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Targeted Killing of *Streptococcus mutans* in Biofilms by a Pheromone Guided Antimicrobial Peptide HP30

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Abstract

Background: *Streptococcus mutans* is a leading cariogenic pathogen of dental caries worldwide. Clinically, eliminating *S. mutans* from dental biofilms using antibiotics is not practical, because these agents indiscriminately kill other members of the resident flora, leading to ecological disruption and other negative clinical consequences. To develop target-specific antimicrobials, we evaluated several fusion peptides and identified a new peptide HP30 that showed a high selectivity for targeted killing of *S. mutans*.

Methods: The selective killing activity of HP30 was evaluated against *S. mutans* and other related oral species in both mono- and dual-species biofilm cultures.

Result: In the dual-species cultures, 80% of *S. mutans* cells were killed, but only 20% of *S. sanguinis* were killed following exposure to HP30 (5.0 μM) for 15 min. Similarly, 80% of *S. mutans* cells were killed but only 5% of *Actinomyces naeuslundii* were killed following the same exposure. The peptide-guided killing was also confirmed in the dual-species biofilms and the killing increased with increasing concentrations of HP30. However, a combination of low concentrations of HP30 with EDTA well maintained the killing activity against *S. mutans* in the biofilms. A *S. mutans* mutant lacking the ComD receptor only showed 20% of killing, while a ComD overexpression strain showed 90% of killing, suggesting that HP30 predominantly binds to the ComD receptor before triggering the selective killing.

Conclusion: New peptide HP30 displays a high selectivity for targeted killing of *S. mutans* due to an improved binding of the peptide to the ComD receptor.

Keywords: Targeted killing; Pheromone-guided antimicrobial peptide; Quorum sensing, Biofilm; *Streptococcus mutans*

Introduction

Streptococcus mutans is a Gram-positive bacterium that lives in dental biofilms consisting of diverse species of microorganisms [1]. Under appropriate environmental conditions, *S. mutans* can rapidly produce acids from fermentable dietary carbohydrates and lower the biofilm pH, initiating demineralization of the tooth surface or tooth decay [2]. *S. mutans* is therefore considered as a primary etiological agent of dental caries worldwide [3]. *S. mutans* can also be a cause of subacute infective endocarditis [4]. Although dental caries is not a life-threatening disease, diagnosing, treating and retreating caries and caries-related diseases is still a major financial burden to public health system. Clinically, eliminating *S. mutans* from dental biofilms using wide-spectrum antimicrobial agents is not practical, because these agents indiscriminately kill the resident microbes, leading to ecological disruption or other clinical negative consequences, such as allergy and development of antibiotic resistance [2,3]. Therefore, prevention of *S. mutans* and its associated diseases requires the development of new strategies that specifically target *S. mutans* without significant disruption of other resident members in dental biofilms.

In recent years, several attempts have been made to achieve target-specific antimicrobial therapy by coupling antibiotics to species-specific monoclonal antibodies or by creating fusion peptides that combine killing domains with bacterial recognition domains [5-8]. These narrow-spectrum antimicrobials can selectively target specific pathogens without significant effect on the other members of the resident microflora. Among these antimicrobials, pheromone-guided antimicrobial peptides (PG-AMPs) have been found to show the greater selectivity for targeted killing of bacteria. It is well known that quorum-sensing signal pheromones can specifically bind to the same bacterial species that produce them and function at nano-molar concentrations for coordinated

activities [9,10]. The rationale of creating such antimicrobial peptides is based on the addition of a targeting domain of a quorum-sensing signal pheromone from a target pathogen to the killing domain of a known antimicrobial peptide [11,12]. Both domains are fused via a short linker to generate a new hybrid peptide without detrimental change of their activities. Design of such peptides requires identification of two functionally independent molecules, a killing domain of a known AMP and a targeting domain of a species-specific, high-affinity binding molecule, such as quorum sensing signal pheromone [11,12]. The major advantage of such PG-AMPs is that the targeting domain can guide the fusion peptide to bind selectively to the target pathogen, leading to selective killing. Such pheromone-guided antimicrobial peptides have been successfully developed against various bacterial pathogens, including MRSA *S. aureus*, vancomycin- resistant *E. faecalis, Pseudomonas* spp and cariogenic pathogen *S. mutans* [6-8,11,12].

We previously constructed a peptide library and identified a salt-resistant peptide, IMB-2, which selectively killed *S. mutans* [12]. However, this peptide also showed a low level of non-specific killing in mixed cultures. To improve the selectivity, we screened and evaluated several more peptides and identified a new peptide HP30 that shows significant improvement in selective killing of *S. mutans*. We also explored the potential mechanism of such improved selectivity in the targeted killing by analyzing the ComD protein, the receptor of the quorum sensing signal pheromone of *S. mutans*. In addition, we found that a combination of a low concentration of HP30 with EDTA significantly enhanced selective killing of *S. mutans* cells in mixed biofilms. Here, we report the results of our evaluation of this peptide against *S. mutans* and other related oral species.

Methods and Materials

Bacterial strains, media and growth conditions

Bacterial strains used in this study were *S. mutans* UA159 (wt) and its derived strains, a *comD* deletion mutant (ΔcomD), a ComD overexpression strain (ComD-Ox) and a His-tagged *comD* positive control strain (ComD+). Five *S. mutant* strains were also included to determine MIC and MBC (Table 1). Three oral species, *S. sanguinis* SK108, *S. mitis* SK142 and *Actinomyces naeslundii* WVU627 were used to develop dual-species cultures. All the strains were grown on blood agar plates or in Brain-Heart Infusion (BHI) broth at 37 °C anaerobically, unless otherwise specified.

Species or Strain	MIC (μM)	MBC (μM)
S. mutans UA159	2.5	5.0
S. mutans GS5	2.5	5.0
S. mutans NG8	2.5	5.0
S. mutans BM71	2.5	5.0
S. mutans KK23	2.5	5.0
S. mutans R221	2.5	5.0
S. sanguinis SK108	10	20
S. mitis SK142	10	20
A. naeuslundii WVU627	20	80

Table 1: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of HP30 against Oral Bacterial Strains

Peptide synthesis

HP30 (SGSLSTFFRLFNRGGGWGHFFKKAAHVGKLNH$_2$) was chemically synthesized and C-terminally amidated to reduce its end charge and increase the stability [12]. The peptide was purified (purity > 90%) by reversed-phase high-pressure liquid chromatography and its molecular mass was confirmed by mass spectrometry (BioBasic Inc. Ontario). The peptide was dissolved in sterile distilled water as a stock solution and further diluted to final concentrations as required in experiments.

MIC and MBC tests

Minimum inhibitory concentration (MIC) of HP30 was determined against bacterial strains by a broth micro-dilution method. Briefly, 10 μl of mid-log phase cells (≈10^6 CFU/ml) was inoculated into 200 μl/well of BHI containing two-fold serial dilutions of the peptide in a 96-well microtiter plate. Wells with erythromycin (10 μg/ml) were used as positive controls, while wells without addition of HP30 or erythromycin were used as negative controls. The cultures were incubated anaerobically at 37 °C for 20 hours (for streptococcal strains) and for 40 hours (for *Actinomyces naeslundii* WVU627). The optical density of the cultures was read at 590 nm using a microtiter plate reader (Synergy HT, Biotek, USA). Aliquots (20 μl) of samples were further taken from these cultures to determine MBC by viable cell count as described previously [12].

Selective killing assay

To determine selective killing activity of the peptide, we performed time-killing experiments against *S. mutans* strains, *S. sanguinis* SK108, *S. mitis* SK142 and *A. nauslundii* WVU627 in single- and dual-species cultures. Briefly, the mid-log phase cultures of all the

strains were adjusted to the same cell density. The killing was initiated by adding aliquots of cell suspension ($\approx 10^6$ CFU/ml) into the medium containing HP30 in a 96-well microtiter plate. A one-to-one ratio of two species was used in dual-species cultures. Aliquots (20 µl) of samples were taken at 5 and 15 min for viable cell counts by spreading cells on BHI agar plates (single-species) or on blood agar plates (dual-species). The plates were incubated anaerobically at 37 °C for 20 or 40 hours respectively before evaluation of the results. S. mutans was distinguished from other species by examining their colony morphology on blood agar plates. Percent survivals were determined by calculating the number of survivors versus total CFU/ml. The targeted killing of S. mutans was also examined under a fluorescence microscope (Leica AF7000) using a Live/Dead BacLight bacterial viability kit (Invitrogen).

Killing activity against biofilms

The killing activity of HP30 against S. mutans biofilms was examined by a microtiter plate method [12]. Biofilms were initiated by inoculating 10 µl of the mid-log phase cells ($\approx 10^5$ CFU/ml) into 200 µl of 2 X diluted BHI broth supplemented with 10 mM glucose in a 96-well microtiter plate. The cultures were incubated anaerobically at 37 °C for 24 hours. Planktonic cells were removed and added carefully with the same volume of fresh medium containing HP30 for 5 and 15 min. Biofilm cells were washed off from the surfaces by pipetting using 10 mM phosphate buffer. The cell suspensions were serially diluted and spread on agar plates to determine percent survival.

Dual-species biofilms were also developed to assay selective killing of the peptide against biofilms consisting of S. mutans UA159 co-cultured with S. sanguis SK108 or A. naeslundii WVU627. Dual-species biofilms were initiated by inoculating 1:1 ratio of two species in 2X diluted BHI broth plus 10 mM glucose and 0.1% hog gastric mucin (Sigma). After 24-hour incubation, the biofilms were treated by the same ways as above. Biofilm samples were taken at 5 and 15 min, serially diluted and spread on both BHI and blood agar plates to determine survival percentages of individual species.

Detection of the ComD protein by Western blotting

Three S. mutans strains, which were previously constructed for Western analysis of the ComD protein of S. mutans [13], were used to monitor the effects of existence of ComD protein on selective killing of HP30. These strains included a His-tagged comD control strain that expressed a wild type ComD (ComD+), a comD deletion mutant ΔcomD (ComD-) and a ComD-Ox that over-expressed a ComD protein. All the strains were grown in BHI medium with use of competence-stimulating peptide or CSP (1 µM). When the cultures reached mid-log phase ($OD_{600} \approx 0.5$), aliquots of the samples were taken to prepare the crude cell lysates. The samples of the membrane fractions from individual strains were resolved on SDS-PAGE gels and transferred to polyvinylidenedifluoride (PVDF) membrane for Western blot analysis using an anti-His-tag antibody [13]. The membranes were detected with an AP (alkaline phosphatase) detection reagent (Novagen). The protein images on the membranes were examined and photographed using FluorChem SP image system (Alpha Innotech, Calif. USA).

Assay for the stability of HP30 in saliva

Unstimulated whole saliva was collected from six healthy volunteers by following a protocol of the Dalhousie Health Sciences Research Ethics Board. The saliva samples were centrifuged at 5,000 x g at 4 °C to remove food debris. The supernatants were passed through 0.2 µm filters, pooled and stored at -20 °C. Killing activity of HP30 was assayed in the presence of saliva with or without 2 mM EDTA. Viable cell counts were performed to determine percent survivals. The stability of HP30 in saliva was determined by incubating aliquots (15 µg) of HP30 in 100 µl of saliva with or without 2 mM EDTA at 37 °C. Aliquots (15 µl) of sample were taken at 0, 5, 10 and 15 min, boiled and analyzed with 15% SDS-PAGE gels (BioRad). A peptide sample without saliva was used as a control. The gels were stained with Coomassie blue and the peptide bands were scanned by a densitometry for calculation of % degradation compared to 0-min sample.

Statistical analysis

All killing assays were performed in triplicate in two independent experiments. The results were analyzed by Student's t test and $P \leq 0.05$ was considered statistically significant.

Results

HP30 shows an enhanced selectivity for targeted killing of S. mutans cells

We first examined MIC and MBC of HP30 against S. mutans strains and several related oral species in dental biofilms. The results confirmed that HP30 displayed a potent bactericidal activity against S. mutans cells with a MIC of as low as 2.5 µM. There was little or no variation in MIC among S. mutans strains (Table 1). We then examined the killing kinetics of HP30 (MBC 5.0 µM) against S. mutans UA159 and other oral species, S. sanguinis SK108, S. mitis SK142 and A. naeslundii WVU627, grown in monocultures. The results showed that 82% of S. mutans cells were killed following exposure to HP30 (5.0 µM) for 15 min (Figure 1). However, only 20% of S. sanguinis, 18% of S. mitis and 5% of A. naeslundii were killed following the same exposure to HP30. The results clearly demonstrate that HP30 is highly effective to trigger selective killing of S. mutans cells, but less likely kills other oral species.
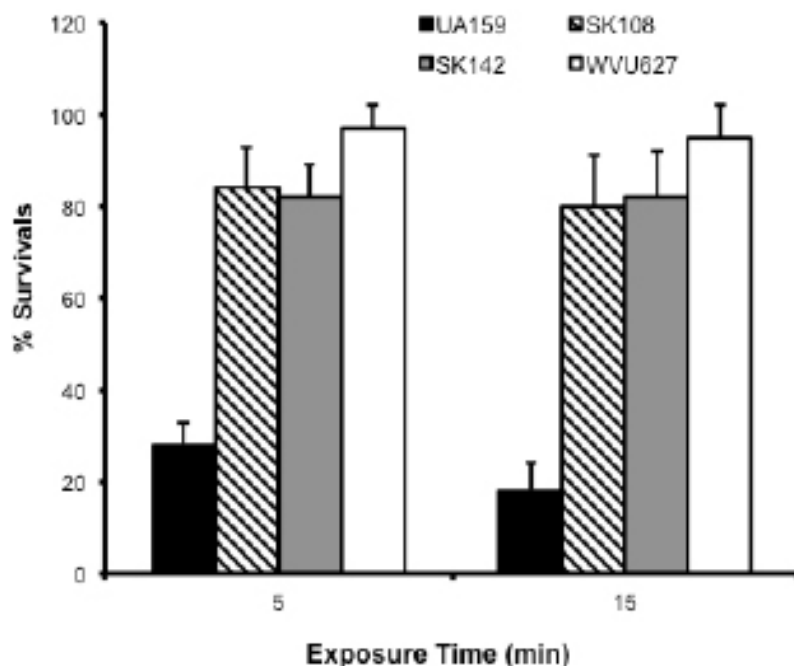
Figure 1: Killing activities of HP30 (5.0 μM) against *S. mutans* UA159 (UA159), *S. sanguinis* SK108 (SK108), *S. mitis* (SK142) and *A. naeuslundii* WVU627 (WVU627) in single-species planktonic cultures. *S. mutans* could be distinguished from *S. sanguinis* and *S. mitis* by colony morphology and haemolysis on blood agar plates.

To further confirm the selectivity of this peptide, we examined the killing activity of HP30 (5.0 μM) against *S. mutans* cells in dual-species cultures, which contained a 1:1 ratio of mid-log phase cells of *S. mutans* UA159 with either *S. sanguinis* SK108 or *A.naeslundii* WVU627. In the mixed culture of *S. mutans* and *S. sanguinis*, nearly 80% of *S. mutans* cells were killed following exposure to HP30 for 15 min, but less than 20% of *S. sanguinis* cells were killed following the same exposure (Figure 2A). Similarly, HP30 killed 82% of *S. mutans* cells but killed less than 5% of *A. naeslundii* cells in dual-species culture following the same exposure (Figure 2B). The results confirm that HP30 displays a high selectivity to kill *S. mutans* cells in the dual-species mixed cultures.
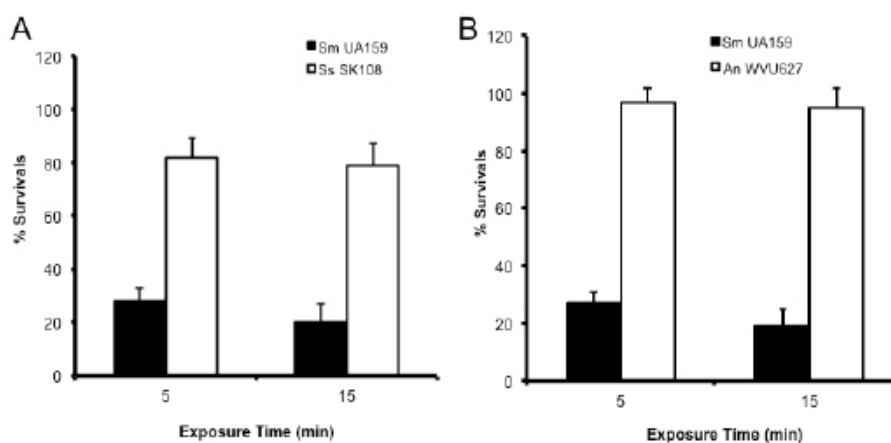

Figure 2: Selective killing activity of HP30 (5 μM) against *S. mutans* in dual-species cultures consisting of *S. mutans* UA159 with either *S. sanguinis* SK108 (**A**) or with *A. naeuslundii* WVU627 (**B**).

HP30 is also effective to kill S. mutans cells in the dual-species biofilms

Next, we examined selective killing of HP30 against *S. mutans* in dual-species biofilms, which consisted of *S. mutans* UA159 with either *S. sanguinis* SK108 or *A. naeslundii* WVU627. The dual-species biofilms (one-day old) grown on 96-well microtiter plates were treated with an increasing concentration of HP30. The results showed that nearly 70% of *S. mutans* cells in the biofilms were killed following exposure to HP30 (5.0 μM) for 15 min. In contrast, only 8% of *S. sanguinis* or 2% of *A. naeslundii* in the biofilms were killed (P < 0.01) following the same exposure (Figure 3). However, the targeted killing of *S. mutans* cells in the dual-species biofilms increased with increasing concentrations of HP30, suggesting that HP30 was highly effective to target *S. mutans* cells in the dual-species biofilms. To increase the efficacy of the selective killing with use of a low concentration of HP30, we combined 5 μM of HP30 with 2 mM of EDTA to treat the biofilms. The results revealed that 15-min treatment of HP30 together with EDTA killed nearly 100% of *S. mutans* cells in the dual-species biofilms (Figure 4), while the same treatment did not increase killing of

other species in the dual-species biofilms. Notably, 2 mM of EDTA alone caused little effect on the viability of *S. mutans* and other species tested (P < 0.001). The result suggests that a combination of low concentrations of HP30 and EDTA showed an additive targeted killing of *S. mutans* grown in the biofilms.
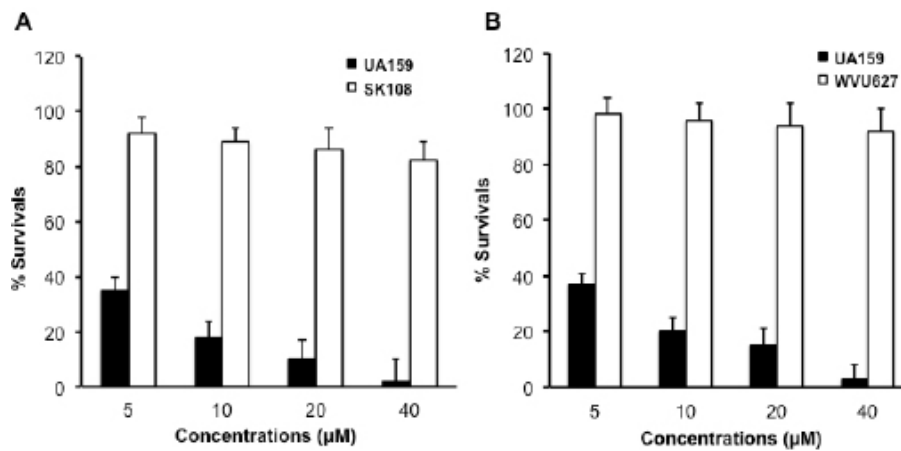

Figure 3: Selective killing activity of HP30 against *S. mutans* in dual-species biofilms consisting of *S. mutans* UA159 with either *S. sanguinis* SK108 **(A)** or with *A. naeuslundii* WVU627 **(B).**
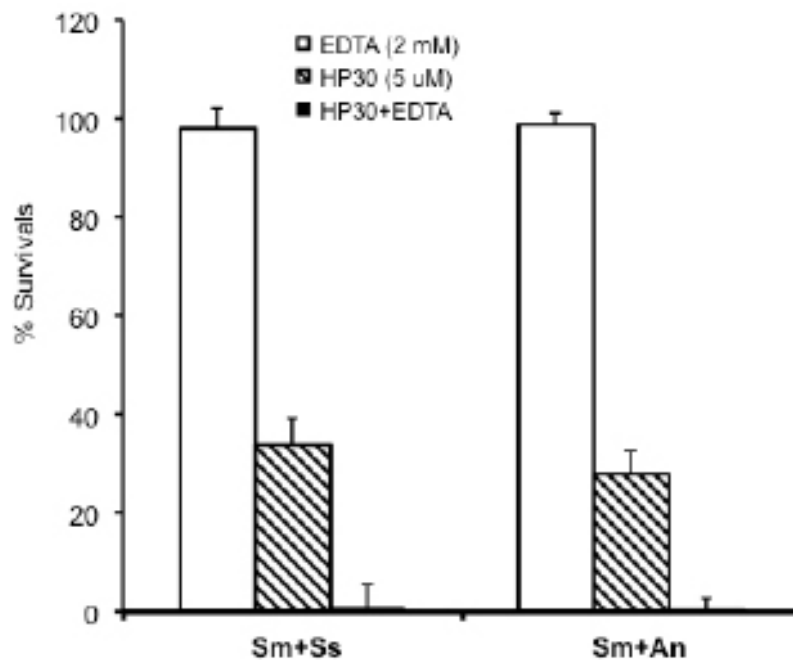

Figure 4: Killing activity of HP30 (5 μM) combined with EDTA (2 mM) against *S. mutans* cells in dual-species biofilms consisting of *S. mutans* UA159 (Sm) with either *S. sanguinis* SK108 (Ss) or with *A.naeuslundii* WVU627 (An). Only survival percentages of *S. mutans* are plotted, but the percentages of other species (Ss or An) are not included.

HP30 predominantly binds to the ComD receptor protein to trigger selective killing

Our results clearly demonstrate that HP30 displays a high selectivity for targeted killing of *S. mutans* cells in both mono- and dual-species mixed cultures. However, the question whether or not HP30 needs to bind to the *ComD* receptor protein before it triggers selective killing remained unclear. To explore this question, we used three previously constructed *S. mutans* strains [13], including a *comD* deletion mutant Δ*comD* (ComD⁻), a strain that over-expresses *ComD* (ComD-Ox) and a positive control strain (ComD+), to examine the effects of *comD* expression on selective killing of *S. mutans*. By Western blot analysis, we first confirmed the existence of a 51-kDa protein in the membrane fractions of both ComD-Ox and positive control strains (Figure 5A), but not in the membrane fraction of Δ*comD* mutant (ComD⁻). It was noted that the ComD-Ox strain showed a slightly higher level (≈ 2.5-fold, estimated by a densitometry) of *ComD* protein than the control strain (ComD+). The results suggest that these three strains were suitable to examine their response to targeted killing by HP30. The results from killing assays showed that compared to the parent strain UA159 (80% of killing), nearly 95% of the ComD-Ox cells were killed following exposure to 5.0 μM of HP30 for 15 min. In contrast, only 10% of Δ*comD* mutant cells were killed (P < 0.01) following the same exposure. The results suggest that binding to

ComD receptor is crucial for HP30 to trigger selective killing of *S. mutans*. We further examined selective killing of HP30 against these strains by Live/Dead *BacLight* bacterial viability assay using a fluorescence microscopy (Figure 5B-a, b, c). The results confirmed that HP30 resulted in the greater killing of *S. mutans* cells that expressed or over-expressed the *ComD* receptor (a and b).
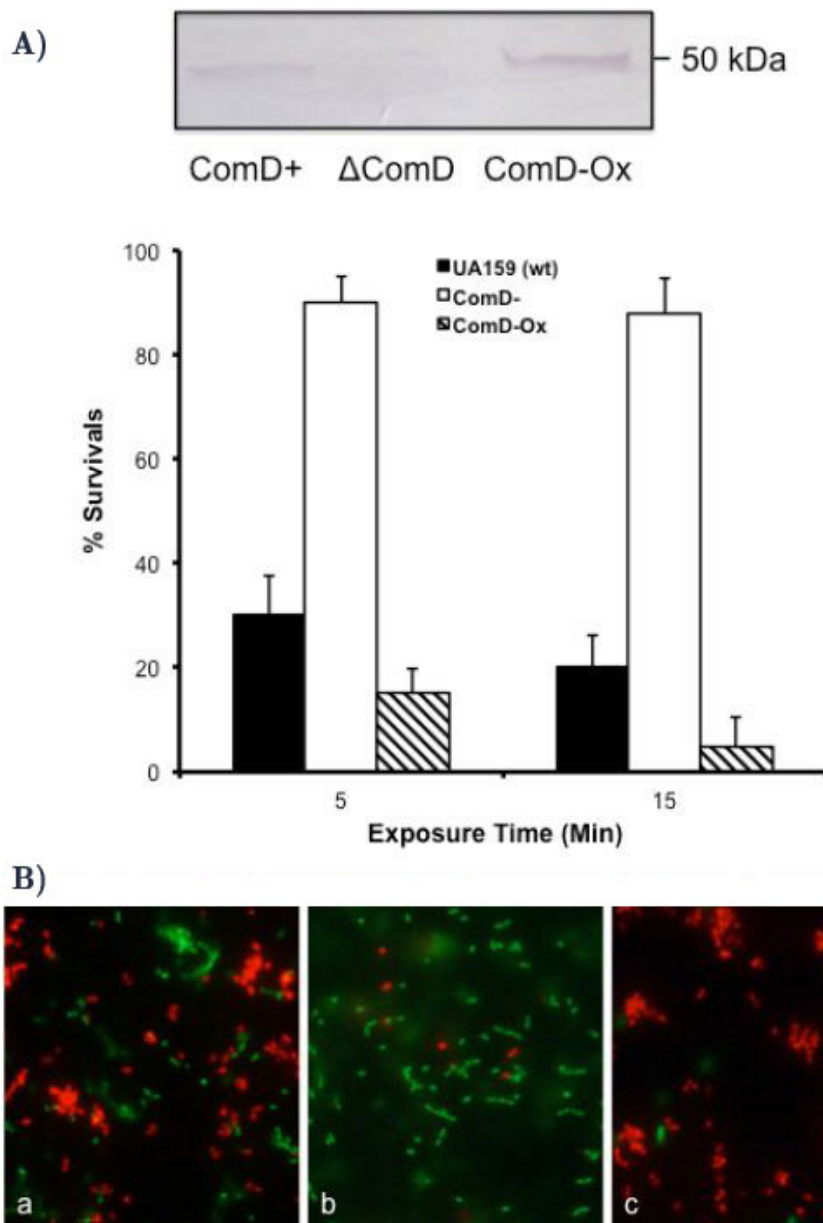

Figure 5: Killing activities of HP30 against *S. mutans* strains, including a positive control strain (ComD+), a comD deletion mutant (Δ*comD*) and a *ComD* overexpression strain (ComD-Ox). **A-up.** Western blotting confirmation of the His-tagged *ComD* protein in strains ComD+, Δ*ComD* and ComD-Ox. **A-down.** Killing activities of HP30 against *S. mutans* UA159 (wt), Δ*comD* (ComD−) and ComD-Ox. **B.** Fluorescence microscopy of targeted killing of *S. mutans* biofilm cells following exposure to HP30 (5.0 μM) for 15 minutes. The cells are labelled with Live/Dead BacLight bacterial viability dye (viable cells are green and dead cells are red). **a.** *S. mutans* UA159 (wt), **b.** Δ*comD* (ComD−) strain and **c.** ComD-Ox strain.

The stability of HP30 in the presence of human saliva

We then examined the killing activity of HP30 against *S. mutans* cells in the presence of human saliva. The results showed that in the presence of saliva only 50% of *S. mutans* cells were killed following exposure to HP30 for 15 min (Figure 6A). Compared to THYE medium only, this indicated a significant decrease ($P < 0.05$) in the killing activity, suggesting that the presence of saliva decreases the killing activity of HP30. However, adding 2 mM of EDTA into the saliva did not only prevent saliva-induced decrease of the killing activity but also enhanced the killing activity of HP30 ($P < 0.01$). The results suggest that a low concentration of EDTA appears to stabilize and enhance the killing activity of HP30 in the presence of saliva. To further confirm this observation, we examined the stability of HP30 in the presence of saliva by incubating an aliquot of the peptide (15 μg) in saliva at 37 ºC followed by SDS-PAGE analysis of the peptide profile. The results showed that exposure of the peptide to saliva without EDTA resulted in progressive degradation during 15-min incubation (Figure 6B), lanes 3-6). However, adding 2.0 mM EDTA into the saliva

significantly reduced the degradation of HP30 (lane 7-10), which was consistent with the results of the killing activity observed.
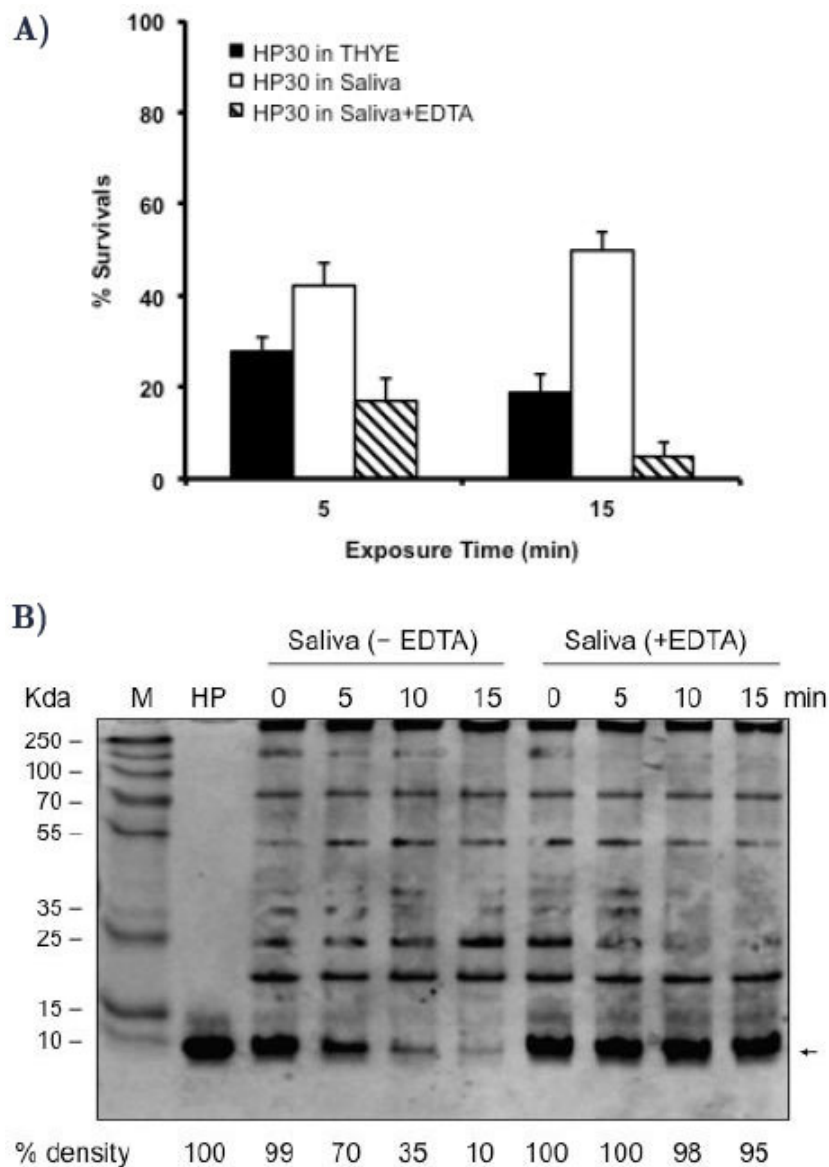

Figure 6: The killing activity and stability of HP30 (5 μM) in the presence of human saliva. **A.** The survival percentages of *S. mutans* cells in saliva exposed to HP30 with or without addition of EDTA (2 mM). **B.** SDS-PAGE analysis of the peptide profiles in the presence of saliva with or without addition of 2 mM EDTA. The peptide bands were scanned and quantitated by a densitometry to calculate % density. Lane 1: a low molecular weight marker; Lane 2: HP30 only (≈ 4.5 KDa) as a blank control; Lanes 3-6: HP30 exposed to saliva without 2 mM EDTA (− EDTA) for 0, 5, 10 and 15 min; Lanes 7-10: HP30 exposed to saliva with EDTA (+ EDTA) for 0, 5, 10 and 15 min. Arrow indicates HP30.

Discussion

In this study, we investigated selective killing activity of a new PG-AMP, HP30, against a leading cariogenic pathogen, *S. mutans*, and other species, in both mono- and dual-species cultures. Our goal was to identify and validate a target-specific antimicrobial agent that selectively kills *S. mutans* cells with minimal disruption of other species in a biofilm community. The results demonstrate that HP30 shows an improved selectivity to target *S. mutans* cells for killing in both planktonic and biofilm cultures, although its killing activity in monocultures is nearly the same as a previously reported PG-AMP, IMB-2 [12]. The results from this study suggest that the improved selectivity in targeted killing likely results from an improved binding of HP30 to the *ComD* receptor protein of *S. mutans*. This suggestion is strongly supported by several lines of evidence. First, the enhanced killing activity of HP30 against *S. mutans* is clearly observed in both mono-and dual-species cultures, suggesting that the killing activity is species-specific. Second, a deletion of *comD* that encodes the CSP receptor results in significant reduction (nearly 80%) in the killing activity of S. mutans. In contrast, the over-expression of the *comD* significantly increases its killing activity (up to 90%), suggesting that the existence of an intact ComD receptor protein is the key requirement for the selective killing of *S. mutans*. This also suggests that HP30 predominantly binds to the ComD receptor protein before it triggers the selective killing. Third, HP30 was designed in such a way that its target domain contains five extra residues, SGSLS, at its N-terminal arm, which makes the N-terminus of

HP30 identical to that of CSP (Table 2). Our previous study by structure-activity analysis of *S. mutans* CSP showed that the core hydrophobic α-helix of CSP along with its N-terminus was essential for peptide binding to the *ComD* receptor [12]. Thus, adding the *N*-terminal five original residues to the fusion peptide would increase the binding of the fusion peptide to the ComD receptor. It is therefore reasonable to assume that HP30 may bind more effectively to the *ComD* receptor than IMB-2 or IMB2 (H/S14), because it retains the intact N-terminus and core hydrophobic α-helix that promote the binding to the *ComD* receptor. We also speculate that HP30 triggers the killing of *S. mutans* cells immediately after it binds to the *ComD* receptor protein. This speculation is based on the fact that HP30 shows a highly selective killing activity against *S. mutans* cells, even in the mixed-species biofilms.

Name	Fusion Peptide (targeting domain – linker – killing domain)	MIC(μM)
CSP-18*	SGSLSTFFRLFNRSFTQA	NA
CSP-21*	SGSLSTFFRLFNRSFTQAñLGK	NA
IMB-2	TFFRLFNR –GG– GWGSFFKKAAHVGKL•NH2	2.8
IMB2-(H/S14)	TFFRLFNR –GG– GWGHFFKKAAHVGKL•NH2	2.8
HP30	SGSLSTFFRLFNR –GG– GWGHFFKKAAHVGKL•NH2	2.5
C16G2	TFFRLFNRSFTQAñLGK –GGG– KNLRHRKGIHIIKKY	5.2

*: CSP-18: competence-stimulating peptide from *S. mutans* after C-terminal cleavage (ñ) by SepM; CSP-21: CSP before the C-terminal cleavage (ñ); Underline represents the core hydrophobic α-helical structure (minimal targeting sequence of CSP); H/S14 (**H**): His-to-Ser14 substitution. NH_2: the C-terminal amidation (to increases the stability). MIC: minimum inhibitory concentration. NA: not applicable.
Table 2: The Peptide Sequences from Known PG-AMPs Specific to *S. mutans*

Despite the high efficacy in targeted killing of *S. mutans*, the exact mechanism how HP30 triggers the killing of *S. mutans* remains unclear. The evidence from this study suggests that, as the originally designed, HP30 likely binds to the *ComD* receptor by its targeting domain (the *N*-terminal arm) before it rapidly triggers the killing by its killing domain (the *C*-terminal arm). However, Kaplan *et al.* report a similar PG-AMP, C16G2, which contains a portion (the core hydrophobic α-helix and the *C*-terminus) of *S. mutans* CSP (Table 2) [14]. These researchers have found that C16G2 also kills *S. mutans* cells in a species-specific manner, but the killing by C16G2 is independent on the *ComD* receptor. Their work suggests that C16G2 may involve the membrane partition steps that enable C16G2 to bind and retain on the *S. mutans* surface. Such retention of C16G2 on the cell surface then results in loss of membrane potential ($\Delta\Psi$) of *S. mutans* cells, triggering the cell death at a level of potency similar to a peptide antibiotic melittin B [14]. This suggests that the *ComD* receptor, which is supposed to be the binding site for the peptide, plays little or no role in targeting of C16G2 to the surface of *S. mutans* cells. This divergence was not very clear until the recent discovery that *S. mutans* can produce an extracellular protease SepM that is required for the cleavage of *C*-terminal three residues, LGK, of CSP-21 to generate a functional CSP-18 [14] (Table 2). Since C16G2 contains an intact *C*-terminus of CSP-21, including LGK, it suggests that the *C*-terminal killing domain of C16G2 could be cleaved off from the cleavage site by SepM, resulting in release of two independent peptide moieties, the targeting domain and killing domain [15]. In contract, the targeting domain of HP30 or IMB-2 does not have the *C*-terminal three residues, LGK, this implies that the *C*-terminal killing domain of HP30 and IMB-2 would not be subject to the cleavage by SepM. Therefore, the discovery of protease SepM may explain the divergence in the action mechanisms of these known fusion peptides, C16G2 and HP30. Thus, the action mechanism of C16G2 observed by Kaplan *et al.* can be interpreted as the action mode by the killing domain alone, which is truly consistent with their observation [14]. Nevertheless, this does not exclude the possibility that peptides HP30 and IMB-2 may also involve a low level of non-specific binding to the surface of *S. mutans*, since it kills 5-10% of the ComD deletion mutant cells.

It is well known that biofilms in nature inherently resist treatment of antimicrobial agents by a number of mechanisms, including drug diffusion limitation in biofilms [16]. This often requires increasing concentrations of the antimicrobial agents to achieve expected results, but the higher doses of an antimicrobial also increase negative or side effects of the drug [17]. To circumvent this problem, some strategies have been proposed to improve killing activity of an antimicrobial agent to treat biofilm infections without use of too much drugs. Among these strategies, EDTA (ethylenediaminetetraacetic acid), a well-known chelating agent, is frequently reported to show anti-biofilm activity due to its chelation of divalent cations required for stabilizing biofilm matrix [18,19]. EDTA is found to increase sensitivity of bacteria to killing by antibiotics, showing synergic effect on biofilms. For example, EDTA combined with minocycline is effective against biofilms formed by *Staphylococcus epidermidis, S. aureus* and *Pseudomonas aeruginosa* [20]. At a higher concentration, EDTA can directly result in bacterial viability loss [19]. In our study, we also confirm that a low concentration (2 mM) of EDTA combined with HP30 (5 μM) does not only increase the stability of the peptide in the presence of saliva but also enhances selective killing of *S. mutans* cells grown in biofilms. Our work highly recommends such an application by combining a low concentration of EDTA with a selected antimicrobial agent, such as HP30, to deal with a biofilm disease caused by a pathogen living along with the resident microflora.

In conclusion, we have evaluated a novel PG-AMP HP30 that shows an improved selectivity to kills *S. mutans*. The evidence suggests that the improved selectivity in the targeted killing results from an improved binding of HP30 to the *ComD* receptor of *S.*

mutans. Our work also confirms that a combination of a low concentration of EDTA with HP30 significantly enhances the killing activity of HP30 against *S. mutans* in biofilms. Such a combination makes HP30 more effectively target *S. mutans* and extend its killing activity in the presence of saliva. Because of its improved selectivity and effectiveness, HP30 shows good promise for future development as a target-specific antimicrobial agent against *S. mutans* in dental biofilms.

Acknowledgment

Conflict of Interest

The authors declare that they have no competing financial interest.

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