminoglycosides (as is well known and which has been confirmed here for the strain of S. aureus used). In this context, it is interesting that alkalinization of lysosomes has been associated with improved intracellular activities of aminoglycosides (38). Yet, the activity of oritavancin is unaffected by acidity (as shown in a previous publication [64] and confirmed here), which indicates that effects other than pH, such as binding to intralysosomal constituents, need to be taken into consideration. In a broader context, a lack of true bioavailability and the defeating effect of the local physicochemical conditions on activity probably explain why cell accumulation per se is not necessarily predictive of intracellular efficacy for most antibiotics. This even appears to be the case for drugs with apparent large bioavailabilities, such as the fluoroquinolones. Indeed, fluoroquinolones show considerably less activity than is anticipated from their level of cellular accumulation, as demonstrated here and in other recent studies (2, 48, 56). Macrolides may also suffer from the same effects, but their bacteriostatic character is probably the most critical determinant in their lack of an intracellular killing effect. Conversely, the bactericidal effects of β-lactams against intracellular S. aureus when these compounds are used at large extracellular concentrations, as seen here for oxacillin and in previous studies with ampicillin and meropenem (56), not only could be due to the fact that these drugs may reach intracellular concentrations that eventually reach far above their MICs but could also be due to the production of cellular factors that enhance their activities (37, 45, 67).

Our results with linezolid and rifampin require attention,
since both drugs are usually recommended for the treatment of difficult-to-treat staphylococcal infections, but they failed to demonstrate significant intracellular bactericidal effects in our study. This observation is actually not surprising for linezolid, which is essentially bacteriostatic and which does not accumulate in macrophages. Conversely, the weak intracellular activity of rifampin, while it is marked after 3 to 6 h, does not progress over time thereafter, showing the importance of taking this parameter into account when different antibiotics are compared.

The present study used only one strain of fully susceptible S. aureus, which may be considered a major limitation for extrapolation of the findings of this study to clinical situations. Actually, the strain studied here has been widely used for the evaluation of the in vitro activities of new antibiotics in broth (61) as well as in phagocytes (24). The choice of a unique, well-characterized strain was actually essential for addressing the question of antibiotic intracellular activity per se and avoiding the blurring of the results because of other factors that can modulate the intracellular response to antibiotics, such as virulence and variations in the expression of resistance mechanisms. Given this caveat and pending further studies with clinical strains, the data presented in this paper may provide unambiguous pharmacological support to the use of new quinolones (7, 17) or oritavancin (40), as an alternative to ß-lactams (68), for the treatment of recurrent S. aureus infections, provided that sufficient extracellular concentration/MIC ratios are obtained for a sufficient period of time. These conditions may not be obtainable for more toxic drugs such as aminglycosides or conventional glycopeptides and will not be met with bacteriostatic antibiotics. We also suggest that in vitro models are useful for the appropriate design of animal and clinical studies aimed at evaluating the efficacies of antibiotics against intracellular pathogens, provided that they are made as relevant to the in vivo situation as possible in terms of the drug concentration and the duration of exposure.

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