Activity of Synthetic Antimicrobial Peptide GH12 against Oral Streptococci

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Key Words
Antimicrobial cationic peptide · Bacterial sensitivity test · Biofilms · Confocal laser scanning microscopy · Dental caries · Scanning electron microscopy · Secondary protein structure · Streptococcus

Abstract
Controlling the growth of cariogenic microorganisms such as oral streptococci is an adjunct therapy for caries-active individuals to prevent and treat caries. Here we investigated the antimicrobial activity of the synthetic amphipathic α-helical antimicrobial peptide GH12 (GLLWHLLHLLH-NH₂) against oral streptococci in vitro. Circular dichroism studies showed that GH12 takes on an α-helical conformation in the presence of membrane-mimicking solvents, and reversed-phase high-performance liquid chromatography studies showed that GH12 remains stable in saliva. The peptide showed bactericidal activity against oral streptococci, with minimum inhibitory concentrations ranging from 6.7 to 32.0 μg/ml. GH12 concentrations 4-fold higher than the minimum bactericidal concentration completely killed oral streptococci within 20 min. Treating oral streptococci with GH12 caused noticeable changes in bacterial viability and morphology based on confocal laser scanning microscopy and scanning electron microscopy. Effects of GH12 on biofilm formation and on viability of mature biofilm were quantified by crystal violet staining and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. GH12 effectively inhibited biofilm formation and metabolic activity in biofilms of oral streptococci, especially S. mutans, S. sobrinus and S. salivarius. These results suggest that GH12 shows rapid and strong antimicrobial activity against oral streptococci in vitro, opening the door to preclinical and clinical studies to explore its potential for caries prevention and treatment.

Dental caries is a major, chronic oral health problem all over the world, affecting 60–90% of schoolchildren and most adults in industrialized countries [Petersen, 2003]. Dental caries results from interactions over time between acid-producing bacteria, diet, and host factors including teeth and saliva [Selwitz et al., 2007]. Frequent and/or excessive consumption of cariogenic dietary carbohydrate (especially sucrose) is crucial in caries causation because dietary carbohydrate can be metabolized by oral bacteria and serves as a substrate for the extra- and intracellular polysaccharide synthesis, thus stimulating the proliferation and adhesive qualities of cariogenic bacteria and allowing them to produce acid [Sheiham and James, 2015]. Restriction of sucrose intake, although indispensable, would be unlikely to prevent caries thoroughly, especially if frequent starch intake persisted.
Streptococci are widely distributed in the oral cavity, and they include soft-tissue colonizers, such as *Streptococcus salivarius*, *S. oralis* and *S. mitis*, as well as hard-surface colonizers, such as *S. mutans*, *S. sobrinus* and *S. gordonii* [Lemos et al., 2005]. They are closely associated with oral health [Barbosa da Silva et al., 2008; Martínez-Martínez et al., 2011], and they constitute up to 80% of early plaque biofilms [Rosan and Lamont, 2000]. Therefore, inhibiting the growth of cariogenic oral streptococci is still promising to be used as an adjunct therapy for caries control for a limited period of time and especially for high caries risk or caries-active individuals.

Antimicrobial peptides are ubiquitous, naturally occurring peptides with potent antibacterial, antifungal, and antiviral properties. They are present in insects, plants and animals including humans, reflecting their fundamental role in the evolution of complex multicellular organisms [Zasloff, 2002; Reddy et al., 2004]. Antimicrobial peptides are classified into four groups based on their secondary structure: α-helical, β-sheet, loop and extended [Powers and Hancock, 2003]. Though thousands of antimicrobial peptides have been described, most structural and biochemical studies have focused on amphipathic α-helical peptides [Dennison et al., 2005; Lan et al., 2010; Liu et al., 2013]. A broad range of antimicrobial peptides has been synthesized in the laboratory using diverse approaches including sequence modifications, minimalist methods, combinatorial libraries, and template-assisted methods [Giangaspero et al., 2001; Zelezetsky and Tossi, 2006]. Some of them have shown potential in vitro for inhibiting oral pathogens, including oral streptococci [Liu et al., 2011; Shang et al., 2014].

He et al. [2007] generated several synthetic antimicrobial peptides that showed robust killing activity against several oral streptococci. Da Silva et al. [2013] designed the 19-residue synthetic antimicrobial peptide Lys-a1 and showed that it has substantial antimicrobial and antibiofilm activity against oral pathogenic streptococci. Shang et al. [2014] created a synthetic peptide called L-K6 from the naturally occurring peptide temporin-1CEb, and showed that it had good antimicrobial activity against oral pathogens and that it inhibited *S. mutans* biofilm formation. Despite these successes, current peptides can be unstable, expensive to produce, and ineffective because of microbial resistance [Giuliani et al., 2007]. Thus, studies are needed to identify stable antimicrobial peptides with antibacterial activity against cariogenic pathogens and minimal length in order to minimize production costs.

Here we describe a novel amphipathic α-helical antimicrobial peptide GH12 containing only 12 amino acids. The peptide was designed using minimalist approaches on the basis of a helical wheel projection. The purpose of this study was to evaluate the stability of GH12 and assess its antibacterial and antibiofilm activity against oral streptococci. Secondary structure and stability of GH12 were assessed using circular dichroism and reversed-phase high-performance liquid chromatography (HPLC), and antibacterial activity was investigated in vitro.

**Materials and Methods**

**GH12 Synthesis and Purification**

GH12 peptide (GLWHLHHLH-NH₂) was synthesized by GL Biochemistry (Shanghai, China) using a standard Fmoc solid-phase peptide synthesis protocol. The purified peptide (>99.00%) was completely dissolved in sterile deionized water at a concentration of 5,120.0 μg/ml, sterilized by passage through a 0.22-μm syringe filter and stored in a freezer at –20°C.

**Secondary Structure and Stability of GH12**

The secondary structure of GH12 was investigated at room temperature in different solvents using CD spectroscopy (J-1500; Jasco, Tokyo, Japan) and a quartz cell with 0.1 cm path length. GH12 was dissolved to a final concentration of 200.0 μg/ml in 20.0 mM sodium phosphate buffer (pH 7.0) alone or supplemented with 25 mM sodium dodecyl sulfate (SDS), or 50% (v/v) trifluoroethanol (TFE). Spectra were recorded by scanning wavelength from 190 to 240 nm at 100 nm/min; mean residue ellipticity (θ M; degrees · m⁻¹ · cm⁻¹) was averaged over 10 scans for each sample [Wiradharmada et al., 2011a]. Spectra were analyzed using CDPro (http://lamar.colostate.edu/~sreeram/CDPro/main.html). Fractions of different types of secondary structure were calculated using three programs within the software (SEC-LCON3, CONTINLL, and CDSSTR). The results of the three methods were averaged to give the final fractions.

The stability of GH12 in human saliva was analyzed by reversed-phase HPLC as described [Na et al., 2007], with some modifications. Whole human saliva was collected from 3 healthy volunteers and immediately centrifuged at 4,200 g for 20 min at 4°C, and the supernatant was filtered through a 0.22-μm syringe filter. Saliva was incubated at 37°C for 1 h to activate proteinases. GH12 previously dissolved in deionized water as described above was added to 9 volumes of saliva to a final concentration of 512.0 μg/ml. The mixture was incubated at 37°C, and samples were collected at 1, 2, 5, 10, 20, and 30 min. GH12 was isolated on a C₁₈ column (3.0 × 100 mm, 1.8 μm) using a 55:45 mixture of mobile phase A (0.1% trifluoroacetic acid in water) and mobile phase B (0.1% trifluoroacetic acid in acetonitrile). Total run time was 12 min at a flow rate of 0.3 ml/min. Injection volume was 2 μl, and chromatograms were recorded at 220 nm. 512.0 μg/ml of GH12 in...
deionized water served as a positive control, and saliva without GH12 served as negative control. Peak areas of GH12 at each time point were compared with the peak area of the positive control to determine the amount of remaining GH12.

**Bacterial Strains and Culture**

The oral streptococci S. mutans UA159, S. mitis ATCC6249, S. gordonii DLI, S. sanguinis ATCC10556, S. salivarius ATCC13419, and S. sobrinus 6715 were provided by the State Key Laboratory of Oral Diseases at Sichuan University (Chengdu, China) and stored at –20°C. Single bacterial colonies were inoculated for 24 h into 10 ml of brain-heart infusion (BHI) broth (Oxoid Ltd., Basingstoke, UK) under anaerobic conditions (80% N₂, 10% CO₂ and 10% H₂). Culture aliquots (100.0 μl) were further incubated overnight in 10.0 ml of BHI broth until they reached the stationary phase. This subculturing step can lead to better microbial growth and development [Cardoso Sa et al., 2012]. Finally, overnight bacterial suspensions were adjusted to the desired concentration with BHI broth for susceptibility assays and time kill assays, or with BHI broth supplemented with 1.00% (w/v) sucrose for biofilm susceptibility assays according to previous studies [Zhou et al., 2013; Shang et al., 2014]. Meanwhile, bacterial suspensions in mid-log phase of growth were observed under confocal laser scanning microscopy [Imazato et al., 2008] or scanning electron microscopy [Tao et al., 2011] to ensure that bacteria were in high viability, thus minimizing the interference of the dead bacteria before the treatment of GH12.

**Bacterial Susceptibility Assay**

Antimicrobial activity assays were performed in 96-well U-bottomed microtiter plates using the 2-fold serial dilution test following the recommendations of the Clinical and Laboratory Standards Institute with modifications [Cardoso Sa et al., 2012; Joycharat et al., 2013; Clinical and Laboratory Standards Institute, 2014]. For assays to determine the minimum inhibitory concentration (MIC), 2-fold serial dilutions of GH12 were prepared with sterile deionized water to achieve concentrations ranging from 5,120 to 5.0 μg/ml in a volume of 20.0 μl. To these peptide dilutions were added 80.0 μl of BHI broth and 100.0 μl of bacterial culture adjusted to 2.0 × 10⁶ colony-forming units (CFU)/ml. Thus, each microwell contained final GH12 concentrations ranging from 512.0 to 0.5 μg/ml in a final volume of 200.0 μl and a final bacterial concentration of 1.0 × 10⁶ CFU/ml. This bacterial concentration is consistent with average concentrations of bacterial susceptibility of 1.0 × 10⁶ CFU/ml. This bacterial concentration capable of visually inhibiting bacterial growth after overnight incubation.

For assays to determine the minimum bactericidal concentration (MBC), aliquots (50.0 μl) were taken from microc wells in the MIC assays described above that showed no visual bacterial growth and transferred into 2.0 ml of sterile BHI broth. These cultures were incubated anaerobically at 37°C for 24 h. The lowest GH12 concentration that completely inhibited bacterial growth in the BHI medium was recorded as the MBC. MIC and MBC assays were performed 3 times for all strains.

**Time Kill Assay**

The time kill assay was conducted to assess the short-term bactericidal effect of GH12 against oral streptococci [Li et al., 2013]. Briefly, GH12 solution (200.0 μl) and fresh sterile BHI broth (800.0 μl) were added to 1 ml of bacterial culture containing approximately 2.0 × 10⁶ CFU/ml, yielding final peptide concentrations equal to 100.00, 200.00 or 400.00% of the MBC in a final volume of 2.0 ml. Aliquots (10.0 μl) of the suspension were withdrawn after 0, 1, 5, 10, 20, 30, 60, 120, 240 and 360 min of incubation at 37°C; aliquots were 10-fold serially diluted, plated on BHI agar, and incubated anaerobically at 37°C for 48 h to allow CFU determination. Time kill kinetic curves for 6 strains were constructed by plotting log₁₀ CFU per milliliter versus incubation time over 6 h. CHX was used at its MBC for strains. All assays were performed in triplicate on 3 different occasions.

**Confocal Laser Scanning Microscopy**

The LIVE/DEAD BacLight™ Bacterial Viability Kit (L-7012; Molecular Probes, Eugene, Oreg., USA) was used to assess bacterial viability after GH12 treatment. This kit relies on a mixture of the dyes SYTO™9 and propidium iodide to stain bacteria with intact cell membranes fluorescent green (live bacteria) and to stain bacteria with damaged membranes fluorescent red (dead bacteria). Bacteria in the mid-log phase of growth were harvested by centrifugation at 4,500 g for 5 min and diluted in 0.85% NaCl to an approximate concentration of 2.0 × 10⁹ CFU/ml. The bacterial suspensions were incubated anaerobically at 37°C in the absence or presence of GH12 at a final concentration of 64.0 μg/ml for 5 min, 20 min, 1 h and 3 h. CHX (0.12%, v/v) served as a positive control and 0.85% NaCl as a negative control. Suspensions were centrifuged again, and the pellet was washed twice and resuspended in 0.85% NaCl. Equal proportions of SYTO™9 and propidium iodide were mixed and added to the bacterial suspension, which was then left standing in the dark for 15 min at room temperature. An aliquot (5.0 μl) of the stained bacterial suspension was trapped between a glass microscope slide and a square coverslip, then observed under a confocal laser scanning microscope (TCS SP2; Leica Microsystems, Wetzlar, Hessen, Germany) using a ×63 oil objective lens. Excitation and emission wavelengths were 488 and 500 nm for SYTO™9, and 488 and 635 nm for propidium iodide. Three fields of view were photographed for each sample, and the areas stained green or red were measured by Image-Pro® Plus 6.0 to calculate the percentage of dead cells.

**Scanning Electron Microscopy**

Electron microscopy was used to analyze bacterial morphology in the presence of GH12. Bacterial cultures in the mid-log phase of growth were treated with GH12 at a final concentration of 64.0 μg/ml or CHX at a final concentration of 0.12% (w/v), and incubated anaerobically for 24 h at 37°C [Tao et al., 2011]. Cells were pelleted by centrifugation at 4,500 g for 5 min, washed twice and resuspended in phosphate-buffered saline (PBS). An aliquot (5.0 μl) of bacterial suspension was deposited on a clean sterile glass slide and air-dried at 37°C. Samples were fixed overnight in 2.50% glutaraldehyde solution, rinsed twice with PBS and dehydrated in a graded series of ethanol solutions (35.00, 50.00, and 75.00% for 30 min each; then two cycles of 90.00 and 100.00% for 30 min each) [Weber et al., 2014]. Samples were desiccated, sputter-coated with gold, and observed on a scanning electron microscope (Inspect F; FEI, Eindhoven, The Netherlands) at 20.0 kV.
Biofilm Susceptibility Assay

Effects of GH12 on oral streptococcal biofilm formation were examined in 96-well flat-bottom microtiter plates using a microdilution method as described [Mataraci and Dosler, 2012] with modifications. Briefly, bacteria were grown in BHI broth supplemented with 1.00% (w/v) sucrose and GH12 at final concentrations ranging from 512.0 to 0.5 μg/ml such that bacteria were present at a final concentration of 1.0 × 10^6 CFU/ml as described for the bacterial susceptibility assay. Following anaerobic incubation at 37°C for 24 h, waste medium was carefully decanted, and all wells were washed 3 times with 200.0 μl of PBS and fixed with methanol for 15 min. After fixation, wells were stained with 0.10% (w/v) crystal violet for 5 min. The wells were gently rinsed with water to remove the excess dye and air-dried for 1 h. Subsequently, 200.0 μl of 95.00% ethanol was added to each well, the plate was shaken at room temperature for 30 min and the absorbance at 595 nm was measured on a microplate spectrophotometer (Multiskan GO; Thermo Scientific, USA) to determine biofilm biomass. The minimum biofilm inhibition concentration (MBIC) was defined as the lowest GH12 concentration that inhibited biofilm formation by at least 50.00% (MBIC 50 ) or 90.00% (MBIC 90 ) compared with the untreated control [Xu et al., 2011; Shang et al., 2014].

The effects of GH12 on established biofilms were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining method as described [Kwieciński et al., 2009; Cheng et al., 2013]. Bacterial suspension (200.0 μl, 1.0 × 10^6 CFU/ml) was incubated anaerobically in 96-well flat-bottom microtiter plates at 37°C for 24 h. After biofilm formation, wells were washed 3 times with sterile PBS to remove nonadherent cells. 20.0 μl of GH12 (final concentrations ranging from 512.0 to 0.5 μg/ml) and 180.0 μl of fresh BHI broth were then added to the biofilms, which were incubated at 37°C for 24 h. After incubation, the biofilms were washed 3 times with PBS. The metabolic activity of the biofilms was determined by adding 200.0 μl of 500.0 μg/ml MTT in PBS. Following incubation at 37°C for 2 h, the MTT solution was replaced with 200.0 μl of dimethyl sulfoxide to dissolve the formazan crystals, and the absorbance was measured at 540 nm. The minimum biofilm eradication concentration (MBEC) was defined as the lowest concentration of GH12 causing at least 50.00% (MBEC 50 ) or 90.00% (MBEC 90 ) reduction in metabolic activity compared with the untreated control. In both assays, biofilms were treated with CHX as a positive control. Untreated control wells were incubated without GH12. Each assay was run in triplicate with 3 replicates per concentration for all strains. Absorbance values obtained from 3 independent experiments were averaged to calculate the final MBIC or MBEC.

Statistical Analysis

All experiments were performed in triplicate, and statistical analysis was performed using GraphPad Prism® 5.01 for Windows. To compare the means among groups, one-way ANOVA was conducted, followed by Tukey’s multiple comparisons test. Student’s t test was used to assess the significance of differences between GH12 and CHX treatment. p < 0.05 was defined as the threshold of significance.

Table 1. MIC and MBC values of GH12 and CHX against oral streptococci (means ± SD, n = 3)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>MIC, μg/ml</th>
<th>MBC, μg/ml</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>GH12</td>
<td>CHX</td>
</tr>
<tr>
<td>S. mutans</td>
<td>UA159</td>
<td>6.7 ± 2.3^A, A</td>
<td>1.0 ± 0.0^A, A</td>
</tr>
<tr>
<td>S. mitis</td>
<td>ATCC6249</td>
<td>32.0 ± 0.0^A, A</td>
<td>13.3 ± 4.6^B, B</td>
</tr>
<tr>
<td>S. gordonii</td>
<td>DLI</td>
<td>8.0 ± 0.0^A, A</td>
<td>3.3 ± 1.2^B, B</td>
</tr>
<tr>
<td>S. salivarius</td>
<td>ATCC13419</td>
<td>8.0 ± 0.0^A, A</td>
<td>3.3 ± 1.2^B, B</td>
</tr>
<tr>
<td>S. sanguinis</td>
<td>ATCC10556</td>
<td>8.0 ± 0.0^A, A</td>
<td>2.0 ± 0.0^B, B</td>
</tr>
<tr>
<td>S. sobrinus</td>
<td>6715</td>
<td>16.0 ± 0.0^B, A</td>
<td>2.0 ± 0.0^B, B</td>
</tr>
</tbody>
</table>

Different superscript lower case letters within a column indicate significant differences between strains. Different superscript capital letters in a row indicate significant differences between the values for GH12 and CHX (p < 0.05).
Results

Secondary Structure and Stability of GH12
GH12 was dissolved in TFE, which is a hydrophobic solvent that stabilizes helices, or in buffer containing SDS micelles, which mimic the amphiphilic structure of the natural bilayer membrane. The CD spectra of GH12 in SDS and TFE showed a strong α-helical structure, with a single peak at 195 nm and a double peak at 208 and 222 nm (fig. 1); the α-helical structure was 80.87% in SDS and 81.33% in TFE. However, the spectrum of GH12 dissolved in sodium phosphate buffer alone showed a much smaller peak at 196 nm, and the overall spectrum was more similar to that of a β-sheet structure. Indeed, analysis of the spec-

Fig. 2. HPLC chromatograms of GH12 in deionized water (a), GH12 incubated in human saliva for 5 min at 37 °C (b), GH12 incubated in human saliva for 30 min at 37 °C (c) and degradation curve of GH12 (Rt = 5.7 min) in human saliva for 30 min at 37 °C (d). The GH12 peak is shown with its area.
trum suggested only 22.23% of α-helical structure, with concomitantly higher proportions of β-sheet (24.30%).

Reversed-phase HPLC chromatograms of GH12 in de-ionized water and after incubation in saliva for 5 or 30 min are shown in figure 2a–c. GH12 was found at a retention time of 5.7 min (fig. 2a), and no peaks specific to saliva were detected under these experimental conditions. Even after 30-min incubation in saliva, no significant GH12 degradation products were detected (fig. 2c). The peak area of GH12 at each time point was >99.80% of the initial amount (fig. 2d).

**Bacterial Susceptibility**

GH12 was effective at inhibiting and killing all bacterial strains; MIC values ranged from 6.7 to 32.0 μg/ml and MBC values from 8.0 to 64.0 μg/ml (table 1). GH12 ex-
erted stronger antibacterial activity against *S. mutans*, *S. gordonii*, *S. sanguinis* and *S. salivarius*. The MIC for these strains was 6.7–8.0 μg/ml, and the MBC was 8.0–16.0 μg/ml; these ranges were significantly different from the corresponding ranges of 1.0–3.3 and 4.0–8.0 μg/ml for the CHX control (p < 0.05), except the MIC of *S. mutans*. GH12 was significantly less effective against *S. mitis*, for which the MIC was 32.0 μg/ml and the MBC 64.0 μg/ml, than against the other strains (p < 0.05). *S. mitis* was also significantly more resistant to CHX; the MIC was 13.3 μg/ml, and the MBC 16.0 μg/ml.

**Time Kill Assay**

The short-term bactericidal activity of GH12 against oral streptococci depended strongly on peptide concentration (fig. 3a–f). For most strains, a peptide concentration equal to the MBC caused an approximately 3-log reduction within 1 h and complete elimination within 3 h. The exception was *S. gordonii*, which required a substantially longer killing time of 6 h (fig. 3c). In contrast, none of the strains was completely killed after 6-hour treatment with CHX at a concentration equal to the MBC. Bacterial numbers after treatment with the respective MBC of GH12 or CHX were similar at 1 and 5 min (p < 0.05), but numbers of *S. sanguinis* and *S. salivarius* were significantly lower after GH12 treatment than after CHX treatment for 10 min (p < 0.05). Using a GH12 concentration that was 200.00% of the MBC led to a >1-log reduction in the population of planktonic bacteria for all strains at 5 min. When GH12 was used at 400.00% of the MBC, it caused a 1-log reduction within 1 min and a 2- to 3-log reduction within 5 min. Complete killing was observed within 1 h when the peptide concentration was 200.00% of the MBC, and within 20 min when it was 400.00% of the MBC. A GH12 concentration of only 8.0 μg/ml eliminated *S. mutans* within 3 h, and higher concentrations accelerated killing. A GH12 concentration of 32.0 μg/ml completely eliminated *S. mutans* and *S. sanguinis* within 20 min, and led to 1-log reductions in the numbers of *S. mutans* and *S. salivarius* within 1 min and 2-log reductions within 5 min. A GH12 concentration of 64.0 μg/ml led to a >1-log reduction in the population of planktonic bacteria within 5 min, completely killing *S. mutans*, *S. salivarius*, *S. sanguinis*, *S. sobrinus* and *S. gordonii* within 1 h and causing a 3-log reduction in the number of viable *S. mitis* within the same period (fig. 3b).

**Viability of Oral Streptococci Based on Confocal Microscopy**

GH12 showed a clear bactericidal effect in the live/dead staining assays based on confocal laser scanning microscopy (fig. 4). In the absence of GH12, most bacteria stained green (viable), while a substantial number of bacteria stained red (dead) after exposure to 64.0 μg/ml GH12 or CHX (0.12%, w/v), consistent with quantitative image analysis (fig. 5). All strains treated with 0.12% CHX stained red with no viable cells only after 5 min, and quantitative analysis showed that the percentage of dead bacteria reached almost 100%. After treatment with GH12, the number of dead cells also increased rapidly with time. The dead bacteria of *S. mutans*, *S. salivarius* and *S. sanguinis* increased to about 90.00% after 5 min, which was not significantly different with CHX control (p > 0.05). Nearly all bacteria stained red by 20 min with few or no green cells visible: 91.82% of *S. mitis* were dead after 20 min, and significantly greater proportions of the other 5 strains were killed (p < 0.05). After exposure to GH12 for 3 h, >99% of all 6 strains were eliminated.

**Morphology of Oral Streptococci Based on Scanning Electron Microscopy**

Morphological changes in oral streptococci after treatment with GH12 are shown in figure 6. In the absence of peptide, bacteria showed a regular, smooth surface with only occasional cell lysis and debris (fig. 6a, d, g, j, m, p). Exposure to 64.0 μg/ml of GH12 or 0.12% CHX significantly altered bacterial morphology: cells lost their spherical shape and became noticeably smaller, holes and ruptures appeared on the cell surface, and areas of cytoplasmic outpouring, cell shrinkage and lysis became visible (fig. 6b, c, e, f, h, i, k, l, n, o, q, r).

**Biofilm Susceptibility**

In addition to effective inhibition of planktonic bacteria, GH12 was able to inhibit the formation of biofilms formed by oral streptococci (table 2), as well as the viability of those biofilms (table 3). GH12 at 8 μg/ml inhibited biofilm formed by *S. mutans*, *S. salivarius* or *S. sobrinus* by at least 50.00%. The MBIC50 was 21.3–26.7 μg/ml for *S. mitis*, *S. gordonii* and *S. sanguinis*; the values for *S. mitis* and *S. gordonii* were not significantly higher than the corresponding range of 3.3–13.3 μg/ml for CHX (p > 0.05). GH12 at 64.0 μg/ml inhibited biofilm formation of all strains by more than 90.00%, and the MBIC90 of GH12 was only 13.3 μg/ml against *S. mutans* and 16.0 μg/ml against *S. sobrinus*. The MBEC90 of GH12 against *S. mutans* and *S. salivarius* was 26.7–32.0 μg/ml, while the MBEC90 against the two strains was 64.0 μg/ml. Both GH12 and CHX showed the same MBEC50 against *S. mutans*. GH12 at 128.0 μg/ml caused at least...
50.00% reduction of viable bacteria in single-strain biofilms formed by *S. gordonii* or *S. sobrinus*, the peptide at 512 μg/ml caused >90.00% reduction. GH12 was less effective against *S. mitis* and *S. sanguinis* biofilms, for which the MBEC<sub>50</sub> was 512.0 μg/ml and the MBEC<sub>90</sub> >512.0 μg/ml.

### Discussion

Several amphipathic antimicrobial peptides that can inhibit or kill the growth of oral streptococci have been developed, but they show disadvantages such as low efficacy, instability or costly production. Here we describe the activity of GH12 against oral streptococci.
Fig. 5. Percentage of dead bacteria after treatment with 64.0 μg/ml GH12 for various time periods based on Image-Pro Plus 6.0 quantitation (means ± SD, n = 3): S. mutans (a), S. mitis (b), S. gordonii (c), S. salivarius (d), S. sanguinis (e), and S. sobrinus (f). 0.85% NaCl was used as a negative control, and CHX (0.12%, w/v) as a positive control. Different superscript letters at one time point indicate significant differences between treatments.

Fig. 6. Representative scanning electron micrographs of S. mutans (a–c), S. mitis (d–f), S. gordonii (g–i), S. salivarius (j–l), S. sanguinis (m–o) and S. sobrinus (p–r) after 24-hour incubation in the presence of 64.0 μg/ml GH12 (b, e, h, k, n, q), 0.12% (w/v) CHX (c, f, i, l, o, r) or in their absence (a, d, g, j, m, p).
the short synthetic peptide GH12 and provide in vitro evidence that it adopts an α-helical structure in environments that mimic the cell membrane, remains stable in human saliva without significant degradation, and inhibits and kills 6 strains of oral streptococci with different efficacies.

GH12 was designed to be positively charged and to have a high propensity to fold into amphipathic α-helical structures in hydrophobic environments. Therefore, we designed the peptide with hydrophobic amino acids (leucine) along one side of the helical axis and cationic amino acids (histidine) along the other side. CD spectra of GH12 in the presence of solvents mimicking the cell membrane showed a stable, >80.00% helical secondary structure, confirming the validity of our design approach. Similar synthetic peptides that mimic the folding behavior of naturally occurring α-helical antimicrobial peptides, such as VGR16, (FFRR)3, (LLRR)3, and (LLKK)3, show broad-spectrum antimicrobial activity against bacteria and yeast [Ma et al., 2011; Wiradharma et al., 2011b]. We placed a tryptophan residue at position 4 in the GH12 sequence; this position lies at the interface between the start of the hydrophobic side and the end of the hydrophilic side on a helical wheel projection. Such a substitution has been shown to stabilize the helical structure and improve antimicrobial activity [Lee et al., 2011]. HPLC assays showed that GH12 was not significantly degraded even after 30-min incubation in saliva. This suggests that GH12 can be locally applied during oral treatment.

The MIC is considered the ‘gold standard’ for measuring antimicrobial efficacy [Andrews, 2001]. GH12 showed quite low MICs (6.7–32.0 μg/ml) against the 6 strains of oral streptococci.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>MBIC50, μg/ml</th>
<th>MBIC90, μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GH12</td>
<td>CHX</td>
</tr>
<tr>
<td>S. mutans</td>
<td>UA159</td>
<td>8.0±0.0 A, A</td>
<td>0.8±0.3 B, B</td>
</tr>
<tr>
<td>S. mitis</td>
<td>ATCC6249</td>
<td>26.7±9.2 B, A</td>
<td>13.3±4.6 A, A</td>
</tr>
<tr>
<td>S. gordonii</td>
<td>DL1</td>
<td>21.3±9.2 B, A</td>
<td>6.7±2.3 C, A</td>
</tr>
<tr>
<td>S. salivarius</td>
<td>ATCC13419</td>
<td>8.0±0.0 B, A</td>
<td>1.7±0.6 B, A</td>
</tr>
<tr>
<td>S. sanguinis</td>
<td>ATCC10556</td>
<td>26.7±9.2 B, A</td>
<td>3.3±1.2 C, B</td>
</tr>
<tr>
<td>S. sobrinus</td>
<td>6715</td>
<td>8.0±0.0 A, A</td>
<td>1.7±0.6 B, B</td>
</tr>
</tbody>
</table>

Different superscript lower case letters within a column indicate significant differences between strains. Different superscript capital letters in a row indicate significant differences between the values for GH12 and CHX (p < 0.05).
oral streptococci, as well as MBCs (8.0–64.0 μg/ml) indicating high efficacy. The MICs and MBCs of GH12 against oral streptococci were significantly higher than those of CHX under the same conditions (p < 0.05) – except in the case of S. mutans – but lower than those of the antimicrobial peptides KSL [Liu et al., 2011] and nisin [Tong et al., 2010].

Time kill assays, which measure how fast an antimicrobial agent kills bacteria, showed that GH12 worked quickly against the tested strains, and that the killing time decreased to only 5 or 1 min with increasing peptide concentration. A GH12 concentration of 64.0 μg/ml reduced the population of planktonic bacteria >1 log within 5 min. In live/dead staining assays based on confocal laser scanning microscopy, the bacterial suspensions at high bacterial concentration (approx. 1.0 × 10^8 CFU/ml) were incubated in the presence of GH12 at a final concentration of 64.0 μg/ml for 5 min, 20 min, 1 and 3 h since the bacterial susceptibility assay suggested that 64.0 μg/ml was the lowest concentration that exerted a bactericidal effect to all the 6 strains of oral streptococci, and the time kill assay showed that a GH12 concentration of 64.0 μg/ml led to >1 log reduction in the population of planktonic bacteria within 5 min, completely killing S. mutans, S. gordonii, S. salivarius and S. sanguinis within 20 min, killing S. sobrinus within 1 h and causing a 3-log reduction in the number of viable S. mitis within the same period. At the time point of 3 h, all strains were completely eliminated. Therefore, we chose 64.0 μg/ml as the concentration of GH12 and 5 min, 20 min, 1 and 3 h as the time points for observation. Consistent with the time kill assays, 64.0 μg/ml of GH12 caused substantial bactericidal activity even within 5 min in live/dead staining assays. The rapid activity of GH12 is important for clinical applications, since the typical duration of oral rinsing is 0.5–2 min. Rapid action is also important if the peptide is applied at home as a component of toothpaste, in which case contact time may be only 2–5 min [Ding et al., 2014]. The rapid activity of GH12 in vitro increases its potential for practical applications.

Cariogenic bacteria generally grow in biofilms deposited on the tooth surface, so antacaries agents should effectively inhibit new biofilm formation as well as reduce the viability of existing biofilms, rather than only inhibiting planktonic bacteria. Biofilm formation assays showed that GH12 was effective in preventing biofilm formation of cariogenic oral streptococci like S. mutans and S. sobrinus, with MBIC50 values of 8.0 μg/ml and MBIC90 values of 13.3–16.0 μg/ml. MBECS50 and MBECS90 of GH12 were significantly lower against biofilms of S. mutans, S. salivarius or S. sobrinus than against biofilms of the other 3 strains (p < 0.05). S. mutans has been implicated in the initial stage of pits and fissures caries, while S. sobrinus is closely related to the severity of dental caries. Both species are abundant in cariogenic dental plaque [Okada et al., 2005; Martínez-Martínez et al., 2011]. Cluster analysis of subjects with active caries showed that several had high levels of S. vestibularis or S. salivarius [Gross et al., 2012], and S. salivarius has been shown to be strongly cariogenic in animal studies, although less so than S. mutans [Drucker et al., 1984; Willcox et al., 1991]. Oral streptococci, including S. sanguinis, S. gordonii, and S. mitis, are the dominant ‘pioneer’ species in dental biofilm formation [Ritz, 1967], but they are associated with healthy tooth surfaces [Ge et al., 2008; Gross et al., 2012]. Our results suggest that GH12 more selectively inhibits the growth and metabolism of highly cariogenic streptococci in dental plaque, without strongly affecting streptococci of lower cariogenicity. This increases the potential of GH12 for clinical use, since antimicrobial agents that are selective to cariogenic oral streptococci may be better for inhibiting caries while still maintaining the ecological balance of resident ‘healthy’ oral microbes in the biofilm [Pepperney and Chikindas, 2011; Marsh et al., 2015].

How GH12 and other antimicrobial peptides kill microbes remains unclear [Zasloff, 2002]. The observed loss of membrane integrity after exposing S. mutans, S. salivarius and S. sanguinis to GH12 (fig. 6b, k, n) suggests that the peptide may exert biocidal activity by interacting with the membrane surface. The outermost surface of the bacterial phospholipid bilayer contains abundant negatively charged phospholipids [Zasloff, 2002; Yeaman and Yount, 2003]. Gram-positive bacteria such as oral streptococci also have a negatively charged cell wall due to the presence of peptidoglycan and teichoic acids, which facilitates the accumulation of cationic antimicrobial peptides there [Tossi et al., 2000]. Like other α-helical antimicrobial peptides, GH12 may traverse the cell wall by virtue of its amphipathicity; after reaching the cell membrane, the hydrophilic face of the molecules is attracted to the negatively charged phospholipid head groups, while the hydrophobic face inserts into the bilayer [Rotem and Mor, 2009], giving rise to holes and ruptures on the membrane surface. Consistent with this model, we observed holes and ruptures, as well as concomitant cytoplasmic leakage by scanning electron microscopy (fig. 6b, e, n, k). However, GH12 caused cell shrinkage and lysis of S. gordonii and S. sobrinus without creating obvious holes in the membrane (fig. 6h, q). A third situation was observed in which some bacterial cells retained their normal
shape despite being killed. These results raise the possibility that GH12 may kill bacterial cells by multiple pathways, both at the membrane and intracellularly. This is consistent with growing evidence that antimicrobial peptides can inhibit DNA-, RNA- and protein-dependent intracellular processes [Brogden, 2005]. Therefore, it may be that GH12 can work extra- and intracellularly, similarly to how the cationic amphipathic α-helical antimicrobial peptide pleurocidin works. While pleurocidin at low concentrations inhibits synthesis of DNA, RNA and proteins [Patrzykat et al., 2002], the peptide at high concentrations disrupts the membrane [Mason et al., 2006]. However, whether the morphological changes caused by GH12 were dose-dependent or not was still unknown in the present study, and further studies are needed to explore how GH12 kills microbes clearly.

The various species of oral streptococci tested in our study showed differential sensitivity to GH12 under the same experimental conditions. Combining the bacterial susceptibility assay results with the killing speed derived from time kill curves and live/dead staining assays, we conclude that S. mitis was the least sensitive to GH12. Since GH12 is thought to act on bacterial membranes in a nonspecific manner based on polarity and electrostatics, strain-specific differences in sensitivity may reflect differences in cell wall and membrane composition that affect the surface negative charge [Whiley and Beighton, 1998; Yeaman and Yount, 2003] or differences in molecules that interact with GH12 before membrane permeabilization [Nishimura et al., 2004]. Moreover, biofilm susceptibility assays showed that biofilms of the highly cariogenic oral streptococci like S. mutans, S. sobrinus and S. salivarius were more sensitive to GH12 than biofilms of other strains, which may reflect differences in biofilm structure, composition, dynamics or growth rate [Donlan and Costerton, 2002].

CHX is a synthetic cationic bis-guanide, and it exerted antimicrobial activity by interacting with phospholipids and lipopolysaccharides on the cell membrane of bacteria and then entering the cell through some types of transport mechanism, thereby destroying the cells’ osmotic equilibrium and causing leakage of intracellular components (fig. 6c, f, i, l, o, r) [Mohammadi and Abbott, 2009]. Currently, CHX as the most common anticaries agent has been regarded as a primary component in rinses, gels or varnishes and plays an important role in caries prevention [Tong et al., 2011]. However, it may cause undesirable side effects such as vomiting and tooth stains. Compared with CHX, although the antimicrobial effect of GH12 might not be so strong as for the results of bacterial susceptibility assays and biofilm susceptibility assays, the short-term bactericidal effect of GH12 against oral streptococci was excellent according to the time kill assays and live/dead staining assays. Higher concentrations of GH12 might improve the short-term bactericidal effect, and further optimization of GH12 may reduce MIC, MBC, MBIC and MBEC even more, which would increase the potential of GH12 to inhibit antimicrobial growth in vivo.

Here we show evidence that the synthetic amphipathic α-helical antimicrobial peptide GH12 remains stable in human saliva and exerts fast, efficient bactericidal activity against oral streptococci associated with dental caries in vitro. These results suggest that GH12 merits further testing in preclinical and clinical trials as an antimicrobial agent to prevent and treat dental caries. Future studies should explore the cytotoxicity of GH12 and its activity against other oral bacteria and their caries-associated virulence factors.

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Author Contributions

Conceived and designed the experiments: Zhang L., Tu H., Zhou X. Performed the experiments: Tu H., Fan Y., Lv X., Han S. Analyzed the data: Tu H., Han S. Wrote the manuscript: Tu H., Fan Y., Lv X. Revised the manuscript: Zhang L., Zhou X.

Disclosure Statement

The authors declare that there is no conflict of interest.

References


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