Oral Microbiology Immunology 2009: 24: 170–172
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ORAL MICROBIOLOGY AND IMMUNOLOGY

Short communication

Ceragenin CSA-13 exhibits antimicrobial activity against cariogenic and periodontopathic bacteria

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Isogai E, Isogai H, Takahashi K, Okumura K, Savage PB. Ceragenin CSA-13 exhibits antimicrobial activity against cariogenic and periodontopathic bacteria. Oral Microbiol Immunol 2009: 24: 170–172. © 2009 John Wiley & Sons A/S.

Introduction: Ceragenin CSA-13 is a bile-acid-based mimic of endogenous antimicrobial peptides and shares a mechanism of action with many of these antimicrobial agents. Because CSA-13 is not peptide based, it is not a substrate for the proteases that are found in the oral cavity, which are capable of degrading antimicrobial peptides. Furthermore, the simplicity of the ceragenins makes them easier to prepare and purify than antimicrobial peptides. In this study, we examined the antimicrobial activities of CSA-13 against oral pathogens and found that this compound was bactericidal against all of the strains tested.

Methods: The strains used were isolates of *Streptococcus mutans* and *Porphyromonas* species. Minimum inhibitory concentrations (MIC) were determined using agar dilution methods. In susceptibility testing, viable counts were determined after incubation with CSA-13.

Results: CSA-13 was potent against all 23 strains tested with MICs of 1–8 μg/ml for *S. mutans* and 1–16 μg/ml for 24 strains of the genus *Porphyromonas*. The MIC_{50} was 2 and the MIC_{90} was 8 μg/ml for *S. mutans*. MIC ranges for protease-positive *P. gingivalis* and *P. cangingivalis* were 2–16 μg/ml, and 1–2 μg/ml for protease-negative *P. circumdentaria.* CSA-13 interacted with lipopolysaccharide-sensitized erythrocytes at a concentration of 5.0–20.0 μg/ml.

Conclusion: CSA-13 displays broad-spectrum activity against cariogenic and periodontopathic bacteria. CSA-13 was effective against protease-positive *Porphyromonas*. It was shown to bind to erythrocytes coated with lipopolysaccharide and lipoteichoic acid from diverse bacterial strains. These results suggest that CSA-13 may be useful for the prevention and treatment of oral microbial diseases.

Key words: antimicrobial activity; ceragenin CSA-13; lipopolysaccharide-binding activity; *Porphyromonas*; *Streptococcus mutans*

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Accepted for publication April 30, 2008

Antibiotic resistance has been reported in various species from the oral microbiota (7). For example, numerous tetracycline-resistance determinants (e.g. *tetB*, *tetQ*, and *tetW*) have been identified in the oral microbiota. The rise of multidrug resistance has prompted renewed interest in the development of new antimicrobial agents targeting novel sites that may circumvent resistance. One frequently studied target is the bacterial membrane, which is an appealing target given that most structural elements are conserved and resistance to membrane-targeting antibiotics would require major changes in the membrane structure (9). Many agents that target the bacterial membrane are cationic, facially amphiphilic molecules, including endogenous antimicrobial peptides such as CAP18/LL-37 (4, 6). We reported that the peptides comprising the 27 amino acids in the C-terminal domain of CAP18/LL-37 killed *Porphyromonas gingivalis*, *P. circumdentaria*, and other oral anaerobic bacteria and that CAP18/LL-37-derived antimicrobial peptides with individual amino acids replaced by

hydrophobic and cationic amino acid residues showed more potent activities than the wild-type peptide (4). Obtaining high-quality synthetic peptide was straightforward but the cost was high (ca. 1 mg/$100). Many antimicrobial peptides are difficult to synthesize and purify because of their complexity and size (10). In addition, antimicrobial peptides can be substrates for proteases, which limit their *in vivo* half-lives.

The ceragenins, designed to mimic the activities of antimicrobial peptides, are a new type of antimicrobial agent (1, 2). A lead ceragenin, CSA-13, is relatively simple to prepare and purify on a large scale (2), and is stable under physiological conditions. There are no bonds in CSA-13 that would be expected to be cleaved under physiological conditions (e.g. no amides or esters), and no loss of antibacterial properties has been observed after prolonged storage in aqueous solution.

To better understand the antibacterial activities of CSA-13, we compared the activities of CSA-13 with those of two representative cathelicidins (CAP18/LL-37 and BMAP-28) against oral pathogens. Oral cariogenic and periodontopathic bacteria were comprised of multiple isolates of *Streptococcus mutans* (anaerobic bacteria) and *Porphyromonas* spp. (obligate anaerobic bacteria). Clinical isolates of *S. mutans* (24), *P. gingivalis* (12), *P. cangingivalis* (2), and *P. circumdentaria* (10) were used for the tests. *S. mutans* Ingbritt, *P. gingivalis* 381, *P. gingivalis* W83, and *P. circumdentaria* NCTC 12469 were also used. All strains were grown in brain–heart infusion liquid medium (BHI liquid medium; BBL Co., Cockeysville, MD) under anaerobic conditions (gas mix: 15% H_2, 15% CO_2, 70% N_2) at 37°C for 24 h.

Ceragenin CSA-13 (Fig. 1) was prepared as described previously (9). Cationic antimicrobial peptides, CAP18/LL-37 and BMAP-28, were synthesized, purified, and characterized by the Peptide Institute, Inc. (Osaka, Japan), according to a previously described method (4). The active domain of CAP18/LL-37 was synthesized as a C-terminal amide of 27 amino acids ($hCAP18_{109–135}$: FRKSKEKIGKEFKRIVQRIKDFLRNLV). The BMAP-28 active domain was also synthesized as a C-terminal 27-residue amidated peptide (GGLRSLGRKILRAWKKYGPIIVPIIRI-am).

Two assays were used to determine bacterial susceptibility to CSA-13 and the antimicrobial peptides: (1) an agar dilution method for the determination of minimum inhibitory concentrations (MIC) and (2) a susceptibility test for quantification of viable bacteria at each concentration. The 50 and 90% minimum inhibition concentrations (MIC_{50} and MIC_{90}) and the minimal bactericidal concentration (MBC) were determined using agar (BHI agar; Difco Laboratories, Detroit, MI) dilution methods according to the Clinical and Laboratory Standards Institute guidelines. Values of MIC were defined as the lowest concentration of the active agent causing at least 99.9% reduction of the number of viable cells, as compared to controls, for *S. mutans* Ingbritt, *P. gingivalis* 381, and *P. circumdentaria* NCTC12469. The antimicrobial control for all oral bacteria was chlorhexidine gluconate (1000 μg/ml). Additionally, each sample was centrifuged at 1,500 ***g*** for 10 min to remove the antimicrobial agent and the bacteria were resuspended in BHI liquid medium for the determination of MBC; the MBC was defined as the lowest concentration of the active agent resulting in no growth of the bacteria in the medium after anaerobic incubation for 48 h.

In susceptibility testing, bacteria were incubated with CSA-13 in HEPES–Hanks' balanced salt solution (HBSS, pH 7.4) anaerobically (37°C for 1 h), and viable counts were determined by plating from each tube onto BHI agar with 7% horse blood. Bacterial inocula were early-logarithmic-phase cultures, washed twice in HBSS, resuspended in the same buffer, and adjusted to a final concentration of 1×10^4 to -2×10^4 colony-fomring units/ml. To 500 μl of bacterial suspension, 500 μl of active solution (final peptide concentrations: 20, 10, 5, 2.5, 1.2, 0.6, 0.3, and 0.15 μg/ml for *S. mutans* Ingbritt, *P. gingivalis* 381, and *P. circumdentaria* NCTC12469, 20 μg/ml for the other species) was added and incubated at 37°C for 30 min under anaerobic conditions. Aliquots (100 μl) were removed from the tubes, diluted, and plated onto the BHI agar plates containing 7% horse blood. The colony-forming units/ml were assessed after the cells had grown for 72 h at 37°C under the anaerobic conditions.

After cultivation of *P. gingivalis* 381, *P. gingivalis* ATCC33277, and *P. circumdentaria* NCTC12469, the cells were harvested by centrifugation and treated with hot phenol by a previously described method (5, 8). Purified lipopolysaccharide (LPS) was used for sensitization of erythrocytes. The LPS from *Salmonella minnesota* R595, *Escherichia coli* O111:B4, and *Shigella flexneri* serotype 1A were purchased from Difco Co.Ltd. and were used as controls. Cell wall or lipoteichoic acid from *S. oralis* was also prepared for the binding assay.

The LPS-binding activity was examined by the method described previously (4). One milliliter of 1% erythrocytes (human O-type) was sensitized by incubation with 0.2 ml of various LPS solutions (100 μg/ml in HBSS). Fifty microliters of a 1.0% suspension of sensitized erythrocytes was mixed with an equal volume of a twofold serial dilution of the antimicrobial agents in a U-bottomed microtiter plate and incubated at 37°C for 1 h. LPS-binding activity of the antimicrobial agents was expressed as the minimum agglutinating concentration or minimal hemolytic concentration (MAC/MHC).

CSA-13 displayed potent antimicrobial activity against all 23 strains tested with MICs of 1–8 μg/ml for *S. mutans* and 2–16 μg/ml for the genus *Porphyromonas* (Table 1). The MIC_{50}, MIC_{90}, and MBC values of CSA-13 were 2, 8, and 8 μg/ml, respectively, for *S. mutans*. The susceptibility testing of the clinical isolates of *P. gingivalis* gave MIC_{50}, MIC_{90}, and MBC values for CSA-13 of 8, 16, and 16 μg/ml, respectively. Protease-positive *P. gingivalis* and *P. cangingivalis* were eliminated at 2–16 μg/ml, and protease-negative *P. circumdentaria* was killed at 1–2 μg/ml.

We compared the dose-dependent killing activity of CSA-13 and $hCAP18_{109–135}$. The CSA-13 and $hCAP18_{109–135}$ demon-

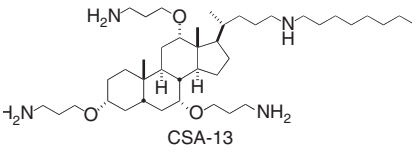

Fig. 1. The chemical structure of CSA-13 (molecular weight 822.94).

Table 1. Minimum inhibitory concentration (MIC) values of CSA-13

Clinical isolates	MIC range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MBC (μg/ml)
S. mutans (n = 24)	1–8	2	8	8
P. gingivalis (n = 12)	2–16	8	16	16
P. cangingivalis (n = 2)	2–16	–	–	–
P. circumdentaria (n = 10)	1–2	1.5	2	2

MBC, minimum bactericidal concentration; MIC_{50}, 50% MIC; MIC_{90}, 90% MIC.

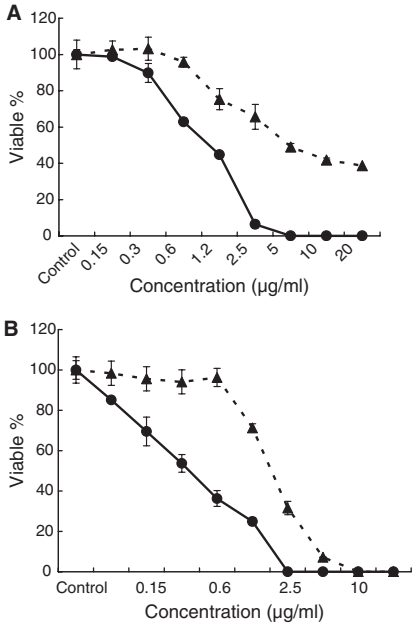

Fig. 2. Susceptibility of *Streptococcus mutans* Ingbritt to CSA-13 or $hCAP18_{109–135}$ peptide. The points and error bars are expressed as the mean and standard deviation of three independent assays. (A) *S. mutans* Ingbritt, (B) *Porphyromonas gingivalis* W83. •, CSA-13; ▲, $hCAP18_{109–135}$.

strated bactericidal effects against *S. mutans* Ingbritt (Fig. 2A). The bacterium was susceptible to CSA-13 and the MIC was 5 μg/ml, while the MIC for $hCAP18_{109–135}$ was more than 20 μg/ml. At that concentration, 39% of *S. mutans* survived. As shown in (Fig. 2B), both the ceragenin and $hCAP18_{109–135}$ demonstrated bactericidal effects against *P. gingivalis* W83. The *P. gingivalis* W83 was susceptible to CSA-13 and $hCAP18_{109–135}$ and the MIC values were 2.5 and 10 μg/ml, respectively.

The LPS binding activity, defined as the MAC, was expressed as the lowest concentration of peptide that could agglutinate LPS-sensitized red blood cells. CSA-13 associated with the LPS-sensitized cells at a concentration range of 5.0–20.0 μg/ml (Table 2). The MAC of cathelicidin peptides was 1.3–20 μg/ml for $hCAP18_{109–135}$ and 2.5–5 μg/ml for BMAP-28. The peptides had MACs of 10 μg/ml for cell wall or LTA-coated red blood cells and CSA-13 had a MAC of 5 μg/ml for cell wall or LTA coated red blood cells.

Table 2. Binding activity of CSA-13 and cathelicidine family cationic peptides

	Hemagglutination: MAC (μg/ml)		
Bacterial component	CSA-13	Human $hCAP18_{109–135}$	Bovine BMAP-28
LPS from *P. gingivalis* 381	10.0	5.0	2.5
LPS from *P. gingivalis* ATCC33277	10.0	2.5	5.0
LPS from *P. circumdentaria* NCTC12469	10.0	2.5	5.0
LPS from *S. minnesota* R595	10.0	1.2	5.0
LPS from *E. coli* O111:B4	5.0	5.0	2.5
LPS from *S. flexneri* serotype 1A	20.0	20.0	5.0
Cell wall from *S. oralis* 113-20	10.0	5.0	5.0
LTA from *S. oralis* 113-20	10.0	5.0	5.0

LPS, lipopolysaccharide; LTA, lipoteichoic acid; MAC, minimum agglutinating concentration.

Susceptibility data from our study demonstrated that CSA-13 has strong bactericidal activity against oral pathogens. In the testing, dose-dependent studies demonstrated that CSA-13 was a more potent agent than $hCAP18_{109–135}$. *S. mutans* in particular was not susceptible to $hCAP18_{109–135}$ over the 1-h incubation time, while the bacterium was effectively killed by CSA-13. CSA-13 has a broad spectrum of activity against cariogenic and periodontopathic bacteria. It is effective against protease-positive *Porphyromonas* and it has been reported that *P. gingivalis* strain W53 was resistant at the concentration of 100 μg/ml CAP18/LL-37 (3). This makes the activity of CSA-13 against all isolates from this genus even more noteworthy.

In innate immunity, antimicrobial peptides are important for protection of the oral cavity (11). This study demonstrates significant activities of CSA-13 against clinically important periodontal microorganisms. CSA-13 has a net positive charge that is electrostatically attracted to negatively charged bacterial membranes and has a high binding activity for LPS and LTA, similar to cationic peptides in innate immunity. From the cationic characteristics, CSA-13 has a mechanism of action that is also seen in cationic peptides which form part of the body's innate immune system, and CSA-13 may prove useful for the treatment and prevention of oral diseases such as caries and periodontitis.

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