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SMAP29 congeners demonstrate activity against oral bacteria and reduced toxicity against oral keratinocytes

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Introduction: Cathelicidins are antimicrobial peptides found in epithelial and mucosal tissues as well as the secondary granules of neutrophils. SMAP29, a sheep cathelicidin, has differential antimicrobial properties against various pathogens, including periodontal organisms. The purpose of this study was to evaluate the antimicrobial properties and cytotoxicity of SMAP29, SMAP28, and three congeners (SMAP18A, SMAP18D, and SMAP14A).
Methods: The peptides at concentrations ranging from 0.25 to 250 μg/ml were tested for their activity against multiple strains of *Streptococcus mutans*, *Streptococcus sanguis*, *Actinomyces israelii*, *Actinomyces naeslundii*, *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Peptostreptococcus micros*, and *Porphyromonas gingivalis* using a radial diffusion assay. Cytotoxicity of keratinocytes was evaluated by measuring lactate dehydrogenase release after incubation with the individual peptides.
Results: SMAP28, thought to be the biologically active peptide, was the most potent antimicrobial (range of minimum inhibitory concentrations 0.06–7.03 μg/ml, $P < 0.05$); however, the activity of SMAP28 and SMAP29 was strongly associated ($r = 0.933$). The congeners also demonstrated antimicrobial activity against the bacteria tested (range of minimum inhibitory concnetrations 0.21–79 μg/ml). Overall, *F. nucleatum* was the most susceptible organism, while *P. gingivalis* was the least susceptible. Keratinocyte cytotoxicity was dependent on peptide length and dose. SMAP28 was the most cytotoxic, while SMAP14A was the least cytotoxic.
Conclusion: The antimicrobial activities against oral microorganisms and the minimal toxicity seen in this study suggest that the congeners of SMAP29 may serve as an alternative to traditional antibiotics in the prevention and treatment of periodontal and other oral diseases.

Key words: antimicrobial peptides; cathelicidins; keratinocytes; oral bacteria; SMAP29

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Cathelicidins are endogenous antimicrobial peptides found in many species, including humans. They are localized throughout the epithelial and mucosal tissues, including the oral cavity and saliva (23), and are produced in bone marrow and stored in the secondary (specific) granules of neutrophils (22). The structure of cathelicidins comprises either α-helical or β-pleated sheets (35), some of which are proline-rich and tryptophan-rich structures (11, 36). They are two-part molecules containing a 'cathelin' domain, at the N terminus, which is conserved among species and an antimicrobial peptide domain, at the C terminus, which is highly variable in structure and responsible for their differential activity (26, 35).

The antimicrobial activity of cathelicidins has been reported for various pathogens. Brogden et al. showed that mouse,

rabbit, and sheep cathelicidins were effective against nine ovine pathogens (4). Skerlavaj et al. showed that SMAP29, a sheep myeloid antimicrobial peptide of 29 amino acids, was effective against methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VREF) isolates, and mucoid *Pseudomonas aeruginosa* (29). In addition, SMAP29 was active against pathogenic fungi (2), including *Cryptococcus neoformans* (29). Travis et al. evaluated the activity of cathelicidins from five mammalian sources (human, sheep, rabbit, rat, and mouse), against *P. aeruginosa*, *Escherichia coli*, *S. aureus*, and MRSA. The rabbit cathelicidin, CAP18, and the sheep cathelicidin, SMAP29, were the most effective, at both high and low salt concentrations (31).

Our laboratory previously compared the antimicrobial activity of human (LL37), sheep (SMAP29), and rabbit (CAP18) cathelicidins against three periodontal pathogens, *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis*, and found that SMAP29 demonstrated the greatest antimicrobial activity (12). In a comparative study, we found that SMAP29 demonstrated activity against multiple strains of oral aerobes and anaerobes and was globally more effective than the β-defensins, HBD-2 and HBD-3, against the anaerobic bacteria (16).

Studies have also evaluated truncations of the cathelicidins for their activity and potential toxicity. For example, Travis et al., tested CAP18 (rabbit cathelicidin) and derivatives and correlated their physiochemical properties with their antimicrobial activity (31). SMAP29 derivatives (ovispirins 1, 2 and 3) were tested by Saiman et al. and found to be nearly as active as SMAP29 against antibiotic-resistant pathogens (27). In another study, SMAP28 (believed to be the native form of SMAP29) showed increased activity when compared to SMAP29; however, it was also hemolytic (17, 29). The derivatives (ovispirins), on the other hand, demonstrated decreased antimicrobial activities but showed very little hemolytic activity (1, 17), similar to that of SMAP29 (31). Sawai et al. further manipulated the ovispirin sequence and produced a novispirin of which the cytotoxicity against human erythrocytes was greatly attenuated, but the antimicrobial properties were maintained (28).

Based on our previous work, and the importance of evaluating the activities and toxicity of mammalian cathelicidins for their therapeutic potential in the oral cavity, the purpose of the present study was to compare the antimicrobial activity of SMAP29 and several congeners against a panel of oral organisms and to assess their toxicity against keratinocytes. Multiple strains of each species were included to evaluate strain specificity.

Materials and methods

Peptides

SMAP29 (a sheep cathelicidin), SMAP28, thought to be the biologically active form of SMAP29 (29), and three shorter SMAP congeners, SMAP18A, SMAP18D, and SMAP14A (these have also been referred to as ovispirins 3, 1, and 2, respectively, in other studies), were utilized in this study (Table 1). The predicted protein properties for each of the cathelicidins are presented in Table 2. The peptides were synthesized as previously reported (4, 31). SMAP29 and SMAP28 are referred to as the parent peptides in this study.

Bacterial species, strains, and growth conditions

Our bacterial collection was composed of American Type Culture Collection (ATCC), laboratory, and clinical strains (Table 3). *A. actinomycetemcomitans*, *Streptococcus sanguis*, and *Streptococcus mutans* were grown in tryptic soy broth (Difco Laboratories, Detroit, MI) with 0.6% yeast extract (Difco); *F. nucleatum* was grown in Schaedler broth (Difco); *P. gingivalis* was grown in tryptic soy broth (Difco) supplemented with 5 μg/ml hemin (Sigma, St Louis, MO); *Peptostreptococcus micros* was grown in brain–heart infusion (Difco) with 0.5% neopeptone and 5 μg/ml hemin (Sigma); and *Actinomyces naeslundii* and *Actinomyces israelii* were grown in brain–heart infusion (Difco). *S. sanguis*, *S. mutans*, *A. naeslundii*, and *A. israelii* (aerobic bacteria) were grown in 5% CO_2. *A. actinomycetemcomitans*, *F. nucleatum*, *P. micros*, and *P. gingivalis* (anaerobic bacteria) were grown in 85% N_2, 10% H_2, and 5% CO_2. At least three strains of each bacterial species were tested for susceptibility to the five peptides (Table 3).

Radial diffusion assay

Radial diffusion assays were performed as previously described (16, 21). Briefly, bacteria were grown in their appropriate media overnight as described above, centrifuged at 7500 ***g*** for 15 min, rinsed with fresh medium, and resuspended in 10 mM sodium phosphate, pH 7.4. An underlay gel was prepared that comprised a mixture of 1% agarose in 10 mM sodium phosphate (pH 7.4) containing 4×10^6 bacteria. The mixture was immediately poured into a square Petri dish and allowed to solidify before 3-mm diameter wells were punched in the agar. The peptides were diluted in 0.01% acetic acid and 0.1% human serum albumin, and 5 μl was added to each well at concentrations ranging from 250 to 0.25 μg/ml. A control

Table 1. Amino acid sequences of SMAP29 and its four congeners, as well as predicted molecular weights[1]

Peptide	#AA	Amino-acid sequence	MW (Da)
SMAP29	29	RGLRRLGRKIAHGVKKYGPTVLRIIRIAG	3256
SMAP28	28	RGLRRLGRKIAHGVKKYGPTVLRIIRIA-NH2	3199
SMAP18A (ovispirin 3)	18	KNLRRI I RKI I H I I KKYG-NH2	2263
SMAP18D (ovispirin 1)	18	KNLRRI I RKI I H I I KKYG	2263
SMAP14A (ovispirin 2)	14	LRRI I RKI I H I I KK-NH2	1800

#AA, number of amino acids; [1]MW, molecular weight, obtained from http://aps.unmc.edu/AP/main.php

Table 2. Predicted peptide properties of SMAP29 and congeners[1]

Properties	SMAP29	SMAP28	SMAP18A	SMAP18D	SMAP14A
No. of hydrophobic residues	11	11	8	8	7
No. of negatively charged amino acids	0	0	0	0	0
Total hydrophobic ratio (%)	37	39	38	38	50
Total net charge (+/–)	+10	+11	+9	+8	+8
Net positive charge/residue	2.90	2.50	2.00	2.25	1.75
Helical structure potential	Yes	Yes	Yes	Yes	Yes
Boman index (kcal/mol) (protein-binding potential)	2.16	2.27	2.38	2.38	2.25
Amino group terminations	No	Yes	Yes	No	Yes

[1]Obtained from http://aps.unmc.edu/AP/main.php

Table 3. Susceptibilities (measured as MIC) of oral bacteria to SMAP29 and congeners[1]

Species	Gram-stain reaction	Strain	MIC (μg/ml) SMAP29	SMAP28	SMAP18A	SMAP18D	SMAP14A
A. actinomycetemcomitans	−	**Y4**	3.8	1.5	3.4	4.6	8.3
		246	0.29	0.39	6.90	5.29	3.80
		ATCC 29523	1.79	1.61	5.33	9.29	5.48
F. nucleatum	−	**1594**	1.02	0.91	2.00	1.72	1.70
		1908	0.46	0.36	0.75	0.37	0.81
		ATCC 49256	0.36	0.06	3.6	2.9	7.6
P. gingivalis	−	**W50**	16.1	6.0	16.5	16.5	> 79
		ATCC 33277	5.38	3.22	7.61	9.34	12.43
		ATCC 49417	12.53	7.03	10.34	13.30	15.29
P. micros	+	**8050**	2.84	2.46	14.30	9.39	1.90
		2903-02	1.79	1.17	5.14	4.48	3.30
		97-1502	1.08	0.69	3.80	3.19	4.03
		ATCC 33270	2.69	1.87	6.91	8.09	0.21
A. naeslundii	+	*14B01*	1.42	1.09	3.24	3.91	3.02
		11A01	3.04	1.41	3.10	3.55	3.65
		14B4C	0.88	0.95	2.35	2.19	2.77
A. israelii	+	*9P04*	1.66	1.63	2.63	2.78	2.62
		1P04	2.05	2.12	2.88	2.53	2.52
		5A40	1.92	1.97	2.50	2.83	2.40
S. sanguis	+	**AC59**	2.95	2.53	8.53	8.43	11.87
		P695	2.74	2.37	3.13	3.30	3.05
		NP506	4.28	2.39	7.53	6.19	2.96
S. mutans	+	**Ingbritt 162**	0.97	0.58	2.11	1.00	1.79
		OMZ175	1.39	0.82	1.95	1.60	1.28
		330–5	0.89	0.96	2.63	0.78	1.74
		ATCC 25175	1.72	2.08	5.72	2.79	3.37

[1]Clinical isolates are shown in *italics*. Laboratory strains and ATCC strains are shown in **bold**. Data presented correspond to the mean of triplicate experiments.

well containing only 10 mM sodium phosphate buffer was included on each plate. The plates were incubated under the appropriate aerobic or anaerobic conditions at 37°C for 3 h to allow for peptide diffusion. A 1% agar overlay gel containing medium specific for the organism tested was then poured over the underlay gel. The plates were incubated again in the appropriate conditions for 12–18 h or until zones of inhibition were visible. Zones were measured with a Boley gauge and recorded as radial diffusion units [zone of inhibition (mm) − well diameter (3 mm) × 10]. The minimum inhibitory concentrations (MICs) were calculated after plotting the radial diffusion units relative to the $\log_{10}$ peptide concentration. The concentration at the *x* intercept deduced from this plot was defined as the MIC. Data were obtained in triplicate.

Cytotoxicity assay

A primary gingival keratinocyte cell line was cultured and seeded at 2.5×10^5 cells per well in 12-well plates in keratinocyte basal medium (Clonetics, Biowhittaker, Inc., Walkersville, MD) and 2.5% fetal bovine serum with antibiotic and antimycotic (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin; Gibco, Grand Island, NY) according to established protocols (15, 16). One immortalized keratinocyte cell line (TERT), (kindly provided by Dr Zoya Kurago, Dr A. Klingelhutz and Dr J. Lee, U. Iowa) was grown in keratinocyte serum-free medium (Gibco) with 0.2 ng/ml epidermal growth factor, 30 μg/ml bovine pituitary extract and with antibiotic and antimycotic (Gibco) and was seeded at approximately 1×10^5 cells per well in 12-well plates. Before the exposure, cultures were grown in medium without antibiotic, antimycotic, or fetal bovine serum. When 80% confluency was reached, cells were exposed for 24 h to SMAP29, SMAP28, SMAP18A, SMAP18D or SMAP14A at three different concentrations: 0.1, 5, and 20 μg/ml, in duplicate. Peptide dilutions were done in 0.1% bovine serum albumin and 0.01% acetic acid in sterile water. Supernatants were removed, spun at 7000 ***g*** for 5 min and stored at –80°C. Cytotoxicity assays were performed in triplicate using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) which measures the lysis of the cell by the amount of lactate dehydrogensase (LDH) released into the supernatant. In brief, 50 μl of each sample and 50 μl of the substrate were added to the wells of a 96-well plate and incubated in the dark at room temperature for 30 min. Then, 50 μl of the stop reaction buffer was added to each well and the result was read on a plate reader at 490 nm. Controls were epithelial keratinocyte cells in media alone.

Statistical analysis

Radial diffusion assays and LDH assays were performed in triplicate. The statistical significance of the radial diffusion assay and LDH assay was evaluated using a Student's two-tailed *t*-test. Coefficients of correlation (Pearson coefficients) were calculated to test the association between peptides and were evaluated by a *P*-value representing the chance that random sampling would result in a correlation coefficient as far from zero as (or farther than) the one observed in the experiment, where $P < 0.05$.

Results

Activities of parent peptides against oral bacteria

SMAP29 and SMAP28 had MICs ranging from 0.29 to 16.1 and 0.06 to 7.3 μg/ml, respectively (Table 3). The MICs of these peptides were significantly lower than those of the congeners ($P \leq 0.0006$). When considering all species and all strains tested, SMAP28 was significantly more active than SMAP29 ($P = 0.0003$); however, their antimicrobial activity was highly correlated ($r = 0.8897$).

Activities of congener peptides against oral bacteria

The three congeners, SMAP18A, SMAP18D, and SMAP14A, demonstrated a collective MIC range of 0.21–16.5 μg/ml (excluding *P. gingivalis* W50 which appeared to be resistant to SMAP14A, MIC > 79 μg/ml) (Table 3). Individual MIC ranges for the three congeners were as follows: 0.75–16.5 μg/ml for SMAP18A, 0.37–16.5 μg/ml for SMAP18D, and 0.21–15.29 μg/ml for SMAP14A (Table 3). The three congeners did not perform significantly differently from one another ($P > 0.05$). While the parent peptides showed more activity, overall, the congeners also demonstrated strong antimicrobial activity against the array of organisms tested.

Species susceptibility to peptides

In general, *F. nucleatum* was the most susceptible organism tested (MIC range of 0.06–7.6 μg/ml), while *P. gingivalis* was the least susceptible (MIC range of 3.22 to >79 μg/ml) (Fig. 1 and Table 3). These two organisms represent the range of susceptibility seen in this study. With the exception of *P. gingivalis,* there were no significant differences when comparing the susceptibility of aerobic and anaerobic organisms, periodontal and non-periodontal organisms, clinical and laboratory isolates, or gram-positive and gram-negative organisms. Interestingly, while SMAP29 and SMAP28 demonstrated the most activity, the congeners also showed global antimicrobial activities against the species *A. actinomycetemcomitans*, *F. nucleatum*, *S. mutans*, *A. naeslundii*, and *A. israelii* with MICs all <10 μg/ml. However, only single strains within the species of *P. micros* (8050) and *S. sanguis* (AC59) were outside this range (14.30 and 11.87 μg/ml, respectively).

Strain susceptibility to peptides

There was variable strain-specific susceptibility for the different organisms with the different peptides (Fig. 1 and Table 3). For example, *P. gingivalis* strain W50 demonstrated fairly consistent susceptibility to all peptides (6.0–16.5 μg/ml) except for its resistance to SMAP14A. It was susceptible at the next highest concentration tested (250 μg/ml) (data not shown). While SMAP14A demonstrated the least activity against *P. gingivalis* strain W50 and *S. sanguis* strain AC59, it showed strain-selective potent activity against *P. micros* strain 33270 and enhanced activity against *S. sanguis* NP506, relative to the other congeners tested.

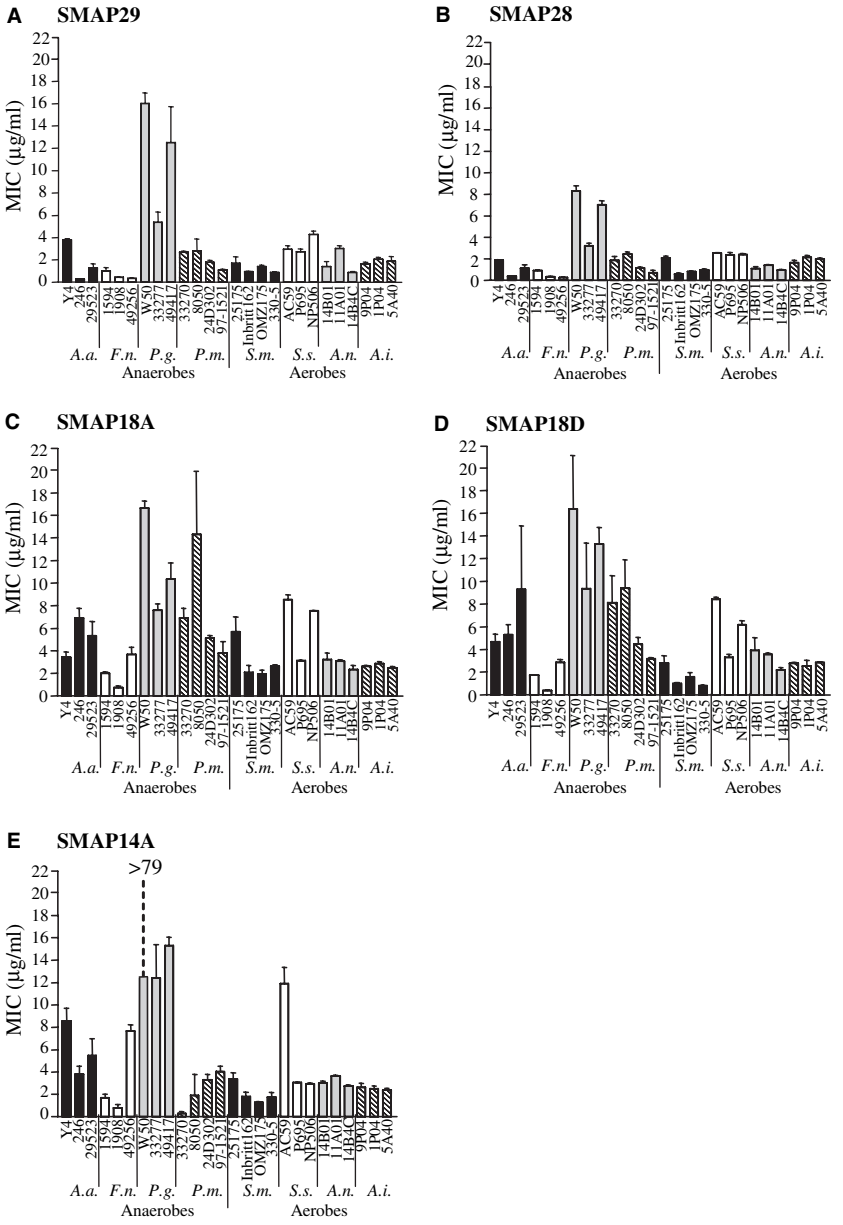

Fig. 1. Susceptibility of aerobic and anaerobic oral bacteria to SMAP29 and congeners. MICs, obtained from radial diffusion assays of SMAP29 (A), SMAP28 (B), SMAP18A, (C), SMAP 18D (D), and SMAP14A (E) against *Actinobacillus actinomycetemcomitans* (*A.a.*), *Fusobacterium nucleatum* (*F.n.*), *Porphyromonas gingivalis* (*P.g.*), *Peptostreptococcus micros* (*P.m.*), *Streptococcus mutans* (*S.m.*), *Streptococcus sanguis* (*S.s.*), *Actinomyces naeslundii* (*A.n.*), and *Actinomyces israelii* (*A.i.*) are presented. Values are means and standard deviations from triplicate assays.

Cytotoxicity of peptides

Cytotoxicity was dependent on peptide length and dose. The higher the concentration of peptide used, the greater the cytotoxicity. This was seen for both the primary gingival keratinocyte cell line and the immortalized TERT keratinocyte cell line (Fig. 2). SMAP28, the peptide with the strongest antimicrobial activity, was also the most cytotoxic towards both cell lines at all peptide concentrations and was significantly different from the control at concentrations as low as 0.1 μg/ml ($P < 0.0001$). The other four peptides evaluated were highly cytotoxic to the primary keratinocyte cell line at 20 μg/ml, but their cytotoxicity dropped off markedly at both 5 and 0.1 μg/ml (Fig. 2A). The two keratinocyte cell lines tested behaved similarly to exposure to the peptides, with the peptides being slightly

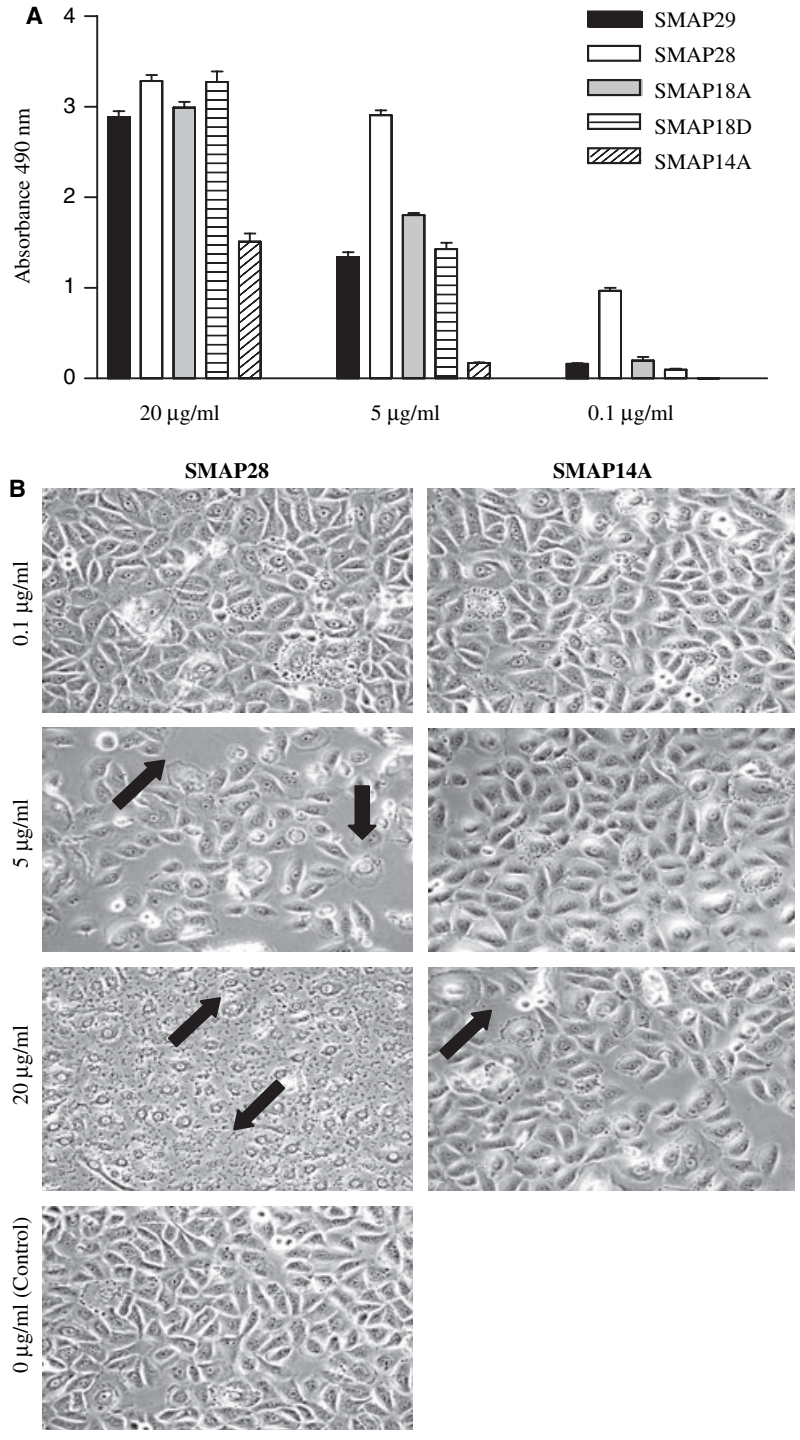

Fig. 2. Cytotoxic effect of SMAP29 and congeners (20, 5, and 0.1 µg/ml) on a TERT immortalized keratinocyte cell line as assessed by LDH assay (A) and light microscopy at 20 × magnification (B) after a 24-h incubation. Arrows represent debris and misshapen cells associated with SMAP28 toxicity. The monolayer retains its cellular integrity when exposed to SMAP14A. In (A) values are means and standard deviations from triplicate assays obtained after subtraction of values from control wells (media alone). Control in (B) describes cells and media without peptide.

more cytotoxic to the immortalized cell line than the primary keratinocyte cell line.

When examined under light microscopy, the integrity of the keratinocytes paralleled the LDH assay results (Fig. 2B). SMAP28 exposure resulted in a massive destruction of the monolayer, with abnormal cell size, shape, and debris, especially obvious with the 20 µg/ml concentration (Fig. 2B). Monolayer disruption was both dose and peptide dependent (Fig. 2B) with SMAP28 being the most destructive. As expected, SMAP14A showed little toxic effect on the keratinocytes and the 20 µg/ml dose did not significantly affect the monolayer and cell appearance when compared to the control (Fig. 2B).

Discussion

This study showed that overall SMAP29, SMAP28, and three SMAP congeners had significant antimicrobial activity against a diverse panel of oral bacteria. With the exception of *P. gingivalis* W50, all the organisms tested were inhibited by the parent peptides and the congeners at low concentrations. SMAP28 was the most effective peptide, significantly more so than its precursor peptide, SMAP29. Importantly, the truncated congeners also showed significant antimicrobial activity against the panel of microorganisms tested suggesting that the full-length peptides are not required for therapeutic application.

SMAP28 differs from SMAP29 in that it is missing one amino acid, the last glycine residue, which is substituted with an amino group (Table 1). This small difference significantly affected the antimicrobial activity, suggesting that even small changes can ultimately affect a peptide's properties.

As a result of substitutions or deletions of amino acids, properties such as hydrophobicity, charge, and length affect peptide function, and probably explain the differences seen in antimicrobial activity among the peptides (Tables 2 and 3). The total net charge appeared to be associated with an increase in antimicrobial activity, as shown by the parent peptides, SMAP29 and SMAP28. This would corroborate the hypothesis that the mechanism of action of these antimicrobial peptides is based on their cationic nature. The addition of amino groups on three of the five peptides increased the net positive charge, hence, potentially enhancing their antimicrobial activity. Interestingly, SMAP18D and SMAP14A (a four-amino-acid truncated congener of SMAP18D) displayed similar antimicrobial profiles (Tables 1 and 3). These results support the hypothesis that net charge is more critical than length in determining antimicrobial activity. Finally, the SMAP congeners utilized in this study have a very similar Boman index (2.16–2.38; Table 2), which suggests similar binding capacities and indicates their multifunctional potential (3). This finding is in agreement with that of Bartlett et al., who showed that the same congeners (with

the exception of SMAP18A, which was not tested) had a capacity to bind lipopolysaccharide that was identical to SMAP29 regardless of the length (1).

Studying the structure and activity of the cathelicidins and derivatives may assist in ascertaining their specific mechanisms of action, largely unknown for most of the natural peptides. Models have been proposed that describe the interaction between the peptides and lipid layers (i.e. the 'carpet' model) (33). Based on these models, it is believed that the peptides cause significant morphological alterations to bacterial surfaces, as shown by scanning electron microscopy (29).

For the purposes of this study, we evaluated bacteria involved at different stages of biofilm formation. These included: early colonizers such as *S. sanguis*, *A. israelii*, and *S. mutans* and *A. naeslundii* (implicated in caries), an intermediate colonizer, *F. nucleatum*, and late colonizers and periodontal pathogens *A. actinomycetemcomitans*, *P. micros*, and *P. gingivalis* (20). While variability in MIC was observed for the different species, all organisms were inhibited, which may have important implications in biofilm development and ultimately in the pathogenesis of caries and periodontal disease.

Contrary to our previous observations, where the antimicrobial activities of the β-defensins were evaluated against these same microorganisms (16), there were no striking differences in the susceptibilities of the aerobic and anaerobic microorganisms to the cathelicidins. However, in both studies, *F. nucleatum* was the most susceptible microorganism while *P. gingivalis* was the least susceptible microorganism (16). These results could be because the two families of antimicrobial peptides share a similar mechanism of action, which is probably linked to their cationic properties. Alternatively, differences in the structural integrity or bacterial byproducts may make the microorganisms more or less susceptible. This helps to explain the strain specificity in both studies. It is known, for example, that select strains within a species may differ in the composition of their cell membranes (i.e. the lipopolysaccharides of *P. gingivalis* and *E. coli*) (9, 24). This could affect the total negative surface charge and result in differential susceptibility to positively charged peptides (10). Second, bacteria such as *P. gingivalis* are known to produce strain-specific proteolytic enzymes, which may degrade the innate peptides (5). Interestingly, *P. gingivalis* is resistant to many important naturally occurring peptides beside the cathelicidins (SMAP29, CAP18, FALL39) (12) and defensins (16, 25), including magainin and Cecropin (7). Despite its resistance, *P. gingivalis* may be unable to colonize if these peptides are killing early and intermediate colonizers, thereby disrupting overall biofilm formation.

SMAP29 and its congeners demonstrate broad-spectrum activity; therefore, one can assume that their mechanism of action relies on non-specific interactions rather than specific ligand/receptor binding. This may be advantageous for the disruption of bacterial cell membranes but may be detrimental to host cells. Cathelicidins are reportedly toxic to human erythrocytes (29). In another study, single amino acid mutations of one of the SMAP29 congeners, SMAP18D, altered the cytotoxic potential against cervical and pulmonary human epithelial cells and erythrocytes, while the antimicrobial activity was unchanged (28). We therefore tested the hypothesis that structural alterations may have an impact on peptide toxicity. Using human gingival keratinocytes as a model, we demonstrated that SMAP28, while demonstrating the highest antimicrobial activity, was also the most cytotoxic. Interestingly, SMAP14A, which retained strong antimicrobial activity, was the least cytotoxic at doses in the range of its MICs. The cytotoxic activities of the congeners correlated with the length of the peptide. The hydrophobic ratio of the peptides has been shown to be critical in host cell toxicity. For example, reduced hydrophobicity results in loss of helicity (19) which, in turn, results in decreased toxicity by the novispirins (18). This corroborates our results because SMAP14A has the lowest number of hydrophobic residues. Another explanation for this may be the enhanced tolerance by the host cells of humans because the shorter sheep peptides contain less foreign sequence.

There are several advantages to utilizing endogenous peptides, such as SMAP29 and especially its shorter congeners, with regard to therapeutic application. They are potentially less allergenic (32), and have a non-specific action on cell membranes with no known receptors. Even if bacteria, particularly pathogens, can develop ways to resist host antimicrobial peptides (7, 10), resistance is less likely compared to traditional antibiotics (13). This is an important feature in their potential application for treatment of periodontal diseases where resistance of periodontal organisms to traditional antibiotics has markedly increased (32). In addition, they display broad-spectrum activity and rapid killing against gram-positive bacteria, gram-negative bacteria, fungi (12, 13, 16, 29), and viruses, including human immunodeficiency virus and herpes simplex virus (14, 34).

Importantly, the cathelicidins exhibit a number of functions in addition to antimicrobial activity. One example is their ability to bind to lipopolysaccharide (30). SMAP29 has two lipopolysaccharide-binding sites and a central hinge (30). Cathelicidins are also involved in other biological processes such as neutralization of lipopolysaccharide toxicity, wound healing (35), chemotaxis of neutrophils, monocytes, lymphocytes, $CD4^+$ T cells, and mast cells (6), and induction following injury or infection (8). The comprehensive analysis of the activities of sheep cathelicidin congeners on host responses are important in evaluating their function(s) as therapeutics in the human oral cavity.

In summary, this study of SMAP29 and its congeners evaluated their structure, cytotoxicity, and antimicrobial activities, allowing us to speculate on their potential therapeutic usefulness and safety in humans. Overall, the smaller derivatives were least cytotoxic, and thus likely to have fewer adverse effects. In addition, the smaller peptides would be less costly to manufacture and allow for easier drug formulation. Further studies are needed to evaluate the activities of these peptides in conjunction with other innate antimicrobials and their effect on other immune molecules if we are to explore their potential for future use in the treatment of oral diseases such as periodontitis and caries.

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