Published in final edited form as:

Mol Oral Microbiol. 2018 December ; 33(6): 430–439. doi:10.1111/omi.12246.

Characterization and development of SAPP as a specific peptidic inhibitor that targets *Porphyromonas gingivalis*

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SUMMARY

Porphyromonas gingivalis is a keystone bacterium in the oral microbial communities that elicits a dysbiosis between the microbiota and the host. Therefore, inhibition of this organism in dental plaques has been one of strategies for preventing and treating chronic periodontitis. We previously identified a Streptococcal ArcA derived Anti-*P. gingivalis* Peptide (SAPP) that *in vitro*, is capable of repressing the expression of several virulence genes in the organism. This leads to a significant reduction in *P. gingivalis* virulence potential, including its ability to colonize on the surface of *Streptococcus gordonii*, to invade human oral epithelial cells, and to produce gingipains. In this study, we showed that SAPP had minimal cytotoxicity to human oral keratinocytes and gingival fibroblasts. We observed that SAPP directly bound to the cell surface of *P. gingivalis*, and that alterations in the sequence at the N-terminus of SAPP diminished its abilities to interact with *P. gingivalis* cells and repressed the expression of virulence genes. Most strikingly, we demonstrated using an *ex-vivo* assay that besides its inhibitory activity against *P. gingivalis* colonization, SAPP could also reduce the levels of several other oral Gram-negative bacteria strongly associated with periodontitis in multispecies biofilms. Our results provide a platform for the development of SAPP-targeted therapeutics against chronic periodontitis.

1. INTRODUCTION

Periodontitis is the 6th most common infection worldwide¹. An estimated 5–20% of the world's population suffers from generalized chronic periodontitis^{2,3}. Based on the National Health and Nutrition Examination Survey (NHANES), the disease affects 47% of adults aged 30 years and older in the United States with different severities^{4,5}.

The prevalence of periodontal infection apparently also increases with age, as 70.1% of adults aged 65 years and older have the disease, according to Centers for Disease Control

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We declare no conflict of interest.

and Prevention. Periodontitis is characterized by the destruction of the supporting tissues of the teeth, including the gingiva, periodontal ligament, and alveolar bone, which is caused by host inflammatory responses to pathogenic oral microbiota. Without treatment, severe periodontitis may lead to tooth loss and difficulties in chewing, which can severely impact the patient's quality of life.

Periodontal diseases and oral bacteria are associated with many systemic diseases and conditions, including coronary artery disease, rheumatoid arthritis, diabetes, pulmonary diseases, cancers of the oro-digestive tract, and Alzheimer's disease^{6–11}. Therefore, prevention and treatment of periodontitis is important for oral and overall health of adults. Chronic periodontitis results from a breakdown in the homeostasis between the periodontal tissue and the surrounding microbes, subsequently leading to uncontrolled inflammation and tissue destruction¹². The loss of tissue homeostasis is initiated by communities of microorganisms colonizing the subgingival area. Approximately 700 species of bacteria representing 13 phyla have been identified in the human oral cavity¹³. Studies using murine models have shown that *P. gingivalis*, a low abundance bacterium, can initiate periodontitis in the context of a multispecies microbial community¹⁴. Therefore, *P. gingivalis* has been suggested as a keystone pathogen that elevates the virulence of the entire microbial community^{14–17}.

P. gingivalis possesses a vast array of effector molecules and systems that enables its colonization and survival in the oral cavity, communication with other bacteria and, ultimately, virulence^{18–21}. Two distinct fimbriae composed of FimA and Mfa1, respectively, are the most well-documented virulence factors of *P. gingivalis*²². Fimbriae contribute to colonization, biofilm formation, cell invasion, bone resorption, and the evasion of the host defense systems^{19,23–32}. Another well-known virulence factor are the gingipains, which include two arginine- and one lysine-specific cysteine proteinases (RgpA, RgpB, and Kgp)³³. So far, all tested *P. gingivalis* strains produce gingipains that are either in membrane associated form or secreted in soluble form³⁴. Gingipains are multi-functional proteins involved in most stages of bacterial physiology and infection. Besides promoting tissue matrix destruction by proteolysis³⁵, gingipains also play an important role in biofilm formation via the C-terminal adhesive regions of RgpA and Kgp, or through profimbrillin processing^{36,37}.

We have previously reported an antagonistic relationship between *P. gingivalis* and *Streptococcus cristatus*. We found that *S. cristatus* arginine deiminase (ArcA) can repress FimA expression in several *P. gingivalis* strains, including strains expressing *fimA* types I, II, and III³⁸. Similar results were later observed by the Davey lab^{39,40}, in which they reported a downregulation of *P. gingivalis* genes encoding the major (*fimA*) and minor (*mfaI*) fimbriae by *Streptococcus intermedius* ArcA. Our data from a peptide array showed that an eleven amino acid peptide with the native sequence of streptococcal ArcA, which we designate streptococcal ArcA derived anti-*P. gingivalis* peptide (SAPP), not only repressed *fimA* and *mfaI* expression but also repressed *mfaI*, *rgpA/B*, and *kgp* expression in *P. gingivalis*⁴¹. Moreover, SAPP does not contain potential residues that electrostatically interact with L-arginine⁴², therefore, it is not likely that this peptide possesses arginine deaminase enzymatic activity.

In this study, we further exploited the effect of SAPP on the colonization of *P. gingivalis* clinical strains in native multi-species biofilms. SAPP structure and its stability in water and diluted human whole saliva were characterized. In addition, we assessed the physiological effects of SAPP in human oral epithelial cells and fibroblasts. Our data show that even at the concentrations much higher than that which reduces virulence gene expression by 50%, SAPP did not affect host cell viability after a 48 h exposure. By comparing levels of *P. gingivalis* in the multispecies microbial biofilms grown in the presence or absence of SAPP, we found that SAPP likely acts efficiently in complex microbial communities and could be exploited for complementary therapy against periodontitis.

2. METHODS

2.1 Bacterial growth conditions and cell lines

P. gingivalis 33277, W83 and mutant strains were cultured from frozen stocks in either Trypticase soy broth (TSB) or on TSB blood agar plates supplemented with yeast extract (1 mg/ml), hemin (5 µg/ml), and menadione (1 µg/ml), and incubated at 37°C in an anaerobic chamber (85% N₂, 10% H₂, 5% CO₂). *S. gordonii* DL1 was grown in Trypticase peptone broth (TPB) supplemented with 0.5% glucose at 37°C under aerobic conditions.

2.2 Peptide synthesis and activity

SAPP and its derivatives were synthesized by Biomatik (Wilmington, DE) and purified with high performance liquid chromatography (HPLC) to achieve 95% purity. The purified peptide was resuspended in nuclease/proteinase-free PBS, aliquoted, and stored at -20°C.

2.3 High-performance liquid chromatography (HPLC) analysis of SAPP

To determine the stability of SAPP in solution, we incubated SAPP (100 µM) in 100 µl H₂O, PBS, and whole saliva at 37°C for 24 h. SAPP in each sample was semi-quantified using LC-MS/MS. Briefly, samples were diluted (1:100) in 10 µl buffer A (0.1% formic acid (v/v) in water). The diluted samples (1 µl) were injected onto a C18 nano-column (0.1 mm x 10 cm, Sigma) and eluted for 30-min using buffer B (0.1% FA in acetonitrile) at a 5 – 20% gradient. MS spectra were acquired in a positive mode on Orbitrap LTQ XL (Thermo Scientific) as follows: 1) full MS in 200–1500 m/z interval, 2) single ion monitoring (SIM) in 331–332 m/z interval, 3) data dependent MS/MS scan fragmenting the most abundant peak from the preceding scan (i.e. 331 m/z parent ion: [M+4H]⁺).

2.4 Circular dichroism (CD) spectroscopy

CD spectra of SAPP (0.75 µM) were collected using a Jasco J-810 spectrometer outfitted with a Peltier temperature control module. The spectra were collected at 25 °C in filter-sterilized distilled water or tri-fluoroethanol using a 1 nm bandwidth at 1 nm intervals with each data point averaged for 1 sec. Data were analyzed using the Jasco spectral analysis program.

2.5 Cytotoxicity and apoptosis assay

Human oral keratinocytes (HOKs) and human periodontal ligament fibroblasts (HPLFs) were purchased from ScienCell Research Laboratories. HPLFs were cultured in fibroblast medium, and HOKs in oral keratinocyte medium using poly-L-lysine coated flasks at 37°C and 5% CO₂, according to manufacturer's instructions. HOKs and HPLFs were then seeded into a six-well plate at a density of 1×10^5 cells/well and cultured for 24 h. The cells were further incubated with growth medium supplemented with SAPP (0, 50, 200 or 800 µM) in a 24 or 48 h time course. The growth medium was then collected for a LDH cytotoxicity assay using a Pierce LDH cytotoxicity assay kit (Thermo Scientific) according to manufacturer's instructions. Absorbance was read at 480 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Rad). To measure the effect of SAPP on HOK and HPLF apoptosis, Apoptotic cells were stained with an Annexin V-FITC apoptosis kit (Invitrogen) and analyzed using a BD FACSCalibur™ flow cytometer and the CellQuest software (BD Biosciences).

2.6 Binding of SAPP to *P. gingivalis*

SAPP labelled at the N-terminus with FITC was used to determine specific interactions between SAPP and *P. gingivalis* cells. *P. gingivalis* strains were grown on glass-bottom microwell dishes (MatTek) for 48 h to create bacterial biofilms. After the removal of unbound bacteria, the biofilms were exposed to the FITC labeled SAPP or its derivatives (5 µM) dissolved in PBS. Free peptides were removed by three washes with PBS/Tween (0.01%). Binding of the SAPP to *P. gingivalis* was observed using a Nikon A1R confocal microscope and quantified by measuring fluorescence density in 10 random areas (24 × 24 mm) with NIS-Elements AR 4.20.

2.7 RNA isolation and qPCR

P. gingivalis were homogenized in Trizol (Invitrogen) and RNA was purified using an RNeasy mini spin column (Qiagen, Valencia, CA) as previously described⁴¹. Primers used are listed in Table S1. Reverse transcription was performed using a Bio-Rad iScript reverse transcription supermix on a TC-3000 thermal cycler (Techne) and a real-time RT-PCR analysis using a QuantiTect SYBR Green RT-PCR kit (Qiagen) on an iCycler MyiQ™ real-time PCR detection system (Bio-Rad) according to manufacturer's instructions. *P. gingivalis* 16S rRNA gene was used for normalization. The expression levels of the investigated genes were determined using the comparative cycle threshold (CT) method. The number of *P. gingivalis* cells in biofilms and released from the biofilms was determined by using qPCR. *P. gingivalis* cells were lysed with lysis solution (solution A; Invitrogen). DNA was extracted using an Easy-DNA kit (Invitrogen).

Bacterial cells in biofilms were counted using a QuantiTect SYBR green PCR kit with their specific 16S rRNA or 23S rRNA gene primers. Standards used to determine bacterial cell numbers were prepared using genomic DNA from bacteria. Purified bacterial DNAs from known bacterial numbers were isolated for a qPCR assay. Three trials were performed on three separate cultures.

2.8 Ex-vivo biofilm assay

The dental plaque sample collection protocol was reviewed and approved by the Meharry Medical College Institutional Review Board (IRB) (approval number 16–08-584). All clinical specimens were collected by clinicians in the Meharry Medical College dental clinic using paper points as previously described⁴³. Chronic periodontitis patients presented with radiographic periodontal bone loss of 4 mm at multiple teeth were selected. A total of 11 subjects, including 8 males and 3 females with a mean age of 47.8 years and an age range from 32 – 66 years, were enrolled.

Immediately after dental sample collection, paper points were placed in TSB (2 ml) to allow bacteria to grow first in an anaerobic chamber for 4 h. Bacteria from each sample were then subcultured in two wells of a six-well plate with and without SAPP (48 µM), and cultured for an additional 48 h to allow native mixed-species biofilms to form. Sessile bacteria were then collected and resuspended in TE buffer, and DNA was extracted using a DNeasy UltraClean Microbial Kit. Bacterial cells in biofilms were enumerated using a QuantiTect SYBR green PCR kit with their specific 16S rRNA or 23S rRNA gene primers. Standards used to determine bacterial cell numbers were prepared using genomic DNA from bacteria. Purified bacterial DNAs from known bacterial numbers were isolated for a qPCR assay. Three trials were performed on three separate cultures.

2.9 Statistical analyses

A Student's *t*-test was used to determine the statistical significance of differences in functions and gene expression of *P. gingivalis* strains grown in the presence or absence of SAPP. A $p < 0.05$ was considered significant.

3. RESULTS

3.1 Structure and stability of SAPP. Structure and stability of SAPP

Secondary structure prediction using Jpred4, Psipre and Jufo9D software suggests that SAPP is at the end of an alpha helix of ArcA and is followed first by a few random coil residues, and then by a beta sheet. The secondary structure of SAPP was analyzed experimentally by circular dichroism (CD) spectroscopy, which showed it exists as a random coil in H₂O (Figure 1). We also examined the effect of addition of the helix inducing agent tri-fluoroethanol (data not shown), which showed that SAPP does not have a significant helical propensity. To determine the stability of SAPP in solution, we incubated SAPP (100 µM) in H₂O or PBS for 24 h, and in diluted whole saliva (PBS:saliva, 1:1) at 37 °C for 24 or 48 h. We then compared the level of SAPP in each sample using LC-MS/MS analysis. As shown in Table 1, SAPP was relatively stable in H₂O and PBS after a 24 h incubation. On the other hand, we observed a gradual reduction of the SAPP signal in diluted whole saliva within a 24 h period, and the signal completely disappeared into the background after 48 h, presumably due to catalytic quantities of peptidases and/or proteases. LC-MS/MS is a semi-quantitative technique, so these results only provide insight into the relative levels of SAPP in these solutions. For example, although the signal is approximately 10-fold smaller, it is not possible to state with confidence that the concentration of SAPP is specifically one tenth

at 24 h. Nonetheless, the trend to loss over time is clear, and conversely, SAPP is found to have a significant life time in human saliva.

3.2 SAPP toxicity

To examine potential cytotoxic effects of SAPP, we examined the plasma membrane integrity of human oral keratinocytes (HOKs) and human periodontal ligament fibroblasts (HPLFs) that had been exposed to SAPP by measuring level of lactate dehydrogenase (LDH) released from damaged cells. As shown in Figure 2A, B, both cells exposed to SAPP at concentrations as high as 800 μ M (approximately 80-times greater than that required to repress expression of *fimA* and *rpg* by 50%, ID₅₀) for as long as 48 h did not exhibit cell membrane damage, when compared to cells without SAPP exposure. HOK and HPLF apoptosis induced by SAPP were also quantitated by propidium iodide (PI) and annexin V-FITC. The proportions of double stained cells including apoptotic and necrotic cells were not altered in HOKs and HPLFs after 24 or 48 h of SAPP treatment, indicating that SAPP has no impact upon cell viability of human oral cells (Figure 2C, D).

3.3 Specific interaction of SAPP and *P. gingivalis* cells

Our previous work demonstrated that *P. gingivalis* directly interacts with *S. cristatus* ArcA, which initiates bacterial cell-cell communication and represses *fimA* expression in *P. gingivalis*⁴¹. To further test if ArcA derived SAPP also binds to *P. gingivalis* cells, we generated fluorescently-labeled SAPP (P33) and its derivatives (P34 and P35), which have one or two residues substituted with alanine, (Table 2) and incubated them (5 μ M) with *P. gingivalis* 33277 attached on saliva coated surfaces. P33 was detected by a confocal microscopy on the *P. gingivalis* surfaces after a 15 min exposure, and a time dependent increase in P33 binding was observed during a 1 h exposure (Figure 3A, B). After a 1 h incubation, attachment of P33 to the *P. gingivalis* colonies had approximately increased 4.6 folds compared to that observed after 15 min incubation. The affinity of P34 and P35 for *P. gingivalis* cells was dramatically lower. We observed a more than 20-fold decreased fluorescence density on the *P. gingivalis* 33277 exposed to P34 and P35 for 1 h, compared to P33 (Figure 3B). Since P34 and P35 failed to bind efficiently to *P. gingivalis* cells, we speculated that they lost their ability to repress expression of virulence genes in *P. gingivalis*. As expected, P33 (16 μ M) repressed expression of *fimA* and *rpgA/B* genes by about 60%, which was consistent with our previous observations⁴¹. However, expression of *fimA* and *rpgA/B* was not regulated in *P. gingivalis* grown in the presence of P34 or P35 (Figure 3C). These data suggest that direct interaction of SAPP and *P. gingivalis* cells is critical for its inhibitory activity, and residues at the N-terminus of SAPP are involved in this interaction.

We further investigated the specificity of P33 binding. As shown in Figure 4, although biofilm formation of *P. gingivalis* W83 was much less than that found in *P. gingivalis* 33277, P33 bound to the cell surfaces of W83 as well, which was consistent with our previous observation on the role of SAPP in inhibiting virulence potential of W83⁴⁴. There is, however, no interaction detected between P33 and *Streptococcus gordonii* GB9. We previously showed using peptide array analysis that two *P. gingivalis* surface proteins, a major immunodominant antigen of *P. gingivalis* (RagB, PGN_0294) and a protein of the MotA/TolQ/ExbB proton channel family (PGN_0806), interacted with ArcA of *S.*

*cristatus*⁴¹. Our results from this study demonstrated that P33 failed to interact with two *P. gingivalis* mutants with deficiency in RagB or PGN_0806 (RagBE and 0806E) (Figure 4), providing further evidence for the involvement of these two surface proteins in the inhibitory activity of SAPP.

3.4 Ex-vivo biofilm inhibition by SAPP

To test if SAPP can function as an efficient inhibitor in complex multispecies microbial communities, we retrieved dental plaque samples, using paper points from 11 periodontitis patients, and suspended the microorganisms in *P. gingivalis* growth medium (TSB). The dental plaque sample from each patient was divided into two wells on a six-well plate coated with human whole saliva in the presence or absence of SAPP (48 μ M) for 48 h. Bacteria bound on well surfaces were collected, and their DNA was extracted. The number of sessile bacteria was determined using qPCR with specific *16S rRNA* or *23S rRNA* primers (Table S1). *P. gingivalis* was detected in all 11 samples. The amount of *P. gingivalis* detected in the multi-species biofilms in wells with SAPP ($1.2 \times 10^4 \pm 6.5 \times 10^3$) were significantly lower than those found in wells without SAPP ($6.5 \times 10^3 \pm 4 \times 10^3$, $p=0.036$) (Figure 5A). Out of 11 samples, 10 samples grown with SAPP exhibited more than 50% decrease in the number of sessile *P. gingivalis* cells compared to those grown without SAPP (Figure 5B). The inhibitory activity of SAPP against *P. gingivalis* binding in the multispecies biofilms ranged from 25–95%. To test if this inhibitory activity is strain-specific, qPCR was conducted using specific primers to determine the *fimA* genotypes (I, II, III, or IV) of *P. gingivalis* in each sample. All four types of *P. gingivalis fimA* were detected in the samples (Figure 5B), indicating that SAPP can inhibit colonization of *P. gingivalis* strains regardless of FimA expression.

Levels of several other oral bacteria in the biofilms were also determined using specific primers corresponding to their respective *16S rRNA* or *23S rRNA* sequences (Table S1). We observed a similar pattern of inhibition in response to SAPP treatment for *Tannerella forsythia* ($2 \times 10^4 \pm 5.9 \times 10^3$ vs $1.1 \times 10^4 \pm 3.8 \times 10^3$, $P=0.004$), *Treponema denticola* ($3.5 \times 10^2 \pm 82$ vs $2.1 \times 10^3 \pm 55$, $P=0.012$), and *Fusobacterium nucleatum* ($1.3 \times 10^5 \pm 4.5 \times 10^4$ vs $8.4 \times 10^4 \pm 3 \times 10^4$, $P=0.022$) (Figure 6A-C). *T. forsythia* and *F. nucleatum* were also detected in 11 samples, while *T. denticola* was only found in 7 samples. It should be noted that the level of *T. denticola* detected was much lower than that of *T. forsythia*. However, we did not detect any significant difference in the numbers of *Aggregatibacter actinomycetemcomitans* and *Streptococcus* in biofilms cultured in the presence or absence of SAPP (Figure 6D, E). These data suggest that SAPP can effect colonization of other periodontal pathogens in highly orchestrated oral microbial biofilms⁴⁵. Although the inhibition of colonization of specific bacteria associated with chronic periodontitis is not understood, *P. gingivalis*, a keystone bacterium, likely plays an important role in the composition of multispecies biofilms.

4. DISCUSSION

Current periodontitis treatment involves surgical- and nonsurgical mechanical therapies, which are usually effective in relieving local inflammation. Systemic or local administration

of antibiotics may be used to treat severe and refractory manifestations of the disease^{46–48}. These treatment strategies are often only temporarily effective and recurrence of the disease is common^{49,50}, possibly due to the re-emergence of pathogens such as *P. gingivalis*⁵¹. Our previous work demonstrated that SAPP represses expression of gingipains and fimbrial proteins in *P. gingivalis in vitro*. As a result, formation of single and dual species biofilms by *P. gingivalis* is greatly reduced^{41,44}. Moreover, SAPP also appears to inhibit gingipain activities and modulate innate immune responses induced by *P. gingivalis*. Therefore, the discovery of SAPP suggests an important avenue for development of novel anti-*P. gingivalis* agents.

Cytotoxicity and stability are critical factors when considering a potential drug candidate. Our data show that SAPP only acted on *P. gingivalis* cells but not on HOKs and HPLFs. Thus, host cell viability was not affected even at concentrations that were 80-times greater than that required to repress expression of *fimA* and *rpg* by 50%. A disadvantage of using peptides as oral topical therapeutic agents is that they are susceptible to salivary proteases and peptidases. One such example is a fungicidal peptide histatin 5. Histatin 5 is a 24 amino acid peptide, and it can be rapidly digested into as many as 19 fragments in diluted whole saliva (saliva:PBS, 1:10)⁵². We measured the level of SAPP in H₂O, PBS, and diluted whole saliva (saliva:PBS, 1:1) in a series of time points. SAPP is stable in H₂O and PBS for 24 h, although it was gradually degraded in the saliva, SAPP signal could still be detected during a 24 h incubation, suggesting that SAPP is relatively stable in saliva. In addition, we demonstrated that SAPP rapidly bound to *P. gingivalis in vitro*, and that most *P. gingivalis* colonies were interacting with SAPP after a 1 h exposure. This interaction may protect SAPP from degradation by salivary proteases and peptidases. Its low cytotoxicity to primary oral cells and its stability in human saliva make SAPP a strong candidate for therapeutic periodontitis treatment.

We previously reported a *P. gingivalis* prevalence of 65% in periodontitis patients as detected by PCR⁴³. Others have also reported that ‘red complex’ bacteria *i.e.* *P. gingivalis*, *T. forsythia*, and *T. denticola* were simultaneously detected in 57–75% of dental samples obtained from chronic periodontitis patients^{53,54}. In this study, we detected *P. gingivalis* and *T. forsythia* in all dental plaque samples. The higher detection rates may be due to amplification of these bacteria in the samples under anaerobic conditions for 48 h. It should be noted that there is a limitation to this *ex-vivo* assay, as it enriches certain bacterial species that can grow under conditions similar to *P. gingivalis*, such as *T. forsythia* and *F. nucleatum*. We detected *T. denticola* in only 7 samples, and the amount of *T. denticola* in biofilms was lower than other oral bacteria tested, which may due to limited growth of *T. denticola* under the experimental conditions. Nevertheless, the *ex-vivo* assay has an edge over *in vitro* assays, as the former tests the ability of clinical strains periodontal organisms to colonize in multispecies microbial communities.

As expected, SAPP treatment led to significant reduction in the level of *P. gingivalis* cells in microbial biofilms. Intriguingly, levels of *P. gingivalis*, *T. forsythia*, *T. denticola*, and *F. nucleatum* in the biofilms were also decreased after SAPP treatment. We postulate that SAPP may not have any direct impact on *T. forsythia*, *T. denticola*, and *F. nucleatum*; rather, the decrease in their levels in the biofilms likely results from the reduction in the level of *P.*

gingivalis in the biofilms. In fact, clinical studies conducted by several independent laboratories have uncovered a strong co-occurrence relationship among *P. gingivalis*, *T. forsythia*, *T. denticola*, and *F. nucleatum* in subgingival plaques^{53–55}. The close relationship may be a result of specific interactions between/among *P. gingivalis* and other oral bacteria associated with chronic periodontitis. A study by Hashimoto et al. revealed coaggregation between *P. gingivalis* and *T. denticola*, which is mediated by *P. gingivalis* fimbrial protein and *T. denticola* dentilisin⁵⁶. *T. denticola* is also known to interact with *T. forsythia* through recognition of the leucine-rich repeat protein LrrA of *T. denticola* by the leucine-rich repeat protein BspA of *T. forsythia*⁵⁷. Moreover, the co-aggregation of *P. gingivalis* and *F. nucleatum* is well documented, and there is evidence that the co-aggregation involves a galactoside of *P. gingivalis* and a lectin of *F. nucleatum*⁵⁸. As a keystone pathogen, *P. gingivalis* is expected to play a vital role in the community composition and to facilitate the complex interaction among various species of bacteria in the oral microbial communities. This notion is supported by a rabbit model of periodontal disease reported by Hasturk *et al.*⁵⁹. In this model, *P. gingivalis* was introduced to the rabbit oral microflora as an exogenous pathogen. The presence of *P. gingivalis* led to more anaerobic flora in the oral cavity of rabbits and to detectable *F. nucleatum*. Here, we provide additional evidence that *P. gingivalis* modulates the levels of important periodontal organisms. Therefore, the ability of SAPP to inhibit *P. gingivalis* colonization in multispecies microbial biofilms makes it a promising therapeutic agent against chronic periodontitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by NIH grants DE025332 (HX), DE012505 and DE023193 (RJL) from NIDCR, and GM118089 (WJC) from NIGMS. This work is also in part by Meharry RCMC and MeTrc grants MD007593, MD007586 from NIMHD, and the Vanderbilt CTSA grant UL1TR002243 from NCATS/NIH. The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

We thank Lauren E. Salay for help with the circular dichroism experiments, the Fluorescence Microscopy Morphology Core, the Proteomics Core, and the FACS Core at Meharry for technical support and data analysis, and the Meharry Office for Scientific Editing.

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Figure 1. Secondary structure analysis of SAPP by circular dichroism.

Far-UV CD spectrum of 0.75 μM SAPP acquired at room temperature in H_2O . The spectrum shows that SAPP is disordered in solution with no significant population of helical or β -sheet secondary structure.

Figure 2. Determination of SAPP-induced cell membrane damage and cell death in HOKs and HPLFs.

HOKs and HPLFs were treated SAPP (0, 50, 200, and 800 μM) for 24 or 48 h. Cytotoxicity was determined by measuring LDH levels in the culture supernatant of HOKs and HPLFs (A and B). SAPP-induced cell death was quantitated using an Annexin V-FITC apoptosis kit (C and D). Each bar represents the mean $\pm$ standard deviation of LDH levels or numbers of apoptotic and necrotic cells. Data are the mean of at least three independent experiments.

Figure 3. Binding of fluorescence labeled SAPP to *P. gingivalis*.

(A) *P. gingivalis* 33277 biofilms were formed on saliva-coated glass and exposed to FITC-labeled SAPP (P33, 5 μ M) for 0, 15, 30, and 60 min or its FITC-labeled derivatives (P34 and P35, 5 μ M) for 1 h. Bacterial associated peptides were visualized using the confocal microscope. (B) Each bar represents average fluorescence intensity in 10 random areas (24×24 mm). Asterisks indicate significant difference in fluorescence intensities detected for samples exposed to the peptides, compared to that detected in the sample without exposure. (C) Each bar represents the relative expression level of *fimA* or *rgpA/B* in *P. gingivalis* 33277 grown with FITC-labeled SAPP (P33, 16 μ M) or its derivatives (P34 and P35, 16 μ M), which was compared to that in 33277 grown without SAPP (1 unit). Means and standard deviations were from three independent experiments. Asterisks indicate statistically significance between expression levels of genes in *P. gingivalis* 33277 grown with and without SAPP (control) ($P < 0.05$; *t* test).

Figure 4. Affinity of SAPP to bacterial surfaces.

The binding of SAPP to bacterial strains was assessed using confocal microscopy. FITC-labeled SAPP (P33, 5 μ M) was incubated with *P. gingivalis* strain W83, *S. gordonii* G9B, or two *P. gingivalis* mutants (0806E and RagBE) for 1 h.

Figure 5. Attachment of *P. gingivalis* in multispecies biofilms.

(A) Multispecies biofilms were created using dental plaque samples retrieved from 11 chronic periodontitis patients, with sample from each patient divided into two cultures and incubated in the presence or absence of SAPP (48 μM). The level of *P. gingivalis* in the biofilms was determined using qPCR with *P. gingivalis* 16S rRNA primers. Mean and standard error of *P. gingivalis* cells detected in the biofilms are presented, and an asterisk indicates a significant difference between the cultures incubated with or without SAPP ($p < 0.05$; *t* test). (B) Comparison of *P. gingivalis* levels detected in the biofilms in the presence and absence of SAPP in each patient sample. Level of *P. gingivalis* in the biofilm without SAPP exposure is considered as 100%. The *fimA* genotype detected in each patient sample is indicated.

Figure 6. Determination of other oral bacterial levels in the biofilms generated using an *ex-vivo* assay.

Levels of *T. forsythia* (A), *T. denticola* (B), *F. nucleatum* (C), *A. actinomycetemcomitans* (D), and *S. gordonii* (E) in the biofilms were determined using qPCR with the corresponding specific *16S rRNA* or *23S rRNA* primers. Asterisks indicate statistical difference between two cultures of each sample grown with or without SAPP ($P < 0.05$; *t*-test).

Table 1Determination of SAPP level in solutions ^a

SAPP treated with	Treating time (hour)	Signal intensity of 331.45 m/z peak
H ₂ O	0	$4.8 \times 10^6 \pm 4.5 \times 10^5$
H ₂ O	24	$2.1 \times 10^6 \pm 1.3 \times 10^5$
PBS	24	$4.6 \times 10^6 \pm 4.2 \times 10^5$
Saliva	0	$3.9 \times 10^6 \pm 3.1 \times 10^5$
Saliva	0.5	$3.6 \times 10^6 \pm 4.0 \times 10^5$
Saliva	1	$2.4 \times 10^6 \pm 2.2 \times 10^5$
Saliva	2	$1.7 \times 10^6 \pm 4.2 \times 10^5$
Saliva	4	$8.8 \times 10^5 \pm 2.7 \times 10^4$
Saliva	24	$4.2 \times 10^5 \pm 3.9 \times 10^4$
Saliva	48	$6.0 \times 10^3 \pm 4.1 \times 10^2$

^aSAPP level was determined using a LC-MS/MS analysis. Values represent the means $\pm$ the standard deviations of three independent experiments.

Table 2

Peptide characteristics

Peptide	Sequence	Purity (%)	MW	Charge (pH 7)	pI
SAPP ⁴¹	NIFKKNVGFKK	98.57	1322.63	4	11.28
P33	FITC-NIFKKNVGFKK	96.37	1697.00	3	11.10
P34	FITC-NIAKKNVGFKK ^a	98.93	1620.90	3	11.10
P35	FITC-NIAAKNVGFKK ^b	97.10	1563.81	2	10.80

^a the third amino acid (F) at the N-terminus of SAPP is substituted with alanine (bold).

^b the third and fourth amino acids (F and K) at the N-terminus of SAPP are substituted with two alanine (bold).