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Communication: Antimicrobial Activity of SMAP28 with a Targeting Domain for *Porphyromonas gingivalis*

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Abstract

Antibiotic therapy is often used with mechanical therapy to treat periodontal disease. However, complications associated with antibiotic use can occur. A 'bacteria-specific' targeted approach would eliminate some of these complications and kill specific periodontopathogens without harming the commensal bacteria. One such approach is to couple antimicrobial peptides to a ligand, pheromone, or antibody specific for the periodontopathogen, *Porphyromonas gingivalis*. To assess the feasibility of this approach, we attached PQGPPQ, a peptide from proline-rich protein 1 to either the N-terminus of SMAP28 (peptide ZS37-37) or the C-terminus of SMAP28 (peptide ZS37-38) to see whether it has potential as a carrier ligand to deliver SMAP28 to the surface of *P. gingivalis*. For *Escherichia coli* and *Aggregatibacter actinomycetemcomitans*, the median minimal inhibitory concentration (MIC) of ZS37-37 was higher than the median of SMAP28 alone, although the median MIC of ZS37-38 was lower than that of SMAP28 alone. For *P. gingivalis*, there was no difference in the median MIC values. For *S. aureus*, the median MIC was higher for ZS37-37 and ZS37-38 compared to SMAP28 alone, particularly for ZS37-38. For *Fusobacterium nucleatum*, the median MIC values were equal for ZS37-37 and ZS37-38 and higher than the median MIC for SMAP28 alone. Attaching PQGPPQ to SMAP28 did not greatly increase the antimicrobial activity of ZS37-37 or ZS37-38 for *P. gingivalis* nor substantially decrease the antimicrobial activity of ZS37-37 or ZS37-38 for the four other microorganisms tested. This is an initial step to develop a selective antimicrobial agent that has 'targeted' antimicrobial activity without adverse reactions often associated with the use of broad-spectrum antibiotics.

Keywords

Porphyromonas gingivalis; SMAP28; Periodontal disease; Targeted antimicrobial activity

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Introduction

Antibiotics are often used as an adjunct to mechanical therapy in the treatment of periodontal disease. Periodontal attachment levels post-therapy are much better in patients who have received systemically administered antibiotics than patients who have not. Occasionally, complications can occur [1]. In some individuals, β -lactam antibiotics can induce nausea, vomiting, abdominal discomfort, diarrhea, allergic skin rashes, and drug fever. Aminoglycosides can induce neurotoxicity, nephrotoxicity, and vestibular toxicity. Glycopeptides can induce infusion site phlebitis, and chloramphenicol can induce bone marrow toxicity. The use of some antibiotics also reduces the normal commensal microbiota and secondary *C. albicans* infections on mucosal surfaces or *Clostridium difficile* infections in the gastrointestinal tract develop. Therefore, a 'bacteria-specific' targeted approach is needed that (1) eliminates complications and side effects, (2) kills specific periodontopathogens in complex polymicrobial environments, and (3) does not kill or reduce the types and amounts of normal commensal bacteria.

Antimicrobial peptides represent an emerging class of antibiotics that rapidly lyse susceptible bacteria, do not induce bacterial resistance, are easy to synthesize in large quantities, work in synergy with other antimicrobial agents, and have very few side effects. Sheep myeloid antimicrobial peptide (SMAP) 28 and 29 are peptides widely studied for their broad-spectrum antimicrobial activity against a variety of bacteria [2,3], including periodontal pathogens [4]. SMAP29 is the peptide deduced from the nucleotide sequence and SMAP28 is the biologically active form with a C-terminal amide [3]. Alone, SMAP28 has broad-spectrum antimicrobial activity. However, when coupled to an outer surface-specific antibody, we found that a *P. gingivalis* IgG-SMAP28 conjugate had narrow-spectrum, 'targeted' antimicrobial activity against *P. gingivalis* in an artificially generated microbial community containing equal colony forming units of *P. gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Peptostreptococcus micros* [5].

In this study, we attached PQGPPQ, a peptide from proline-rich protein 1 to the N-terminus or C-terminus of SMAP28 to see whether it has potential as a targeting ligand to deliver SMAP28 to the surface of *P. gingivalis*. *P. gingivalis* fimbriae specifically bind proline-rich protein 1 through protein-protein interactions [6,7]. Enzymatically degraded proline-rich protein 1 (150 residues) contain binding domains to *P. gingivalis* fimbriae, particularly peptide 1–74, peptide 75–129, and peptide 130–150. Peptides 75–129 and 130–150 inhibit whole-cell binding of *P. gingivalis* to proline-rich protein 1-coated hydroxyapatite beads. Smaller fragments of these domains have activity. Peptide GRPQGPQ inhibits fimbrial binding to proline-rich protein 1-coated hydroxyapatite beads by 97%. Peptide PQGPPQ is as inhibitory as peptide GRPQGPQ and likely the minimal active segment for binding to *P. gingivalis* fimbriae [7].

Materials and Methods

Synthesis of SMAP28

SMAP28 (RGLRRLGRKIAHG VKKYGPTVLRIRIA-NH₂), ZS37-37 (PQGPPQRGLRRLGRKIAHG VKKYGPTVLRIRIA-NH₂), and ZS37-38 (RGLRRLGRKIAHG VKKYGPTVLRIRIAGPQGPPQ) were synthesized by Neo-MPS, Inc. (San Diego, CA) as previously described and suspended in 0.01% acetic acid [2]. The properties of these peptides are listed in Table 1. The net charges were obtained from The Antimicrobial Peptide Database (<http://aps.unmc.edu/AP/main.php>) [8,9]. The theoretical pI and the predicted monoisotopic mass were determined using the Expert Protein Analysis System (ExPASy; <http://ca.expasy.org>). Attaching PQGPPQ, a peptide from proline-rich protein 1, to

the N-terminus or the C-terminus of SMAP28 only decreased the charge of ZS37-38 by one and did not change the theoretical pI of ZS37-37 or ZS37-38.

Cultivation of Microorganisms

Laboratory strains of *Escherichia coli* and *Staphylococcus aureus* and oral strains of *P. gingivalis*, *Fusobacterium nucleatum*, and *A. actinomycetemcomitans* were used. *E. coli* (ATCC 12795) and *S. aureus* (ATCC 29213) were grown for 3 h in Mueller–Hinton broth at 37°C. The bacterial cell suspensions were adjusted to a density containing approximately 1×10^8 CFU/ml (0.108 OD, 600 nm, Spectronic 20D+, Thermo Fisher Scientific, Inc., Waltham, MA) and diluted with media to contain 10^5 CFU/ml. Oral strains of *P. gingivalis*, *F. nucleatum*, and *A. actinomycetemcomitans* do not grow as readily as *E. coli* and *S. aureus*. In order to use these microorganisms in the microdilution assay, the appropriate starting concentrations in the assay inoculum were determined from preliminary growth curve studies in the microtiter plates. *P. gingivalis* strain 381 (obtained from Ann Progulsk-Fox, Department of Oral Biology, University of Florida, Gainesville, FL) was grown in tryptic soy broth (Difco Laboratories, Detroit, MI) supplemented with 5 µg/ml hemin (Sigma, St. Louis, MO) and vitamin K (Sigma, St. Louis, MO) for 72 h at 37°C in an anaerobic chamber. The bacterial cell suspension was adjusted to 1×10^8 CFU/ml and diluted with pre-reduced media to 10^6 CFU/ml *P. gingivalis*. *F. nucleatum* (ATCC 25586) was grown in Schaedler's broth (Difco Laboratories, Detroit, MI) for 72 h at 37°C in an anaerobic chamber. The bacterial cell suspension was adjusted to 1×10^8 CFU/ml and diluted with pre-reduced media to 10^7 CFU/ml *F. nucleatum*. *A. actinomycetemcomitans* (ATCC 43719) was grown in trypticase soy broth (Difco Laboratories, Detroit, MI) supplemented with 0.6% yeast extract (Remel, Lenexa, KS) for 48 h at 37°C in a 5% CO₂ incubator. The bacterial cell suspension was adjusted to 1×10^8 CFU/ml and diluted with pre-reduced media to contain 10^5 CFU/ml *A. actinomycetemcomitans*.

Antimicrobial Assay

A broth microdilution assay was used to determine the minimal inhibitory concentration (MIC) of SMAP28, ZS37-37, and ZS37-38 (Table 1) for aerobic and anaerobic bacteria as previously described in our laboratory [2,5]. Neither the N-terminal and C-terminal peptides of SMAP28 nor the PQGPPQ peptide alone were examined. Briefly, peptides were diluted in 0.01% acetic acid (0.16–80.00 µg/ml) and added to microtiter plates (Immunolon 1 microtiter plates, Thomas Scientific, Swedesboro, NJ). Acetic acid (0.01%) was added to control wells. Cultures in their respective concentrations and media were added. Media without microorganisms was added to wells containing 0.01% acetic acid and used as the plate blanks. After incubation for 24 and 48 h at 37°C in the appropriate conditions for each micro-organism, the optical density of bacterial growth was determined (Spectromax Microplate Reader, Molecular Devices Corp., Sunnyvale, CA). The minimal inhibitory concentration (e.g., the lowest concentration of peptide that reduced growth by more than 50% of control wells) was determined. MICs were performed in triplicate, and the data were reported as the median of these values.

Statistical Analysis

A Kruskal–Wallis exact test was used to test the equality of the median MIC of three replicates defined by the addition of PQGPPQ to the N-terminus (ZS37-37) and C-terminus (ZS37-38) of SMAP28 and SMAP28 alone. The Kruskal–Wallis exact test was applied separately to MIC data from five bacteria: *E. coli*, *S. aureus*, *P. gingivalis*, *F. nucleatum*, and *A. actinomycetemcomitans*. For each bacterium, there were a total of nine observations, three in each group. Exact nonparametric procedures were selected for this analysis due to differences in variances among groups and modest sample sizes. All statistical analysis utilized SAS statistical software and the type I error was set at $\alpha = 0.05$.

Results

SMAP28 is a potent antimicrobial peptide and MICs for the five microorganisms ranged from 0.8 µg/ml for *F. nucleatum* to 6.4 µg/ml for *P. gingivalis*. The Kruskal–Wallis exact test showed significant group differences in the median MIC values for *E. coli* ($P = 0.0107$), *S. aureus* ($P = 0.0036$), *F. nucleatum* ($P = 0.0357$), and *A. actinomycetemcomitans* ($P = 0.0107$) (Table 2). Although statistically significant differences were observed, the MICs of SMAP28, ZS37-37, and ZS37-38 are in a narrow range for *E. coli*, *A. actinomycetemcomitans*, and *F. nucleatum*.

There were no such differences in the median MIC values for *P. gingivalis*. Adding the PQGPPQ peptide at the N-terminal or C-terminal end of SMAP28 induced variability, and there were no statistically significant differences among the MICs of SMAP28, ZS37-37 and ZS37-38.

The scope of the statistical analysis of these preliminary data was limited by number of replicates, three in each group per bacterium. This limitation prevented further exploration into specific pairwise comparison of treatment versus control differences. However, the patterns observed were as follows: for *E. coli* and *A. actinomycetemcomitans*, the median MIC of PQGPPQ added to the N-terminus of SMAP28 was higher than the median of SMAP28 alone, although the median MIC of PQGPPQ added to the C-terminus of SMAP28 was lower than that of SMAP28 alone. In the case of *S. aureus*, the median MICs of SMAP28 and ZS37-37 are almost equal, but much lower than that of ZS37-38. The median MIC values were equal for PQGPPQ added to both the N-terminus and C-terminus of SMAP28 and higher than the median MIC for SMAP28 alone for *F. nucleatum*.

Discussion

We are interested in developing a ‘bacteria-specific’ antimicrobial peptide that kills only one microorganism, like *P. gingivalis*, in a complex polymicrobial environment associated with supragingival and subgingival plaque. Such an approach would eliminate *P. gingivalis* without altering the types and amounts of normal commensal bacteria and would reduce complications and side effects often associated with the use of broad-spectrum antibiotics.

Recently, we used affinity-purified IgG antibodies as our carrier to deliver SMAP28 to the surface of *P. gingivalis* [5]. This delivery system worked well; the preparation specifically killed *P. gingivalis* in an artificially generated microbial community. However, the *P. gingivalis* IgG–SMAP28 conjugate was difficult to make. SMAP28 was synthesized; the maleimide linker was attached; IgG antibody was affinity-purified from the antiserum and concentrated; and the SMAP28-maleimide linker was attached to the affinity-purified *P. gingivalis* IgG. There were a few concerns. It was difficult to control the number of maleimide linkers attaching to SMAP28 (from 1 to 4), and it was difficult to control the number of SMAP28-maleimide molecules linked to the IgG. Therefore, we desired to seek another approach.

One concept is to engineer a synthetic peptide containing both a targeting domain and an antimicrobial domain. This concept has been used successfully by others. For example, Qiu et al. [10,11] increased the specificity of the channel-forming domain of colicin Ia by attaching staphylococcal or enterococcal pheromones. One peptide, prepared by fusing staphylococcal AgrD1 pheromone to colicin Ia, was bactericidal against methicillin-sensitive and methicillin-resistant *S. aureus*, but not against *Staphylococcus epidermidis* or *Streptococcus pneumoniae*. Peptide-treated mice survived a lethal challenge of methicillin-resistant *S. aureus*. A second peptide, prepared by fusing the enterococcal cCF10 pheromone to colicin Ia increased membrane permeability and was bactericidal against vancomycin-resistant

Enterococcus faecalis. Peptide-treated mice also survived a lethal challenge with vancomycin-resistant *E. faecalis*.

This approach has not been tried with *P. gingivalis*. Suitable ligands for receptors on *P. gingivalis* could include hemin fragments that target hemoglobin receptor HmuR; fragments of proline-rich protein 1 that targets fimbriae; *Streptococcus gordonii* SspB that targets a 100-KDa outer membrane protein on *P. gingivalis*; lectins to *P. gingivalis* capsular polysaccharides; lytic phage peptides; siderophores; and other ligands for bacterial surface receptors such as glycoproteins and lactoferrin. The use of some molecules as carriers is simply not feasible. Antibodies, although specific, could cross-react with other bacteria or agglutinate *P. gingivalis*. Hemin would be difficult and time-consuming to produce. Glycoproteins are complex molecules and could also be difficult or time-consuming to isolate or produce on a large scale. *S. gordonii* SspB is a large protein, which would have some potential as a carrier if a *P. gingivalis*-binding epitope could be identified.

Peptide GRPQGPPQ from proline-rich protein 1 inhibits fimbrial binding of *P. gingivalis* to proline-rich protein 1-coated hydroxyapatite beads by 97%. Peptide PQGPPQ is as inhibitory as peptide GRPQGPPQ and likely the minimal active segment for binding to *P. gingivalis* fimbriae [7]. Based on this work, we assessed PQGPPQ as a targeting ligand to deliver SMAP28 to the surface of *P. gingivalis*.

In summary, attaching PQGPPQ to SMAP28 did not greatly increase the antimicrobial activity of ZS37-37 or ZS37-38 for *P. gingivalis* nor substantially decrease the antimicrobial activity of ZS37-37 or ZS37-38 for the four other microorganisms tested. Additional work is needed to identify other targeting domains that have increased specific antimicrobial activity against *P. gingivalis* and decreased antimicrobial activity against other oral micro-organisms. Also, targeting SMAP28 via a carrier that recognizes a more conserved *P. gingivalis* molecule would be more promising. Due to the significant diversity of the fimbriin (*fimA*) gene, it is not certain whether ZS37-37 or ZS37-38 would target nonfimbriated strains of *P. gingivalis* or those strains of *P. gingivalis* found in dental biofilms.

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Table 1

Characteristics of SMAP28, ZS37-37, and ZS37-38. Peptide ZS37-37 has PQGPPQ at the N-terminus of SMAP28 and ZS37-38 has PQGPPQ at the C-terminus of SMAP28

Peptide	Number of amino acid residues	Predicted monoisotopic mass (M + H) ⁺	Charge (theoretical pI)
SMAP28 ^a	28	3197.01	+11 (12.31)
ZS37-37 ^b	34	3801.31	+11 (12.31)
ZS37-38 ^c	35	3858.33	+10 (12.31)

^aRGLRRLGRKIAHG VKKYGPTVLRIRIA-NH₂

^bPQGPPQRGLRRLGRKIAHG VKKYGPTVLRIRIA-NH₂

^cRGLRRLGRKIAHG VKKYGPTVLRIRIAGPQGPPQ

Table 2

Median minimal inhibitory concentrations of SMAP29, ZS37-37, and ZS37-38

Bacteria	Minimal inhibitory concentration (µg/ml)				Kruskal–Wallis exact <i>P</i> value
	SMAP28	(ZS37-37) PQGPPQ added to N-terminus	(ZS37-38) PQGPPQ added to C-terminus		
	Median	Median	Median		
Laboratory strains					
<i>E. coli</i>	3.1	3.4	2.6	0.0107*	
Oral strains					
<i>P. gingivalis</i>	4.8	10.8	16.4	0.7500*	
<i>F. nucleatum</i>	0.8	1.6	1.6	0.0357*	
<i>A. actinomycetemcomitans</i>	1.6	3.1	0.4	0.0107*	

Peptide ZS37-37 has PQGPPQ at the N-terminus of SMAP28, and ZS37-38 has PQGPPQ at the C-terminus of SMAP28. PQGPPQ is the minimal active segment of salivary acidic proline-rich protein 1 that binds to *P. gingivalis* fimbriae [7]

* Significance probability associated with the Kruskal–Wallis exact test of the null hypothesis. There is no difference between the group medians