

## Combined effect of pH and concentration on the activities of gentamicin and oxacillin against *Staphylococcus aureus* in pharmacodynamic models of extracellular and intracellular infections

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**Background:** *Staphylococcus aureus* survives in acid media, including phagolysosomes. Conflicting *in vitro/in vivo* data exist on its susceptibility to antibiotics in such environments.

**Methods:** Oxacillin and gentamicin activities against methicillin-susceptible *S. aureus* ATCC 25923 were compared extracellularly (broth; different pH) and assessed intracellularly (THP-1 macrophages), using a pharmacological approach (antibiotic concentrations: 0.01–1000 × MIC). Antibiotic cellular contents were determined by microbiological assay.

**Results:** MICs and MBCs increased 72-fold for gentamicin, and decreased 8-fold for oxacillin between pH 7.4 and 5.0. Plots of log<sub>10</sub> colony-forming unit changes at 24 h versus log<sub>10</sub> of antibiotic concentration followed sigmoidal shapes, allowing calculation of EC<sub>50</sub> (relative potency) and apparent E<sub>max</sub> (relative efficacy) in all conditions. In broth, the EC<sub>50</sub> of gentamicin rose 316-fold and that of oxacillin decreased 15-fold with unchanged apparent E<sub>max</sub> [–5 log (limit of detection)] between pH 7.4 and 5. Intracellularly, EC<sub>50</sub>s were similar to those observed extracellularly at pH 7.4, but E<sub>max</sub> values were much lower (–1 log) for both antibiotics. Calculations based on the assumed pH in phagolysosomes (5.4) and on local accumulation of antibiotics (gentamicin, 23-fold; oxacillin, 0.05-fold) suggest that the contrasting effects of acid pH on relative potencies of gentamicin and oxacillin could be almost exactly compensated for by differences in accumulation.

**Conclusions:** The weak activity of gentamicin and oxacillin towards intraphagocytic *S. aureus* compared with extracellular forms is not related to an overall decrease of their relative potencies but to impaired efficacy, suggesting the need for new approaches to improve the eradication of intracellular *S. aureus*.

Keywords: acid pH, β-lactams, aminoglycosides, pharmacodynamics, antibiotic accumulation

### Introduction

*Staphylococcus aureus* is a widespread pathogenic bacterium capable of surviving and multiplying in hostile environments. It shows a high tolerance to variations in pH,<sup>1</sup> which confers an advantage for colonizing body sites characterized by a mildly acidic pH, like skin, mouth, vagina, urine and abscesses,<sup>2</sup> where it may cause severe infections. *Staphylococcus aureus* is also able to survive and thrive intracellularly in acidic compartments such as the phagolysosomes of phagocytic cells,<sup>3,4</sup> and this property is considered important to explain the recurrent and relapsing character of many staphylococcal infections.<sup>5–8</sup>

An acid environment is known to impair the activity of many antibiotics. For macrolides and aminoglycosides, lowering the pH markedly increases their MICs,<sup>9–11</sup> which has been considered as a main reason for treatment failures in infections affecting tissues or biological fluids where pH is acidic,<sup>12</sup> and for poor efficacy against intracellular forms of *S. aureus*.<sup>11,13</sup> Yet, acid pH may have the opposite effect for other antibiotics, as evidenced by decreased MICs of β-lactams at acidic versus neutral pH.<sup>11</sup> Understanding these contrasting effects of pH on the activity of aminoglycosides and β-lactams against *S. aureus* may prove critical for a correct evaluation of their potential usefulness in the treatment of infections where intracellular survival

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## Antibiotic susceptibility of *Staphylococcus aureus* at acid pH

is likely to play a critical role. In the present work, we have used gentamicin and oxacillin to systematically compare the influence of pH towards *S. aureus* in broth and to examine their activities against its intracellular forms. We used a pharmacological model<sup>14</sup> in which bacteria and cells were exposed to a wide range of drug concentrations for up to 24 h, allowing us to obtain detailed information on dose–effect relationships, while being able to draw microbiological and clinically relevant conclusions.

### Methods

#### *Bacterial strain and determination of extracellular activity of antibiotics*

All experiments were performed using a methicillin-susceptible strain of *S. aureus* (ATCC 25923) obtained from the American Tissue Cell Collection (Manassas, VA, USA). MICs and MBCs were determined in Mueller–Hinton broth or agar, respectively, adjusted to specific pH values by addition of 2 M HCl or NaOH (the pH being checked before and after incubation). Killing curve experiments were performed as described previously.<sup>14</sup>

#### *Cell infection and determination of intracellular activity and cellular accumulation of antibiotics*

All experiments were conducted with THP-1 macrophages, exactly as described previously.<sup>11,14</sup> Cell associated antibiotics were assayed by a microbiological method (disc diffusion), with *Bacillus subtilis* ATCC 6633 as test-organism and antibiotic medium #11 adjusted at pH 8 for gentamicin, and *S. aureus* ATCC 25923 and antibiotic medium #11 adjusted at pH 5 for oxacillin. The apparent cellular/extracellular concentration ratio of antibiotics ( $C_i/C_e$ ) was calculated as previously described.<sup>11,14</sup>

#### *Analysis of the dose–response curves and statistical analysis*

Data from the dose–response experiments were used to derive a pharmacological model based on the Hill equation (response versus  $\log_{10}$  of drug concentration, using a slope factor of 1), which allowed calculation of the maximal efficacy,  $E_{\max}$ , being the drug concentration causing a maximal effect, and the relative potency,  $EC_{50}$ , defined as the drug concentration causing a response half-way between the effect in absence of drug ( $E_0$ ) and  $E_{\max}$ ; these are two key pharmacological descriptors of the activity of most drugs.<sup>15</sup> (Details and application of these to different classes of antibiotics acting on *S. aureus* have been given earlier.)<sup>11</sup> In this analysis, the following points should be borne in mind:

- (i) Because antimicrobial effects, like those of all chemotherapeutic agents, consist of a fractional reduction of an original inoculum, the  $\log_{10}$  of the colony count reduction needs to be used as descriptor of the response for curve-fitting analysis.
- (ii)  $E_{\max}$  values of antimicrobial agents are—by definition and in contrast to most non-chemotherapeutic agents—negative numbers, since they pertain to decreases in bacterial counts; a larger activity is therefore, strictly speaking, associated with a smaller  $E_{\max}$ . Since this is rather

counterintuitive, we use the term ‘maximal activity’ to define the maximal reduction of bacterial counts observed.

- (iii) Our limit of detection is a reduction of approximately  $-5 \log$  cfu compared with the original inoculum; since this reduction of colony-forming units was always reached for bacteria grown in broth when exposed to increasing concentrations of oxacillin or gentamicin;  $E_{\max}$  for extracellular activity had to be arbitrarily set to that value for the purpose of our analysis.<sup>11</sup>  $E_{\max}$  values given here are, therefore, not meant to describe the maximal activity that could be observed for the drug if other experimental conditions had been used (such as the use of a more concentrated initial inoculum).

All curve fittings and determinations of the  $E_{\max}$  and  $EC_{50}$  values were made using GraphPad Prism<sup>®</sup> version 4.02 for Windows (GraphPad Prism Software, San Diego, CA, USA). The apparent static concentration (drug concentration causing no apparent change compared with the original inoculum) was thereafter determined by graphic interpolation. Analyses of variance (ANOVA), which compare means by splitting the overall observed variance into different parts, were made with GraphPad Instat<sup>®</sup> (GraphPad Prism Software); analyses of covariance (ANCOVA), a method testing whether certain factors have an effect after controlling for quantitative predictors, was made with XLSTAT Pro<sup>®</sup> (version 7.5.2; Addinsoft SARL, Paris, France). Tukey’s test for multiple comparisons was used in both cases.

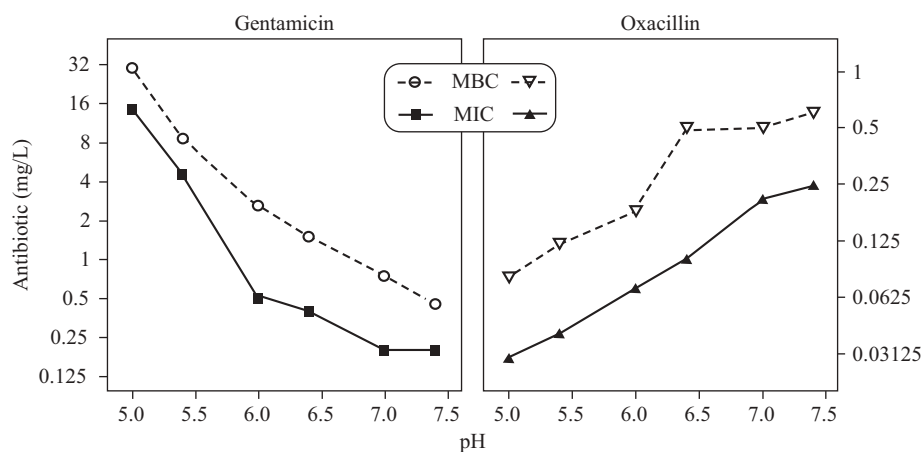
### Materials

Gentamicin was procured from Glaxo-SmithKline-Belgium as the commercial product registered for parenteral use (GEOMYCIN<sup>®</sup>). Oxacillin was purchased from Sigma-Aldrich-Fluka (St Louis, MO, USA) in powder form for microbiological evaluation (potency, 93.9%). Cell culture media and fetal calf serum were purchased from Invitrogen (Paisley, Scotland, UK) and Difco (Sparks, MD, USA). Human serum for opsonization of *S. aureus* was obtained from healthy volunteers and stored at  $-80^{\circ}\text{C}$  as pooled samples until use. Other reagents were purchased from E. Merck AG (Darmstadt, Germany) or Sigma-Aldrich-Fluka.

### Results

#### *Influence of pH on extracellular activities*

Figure 1 shows how the MIC and MBC values of gentamicin and oxacillin are affected when determined in media adjusted to pH values ranging from 7.4 to 5.0. Acid pH drastically reduced the activity of gentamicin, the MIC of which was approx. 70 times higher at pH 5.0 than at pH 7.4. This effect was particularly noticeable between pH 6.0 and 5.0, with the MIC increasing from 0.5 to 14.5 mg/L. In contrast, lowering the pH over the same range markedly and almost linearly increased the activity of oxacillin ( $\sim 10$ -fold decrease in MIC). The MBCs of both drugs varied in parallel to their MICs over the whole range of pH values investigated, remaining systematically 2–4-fold larger than the corresponding MIC, indicating that the bactericidal



**Figure 1.** MICs and MBCs of gentamicin (left-hand panel) and oxacillin (right-hand panel) as a function of the pH of Mueller–Hinton broth (MIC) or agar (MBC). Values are arithmetical means of four determinations.

character of both antibiotics was fully maintained in spite of the overall decrease or increase of intrinsic activity.

In order to further understand the influence of pH on antibacterial intrinsic activity, we performed full dose–response studies at the 24 h time point. We checked that acid pH (5.4 vs. 7.4) only slightly reduced the rate of *S. aureus* growth (3 vs. 2–2.5 log cfu increase in 24 h), without affecting the overall shape of the individual dose–responses. Results are shown in Figure 2. The corresponding pertinent regression parameters, drug response descriptors  $E_{\max}$ ,  $EC_{50}$  (based on the Hill equation), apparent static concentration and statistical analyses are presented in Table 1. When data are examined using mass values for drug concentration, we see that acid pH decreased the relative potency of gentamicin and increased that of oxacillin ( $EC_{50}$ ) without modifying their apparent maximal efficacies ( $E_{\max}$ ). All responses, not only for each antibiotic individually but also when comparing antibiotics, become largely superimposable when data are examined using multiples of the MIC as drug concentration values (Figure 2, lower panels).

#### Intracellular activity and antibiotic accumulation

Dose–response experiments were then performed for both antibiotics against intracellular *S. aureus*, also using the 24 h time point and the same range of drug concentrations as for the extracellular activities. Data are presented in Figure 3, with the pertinent regression parameters, drug response descriptors  $E_{\max}$ ,  $EC_{50}$  (based on the Hill equation), apparent static concentration, and the statistical analyses presented in Table 1. This data revealed a considerable loss of maximal efficacy of the antibiotics against intracellular bacteria ( $E_{\max}$ ) with a slight but significant increase in relative potency ( $EC_{50}$ ).

The cellular accumulation of gentamicin and oxacillin was then measured in infected cells after 24 h of incubation. Because of lack of sensitivity of our microbiological assay, we had to perform these experiments with cells incubated with large extracellular concentrations of antibiotic. To ascertain that accumulation of gentamicin and oxacillin was linearly related to the extracellular concentration, we measured the cell drug contents at increasing extracellular concentrations (50, 100, 150 and 200 mg/L for gentamicin; 300 and 400 mg/L for oxacillin). This

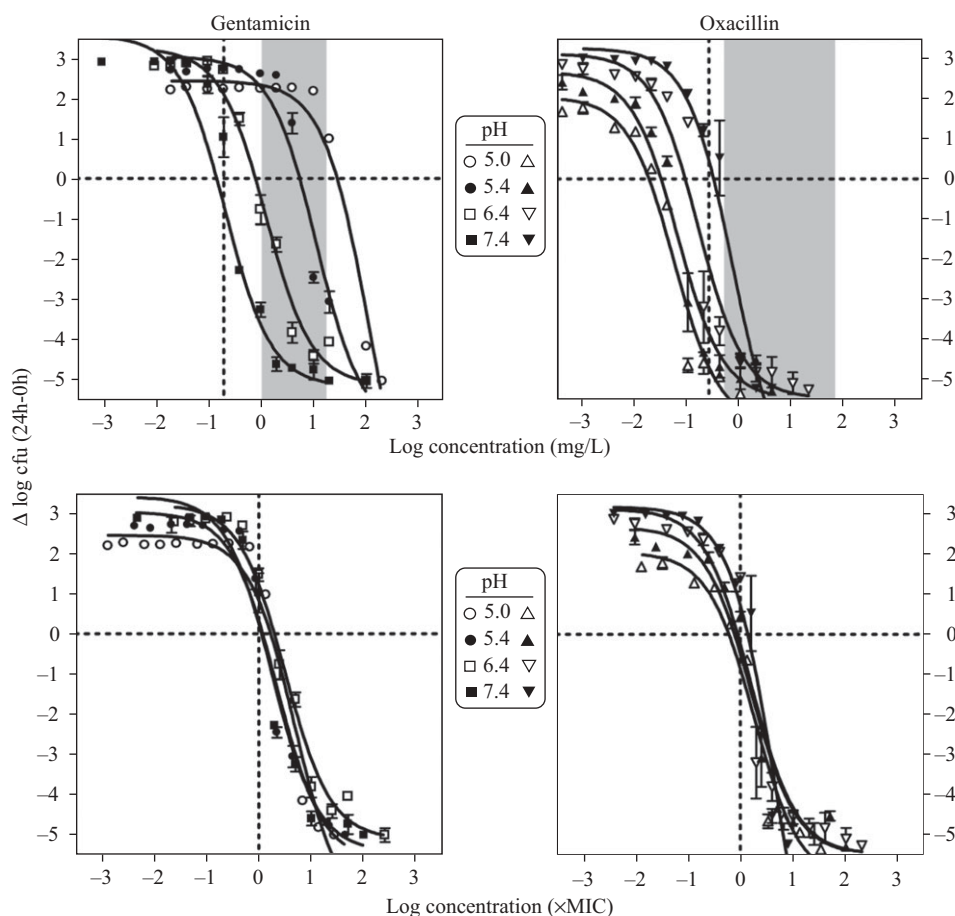
enabled us to calculate the cellular drug content of cells incubated with low concentrations of antibiotics by extrapolation from the values observed at large concentrations. The mean accumulation values, when expressed as the apparent cellular ( $C_c$ ) to extracellular ( $C_e$ ) drug concentration ratios, were  $0.57 \pm 0.20$  (SD) for gentamicin and  $0.05 \pm 0.03$  (SD) for oxacillin; there was no evidence that extracellular concentration influenced these values: regression slopes of  $C_c/C_e$  versus  $C_e$  were 0.0012 (CI:  $-0.0042$  to  $0.0019$ ) and 0.0003 (CI:  $-0.0008$  to  $0.0013$ ) for gentamicin and oxacillin, respectively. These  $C_c/C_e$  ratios were then used to calculate the apparent cellular drug concentration at each extracellular concentration used in our previous experiments.

The data of Figure 3 (upper panels) were then re-plotted taking into account (i) the effect of pH on the MIC (as shown in Figure 1) assuming a pH of 5.4 for phagolysosomes,<sup>16</sup> and (ii) the combination of this effect and the apparent local drug concentration. For the latter parameter, we started from the values of apparent cell concentration as explained above, but assumed that (i) cell-associated gentamicin was localized in the phagolysosomes, as is commonly accepted,<sup>17,18</sup> and that these vacuoles accounted for approx. 2.5% of the cell volume<sup>19</sup> (gentamicin local concentration would then be 40 times larger than deduced from its apparent  $C_c/C_e$  ratio); (ii) cell-associated oxacillin would be uniformly distributed, as suggested from previous studies examining the subcellular distribution of <sup>14</sup>C-labelled penicillin in macrophages.<sup>20</sup> The results of these calculations (Figure 3, lower panels) show that for both gentamicin and oxacillin the effects of pH could be almost entirely compensated for by their respective local accumulation properties. Indeed, when only the acid pH parameter was taken into account, we observed a marked shift of the data over the MIC scale towards lower values for gentamicin and to larger values for oxacillin. Data, however, returned to their original position when local drug concentrations were used to calculate the corresponding multiple of MIC.

## Discussion

Aminoglycosides and  $\beta$ -lactams have long been considered poorly efficient against intracellular bacteria, because of their

## Antibiotic susceptibility of *Staphylococcus aureus* at acid pH



**Figure 2.** Dose–response curves of the extracellular activity of gentamicin (left-hand panels) and oxacillin (right-hand panels) against *Staphylococcus aureus*. The graphs show the change in the number of colony units (log cfu) per mL after 24 h of incubation of *S. aureus* in broth adjusted to different pH values. Data are plotted as a function of the drug concentration expressed in mg/L (upper panels), and in multiples of the MIC of each drug measured at the considered pH (lower panels; see Figure 1 for MIC values). For each diagram, the horizontal dotted lines show a static effect (initial inoculum:  $5.94 \pm 0.05$  log cfu/mL), whereas the vertical dotted lines show the MIC at pH 7.4 for the upper panels, or are set to 0 (corresponding to the log of the MIC at each pH) for the lower panels. The zones highlighted in grey correspond to the serum concentrations ranges (total drug) that can be observed in patients (gentamicin, 1–18 mg/L; oxacillin, 0.5–86 mg/L) after conventional iv administration. Data are means  $\pm$  SD of three independent experiments (most of the SD bars are smaller than the symbols). The limit of detection was  $-5$  log cfu. Curves were constructed by non-linear regression using the Hill equation. See Table 1 for regression parameters, pharmacological and microbiological descriptors, and statistical analyses.

small or slow cellular accumulation (ref. 21 for review), and in the case of aminoglycosides, the confounding effect of the intraphagosomal acid pH on their activity.<sup>22</sup> Yet, both types of antibiotics are used to treat various types of staphylococcal infections.<sup>23–26</sup> Most *in vitro* studies confirmed the inefficacy of  $\beta$ -lactams and aminoglycosides against intracellular *S. aureus* but used short-term exposures and limited concentration ranges.<sup>11</sup> Studies using sufficiently large concentrations and a 24 h exposure time showed significant activity for different  $\beta$ -lactams and for gentamicin.<sup>11,14</sup> The present data go one step further in offering a rational, mechanistic explanation to the apparent contradiction between these various models.

We show first of all that antimicrobial responses are always related to concentration, obeying the classical pharmacology described by the Hill equation.<sup>27</sup> This is not contradictory to what is commonly assumed as being the key pharmacodynamic properties of  $\beta$ -lactams (time-dependency) and aminoglycosides (concentration-dependency).<sup>28</sup> Our conclusions, indeed, are

based on results observed over a much wider range of drug concentrations than usual, and which includes sub-MIC and supra-MIC values. If the observation is limited to the  $C_{\min}$ – $C_{\max}$  range (as shown in Figures 2 and 3), one sees that oxacillin activity is already almost maximal, and therefore appears to be concentration-independent, whereas gentamicin is fully concentration dependent within the same range. (For oxacillin, which is 90% protein-bound, the  $C_{\min}$ – $C_{\max}$  zone may need to be shifted to the left of 1 log<sub>10</sub> unit, since it is generally agreed that only free concentration is related to activity; our model, unfortunately, does not allow analysis in detail of the effect of extracellular protein binding on intracellular activities,<sup>11,14</sup> preventing further examination of this parameter here.) This ‘wide range of drug concentrations’ approach was actually critical to understand how pH affects the activity of antibiotics. The data shows that only relative potencies ( $EC_{50}$ ) and not apparent maximal efficacies ( $E_{\max}$ ) are modified by acid pH, but since activity in broth always reached the limit of detection, we cannot exclude that the

**Table 1.** Pertinent regression parameters<sup>a</sup> (with confidence intervals, CI), and statistical analyses of the dose–response curves illustrated in Figures 2 and 3

Abscissa	pH	Gentamicin					Oxacillin				
		E <sup>b</sup> <sub>max</sub> (CI)	EC <sup>c</sup> <sub>50</sub> (CI)	C <sup>d</sup> <sub>static</sub>	R <sup>2</sup>	ANCOVA	E <sup>b</sup> <sub>max</sub> (CI)	EC <sup>c</sup> <sub>50</sub> (CI)	C <sup>d</sup> <sub>static</sub>	R <sup>2</sup>	ANCOVA
Extracellular activity											
Mass concentration (mg/L) <sup>e</sup>  Multiples of the MIC at the considered pH <sup>f</sup>	7.4	<–5	0.36 (0.21 to 0.62) a;A	0.13	0.976	a,A	<–5	0.73 (0.33 to 1.62) a;B	0.31	0.964	a,B
	6.4	<–5	1.26 (0.83 to 1.91) b;A	0.76	0.985	b,A	<–5	0.16 (0.08 to 0.29) a;B	0.09	0.962	b,B
	5.4	<–5	11.25 (5.63 to 22.48) c;A	5.49	0.962	b,A	<–5	0.06 (0.04 to 0.11) a;B	0.03	0.973	b,B
	5.0	<–5	113.8 (46.21 to 280.0) d;A	28.18	0.977	c,A	<–5	0.05 (0.02 to 0.12) a;B	0.02	0.962	b,B
	7.4	<–5	1.79 (1.04 to 3.07) e;A	1.12	0.976	d,A	<–5	4.77 (1.76 to 12.82) b;B	1.38	0.971	c,B
	6.4	<–5	3.14 (2.06 to 4.79) e;A	2.00	0.985	d,A	<–5	1.55 (0.84 to 2.88) c;B	0.90	0.962	d,A
	5.4	<–5	2.22 (1.23 to 4.00) e;A	1.17	0.969	e,A	<–5	1.58 (0.89 to 2.80) c;A	0.77	0.973	d,B
	5.0	<–5	4.84 (2.33 to 10.01) f;A	1.70	0.972	e,A	<–5	1.82 (0.82 to 4.06) c;B	0.62	0.962	d,B
	Intracellular activity										
Extracellular mass concentration (mg/L) <sup>g</sup>  Extracellular multiples of MIC at pH 7.4 <sup>h</sup>  Extracellular multiples of MIC at pH 5.4 <sup>h</sup>  Extracellular multiples of MIC at pH 5.4 × calculated cellular (oxacillin) or phagolysosomal (gentamicin) accumulation <sup>h</sup>		–1.12 (–1.77 to –0.47)	0.13 (0.06 to 0.28) g;A	3.16	0.927	f,A	–0.69 (–0.85 to –0.53)	0.07 (0.04 to 0.14) d;A	0.17	0.988	e,B
		–1.12 (–1.77 to –0.47)	0.65 (0.30 to 1.42) h;A	1.58	0.927	g,A	–0.69 (–0.85 to –0.53)	0.36 (0.19 to 0.68) e;B	0.83	0.988	f,B
		–1.12 (–1.77 to –0.47)	0.029 (0.013 to 0.06) i;A	0.07	0.927	h,A	–0.69 (–0.85 to –0.53)	3.60 (1.92 to 6.76) f;B	8.13	0.988	g,B
		–1.12 (–1.77 to –0.47)	0.66 (0.30 to 1.44) h;A	1.62	0.927	g,A	–0.69 (–0.85 to –0.53)	0.19 (0.10 to 0.35) e;A	0.43	0.988	h,B

<sup>a</sup>Using all data points from an antibiotic concentration of 0.01–1000 × MIC. Data from samples without antibiotics were not used since there was evidence of an overestimation of the true value of the intracellular counts when the extracellular concentration of antibiotic was lower than 0.01 × MIC.

<sup>b</sup>Decrease in colony units (in log<sub>10</sub> cfu) at time = 24 h from the corresponding original inoculum, as extrapolated for antibiotic concentration = ∞; –5 corresponds to samples yielding 5 counts/plate, which was considered as the lowest practical limit of detection (all samples for which 5 or less counts/plate were observed were, therefore, given arbitrarily a value of –5 log cfu decrease).

<sup>c</sup>Concentration causing a reduction of the inoculum half-way between initial (E<sub>0</sub>) and maximal (E<sub>max</sub>) values, as obtained from the Hill equation (using a slope factor of 1).

<sup>d</sup>Concentration resulting in no apparent bacterial growth (no. of colony-forming units identical to the original inoculum), as determined by graphical intrapolation.

<sup>e</sup>From data of Figure 2, upper panels.

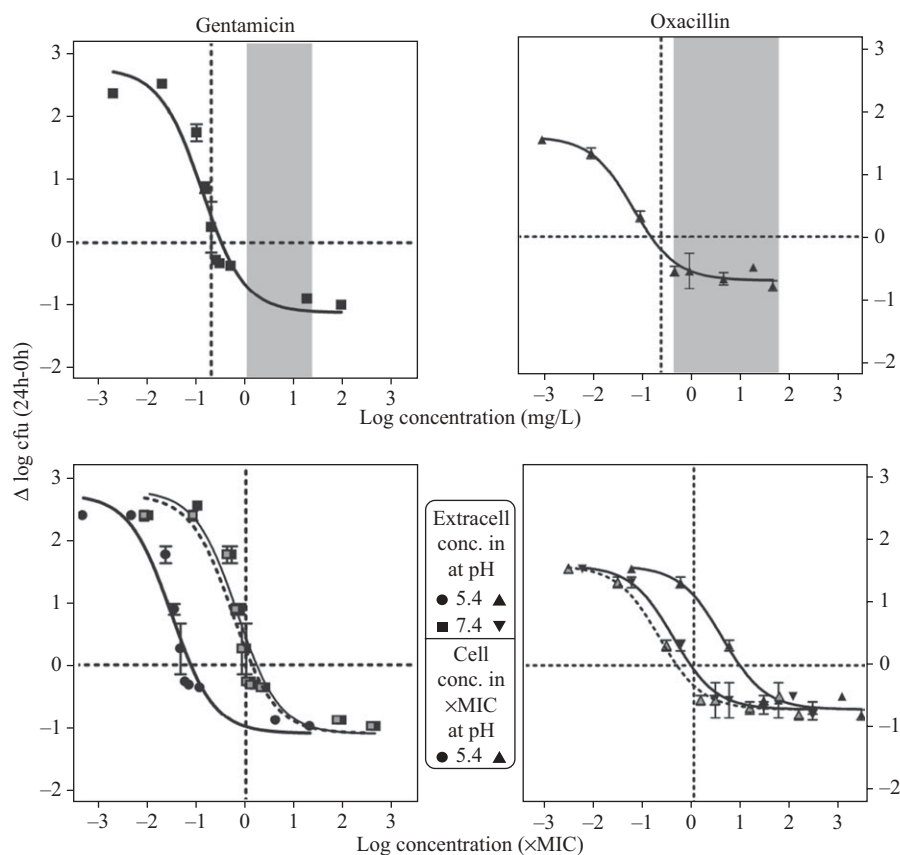
<sup>f</sup>From data of Figure 2, lower panels.

<sup>g</sup>From data of Figure 3, upper panels.

<sup>h</sup>From data of Figure 3, lower panels.

Statistical analyses: (i) Analysis per column (one-way ANOVA with Tukey's test for multiple comparisons between corresponding conditions for each drug), figures with different lower case letters are significantly different from each other ( $P < 0.05$ ). (ii) Analysis per row (unpaired, two-tailed  $t$ -test between gentamicin versus oxacillin for each experimental condition), figures with different upper case letters are significantly different from each other ( $P < 0.05$ ). (iii) Global analysis (ANCOVA) with Tukey's test for multiple comparisons: analysis per column, figures with different lower case letters are significantly different from each other ( $P < 0.05$ ); analysis per row (gentamicin vs. oxacillin), figures with different upper case letters are significantly different from each other ( $P < 0.05$ ).

### Antibiotic susceptibility of *Staphylococcus aureus* at acid pH



**Figure 3.** Dose–response curves of the intracellular activity of gentamicin (left-hand panels) and oxacillin (right-hand panels) against *Staphylococcus aureus* phagocytosed by THP-1 macrophages. The ordinates show the change in the  $\log_{10}$  cfu per mg of cell protein after 24 h of incubation compared with the post-phagocytosis inoculum ( $6.67 \pm 0.07 \log_{10}$  cfu/mg protein; the horizontal dotted line shows, therefore, a static effect). The abscissa shows the drug concentration expressed as follows. Upper panels: actual extracellular concentration ( $\log_{10}$  mg/L); the zones highlighted in grey correspond to the serum concentrations ranges (total drug) that can be observed in patients (gentamicin, 1–18 mg/L; oxacillin, 0.5–86 mg/L) after conventional intravenous administration. Lower panels: (i) actual extracellular concentration (closed symbols and continuous lines) expressed as  $\log_{10} \times \text{MIC}$ , with MIC measured at pH 7.4 (filled squares, gentamicin; filled upside-down triangles, oxacillin) or at pH 5.4 (filled circles, gentamicin; filled triangles, oxacillin); (ii) concentrations assumed to prevail (open symbols and dotted lines) in lysosomes (gentamicin; open circles) or in whole cells (oxacillin; open triangles) expressed as  $\log_{10} \times \text{MIC}$  measured at pH 5.4. For all panels, the vertical dotted line shows the MIC at pH 7.4. Data are means  $\pm$  SD of three independent experiments (most SD bars are smaller than the symbols). The limit of detection was  $-5 \log$ . Curves were constructed by non-linear regression using the Hill equation. See Table 1 for regression parameters, pharmacological and microbiological descriptors, and statistical analyses.

real  $E_{\max}$  is beyond that limit. We therefore suggest that pH acts by modulating the binding and/or target accessibility of gentamicin and oxacillin, and not by making bacteria more or less tolerant to the drugs. Acid pH indeed impairs gentamicin transport into bacteria,<sup>29</sup> probably as a result of its larger ionization at pH 5.4 versus pH 7.4 (the  $\text{pK}_{\text{a}}$ s of the amino groups of gentamicin being between 5.5 and 9).<sup>30</sup> Conversely, the  $\text{pK}_{\text{a}}$  of the carboxylate function in oxacillin (about 2.4)<sup>31</sup> is probably too low to markedly modulate the behaviour of the molecule in the 5.4–7.4 pH range. Yet, we know that bringing the pH to 5.5 increases the affinity of penicillin for its binding proteins (PBPs) based on binding studies to *Escherichia coli* PBPs 1b, 1c, 2 and 3,<sup>32</sup> (the main PBPs in *S. aureus* are PBPs 1, 2, 3 and 4)<sup>33</sup> and on direct measurement of penicillin binding to whole cell wall extracts of non- $\beta$ -lactamase producing methicillin-susceptible *S. aureus* (*S. Lemaire*, F. Van Bambeke, M. P. Mingeot-Leclercq, Y. Ghipczinsky and P. M. Tulkens, unpublished data). A key general conclusion of our studies is therefore that (i) aminoglycosides will exert activity against *S. aureus* in an acidic

environment if their concentration reaches a value that compensates for their decreased relative potency, which is probably what takes place intracellularly through the local accumulation of the drug; and (ii) conversely, the low accumulation of  $\beta$ -lactams in cells can be compensated for by their commensurate increase in relative potency, making these drugs to appear active in spite of their apparently unfavourable cellular pharmacokinetics. Therefore, we see that intracellular activity of aminoglycosides and  $\beta$ -lactams cannot be simply deduced from the study of their cellular accumulation only.

Effects of pH and local concentration, however, fail to explain the poor eradicating capabilities of aminoglycosides and  $\beta$ -lactams towards intracellular *S. aureus*. We see that decreased maximal efficacy ( $E_{\max}$ ), rather than a change in apparent potency ( $\text{EC}_{50}$ ), is probably the critical determinant. Observed for many antibiotics with distinct modes of action,<sup>11</sup> this effect could result from the selection of pre-existing resistant subpopulations, an inaccessibility of part of the population to the drugs, or an increased tolerance to the drugs. Nevertheless, bacteria

collected from cells exposed to large concentrations of gentamicin or oxacillin show an unaltered MIC when retested in broth. Inaccessibility of the drug could result from very local differences in environment not translated into obvious morphological differences. Increased tolerance may result from alteration of the metabolic status of the bacteria, such as formation of the so-called 'small colony variants'<sup>34</sup> that are intrinsically less sensitive to antibiotics.<sup>35</sup> Exposure of *S. aureus* to mild acid pH modifies the expression level of about 400 genes<sup>2</sup> in a similar way to heat shock or behaviour in biofilms,<sup>36,37</sup> two situations where bacteria are poorly susceptible to antibiotics.<sup>38</sup>

Although our conditions of drug exposure are remote from those prevailing in patients, our data may nevertheless help a better understanding of how the activity of antibiotics could be improved in the clinical arena. Thus, strategies aiming at increasing the drug relative potencies (resulting in a lower MIC) and/or their concentrations at the site of infection could be useful for optimizing activity. Reducing local MICs by manipulating lysosomal pH proved efficient for increasing the intracellular activity of aminoglycosides *in vitro*<sup>13</sup> but is difficult to exploit *in vivo*. Selecting molecules with low MICs at acidic pH and optimizing exposure of intracellular bacteria to these drugs by prolonging the time of exposure and using extracellular concentrations as high as possible appear to be straightforward approaches.

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## Transparency declarations

None to declare.

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## Antibiotic susceptibility of *Staphylococcus aureus* at acid pH

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