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Identification of Antimicrobial Peptide Hexamers against Oral Pathogens through Rapid Screening of a Synthetic Combinatorial Peptide Library

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A positional scanning synthetic peptide combinatorial library (PS-SCL) was screened in order to identify antimicrobial peptides against the cariogenic oral bacteria, *Streptococcus mutans*. Activity against *Streptococcus gordonii* and *Aggregatibacter actinomycetemcomitans* was also examined. The library was comprised of six sub-libraries with the format $O_{(1-6)}XXXXX\text{-}NH_2$, where O represents one of 19 amino acids (excluding cysteine) and X represents equimolar mixture of these. Each sub-library was tested for antimicrobial activity against *S. mutans* and evaluated for antimicrobial activity against *S. gordonii* and *A. actinomycetemcomitans*. The effect of peptides was observed using transmission electron microscopy (TEM). Two semi-mixture peptides, $RXXXXN\text{-}NH_2$ (pep-1) and $WXXXXN\text{-}NH_2$ (pep-2), and one positioned peptide, $RRRWRN\text{-}NH_2$ (pep-3), were identified. Pep-1 and pep-2 showed significant antimicrobial activity against Gram positive bacteria (*S. mutans* and *S. gordonii*), but not against Gram negative bacteria (*A. actinomycetemcomitans*). However, pep-3 showed very low antimicrobial activity against all three bacteria. Pep-3 did not form an amphiphilic α-helix, which is a required structure for most antimicrobial peptides. Pep-1 and pep-2 were able to disrupt the membrane of *S. mutans*. Small libraries of biochemically-constrained peptides can be used to generate antimicrobial peptides against *S. mutans* and other oral microbes. Peptides derived from such libraries may be candidate antimicrobial agents for the treatment of oral microorganisms.

Key words: synthetic antimicrobial peptides, *Streptococcus mutans*, oral pathogens, helical wheel, membrane disruption.

Introduction

Dental caries and periodontal disease are common infectious oral diseases and are associated with dental plaque, which can contain cariogenic bacteria such as *Streptococcus mutans* [1-3] and/or periodontopathogenic bacteria such as *Porphyromonas gingivalis* [4-5]. Because controlling these dental plaque bacteria is important for the prevention and treatment of oral diseases, they are often treated with broad-spectrum antiplaque chemical agents such as chlorhexidine [6] or antibiotics such as vancomycin [7-8]. However, the frequent clinical application of these drugs is limited, particularly because chlorhexidine has a bitter taste and can cause staining of the teeth [9-10]. Also, the number of antibiotic-resistant organisms has increased, thereby reducing the efficacy of these antibiotics [11]. Thus, it is

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necessary to develop alternative and clinically useful antiplaque agents that have negligible or no side-effects.

Recently, natural antimicrobial peptides and their synthetic derivatives have attracted attention as potential antibiotic surrogates; these peptides show killing activity against a wide spectrum of bacterial species, including drug-resistant strains, and bacterial resistance is less common [12-15]. The positively charged residues within these peptides, coupled with their secondary structure helix, loop and other conformations, seem to disrupt the negatively charged lipid membrane, leading to the formation of pores, which compromise membrane integrity [15-17]. In addition, they appear to play a role in innate immune and inflammatory responses, such as immune response induction, cytokine release, and chemotaxis [15,19-20]. However, their clinical application is limited because their biological activity is altered within the human body: the peptides are susceptible to proteolytic degradation *in vivo* and fail to achieve high levels of bactericidal activity at physiologic pH values [14,21]. Nevertheless, such antimicrobial peptides may be efficient and safe for the treatment of oral infections because they could be applied directly to pathogenic bacteria within the oral cavity. They would then be degraded by digestive enzymes and absorbed through the intestines along with other nutrients.

In addition to natural antimicrobial peptides and their synthetic derivatives, many investigators have used synthetic combinatorial technology to develop peptide libraries in an attempt to understand their mechanism(s) of action and to improve their antimicrobial activity [22-23]. However, until now, few studies have used synthetic libraries to generate antimicrobial peptides and then examined their effects against oral pathogenic bacteria [24-25].

Combinatorial libraries are an efficient method of generating and identifying peptides with potent biological activity [26-31]. A positional scanning synthetic combinatorial library (PS-SCL) comprises sub-libraries of peptides, in which one position is occupied by a defined amino acid residue and the others by different amino acids. Here, we identified a new synthetic antimicrobial peptide against oral pathogens by screening a PS-SCL comprising hexapeptides, and then examining their antimicrobial activity against *S. mutans*, *S. gordonii*, and *A. actinomycetemcomitans*. We also attempted to elucidate the underlying mechanism of action and assessed their potential use for the prevention/treatment of oral diseases.

Materials and Methods

Bacterial strains and growth media

The bacterial strains used in this study were *Streptococcus mutans* (ATCC 25175), *Streptococcus gordonii* (KCTC 3286), and *A. actinomycetemcomitans* Y4 (KCTC 3698). All microbes were grown in tryptic soy agar (TSA, Difco Laboratories, USA) containing brain heart infusion (BHI) at 37°C in a standing culture. *S. mutans* and *S. gordonii* were grown under aerobic conditions (5% CO_2), whereas *A. actinomycetemcomitans* was grown under anaerobic conditions (100% N_2).

Peptide library and individual peptides

The hexapeptides (hexamers) used in the current study were provided by the Peptide Library Support Facility (PLSF) at POSTECH (Pohang, Korea). The library used here was PS-SPCL [22]. The peptide library was a "one-bead one-peptide" library and was synthesized using a resin that utilizes a polystyrene matrix as a base and polyethyleneglycol as a linker (Novabiochem TG-resin, 01-64-3) [32]. Mixed (X) positions comprised one of 19 different amino acids (excluding cysteine), with the relative ratios adjusted to yield a near-equimolar ratio. Each amino acid was synthesized by C-terminal amidation. A single library comprised 114 tubes (6 positions × 19 amino acids); the total peptide concentration was 30 mM and the concentration of each individual peptide was 12 nM. There were 19 sub-libraries, each containing 2,476,099 peptides.

Individually synthesized peptides were purchased from Peptron (Daejeon, Korea). Each peptide was purified by high-pressure liquid chromatography on a C18 column (Waters 290 separation module, USA) and confirmed by mass spectrometric analysis with an HP1100 series LC/MSD (Hewlett-Packard, Palo Alto, CA, USA).

Both peptide library and individual peptides were used as suggested by the suppliers.

Antimicrobial activity assay

Each test strain of bacteria was seed cultured in 5 ml of rich broth and incubated overnight to an optical density 1.0 at 570 nm, which corresponds to 1×10^8 colony forming units (CFU)/ml. Each bacterial strain was then inoculated into tryptic soy broth (TSB) in sterile 96 well plates to yield a

final volume of 100 μl (a dilution of 1:100). An appropriate volume of peptide stock solution was then added to the first column of the plate (final concentration: 1.5 mM) and then serially diluted across the plate (from 1.5 mM to 0.15 mM). For control, only vehicle was added. The plates were incubated at 37°C under aerobic or anaerobic conditions (depending on the bacterium) for over 18 hours without shaking. The absorbance was measured at 570 nm to quantify bacterial growth. All experiments were performed in triplicate. Inhibitory concentration 50 (IC_{50}) values for the peptides were calculated from the graph derived from the antimicrobial activity assay. IC_{50} values were determined in triplicate.

Transmission electron microscopy

For TEM analysis, overnight cultures of *S. mutans* were inoculated into fresh medium (1:100 dilution) and grown overnight in the presence of the peptide of interest. To prepare sample specimens for TEM, the bacterial suspension was fixed with 2% glutaraldehyde and 4% paraformaldehyde. After dehydration with ethanol, samples were post-fixated in osmium tetroxide to augment the electronic density. The prepared specimens were then washed thoroughly with phosphate or cacodylate buffer to eliminate free fixative and then dehydrated. The infiltration step was performed at room temperature: the specimen was embbed sufficiently in Epon 812 to increase the viscosity of the sample resin. Ultra-thin sections were cut with an ultramicrotome (Sorvall instrument MT6000, Tucson, AZ, USA), and specimens were electronically stained with uranyl acetate and lead citrate. Bacterial morphology was observed by TEM (Hitachi H-600, Hitachi Co., Tokyo, Japan).

Results

Selection of antimicrobial hexapeptides by screening of a peptide library

The PC-SPCL library comprised hexamers with an amidated C-terminus. Specific positions within each peptide were defined, whereas other positions were occupied by one of 19 amino acids from an equimolar mixture (excluding cysteine). The peptide library comprised six sub-libraries and the hexapeptides were represented as $O_{(1-6)}XXXXX\text{-}NH_2$, where O was a specific amino acid and X was one of 19 amino acids from the equimolar mixture. The resulting library was screened for antimicrobial activity against *S. mutans* at two different concentrations, and the most active amino acid at each defined position was identified.

Selection was performed by measuring the antimicrobial activity of each sub-library. The cariogenic bacterium, *S. mutans*, was used as the test model. The sub-library that reduced the growth of *S. mutants* to less than 30% of that of the untreated control (at both concentrations tested) was used for further selection.

We selected peptide libraries in which arginine (R) and trypsin (W) occupied the first position, i.e., $RXXXX\text{-}NH_2$ and $WXXXXX\text{-}NH_2$, respectively. Both of these peptides showed strong growth inhibition at 0.6 mM and 1.5 mM (Fig. 1A). When examining position 2 ($XOXXXX\text{-}NH_2$), we found that R and W were again the most effective, showing high

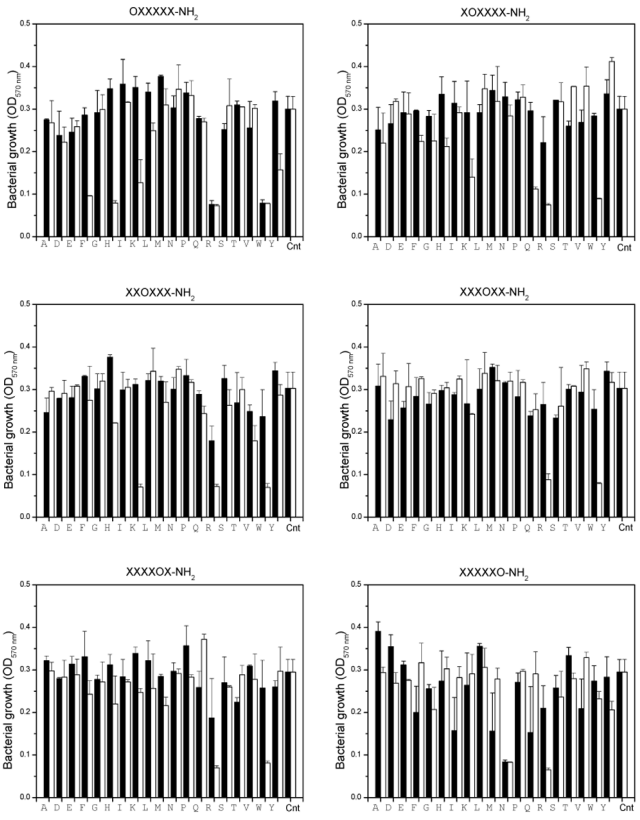

Fig. 1. Inhibition of bacterial growth by the synthetic peptide combinatorial library. *S. mutans* was grown overnight in the presence of peptides at 0.6 (black bars) or 1.5 mM (white bars). The absorbance was then measured at 570 nm. O represents a defined position. The amino acid occupying this position in a specific peptide mixture is shown on the x-axis. The remaining five positions (X) comprise one of 19 amino acids (excluding cysteine). Cnt: control.

antimicrobial activity at 1.5 mM; however, the antimicrobial activity at 0.6 mM was lower (73.7% of the control value for R, and 94.7% of the control value for W). Gln and Lys at position 2 also showed good growth inhibitory effects (Fig. 1B). R and W were again most effective at position 3 (XXOXXX-NH_2). Lysine (K) also showed high growth inhibitory activity at 1.5 mM, but showed little activity at 0.6 mM. Peptides containing R and W at positions 4 and 5 (XXXOXX-NH_2 and XXXXOX-NH_2) were also very effective, whereas asparagine (N) was most effective at position 6 (Ac-XXXXXO-NH_2), followed by R.

These position scanning results suggested that a peptide with R/W at position 1, R/W/K at position 2, R/W/K at position 3, R/W at position 4, R/W at position 5, and R/N at position 6 would be most effective. It is noteworthy that a positively charged amino acid (R) or an uncharged polar amino acid (W) appears in most positions. This suggests that either R or W are essential for antimicrobial activity against *S. mutans*. A peptide selected on the basis of this result would be very hydrophilic and soluble rather than amphiphilic (the latter is thought to be an essential property for antimicrobial activity). Thus, we decided to select three peptides: two containing a mixture of amino acids positions 1 to 6 and one with specific amino acids in all six positions. The results showed that a peptide containing R or W at position 1 and N at position 6 showed high antimicrobial activity at all concentrations tested. Thus, we synthesized three hexapeptides, RXXXXN-NH_2 (pep-1), WXXXXN-NH_2 (pep-2), and RRRWRN-NH_2 (pep-3), for further study.

Activity of the selected hexapeptides against oral microbes

We next tested the activity of the three peptides against *S. mutans*, *S. gordonii*, and *A. actinomycetemcomitans*. The results are presented in Figure 2. Both pep-1 and pep-2 showed significant antimicrobial activity against *S. mutans* at 0.6 mM and 1.5 mM, and the response was dose-dependent (Fig. 2A). However, pep-3 showed no activity against *S. mutans* at the concentrations tested. Antimicrobial activity against *S. gordonii* is shown in Figure 2B. Pep-1 showed antimicrobial against *S. gordonii*, and the response was dose-dependent. Pep-2 also showed some antimicrobial activity. However, pep-3 showed no activity. When we tested the antimicrobial activity of the peptides against *A. actinomycetemcomitans*, none showed significant antimicrobial activity.

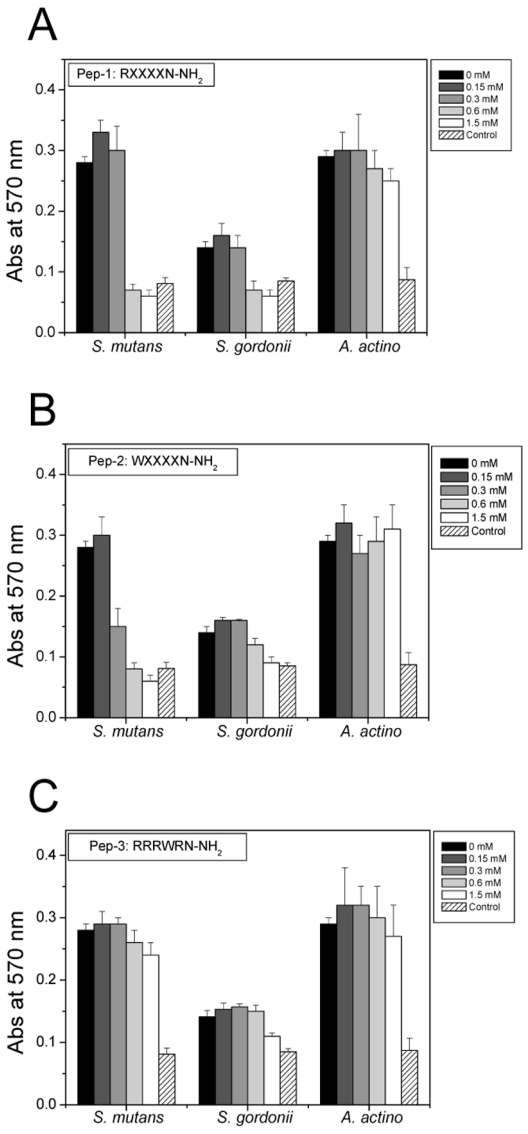

Fig. 2. Antimicrobial activity of pep-1, pep-2, and pep-3 against *S. mutans*, *S. gordonii*, and *A. actinomycetemcomitans*. All experiments were repeated at least three times, and data are expressed as the mean ± standard deviation (SD).

These data were unexpected because the amino acids at these positions within the peptides showed significant antibacterial activity in the previous experiments. We next modelled the α-helical wheels of pep-1, pep-2, and pep-3 (Fig. 3). The overall charge of these peptides was +1/0 for pep-1 and pep-2, and +4 for pep-3. As expected, pep-1 and pep-2 harbored fixed positive or polar amino acids in positions 1 and 6, suggesting that the helical wheel may be amphiphilic if a positively charged or polar amino acid occupied position X_1 and/or position X_4, and a nonpolar amino acid occupied positions X_2 and X_3 (Fig. 3). The overall

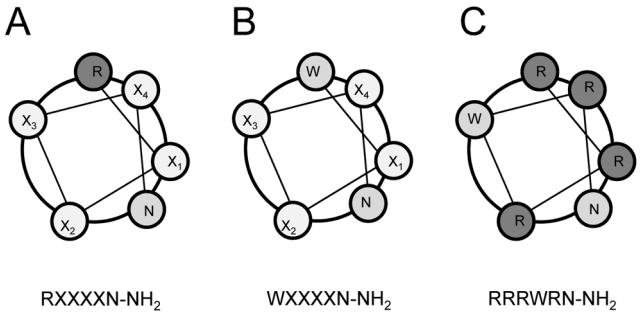

Fig. 3. α-helical wheel projection for representative peptides. Residues are either designated by single letter codes or numbered consecutively from the C- to the N-terminus. Positively charged amino acids are shaded dark grey and polar amino acids are shaded light grey.

charge of pep-3 was +4, as it contained four positively charged amino acids (R). The arginine residues within pep-3 were well dispersed and therefore not localized to one side of the peptide helix. In addition, the other amino acids in pep-3 (W and N) were polar amino acids. Thus, pep-3 was a hydrophilic peptide and not able to form the amphiphilic α-helical structure frequently found in antibacterial peptides [14-17,33]. This absence of amphiphilicity may explain the low antimicrobial activity of pep-3.

Since pep-3 did not show significant antimicrobial activity against the three bacteria tested in this study, we synthesized another peptide, RQWWRN-NH_2 (pep-4), which contained amino acids that showed antimicrobial activity in the PS-SPCL screening experiments (Fig. 1). When we mapped the helical wheel for pep-4, we again found that the positively charged and polar amino acids were well dispersed. Thus, pep-4 did not show activity against the three test strains (data not shown). This supports the idea that pep-3 did not show antimicrobial activity because it lacked an amphiphilic helical structure. We also synthesized hexapeptides, RXXXRN-NH_2 (pep-5) and RXXXWN-NH_2 (pep-6), which contained either R or W at position X_4 of pep-1 (Fig. 3). The extra R or W increased the amphiphilic characteristics of the peptide helix. However, the antimicrobial activity of pep-5 and pep-6 was not as great as that of pep-1 or pep-2 (data not shown). In addition, we screened libraries comprising tri- and quadrapeptides; however, they did not show antimicrobial activity under the experimental conditions used in this study.

Possible mechanism underlying antimicrobial activity

Based on the results obtained above, pep-1 and pep-2 showed the greatest potential for use as antimicrobial agents.

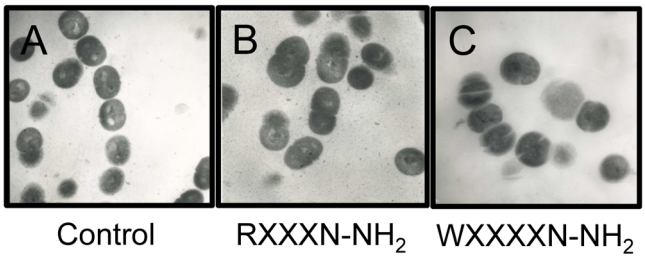

Fig. 4. TEM images of *S. mutants* after incubation with the antimicrobial peptides. Bacteria were incubated overnight with vehicle only (A), 0.6 mM of pep-1 (B) or 0.4 mM of pep-2 (C), and bacterial membranes were observed (mag, ×30,000).

Hence, we used these peptides for further experiments. To investigate possible mechanism of action of these antimicrobial peptides, we examined their effects on the integrity of the bacterial cell membrane. First, we determined the IC_{50} values of pep-1 and pep-2 against *S. mutans*, which was the most susceptible bacterium tested in this study. The IC_{50} values of pep-1 and pep-2 were approximately 0.6 and 0.4 mM, respectively. Figure 4 shows TEM images of *S. mutans* treated with pep-1 and pep-2 at the IC_{50}. Peptide treatment disrupted the bacterial cell membrane (Fig. 4B and C) when compared with the non-treated control (Fig. 4A). Bacteria treated with either pep-1 or pep-2 seemed to stall at a particular divisional stage or became fused with other bacteria. This indicates a malfunction of the membrane. Both peptides showed a similar pattern of bacterial membrane destabilization, suggesting that both may share a similar bactericidal mechanism.

Discussion

The outer-most leaflet of the bacterial membrane contains negatively charged phospholipids; therefore, they present a "negative" surface [34]. These negatively charged molecules create a polyanionic microbial cell surface, which attracts cationic peptides. Generally, antimicrobial peptides are relatively short, positively charged, and amphiphilic [13-16]. The first step of the interaction between amphiphilic antimicrobial peptides and the bacterial membrane is the initial attraction between the cationic peptide and negatively charged components on the outer bacterial envelope, such as the phosphate groups within the lipopolysaccharides of Gram negative bacteria or the lipoteichoic acids on the surface of

Gram positive bacteria. The next steps include displacement of lipids, alteration of membrane structure, and peptide entry into the target bacteria [13-16]. Various mechanisms have been suggested to explain bacterial killing by peptides, including the creation of holes in the membrane that result in the loss of intracellular contents, disrupting the distribution of lipids between the leaflets of the lipid bilayer, which causes loss of membrane function or allows substances to be translocated across the membrane and acting on an internal target [13,16,34-35].

Here, we screened a PC-SPCL library to identify peptides with antimicrobial activity against oral pathogens. The PS-SPCL library has previously been used to identify clinically useful peptides [22,26-28]. We found that hexamers with positively charged or polar amino acids in most positions showed antimicrobial activity against *S. mutans*. We believe that this is due to the positive charge or polar characteristics of the peptide rather than to the position of the positively charged or polar amino acids at a specific location within the peptide. We identified a hexamer, pep-3, which contained four R residues and two polar residues. However, pep-3 did not show the expected level of antimicrobial activity, suggesting that amphiphilicity is required for antimicrobial activity. Pep-1 and -2, which have N and R or N and W at the N- and C-termini, respectively, did show antimicrobial activity (Figs. 2 and 3). This is because the two amino acids (R and N, or W and N) were located on almost opposite sides of the helix; therefore, the helix was amphiphilic. It appears that these semi-amphiphilic peptides were attracted to (and attached to) the bacterial membrane and were able to either disturb its normal function or enter the bacteria.

The results of the present study showed that pep-1 and pep-2 had activity against Gram positive bacteria (*S. mutans* and *S. gordonii*) but not against Gram negative bacteria (*A. actinomycetemcomitans*). This may be due to differences in cell membrane structure. Gram negative bacteria have an inner plasma membrane and an outer membrane, whereas Gram positive bacteria have only plasma membrane. Thus, peptide-mediated pore formation would have a greater effect on Gram positive bacteria, which do not have a periplasmic space to act as a buffer. In addition, it seems that the cationic nature of antimicrobial peptides ensures accumulation at polyanionic microbial cell surfaces that contain acidic polymers such as lipoteichoic acid, which are abundant on the surface of Gram positive cells [13-16]. Further studies are needed to fully elucidate the mechanism(s) underlying the antimicrobial activity of these peptides.

Cytotoxicity is also an issue for drugs used either systemically or locally. In general, the cytotoxicity of antimicrobial peptides is measured in hemolytic assays using mammalian red blood cells. Actually, these peptides do show a degree of cytotoxicity against mammalian cells because, similar to Gram positive bacteria, they also have a single membrane; however, small antimicrobial peptides show lower cytotoxicity than longer peptides [26,36]. Therefore, pep-1 and pep-2 should be evaluated for cytotoxicity against mammalian cells prior to application for clinical use.

We tested the peptides against the oral cariogenic bacterium, *S. mutans*, and the pathogenic bacteria, *S. gordonii* and *A. actinomycetemcomitans*. *S. gordonii* is a component of the normal microbial flora within the human oral cavity and the primary etiological agent of infective endocarditis [37-38]. *A. actinomycetemcomitans* is a Gram negative bacterium that causes localized aggressive periodontitis [39]. It will be useful to examine the three dimentional structure of the petides and the effects of the hexamers identified in the present study against normal bacteria or on oral microbial ecology; however, they could be used to eradicate the oral pathogens described above. In addition, it would be useful to know the nature of antibacterial effect acquired in this study, e.g. bacteriostatic or bactericidal by subsequent investigation.

In conclusion, the present study screened a positioning peptide library to identify potentially useful antimicrobial peptides that are clinically effective against oral pathogens. Two hexamers (pep-1 and pep-2) containing amino acids N and R at the N- and C-termini, respectively, showed significant antimicrobial activity. TEM studies showed that these peptides appear to act by disrupting the bacterial membrane. We believe that pep-1 and -2 show potential as clinically useful antimicrobial agents. Further studies should examine their toxicity to mammalian cells both *in vitro* and *in vivo*.

Acknowledgment

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Conflict of interest

The authors declare that they have no competing interest.

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