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Antimicrobial susceptibility of anaerobic bacteria

A novel antimicrobial peptide against dental-caries-associated bacteria

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ABSTRACT

Dental caries, a highly prevalent oral disease, is primarily caused by pathogenic bacteria infection, and most of them are anaerobic. Herein, we investigated the activity of a designed antimicrobial peptide ZXR-2, and found it showed broad-spectrum activity against a variety of Gram-positive and Gram-negative oral bacteria, particularly the caries-related taxa *Streptococcus mutans*. Time-course killing assays indicated that ZXR-2 killed most bacterial cells within 5 min at 4 × MIC. The mechanism of ZXR-2 involved disruption of cell membranes, as observed by scanning electron microscopy. Moreover, ZXR-2 inhibited the formation of *S. mutans* biofilm, but showed limited hemolytic effect. Based on its potent antimicrobial activity, rapid killing, and inhibition of *S. mutans* biofilm formation, ZXR-2 represents a potential therapeutic for the prevention and treatment of dental caries.

1. Introduction

Dental caries is the most prevalent oral disease and considered as a major public health problem [1]. Clinical and experimental animal studies have indicated that caries is caused by many cariogenic microbes, in which some acidogenic *Streptococcus* sp. are the main caries-associated pathogens. The pathogens that adhere to teeth surface can form dental plaque biofilm firstly. Then, they induce inflammation around teeth, which may gradually develop into dental caries [2–4]. Because of the environment of caries and formation of biofilms, most of the pathogenic bacteria are grown in an anoxic condition. Among those caries-associated bacteria, *Streptococcus mutans* is considered a major pathogen for dental caries due to its acidogenic and aciduric properties. *S. mutans* can consume multiple fermentable sugars to produce acids, which cause dissolution of minerals in the tooth enamel and eventually result in dental caries. In addition, *S. mutans* can form biofilms on the surface of tooth, which make it persistently colonize the tooth surface [5]. Besides *S. mutans*, there are also other oral pathogens, including *Streptococcus sobrinus* and *Porphyromonas gingivalis*, which can provoke diseases of the oral cavity [6]. Therefore, effective control of pathogenic bacteria to prevent dental caries requires broad spectrum antibacterial activity and ability to prevent biofilm formation.

Due to the frequent use of antibiotics, drug-resistant strains have emerged, so novel antimicrobial agents are urgently needed to control bacterial infection. Antimicrobial peptides (AMPs) are considered as potential substitutes of antibiotics owing to their potent activity against a wide spectrum of bacteria, including drug-resistant strains [7–11]. A variety of AMPs also can prevent the formation of biofilm or reduce mature biofilm mass [12,13].

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Researches have shown that the concentrations of histatin-5 and β-defensin-2 (two AMPs) in saliva increase in early childhood caries compared to caries-free group, suggesting a role of AMPs in the defense of oral infection [14,15]. It has been reported that some cariogenic bacteria, including *S. mutans*, are susceptible to AMPs like human β-defensins and to LL-37, a cathelicidin family antimicrobial peptide [16]. Novel AMPs have also been designed to target against *S. mutans* for the treatment of cavities [17,18].

In a previous study, we designed a lytic peptide ZXR-2 (FKIGGFIKKLWRSLLA), which showed potent activity against cancer cells [19]. In order to investigate the antimicrobial activity of this peptide and explore its potential application in the prevention and treatment of dental caries, the antimicrobial and anti-biofilm activity of ZXR-2 against dental-caries-associated bacteria were tested.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Weissella confuse, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus fermentum*, *Lactobacillus rhamnosus*, *Lactobacillus oris*, *Lactobacillus salivarius*, *Lactobacillus gasseri*, *Lactobacillus delbrueckii* and *Lactobacillus mucosae* were cultured in De Man, Rogosa and Sharpe (MRS) broth. *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus gordonii*, and *Streptococcus sanguis* were cultured in brain-heart infusion (BHI) broth. *Porphyromonas gingivalis* was cultured in tryptone (tryptic) soy broth (TSB). All bacterial strains were isolated from clinical and cultured anaerobically at 37 °C using AnaeroGen™ Compacts (Thermo Fisher Scientific Inc. MA, US). And all of them were provided by Peking University School of Stomatology (Beijing, China).

2.2. Peptide synthesis and purification

The peptide, ZXR-2 (FKIGGFIKKLWRSLLA), was synthesized using a solid-phase method with the standard Fmoc chemistry on an automatic peptide synthesizer (CS-Bio 136, CS-Bio Company Inc. USA). The obtained dry, resin-bound peptides were cleaved and side-chain deprotected with a cocktail of Trifluoroacetic acid (TFA): H_2O: thioanisole: phenol: ethanedithiol (82.5:5:5:5:2.5). Then the resin was removed by filtration, and TFA was evaporated under a slow stream of N_2. The peptides were precipitated from the filtrate with cold diethyl ether. The resulting crude peptides were analyzed and purified by reverse-phase high-pressure liquid chromatography (Shimadzu LC-6AD, Cosmosil C18 peptide/protein column). After purification by high performance liquid chromatography (HPLC), organic solution was removed by rotary evaporation, and the generated peptide solution was lyophilized to obtain pure peptide powder, which was used in all experiments. The purity of the peptide was assessed by analytical HPLC and ESI-MS (Thermo LTQ Orbitrap XL equipped with an electrospray ionization source). The results showed that the peptides were synthesized correctly and the purity was >95%.

2.3. Antimicrobial activity assay

Mid-logarithmic (mid-log) phase bacteria (OD570 = 0.6–0.8) were used and cultured anaerobically at 37 °C. First, cell suspension of each bacterial strain (180 μL/well) was seeded into 96-well plates (Costar, Cambridge, MA). Then, peptide stock solution (10 mM) was serially diluted to the desired concentrations (0–320 μM) in corresponding culture broth of each bacterial strain, and 20 μL/well of the peptide solutions were added to make the final concentrations of 0–32 μM. Penicillin was used as the positive control, while the corresponding broth without peptide was used as the negative control. After anaerobic incubation overnight, the absorbance of each well at 570 nm (OD570) was measured using a microplate reader (Multiskan Spectrum, Thermo Fisher Scientific Inc. MA, USA) to evaluate cell growth. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of peptide that prevented bacterial growth. All results were the average of three independent measurements.

2.4. Bacteria killing kinetics

The killing kinetics of ZXR-2 against bacteria associated with dental caries was analyzed using a time-killing assay [18,20]. Mid-log phase bacteria were diluted in their corresponding bacterial growth medium to 10^5 cells/mL and then mixed with 2 × and 4 × MICs of ZXR-2. The untreated bacteria were used as negative control. All samples were anaerobically incubated at 37 °C for 0–4 h. At different indication time points (1, 2, 5, 15, 30, 60, 120 and 240 min), 10 μL bacterial suspensions were collected and diluted with 490 μL growth medium. The samples were spread onto agar plates, incubated anaerobically at 37 °C for 24–48 h, and then the colonies were counted. All assays were conducted in at least triplicate.

2.5. Scanning electron microscopy (SEM) analysis

ZXR-2–induced morphological changes in *S. mutans* and *L. fermenti* were evaluated by SEM (Cambridge S-250MK3, Cambridge, UK). Bacteria were treated with ZXR-2 (20 × MICs or 10 × MICs) for 4 and 24 h anaerobically at 37 °C. After treatment, bacteria were collected by centrifugation at 6000 rpm for 5 min and washed with PBS three times. Then the bacteria were fixed with 2.5% buffered glutaraldehyde for 4 h, washed with PBS three times, and dehydrated using a series of ethanol solutions (30%, 50%, 70%, 85% and 90% once, 100% twice) for 15 min each. Fixed bacteria were deposited on a piece of cover glass and coated with gold. SEM images were obtained using an accelerating voltage of 2 kV.

2.6. Biofilm susceptibility assay

The effect of ZXR-2 on the formation of *S. mutans* biofilm was evaluated by a microdilution method [21–23]. Briefly, two-fold serial dilutions of peptide with BHI (containing 2% sucrose, BHIS) were added to 96-well plates at a volume of 20 μL per well; the final concentrations of peptide ranged from 1 to 32 μM. Bacterial cell suspension (180 μL) was added to each well to a final concentration of 1 × 10^6 CFU/mL, while medium only was added to the blank control. After anaerobically incubated at 37 °C for 24 h, the culture broth was removed and planktonic bacteria were washed out with PBS. The biofilm was stained with 0.1% (w/v) crystal violet (CV) for 5–10 min. Excess dye was removed by rinsing the plate with water. After air-drying at room temperature, the CV dye associated with biofilms was extracted using 33% glacial acetic acid and quantified using a microplate reader by measuring absorbance at 600 nm. The inhibition rate was calculated using the following formula: 1−(A_{600} of the treated biofilm/A_{600} of the control).

The effects of ZXR-2 on 1-day-old biofilm were also determined. Samples (200 μL) of *S. mutans* cells (1 × 10^6 CFU/mL) were transferred to the wells of 96-well plates and then anaerobically incubated for 24 h at 37 °C in an anaerobic environment. Planktonic bacteria were discarded and the biofilm was washed once with PBS. Fresh ZXR-2 containing BHIS medium (final concentration, 0–128 μM) was added to the wells, and the plate was further anaerobically incubated at 37 °C for 24 h. Biofilm mass was also determined using the above CV staining method. All assays were performed in at least triplicate.