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Pleurocidin congeners demonstrate activity against Streptococcus and low toxicity on gingival fibroblasts

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ABSTRACT

Objectives: Fish epidermal antimicrobial peptides, such as pleurocidin, are cathelicidins with broad-spectrum antimicrobial activity against gram negative and gram-positive bacteria, as well as fungi. In the current study, we attempted to optimize peptide bioactivity by sequence modification and assess the antimicrobial activities.
Methods: Fifteen pleurocidin analogues were designed, and the efficacy of pleurocidin congeners against common cariogenic microorganisms was tested; furthermore, we performed a preliminary study of the antimicrobial mechanism. We assayed the minimal inhibitory concentration (MIC), minimal bactericide concentration (MBC) and bactericidal kinetics to determine the cell killing activity. Scanning electron microscopy (SEM) was used to observe the bacterial membrane after treatment with congeners' peptides. Human gingival fibroblasts (HGFs) were also used in toxicity studies.
Results: The MIC and MBC results indicated that peptide congeners had different antimicrobial activities against the tested oral strains. Toxicity studies indicated that several congener peptides had little effect on human gingival fibroblasts (HGFs) with 5 min of *in vitro* treatment.
Conclusion: Our findings suggested that several pleurocidin congeners had the antimicrobial effect against *Streptococcus mutans*, *Streptococcus sanguinis* and *Streptococcus sobrinus*.

1. Introduction

Dental caries is one of the most common infectious diseases in humans, and it is initiated by the formation of dental plaque biofilms. Although antibiotics are not routinely prescribed for dental caries, the aim of antimicrobial agents is to control rather than eliminate dental plaque (Marsh, 2010). Recently, there have been severe criticisms of the indiscriminate use of antibiotics with the development of bacterial resistance (Soares et al., 2012). For this reason, many antimicrobial peptides (AMPs) have been studied for preventing and treating dental caries (Lucchese et al., 2012). The manufacturing costs and poor pharmaceutical and pharmacokinetic properties were the largest obstacles for commercial and clinical applications. Several small molecules peptides were designed and could inhibit *S. mutans* biofilm formation (Liu, Worthington, Melander, & Wu, 2011; Wei, Campagna, & Bobek, 2006). However, although there have been many successful approaches to therapeutic applications (Sullivan et al., 2011), no AMP agent has yet received FDA approval.

To reduce the production costs, the following are two important considerations for commercial development: a shorter size and simpler amino acid composition of the AMPs (Won, Kang, Choi, & Lee, 2011). Then, for clinical development, bioactivity of any such peptide molecule can first be optimized *in vitro*. Towards this goal, our laboratory used pleurocidin, a natural linear cationic α-helical AMP, to develop short AMP variants with favorable bioactivity. Many reports have demonstrated that pleurocidin has antimicrobial activity against various pathogens (Cole, Weis, & Diamond, 1997; Lee & Lee, 2008; Mason, Chotimah, Bertani, & Bechinger, 2006), including cariogenic microorganisms and fungi (Lee et al., 2009) as well as induces significant morphological alterations in bacterial surfaces (Tao et al., 2011). Additionally, the N- and C-terminal regions of pleurocidin have antimicrobial and antifungal activity (Cho, Choi, & Lee, 2012; Lee & Lee, 2010).

Unlike other types of AMPs, such as disulfide-containing defensins and bacteriocins, α-helical peptides, which are small

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linear peptides, are relatively easy to modify and synthesize chemically. Therefore, their mode of action has been extensively studied. The amphipathic α-helical peptides generally adopt a structure that positions the hydrophobic residues on one side and the hydrophilic residues on the other side of the helical axis (Won, Jung, Kim, Seo, & Lee, 2004). Many studies have revealed that the activities of amphipathic α-helical antimicrobial peptides can be influenced by interrelated structural and physicochemical parameters, such as the size, residue arrangement, charge (cationicity), hydrophobicity and amphipathicity (Chou et al., 2008; Giangaspero, Sandri, & Tossi, 2001; Tossi, Sandri, & Giangaspero, 2000). These parameters are tightly related such that altering even one residue in the peptide can result have a significant difference. However, the data generated by different studies are sometimes conflicting, especially in terms of the antimicrobial activity. This is partly because different groups have performed the assays under different conditions.

In this report, our laboratory modified pleurocidin to create a family of 15 related peptides, and we determined their MICs and MBCs under standardized assay conditions. The killing kinetics and membrane change of pleurocidin congeners were also described. In consideration of the potential for clinical application in the oral cavity, the cell toxicity of selected pleurocidin congeners against HGFs was also evaluated.

2. Methods and materials

2.1. Peptide synthesis, purification and preparation

Using pleurocidin as the parent peptide, our laboratory constructed fifteen congener peptides (Table 1). All peptides were commercially synthesized by CL Bio-scientific (Xi'an, China) with purities of 90%, as previously described (Tao et al., 2011). The predicted properties for the peptides are presented in Table 1. Alpha-helix wheel projections for several peptides was showed in Fig. 1. The purity (90%) was considered when preparing the stock solution for all assays. The peptides were dissolved in sterile distilled water as stock solutions (5.12 mg/ml) and further diluted to final concentrations for individual experiments. Aliquots (0.1 ml) were stored at −20 °C.

2.2. Strains and growth conditions

The bacteria used in this study included American Type Culture Collection (ATCC) and laboratory strains of different oral bacteria. *Streptococcus mutans* UA159, *Streptococcus sanguinis* ATCC 10556, and *Streptococcus sobrinus* ATCC 6715 were grown in Brain Heart Infusion (BHI; Difco Laboratories, Detroit, MI) broth overnight at 37 °C prior to use. All strains were grown in an anaerobic incubator with 85% N_2, 10% H_2 and 5% CO_2.

2.3. Determination of antimicrobial activity

A serial two-fold dilution assay was used to determine the MICs and MBCs of all congeners based on methods that followed the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (Brogden, Nordholm, & Ackermann, 2007; Institute, 2010; Mai et al., 2011). Briefly, an aliquot of mid-log-phase cells (~10^5 CFU/ml) was inoculated into 200 μl/well of BHI containing 2-fold serial dilutions of a peptide in a 96-well plate. The final concentration of congeners ranged from 0.125 μg/ml to 512 μg/ml. After mixing, the cultures were incubated at 37 °C anaerobically for 20 h without shaking. Visual verification of microbial sedimentation as well as the absorbance readings (at 600 nm) confirmed the MICs. Afterwards, a 100 μl sample of solution was collected from the from the MIC test bacterial culture medium, serially diluted, and spread on BHI plates for an additional 16 h of incubation; afterwards, the bacterial numbers were counted. The MBCs were defined as the lowest concentration that did not allow for visible growth on plates. Each assay was performed in three independent experiments for all bacteria.

2.4. Congener peptides killing kinetics

The methods for evaluating the killing kinetics of pleurocidin congeners were similar to traditional time-kill experiments, as described previously (Eckert et al., 2006; He et al., 2010). *S. mutans* UA159 was chosen due to the major etiological agent of dental caries. Briefly, *S. mutans* UA159 were grown to log phase and diluted to 10^5 CFU/ml in BHI. Under anaerobic conditions, congeners were added as indicated to the bacteria with time-kill methodology (CLSI M26-A) at concentrations of 8 × MIC at 37 °C. Aliquots of 10 μl of cell suspension were then removed at various intervals (2, 5, 10, 20, 30, 60 and 120 min) and rescued by dilution into growth medium (1:50); they were then immediately put on ice to stop growth. Aliquots of 20–500 μl of rescued cells were spread on the BHI plates, and colony-forming units per milliliter were calculated after overnight incubation at 37 °C under anaerobic conditions. Each assay was repeated in three

Table 1
Amino acid sequence alignment of pleurocidin and its analogue peptides.

Name	Sequence	Hydrophobicity <H>	Hydrophobic moment <μH>	Net charge z
Ple	GWGSFFKKAAHVGKHVGKAALTHYL-NH_2	0.421	0.309	4
Pm1	GWGRFFKKWWRVGKRVGK	0.321	0.756	7
Pm2	GWGRFFKKAARVGKRVGK	0.106	0.646	7
Pm3	GWGKFFKKAAKVGKKVG	0.174	0.623	6
Pm4	GWGKFFKKFFKVGK	0.406	0.744	5
Pm5	GWKKFFKKAAKVGK	0.124	0.665	6
Pm6	GWKKFFKKAKKVGK	0.031	0.682	7
Pm7	GWKKFFKKAKKVWK	0.191	0.804	7
Pm8	GWGKFFKKFFKFGK	0.446	0.755	5
Pm9	GWKKFFKKWKKVWK	0.330	0.943	7
Pm10	WGKFFKKFFKFGK	0.481	0.812	5
Pm11	WFKFFKKFFKKWK	0.578	0.958	6
Pm12	WGKFFKKFFKKGK	0.267	0.777	6
Pm13	FKFFKKFFKKWK	0.438	0.862	6
Pm14	FKFFKKFFKKW	0.568	0.933	5
Pm15	KFFKKFFKKW	0.446	0.986	5