ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2009, p. 4801–4808 Vol. 53, No. 11
0066-4804/09/$12.00 doi:10.1128/AAC.00685-09

Plectasin Shows Intracellular Activity against *Staphylococcus aureus* in Human THP-1 Monocytes and in a Mouse Peritonitis Model[▽]

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Received 19 May 2009/Returned for modification 7 July 2009/Accepted 28 August 2009

Antimicrobial therapy of infections with *Staphylococcus aureus* can pose a challenge due to slow response to therapy and recurrence of infection. These treatment difficulties can partly be explained by intracellular survival of staphylococci, which is why the intracellular activity of antistaphylococcal compounds has received increased attention within recent years. The intracellular activity of plectasin, an antimicrobial peptide, against *S. aureus* was determined both in vitro and in vivo. In vitro studies using THP-1 monocytes showed that some intracellular antibacterial activity of plectasin was maintained (maximal relative efficacy [E_{max}], 1.0- to 1.3-log reduction in CFU) even though efficacy was inferior to that of extracellular killing (E_{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_{max} of a 1.1-log CFU reduction. The parameter most important for activity was fC_{peak}/MIC, where fC_{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 vitro 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.

Staphylococcus aureus is an important causative agent of numerous community- and hospital-acquired infections (21), ranging from minor infections such as dermatitis or wound infections (14, 17) to critical septicemia-related diseases, including osteomyelitis (9), endocarditis (10), and meningitis (31). Treatment of staphylococcal disease can be challenging since the infection sometimes persists or recurs even after prolonged treatment with antimicrobial agents (7, 18–20). Several aspects of bacterial pathogenesis may be involved in the persistence of staphylococcal infections. An important feature reported by several authors is the ability of the bacteria to invade and survive inside the cells (6, 15, 16, 25, 26).

Intracellular accumulation of the bacteria can complicate the use of antimicrobial agents since the activity may be impeded by several factors such as poor drug distribution or increased metabolism inside the cells and the inability of the drug, even if it accumulates at the site of infection, to express its activity against the causative organism. Many antibiotic agents diffuse poorly into the phagocytes or do not enter the same intracellular compartment as the bacteria; furthermore, their antimicrobial activity may be affected unfavorably by the intracellular milieu or changes in bacterial metabolism (4, 37, 38).

Many drugs, including commonly used antistaphylococcal compounds, such as the β-lactamase-resistant β-lactams, aminoglycosides, and vancomycin, exhibit poor intracellular activity against *S. aureus*, some with a relative efficacy as low as −0.5 to −1.0 log CFU (1, 3). Additionally, a rise in incidence of methicillin (meticillin)-resistant *S. aureus* (39) and emergence of multidrug-resistant strains (2) have further complicated the therapy of these infections.

Therefore the need for novel compounds against these types of infections is increasing, with special attention paid to a quantitative evaluation of their activity against intracellular forms of *S. aureus*.

Plectasin is a defensin-type antimicrobial peptide derived from the pezizalean fungus *Pseudoplectania nigrella*. The compound has shown potent antimicrobial effect both in vitro and in vivo against various gram-positive bacteria, including resistant strains of *Streptococcus pneumoniae* and some strains of *S. aureus* (5, 28). Furthermore, plectasin has a novel mode of action (binding the bacterial cell wall precursor lipid II) compared to commonly employed antistaphylococcal compounds (T. Schneider, T. Kruse, R. Wimmer, I. Wiedemann, V. Sass, U. Pag, A. Jansen, A. K. Nielsen, P. H. Mygind, D. S. Raventos, S. Neve, B. Ravn, L. De Maria, L. Kamenova, H. G. Sahl, and H. H. Kristensen, submitted for publication). Of interest, plectasin has been found to be innocuous to eukaryotic cells

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▽ Published ahead of print on 8 September 2009.

(28). 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 [$fT>MIC_{24\ h}$], free area under the concentration-time curve/$MIC_{24\ h}$ [$fAUC/MIC_{24\ h}$], and free peak concentration/MIC [fC_{peak}/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 WDEDDMQCHNHCKSIKGYKGGYCAKGGFVCKCY) 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 1×10^8 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 pH-adjusted 5% mucin solution (M-2378; Sigma-Aldrich) to ensure establishment of infection. Strain E33235 was chosen since the MIC of plectasin is relatively low (2 mg/liter), and ATCC 25923 was applied as a control for the intracellular setup in the THP-1 model. In the bioassay for assessment of protein binding of plectasin, *S. aureus* SA6 (ATCC 29737) was used.

In vitro studies. (i) Extracellular concentration-kill studies. Concentration-kill curve studies were performed as previously described (4). In short, bacteria in exponential growth were resuspended in cation-adjusted Mueller-Hinton broth, and plectasin concentrations from 0.001 to 256 times the MIC were tested. At 0 h control samples without plectasin were spread for calculation of initial CFU load (6.6×10^6 to 9.5×10^6 CFU/ml). The number of viable bacteria was determined after 24 h of incubation at 37°C. All tests were performed in triplicate, and results are stated as means of the three CFU determinations (see Fig. 2).

(ii) Intracellular concentration-kill studies of human THP-1 monocytes. In vitro 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 5×10^5 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 2×10^6 cells/ml.

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 RPMI 1640 medium (10% fetal calf serum) and plectasin was added 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 (1.1×10^6 to 4.0×10^6 CFU/ml). All tests were performed in triplicate, and results are expressed as means of the three CFU determinations (see Fig. 2).

The intracellular activity was calculated as CFU per mg protein to assess the amount of bacteria in relation to the monocyte protein content in each sample. Therefore the remaining sample was used for a protein assay, where samples were sonicated and protein content was quantified as described by Lowry et al. (24).

(iii) 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.

In vivo studies in the mouse peritonitis/sepsis model. (i) Animals. All studies involving use of animals were approved by Novozymes' Laboratory Animal Review Committee and complied with Danish legislation on use of laboratory animals. Female outbred NMRI mice (starting weight: 26 to 30 g) purchased from Harlan Europe were used in all studies. Animals were kept in standard Macrolon type III cages (four to seven per cage), were fed a standard pellet diet (2016; Harlan) ad libitum, and had free access to bottled drinking water. Bedding was aspen wood, and animals had nesting material from Scanbur as environmental enrichment. Animals were acclimatized for 5 to 6 days prior to study initiation.

(ii) 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 (s.c.) with plectasin at four different dose levels: 4.25, 8.5, 17, or 34 mg/kg of body weight. The dose volumes varied from 0.3 to 0.6 ml/animal. Blood samples were collected in uncoated Eppendorf tubes after 5, 10, 20, 30, 40, 60, 120, 180, and 360 min at 17 and 34 mg/kg. Samples of 4.25 and 8.5 mg/kg were taken after 5, 10, 20, 40, 60, 120, and 240 min. Serum was separated and stored at −20°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.

(iii) Calculation of dosing regimens. Doses for the PK/PD study were designed to vary fC_{peak}, $fT>MIC_{24\ h}$, and $fAUC/MIC_{24\ h}$. 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 $fAUC_{24\ h}$ was calculated from mean concentrations using the trapezoidal rule. In Table 2 the $fT>MIC$ and $fAUC$ for the applied dosing regimens are listed.

(iv) Protein binding. The impact of the protein binding capacity of plectasin was an important factor when determining dose regimens. The protein binding to murine and human albumin was assessed by the ultrafiltration method using Centrifree (724-04104 A; Millipore, Bedford, MA). The concentrations of plectasin in the ultrafiltrate were measured by a bioassay using *S. aureus* SA6 ATCC 29737 as the test strain.

(v) Infection 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

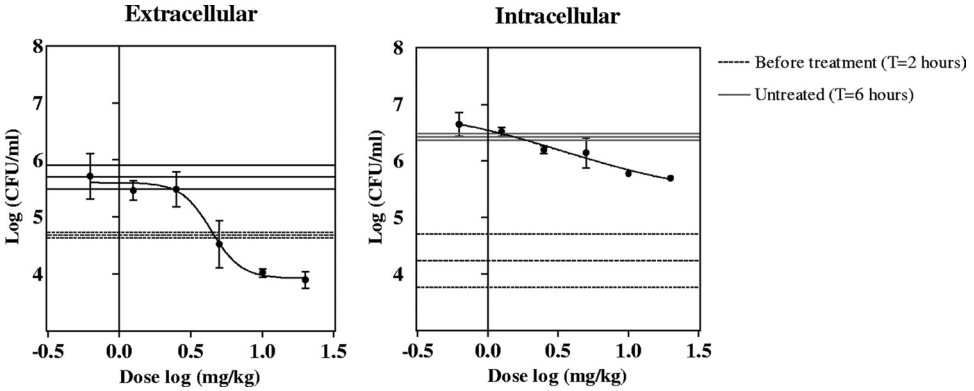

FIG. 1. Extra- and intracellular activity of plectasin in a 6-hour dose-response study using the murine peritonitis model for *S. aureus* E33235. Results are relative to the plectasin dose (abscissa). Horizontal lines (dotted and solid, respectively) show the CFU count before treatment and in untreated control animals at 6 h.

study were performed (Fig. 1). These studies investigated whether the strain was suitable for use in the model and whether plectasin had intracellular effects.

Next, a 24-h PK/PD study was set up to assess intra- and extracellular effects of plectasin and which PK parameters were best correlated to efficacy (see Fig. 3). In brief, mice were inoculated intraperitoneally with a 0.5-ml suspension of *S. aureus* (5×10^7 CFU/ml) in 5% mucin. Two hours after inoculation the animals were treated s.c. with plectasin (dosing volume of 0.3 to 0.6 ml). Thereafter, different dosing regimens ($n = 3$) were applied, with doses ranging from a single dose of 4.25 mg/kg to three doses of 34 mg/kg in 24 h (see Table 2) based on the PK study described below, in order to vary the fC_{peak}, $fT{>}MIC_{24\ h}$, and $fAUC_{24\ h}$ between the treated groups. Six hours after inoculation the untreated control mice were euthanized and peritoneal fluid was collected through a peritoneal wash with 2 ml 0.9% sterile saline. Afterwards, the extra- and intracellular fractions were separated in a separation assay as described below. After 24 h all treated animals were euthanized, and peritoneal fluid collection and separation procedures similar to those for the control animals were performed.

(vi) Separation of intra- and extracellular bacteria in peritoneal fluid. Separation of intra- and extracellular bacteria in peritoneal fluid was performed as a modified version of the procedures described by Sandberg et al. (32). The collected peritoneal fluid from one mouse was diluted 1:1 with Hanks balanced salt solution. The total CFU count of the diluted sample was determined before any further procedures. The diluted sample was then divided into two equal fractions of ~1.5 ml each (fractions A and B). For extracellular CFU quantification, fraction A was centrifuged at $300 \times g$ at room temperature for 10 min and the extracellular CFU count from the supernatant was quantified. For intracellular CFU quantification, lysostaphin (L-7386; Sigma-Aldrich Inc.) was added to fraction B to a final concentration of 15 mg/liter and the fraction was incubated for 15 min at room temperature. The lysostaphin was removed, and the fraction was prepared for CFU quantification as described previously (32). The effect of plectasin treatment was estimated as the decrease in CFU counts in the peritoneal fluid compared to counts for untreated controls (samples after 2 h), and the antibacterial activity was assessed for total, extracellular, and intracellular counts separately.

Data analysis and statistical methods. For in vitro intracellular dose-response studies (analysis of dose-effect relationships), the Hill equation was employed to calculate the maximal relative efficacy (E_{max}), static concentration (C_{static}), and goodness of fit (R^2). These parameters were determined using nonlinear regression. In the Hill equation a slope factor of 1 was employed as described by Barcia-Macay et al. (3). E_{max} was defined as the log change in CFU counts between the postphagocytosis inoculum and CFU after 24 h of treatment, while C_{static} was the concentration (in multiples of the MIC) resulting in no apparent bacterial growth (CFU identical to that of the original inoculum). For statistical analyses, see Table 1. Comparisons of corresponding E_{max} values of extra- and intracellular activities for each compound were performed using the unpaired *t* test ($P < 0.05$). Extracellular concentrations were compared to intracellular concentrations using analysis of covariance of the curve between corresponding parameters for extra- and intracellular activities. The significance level was set at a *P* value of <0.05. Curve fittings and statistical analyses were performed using Prism 5.0 (GraphPad Prism Software, San Diego, CA).

Results from the animal PK/PD studies were also analyzed by using the sigmoidal model (Hill's equation), with E_{max} defined as the difference in CFU in the peritoneal fluid between untreated control animals (2 h after infection) and animals treated for 24 h. The correlations between efficacy and the PK/PD parameters ($fT{>}MIC_{24\ h}$, $fAUC/MIC_{24\ h}$, fC_{peak}/MIC) were calculated by nonlinear regression. The correlation coefficients (R^2) were used to determine the goodness of fit of the curves.

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-kill 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_{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_{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_{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 252923 when data were plotted against multiple of

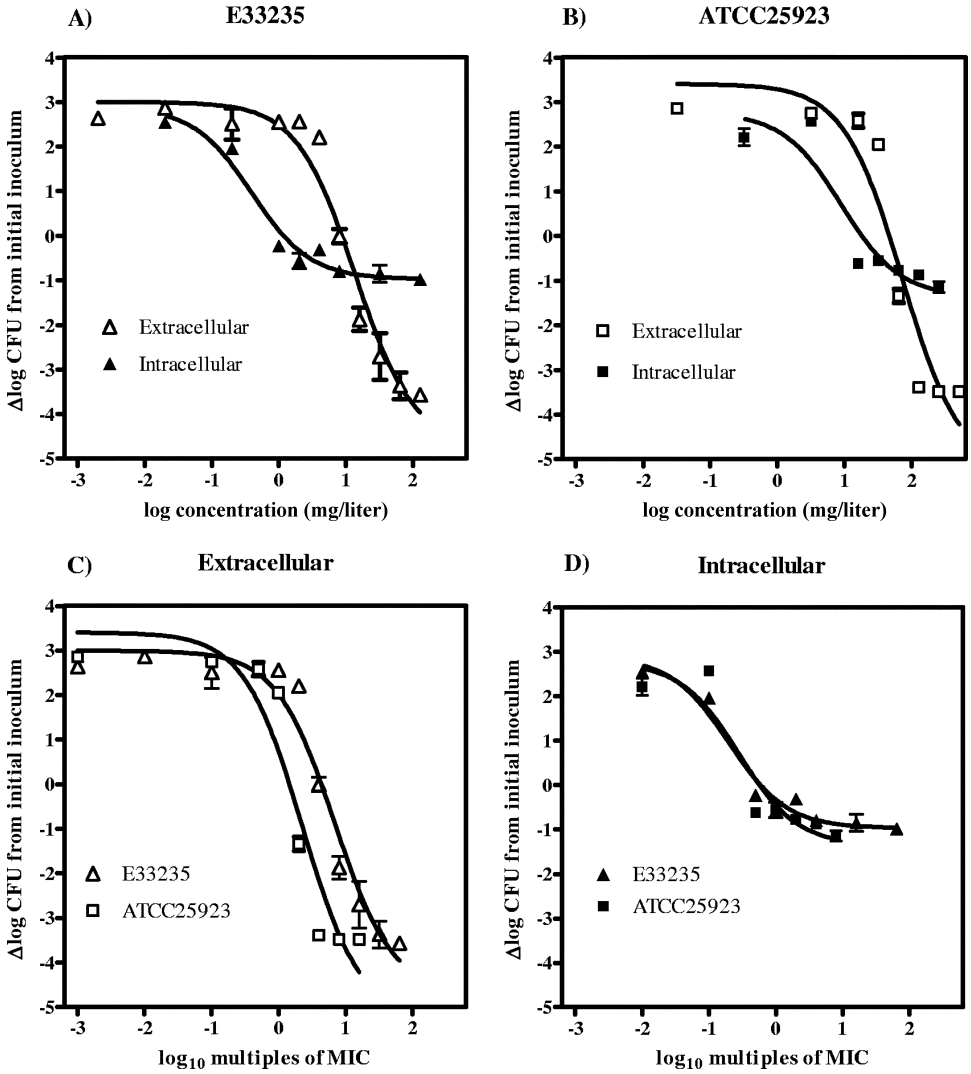

FIG. 2. (A and B) Extra- and intracellular activity of plectasin against *S. aureus* E33235 (A) and *S. aureus* ATCC 25923 (B). The abscissa shows the extracellular concentrations of plectasin applied. The ordinate shows the change in CFU per ml (extracellular) or per mg cell protein (intracellular) 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_{max} and C_{static} from in vitro extra- and intracellular studies of antistaphylococcal activity of plectasin[a]

Strain (MIC [mg/liter]) and type of activity (at 24 h)	E_{max} (log CFU) (CI)[b]	C_{static}[c]	R^2
E33235 (2)			
Intracellular	−1.0 (−1.78 to −0.76)	0.6	0.952
Extracellular	<−4.5* (−5.51 to −3.90)	0.2	0.989
ATCC 25923 (32)			
Intracellular	−1.4 (−1.92 to −0.79)	0.6	0.882
Extracellular	<−4.5* (−6.70 to −3.88)	1.5	0.917

[a] 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.

[b] *, values below the extracellular level of detection (−4.5 log CFU). CI, confidence interval.

[c] Expressed as multiples of the MIC.

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 (≤1% 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 (fAUC/MIC$_{24\ h}$, fT>MIC$_{24\ h}$, and fC$_{peak}$/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

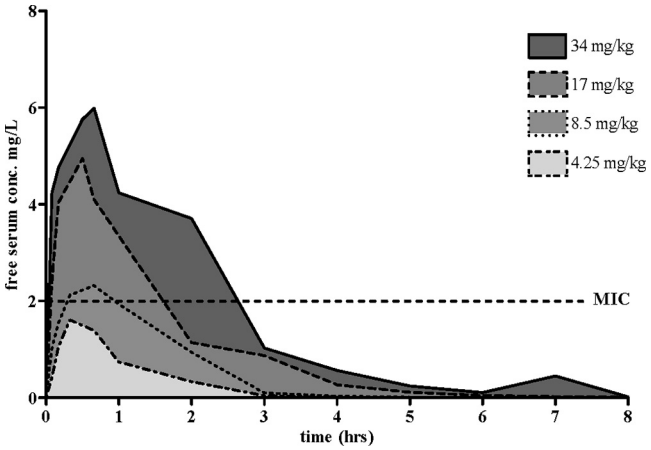

FIG. 3. PK studies. Free serum concentrations (conc) of plectasin in mice after s.c. treatment with doses from 4.25 to 34 mg/kg are shown to illustrate the *f*T>MIC and *f*AUC for the different dosing regimens. The MIC against *S. aureus* E33235 was 2 mg/liter. The lowest dose level did not reach serum concentrations above the MIC (see Table 2).

(2 mg/ml) and the serum elimination half-life was approximately 45 min, *f*AUC and *f*T>MIC could be assessed only within a limited range in animal studies if dosing volumes and intervals were within acceptable limits. The protein binding for plectasin in human and murine serum was determined to 90%. The coefficient of variation was determined to be from 4.2 to 11.4% for interplate variation.

Dose-response study of plectasin versus *S. aureus* in the mouse peritonitis model. An initial comparative study of strains of *S. aureus* showed no significant differences between *S. aureus* E33235 and the *S. aureus* strain previously used in the model with regard to virulence and cell sensibility in the separation assay (data not shown). A dose-response study for *S. aureus* over 6 hours showed an intracellular decrease in CFU of approximately 1 log compared to untreated controls (Fig. 1). However, the intracellular CFU count could not be reduced to levels below the initial inoculum, indicating that the onset of plectasin activity in the intracellular compartment is slower than the extracellular activity.

Twenty-four-hour PK/PD study using the mouse peritonitis model. The 24-hour PK/PD study using the mouse peritonitis model was performed to assess the intra- and extracellular activities of plectasin against *S. aureus* E33235 and to investigate which PK/PD parameters (fAUC/MIC$_{24\ h}$, fT>MIC$_{24\ h}$, and fC$_{peak}$/MIC) best predicted the efficacy of plectasin. Results are shown in Fig. 4.

The differences in activity between treatment groups for total, extracellular, and intracellular CFU counts were compared separately due to the initial differences in CFU and were compared to values for untreated controls. E33235 was chosen as the test strain since it was sensitive to plectasin while the relatively high MIC against ATCC 25923 precluded the use of this strain in vivo.

Some animals treated with the lowest doses of plectasin (4.25 mg/kg) were euthanized before the 24-h study period was completed so that they would not succumb to the infection. CFU counts were not performed, but to avoid an artificially low CFU number by excluding these animals, CFU counts were estimated as equal to those for untreated control animals (at 6 h). The initial inoculum was 7.5×10^7 CFU/ml. The medians of the total bacterial count were 8.1×10^5 (total), 2.8×10^4 (extracellular), and 2.6×10^5 (intracellular) CFU/ml 2 hours after inoculation and 1.1×10^7 (total), 7.6×10^5 (extracellular), and 6.8×10^6 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 fC$_{peak}$/MIC, fT>MIC$_{24\ h}$, and fAUC/MIC$_{24\ h}$. 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 fC$_{peak}$/MIC and efficacy (Fig. 4), both extra- and intracellularly. Regression of the data with the fC$_{peak}$ resulted in the strongest correlation observed (0.86 extracellularly and 0.75 intracellularly). Regression of the dose-response data with the fT>MIC$_{24\ h}$ parameter resulted in a poor fit of the data, with an R^2 value of 0.14 intracellularly. The R^2 for fT>MIC$_{24\ h}$ extracellularly was 0.38. Correlation coefficients (goodness of fit) are shown in Fig. 4. E_{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 fC$_{peak}$ 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_{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_{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[a]

Total dose (mg)	Single dose (mg/kg)	No. of doses in 24 h	*f*T>MIC (h)	*f*AUC/MIC
1.02	34	1	2.64	6.45
2.04	34	2	5.28	12.89
3.06	34	3	7.91	19.34
0.51	17	1	1.61	3.85
0.26	8.5	1	0.95	1.98
0.51	8.5	2	1.89	3.96
1.02	8.5	4	3.79	7.91
1.56	8.5	6	5.68	11.87
0.13	4.25	1	0.00	0.95
0.39	4.25	3	0.00	2.85

[a] 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.

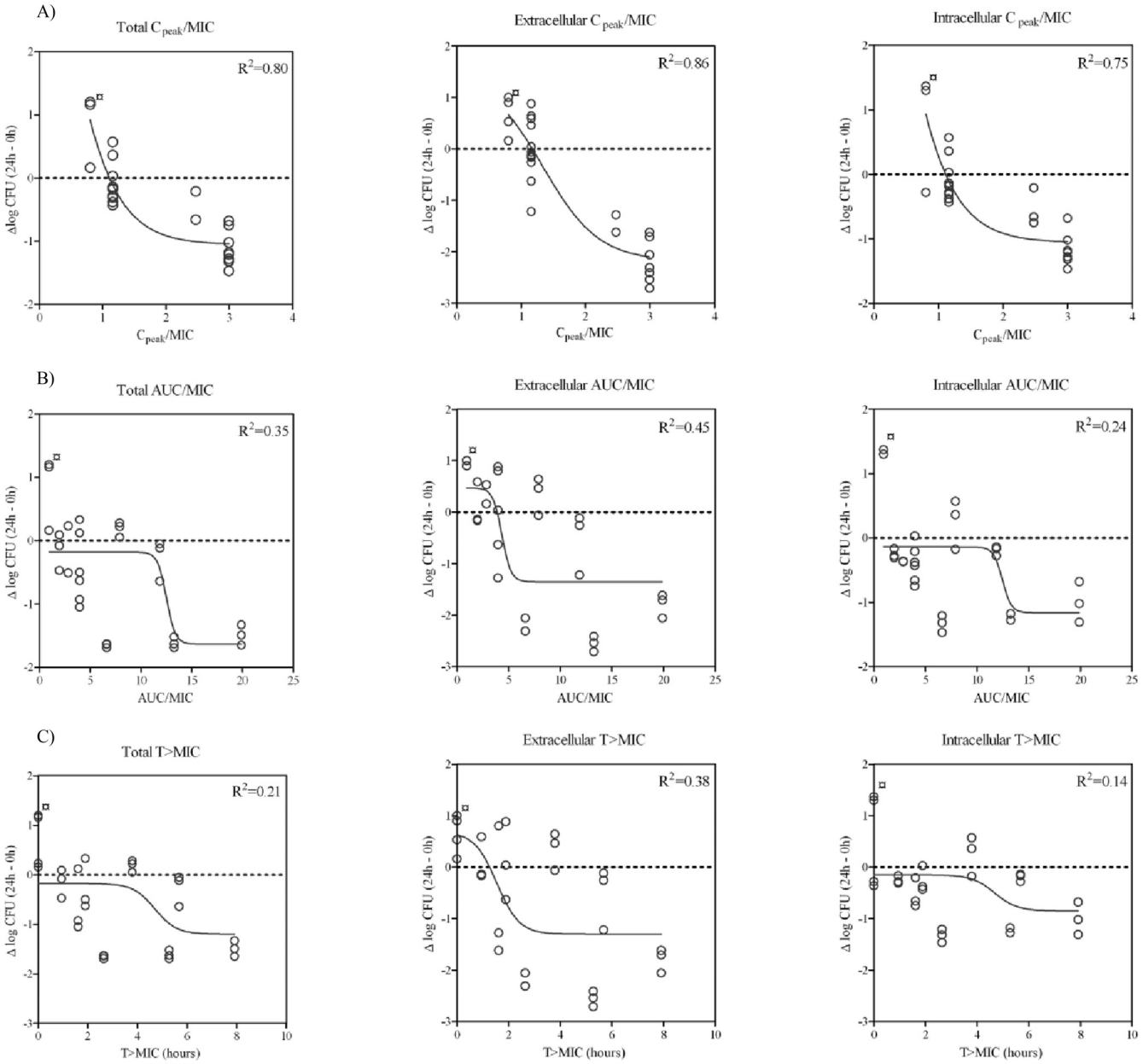

FIG. 4. Correlations between PK parameters for total, extracellular, and intracellular activity of plectasin against *S. aureus* E33235 (MIC = 2 mg/liter) after 24 h. (A) fC_{peak}/MIC; (B) fAUC/$MIC_{24\ h}$; (C) $fT{>}MIC_{24\ h}$. The ordinate displays the change in CFU (corresponding to the E_{max} value) in peritoneal fluid between treated animals (24 h) and untreated controls (at 2 h). CFU counts marked with ¤ are estimated CFU from animals taken out before the 24-h study period was completed.

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 *S. aureus* (>3 log kill). When applying the intracellular human THP-1 monocyte model, it was obvious that the E_{max} of plectasin against intracellular *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_{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_{max} value of −0.5 log CFU (35). Actually, plectasin had an in vitro intracellular activity comparable to previously published results for β-lactams. Lemaire et al. (23) found values for E_{max} against *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_{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 fC_{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. aureus* and methicillin-resistant *S. aureus*, respectively, and found E_{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 $fT{>}MIC_{24\ h}$ and $fAUC/MIC_{24\ h}$.

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. 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 fC_{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. 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.

ACKNOWLEDGMENTS

We thank M.-C. Cambier and M. Vergauwen, Université catholique de Louvain, and J. M. Andersen, Statens Serum Institut, for dedicated technical assistance. K. S. Jensen from Statens Serum Institut is

thanked for fruitful discussions on PK setup and S. Buskov, Novozymes, for assistance with HPLC analyses of serum samples.

This work was supported by a grant from the Danish Ministry of Science, Technology and Innovation and by the STAPHAUR program of the Belgian Region Wallonne (grant no. EP1A320501R052F/415735).

K. S. Brinch and H. H. Kristensen are employed by Novozymes A/S. N. Høiby and N. Frimodt-Møller are members of the Novozymes advisory board associated with the plectasin project.

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