In vitro assessment of antimicrobial peptides as potential agents against several oral bacteria

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Background: Antimicrobial peptides are components of the innate immunity that play an important role in systemic and oral health.

Objectives: The antibacterial activity of the amphibian-derived Kc-S4(1-15)a antimicrobial peptide was tested against oral pathogens associated with caries and periodontitis and compared with the activities of the human-derived antimicrobial peptides LL-37 and dhvar4a.

Methods: Growth inhibition of planktonic bacteria was tested using standard microdilution assays. Live/Dead staining followed by confocal scanning laser microscopy (CSLM) was used to determine the bactericidal effect of Kc-S4(1-15)a on Streptococcus mutans attached to a glass surface or grown as biofilm.

Results: The cariogenic species S. mutans, Streptococcus sobrinus, Lactobacillus paracasei and Actinomyces viscosus were resistant to LL-37 found in the oral cavity. Porphyromonas gingivalis was the species most resistant to the three tested peptides. Kc-S4(1-15)a demonstrated the highest activity against the tested planktonic bacteria. In addition, Kc-S4(1-15)a was bactericidal to surface-attached S. mutans as well as to S. mutans biofilms grown in vitro. However, surface attachment increased S. mutans resistance to the antimicrobial peptide.

Conclusions: Our results support growing evidence suggesting the use of antimicrobial peptides for prevention and treatment of oral disease.

Keywords: oral infection, biofilm, LL-37, dermaseptin, histatin-5

Introduction

Microbial resistance to antibiotics raises the need to develop novel compounds and approaches for microbial control. Antimicrobial peptides offer a promising opportunity to combat microbial strains. Acquisition of resistance to antimicrobial peptides in susceptible strains is slower and less common compared with that developed against other antimicrobial agents. A net positive charge and the ability to adopt an amphipathic structure are properties thought to enhance the affinity of peptides for negatively charged phospholipids at the outer surfaces of bacterial membranes. The peptides interfere with the membrane’s integrity and may further affect cytoplasmic targets. In vertebrates, antimicrobial peptides are synthesized and secreted from phagocytic cells and epithelia and contribute to the innate immunity. The oral cavity, which is colonized by numerous microorganisms, contains a wide selection of antibacterial peptides that play an important role in maintaining its complex ecological homeostasis. Tooth decay and periodontal disease are associated with the presence of dental plaque, a microbial biofilm comprising multiple species anchored to oral surfaces and protected by a self-produced polymeric matrix. Bacterial traits including susceptibility to antimicrobial agents are altered in biofilm compared with planktonic environment. Controlling dental plaque bacteria is important for prevention and treatment of oral diseases.

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Effect of three antimicrobial peptides on selected oral pathogens

Three antimicrobial peptides were tested in the present study. K$_4$-S$_4$(1-15)a is an analogue of dermaseptin S4, a member of the dermaseptin family, originally isolated from tree frog skin. The antibacterial activity of K$_4$-S$_4$(1-15)a was compared with those of two human-derived antibacterial peptides common in the oral cavity, LL-37 is produced and secreted from epithelia and granules of neutrophils. Deficiency of LL-37 in neutrophils and saliva has been correlated with occurrence of periodontal disease. Dhvar4 is a potent derivative of histatin-5, a member of a family of antifungal, histidine-rich proteins, secreted from salivary glands. Dhvar4a, a C-terminally amidated form of dhvar4, was used in our experiments.

Materials and methods

Peptide synthesis

K$_4$-S$_4$(1-15)a (LWKTLKLKLKAAANH$_2$), dhvar4a (KRLFKK-LLFSLRKY-NH$_2$) and LL-37 (LGLDFFRKSKEKGFKRIQVRKDFLRNLVPRTES) were prepared by automated solid phase method applying Fmoc active ester chemistry and subsequently purified by reversed phase HPLC. K$_4$-S$_4$(1-15)a and LL-37 showed a purity level >95% and dhvar4a (Biosight, Israel) >80% as determined by analytical HPLC.

Bacterial strains and growth conditions

Species and strains are listed in Table 1. Actinomyces viscosus, Lactobacillus paracasei, Streptococcus mutans and Streptococcus sobrinus were cultured in brain–heart infusion broth (BHI) (Difco, MD, USA), at 37°C in an atmosphere enriched with 5% CO$_2$. Porphyromonas gingivalis and Fusobacterium nucleatum were grown in Wilkin’s broth (Oxoid, UK) and BHI supplemented with 0.05% glutamate, respectively, in jars containing an anaerobic atmosphere generation system (Oxoid). The antibacterial activity of K$_4$-S$_4$(1-15)a on LL-37 showed a purity level >95% and dhvar4a (Biosight, Israel) >80% as determined by analytical HPLC.

Table 1. Susceptibilities of selected bacteria to the antibacterial peptides K$_4$-S$_4$(1-15)a, dhvar4a and LL-37

<table>
<thead>
<tr>
<th>Organism</th>
<th>MIC (mg/L)</th>
<th>K$_4$-S$_4$(1-15)a</th>
<th>dhvar4a</th>
<th>LL-37</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. mutans DJ1</td>
<td>20</td>
<td>50–100 (100)</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>S. mutans ATCC 27351</td>
<td>5</td>
<td>20–40 (25)</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>S. mutans UA159</td>
<td>20</td>
<td>50–100 (100)</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>S. sobrinus 6715</td>
<td>5</td>
<td>20–40 (30)</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>L. paracasei DJ1</td>
<td>10</td>
<td>100</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>A. viscosus ATCC 43146</td>
<td>5–10 (5)</td>
<td>30–100 (50)</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>A. actinomycetemcomitans Y4</td>
<td>25–50 (50)</td>
<td>200</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>A. actinomycetemcomitans Y4</td>
<td>25–50 (50)</td>
<td>200</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>F. nucleatum ATCC 10953</td>
<td>5</td>
<td>50–100 (50)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>P. gingivalis ATCC 33277</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>P. gingivalis W50</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>E. coli ATCC 25922</td>
<td>5</td>
<td>100</td>
<td>50–100 (75)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Calculated median values are shown in parentheses.

Results and discussion

K$_4$-S$_4$(1-15)a and dhvar4a inhibited growth of the oral streptococci, lactobacilli and A. viscosus at MIC values ranging from 5–20 and 20–100 mg/L, respectively. LL-37 did not inhibit growth of these species. This finding is in agreement with a recent study that found no correlation between dental caries and levels of salivary LL-37, in contrast to other antibacterial peptides.

Among the selected Gram-negative species, F. nucleatum and the enteric E. coli were the most susceptible to the tested antibacterial peptides (Table 1). P. gingivalis was resistant to the peptides at all tested concentrations. Similarly, A. actinomycetemcomitans demonstrated low susceptibility to LL-37 and dhvar4a. K$_4$-S$_4$(1-15)a, however, inhibited A. actinomycetemcomitans growth, but at higher concentrations than those required for inhibiting the other susceptible bacteria. Out of the three peptides, K$_4$-S$_4$(1-15)a demonstrated the highest potency towards the
tested species. Previous studies observed an MIC of $\sim 10$ mg/L and 50–100 mg/L for LL-37 and a shorter analogue, against A. actinomycetemcomitans.\cite{9,10} MIC discrepancies may be attributable to differences in the experimental conditions such as exposure times, media and salt concentrations used in the antibacterial assays.

Dental caries and periodontal disease are associated with bacterial biofilm. Bacteria in biofilm display lower susceptibility to antimicrobial agents compared with planktonic bacteria.\cite{2} As seen in Figure 1, K$_4$S$_4$(1-15)a killed surface-attached S. mutans and inhibited biofilm formation. Killing of the surface-attached bacteria occurred at a higher concentration (50 mg/L) than that required to inhibit planktonic growth (5 mg/L). It appears that attachment of S. mutans to a surface reduces its susceptibility to K$_4$S$_4$(1-15)a. Chlorhexidine digluconate inhibited biofilm formation at 5 mg/L.

The ability of K$_4$S$_4$(1-15)a to affect viability of S. mutans in a mature biofilm was also tested (Figure 1). Following 1 h of exposure to 500 mg/L K$_4$S$_4$(1-15)a, a bactericidal effect was evident throughout the $\sim 100$ µm biofilm. At a concentration 10-fold lower, partial killing effect could be observed, corresponding with the effect of chlorhexidine digluconate (Figure 1).

Information regarding the effects of antibacterial peptides on biofilm compared with planktonic bacteria is limited. Dhvir4 reduced viability of a complex oral biofilm to a lower degree compared with homogeneous planktonic cultures,\cite{2} corresponding with our observations regarding the S. mutans biofilm.

Mechanisms for microbial resistance to polycationic peptides include mutations affecting the membrane structure and charge distribution, modifications in the lipopolysaccharide structure of Gram-negative bacteria and active pumping of peptides out of the cell. P. gingivalis, which is implicated in periodontal diseases, is a highly proteolytic organism that has been found to degrade a variety of antimicrobial peptides.\cite{1} This may account for its resistance to the tested antibacterial peptides.

In conclusion, it seems feasible to employ antibacterial peptides in future therapy of oral infection, as one approach in

![Figure 1. CSLM cross-section images of S. mutans biofilms. Dead cells were stained red, while live cells were stained green using the BacLight LIVE/DEAD viability stain. Numbers in the bottom-left corner of each panel represent % viable cells ± SD (n = 4), as measured by Image ProPlus software. (A–G) Mature biofilms, untreated (A) or exposed for 1 h to 5 (B), 50 (C), 500 (D) mg/L of K$_4$S$_4$(1-15)a or to 5 (E), 50 (F), 500 (G) mg/L CHX. (H–K) Surface-attached S. mutans, untreated (H) or exposed overnight to 5 (I) or 50 (J) mg/L K$_4$S$_4$(1-15)a or to 5 mg/L CHX (K).](image-url)
dealing with the increase in microbial resistance to antibiotics. The oral cavity, being readily accessible for local application, may be particularly suitable for peptide therapy.

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Transparency declarations

None to declare.

References