Targeted Antimicrobial Treatment to Re-establish a Healthy Microbial Flora for Long-term Protection

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ABSTRACT

Streptococcus mutans has been implicated as the major acid-producing (cariogenic) bacterium. Dietary sugars and other factors may cause an imbalance of oral microflora that enables *S. mutans* to become dominant in the multi-species biofilms on the tooth surface, which could lead to dental caries. The application of broad-spectrum antimicrobials often results in re-colonization and re-dominance of *S. mutans* within oral flora, while in contrast, therapies capable of selective elimination of *S. mutans* from oral microbial communities may help to re-establish the normal flora and provide long-term protection. C16G2, a novel synthetic antimicrobial peptide with specificity for *S. mutans*, was found to have robust killing efficacy and selectivity for *S. mutans in vitro*. A subsequent pilot human study found that a single application of C16G2 in the oral cavity (formulated in a mouthrinse vehicle) was associated with a reduction in plaque and salivary *S. mutans*, lactic acid production, and enamel demineralization during the entire 4-day testing period. C16G2 is now being developed as a new anticaries drug.

INTRODUCTION

This is a short review with a specific focus on C16G2, a targeted antimicrobial peptide, which was recently accepted by the US FDA as an Investigational New Drug (IND) to be tested for its safety and clinical efficacy against dental caries. The article will briefly review the rationales behind this new investigative drug, as well as the *in vitro* and *in vivo* data that support its possible efficacy.

CURRENT APPROACHES FOR TREATING DENTAL CARIES AND THEIR POSSIBLE LIMITATIONS

Dental caries is a chronic disease of microbiological origin, and *S. mutans* has been implicated as one of the major etiological pathogens responsible for the majority of caries (Loesche, 1986; Tanzer *et al.*, 2001). Caries arises from dietary sugars (primarily sucrose) that cause acidogenic microbes, such as *S. mutans,* to produce acids that damage tooth structure. This also leads to an imbalance in the oral microflora that enables these cariogenic bacteria to become dominant in the multi-species biofilms found on the tooth surface.

Currently, the surgical approach (drilling and filling) is still the main tool to treat dental caries, while the only chemical intervention approved by the FDA for fighting caries involve the use of fluoride-containing varnishes and toothpastes, which are capable of preventing enamel demineralization and reversing the process through remineralization (Stookey *et al.,* 1993; Donly, 2003). Fluoride has been successful in reducing the incidence and prevalence of dental caries and should remain an important part of any anti-caries regimen, but it has limited efficacy in killing cariogenic bacteria residing in dental plaque, even though there have been reports of its inhibitory effects on bacterial metabolism (Hamilton, 1990). This could be a factor contributing to the persistence of dental caries within populations, despite fluoride's well-documented clinical efficacy (Beighton, 2005; Anderson and Shi, 2006; Milgrom *et al*., 2009).

Controlling caries by reducing the total bacterial load in saliva and plaque through the use of broad-spectrum antibacterial agents can, in theory, reduce caries incidence; however, broad killing of the bacteria alone allows for equal competition between cariogenic bacteria and non-pathogenic organisms to re-establish the biofilms. Consequently, there is no strong clinical evidence supporting the long-term prevention of re-infection of cariogenic bacteria and very few studies examining the impact on caries reduction (Milgrom *et al*., 2009; Vollmer *et al.,* 2010; Young *et al.,* 2010; Papas *et al.*, 2012). The reason for the lack of long-term protection could be tied to the persistence of cariogenic bacteria within the dental plaque and the dynamic balance of the biofilm community between a healthy state and cariogenic state. Individual behaviors with respect to oral hygiene and diet play a major role in determining whether dental plaque remains healthy or cariogenic. If an individual has poor oral hygiene and a diet high in refined sugars, the re-established biofilm will retain a community where cariogenic conditions persist and may dominate.

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Key Words

microbial ecology, microbiology, microbial genetics, caries, dental biofilm, microbiota.

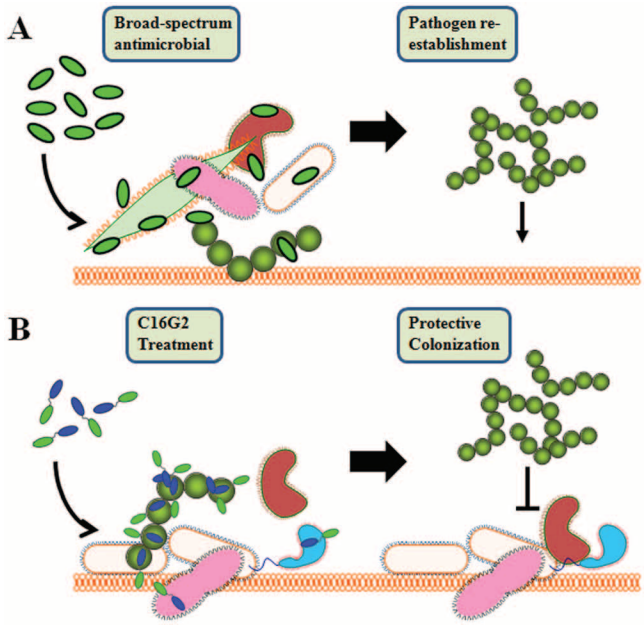

Figure 1. Illustration of targeted antimicrobial treatment for long-term protection. **(A)** Broad-spectrum antimicrobial therapy results in killing of pathogenic and normal flora bacteria. The mucosal surface can be re-colonized by pathogens. **(B)** Targeted STAMP (C16G2) treatment results in selective pathogen removal and protective colonization associated with the remaining and established normal flora.

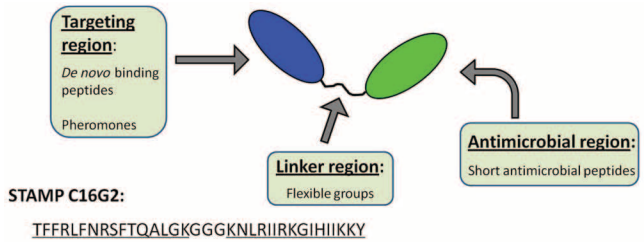

Figure 2. Illustration of the basic structure for the specifically targeted antimicrobial peptides (STAMPs). Adapted from Eckert (2011).

THE RATIONALE BEHIND THE SPECIFICALLY TARGETED ANTIMICROBIAL PEPTIDES

It is known that patients with "healthy" dental plaque displaying low levels of *S. mutans* are resistant to exogenous colonization from cariogenic pathogens and have shown long-term protection from dental caries (Keene and Shklair, 1974; Anderson and Shi, 2006; He *et al*., 2009; Marsh, 2010). Lack of *S. mutans* has been associated with sites of healthy dentition, while progressively increasing levels of S. *mutans* are associated with sites of caries development and cavitation (Becker *et al*., 2002).

Based on these findings, it was suggested that targeted antimicrobial therapies against *S. mutans* may be a good alternative approach to treat dental caries (Eckert *et al.*, 2006). As illustrated in Fig. 1, with the exception of a limited number of oral pathogens (*S. mutans* being a major example), most of the micro-organisms within the indigenous oral microflora are benign or beneficial. The application of broad-spectrum antimicrobials often results in the re-colonization and re-dominance of cariogenic bacteria within the oral flora (Fig. 1A). In contrast, therapies capable of selective elimination of cariogenic bacteria from the oral flora may help to re-establish normal flora to provide long-term protection (Fig. 1B).

To achieve the selective elimination of a particular bacterial species from a multi-species microbial community, a novel technology called Specifically Targeted Antimicrobial Peptides (STAMPs) was developed. As illustrated in Fig. 2, a "STAMP" is a fusion peptide with 2 main domains: a killing domain, made of a non-specific antimicrobial peptide; and a targeting domain, containing a species-specific targeting peptide. The targeting domain provides specific binding to a selected pathogen and facilitates the targeted delivery of an attached antimicrobial peptide.

C16G2 is a STAMP designed with antimicrobial specificity for *S. mutans* with 2 peptide sequences for specific functional domains (Fig. 2). The first is a *S. mutans*-selective 'targeting domain' derived from a fragment of the *S. mutans* competence stimulating peptide (CSP), designated as C16 (or CSP_{C16}), and comprised of amino acids 1 through 16. The second is a "killing domain" derived from a broad-spectrum antimicrobial peptide, designated as G2, and comprised of amino acids 20 through 35. These 2 peptide sequences are conjoined by a sequence of 3 glycine residues comprised of amino acids 17 through 19, which allows both domains to function properly.

THE ROBUST *IN VITRO* KILLING ACTIVITY AND SELECTIVITY OF C16G2 FOR *S. MUTANS*

In the original publication describing C16G2 (Eckert *et al.,* 2006), the group discovered that C16G2 was potent against *S. mutans* grown in a planktonic or biofilm state and did not affect other oral streptococci tested. C16G2 has rapid bactericidal activity against *S. mutans*, killing bacteria within seconds of contact, but is slower against untargeted bacteria, where several minutes to hours are required to reach similar levels of killing (Kaplan *et al*., 2011). Multi-species biofilms from which *S. mutans* has been eliminated by C16G2 resist re-colonization by *S. mutans*, demonstrating the protective colonization effect *in vitro* (Li *et al.,* 2010). Mechanistic studies indicate that C16G2 is bactericidal *via* a cytoplasmic membrane disruption mechanism. Accumulation of C16G2 on the cell surface of *S. mutans* leads to loss of membrane potential, leakage of intracellular adenosine triphosphate (ATP), and loss of membrane integrity, followed by cell death (Kaplan *et al.,* 2011).

THE CLINICAL EFFICACY OF C16G2 IN A PILOT STUDY IN HUMANS

A pilot study was conducted in humans to evaluate the efficacy of C16G2 *in vivo* (Sullivan *et al.,* 2011). The objectives of the study were to assess the effects of a single application of C16G2 on *S. mutans* in saliva and dental plaque, on plaque pH, on lactic acid production after a sucrose challenge, and mineral loss from tooth enamel.

Table. Statistically Significant Improvement *vs.* Placebo in *S. mutans* Levels, Plaque pH, Acid Production, and Enamel Demineralization after C16G2 Rinse

Treatment	Δ *S. mutans* CFU from Baseline, Plaque (x 10^6)	Δ *S. mutans* CFU from Baseline, Saliva (x 10^6)	Resting pH, Plaque	Lactate Concentration (nM/mg plaque)	% Demineralization
Placebo	5.02	1.50	6.68	8.81	23.0
C16G2-treated	–0.906	–0.5	7.22	6.3	–3.73

Study participants arrived at the dental clinic on Day 0 (baseline) without having performed morning oral hygiene, and samples of fasting plaque and whole saliva were collected for analysis of *S. mutans* and plaque pH. Participants then rinsed with a 10% sucrose solution for 2 min, and 8 min later a second plaque sample was collected for pH analysis. Mouth retainers containing 4 ground and polished blocks of bovine enamel were put into the mouth of each participant. Participants then rinsed for 40 sec with either placebo or 8 mg C16G2. They were allowed to leave the clinic and were instructed to wear the retainer at all times, except to dip the mouth retainer in 10% sucrose at 4 different times throughout the day before placing it back into the mouth. Participants returned to the clinic daily on Days 1-4. Plaque and saliva samples were taken before and after a 10% sucrose rinse, and a block of the bovine enamel was also removed and assessed for demineralization on Day 4.

Twelve healthy individuals were enrolled in the study. As summarized in the Table, the results showed that 8 mg C16G2 was able to selectively eliminate *S. mutans* from plaque and saliva while leaving the remaining flora, including closely related non-mutans oral streptococci, relatively undisturbed. In the placebo group, *S. mutans* increased during the course of the study. A reduction of *S. mutans* in the C16G2 group resulted in a higher resting plaque pH, lower lactic acid production, and a significant reduction in enamel mineralization (Table). Based on these findings, the authors concluded that C16G2 produced reductions in plaque and salivary *S. mutans*, lactic acid production, and enamel demineralization. The impact on total plaque bacteria was minimal.

CURRENT DEVELOPMENT STATUS OF C16G2

C16G2 is currently being developed by a Los Angeles-based biotechnology company, C3 Jian Inc. (www.c3-jian.com). An Investigational New Drug (IND) application has been accepted by the US FDA, and the phase I clinical trial is due to start in the summer of 2012.

SUMMARY AND DISCUSSION

It is now clear that dental caries is a microbial-ecology-related disease. Any effective antimicrobial treatment against dental caries should modulate the microbial ecology of dental plaque in a pathogen-targeted manner, since indiscriminant antibacterial killing could lead to the disruption of the ecological balance of normal oral flora and result in persistent pathogenesis and possibly unknown clinical consequences. The STAMP technology offers a novel approach and a promising future for the treatment of microbial infections and dental caries at oral mucosal surfaces, where protective residential flora play a dynamic role in maintaining the health of the host and whose preservation should be maximized.

Given the fact that *S. mutans* is the major cariogenic bacterium, it is likely that C16G2 will demonstrate some anticaries activities if its bioactivities against *S. mutans* are retained *in vivo*. Since *S. mutans* is not the only cariogenic bacterium, a diagnostic device which could instantly detect the level of *S. mutans* in saliva and plaque would be very useful to screen for needy individuals and to monitor treatment efficacy. Such a device is currently being developed by C3 Jian Inc. The biggest unknowns at this point are: (1) how long it takes for *S. mutans* to re-invade and re-establish themselves within the treated oral microbial flora after being initially eliminated by C16G2; and (2) whether elimination of *S. mutans* would lead to the dominance of new oral pathogens. These two questions will be addressed in the IND clinical trials.

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