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Cytotoxicity and the effect of cationic peptide fragments against cariogenic bacteria under planktonic and biofilm conditions

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ABSTRACT

This study evaluated the cytotoxicity and effect of fragments derived from three oral cationic peptides (CP): LL-37, D6-17 and D1-23 against cariogenic bacteria under planktonic and biofilm conditions. For cytotoxicity analysis, two epithelial cell lines were used. The minimum inhibitory concentration and the minimal bactericidal concentration were determined for the CP fragments and the control (chlorhexidine-CHX) against cariogenic bacteria. The fractional inhibitory concentration was obtained for the combinations of CP fragments on *Streptococcus mutans*. Biofilm assays were conducted with the best antimicrobial CP fragment against *S. mutans*. The results indicated that D6-17 was not cytotoxic. D1-23, LL-37 and CHX were not cytotoxic in low concentrations. D1-23 presented the best bactericidal activity against *S. mutans*, *S. mitis* and *S. salivarius*. Combinations of CP fragments did not show a synergic effect. D1-23 presented a higher activity against *S. mutans* biofilm than CHX. It was concluded that D1-23 showed a substantial effect against cariogenic bacteria and low cytotoxicity.

Introduction

Early childhood caries (ECC) represents the most common chronic disease in childhood with a prevalence of around 26% in Brazil (SB Brasil 2010) and 23% in the USA (Dye et al. 2015) among 5–6 year old children, and can be observed in toddlers as young as 12 months of age (SB Brasil 2010; Dye et al. 2015). ECC can progress and lead to severe destruction of primary teeth, causing infection, pain, chewing and speech difficulties, physiological trauma and early dental loss (Losso et al. 2009). Besides the negative effects on health, quality of life and high treatment costs, children who present ECC remain at a high risk for future caries recurrences and under continuous dental interventions, such as topical fluoride/antimicrobial applications (O'Sullivan & Tinanoff 1996).

By virtue of rapid tooth destruction in a short period of time, the association between the following factors has been suggested in the etiology of ECC: frequent intake of a diet rich in fermentable carbohydrates such as sucrose, a high microorganism count and immunological vulnerability (Mattos-Graner et al. 1998, 2001). The bacterial group considered most cariogenic is mutans streptococci, especially *Streptococcus mutans*, one of the primary bacterial colonizers of dental enamel and less frequently *Streptococcus sobrinus* (van Houte et al. 1991; Mattos-Graner et al. 2001, 2014). However, other acidogenic and aciduric species, such as *Lactobacillus* and *Actinomyces*, are involved in the initiation of carious lesions (Sansone et al. 1993; van Houte et al. 1996).

The immune system has several forms of defense against pathogenic microorganisms. In addition to the physical barrier against the entrance of foreign microbes, mucous membranes are a source of potent antimicrobial cationic peptides (AMCP) (Mccormick & Weinberg 2010). They have an inhibitory action against a broad range of bacteria, fungi and enveloped viruses and promote modulation

of the host immune response while maintaining normal microbiota in different niches such as the skin, intestine and oral cavity (Mccormick & Weinberg 2010; Wiesner & Vilcinskas 2010). The main AMCP found in saliva and/or crevicular fluid are α- and β-defensins (hBD) and cathelicidins, produced by epithelial cells (Abiko et al. 2003). Studies have focused on the effect of hBD-3 against important oral pathogens, such as *S. mutans*, *S. sobrinus*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* (Ouhara et al. 2005). The human cationic peptide (hCAP-18) is the only cathelicidin identified in humans, produced by epithelial cells from the lungs, gut, urogenital tract and oral cavity. After secretion, hCAP-18 is broken, by protease activity, to a small peptide called LL-37. This peptide fragment is a multifunctional immune modulator with antibacterial function and the ability to stimulate angiogenesis, skin healing and chemotaxis of inflammatory cells (Mccormick & Weinberg 2010; Wiesner & Vilcinskas 2010).

Limited research has been conducted to determine the effectiveness of synthetic or natural chemotherapeutic agents, individually or in combination, to prevent or reduce the incidence of ECC (Horowitz 1998). There has been recent interest in the use of peptides for the prevention of dental caries (da Silva et al. 2013; Benergossi et al. 2015). Although AMCP have been pointed out as a new class of antibiotics, the long length of their amino acid chain or chemical linkages make their production as a therapeutic agent difficult. These peptides could serve as a template for the design of effective antibiotics for oral application, potentially reducing the cost of production and optimizing their antimicrobial properties (Batoni et al. 2011). Synthetic analogs of AMCP have reached clinical trials to be indicated for patients, as reported for defensin mimetic PMX-30063 and histatin-5 P113 (Gordon et al. 2005). The aim of this study was to evaluate the cytotoxicity and the effect of fragments derived from three oral cationic peptides (CP): LL-37 (from hCAP-18), and D6-17 and D1-23 (from ortholog of β-defensin-3) against cariogenic bacteria under planktonic and biofilm conditions.

Materials and methods

Preparation of peptides and controls

The peptide fragments LL-3(LLGDFFRKSKEKIGK EFKRIVQRIKDFLRNLVPRTES) derived from hCAP-18 (Ji et al. 2007); Def14-1C^V (6-17) or D6-17 (LRKFFARIRGGR) and Defb14-1C^V (1-23) or D1-23 (FLPKTLRKFFARI RGGRAAVLNA) derived from Defb14, the mouse ortholog of human β-defensin-3 (Reynolds et al. 2010), were purchased from Invitrogen (Life Technologies, Carlsbad, CA, USA). Defb14-1C^V is a peptide in which the cysteines have been replaced with alanines, except Cys$_{40}$, which resides at position V of the six-cysteine motif. The synthetic peptides were resuspended in sterile deionized water at 20 mM and stored at −20°C prior to use. Chlorhexidine digluconate (CHX, Sigma Aldrich, St Louis, MO, USA) was used as a control. All subsequent experiments were performed in triplicate, in three independent assays.

Cytotoxicity tests

Epithelial cell cultures

The following cell lines were tested: immortalized human gingival epithelial cell line OBA-9 and skin epidermal HaCaT. The OBA-9 cells were cultured in K-SFM serum-free medium (Life Technologies), containing insulin, epidermal growth factor, fibroblast growth factor and 100 μg ml^{-1} of penicillin G/streptomycin. The HaCaT cells were cultured in Dulbecco's modified Eagle's medium – DMEM (Gibco BRL, Carlsbad, CA, USA) plus 10% fetal calf serum and 100 μg ml^{-1} of penicillin G/streptomycin. Both cell lines were grown until they reached subconfluent density at 37°C in 5% CO_2 (Bedran et al. 2014).

Stimulation of epithelial cells by peptide fragments

The epithelial cells were harvested following trypsin treatment (5 min) (TrypLETM Express; Life Technologies) at 37°C. Proteases were then inactivated by adding 0.3 mg ml^{-1} of trypsin inhibitor and cells were harvested by centrifugation (500 × *g* for 5 min), suspended in fresh medium, seeded in a 96-well microplate (200 μl well^{-1}, 1 × 10^6 cells ml^{-1}) and incubated overnight at 37°C in a 5% CO_2 atmosphere to allow cell adhesion before stimulation. The cells were then stimulated with the peptide fragments (LL-37; D6-17; D1-23) and CHX at concentrations ranging from 1 to 0.001 mM for 24 h at 37°C in 5% CO_2 (Bedran et al. 2014).

Determination of cell viability

A colorimetric MTT cell viability assay (Roche Diagnostics, Mannheim, Germany), using 3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide as the substrate, was used to determine the effect of cationic peptide fragments and controls on cell viability. After exposure to the peptides and controls, the extracts were aspirated and replaced by 90 μl of DMEM or K-SFM plus 10 μl of MTT solution (5 mg ml^{-1} sterile PBS; Sigma Aldrich). Next, the culture medium with the MTT solution was aspirated and replaced with 100 μl of acidified isopropanol solution. Two 50-μl aliquots of each well were transferred to 96-well plates. Cell viability was evaluated using spectrophotometry, being proportional to the absorbance measured at OD$_{570\ nm}$ with an ELISA microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The means were calculated for the groups and transformed into

percentages, which represented the inhibitory effect of the mitochondrial activity of the cells by the peptides/CHX. The negative control (DMEM or K-SFM) was defined as having 100% cell metabolism (Bedran et al. 2014).

DAPI staining

Apoptotic nuclear morphology was observed using 4,6-diamidino-2-phenylindole dihydrochoride (DAPI). HaCaT cells at a density of 2×10^5 cells well^{-1} were placed on 24-well slides and treated with 0.1 mM of each peptide fragment or CHX for 24 h. Next, cells were washed with phosphate-buffered saline (PBS) and stained with DAPI solution, as described previously (Lai et al. 2011). After staining, the cells were examined and photographed using a fluorescence microscope (Leica, DM5500 B, Wetzer, Hesse, Germany).

Antimicrobial tests

Bacterial conditions

The following bacterial strains used in the present study were kindly provided by the Oswaldo Cruz Foundation (FIOCRUZ – Rio de Janeiro, São Paulo, Brazil): *Streptococcus mutans* (ATCC −25175), *S. mitis* (ATCC 4945), *S. oralis* (IAL -1676), *S. sanguinis* (ATCC 10557), *S. salivarius* (ATCC 7073), *Lactobacillus acidophilus* (ATCC 4356), *L. paracasei* (ATCC 335), *L. rhamnosus* (ATCC 9595), *L. brevis* (ATCC 367), *L. fermentum* (ATCC 9338) and *Actinomyces israelii* (ATCC 12102). Clinical *S. mutans* strains 1 and 2 (CS1 and CS2) were kindly provided by Renata Mattos-Graner and were previously isolated from ECC children and characterized as highly cariogenic (Mattos-Graner et al. 2004). The purity of the strains was confirmed by Gram stain. Microbial suspensions were prepared from a culture previously grown in mitis salivarius agar base (Difco Laboratories, Detroit, MI, USA) with 0.2 U mg ml^{-1} bacitracin (Sigma-Aldrich) for *S. mutans* strains, mitis salivarius agar (Difco Laboratories) for the other *Streptococcus* strains (Difco Laboratories), Rogosa agar (Difco Laboratories) for *Lactobacillus* and brain heart infusion (BHI) agar (Difco Laboratories) for *Actinomyces* and incubated at 37°C for 24 h in 5% CO_2. Growth curve assays were performed for each bacterium in order to determine the OD at the mid-log phase with ~ $5–10 \times 10^8$ CFU ml^{-1} to be used in subsequent experiments. The absorbance was measured using a microplate reader (Eon Microplate Spectrophotometer, Biotek Instruments, Winooski, VT, USA) to assess cell density.

Determination of MIC and MBC

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by the broth microdilution method, in 96-well microtiter plates, following the criteria previously described by the Clinical Laboratory Standards Institute M7-A9 (CLSI 2012) for bacteria. Bacterial cell cultures at the mid-log phase were harvested by centrifugation (Hanil Combi centrifuge, 514R) for 10 min, at $3,000 \times g$, the supernatant was discarded and the pellet re-suspended in Mueller–Hinton broth (Difco Laboratories). The final concentration of bacterial suspension in the wells was $5–10 \times 10^5$ CFU ml^{-1}. The fragments of cationic peptides were serially diluted in sterile deionized water at concentrations ranging from 1 to 0.001 mM. Next, a bacterial suspension was inoculated into each well. The plates were incubated at 37°C for 24 h in 5% CO_2. After this, 15 μl of 0.01% resazurin stain (Sigma-Aldrich) was applied to each well and incubated for 4 h to determine cell viability (Hahnel et al. 2012). After that, wells corresponding to MIC and at least three previous wells were homogenized, serially diluted and plated on Mueller–Hinton agar to determine the MBC. The plates were incubated at 37°C for 24 h in 5% CO_2. The number of colony forming units ml^{-1} (CFU ml^{-1}) of bacteria was determined. The MBC was considered when the peptides killed 99.9% of the tested bacterial culture. CHX and the culture medium were used as positive and negative controls, respectively.

Determination of FIC

The combined effects of peptide fragments were evaluated by the fractional inhibitory concentration (FIC) index, using the checkerboard assays as previously described (Tong et al. 2011). Briefly, the rows of a 96-well microplate contained the same concentrations of one of the peptide fragments (or two peptides, for triple combinations), diluted from 1 to 0.001 mM along the *y*-axis. The column contained the same concentration of another peptide fragment, diluted from 1 to 0.001 mM along the *x*-axis. After incubation for 24 h at 37°C in 5% CO_2, the plates were stained with resazurin for 4 h. The FIC index was calculated according to the equation: FIC index = FIC A (MIC of antimicrobial A in combination/MIC of A alone) + FIC B (MIC of antimicrobial B in combination/MIC of B alone). The FIC values were interpreted as synergy if the values were ≤ 0.5, no interaction if the values were between 0.5 and 4.0 and antagonism if the values were > 4.0.

Biofilm assays

Biofilm assays were conducted with the peptide fragment, which demonstrated the greatest bactericidal effect against *S. mutans* strains (D1-23). This part of the study was reviewed and approved by the Animal and Human Research Ethics Committee of Araçatuba Dental School, Universidade Estadual Paulista, Brazil (Protocols: 198/2013 and #CAAE 13079213.4.0000.5420). These

assays were based on the study of Ccahuana-Vásquez and Cury (2010) with some modifications. Enamel blocks (2 mm × 2 mm × 2 mm) from bovine incisor teeth were cut and sequentially polished and selected through measurement of surface free energy (Drop Shape Analyzer – DSA100, Krüss GmbH, Hamburg, Germany) (Brambilla et al. 2012). The mean ± standard deviation (SD) of the free energy on the enamel blocks was 114 ± 15 mM m^{-1} and they were carefully randomized and distributed into three groups ($n = 6$): a negative control (culture medium), D1-23 and CHX. The enamel blocks had been previously sterilized in water in glass tubes at 121°C for 30 min and their sterility was tested before use (Amaechi et al. 1998). The enamel blocks were fixed with double sided tape to the bottom of sterile polystyrene 96-well microplates, with a U-shaped base, and pretreated with 200 μl of the stimulated saliva per well for 4 h at 37°C in 5% CO_2 (coating phase). The saliva was previously centrifuged at 3,000 × g for 10 min and the supernatant filtered through a 0.22-μm membrane filter (Corning Inc., Corning, NY, USA). After the incubation time, the saliva was removed and 10 μl of each microorganism suspension (~5–10 × 10^6 CFU ml^{-1}) was inoculated into each well containing 90 μl of BHI broth supplemented with 1% sucrose. The plates were incubated at 37° in a 5% CO_2 atmosphere. After 48 h, the culture medium was removed and the wells were washed with sterile saline (0.9% NaCl) for subsequent addition of 200 μl of D1-23, CHX and water. The concentrations used for these assays were 2 and 10 times higher than the MBC concentration. The microplates were incubated under the same conditions for 24 h. Specimens were carefully removed from the wells, washed in saline and individually transferred to microtubes containing 1 ml of saline and sonicated at 7W for 30 s (Branson, Sonifier 50, Danbury, CT, USA) to detach cells from the biofilm formed on the enamel specimens (Ccahuana-Vásquez & Cury 2010). Aliquots of the suspension were diluted and inoculated in BHI agar. The plates were incubated for 48 h at 37°C, with 5% CO_2. After this period, bacterial colonies were counted and expressed in CFU ml^{-1}.

Confocal laser scanning microscopy (CLSM)
Biofilm assays for CLSM analysis were conducted with *S. mutans* ATCC testing the peptide that showed the best antimicrobial activity on MIC/MLC assays. Enamel blocks ($n = 6$) measuring 3 mm × 3 mm × 0.5 mm were inserted in wells, as described above and the biofilm formed over 48 h was exposed to peptide (D1-23) or CHX at 10× the MBC concentration for 24 h. After this period, enamel blocks were washed once with sterile deionized water and stained with 100 μl of fluorescent Live/Dead BacLight Bacterial Viability stain (L13152, Molecular Probes, Eugene, OR, USA) containing SYTO 9 and propidium iodide,

according to the manufacturer's instructions. The excitation/emission wavelengths were 480/500 nm for SYTO 9 and 490/635 nm for propidium iodide. Two additional uninfected specimens were stained using the same protocol and used as negative controls. Fluorescence from the stained cells was viewed by CLSM (Leica TCS SP5, Leica Microsystems, Lincolnshire, IL, USA), using a 63 × oil immersion lens. CLSM images were acquired using software (LAS AF Leica Microsystems) at a resolution of 1,024 by 1,024 pixels. Ten-micrometer-deep scans (0.2-μm step size) were obtained from two randomly selected places with the CLSM. In order to analyze the Live/Dead cells ratios on the enamel slices, all scans were reconstructed in a 3-D model by the same software. The quantification of red fluorescence ratio in relation to green-and-red fluorescence and biofilm thickness were determined by software ImageJ 1.48 (NIH, Bethesda, MA, USA) (Lee et al. 2013).

Statistical analysis
Data from the cytotoxicity assays were analyzed using the ANOVA/Tukey tests in order to compare the effects of the peptide fragments on epithelial cells, considering each concentration separately. Box-whisker plots were drawn to represent the distribution of non-parametric data obtained in the biofilm assays and Mann–Whitney tests were applied to compare D1-23 with CHX for each *S. mutans* strain. CLSM data (quantification of dead cells in relation to the total cells and biofilm thickness) were converted to mean/SD and analyzed using ANOVA and Tukey tests ($p < 0.05$). SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used to run the statistical analysis.

Results

Cytotoxicity tests
Figures 1 and 2 present the percentage metabolism of HaCat and OBA-9, respectively. After exposure for 24 h, D6-17 did not affect the cell metabolism of either epithelial line at the concentrations tested. D1-23 showed toxicity at concentrations > 0.2 mM for both epithelial cells. LL-37 and CHX were the most cytotoxic peptides, demonstrating toxicity at concentrations > 0.02 mM for both cells. Figure 3 shows representative fluorescence microscope images of HaCaT cells exposed to peptide fragments at 0.1 mM. At higher intensity, DAPI-stained cells and the presence of fragmented nuclei, as a consequent of nuclear condensation and chromatin shrinkage, were observed for the LL-37 group and cells undergoing apoptosis in the CHX group. Cells treated with D1-23 and D6-17 had a similar morphology to the control group (culture medium).
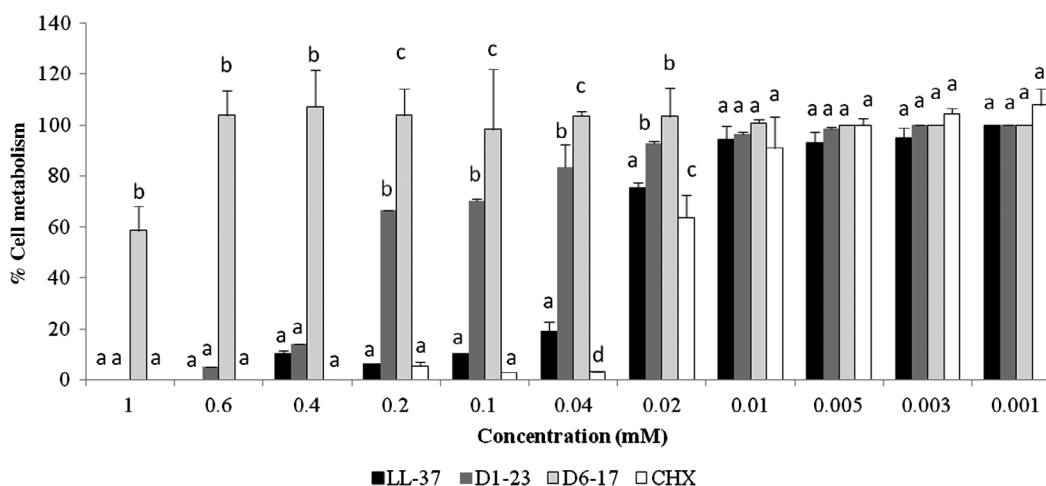
Figure 1. Mean (bars = SD) of the percentage of HaCat cell metabolism (MTT assay) after exposure to CHX and peptide fragments. Different lower case letters show statistical differences among the groups, considering each concentration separately, according to ANOVA and Tukey tests.

Figure 2. Mean (bars = SD) of the percentage of OBA-9 cell metabolism (MTT assay) after exposure to CHX and peptide fragments. Different lower case letters show statistical differences among the groups, considering each concentration separately, according to ANOVA and Tukey tests.
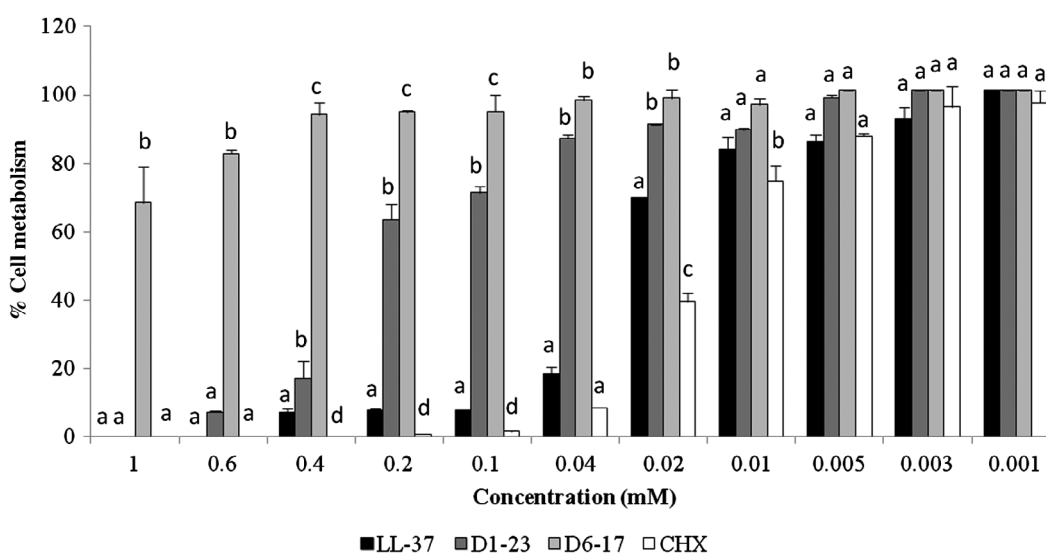
Antimicrobial activity (planktonic condition)

Table 1 shows the MIC and MBC values obtained for the cationic peptide fragments. CHX demonstrated the best antibacterial effect against all the bacteria tested. Among the peptide fragments, D1-23 demonstrated the best bactericidal activity against *S. mutans* strains, *S. mitis* and *S. salivarius* with MIC values ranging from 0.003 to 0.1 mM and MBC ranging from 0.005 to 0.2 mM. D1-23 did not have an effect on *S. oralis* or *S. sanguinis*, but it showed better results against *Lactobacillus* spp. and *Actinomyces israelii* (MIC/MBC range: 0.003–0.4 mM). LL-37 demonstrated a superior effect against the

Lactobacillus and *Actinomyces* species tested; however, its efficacy against *Streptococcus* spp. was less than D1-23. D6-17 showed bactericidal activity only against *S. mutans* strains, *L. brevis and L. fermentum*. Table 2 presents the FIC values for peptides combinations and no synergic effect was observed against *S. mutans*.

Effect against S. mutans *biofilm*

Box-whisker plots showed a reduction in the percentage of *S. mutans* after exposure for 24 h to D1-23 and CHX. Both agents improved their activity against biofilm of all *S. mutans* strains with an increase in concentration (2 to
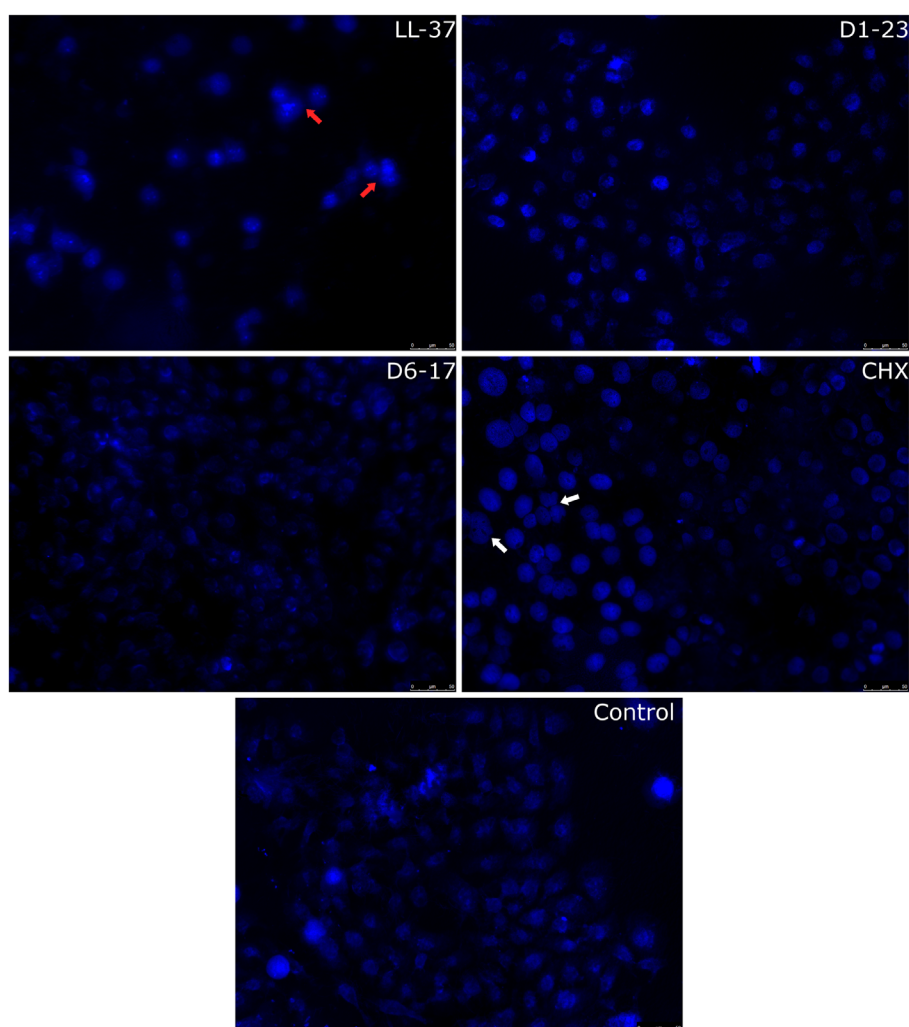
Figure 3. Fluorescence microscope images (×200) of HaCaT cells treated with peptide fragments (LL-37, D1-23 and D6-17) and CHX for 24 h. Red arrows show a higher intensity of DAPI-staining cells and the presence of fragmented nuclei observed for the LL-37 group. White arrows show cells with apoptosis in the CHX group. Control = the culture medium (DMEM).

Table 1. The MIC (and MBC)[†] values obtained for the cationic peptide fragments against oral bacteria.

	LL-37 mM	D1-23 mM	D6-17 mM	CHX mM
S. mutans (ATCC)	0.1 (0.2)	0.01 (0.02)	0.1 (0.2)	0.001 (0.003)
S. m clinical strain 1 (CS1)	0.2 (0.2)	0.003 (0.005)	0.4 (0.4)	0.001 (0.001)
S. m clinical strain 2 (CS2)	0.1 (0.1)	0.01 (0.01)	0.1 (0.2)	0.001 (0.003)
S. salivarius	0.005 (0.01)	0.005 (0.01)	0.4 (0.6)	0.001 (0.005)
S. mitis	0.01 (0.02)	0.1 (0.2)	0.6 (> 1)	0.005 (0.005)
S. oralis	0.2 (0.4)	> 1(> 1)	1(> 1)	0.02 (0.02)
S. sanguinis	> 1(> 1)	> 1(> 1)	> 1(> 1)	0.01 (0.02)
L. acidophilus	0.01 (0.01)	0.4 (0.4)	> 1(> 1)	0.003 (0.02)
L. casei	0.02 (0.2)	0.4 (> 1)	> 1(> 1)	0.003 (0.02)
L. rhamnosus	0.01 (0.01)	0.4 (0.4)	> 1 (> 1)	0.003 (0.003)
L. brevis	0.001 (0.001)	0.003 (0.003)	0.02 (0.02)	0.001 (0.001)
L. fermentum	0.001 (0.003)	0.005 (0.02)	0.4 (0.6)	0.001 (0.005)
Actinomyces israelii	0.005 (0.04)	0.02 (0.2)	0.4 (> 1)	0.005 (0.02)

[†]MIC – Minimum inhibitory concentration; MBC – minimal bactericidal concentration.

$10 \times$ MBC). At $2 \times$ MBC, CHX presented a similar effect for all the strains tested. D1-23 at $2 \times$ MBC was superior to CHX only against *S. mutans* ATCC. This strain was more sensitive to D1-23 than the other *S. mutans* strains, showing the highest bacterial reduction at $10 \times$ MBC. D1-23 demonstrated a better effect against *S. mutans* biofilms than CHX at $10 \times$ MBC, except for *S. mutans* CS1 (Figure 4). Representative images obtained from CLSM

Table 2. Values of FIC obtained by combinations of fragments of cationic peptides against *S. mutans* (ATCC 25175).

	FIC*	Effect
Double combination		
D6-17+D1-23	2.4	No interaction
D6-17+LL37	2.4	No interaction
D1-23+LL-37	2.1	No interaction
Triple combination		
D6-17+D1-23+LL-37	2.5	No interaction

*FIC index = FIC A + FIC B = (MIC of antimicrobial A in combination/MIC of A alone) + (MIC of antimicrobial B in combination/MIC of B alone). Synergism was defined as an FIC index # 0.5; no interaction as an FIC index of 0.5–4.0 and antagonism as an FIC index > 4.0.

analysis from *S. mutans* biofilm on enamel blocks are shown in Figure 5A. The D1-23 and CHX images show more dead cells (red points) when compared to the control (culture medium) images which showed mostly live cells (green points). D1-23 demonstrated greater activity against *S. mutans* biofilms than CHX and the control groups, with an average of 71% red cells, indicating cell death (Figure 5B). The biofilm thickness decreased in the D1-23 group compared to the CHX and control groups, as shown in Figure 6A and B.

Discussion

The aim of this study was to identify smaller peptide sequences with the same or better antimicrobial activity than the original oral cationic peptides, with no cytotoxicity, thereby reducing costs and difficulties with

synthesis and as well as other limitations. In the present study, three cationic peptide-derived fragments were evaluated: D6-17, D1-23 and LL37 with charge of +5, +7 and +6, pH.7.0, respectively (Dürr et al. 2006; Reynolds et al. 2010). Reynolds et al. (2010) synthesized several overlapping fragments of Defb14 (mouse ortholog of human β-defensin 3) and Defb14-1C^v (peptide with cysteines replaced with alanines except Cys$_{40}$, which resides at position V of the six-cysteine motif) and determined planktonic antimicrobial activity. Defb14-1C^v (D1-23) and Defb14-1C^v (D6-17) had the best MBCs against Gram-positive and Gram-negative bacterial strains. LL-37 was also chosen for this study based on its antimicrobial activity reported in previous studies (Gordon et al. 2005; Ouhara et al. 2005).

One limitation to the use of peptides as future drugs is their toxicity to the host cell at therapeutic concentrations. In the present study, D6-17 did not affect cell metabolism and D1-23 demonstrated good results up to 0.2 mM for both cell lines. It is well known that cationic peptides not only interact with microorganisms, but also with eukaryotic cells, causing toxicity. Hydrophobic interactions occur between anionic phosphatidylglycerol from bacterial cell membranes and with zwitterionic phosphatidylcholine and phosphatidylethanolamine from mammalian cells (Yeaman & Yount 2003). The cytotoxicity of hBD-3 fragments is related to hydrophobicity, which decreases as the length of the peptide decreases (Reynolds et al. 2010). D1-23 was more cytotoxic than D6-17 as it possesses a longer N-terminal length and consequently higher hydrophobicity (Ji et al. 2007). The present results are in
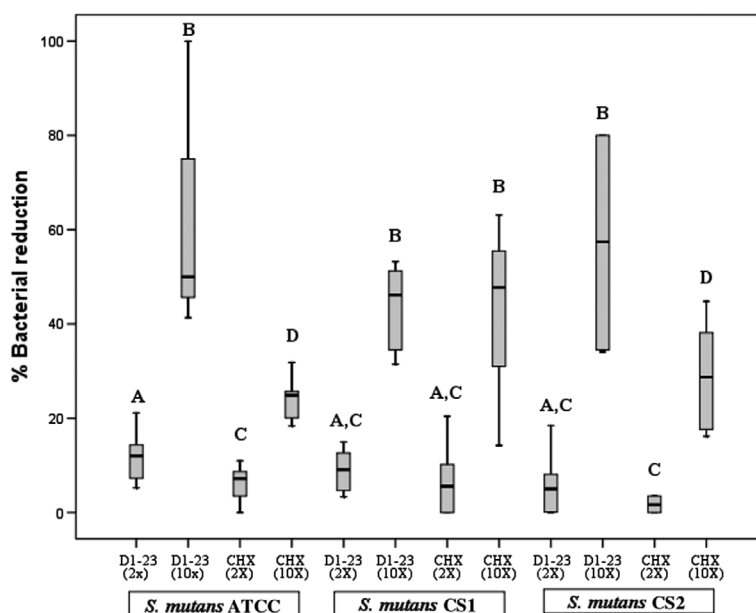

Figure 4. Box-whisker plots of the activity of D1-23 (2 × MBC and 10 × MBC) and CHX (2 × MBC and 10 × MBC) against biofilms of *S. mutans* ATCC and clinical strains (CS1 and CS2). Different upper case letters show statistical differences among the groups, considering each strain separately, according to Mann–Whitney tests. Bars indicate minimum and maximum values. Boxes indicate lower and upper quartiles. The line in the middle of the boxes is the median.
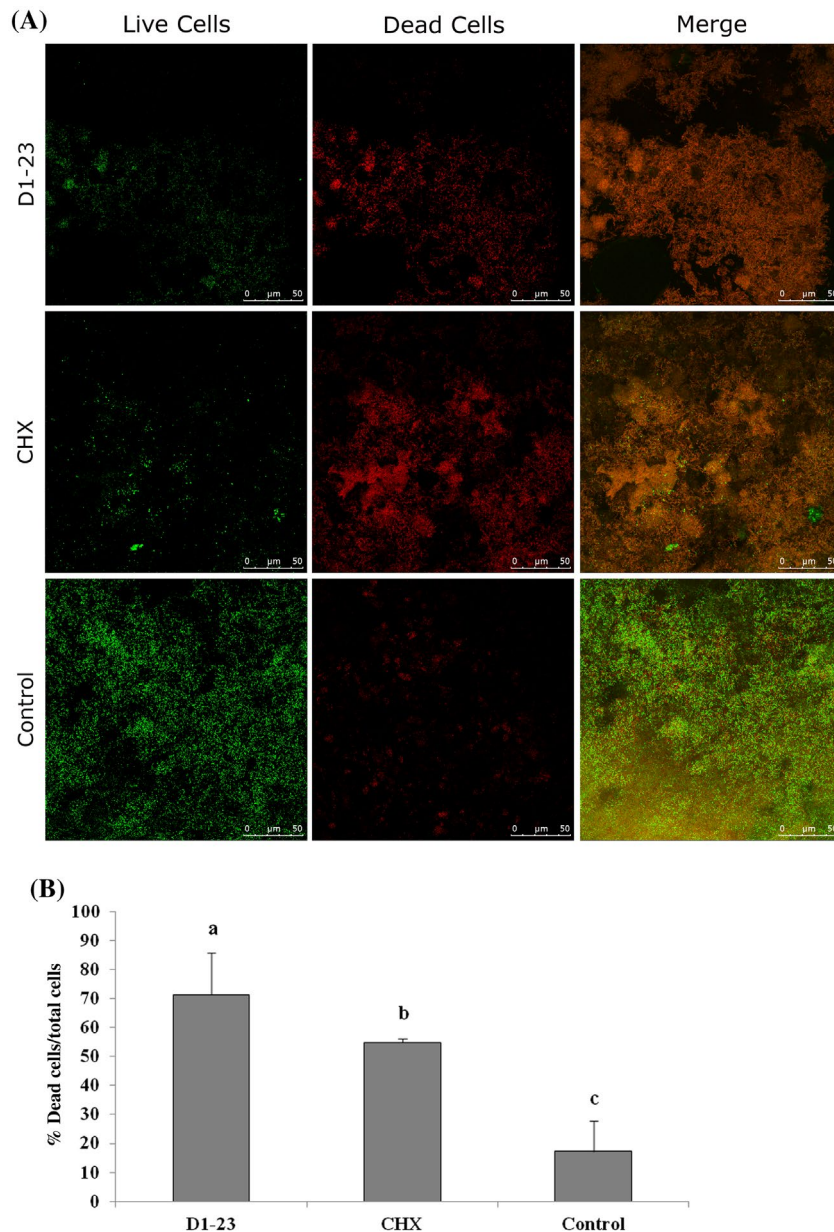
(A)

| | Live Cells | Dead Cells | Merge |

(B)

Figure 5. CLSM images of *S. mutans* biofilms on enamel blocks after exposure to D1-23 and CHX. (A) Representative images (×63 immersion) of dead cells and live cells and merge images of *S. mutans* biofilms. D1-23 and CHX showed higher quantities of dead cells (red points) when compared to the control (no treatment) which presented a strong predominance of live cells (green points). (B) Means (bars – SDs) of the percentage of dead cells obtained after CLSM analysis of *S. mutans* biofilm. Different lower case letters show statistical differences among the groups, according to ANOVA and Tukey tests ($p < 0.05$).

agreement with those of Klüver et al. (2005), who tested the cytotoxicity of hBD-3 peptides with three disulfide bonds and the analogs containing alanine and tryptophan for the cysteine residues. They showed that replacement of cysteine residues with alanine caused a reduction in the overall hydrophobicity, making the analogs less cytotoxic. Alanine has one methyl group in its side chain while cysteine has a sulfhydryl. D1-23 and D6-17 are alanine-substituted hBD-3 variants demonstrating an advantage in relation to disulfide bonds in peptides or analogs containing tryptophan, which exhibit a markedly

higher potency in reducing human monocytic cell viability (Yeaman & Yount 2003). In contrast, Liu et al. (2008) also tested the cytotoxicity of linear analogs of hBD3 and, regardless of their hydrophobicity, they showed reduced epithelial toxicity when compared with wild-type hBD3 in the concentration range of 6.25–200 µg ml⁻¹.

In the present study, LL-37 was the most toxic peptide fragment to epithelial cells, with a reduction in cytotoxicity at concentrations < 0.02 mM. DAPI fluorescence analysis confirmed the toxic effect of LL-37 at 1 mM causing fragmentation of cell nuclei. The effect of LL-37 on
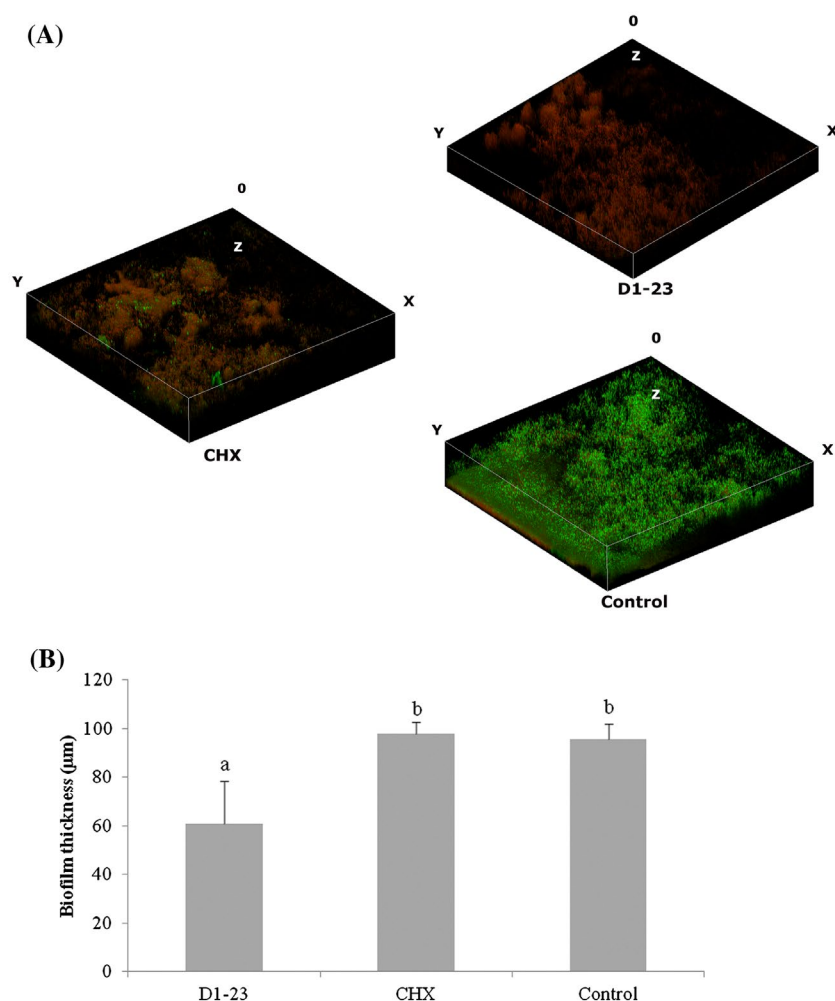
(A)

(B)

Figure 6. Biofilm thickness. (A) Representative 3-D CLSM images of *S. mutans* biofilms after exposure to D1-23 and CHX for 24 h. (B) Means (bars – SDs) of biofilm thickness obtained after CLSM analysis of *S. mutans* biofilm. Different lower case letters show statistical differences among the groups, according to ANOVA and Tukey tests ($p < 0.05$). Control = no treatment.

mammalian cells, but not epithelial cells, was first studied by Johansson et al. (1998) who observed cytotoxicity at 13–25 µM, which gradually increased at higher concentrations. In a culture of gingival epithelial cells (HGEC), similar to OBA-9 lines, obtained from gingival tissue overlying impacted third molars of patients, doses of LL-37 up to 6 µM did not significantly decrease the percentage survival of HGEC (Montreekachon et al. 2014). In the human body, a high concentration of LL-37 is controlled by its binding to plasma proteins, such as apolipoprotein A-I, reducing both cytotoxicity and antimicrobial activity (Ciornei et al. 2005). Studies have focused on more active fragments or analogs of LL-37 with a less cytotoxic effect (Johansson et al. 1998; Ciornei et al. 2005). All peptide fragments were less cytotoxic than CHX solution. Images of DAPI stained samples showed apoptosis in epithelial cells treated with CHX, as observed in different types of cells in other studies (Giannelli et al. 2008; Rocha et al. 2014). CHX has been implicated as an apoptosis-promoting

agent because it induces disturbances of mitochondrial function, and increases in intracellular Ca^{+2} and oxidative stress (Giannelli et al. 2008).

For the present study, some early colonizers (*S. sanguinis*, *S. mitis*, *S. oralis*, and *Actinomyces* spp.) and late colonizers (*S. mutans*, *S. sobrinus*, and *Lactobacillus* spp.) related to dental biofilm formation were chosen to test the antimicrobial activity of cationic peptide fragments. Among the fragments of peptides, D1-23 demonstrated the best bactericidal activity against *S. mutans* strains, *S. mitis* and *S. salivarius* and good results against *Lactobacillus* spp. and *Actinomyces israelii*. Reynolds et al. (2010) discovered that the 23-amino-acid N-terminal half of Defb14-1CV is a potent antimicrobial region while the C-terminal half is not. The regions containing the amino acids 1–10 and 6–17 demonstrated a potent bactericidal effect; however, D6-17 showed a strong inhibitory action against Gram-negative strains (Reynolds et al. 2010). In the present study, D1-23 was more effective than D6-17 against cariogenic

bacteria, confirming the better efficacy of D1-23 on Gram-positive bacteria compared to other Defb14 peptide fragments (Reynolds et al. 2010). Biofilm reduction superior to that of CHX solution was observed for D1-23 at 10 × MBC against *S. mutans* strains, except for *S. mutans* CS1. Confocal analysis also showed a greater effect against *S. mutans* biofilms and thinner biofilms for D1-23 treatment when compared to CHX and the control groups. The effect of antimicrobial agents on biofilms depends on several factors, such as depletion of the treatment in the fluid phase, penetration of the antibiotics and the physiology (stages of growth) of the biofilms. The first and last factors are probably not general causes of biofilm tolerance in *in vitro* models. However, penetration times could interfere in the ability of antibiotics to reduce biofilm biomass. Conversely, penetration times do not increase with the molecular weight of the antimicrobial agent. Even large antibiotics and antimicrobial peptides can penetrate a biofilm within a few minutes. Some examples of large agents that penetrate rapidly within biofilms are vancomycin (0.5 min), daptomycin (1.5 min), and nisin (4–10 min) (Stewart et al. 2015).

There are no previous studies evaluating the effect of D1-23 and D6-17 against biofilms. However, the original human form, hBD-3, exhibited more antibacterial activity against mature multispecies biofilms of *S. mutans*, *A. naeslundii*, *L. salivarius* and *E. faecalis* than CHX (Lee et al. 2013). Both the structure and the sequence are important for the antimicrobial activity of these β-defensin derivatives (Reynolds et al. 2010). The mechanism of action of defensins is associated with peptide binding to the bacterial cell membrane. Ionic interaction of cationic defensins with negatively charged phospholipids causes permeabilization and cell lysis (Abiko et al. 2003; Ganz 2003). Sahl et al. (2005) found that membrane depolarization contributes to rapid killing of a significant number of bacterial cells within a culture. However, subpopulations appear to survive and grow or are killed by other activities of the peptides, such as the activation of cell wall lytic enzymes.

Another important cationic peptide tested in the present study was LL-37. This peptide demonstrated a superior effect against the *Lactobacillus* and *Actinomyces* species tested; however, its action on *Streptococcus* spp. was lower when compared to D1-23. LL-37 acts on the outer membrane of bacterial cells binding with positively charged amino acids in contact with the head groups of the phospholipids. The accumulation of peptides causes small toroidal pores that lead to severe leakage. Additionally, the inner membrane is covered in a carpet-like manner, allowing intracellular targets such as DNA binding with LL-37. Electrostatic interaction with protein complexes responsible for electron transport may also occur with LL-37, generating ATP, which could lead to the disruption of membrane homeostasis (reviewed by Vandamme et al 2012). Ouhara et al. (2005) evaluated the inhibitory effect of LL-37 on the cariogenic bacteria *S. mutans*, *S. sobrinus*, *S. salivarius*, *S. sanguinis*, *S. mitis* and *L. casei* and found MICs ranging from 25–50 μg ml^{-1} (~0.01 mM), lower than obtained in the present study. In contrast to the present study, the authors found a superior effect of LL-37 against streptococci when compared to *L. casei*. In the present study, synergism was not observed among peptide fragments. LL-37 and hBD-3 had a synergic effect on killing *S. aureus* at pH 8.0 and 7.4 that was eliminated at pH 6.8 (Abou Alaiwa et al. 2014). *S. mutans* is considered highly acidogenic (van Houte et al. 1991; Mattos-Graner et al. 2014) and the pH of the culture medium could have interfered in the synergism of the peptides.

There is a demand for novel antimicrobials due to the current trend of loss of potency of commonly used antibiotics. Peptides could be an alternative to conventional antimicrobials because they selectively target prokaryotes and minimally trigger the emergence of microbial resistance. However, native peptides tend to be easily degraded, are expensive to produce and have been shown to be toxic in their active forms (Abiko et al. 2003; Wiesner & Vilcinskas 2010; Batoni et al. 2011). The design of synthetic fragments of peptides with a broad spectrum of action against bacterial pathogens, low toxicity to the host and low production costs could be useful for oral application as a method for caries prevention.

Conclusion

Of the limited number of peptide fragments tested in the present study, D1-23 showed good efficacy against cariogenic bacteria under planktonic and biofilm conditions and a low toxicity to epithelial cells.

Disclosure statement

No potential conflict of interest was reported by the authors.

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