High *in vitro* antimicrobial activity of synthetic antimicrobial peptidomimetics against staphylococcal biofilms

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**Objectives:** The aim of the study was to investigate the antimicrobial effect of different antibiotics and synthetic antimicrobial peptidomimetics (SAMPs) on staphylococcal biofilms.

**Methods:** Biofilms of six staphylococcal strains (two *Staphylococcus haemolyticus*, two *Staphylococcus epidermidis* and two *Staphylococcus aureus* isolates) were grown for 24 h in microtitre plates. They were washed and treated for 24 h with different concentrations of linezolid, tetracycline, rifampicin and vancomycin and four different SAMPs. After treatment, the redox indicator Alamar Blue was used to quantify metabolic activity of bacteria in biofilms, and confocal laser scanning microscopy with LIVE/DEAD staining was used to further elucidate any effects.

**Results:** At MIC levels, rifampicin and tetracycline showed a marked reduction of metabolic activity in the *S. epidermidis* and *S. haemolyticus* biofilm. Linezolid had a moderate effect and vancomycin had a poor effect. MIC ×10 and MIC ×100 improved the antimicrobial activity of all antibiotics, especially vancomycin. However, metabolic activity was not completely suppressed in strong biofilm-producing strains. At MIC ×10, the three most effective SAMPs (Ltx5, Ltx9 and Ltx10) were able to completely eliminate metabolic activity in the *S. epidermidis* and *S. haemolyticus* biofilms, which was also confirmed by complete cell death using confocal laser scanning microscopy investigations. Although none of the Ltx SAMPs could fully suppress metabolic activity in the *S. aureus* biofilm, their effect was superior to all tested antibiotics.

**Conclusions:** SAMPs had superior antimicrobial activity in staphylococcal biofilms compared with conventional antibiotics and are potential new therapeutic agents for biofilm-associated infections.

Keywords: Alamar Blue, biofilm inhibitory concentrations, staphylococci

**Introduction**

Biofilms are defined as microbial-derived sessile communities attached to a surface and embedded in a self-produced polymeric matrix. They play a central role in the pathogenesis of serious infections caused by *Staphylococcus aureus* and coagulase-negative staphylococci, i.e. chronic wound infections and medical device-related infections.⁰−⁷ While there is intense research activity in the field of *Staphylococcus epidermidis* and *S. aureus* biofilms,⁸−⁹ far less is known about the biofilm produced by *Staphylococcus haemolyticus*.

Bacteria grown in biofilms are more tolerant to antimicrobial agents than their planktonic counterparts.⁰−⁸ Susceptibility testing of planktonic bacteria may fail to predict *in vivo* resistance of device-related infections to antimicrobial agents.⁹ Standardized laboratory models to test antimicrobial agents in biofilms are still lacking, although a broad range of models for quantifying treated versus untreated biofilms have been described. In most of these models, the quantification of biofilm is done by conventional plating after disruption of the biofilm.¹⁰ These methods are labour-intensive and slow, and the process of disrupting the biofilm can be incomplete or kill cells so that the
number of colonies does not necessarily reflect the number of viable bacteria in the biofilm. Indirect methods are based on quantification of biomass (both living and dead cells), viability assays (living cells) and matrix quantification.\textsuperscript{11–15}

The rising number of infections caused by bacterial isolates resistant to conventional antibiotics has lead to an intense search for novel antibiotics. Cationic antimicrobial peptides (CAPs) are widespread in nature and play an important role as part of innate immunity. In general, CAPs are fairly large molecules that carry a net positive charge and contain ~50% hydrophobic residues.\textsuperscript{16–18} Their mode of action involves binding to negatively charged structural molecules on the microbial membrane. Once bound, CAPs form pores that increase the cell membrane permeability and ultimately lead to cell lysis. There is also evidence for other antimicrobial mechanisms such as interaction with intracellular targets,\textsuperscript{19,20} induction of the host immune response\textsuperscript{21} and activation of autolytic enzymes.\textsuperscript{22} CAPs have a broad spectrum of antimicrobial activity and development of resistance is rare.\textsuperscript{23,24} Unfortunately, CAPs are difficult and expensive to produce in large quantities and are usually sensitive to protease digestion.\textsuperscript{25} Modifications of CAPs have resulted in the development of extremely short synthetic antimicrobial peptidomimetics, also called SAMPs.\textsuperscript{26} SAMPs mimic the effect of CAPs, but have improved pharmacokinetic properties and are thus a promising new group of antimicrobial substances.\textsuperscript{26,27}

The primary aim of this study was to investigate the antimicrobial activity of clinically relevant antibiotics and newly designed SAMPs against biofilms of three different staphylococcal species. Second, we wanted to evaluate a simple screening method to quantify the metabolic activity of biofilms before and after the biofilm had been subjected to treatment with antimicrobial agents.

Materials and methods

Bacterial strains and growth conditions

The six staphylococcal strains (two \textit{S. epidermidis}, two \textit{S. haemolyticus} and two \textit{S. aureus}) used in this study were selected based on their previously known biofilm forming capacity (Table 1). Bacteria were grown overnight at 37°C in cation-adjusted Mueller–Hinton II broth (MHIIB).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>MIC antibiotics (mg/L)</th>
<th>MIC SAMPs (mg/L)</th>
<th>Biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Source</td>
<td>RIF</td>
<td>VAN</td>
<td>TET</td>
</tr>
<tr>
<td>SH TUH 51-03</td>
<td>blood culture</td>
<td>&lt;0.016</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>SH TUH 51-07</td>
<td>blood culture</td>
<td>0.016</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>SE TUH 08-16</td>
<td>blood culture</td>
<td>0.016</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>SE RP62A ATCC 35984</td>
<td>blood culture</td>
<td>&lt;0.016</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>SA PIA 9</td>
<td>joint fluid</td>
<td>&lt;0.016</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>SA PIA 90</td>
<td>joint fluid</td>
<td>0.016</td>
<td>2</td>
<td>0.5</td>
</tr>
</tbody>
</table>

RIF, rifampicin; VAN, vancomycin; TET, tetracycline; LZD, linezolid; GEN, gentamicin; OXA, oxacillin; SH, \textit{S. haemolyticus}; SE, \textit{S. epidermidis}; SA, \textit{S. aureus}.

\textsuperscript{a}PCR detection of \textit{icaD} as a marker of the operon.

Antibiotics, SAMPs and susceptibility testing under planktonic growth conditions

We determined the MICs of oxacillin, gentamicin, tetracycline, vancomycin and linezolid using Etest (AB Biodisk, Solna, Sweden) and MICs of rifampicin using broth microdilution assay.\textsuperscript{28} Breakpoints were interpreted according to EUCAST criteria.\textsuperscript{29} We selected four different SAMPs (Ltx5, Ltx9, Ltx10 and Kp14; Lytix Biopharma, Tromsø, Norway) based on previously known antimicrobial activities and determined their exact MICs with broth microdilution assay. Kp14 was included to represent an SAMP with a low antimicrobial activity. All four SAMPs are tripeptides with two arginine residues providing their cationic moties (Figure 1). The lipophilic bulk is provided by a modified tryptophan derivate (Ltx5, Ltx9 and Ltx10) or 4'-phenyl-phenylalanine (Kp14). The difference between the compounds in the Ltx series is the size of the C-terminal modification; Ltx5 has the smallest and Ltx9 has the largest C-terminal modification (Figure 1). The molecular weights of the SAMPs are in the range of 700–800 Da.

Biofilm formation and quantification of activity against biofilms

Biofilm formation was induced in 96-well flat-bottomed microtitre plates (Nunclon Surface, NUNC, Roskilde, Denmark). First, overnight cultures were diluted 1:100 in MHIIB (\textit{S. epidermidis} and \textit{S. haemolyticus}) or tryptic soy broth with 5% glucose and 5% NaCl (\textit{S. aureus}). An aliquot of 200 µL of this bacterial suspension (10^7 cfu/mL) was added to each well and incubated for 24 h at 37°C. After 24 h, the wells were carefully washed twice with phosphate-buffered saline (PBS) to remove planktonic bacteria. The washing procedure was evaluated by measuring metabolic activity of the PBS with the Alamar Blue (AB) method, described in detail below.\textsuperscript{12} DNA extractions and PCRs for \textit{icaD}, as a marker for the \textit{ica} operon, were carried out as reported previously.\textsuperscript{30}

The washed biofilms were subjected to treatment with antibiotics or SAMPs at different concentrations. Stock solutions of tetracycline (Sigma Aldrich), vancomycin (Alpharma) and linezolid (Pfizer) were diluted in MHIIB to 5, 50 and 500 mg/L, and rifampicin (Sigma Aldrich) was diluted in MHIIB to 0.01, 0.1 and 1 mg/L. Trifluoroacetate salts of the SAMPs were dissolved in sterile water and diluted to 5, 50 and 500 mg/L in MHIIB. Antibiotics or SAMPs (200 µL), at different concentrations, were added to each well and incubated for 24 h at 37°C. Positive controls were untreated biofilms with only 200 µL of MHIIB added. Negative controls were only
200 μL of MHIIB, with no bacteria added. We quantified the metabolic activity of biofilm with a slightly modified method previously described by Pettit et al. Briefly, the wells were washed twice with PBS. We then added 250 μL of MHIIB with 5% AB (Invitrogen, Carlsbad, CA, USA) to each well. AB is a redox indicator that both fluoresces and changes colour in response to chemical reduction. The extent of reduction is a reflection of bacterial cell viability.

After 1 h of incubation at 37°C, absorbance was recorded at 570 and 600 nm using a Versamax tuneable microplate reader (Molecular Devices, Sunnyvale, CA, USA). All assays were performed three times with eight parallels. The highest and lowest values of each run were excluded from the analyses, and the remaining 18 values were averaged.

The biofilm method quantifying metabolic activity was compared with a standard semi-quantitative biomass quantification method in 96-well microtitre plates. For these experiments, we grew 24 h biofilms of all six staphylococcal strains and analysed metabolic activity with AB, as described earlier. Biomass quantification of the 24 h biofilms was performed by staining the biofilm with Crystal Violet (CV). After staining, ethanol/acetone (70:30) was added to each well to dissolve remaining CV along the walls of the wells. The optical density was then recorded at 570 nm using a spectrophotometer.

**Biofilm imaging**

Aliquots (1 mL) of MHIIB-diluted overnight culture were used to grow *S. haemolyticus* TUH 51-07 biofilm on plastic coverslides (Thermanox, cell culture treated on one side, NUNC, Roskilde, Denmark) in 24-well dishes (Falcon 3047, Becton Dickinson, NJ, USA) for 24 h. The coverslides were then washed carefully with PBS, moved to a new plate and treated for 24 h with 50 and 500 mg/L tetracycline, 50 and 500 mg/L vancomycin or 50 and 500 mg/L Ltx5. The coverslides were washed again with 9% NaCl and stained with a LIVE/DEAD kit (Invitrogen Molecular Probes, Eugene, OR, USA) following the manufacturer’s instructions. This stain contains SYTO 9 (green fluorescent) and propidium iodide (PI; red fluorescent), both binding to DNA. When used alone, the SYTO 9 generally stains all bacteria in a population, both those with intact and those with damaged membranes. In contrast, PI penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain green fluorescence when both dyes are present. We examined treated and untreated biofilms with a Leica TCS SP5 (Leica Microsystems CMS GmbH, Mannheim, Germany) confocal laser scanning microscope (CLSM). Images were obtained using a 63 × 1.2 NA HCX PL APO water immersion lens. For detection of SYTO 9 (green channel), we used the 488 nm line of the argon laser and a detection bandwidth of 495–515 nm. For PI detection (red channel), we used the 561 nm line and a detection bandwidth of 615–660 nm. The two fluorescent signals were collected sequentially at 400 Hz. Image analyses and export were performed in a Leica LAS AF version 1.8.2.

**Statistical analysis and evaluations**

The percent reduction of AB was calculated according to the manufacturer’s formula (Invitrogen). We calculated mean and standard deviations (SD) of all repeated measurements. Pearson’s two-tailed correlation between the AB and CV methods was calculated on averaged data from all six staphylococcal strains. Statistical analysis was performed with SPSS for Windows software version 14.0.

No consensus exist regarding the interpretation of minimal biofilm inhibitory concentrations (MBICs) using the AB method. We present the crude percentage values of AB reduction, including positive and negative controls. We define two levels of antimicrobial suppression of metabolic activity. A strong suppression was obtained if an agent, after adjusting for the negative control, at a certain concentration caused ≥75% reduction of AB compared with the positive control. A complete suppression was obtained if an agent at a certain concentration caused a reduction of AB ≤ negative control value + 2SD.

**Results**

Table 1 summarizes MICs of the antibiotics and SAMPs. All six strains were susceptible to vancomycin, linezolid, rifampicin, vancomycin and tetracycline. The two *S. aureus* strains were
susceptible to gentamicin and oxacillin, while the four other staphylococcal strains were resistant to these agents. MICs of the SAMPs were in general higher than the MICs of the antibiotics.

There was a strong correlation ($R = 0.939$, $P = 0.002$) between biomass quantified by CV staining and biofilm metabolic activity quantified by AB reduction in the 24 h old biofilm (Figure 2). There was negligible metabolic activity in the PBS after the washing, indicating almost complete removal of planktonic bacteria from the wells (data not shown).

Figures 3 and 4 show the percentages of AB reduction in untreated and treated biofilms. With few exceptions, the tested antibiotics reduced metabolic activity of all strains at concentrations around MICs. With higher antibiotic concentrations, roughly $10–20 \times$ MIC, all antibiotics caused a strong suppression of metabolic activity, except in *S. aureus* PIA 9. However, only tetracycline was able to cause a complete suppression of metabolic activity in one strain (*S. aureus* PIA 90). None of the antibiotics caused $\geq 50\%$ AB reduction in the *S. aureus* PIA 9 biofilm. This strain seemed to create a biofilm completely resistant to vancomycin. Ltx5, Ltx9 and Ltx10 caused a strong or complete suppression of metabolic activity in all biofilms at concentrations of 50 mg/L, except in *S. aureus* PIA 9. In some strains, even a concentration of 5 mg/L was sufficient to cause complete suppression. Under planktonic growth conditions, high concentrations (64–256 mg/L) of Kp14 were needed to inhibit growth in all strains (Table 1). The biofilm susceptibility assay verified the poor antimicrobial activity of KP14 compared with the Ltx SAMPs (Figure 4). However, Kp14 at a concentration of 500 mg/L was

![Figure 2. The 24 h biofilm of six different staphylococcal strains. Quantification of biomass with CV (top panel) and quantification of metabolic activity with AB (bottom panel).](image-url)
Figure 3. Effect of 24 h treatment with rifampicin, linezolid, tetracycline and vancomycin on 24 h biofilm of six different staphylococcal strains. Values are means of three experiments ± SD. *Strong suppression of metabolic activity. **Complete suppression of metabolic activity.
Figure 4. Effect of 24 h treatment with four different SAMPs on 24 h biofilm of six different staphylococcal strains. Values are means of three experiments ± SD. *Strong suppression of metabolic activity. **Complete suppression of metabolic activity.
able to suppress metabolic activity completely in *S. haemolyticus* and *S. epidermidis* biofilms and was thus still more effective than the tested antibiotics. Ltx9 and Ltx10 seemed to have a lower antimicrobial activity at 500 mg/L compared with 50 mg/L. However, at 500 mg/L, both these SAMPs, in contrast to Ltx5, showed visual turbidity probably due to the lower aqueous solubility of Ltx9 and Ltx10 compared with Ltx5 (J. S. S. and W. S., unpublished data).

Figure 5 shows confocal microscopy pictures of an *S. haemolyticus* TUH 51-07 biofilm using LIVE/DEAD staining. As expected, the untreated biofilm showed green cells with intact cell membranes. In the biofilm subjected to treatment with Ltx5 at a concentration of 50 mg/L, and especially 500 mg/L, almost all cells are stained red, indicating dead bacteria. In biofilm subjected to treatment with 500 mg/L tetracycline, a significant part of the cells remain green indicating live bacteria with intact cell membranes. Treatment of the biofilm with vancomycin (Figure 5d) at a concentration around the peak values obtained in clinical practice (50 mg/L) showed predominantly green cells (live organisms).

**Discussion**

The primary aim of the study was to investigate the effect of different antibiotics and SAMPs on preformed staphylococcal biofilm using a simple screening method to quantify *in vitro* biofilm. We consider the viability of biofilm cells as most important when evaluating the effect of antimicrobial agents. We therefore chose a quantification model based on the reduction of AB by metabolically active cells. This method has shown excellent applicability as it is simple, fast, non-toxic and suitable for high-throughput quantification of biofilms grown in microtitre plates. In our study, experiments were performed on three different dates with eight parallels and showed a good reproducibility (Figures 3 and 4). The AB method was able to detect dose-dependent differences in the effect of antibiotics and SAMPs. Like other investigators, we found a strong correlation between the amount of biofilm mass quantified with CV and the metabolic activity quantified with AB in untreated biofilms.

Some authors have defined a drug concentration resulting in ≥50% reduction of AB as the MBIC. In our study, we did
not aim to find the exact MBIC cut-off as we used only three different drug concentrations, in 10-fold increasing steps. It is well known that after bacterial biofilms have been exposed to antibiotics bacterial re-growth is the rule.\(^{23}\) We therefore used stricter criteria defining strong and complete suppression of metabolic activity. As others, we believe that the AB method is an attractive candidate for a standard method of biofilm susceptibility testing.\(^{12}\) However, more data, including animal experiments and clinical experience in biofilm associated infections, are needed to define broadly applicable and clinical useful MBIC cut-offs.

Previous studies have reported promising therapeutic potential of the SAMPs used in this study when tested on several clinically relevant, multiresistant bacteria.\(^{26,27}\) Our hypothesis was that these SAMPs also would be more effective than conventional antibiotics in killing staphylococci in biofilms. All Ltx SAMPs were clearly more effective in reducing metabolic activity in staphylococcal biofilms at low concentrations compared with antibiotics, even though they generally had higher MICs under planktonic growth conditions. Under planktonic growth conditions, all strains used in this study were sensitive to vancomycin, linezolid, rifampicin and tetracycline. Poor antimicrobial activity of vancomycin on staphylococcal biofilms has been reported previously.\(^{12,23}\) In our study, 50 mg/L vancomycin exerted a strong suppression of metabolic activity on mature biofilms from four out of the six strains tested. Still, CLSM confirmed that most bacteria were not killed by this concentration. In general, antibiotics were rarely able to cause a complete suppression of metabolic activity. In contrast, SAMPs were frequently able to suppress metabolic activity completely, indicating effective killing. Images obtained by the CLSM further supported this finding. Treatment with 500 mg/L Ltx5 caused membrane damage of all cells, indicating cell lysis in the *S. haemolyticus* biofilm. Previous studies have also showed that the SAMPs used in this study affect the bacterial membrane integrity and lead to cell lysis in a concentration dependent manner.\(^{26,27}\) Biofilms treated with 500 mg/L tetracycline still contained a significant number of living cells, as recorded by LIVE/DEAD staining, even though there was hardly any measurable metabolic activity in the corresponding biofilm assay.

There are alternative ways to interpret this discrepancy between findings from our confocal pictures and biofilm assays. First, although bacterial cells still had an intact cell membrane after treatment with tetracycline, they might have extremely low metabolic activity due to its bacteriostatic effect. Second, biofilm grown on the cover slides used for CLSM may show an increased tolerance to tetracycline compared with biofilm grown in the wells of a microtitre plate. It is well known that the environment and growth conditions may affect the architecture of a biofilm\(^{24}\) and that differences in this architecture can affect the sensitivity of the cells to antimicrobial agents.\(^{35}\) Third, there is a possibility that the correlation between the reduction of AB and metabolic activity is non-linear and that values in the low ranges of percentage AB reduction (e.g. after treatment with 500 mg/L tetracycline) underestimate the metabolic activity.

Our results indicate that when treatment with 500 mg/L Ltx5 causes a complete suppression of metabolic activity, all cell membranes are also disrupted and bacterial cells are expected to be dead. Furthermore, the antimicrobial properties of SAMPs seem to be similar on bacteria embedded in a biofilm and planktonic bacteria. We believe that the superior antimicrobial effect of the Ltx SAMPs compared with antibiotics is the result of lysis of staphylococci when these agents are used above a certain concentration. It seems that Ltx SAMPs cause damage of the bacterial cell membranes even in slow growing or dormant bacteria embedded in a biofilm. In contrast, the antimicrobial agents used in this study predominantly affect growing bacteria by inhibiting their cell wall development (vancomycin) or by inhibition of their protein synthesis (linezolid, rifampicin and tetracycline).

Other groups have investigated the effect of synthetic and natural CAPs on staphylococcal biofilms.\(^{14,25,36,38}\) They found that different antimicrobial peptides could be used to prevent biofilm formation or treat young biofilms. However, only one group found an antimicrobial peptide, based on the structure of magainin, to be effective in killing different bacteria in a 24 h biofilm.\(^{25}\) To our knowledge, there are no reports on CAPs or SAMPs that effectively kill staphylococci in 24 h biofilms.

Low concentrations of both antibiotics and SAMPs had a better antimicrobial effect on weak biofilm producing strains than on strong biofilm producers. Both the architecture of a thin biofilm, the total number of bacteria in such a biofilm and probably a lower proportion of bacteria with slow growth may explain this finding. Surprisingly, others found no correlation between the degree of biofilm production and antibiotic susceptibility.\(^{38,39}\) Differences in the definition of weak or strong biofilm producers could be one explanation.

Biofilm producing *S. aureus* strains were more difficult to treat with SAMPs than *S. epidermidis* and *S. haemolyticus*, although the MICs under planktonic conditions did not differ among these three species. None of the SAMPs were able to suppress *S. aureus* PIA9 biofilm metabolic activity completely. Resistance or tolerance to defensins and other human antimicrobial peptides in *S. aureus* has also been reported previously, but these antimicrobial substances were tested in planktonic bacteria, not in biofilm.\(^{38,40,41}\) Interestingly, in *S. aureus*, an active glycopeptide resistance-associated two component regulatory system, GraRS, mediates resistance to CAPs under planktonic growth conditions. GraRS is also involved in up-regulation of biofilm production.\(^{22}\) However, the three Ltx SAMPs used in our study all showed good antimicrobial activity against *S. aureus* under planktonic growth conditions. There is some evidence that in *Escherichia coli*, the biofilm formation itself can induce tolerance to CAPs due to changes in intra-biofilm physiochemical gradients.\(^{35}\) We do not have sufficient data to examine whether some strains of *S. aureus* behave in a similar manner.

We conclude that the Ltx SAMPs used in this study have superior antimicrobial activity in staphylococcal biofilms compared with conventional antibiotics. Ltx SAMPs are potential new therapeutic agents in biofilm-associated infections. They could be especially attractive for topical treatment of chronic wound infections. Currently, a Phase 1 study of Ltx9 for local therapy of post-operative wound infections is being planned. The clinical applicability of SAMPs to prevent medical device associated staphylococcal infections warrants future *in vivo* studies.

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Transparency declarations
J. S. S. and W. S. declare competing financial interests. J. S. S. is CSO for infectives and W. S. is a scientist at Lytix Biopharma AS. Lytix Biopharma AS is a privately held Norwegian pharmaceutical company dedicated to the discovery and development of novel treatments for drug-resistant infectious diseases and cancer. None of the other authors declares any conflict of interest.

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