In vitro activity of novel biomimetic compounds against oral *Candida* strains.

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ABSTRACT

Background: Non-peptidic analogs that mimic the properties of the antimicrobial peptides (AMPs) have been developed. These synthetic analogs have many advantages over peptides because of their small size, which increases their stability and tissue penetration, and ability to fine tune their structure for optimization of potency and safety. To investigate antifungal properties, several series of mimetics (MW <1,000) were screened against oral *Candida* strains. One phenylalkyne and several arylamide compounds that were previously shown to have reduced mammalian cell cytotoxicities were found to be active against *C. albicans* and other *Candida* species , **Methods.** Minimum Inhibitory concentrations (MIC) for blastoconidia were determined using a standard broth microdilution. MIC against hyphae were defined as the lowest concentration where hyphae were no longer visible microscopically. **Results.** MICs for the phenylalkyne PMX70004 and the arylamide PMX30016 were 0.5-1.0µg/ml against blastoconidia of azole-susceptible strains of *C. albicans*, *C. dubliniensis*, *C. galabrata*, *C. parapsilosis*, and *C. tropicalis*, and against azole-resistant strains of *C. albicans* and *C. krusei*. MIC against hyphae of *C. albicans* were 4µg/ml (PMX70004) and 0.5µg/ml (PMX30016). A four-fold increase in the MIC occurred with 50% saliva. Synergistic activity was observed with the antifungal drug Itraconazole. Compounds demonstrated rapid cidal activity, where > 2 log killing was observed after 10 min at 10µg/ml. No increase in MIC was observed after 20 passages at sub-MIC concentrations. **Conclusions.** Both compounds demonstrated broad spectrum *in vitro* activity against drug susceptible and resistant stains of *Candida* in both blastoconidial and hyphal forms. Together with their low cytotoxicity, synergy with a standard antifungal agent, and lack of resistance suggests they are good candidates for further development for treatment of *Candida* infections.

INTRODUCTION

Oral candidiasis, an infection mainly caused by the fungus *C. albicans*, is an increasing problem in AIDS and other immuno-compromising diseases. This disease, which is caused by normal flora in the human body, can take many forms. The most common form of oral candidiasis is pseudomembranous candidasis, which is characterized by white patches on the oral mucosa surface. Another frequently seen form is denture stomatitis, which commonly affects edentulous individuals. This is caused by candida growing as a biofilm on the denture, and it is highly resistant to standard oral antifungal treatments such as Nystatin. Oral candidiasis can cause painful lesions, poor nutrition, and may even lead to death. It is a major cause of morbidity in the immunocompromised because it can predispose these patients to esophageal candidiasis, an invasive form with a higher risk for fatal, disseminated infection.

Although there are many antifungal treatments availabale, the long term use of these compounds in an immunocompromised patient has led to a significant rise in antifungal resistant organisms. Therefore, the discovery of novel agents that do not exhibit this problem will positively impact human health. Research has shown that antimicrobial peptides' mechanism of action prevents microbial responses that lead to resistance against toxic substances. These compounds work by forming a pore in the membrane of microorganisms, leading to the instability of the cell membrane as well as the leakage of important cell metabolites. In addition, these peptides can discriminate between a microbial cell and an animal cell due to charge differences. Small molecule mimetics of antimicrobial peptides have greater advantages in that they are less expensive, have enhanced tissue distribution, and because their physical properties can be manipulated to enhance potency and safety. The purpose of this project is to test the effectiveness of two antimicrobial peptide mimetics, PMX 30016 and mPE, in order to develop novel therapies for oral candidiasis.

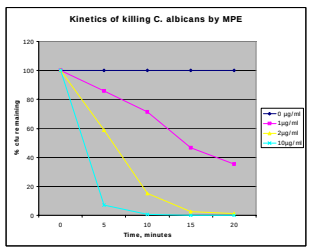
MIC ANALYSIS

MIC (24hr) (µg/ml)

Species/strain	PMX30016* MIC	mPE** MIC
C. albicans ATCC 90028	4.0	1.0
C. Albicans *ATCC 90028* Hyphae	8.0	4.0
C. albicans 44	2.0	0.5
C. albicans M70	2.0	0.5
C. dubliniensis NCPF3949	8.0	0.5
C. glabrata ATCC 90030	8.0	1.0
C. krusei ATCC 6258	4.0	1.0
C. parapsilosis ATCC 22019	4.0	1.0
C. tropicalis ATCC 750	4.0	1.0

*data obtained in subsequent experiments
**from Beckloff et al., Antimicrob. Agents Chemother., 51:4125, 2007

KINETICS OF ACTIVITY AGAINST *C.ALBICANS* 90028



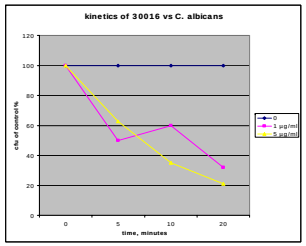


Figure 2: Kinetics of candidacidal activity of mPE and PMX 30016. Liquid cultures of *C. albicans*, diluted to 1 x 10^6 cfu/mL, were incubated with compounds at the indicated concentrations for times of 0, 5, 10, 15, 20 mins, followed by plating on YPD agar to quantify number of colonies remaining. Results are presented as percent viable colonies remaining after treatment.

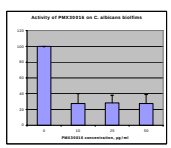
SYNERGY ASSAYS

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

Table 1: Synergy Assays. Standard checkerboard assays of PMX30016 and mPE were done to quantify the fractional inhibitory concentration when used in combination with chlorhexidine or Itraconazole. An assay was also used to check the activity of mPE in conjunction with PMX 30016. Fractional Inhibitory Concentrations determine whether activities between two drugs are synergistic or additive (Antagonistic), where an FIC<1 is synergy and an FIC>1 is antagonistic. The following equation is used to calculate FIC: FIC index = FIC(A) + FIC(B) = [A]/MIC(A) + [B]/MIC(B) , where [A] is the lowest inhibitory concentration of compound A in the presence of compound B, MIC (A) is the MIC of compound A alone, and FIC(A) is the FIC of compound A alone. [B], MIC (B) and FIC (B) are values corresponding to the compound B.

DENTURE BIOFILM MODEL

A



B

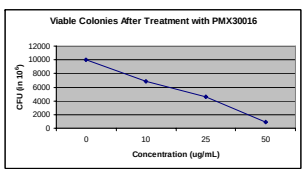


Figure 6: Dose response of PMX 30016 in a denture *C. albicans* biofilm model. In this study, fungi were seeded onto strips of poly(methyl methacrylate). Biofilms were allowed to develop for 72 hrs, and the strips were then incubated in increasing concentrations of PMX30016 over a 48 hr time period. Activities of biofilms were measured using an XTT solution, which is converted to a colored product in the presence of metabolic activity. The OD of these solutions at a wavelength of 450 was then measured and plotted against the respective concentration, shown in A. Data in A are a mean of two independent experiments (error bars=range). In addition to the XTT assay, the biofilm was scraped from each of the individual strips from one of the experiments into PBS, and dilutions were grown on YPD agar to count the amount of viable colonies present (B).

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EFFECT OF SALIVA

MIC assays were carried out against C. albicans for mPE and PMX 30016 in the presence of pooled, clarified, filtered saliva. The results shown below demonstrate a limited inhibition in the presence of 50% saliva for mPE and some synergy with PMX30016

SALIVA CONCENTRATION (%)	Fold change in MIC mPE	PMX30016
0	1	1
10	2	1
25	2-4	0.5-1
50	4	0.5

DEVELOPMENT OF RESISTANCE

To determine if C. albicans would readily develop resistance to the mimetics, blastoconidia were grown in liquid culture in YPD broth in 0.5X MIC mPE. After each passage in the same concentration mPE, MIC assays were carried out. No change in the MIC was observed after 20 passages at this subMIC concentration, suggesting that these conditions do not lead to resistance.

DISCUSSION

Our results indicate that the antimicrobial peptide mimetics mPE and PMX 30016 are very effective against the main species of Candida found in oral infections, as well as other species that have developed in resistance to currently present antifungal drugs. The MIC study shows that both compounds are similarly active against a variety of species of Candida. Kinetics studies demonstrate that both compounds act rapidly at low concentrations. In synergy assays we observed potent synergy between both compounds and Itraconazole, suggesting that a combination therapy could be useful. Furthermore, while no synergy was observed either between the two compounds or with Chlorhexidine, there was no antagonism, further supporting the use of multiple therapies.

In this study we used a denture biofilm model, which allowed for candida to be grown in a more complex environment, thereby better representing the environment of the mouth. XTT studies showed that the metabolic activity of *C.albicans* decreased as the concentration of PMX 30016 increased. In addition, electron microscopy also supports the fact that the amount of biofilm decreases with increasing concentrations of PMX 30016 (data not shown).

The results with saliva indicate that either compound could be used as a topical treatment in the oral cavity with little reduction in efficacy. Furthermore, the preliminary result with mPE suggests that unlike other antifungals, these mimetics do not readily lead to resistant strains of the organisms.

CONCLUSION

- **PMX 30016 and mPE prove to be effective agents against *C.albicans* and other candidal species in vitro.**
- **The activity of PMX 30016 and mPE are synergistic with the antifungal agent Itraconazole.**
- **PMX 30016 shows antifungal activity in a denture biofilm model.**
- **PMX 30016 is active even in the presence of saliva.**
- ***C. albicans* does not readily develop resistance to mPE.**

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