plectasin has been found to be innocuous to eukaryotic cells (5, 28). Furthermore, plectasin has a novel mode of action and some intracellular antibacterial activity of plectasin was maintained (maximal relative efficacy \( E_{\text{max}} \), 1.0- to 1.3-log reduction in CFU) even though efficacy was inferior to that of extracellular killing \( E_{\text{max}} > 4.5\)-log CFU reduction). Animal studies included a novel use of the mouse peritonitis model, exploiting extra- and intracellular differentiation assays, and assessment of the correlations between activity and pharmacokinetic (PK) parameters. The intracellular activity of plectasin was in accordance with the in vitro studies, with an \( E_{\text{max}} \) of a 1.1-log CFU reduction. The parameter most important for activity was \( FC_{\text{peak}}/\text{MIC} \), where \( FC_{\text{peak}} \) is the free peak concentration. These findings stress the importance of performing studies of extra- and intracellular activity since these features cannot be predicted from traditional MIC and killing kinetic studies. Application of both the THP-1 and the mouse peritonitis models showed that the in vitro results were similar to findings in the in vivo model with respect to demonstration of intracellular activity. Therefore the in vivo model was a good screening model for intracellular activity. However, animal models should be applied if further information on activity, PK/pharmacodynamic parameters, and optimal dosing regimens is required.

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Plectasin was discovered by Novozymes in 2002, and a variant is at present in the preclinical phases of drug development, with expected first dose in humans in 2010.

While in vitro models using either human or animal cell lines have been developed to study the intracellular activity of antibiotics (4, 12, 13, 15, 38), we lack effective animal models. Sandberg et al. recently described a model where both the extra- and intracellular antimicrobial effects on \( S. \) aureus were studied in a modified version of the mouse peritonitis model (32).

Combining the above-mentioned methods performed in cell cultures and in animal models may provide valuable information on the intracellular properties of an antimicrobial peptide. Therefore the objective of this study was to apply both in vitro and in vivo methods when testing the intracellular effect of plectasin against \( S. \) aureus. Furthermore, the correlation between in vitro and in vivo results and the intracellular effects of plectasin and impact of pharmacokinetic (PK)/pharmacodynamic (PD) parameters (free time above MIC at 24 h \( [T > \text{MIC}_{24 \text{ h}}] \), free area under the concentration-time curve \( \text{MIC}_{24 \text{ h}} [\text{AUC/MIC}_{24 \text{ h}}] \), and free peak concentration/MIC \( [\text{C}_{\text{peak}}/\text{MIC}] \) ) were investigated. To our knowledge these studies represent the first application of a combined in vitro and in vivo approach to the study of the PD properties governing the intracellular activity of a peptidic antibiotic.

(Parts of this study were presented at the 48th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, 25 to 28 October 2008.)

**MATERIALS AND METHODS**

**Plectasin.** Wild-type plectasin (NZ2000) (amino acid sequence: GFGCNGP WDDEDDMQCNHC9KGYGGYAKGGFVCKCY) was used in animal and in vitro studies was diluted to a concentration of 2 mg/ml in 50 mM acetate and 500 mM NaCl, pH 4. The solution pH was adjusted to 5.0 with 10 mM trisodium phosphate and NaCl (9 g/liter). The molecular mass was 4.4 kDa, and the purity was determined to be 96.9% by high-pressure liquid chromatography (HPLC).

Mygind et al. (28) have described the antimicrobial activity as well as the cloning, purification, and structural features of plectasin in detail.

**Bacterial strains and susceptibility studies.** In all in vitro killing kinetics studies, Staphylococcus aureus strain E33235 (methicillin-susceptible \( S. \) aureus; clinical bacteremia isolate from Statens Serum Institut) and \( S. \) aureus ATCC 25923 were used. MICs were determined in Mueller-Hinton broth at pH 5.4 and 7.4 using a standard microtiter tray method according to recommendations provided by the Clinical and Laboratory Standards Institute (8) as earlier described (36). For the in vivo studies \( S. \) aureus strain E33235 was used in all experiments. The bacteria were grown on 5% blood plates at 37°C. Colonies were suspended in saline to approximately \( 1 \times 10^8 \text{ CFU/ml} \) (optical density at 546 nm = 0.13). After 1:10 dilution in 0.9% NaCl, the bacterial load was quantified by CFU counts. The bacterial inoculum for the mouse peritonitis model was prepared in a sterile 1:10 dilution in 0.9% NaCl, the bacterial load was quantified by CFU counts. The in vivo studies of the intracellular activity of THP-1 monocytes were performed as earlier described (3). In short, a bacterial culture in exponential growth was centrifuged, and the pellet was resuspended in RPMI 1640 (10% human serum) (Lonza, Walkersville, MD). The suspension was incubated for 45 min at 37°C to allow opsonization of bacteria and subsequently adjusted to a concentration of \( 5 \times 10^6 \text{ CFU/ml} \). Human THP-1 monocytes (ATCC TIB-202; LCG Promochem Ltd., Teddington, United Kingdom) were cultivated in RPMI 1640 medium (10% fetal calf serum) as previously described (34). The cells were adjusted to \( 2 \times 10^5 \text{ cells/ml} \). The suspension of cells was injected subcutaneously with \( 0.2 \) to \( 0.6 \) ml/animal. Blood samples were collected in uncoated Eppendorf tubes at 0, 0.5, 1, and 2 h.

Infection of the monocytes was performed by replacing the culture medium with the bacterial suspension in a bacterium-to-monocyte ratio of 4:1. The suspension of cells and bacteria was incubated at 37°C and 5% \( CO_2 \) for 1 hour to allow phagocytosis. The monocytes were then washed with phosphate-buffered saline (PBS) containing 50 mg/liter gentamicin. After 45 min gentamicin was removed by washing with PBS. After the last wash cells were resuspended in fresh medium. Cells were kept at a concentration of from 0.01 to 128 times the MIC. Samples were incubated at 37°C and 5% \( CO_2 \) and after 24 h CFU counts were performed. After incubation samples were spun down, washed in PBS, and spun down again, and cells were lysed in sterile water to release intracellular bacteria before spreading dilution on Trypticase soy agar plates for CFU determination. At 0 h, CFU in control samples without plectasin were spread to determine initial CFU concentration (1.1 \( \times 10^6 \) to \( 4.0 \times 10^6 \text{ CFU/ml} \)). All tests were performed in triplicate, and results were expressed as means of three CFU determinations (see Fig. 2).

**PK study.** A single-dose serum PK study with plectasin was performed to determine relevant doses for the PK/PD peritonitis study. Mice (three per time point) were injected subcutaneously with \( 0.2 \) to \( 0.6 \) mg/kg of body weight. The dose volumes varied from 4.25, 8.5, 17, or 34 mg/kg. The dose levels varied from 0.3 to 0.6 mg/liter. Blood samples were collected in uncoted Eppendorf tubes after 5, 10, 20, 30, 60, 120, 180, and 360 min at 17 and 34 mg/kg. Samples of 4.25 and 8.5 mg/kg were collected after 5, 10, 20, 40, 60, 120, and 240 min. Serum was separated and stored at \(-20^\circ \text{C}\) until analysis. Analyses of total serum concentrations of plectasin were performed by HPLC, and the free fraction was then estimated based on the calculated percentage of protein binding.

**Cell viability.** To ensure that results for intracellular activity were not affected by a cytotoxic effect of plectasin on the THP-1 cells, the cell viability after 24 h of incubation with plectasin (1 to 256 mg/liter) was assessed by the trypan blue exclusion test.

**Calculation of dosing regimens.** Doses for the PK/PD study were designed to vary \( f_{\text{peak}}/\text{MIC}_{24 \text{ h}} \), \( T_{\geq \text{MIC}_{24 \text{ h}}} \), and \( \text{AUC/MIC}_{24 \text{ h}} \). The PK values were based on the PK study described using the free drug concentrations defined by Mouton et al. (27). Through extrapolations of data the exponential equation, which described the final concentration curve for the given dose, was found. This equation was used to extrapolate the concentration curve to the time (8 or 12 h). These data were analyzed in GraphPad Prism, and the PK parameters were calculated by using a noncompartmental model. The \( \text{AUC}_{\text{peak}}/\text{MIC} \) was calculated from mean concentrations using the trapezoidal rule. In Table 2 the \( T_{\geq \text{MIC}} \) and \( \text{AUC} \) for the applied dosing regimens are listed.

**Infective and treatment of animals.** The animal model was a modified version of the previously described mouse peritonitis model (11). Initially, a study comparing \( S. \) aureus E33235 with the strain previously used in the model (E19977, clinical isolate; Statens Serum Institut) and a 6-hour dose-response
The intracellular activity of plectasin was assessed by phagocytosed by human THP-1 monocytes. The intracellular activity of plectasin was assessed by 24-hour dose-response studies of S. aureus phagocytosed by human THP-1 monocytes using a wide range of extracellular concentrations (0.01 to 0.128 times the MIC). Results from the studies are shown in Fig. 2A and B and Table 1, where data are plotted against the weight concentrations of plectasin.

The killing effect was decreased compared to the extracellular effect, and the $E_{\text{max}}$ values for plectasin against E33235 and ATCC 25923 after 24 h were $-1.0$ log CFU and $-1.3$ log CFU, respectively. Some concentration dependency was still observed, even though the activity had a tendency to level off at drug concentrations above four times the MIC. The static $C_{\text{static}}$ varied from 0.25 (E33235) to 1.5 (ATCC 25923) times the MIC.

### RESULTS

In vitro studies. (i) Susceptibility studies. MICs were determined at both neutral and acidic pHs, since the intracellular pH is often markedly lower than that of the extracellular compartments. The MICs for plectasin against Staphylococcus aureus E33235 were determined to be 2 mg/liter at pH 7.4 and 4 mg/liter at pH 5.4. For S. aureus ATCC 25923 the MIC was 32 mg/liter at both pH 5.4 and 7.4.

(ii) Extracellular concentration-killing studies. The extracellular killing effect of plectasin against S. aureus was tested by applying concentrations from 0.001 to 256 times the MIC over a 24-hour period. Results are shown in Fig. 2 and Table 1. Plectasin had a potent bactericidal effect (defined by CLSI as $>3$-log decrease in CFU compared to the initial inoculum) (8). $E_{\text{max}}$ values were calculated to be $-4.5$ log CFU (limit of detection) for both E33235 and ATCC 25923 after 24 h. The rate of killing was concentration dependent. $C_{\text{static}}$ varied from 0.25 (E33235) to 1.5 (ATCC 25923) times the MIC.

(iii) Intracellular effect studies with human THP-1 monocytes. The intracellular activity of plectasin was assessed by 24-hour dose-response studies of S. aureus phagocytosed by human THP-1 monocytes using a wide range of extracellular concentrations (0.01 to 128 times the MIC). Results from the studies are shown in Fig. 2A and B and Table 1, where data are plotted against the weight concentrations of plectasin.

The killing effect was decreased compared to the extracellular effect, and the $E_{\text{max}}$ values for plectasin against E33235 and ATCC 25923 after 24 h were $-1.0$ log CFU and $-1.3$ log CFU, respectively. Some concentration dependency was still observed, even though the activity had a tendency to level off at drug concentrations above four times the MIC. The static dose was related to the MIC of plectasin against each of the two strains tested. Of interest, plectasin showed indistinguishable effects against the intracellular forms of E33235 and ATCC 25923 when data were plotted against multiple of...
concentrations (Fig. 2C and D). The corresponding static concentrations were 2.5- (ATCC 25293) to 6.6-fold (E33235) lower against the intracellular forms than against the extracellular ones.

(iv) Cell viability. The viability of THP-1 monocytes after 24 h of treatment with plectasin was assessed by trypan blue staining. No changes in the number of live cells were observed when testing plectasin concentrations from 1 to 256 mg/liter. All conditions resulted in a live/dead ratio of approximately 100:1 (\( <0.01 \) dead cells), corresponding to an unchanged cell viability compared to the negative control sample.

(v) PK studies and protein binding. Single-dose PK studies were performed in order to determine the parameters based on the free drug concentrations \( (f_{\text{AUC/MIC}_{24 \ h}}/f > \text{MIC}_{24 \ h}) \) and \( f_{\text{peak/MIC}} \) assessed in the in vivo PK/PD study. The results from the PK study are shown in Fig. 3 and Table 2.

Since the solubility of plectasin was low at physiological pH concentrations (Fig. 2C and D). The corresponding static concentrations were 2.5- (ATCC 25293) to 6.6-fold (E33235) lower against the intracellular forms than against the extracellular ones.

FIG. 2. (A and B) Extra- and intracellular activity of plectasin against S. aureus E33235 (A) and S. aureus ATCC 25923 (B). The abscissa shows the extracellular concentrations of plectasin applied. The ordinate shows the change in CFU per ml (extracellular) or per mg cell protein (intracellular) at 24 h compared to the initial inoculum. A sigmoidal function was applied for regression analysis. (C and D) Extracellular and intracellular activity of plectasin.

### Table 1. \( E_{\text{max}} \) and \( C_{\text{static}} \) from in vitro extra- and intracellular studies of antistaphylococcal activity of plectasin

<table>
<thead>
<tr>
<th>Strain (MIC [mg/liter]) and type of activity (at 24 h)</th>
<th>( E_{\text{max}} ) (log CFU) (CI)</th>
<th>( C_{\text{static}} )</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>E33235 (2)</td>
<td>Intra: -1.0 ((-1.78 ) to (-0.76))</td>
<td>0.6</td>
<td>0.952</td>
</tr>
<tr>
<td></td>
<td>Extr: (-4.5^*) ((-5.51 ) to (-3.90))</td>
<td>0.2</td>
<td>0.989</td>
</tr>
<tr>
<td>ATCC 25923 (32)</td>
<td>Intra: -1.4 ((-1.92 ) to (-0.79))</td>
<td>0.6</td>
<td>0.882</td>
</tr>
<tr>
<td></td>
<td>Extr: (-4.5^*) ((-6.70 ) to (-3.88))</td>
<td>1.5</td>
<td>0.917</td>
</tr>
</tbody>
</table>

\( ^* \) All data points (see Fig. 2) were used for the regression analysis. P values, determined by analysis of covariance between curves for extra- and intracellular concentrations (there was no significant difference between the curves for intracellular activity between E33235 and ATCC 25923 when plotted against multiples of their MIC), were \( <0.0001 \) for both strains.

\( ^* \) values below the extracellular level of detection (\(-4.5 \) log CFU). CI, confidence interval.

\( ^* \) Expressed as multiples of the MIC.
CFU counts were estimated as equal to those for untreated control animals (at 6 h). The initial inoculum was $7.5 \times 10^7$ CFU/ml. The medians of the total bacterial count were $8.1 \times 10^8$ (total), $2.8 \times 10^8$ (extracellular), and $2.6 \times 10^7$ (intracellular) CFU/ml 2 hours after inoculation and $1.1 \times 10^7$ (total), $7.6 \times 10^5$ (extracellular), and $6.8 \times 10^6$ CFU/ml (intracellular) 6 hours after inoculation in untreated control animals.

**Influence of PK and PD parameters on antimicrobial effect.** Dose-response data were analyzed to examine the impact of the PK/PD parameters by relating the number of bacteria from peritoneal fluid in treated animals to $f_{C_{\text{peak}}}/\text{MIC}$, $T_{\text{MIC24 h}}$, and $f_{\text{AUC/MIC24 h}}$. To determine the PK/PD relationships, the correlations (Hill’s) in both the extra- and intracellular compartment were calculated and the effect was defined as the decrease in CFU in the peritoneal fluid 24 h after the first treatment compared to CFU in untreated control animals (at 2 h).

The results showed that infection outcome was highly affected by the size of the first dose, with a strong correlation between $f_{C_{\text{peak}}}/\text{MIC}$ and efficacy (Fig. 4), both extra- and intracellularly. Regression of the data with the $f_{C_{\text{peak}}}$ resulted in the strongest correlation observed (0.86 extracellularly and 0.75 intracellularly). Regression of the dose-response data with the $f_{T>\text{MIC24 h}}$ parameter resulted in a poor fit of the data, with an $R^2$ value of 0.14 intracellularly. The $R^2$ for $f_{T>\text{MIC24 h}}$ extracellularly was 0.38. Correlation coefficients (goodness of fit) are shown in Fig. 4. $E_{\text{max}}$ values were estimated to be $-1.1$ log CFU intracellularly and $-2.2$ log CFU extracellularly.

**Influence of dose size (one large dose versus multiple small doses) on intra- and extracellular effect.** As indicated by $f_{C_{\text{peak}}}$ being the most important factor for efficacy, a pronounced difference between dosing regimens, e.g., a single dose of 17 mg/kg compared to four doses of 4.25 mg/kg, was observed. In the latter case slight regrowth of the bacteria was seen, with $E_{\text{max}}$ Values of 0.4 and 0.3 log CFU extra- and intracellularly, respectively, since plectasin concentrations in serum did not exceed the MIC (Fig. 3). The values for single dosing with a high concentration (17 mg/kg) of plectasin resulted in $E_{\text{max}}$ values of $-1.3$ (intracellular) and $-2.2$ (extracellular).

---

**Table 2. Treatment regimens and resulting PK/PD parameters for S. aureus E33235 in the mouse peritonitis study**

<table>
<thead>
<tr>
<th>Total dose (mg)</th>
<th>Single dose (mg/kg)</th>
<th>No. of doses in 24 h</th>
<th>$f_{T&gt;\text{MIC}}$ (h)</th>
<th>$f_{\text{AUC/MIC}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.02</td>
<td>34</td>
<td>1</td>
<td>2.64</td>
<td>6.45</td>
</tr>
<tr>
<td>2.04</td>
<td>34</td>
<td>2</td>
<td>5.28</td>
<td>12.89</td>
</tr>
<tr>
<td>3.06</td>
<td>34</td>
<td>3</td>
<td>7.91</td>
<td>19.34</td>
</tr>
<tr>
<td>0.51</td>
<td>17</td>
<td>1</td>
<td>1.61</td>
<td>3.85</td>
</tr>
<tr>
<td>0.26</td>
<td>8.5</td>
<td>1</td>
<td>0.95</td>
<td>1.98</td>
</tr>
<tr>
<td>0.51</td>
<td>8.5</td>
<td>2</td>
<td>1.89</td>
<td>3.30</td>
</tr>
<tr>
<td>1.02</td>
<td>8.5</td>
<td>4</td>
<td>3.79</td>
<td>7.91</td>
</tr>
<tr>
<td>1.56</td>
<td>8.5</td>
<td>6</td>
<td>5.68</td>
<td>11.87</td>
</tr>
<tr>
<td>0.13</td>
<td>4.25</td>
<td>1</td>
<td>0.00</td>
<td>0.95</td>
</tr>
<tr>
<td>0.39</td>
<td>4.25</td>
<td>3</td>
<td>0.00</td>
<td>2.85</td>
</tr>
</tbody>
</table>

<sup>a</sup> PK/PD parameters were calculated based on free drug concentrations (27) using GraphPad Prism. MIC for E33235 was 2 mg/liter. There were three mice per group.
DISCUSSION

The data presented in this study represent the first evaluation of the intracellular antistaphylococcal properties of an antimicrobial peptide by application of both in vitro and in vivo methods. Plectasin exhibited extracellular bactericidal activity in vitro against \textit{S. aureus} (H11022 3 log kill). When applying the intracellular human THP-1 monocyte model, it was obvious that the \(E_{\text{max}}\) of plectasin against intracellular \textit{S. aureus} was reduced, with values of about 1 log CFU compared to values of more than 4.5 log CFU for extracellular bacteria. Such a decrease in relative efficacy has been observed in all previous studies of intracellular activity of antistaphylococcal compounds, and when comparing results to previous studies, it was found that the in vitro intracellular activity of plectasin was more pronounced than those of both vancomycin (\(E_{\text{max}}\) of \(-0.5\) log CFU) and linezolid, which are compounds commonly used against difficult-to-treat staphylococcal infections (3, 22). Also, studies of a macrolide (azithromycin) revealed inferior relative efficacy, with an \(E_{\text{max}}\) value of \(-0.5\) log CFU (35).

Actually, plectasin had an in vitro intracellular activity comparable to previously published results for \(\beta\)-lactams. Lemaire et al. (23) found values for \(E_{\text{max}}\) against \textit{S. aureus} ATCC 25923...
for β-lactams of between $-0.68 \log$ CFU (oxacillin) and $-0.90 \log$ CFU (imipenem).

These earlier studies of conventional antibiotics also found a much lower intracellular activity than would have been expected from extracellular killing kinetics and from the levels of intracellular accumulation, indicating a low correlation between the intracellular activity and the level of accumulation (4, 12, 29, 30, 36). Furthermore, the phenomenon of decreased relative efficacies could be caused by several factors such as acidic pH conditions, binding to intracellular proteins or other constituents, or changes in the metabolism of the bacteria (4, 36). Conversely, we see that the relative potencies of plectasin (defined by its 50% effective concentration and $C_{\text{static}}$ values) are more favorable against the intracellular than the extracellular forms. This may be related to the intracellular accumulation of the antibiotic, a point that will need to be examined experimentally. It is, nevertheless, remarkable that, despite their marked difference in intrinsic susceptibilities, the two strains tested in the THP-1 monocyte model behaved in indistinguishable manners when challenged intracellularly with equipotent extracellular concentrations. This indicates that, for a given drug, intrinsic activity is the driving force that determines the relative potency of activity, suggesting that MIC can be used as a useful indicator in this context.

In the mouse peritonitis model the main conclusions were that the extracellular activity of plectasin was highly concentration dependent whereas the intracellular activity showed some concentration dependency up to around eight times the MIC. Both extracellular killing and intracellular killing were mainly dependent on a high $f_{C_{\text{peak}}}$, whereas small frequent doses resulted in a much poorer effect of treatment. As observed in the THP-1 monocytes, intracellular bacterial killing was decreased compared to extracellular killing but a certain level of activity remained intact, with a maximum decrease in CFU of above 1 log.

Since the in vivo model was developed recently, only few data were available for comparison. Sandberg et al. tested dicloxacillin and rifampin (rifampicin), compounds often used for treatment of methicillin-susceptible $S. \text{aureus}$ and methicillin-resistant $S. \text{aureus}$, respectively, and found $E_{\text{max}}$ values inferior to those for plectasin treatment, with only a static effect on or even regrowth of bacteria after 19 h of treatment (32). Due to a relatively low solubility in combination with a serum elimination half-life of plectasin of approximately 45 min, it was not possible to vary doses enough to obtain a larger span in $f_{C_{\text{peak}}}>MIC_{24 \text{h}}$ and $f_{\text{AUC}}/\text{MIC}_{24 \text{h}}$.

Direct comparisons between in vitro and in vivo results were complicated by several factors. In the THP-1 monocytes the bacteria and cells were continuously exposed to the antibiotics, whereas the animals in the peritonitis studies were injected one to six times over the 24-h study period. Furthermore, the in vitro model could not evaluate the influence of protein binding. A major limitation to the in vitro model was that the experiments were performed with an immortalized cell line, which may behave differently from normal monocytes/macrophages. Therefore data could not be directly extrapolated to the situations prevailing in humans upon infection by $S. \text{aureus}$. This limitation also affects the in vivo model used here, as staphylococcal disease involves many other types of cells and tissues (16, 26). The in vivo model, however, allows the immune response of the host, which is known to influence the course of infection, to be taken into account to a certain extent.

An advantage of the in vivo model was that the PK parameters could be determined and the intracellular activity assessed in a whole-body system including a functional immune system. When analyzing results from the in vivo model, the dynamic nature of infection should therefore also be considered. As opposed to results for the THP-1 model, the amount of intracellular bacteria was not static in vivo, since intracellular growth as well as phagocytosis and cell lysis takes place throughout the course of the infection. Also the rapid extracellular effect of plectasin could impact the intracellular activity, as many bacteria were expected to be killed before entering the monocytes. Therefore, further studies investigating the dynamics of the intracellular/extracellular bacteria not only after 24 h but also at time intervals during the 24-hour period would provide valuable information on the effect of plectasin on these types of infection.

Even though several aspects were found to influence the course of infection differently in vivo and in vitro, our results demonstrated many similarities in results from the two studies. These findings indicate that the THP-1 model is useful as a first screening tool for intracellular activity, yet the animal models are needed for gaining more extended knowledge on drug efficacy and PK/PD parameters and their impact on the optimal dosing regimens required for progressing from preclinical to clinical investigations.

When comparing our results with previous studies of intracellular activity of antibiotics, it is important to remember that until now plectasin has been applied only in animal and in vitro models and that the human $f_{C_{\text{peak}}}$ is yet to be defined. Therefore direct comparisons to conventional antimicrobial compounds cannot be performed, as it is unknown whether the human serum concentrations of plectasin optimal for intracellular activity can be obtained.

Plectasin has proven to exhibit a pronounced extracellular antistaphylococcal activity. Even though the activity is reduced intracellularly, we have shown, both in vitro and in vivo, that a level of intracellular activity comparable or superior to previously published results on commercially available antistaphylococcal compounds is retained. Both the present and the aforementioned studies of intracellular antistaphylococcal activity have shown that potent activity in traditional killing kinetics assays is not necessarily predictive of intracellular efficacy. These results therefore raise questions about the usefulness of many antibiotics traditionally used in treatment of infections where intracellular staphylococci play a role and stress the importance of addressing these issues in clinical trials in cases of treatment failure. Additional studies are required to gain further insight of the mechanisms of plectasin against intracellular $S. \text{aureus}$. Future studies should include testing against a wider range of staphylococcal strains, use of different cell types, and assessment of the cellular uptake and subcellular distribution of plectasin.

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