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Efficacy of a novel antimicrobial peptide against periodontal pathogens in both planktonic and polymicrobial biofilm states

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ABSTRACT

Streptococcus gordonii, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* represent the early, middle and late colonizers of the bacterial accretion in dental plaque biofilms. These sessile communities constitute a protected mode of growth that promotes survival in a hostile environment. This study describes a novel and unrecognized role for a synthetic cationic antimicrobial peptide, Nal-P-113, which inhibits and kills periodontal bacteria in planktonic state, inhibits the formation of biofilms and eradicates polymicrobial biofilms. Nal-P-113 is also stable in saliva, serum and saline solution. At a concentration less than 320 μg/mL which is harmless to normal oral cells, Nal-P-113 can kill bacteria in planktonic state. At a concentration of antimicrobial peptide Nal-P-113 (1280 μg/mL) which only causes slight damages to normal oral cells is needed to kill bacteria in biofilm state. It is worth mentioning that this concentration of Nal-P-113 is harmless to rat oral mucosa compared to chlorhexidine. The mechanism of Nal-P-113 inhibiting and killing periodontal bacteria might rely on the abilities to permeabilize and/or to form pores within the cytoplasmic membranes, thus causes the death of bacteria. Here, we provided a novel and stable antimicrobial peptide with very low mammalian cytotoxicity, which can inhibit and kill periodontal bacteria in both planktonic and polymicrobial biofilm states.

Statement of Significance

Nal-P-113 is a potent antimicrobial peptide with strong antimicrobial ability, improved deficiency compared with other antibacterial peptides, and remains stable in phosphate buffered saline, saliva, brain-heart infusion medium and bovine calf serum. Nal-P-113 exhibits a broad spectrum of bacteriocidal activity with excellent eradicating capability on oral pathogens and the respective biofilms. In this study, we used propidium iodide staining, scanning electron microscopy and transmission electron microscopy to confirm that Nal-P-113 can perforate plasmalemma thereby resulting in the death of oral pathogens and disintegrate the respective biofilms. Nal-P-113 also showed effective anti-plaque biofilms and cytotoxicity in the rat periodontitis model. No adverse effects can be observed on the gingivomucosa tissue. In short, the antimicrobial peptide Nal-P-113 presented to be an effective yet have low mammalian cytotoxicity agent with potential application in the clinic. This study provides a proof of concept in applying antimicrobial peptides in the clinical perspective.

1. Introduction

Periodontal diseases are major public health concerns due to their high prevalence in general population. While epidemiological studies showed that certain countries like Sweden and Switzerland have low prevalence of periodontal disease [1,2], in many developed countries like Germany, the prevalence rate of periodontitis can reach as high as 70.9% in 35–44-year-old adults and 87.4% in 75–84-year-old seniors despite their advanced healthcare system [3]. Similarly, according to the National Health Service, it was estimated that more than 50% of the US adult population are affected by periodontal disease at certain degree and approximately 15% of UK population have been diagnosed with severe periodontitis [4].

Periodontitis is a chronic polymicrobial disease of the gums and causes inflammation in its milder form. As the disease worsens, periodontal tissues become severely inflamed and can eventually progress to tooth loss. Periodontitis causes plaque accumulation

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and deposit in the gap between joining epithelium and teeth, leading to the formation of inflammation and periodontal pocket. The threat of periodontal disease reaches beyond the border of oral hygiene increasing evidences suggested potential correlation between periodontal disease and the risk of cardiovascular disease, diabetes and respiratory complication. Maternal periodontitis can even lead to pre-term birth (PTB) and low birth weight (LBW). Unfortunately, treatments for periodontal diseases are relatively costly compared to other diseases, causing an economic burden of £1.6 billion per annum in UK alone [4]. Therefore, searching for better means to battle periodontitis can significantly contribute to public health and social-economic development.

Microbial biofilms formation is the major etiological agent for dental disease. Colonization of bacteria around the teeth and periodontal tissue causes severe inflammation. There are nearly 700 bacterial species that are postulated to colonize the surface of the oral cavity. Several studies have reported that potential pathogenic species may coexist through commensalism and form the multi-specie microbial community [5]. *Streptococcus gordonii* (*S.g*), *Fusobacterium nucleatum* (*F.n*) and *Porphyromonas gingivalis* (*P.g*) are the three bacteria species that typically colonize bacterial accretion at early, middle and late stages of dental plaque biofilms formation, respectively [5]. *S. gordonii* is known as one of the initial colonizing bacterium on tooth surfaces and functions as an anchor for subsequent attachment of other species to establish complex oral biofilms [6]. *F. nucleatum* comes into the picture later because it needs to bind the early colonizer such as *S. gordonii* Challis CH1 [7]. An additional role of *F. nucleatum* is to further reduce the oxygen level to which other anaerobic pathogenic organisms like *P. gingivalis* can thrive [8]. *P. gingivalis*, an important pathogenic factor of chronic periodontitis, can adhere to either *F. nucleatum* or *S. gordonii* [9,10] and subsequently form metabolically compatible mature dental plaque biofilms.

Numerous studies indicated that the removal of tooth surface dental plaque biofilms is an effective approach for periodontitis treatment [11]. However, this approach faces several difficulties. Firstly, biofilms in oral cavity and periodontal pocket is highly resistant to antibiotic treatment due to its complex structure. Secondly, the abuse of antibiotics in recent years has led to the emerging of drug-resistant bacterial strains [12]. Currently, the first line treatment for periodontitis is metronidazole. However, the beneficial effects of metronidazole are accompanied with undesirable side effects including diarrhea, vomiting, metallic taste, headache and dizziness. Chlorhexidine is another potent anti-plaque chemical agent but its clinical application is limited by bitter taste and teeth stain [13,14]. Therefore, development of alternative treatments is in urgent need to remove periodontal pathogens [15–17].

Antimicrobial peptides (AMP) are emerging as promising antimicrobial agents due to their rapid bactericidal activities [18]. Up-to-date, three models have been widely described for the mode of action of antimicrobial peptides [19,20]. Barrel-stave model proposed that individual peptide molecules will form an aqueous pore with the acyl chains of lipid in the membrane, which favor the aggregation of the peptides on the membrane surface and induce the conformational phase transition and thinning of the membrane. Thus, they kill the microbe by leakage of intracellular components. In the toroidal pores model, peptide inserts or induces a positive curvature strain in the membrane and resulting in the formation of a toroidal pore that can cause micellization of the membrane. Alternatively, the peptides may insert into bacterial membranes by straddling the interface of the hydrophilic head groups and the fatty acyl chains of membrane phospholipids [21]. After insertion, the breakdown of membrane integrity could be accomplished via the swamping of membrane charge by "carpet" peptides at the interface, the detergent-like dissolution of membrane, or the formation of peptide-lipid aggregations within the bilayer [22], which also leads to bacterial cell lysis and thereby cell death [23].

However, the antibacterial activity of cationic AMP is severely compromised under physiological condition due to the high salt concentration present in saliva, serum and other body fluids, under which condition the interaction between AMP and bacteria membrane becomes interrupted. AMP can also be rendered ineffective when bound with anionic protein or glycan which shield the peptide binding site of the bacteria. Additionally, bacteria like *P. gingivalis*, evolve defensive mechanism, such as proteases that quickly degrade AMP. Apart from the barriers mentioned above, the safety profile of AMP remains unclear and more studies are needed to investigate any potential cytotoxicity to host cells.

P-113 (AKRHHGYKRKFH-NH2) is a 12-amino-acid histidine-rich peptide derived from the saliva protein histatin 5. It has been demonstrated that P-113 is bactericidal and fungicidal in oral cavity against clinically important microorganisms such as *Streptococci*, *Staphylococci*, *Pseudomonas* spp. and *Candida albicans* [21–25]. However, studies reported that the efficacy of P-113 was greatly reduced under high salt concentration [26–28]. Moreover, similar to other antimicrobial peptides, antimicrobial potency of P-113 becomes significantly compromised in biological fluid such as plasma, serum, saliva and sputum [22,29–31]. Previously, we have successfully increased salt resistance of antimicrobial peptides by replacing tryptophan or histidine residues with the bulky amino acids β-naphthylalanine and β-(4,4′-biphenyl)alanine [32]. The variant, Nal-P-113, with β-naphthylalanine substitution, retained bactericidal activity even at high salt concentration [32].

Here, we investigated the bacteriostatic and bactericidal ability of Nal-P-113 against major pathogenic bacteria strains involved in the oral plaque biofilm formation. Nal-P-113 showed significant bactericidal activity on *S. gordonii*, *F. nucleatum* and *P. gingivalis* in both planktonic and polymicrobial biofilm states. Cellular and biofilm structure were severely disrupted by Nal-P-113 treatment in both planktonic and biofilm state. Furthermore, Nal-P-113 demonstrated favorable safety profile because it causes minimal damage to periodontal ligament stem cells and is harmless to rat gingival mucosa at biological active concentration. Our results provide a basis to further develop Nal-P-113 as a clinical agent to fight against periodontitis caused by oral pathogens.

2. Materials and methods

2.1. Chemicals

Nal-P-113, Ac-AKR-Nal-Nal-GYKRKF-Nal-NH_2, was synthesized by Institute of Biotechnology and Department of Medical Science, National Tsing Hua University [32,33]. The identity of the peptides was checked by electrospray mass spectroscopy, and the purity was assessed by high-performance liquid chromatography (HPLC). Peptide concentration was determined with a UV spectrophotometer (280 nm). Rhodamin B was conjugated to the N terminal amine of Nal-P-113 (Labeled by China Peptides, China). Chlorhexidine and metronidazole were purchased from Sigma (CA). Penicillin was obtained from MD Bio (Taiwan, China).

2.2. Bacterial strains

F. nucleatum ATCC25586 was purchased from ATCC (VA). *S. gordonii* Challis CH1 and *P. gingivalis* W83 were obtained from the Department of Oral Biology at China Medical University. *F. nucleatum* ATCC25586 and *P. gingivalis* W83 were cultured in freshly prepared brain heart infusion (BHI) agar plate (Difco Laboratories, MI) and supplemented with 5% sterile defibrinated sheep blood, 1%

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