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Activity of antimicrobial peptide mimetics in the oral cavity: I. Activity against biofilms of *Candida albicans*

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SUMMARY

Naturally occurring antimicrobial peptides hold promise as therapeutic agents against oral pathogens such as *Candida albicans* but numerous difficulties have slowed their development. Synthetic, non-peptidic analogs that mimic the properties of these peptides have many advantages and exhibit potent, selective antimicrobial activity. Several series of mimetics (with molecular weight < 1000) were developed and screened against oral *Candida* strains as a proof-of-principle for their antifungal properties. One phenylalkyne and several arylamide compounds with reduced mammalian cytotoxicities were found to be active against *C. albicans*. These compounds demonstrated rapid fungicidal activity in liquid culture even in the presence of saliva, and demonstrated synergy with standard antifungal agents. When assayed against biofilms grown on denture acrylic, the compounds exhibited potent fungicidal activity as measured by metabolic and fluorescent viability assays. Repeated passages in sub-minimum inhibitory concentration levels did not lead to resistant *Candida*, in contrast to fluconazole. Our results demonstrate the proof-of principle for the use of these compounds as anti-*Candida* agents, and their further testing is warranted as novel anti-*Candida* therapies.

INTRODUCTION

Oral infections with *Candida*, oropharyngeal candidiasis, were observed in 90% of patients undergoing chemotherapy for acute leukemia (Rodu *et al.*, 1988), and in 95% of patients infected with HIV (Dupont *et al.*, 1992). Although the introduction of highly active antiretroviral therapy has reduced these numbers in HIV-infected patients, the occurrence is still very high. In denture stomatitis, which commonly affects edentulous individuals (Webb *et al.*, 1998), *Candida* grows as a biofilm on the bioprosthetic materials used to make dentures (Edgerton *et al.*, 1993), and is highly resistant to standard antifungal agents (Chandra *et al.*, 2001a). Treatment of candidiasis is either with topical antifungal agents such as Nystatin, or with systemic agents, including azoles, such as fluconazole or itraconazole, or echinocanadins, such as capsofungin. With the recurrence of oropharyngeal candidiasis in HIV-infected patients, long-term treatments have led to a significant rise in antifungal-resistant organisms (for review, see Ghannoum & Rice, 1999).

With the initial discovery of naturally occurring, broad-spectrum antimicrobial peptides (AMPs) such as defensins and magainins (reviewed in Diamond *et al.*, 2009), activity against *Candida albicans* was examined as a potential alternative to standard antifungal treatments. The AMPs exhibit potent *in vitro*

anti-candidal activities under normal conditions (Selsted *et al.*, 1985; Zasloff, 1987; Benincasa *et al.*, 2006). Examination of human saliva also identified naturally occurring peptides known as histatins with anti-candidal activity *in vitro* (Oppenheim *et al.*, 1988), and synergy with antifungals such as azoles (Wakabayashi *et al.*, 1996). Synthetic peptides also exhibit potent activity against *Candida* species (Nikawa *et al.*, 2004; Burrows *et al.*, 2006), and using a mouse model of candidiasis, peptides derived from lactoferrin exhibited activity *in vivo* (Takakura *et al.*, 2003), suggesting that molecules derived from AMPs would be useful drugs with which to treat these infections. However, *Candida* species demonstrate an innate immune evasion strategy of proteolytic cleavage of AMPs (Meiller *et al.*, 2009).

Given their very broad specificity, amphiphilic AMPs appear to be ideal therapeutic agents. However, significant pharmaceutical issues, including poor tissue distribution, systemic toxicity and difficulty and expense of manufacturing, have severely hampered clinical progress. To address this problem, researchers have developed different types of small molecule peptide mimetics. These include peptoids, β-peptides, arylamide oligomers and phenylene ethynylenes (reviewed in Rotem & Mor, 2009). We have recently described a series of non-peptidic analogues that have many advantages over peptides because of their small size, which increases stability and enhances tissue distribution, and ability to fine-tune their physical properties for optimization of potency and safety (reviewed in Som *et al.*, 2008). These include the phenylethynylene derivative, mPE (PMX70004), which exhibits broad-spectrum antimicrobial activity against oral pathogens (Beckloff *et al.*, 2007), including three different strains of *C. albicans* and five non-albicans *Candida* species (*C. glabrata*, *C. dubliniensis*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*). Two other classes, based on arylamide and arylurea scaffolds, also demonstrate broad-spectrum antimicrobial activity (Liu *et al.*, 2004; Tang *et al.*, 2005, 2006). Together, they represent a class of AMP mimetics that exhibit the activity of AMPs, without the protease sensitivity and expense of the peptides. To assess their potential as antifungal therapies for oral *Candida* infections, we examined as a proof-of-principle the antifungal activities of representatives of the three classes of mimetics on *C. albicans*, both in planktonic states and in biofilms on denture material.

METHODS

Yeast strain

Candida albicans ATCC 90028 (obtained from the laboratory of Dr David Perlin, PHR/UMDNJ) were used for all assays and are cultured on YPD agar (1% yeast extract, 2% peptone, 2% dextrose, pH 5.7) at 37°C. For liquid assays, single colonies were dispersed in RPMI-1640 (Mediatech, Manassas, VA) with 3-(*n*-morpholino)propanesulfonic acid (MOPS), pH 7.0 at a concentration of 2.5×10^6 colony-forming units (CFU) ml^{-1}.

Peptide mimetic compounds

Twelve different peptide mimetic compounds developed by PolyMedix, Inc. (Radnor, PA) were tested in antimicrobial screens [minimum inhibitory concnetration (MIC) assay]. Further comprehensive assays were performed on the following four compounds, mPE (PMX70004), PMX30016, PMX000519 and PMX10149 (for structures, see Supporting Information Fig. S1), all of which exhibited MIC values below 8 μg ml^{-1}. All compounds were dissolved in dimethylsulfoxide (Sigma, St Louis, MO) at the stock concentration of 20 mg ml^{-1}, and stored at −20°C.

MIC assay

Assays were carried out in 96-well plates using the NCCLS method as previously described (Beckloff *et al.*, 2007). Mimetic compounds were diluted in 50 μl RPMI/MOPS in a 96-well plate (Tissue-culture-treated, BD Falcon, Bedford, MA). Suspensions of *Candida* (50 μl) were added to each well, and the plates were then incubated at 37°C in a humidified chamber for a period of 24 h. The MIC was determined as the first well without turbidity in the broth for the fungicide (mimetic compounds), or the first well without an increase of OD at 600 nm for fluconazole. A sample (25 μl) from the well defined as the MIC, plus samples from three wells with higher concentrations, were plated onto YPD agar. Colonies were counted after 24 h. The minimal fungicidal concentration (MFC) is defined as the lowest concentration at which no colonies are observed. All MIC/MFC assays were performed in duplicate.

Fungicidal kinetics

Fresh cultures of *Candida* were resuspended in RPMI-MOPS at a concentration of 2.5×10^6 CFU ml^{-1}. Samples (500 μl) were incubated in the presence of the mimetic at 37°C, and aliquots were removed at the indicated timepoints, diluted in phosphate-buffered saline and plated on YPD agar for colony counts after 24 h of growth at 37°C.

Resistance assays

Fresh cultures of *Candida* (2.5×10^6 CFU ml^{-1}) were grown in 3 ml RPMI-1640-MOPS in the presence of 0.5 × MIC of each compound at 37°C in a shaking incubator for 24 h. Then, 50 μl of the culture was plated on YPD agar and incubated at 37°C overnight. Colonies were resuspended in RPMI-MOPS and cultured under the same conditions in 0.5 × MIC. This culturing procedure was repeated serially for 20 passages. The MIC assays were performed at every other passage.

Analysis of *C. albicans* biofilms

The denture biofilm model was carried out as described previously (Chandra *et al.*, 2001a,b, 2009). Briefly, 8 × 6-cm double-thickness strips of medium thickness pink dental base plate wax (Benco Dental, Wilkes-Barre, PA) were pressed onto a wet paper towel heated on a hot plate to 65°C to give the pressed side a slightly rough surface texture. The wax strips were packed, flasked and processed into denture base acrylic (Lucitone 199® Dentsply International, Milford, DE). The resulting strips of denture acrylic had a smooth surface and a textured surface that resembled the tissue-facing aspect of a denture. These strips were then cut into 2-cm squares using a low-speed saw (Isomet®; Buehler Ltd., Lake Bluff, IL) and diamond blade with water lubricant. The cut surfaces were then polished using 600-grit silicon carbide paper (also from Buehler Ltd.) and water lubricant. The acrylic squares were stored in sterile water until use.

The denture squares were pre-coated with pooled ($n = 3$), clarified human saliva for 30 min at 37°C. Saliva was removed, and *C. albicans* was diluted to a concentration of 10^6 CFU ml^{-1}, and 100 μl was placed on the squares. The squares were incubated at 37°C in a humid chamber for 3 h. Non-adherent cells were removed, and the squares were further incubated in six-well plates in 2.5 ml RPMI-MOPS medium for 72 h at 37°C in 100% humidity. For treatment, medium was replaced with fresh RPMI-MOPS containing the peptide mimetics. To quantify viability, an XTT assay was carried out using the TOX-2 kit (Sigma) diluted according to the manufacturer's instructions in RPMI without Phenol red, and incubated for 3 h at 37°C. Metabolic activity was quantified by measuring optical density at 450 and 600 nm. Parallel cultures were scraped with a rubber scraper, resuspended in phosphate-buffered saline, and plated on YPD-agar to quantify viable colonies.

To visualize activity microscopically, *Candida* are cultured on YPD-agar plates to log phase and resuspended in RPMI-MOPS at a density of 10^8 cells ml^{-1}. Pooled, clarified human saliva was added to Lab-Tek chamber slides (Nunc, Rochester, NY) for 3 h. Then, 200 μl of the resuspended *Candida* was added to the chamber after the removal of saliva. Cells were incubated for 3 h, and non-adherent cells were removed, and the slides were washed with RPMI-MOPS. The slides were cultured for a period of 72 h before the treatment. The biofilms were visualized using the LIVE/DEAD *Bac*Light Bacterial Viability kit (InVitrogen, Carlsbad, CA) as described (Jin *et al.*, 2005). Stained slides were incubated at 30°C in a dark chamber for 20 min and the pictures were captured using a Zeiss LSM 510 on Zeiss Axiovert 100M Base (Zeiss, Thornwood, NY).

RESULTS

Fungicidal activity of mimetics against *C. albicans*

After assessing the MIC of 12 different compounds, the fungicidal kinetics of the four most active compounds was tested at 50 μg ml^{-1} (Fig. 1A). As no reduction in CFU was observed by 10 min with PMX30016, only the three remaining compounds were also tested at 10 μg ml^{-1} (Fig. 1B). Of the compounds tested, mPE exhibits the most rapid killing, with a three-log reduction in CFU after 1–5 min at 50 μg ml^{-1}. When this compound was incubated with *C. albicans* hyphae, no viable cells were visible at concentrations higher than 32 μg ml^{-1} after a 24-h incubation (data not shown), indicating that the compound could also kill cells in this form.

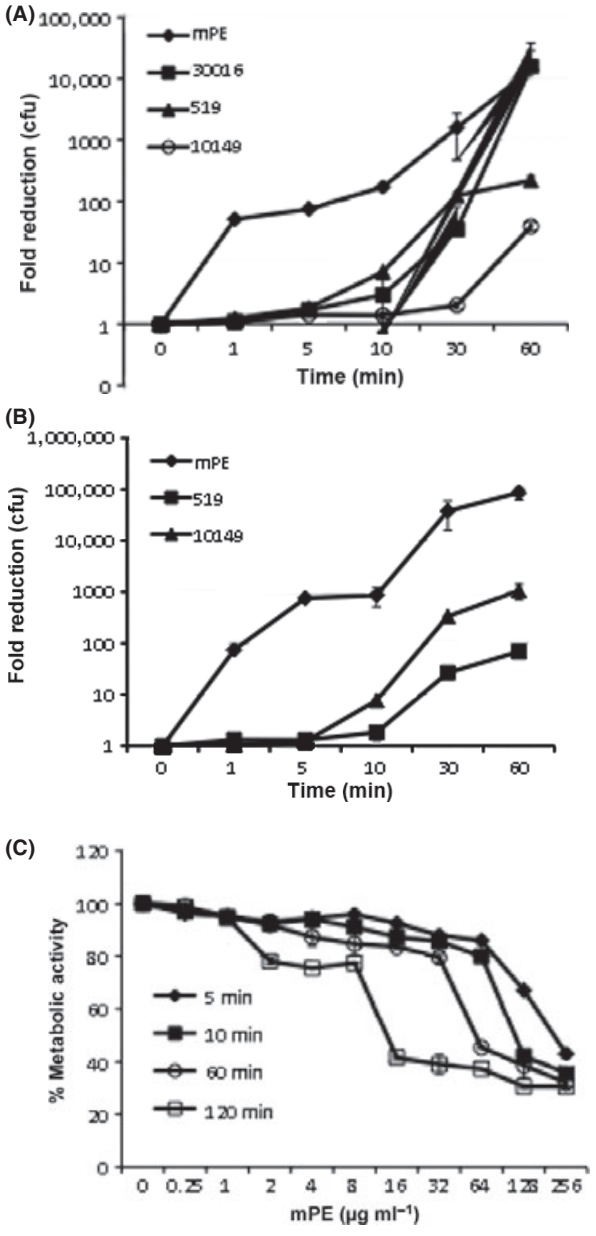

Figure 1 Kinetics of activity against *Candida albicans*: 5×10^5 CFU *C. albicans* ATCC90028 were incubated with the indicated compounds at either 50 μg ml^{-1} (A) or 10 μg ml^{-1} (B) for the times indicated. Viable CFU were quantified by plating. Results shown are the mean fold reduction in CFU ± SD of three independent experiments.

Activity against *C. albicans* biofilms

To examine the ability of the compounds to kill *C. albicans* in a biofilm, cells were grown on saliva-coated squares of polymethylmethacrylate as described elsewhere (Chandra *et al.*, 2001a,b). They were then treated with increasing concentrations of either mPE or PMX30016 for 24 h, and cell viability was measured by XTT assay. The results in Fig. 2A clearly demonstrate that incubation with both compounds resulted in a significant reduction of viable *C. albicans* in this model of *Candida* infection. To confirm that the reduction in the metabolic activity measured by the XTT assay reflects candidacidal activity, a parallel biofilm culture treated with PMX30016 was scraped from the denture material, dispersed by vortex and plated to determine viable blastoconidia. The results in Fig. 2B show a direct relationship between metabolic activity as measured by XTT assay and viable organisms.

To visualize the killing, we treated simple biofilms of *C. albicans* grown on saliva-coated plastic with 0, 50 or 100 μg ml^{-1} mPE for 24 h. The medium was removed, and the *Candida* were stained with SYTO9 and propidium iodide and visualized using fluorescence microscopy. Although this stain is usually used to visualize bacterial viability, it has been demonstrated to be effective in studying *Candida* biofilms as well (Jin *et al.*, 2005). In this case, the green dye (SYTO9) stains cells regardless of viability, while the red dye (propidium iodide) stains only cells with compromised membranes. The results in Fig. 2C demonstrate that mPE treatment results in increased membrane permeability, which allows the red fluorescent stain entrance into the fungi. Further examination of biofilms that were treated with 100 μg ml^{-1} mPE for 0, 1 or 5 min showed the rapid development of membrane permeability, with visible staining within 1 min of exposure (Fig. 2D).

Development of resistance

To determine whether growth in sub-MIC concentrations of the drugs would lead to resistant organisms, we grew *C. albicans* 90028 in 0.5 × MIC of the compounds (or fluconazole as a positive control) for 20 serial passages. After every second passage, the MIC was quantified on the growing organisms. The results shown in Fig. 3 demonstrate that no resistance developed over the passages, in contrast to fluconazole, which rapidly led to resistance.

Factors that modify *in vitro* activity

A variety of physiological factors, including salt, serum proteins and factors found in saliva, are known

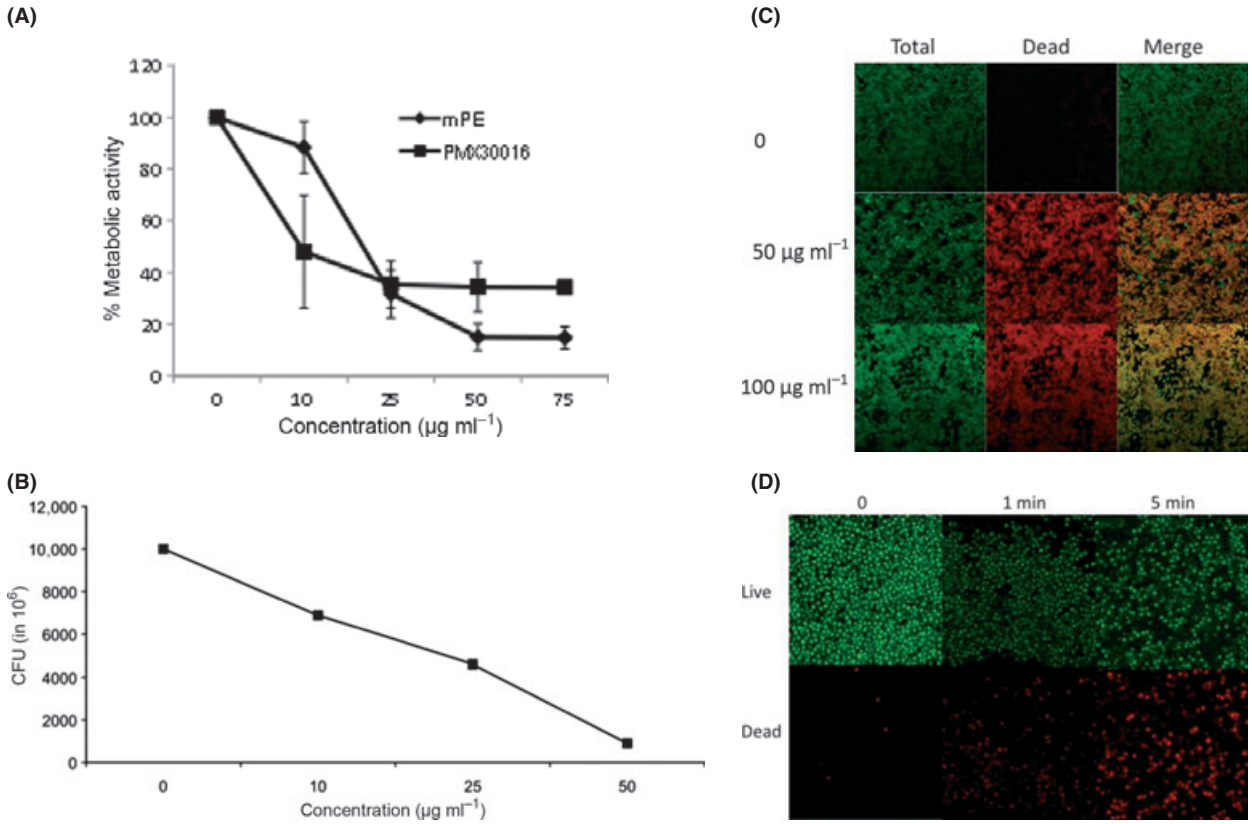

Figure 2 Activity of peptide mimetics against biofilms of *Candida albicans*. Biofilms were grown on saliva-coated denture material (A, B) or in saliva-coated wells of a chamber slide (C, D) for 3 days. Compounds were added to the growth medium for 24 h, and metabolic activity was measured by XTT assay ($n = 3$; results are mean ± SD) (A). In (B) activity was measured by incubating biofilms with PMX30016, followed by plating the fungi to quantify viable organisms (results shown are representative of three independent experiments). Visualization of killing was carried out by Live/Dead staining followed by confocal fluorescent microscopy (C). (D) Time course of exposure to 100 μg ml^{-1} mPE with Live/Dead staining.

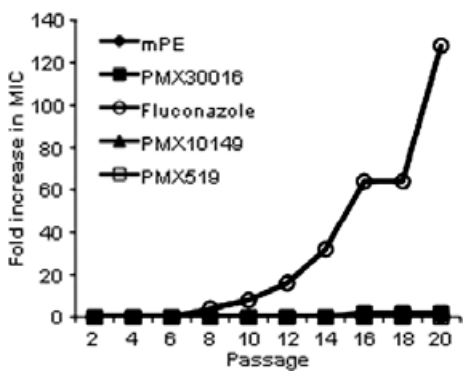

Figure 3 Development of resistance to mimetics. *Candida albicans* 90028 was grown in 0.5 × minimum inhibitory concentration (MIC) of the compounds listed, at 35°C. At every second passage, a sample was tested for MIC by standard methods.

Table 1 *In vitro* activity of mimetics in saliva

Saliva concentration (%)	Fold change in MIC			
	mPE	PMX30016	PMX519	PMX10149
0	0	0	0	0
10	0.5	0	0	0
25	0.5	0.5–0	1	0
50	0.5	0.5	1	0

MIC, minimum inhibitory concnetration.

to inhibit antimicrobial activity. For this reason, we determined the effect of these factors on the activity of the compounds against *C. albicans*. Our results shown in Table 1 demonstrate that there was no

Table 2 Synergistic activity of mimetics

Compound 1	Compound 2	FIC Index
mPE	Itraconazole	0.5
PMX30016	Itraconazole	0.2
PMX30016	Chlorhexidine	1
mPE	Chlorhexidine	1
mPE	PMX30016	1

FIC, fractional inhibitory concentration.

inhibition observed by saliva (and actually some increase in activity). These results support the further examination of the compounds as oral anti-fungal drugs. Whereas other antimicrobial peptides exhibit reduced anti-candidal activity in the presence of salt (Vylkova *et al.*, 2007b), our assays are carried out in physiological salt (154 mM), demonstrating that there is no detrimental effect of salt on this activity.

To examine the potential for combination therapy with other antifungals, we determined the activity of mPE and PMX30016 in the presence of the common oral antiseptic agent chlorhexidine and the standard antifungal agent itraconazole. The results in Table 2 demonstrate that the compound exhibits potent synergistic activity (i.e. a fractional inhibitory concentration index < 1) with itraconazole, suggesting that it could be used in conjunction with this compound.

DISCUSSION

Oral candidiasis is routinely treated with topical and systemic antifungal agents including azoles such as fluconazole and itraconazole (Vazquez, 2000). However, resistance to these agents is common, and together with the immunodeficiency that is often found in these patients, the failure of standard therapies suggests a need for novel strategies. Since their discovery, AMPs have been suggested as a useful source of antimicrobial therapeutic agents, especially because of the low development of resistance (Diamond *et al.*, 2009). Histatins are naturally occurring antifungal peptides found in human saliva, that exhibit potent activity against *C. albicans* (Oppenheim *et al.*, 1988; Helmerhorst *et al.*, 1997). Anti-candidal activity of other AMPs such as β-defensins (Vylkova *et al.*, 2007a,b), cathelicidins (Benincasa *et al.*, 2006) and piscidins (Sung *et al.*, 2008) has also been described. Indeed, the normal expression of β-defensins may be necessary for natural defense against *Candida* growth (Conti *et al.*, 2009). However, numerous technical and biological issues have made their development into useful drugs difficult. We have previously demonstrated that small mimetics based on AMP structures are highly active against a wide variety of bacterial species, including biofilms of *Streptococcus mutans* (Tew *et al.*, 2006; Beckloff *et al.*, 2007). Here we show proof-of-principle for these compounds exhibiting potent antifungal activity against *Candida* species, in both planktonic and biofilm forms, and being active in the presence of saliva.

The rapidity with which killing is observed, together with the evidence that membrane permeability occurs with a short exposure, suggests that these mimetics, like their AMP models, act on membranes, although this might not be the primary target of some peptides (for review see Brogden, 2005). Indeed, the phenylethynylene derivative we use here, mPE (Arnt *et al.*, 2006; Nusslein *et al.*, 2006), is able to cause leakage of dyes from phospholipid vesicles, at concentrations similar to those required to kill bacteria. It also promotes the loss of the membrane potential in bacteria at concentrations and over a time course that are consistent with its antimicrobial activity. Furthermore, the lack of resistance development after 20 passages at sub-MIC levels, similar to what is found with many peptides, is suggestive of physical action on membranes, as opposed to intracellular activity against a metabolic target such as is seen with histatins (Kavanagh & Dowd, 2004). This is supported by recent work using short antimicrobial peptides, demonstrating membrane activity as determined by dye-leakage and membrane depolarization experiments, as well as visualization by electron microscopy (Zhou *et al.*, 2010). Further studies on the mechanism of action of these mimetics, both against single organisms and on the biofilms, will allow a direct comparison with peptides, and will lead to a greater understanding of their activity allowing the design of optimal structures.

Together, our results support the development of these compounds as therapies against oral infections such as candidiasis. The low toxicities against mammalian cells (Tew *et al.*, 2006), activity against biofilms grown on denture materials, and the lack of resistance found with the mimetics are important features for new anti-*Candida* agents.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Structures of the compounds mPE (PMX70004), PMX30016, PMX000519 and PMX10149.

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