Effect of caspofungin and micafungin in combination with farnesol against *Candida parapsilosis* biofilms

Renátó Kovács[1*], Aliz Bozó[1], Rudolf Gesztelyi[2], Marianna Domán[1], Gábor Kardos[1], Fruzsina Nagy[1], Zoltán Tóth[1], László Majoros[1]

[1]Department of Medical Microbiology, University of Debrecen, Hungary

[2]Department of Pharmacology and Pharmacodynamics, University of Debrecen, Hungary.

Keywords: echinocandin, time-kill experiment, FIC index, drug interaction, nonlinear regression

*Corresponding author: Renátó Kovács, Department of Medical Microbiology, University of Debrecen, 4032 Debrecen, Nagyerdei krt. 98., Hungary

Phone: 00-36-52-255-425, Fax: 00-36-52-255-424;

e-mail: kovacs.renato@med.unideb.hu

Abstract

The *in vitro* activity of caspofungin and micafungin was determined with and without farnesol in RPMI-1640 against *Candida parapsilosis* biofilms. Drug interactions were examined using the XTT colorimetric assay-based broth microdilution checkerboard method. Drug-drug interactions were assessed utilizing a fractional inhibitory concentration index (FICI), Bliss independence and a comparison of time-kill curves. The median sessile MICs of five *C. parapsilosis* clinical isolates ranged between 32-256 mg/L, 16-512 mg/L and >300 μM for caspofungin, micafungin and farnesol, respectively. The median MICs for caspofungin and micafungin in combination with farnesol showed 8-64- and 4-64-fold decrease, respectively. Paradoxical growth noticed with both echinocandins was eliminated by farnesol. Based on FICIs, synergism was observed for caspofungin (range of median FICIs: 0.155-0.5) and micafungin (range of median FICIs: 0.093-0.5). Concordantly, MacSynergy analysis and global fitting of nonlinear regression based on a Bliss independence model showed synergism for caspofungin and micafungin, as well. In line with FICI findings and the Bliss independence model, synergistic interactions were confirmed by time-kill experiments. The metabolic activity of fungal cells was significantly inhibited by caspofungin+farnesol at all three tested combinations (4mg/L+75μM, 8mg/L+75μM, 16mg/L+75μM) between 3-24 hours compared with control ($P<0.05$-0.001). Significant inhibition was observed for micafungin+farnesol between 3-12 hours ($P<0.001$) but not at 24 hours. Despite the favorable effect of farnesol in combination with echinocandins, further *in vivo* studies are needed to confirm its therapeutic advantage in catheter-associated infections caused by *C. parapsilosis.*

1. Introduction

The frequent usage of intravascular devices predispose patients to invasive *Candida parapsilosis* infection due to the high rate of biofilm production by this pathogen, which may reach 86% [1]. Although infections caused by *C. parapsilosis* show the highest 90-day survival rate (70.7%) among the five most important *Candida* species, higher mortality appears to be associated with biofilm formation with mortality rates of 70% and 45.7% for biofilm producer and non-producer *Candida* species, respectively [2,3].

According to the guidelines of the Infectious Diseases Society of America, catheter removal is recommended in non-neutropenic patients for catheter-related bloodstream infections caused by *Candida* species [4]. However, for patients with poor venous access, an alternative, catheter salvage therapy may be a better option [5]. Data in a recent study showed that central venous catheter removal did not have a significant impact on the survival rate in surgical patients with candidemia caused by *C. parapsilosis* [6]. Therefore, administration of an antifungal lock solution might improve the outcome of biofilm-related infections caused by *C. parapsilosis* [7]. Since the number of biofilm-active antifungal drugs is limited (primarily echinocandins and lipid-associated formulations of amphotericin B), the discovery of new compounds with anti-biofilm activity has become more important in the last few years [8].

Farnesol is an isoprenoid quorum-sensing molecule, which inhibits the yeast-to-hypha transition in *C. albicans* and consequently blocks biofilm formation [9,10]. *C. albicans* produces the highest level of farnesol (35.6±16.5 μM) while *C. parapsilosis* releases less than 1 μM [11]. Nevertheless, exogenous farnesol can decrease biofilm formation in *C. parapsilosis*, probably by a pathway different from that of *C. albicans*, as *C. parapsilosis* does not form true hyphae [12,13].

Until recently little was known about drug interactions between traditional antifungal agents and farnesol against non-albicans *Candida* species. Hence, our aim in this study was to examine the *in vitro* interaction between two echinocandins (caspofungin, micafungin) and farnesol against *C. parapsilosis* biofilms.

This work was partly presented at 7th Trends in Medical Mycology meeting, 9-12 October 2015, Lisbon, Portugal, poster number: P073

2. Materials and methods

2.1 Organisms

In our preliminary experiments, twelve out of twenty-six clinical *C. parapsilosis* isolates formed biofilms; out of which five strains derived from blood culture were used in this study (16641, 17432, 17818, 10252, 9613) together with ATCC 22019 as a reference strain. Clinical isolates were identified by APID32C panel as well as matrix-assisted laser desorption/ionization time of flight mass spectrometry (Microflex, Bruker Daltronics, Bremen, Germany). Biofilm production of examined *C. parapsilosis* isolates was verified using a crystal violet assay as previously described [14].

2.2 Susceptibility testing of planktonic cells

MICs of caspofungin (Sigma, Budapest, Hungary, pure powder), micafungin (Astellas, pure powder) and farnesol (Sigma, Budapest, Hungary) were carried out by broth microdilution method in RPMI-1640 (with L-glutamine and without bicarbonate, pH 7.0 with MOPS; Sigma, Budapest, Hungary) according to the CLSI standard M27-A3 protocol [15].

Final drug concentrations ranged between 0.06-4 mg/L for caspofungin and micafungin, as well as 1.17-300 μM for farnesol. Farnesol was obtained as 3M stock solution that was diluted to a 30 mM working stock solution in 100% methanol. The working concentrations for drug were prepared in RPMI-1640. Each drug-free control well contained 1% (vol/vol) methanol [16].

The inoculum was 0.5-2.5x10^3 cells/mL. The plates were incubated for 24 hours at 37 °C. MICs were detected based on turbidity (492 nm) as at least 50% growth reduction compared

with the antifungal free control [16]. Percent of change of turbidity was calculated on the basis of absorbance (A) as 100% × (A_{well} − $A_{background}$)/($A_{drug\text{-}free\ well}$ − $A_{background}$). The background was measured from the fungus-free well [16,17].

2.3 Biofilm formation

Biofilms were grown according to the method described by Pierce and colleagues with some modifications [17]. Isolates were subcultured on Sabouraud dextrose agar. Fungal cells were harvested by centrifugation (3000g for 5 minutes) and washed three times in sterile physiological saline. After the final washing, the pellets were resuspended in approximately 5-6 mL physiological saline and counted using Burker's chamber. The final density of suspension was $1x10^6$ CFU/mL in RPMI-1640, as confirmed by quantitative culture as well [17].

A total of 100 μL of the *C. parapsilosis* suspension were pipetted into polystyrene flat-bottom 96-well microtitre plates then sealed with parafilm and incubated statically for 24 hours at 37 °C. After the incubation time, the medium was removed and the plates were washed three times [17].

2.4 Susceptibility testing of biofilms

Based on our preliminary results the examined concentrations for MIC determination in biofilms were 4-256 mg/L, 2-512 mg/L and 1.17-300 μM for caspofungin, micafungin and farnesol, respectively. Caspofungin and micafungin concentrations ranged between 0.5-32 mg/L for the sessile ATCC 22019 reference strain.

To determine the biofilm MICs, one-day-old biofilms were washed three times with 200 μL

sterile physiological saline. Different drug concentrations were added to preformed biofilms then the plates were incubated for 24 hours at 37 °C and the MICs were defined as the lowest concentration that induced at least 50% reduction in metabolic activity of fungal cells.

Metabolic activity was quantified by XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay, as previously described [16,17]. XTT solution (0.5 g/L) was supplemented with menadione (10 mM prepared in 100% acetone) to a final concentration of 1 μM. Drugs were removed prior to assay of metabolic activity by washing with physiological saline. Afterwards 100 μL aliquot of XTT/menadione solution was added to each well containing the prewashed biofilms as well as to negative control wells. Plates were covered with aluminium foil and incubated in darkness for 2 hours at 37 °C. After incubation, 80 μL supernatant from each well was measured photometrically at 492/620 nm. The percentage change of metabolic activity was calculated in the same way as changing of turbidity for planktonic cells. [16,17].

2.5 Interactions between farnesol and echinocandins

Drug interactions were measured by a two-dimensional broth microdilution checkerboard assay both for planktonic and sessile cells [16,18,19]. Based on our preliminary results the examined concentration ranges were the same as mentioned above at planktonic and sessile MIC determination.

A fractional inhibitory concentration index (FICI) was used to assess the drug interactions between farnesol and echinocandins: $\Sigma FIC=FICA+FICB=MIC_A^{comb}/MIC_A^{alone}+MIC_B^{comb}/MIC_B^{alone}$, where MIC_A^{alone} and MIC_B^{alone} are the MIC values of agents A and B used alone and MIC_A^{comb} and MIC_B^{comb} are the MICs of agents A and B when acting in combination, respectively. FICI was defined as the lowest

ΣFIC [16,18,19]. The MIC values of the drugs alone and of all isoeffective combinations were determined as the lowest drug concentrations showing at least 50% reduction of turbidity for planktonic and at least 50% reduction of metabolic activity for sessile cells compared with the untreated controls. Off-scale MIC values were converted to the next highest two-fold concentration. Synergism was defined as FICI ≤0.5, indifferent interaction as FICI between >0.5 and 4 and antagonism as FICI >4 [16,18,19].

To analyse drug-drug interactions further, we built Bliss independence models (BIs) using MacSynergy II software [20]. BI calculates the difference (ΔE) of predicted percentage of growth (E_{ind}) and experimental observed percentage of growth (E_{exp}) to define the interaction of the combination of each drug. E_{ind} is calculated by the equation: $E_{ind}=E_A \times E_B$ where E_{ind} is the predicted percentage of growth that defines the effect of combination where the drugs are acting alone. E_A and E_B are the experimental percentages of growth with each drug acting alone. The synergy volumes were calculated at the 95% level of confidence. The obtained ΔE values of each combination were represented as the *z* axis in 3-D plot [16,18]. The volumes of synergy or antagonism were given in units of $\mu M^2\%$, which are analogous to the units for area under a dose-response curve in the two-dimensional graph. MacSynergy II defines $\mu M^2\%$ threshold values in log volume between >2 to 5, 5 to 9 and >9 for minor synergy, moderate synergy and strong synergy, respectively. The corresponding negative values define minor, moderate and strong antagonism, respectively. Values between -2 and 2 $\mu M^2\%$ are considered as indifferent interaction. When a small number of drug concentration pairs results in antagonistic interaction in a generally synergistic combination, the applied terminology is "synergy for most combinations" [20].

Another procedure following the Bliss independence model, used in the present work for biofilms, was a global fitting of nonlinear regression model (recommended by the GraphPad

Software, Inc.). Curve fitting and statistical analysis were performed using GraphPad Prism 6.05, while other calculations were made by means of Microsoft Office Excel 2013.

2.6 Time-kill experiments

The findings of the checkerboard 96-well plates were confirmed by time-kill investigations for sessile *C. parapsilosis* cells. Based on the results of XTT assay, three caspofungin and micafungin concentrations were chosen (4, 8 and 16 mg/L) and their anti-biofilm effect alone and in combination with 75 μM farnesol was examined. One-day-old biofilms were washed three times with 200 μL physiological saline, then the different drug concentrations in RPMI-1640 were added to wells assigned to endpoints 3, 6, 9, 12 and 24 hours. After 3, 6, 9, 12 and 24 hours incubation, the corresponding wells were washed and the metabolic activity of the biofilm was measured as described above. Baseline metabolic activity was measured without adding the drug. Time-kill curves were prepared from the measured metabolic activity values using GraphPad Prism 6.05.

2.7 Data analysis

In time-kill experiments, one-way ANOVA with Dunnett's post-testing was used to analyse the metabolic activity reduction exerted by drugs alone and in combinations compared to control.

3. Results

The tested planktonic *C. parapsilosis* isolates were classified either susceptible or intermediate to caspofungin and micafungin according to CLSI breakpoints [21]. The median value and ranges of MIC values for planktonic and sessile *C. parapsilosis* cells are shown in Table 1 and Table 2. The median MICs for micafungin against sessile cells showed a wider range compared to caspofungin. The measured median MIC value against the sessile strain ATCC 22019 for caspofungin and micafungin was equal to that of the planktonic form (2 mg/L and 1 mg/L, respectively) (Table 2).

The median MIC values observed for caspofungin and micafungin in combination with farnesol showed a 2-64-fold decrease against planktonic cells, respectively (Table 1). A similar reduction in median MICs was observed for sessile cells (8-64-fold and 4-64-fold for caspofungin and micafungin, respectively) (Table 2). In the case of planktonic ATCC 22019, a 2- to 16-fold MIC decrease was detected while a 2-fold MIC reduction was observed for sessile cells (Table 1, Table 2). For planktonic cells farnesol MIC values were reduced 4-32-fold in combination with caspofungin, while in combination with micafungin farnesol MICs were reduced 16-256-fold. Similarly reductions in echinocandin MICs were observed with farnesol against sessile cells (4-32-fold and 8-16-fold, with caspofungin and micafungin respectively).

Table 3 and Table 4 summarize the *in vitro* interactions between farnesol and the two echinocandins based on the FICI. Antagonism was never observed (all ΣFICs <4). For planktonic cells based on FICI, synergy with caspofungin and farnesol was observed only against a single isolate (17432), however, for the combination of micafungin with farnesol synergy was demonstrated against all planktonic *C. parapsilosis* except for ATCC 22019 (Table 3).

Synergy between farnesol and micafungin as well as farnesol and caspofungin was observed for all clinical isolates when grown in biofilm (Table 4). The level of synergy was variable; the lowest level of synergy was noticed for isolate 16641 in both cases (median FICI was 0.5) (Table 4). Neither drug combinations showed synergy against the isolate ATCC 22019 in either planktonic or sessile form.

Interactions between echinocandins and farnesol against planktonic cells using MacSynergy II are shown in Table 3. Synergy for most combinations was observed both with caspofungin and micafungin (Figure 1 A-B). In accordance with FICI findings, synergism was detected using the MacSynergy software for all sessile clinical *C. parapsilosis* isolates (Table 4 Figure 1 C-D). The interactions were variable for both planktonic and sessile strain ATCC 22019; based on FICI calculations indifferent interactions were observed for both drugs. However, using MacSynergy calculations, the interaction between both echinocandins and farnesol was synergistic.

In time-kill experiments, caspofungin alone did not inhibit significantly the metabolic activity of sessile clinical isolates in the first three hours compared with the control biofilms. However, significant reduction was observed at 6, 6-9 and 6-24 hours with 4 mg/L, 8 mg/L and 16 mg/L concentrations, respectively ($P<0.05$-0.001) (Figure 2). In combination with 75 μM farnesol, a significant reduction of metabolic activity was observed for all sessile clinical *C. parapsilosis* isolates at each tested time point ($P<0.05$-0.001) (Figure 2).

The metabolic activity of biofilms of all five *C. parapsilosis* isolates were inhibited by the three tested micafungin concentrations in the first 9 hours ($P<0.05$-0.001). In addition, significant reduction of metabolic activity was observed with 16 mg/L micafungin even at 12 hours ($P<0.001$). At 24 hours, metabolic activity increased to similar levels to controls (Figure 2).

In the presence of 75 μM farnesol, 4 mg/L micafungin triggered a marked reduction in

metabolic activity in the first 6 hours for the five clinical isolates. This reduction continued in the first 9 hours with 8 mg/L micafungin+75 μM farnesol and 16 mg/L micafungin+75 μM farnesol, respectively. However, after nine hours the metabolic activity of fungal cells increased steadily; reaching similar levels to controls. In spite of this increase, the metabolic activity of sessile fungal cells was significantly lower for all of three combinations between 3 and 12 hours compared with control ($P<0.001$). At 24 hours, significant differences were never observed between the metabolic activity of fungal cells treated with combinations compared with control fungal cells (Figure 2).

Global fitting revealed paradoxical growth between 32-128 mg/L and 64-512 mg/L concentrations for caspofungin and micafungin, respectively, which was eliminated by addition of farnesol. The $logEC_{50}$ of caspofungin in combination with farnesol was -4.438, while this value was -4.552 for micafungin+farnesol. The $logEC_{50}$ of farnesol was -2.989 and -3.115 in combination with caspofungin and micafungin, respectively. Global fitting suggested potential synergistic interactions at high concentrations from 64 mg/L+75 μM farnesol and 128 mg/L+75 μM farnesol for caspofungin and micafungin, respectively. At lower concentrations an additive effect was observed.

4. Discussion

The use of central venous catheters poses a severe risk of device-related infections caused by *C. parapsilosis*, moreover, the formed biofilm may be a potential source of invasive *Candida* infection [22]. Although catheter removal is recommended for catheter-associated infections caused by *Candida* species, in certain situations this procedure does not always result in a better outcome [6]. Therefore, an antifungal lock solution or drug combinations may have a role in treatment of infections related to *Candida* biofilms.

In previous *in vitro* studies, potential lock concentrations were compared against *C. parapsilosis* biofilms. Amphotericin B deoxycholate and caspofungin alone demonstrated complete inhibition against *C. parapsilosis* biofilms. However, only one strain was used in that study [23]. In another study, amphotericin B lipid complex supplemented with EDTA was more effective compared with amphotericin B lipid complex or EDTA alone [24].

Another potential therapeutic strategy in antifungal catheter lock therapy may be the disruption of fungal quorum sensing. In 2001, farnesol was described as the first eukaryotic quorum-sensing molecule in *C. albicans* [10]. To date, its exact mode of action in *Candida* species remains little known, nevertheless, it is described as having a pivotal role in blocking filamentation and biofilm formation through the direct inhibition of Ras1 and the adenylyl-cyclase-cAMP-PKA-Efg1 pathway in *C. albicans* [25]. In addition to inhibition of biofilm production, exogenous farnesol can inhibit growth (>50 μM), and also influence lipid metabolism, alter the lipid polarization and has an effect on genes of amino acid biosynthesis and ribosome biogenesis in *C. parapsilosis* [9,12,14].

Cordeiro *et al*. observed significant MIC reductions against planktonic *C. parapsilosis* strains with higher MIC (2 mg/L) when farnesol and caspofungin were combined. The published FICI range (0.124-0.5) indicated synergistic interaction. Similar effect was observed for

fluconazole+farnesol and itraconazole+farnesol, as well as partly for amphotericin B+farnesol [26].

Unlike the findings of Cordeiro *et al*., we observed variable results regarding the interaction of caspofungin+farnesol against planktonic *C. parapsilosis* [26]. However, our calculated median FICI values were very close to the synergy threshold and the MacSynergy tests resulted in synergy for most combinations. Nevertheless, our biofilm-based results demonstrated consistent synergistic interactions for both caspofungin+farnesol and micafungin+farnesol combinations against all tested sessile clinical isolates. It is noteworthy that indifferent interactions were observed for planktonic and sessile cells of the ATCC 22019 strain based on FICI, probably due to its poorer biofilm production; synergy was detected only with MacSynergy analysis in case of most tested combinations [27].

Notably, based on FICI values and MacSynergy II analysis, higher level of synergy was observed for biofilms compared with planktonic cells both for caspofungin and micafungin. Moreover, the presence of synergy for biofilms was confirmed by time-kill investigations as well. A significant reduction in metabolic activity was detected at all tested combinations at all time points for caspofungin while significant inhibition in metabolism was observed only in the first 12 hours for micafungin.

Farnesol consistently enhanced the activity of caspofungin and micafungin against one-day-old *C. parapsilosis* biofilms, as concordantly shown in two independent experimental settings (checkerboard dilution and time-kill).

Previously, synergistic interactions exerted by farnesol with micafungin were described against *C. albicans* SC5314 biofilm using FICI and Bliss independence model [16]. A similar phenomenon was observed for combinations of farnesol and fluconazole. The interaction between farnesol and amphotericin B was variable, because there was no interaction based on FICI indices, while synergism was detected according to Bliss independence model [16].

Similar variability was observed in case of nonlinear regression models in this study, where indifferent interaction was suggested at some lower concentrations, which were found synergistic with other analyses. This variability was not found when analysing higher concentrations. Existence of such variability points to the necessity to use multiple analytic approaches in parallel when examining drug interactions.

Although farnesol may potentiate the activity of echinocandins against *C. parapsilosis* biofilms, disruption of quorum sensing may have unfavourable effects as well. It may trigger dispersion, which may lead to disseminated *Candida* infection [28]. Furthermore, biofilm may cover the catheter tip or outer surface therefore both antifungal lock and systemic antifungal therapy may be required [22]. These issues need attention before the clinical utility of farnesol can be properly assessed.

In conclusion, it is the first study that examines the effect of farnesol in combination with echinocandins against biofilm of a non-albicans species. Based on these results, farnesol showed synergistic interactions with caspofungin and micafungin against one-day-old *C. parapsilosis* biofilms. Farnesol may be a potential adjuvant in a catheter lock solution, but further *in vivo* studies are needed to assess safety and to confirm efficacy.

Acknowledgements

L. Majoros received conference travel grants from MSD, Astellas and Pfizer.

Micafungin pure powder was kindly provided by Astellas.

R. Kovács supported by the TÁMOP 4.2.4 A/2-11-1-2012-0001 National Excellence Program-Elaborating and operating an inland student and researcher personal support system. The project was subsidized by European Union and co-financed by the European Social Fund.

The authors thank David S. Perlin for a critical review of manuscript.

Ethical approval: not required

References

[1] Shin JH, Kee SJ, Shin MG, Kim SH, Shin DH, Lee SK et al. Biofilm production by isolates of *Candida* species recovered from nonneutropenic patients: comparison of bloodstream isolates with isolates from other sources. J Clin Microbiol. 2002;40:1244-8.

[2] Tumbarello M, Posteraro B, Trecarichi EM, Fiori B, Rossi M, Porta R et al. Biofilm production by *Candida* species and inadequate antifungal therapy as predictors of mortality for patients with candidemia. J Clin Microbiol. 2007;45:1843-50.

[3] Pfaller MA, Andes DR, Diekema DJ, Horn DL, Reboli AC, Rotstein C et al. Epidemiology and outcomes of invasive candidiasis due to non-albicans species of *Candida* in 2,496 patients: data from the Prospective Antifungal Therapy (PATH) registry 2004-2008. PLoS One. 2014;9:e101510.

[4] Mermel LA, Allon M, Bouza E, Craven DE, Flynn P, O'Grady NP et al. Clinical practice guidelines for the diagnosis and management of intravascular catheter-related infection: 2009 Update by the Infectious Diseases Society of America. Clin Infect Dis. 2009;49:1-45.

[5] Vassallo M, Dunais B, Roger PM. Antimicrobial lock therapy in central-line associated bloodstream infections: a systematic review. Infection. 2015 Feb 6. [Epub ahead of print]

[6] Klingspor L, Tortorano AM, Peman J, Willinger B, Hamal P, Sendid B et al. Invasive *Candida* infections in surgical patients in intensive care units: a prospective, multicentre survey initiated by the European Confederation of Medical Mycology (ECMM) (2006-2008). Clin Microbiol Infect. 2015;21:87.e1-87.e10.

[7] Walraven CJ, Lee SA. Antifungal lock therapy. Antimicrob Agents Chemother. 2013;57:1-8.

[8] Pierce CG, Srinivasan A, Uppuluri P, Ramasubramanian AK, López-Ribot JL. Antifungal therapy with an emphasis on biofilms. Curr Opin Pharmacol. 2013;13:726-30.

[9] Langford ML, Atkin AL, Nickerson KW. Cellular interactions of farnesol, a quorum-

sensing molecule produced by *Candida albicans*. Future Microbiol. 2009;4:1353-62.

[10] Hornby JM, Jensen EC, Lisec AD, Tasto JJ, Jahnke B, Shoemaker R et al. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. Appl Environ Microbiol. 2001;67:2982-92.

[11] Weber K, Sohr R, Schulz B, Fleischhacker M, Ruhnke M. Secretion of E,E-farnesol and biofilm formation in eight different *Candida* species. Antimicrob Agents Chemother. 2008;52:1859-61

[12] Rossignol T, Logue ME, Reynolds K, Grenon M, Lowndes NF, Butler G. Transcriptional response of *Candida parapsilosis* following exposure to farnesol. Antimicrob Agents Chemother. 2007;51:2304-12.

[13] Laffey SF, Butler G. Phenotype switching affects biofilm formation by *Candida parapsilosis*. Microbiology. 2005;151:1073-81.

[14] Marcos-Zambrano LJ, Escribano P, Bouza E, Guinea J. Production of biofilm by *Candida* and non-*Candida* spp. isolates causing fungemia: comparison of biomass production and metabolic activity and development of cut-off points. Int J Med Microbiol. 2014;304:1192-8.

[15] Clinical and Laboratory Standards Institute.: Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard, 3rd ed. M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA, USA, 2008.

[16] Katragkou A, McCarthy M, Alexander EL, Antachopoulos C, Meletiadis J, Jabra-Rizk MA et al. *In vitro* interactions between farnesol and fluconazole, amphotericin B or micafungin against *Candida albicans* biofilms. J Antimicrob Chemother. 2015;70:470-8.

[17] Pierce CG, Uppuluri P, Tristan AR, Wormley FL Jr, Mowat E, Ramage G et al. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nat Protoc. 2008;3:1494-500.

[18] Meletiadis J, Verweij PE, TeDorsthorst DT, Meis JF, Mouton JW. Assessing *in vitro* combinations of antifungal drugs against yeasts and filamentous fungi: comparison of different drug interaction models. Med Mycol. 2005;43:133-52.

[19] Meletiadis J, Pournaras S, Roilides E, Walsh TJ. Defining fractional inhibitory concentration index cutoffs for additive interactions based on self-drug additive combinations, Monte Carlo simulation analysis, and *in vitro-in vivo* correlation data for antifungal drug combinations against *Aspergillus fumigatus*. Antimicrob Agents Chemother. 2010;54:602-9.

[20] Prichard, MN., and C. Shipman, Jr. 1990. A three-dimensional model to analyze drug-drug interactions. Antivir. Res. 14:181-206.

[21] Pfaller MA, Diekema DJ, Andes D, Arendrup MC, Brown SD, Lockhart SR et al. Clinical breakpoints for the echinocandins and *Candida* revisited: integration of molecular, clinical, and microbiological data to arrive at species-specific interpretive criteria. Drug Resist Updat. 201;14:164-76.

[22] Nett JE. Future directions for anti-biofilm therapeutics targeting *Candida*. Expert Rev Anti Infect Ther. 2014;12:375-82.

[23] Öncü S. *In vitro* effectiveness of antifungal lock solutions on catheters infected with *Candida* species. J Infect Chemother. 2011;17:634-9.

[24] Raad II, Hachem RY, Hanna HA, Fang X, Jiang Y, Dvorak T et al. Role of ethylene diamine tetra-acetic acid (EDTA) in catheter lock solutions: EDTA enhances the antifungal activity of amphotericin B lipid complex against *Candida* embedded in biofilm. Int J Antimicrob Agents. 2008;32:515-8.

[25] Davis-Hanna A, Piispanen AE, Stateva LI, Hogan DA. Farnesol and dodecanol effects on the *Candida albicans* Ras1-cAMP signalling pathway and the regulation of morphogenesis. Mol Microbiol. 2008;67:47-62.

[26] Cordeiro RA, Teixeira CE, Brilhante RS, Castelo-Branco DS, Paiva MA, Giffoni Leite JJ et al. Minimum inhibitory concentrations of amphotericin B, azoles and caspofungin against *Candida* species are reduced by farnesol. Med Mycol. 2013;51:53-9.

[27] Tavanti A, Hensgens LA, Mogavero S, Majoros L, Senesi S, Campa M. Genotypic and phenotypic properties of *Candida parapsilosis* sensu strictu strains isolated from different geographic regions and body sites. BMC Microbiol. 2010;10:203.

[28] Uppuluri P, Chaturvedi AK, Srinivasan A, Banerjee M, Ramasubramaniam AK, Köhler JR et al. Dispersion as an important step in the *Candida albicans* biofilm developmental cycle. PLoS Pathog. 2010;6:e1000828.

Figure 1

Effect of farnesol in combination with caspofungin (A and C) and micafungin (B and D) against planktonic (A and B) and sessile (C and D) cells of a representative isolate *C. parapsilosis* 17432 using MacSynergy II analysis. Peaks of positive values represent synergy, while negative values indicate antagonism.

Figure 2

Time-kill curves of caspofungin (A) and micafungin (B) alone and in combination with farnesol against biofilm-derived five *C. parapsilosis* clinical isolates. Each time point represents the mean±SEM (error bars, standard error of mean) of the five isolates.

Table 1 Minimum inhibitory concentrations (MICs) of caspofungin and micafungin alone and in combination with farnesol against *C. parapsilosis* planktonic cells.

Drug	Isolate	Median MIC (range) of drug used (50% O.D. reduction in turbidity)			
		Alone		In combination	
		Echinocandin (mg/L)	Farnesol (µM)	Echinocandin (mg/L)	Farnesol (µM)
CAS	16641	4	300 (300->300)	0.12 (0.12-2)	37.5 (37.5-75)
	17432	4	150	0.06	18.75
	17818	4 (2-4)	150	0.06 (0.03-0.12)	37.5 (1.17-75)
	10252	4	>300	2	18.75
	9613	2 (2-4)	300 (150->300)	0.06 (0.06-2)	75 (37.5-150)
	ATCC 22019	2	75 (75-150)	1 (0.25-1)	18.75 (18.75-37.5)
MICA	16641	2 (1-4)	300 (150->300)	0.5 (0.06-0.5)	9.38 (1.17-18.75)
	17432	4	>300 (150->300)	0.06 (0.03-0.5)	2.34 (2.35-4.7)
	17818	2 (2-4)	300 (150-300)	0.12 (0.06-2)	9.38 (4.7-9.38)
	10252	2 (2-4)	>300	1 (0.25-1)	18.75 (4.7-18.75)
	9613	4 (2-4)	>300 (150->300)	0.5 (0.12-1)	37.5 (1.17-37.5)
	ATCC 22019	1	75	0.06 (0.06-0.12)	18.75

Table 2 Minimum inhibitory concentrations (MICs) of caspofungin and micafungin alone and in combination with farnesol against *C. parapsilosis* biofilms.

Drug	Isolate	Median MIC (range) of drug used (50% O.D. reduction in metabolic activity)			
		Alone		In combination	
		Echinocandin (mg/L)	Farnesol (μM)	Echinocandin (mg/L)	Farnesol (μM)
CAS	16641	32 (16-64)	>300	4 (4-8)	18.75
	17432	256	>300	4	150 (37.5-150)
	17818	256	>300	4	18.75 (18.75-75)
	10252	256	>300	4	75 (37.5-150)
	9613	256 (256-512)	>300	4	75 (37.5-75)
	ATCC 22019	2 (2-4)	>300	1 (1-2)	18.75 (4.7-75)
MICA	16641	16 (16-32)	>300	4 (2-4)	75 (2.35-75)
	17432	256 (256-512)	>300	4 (2-8)	37.5 (37.5-150)
	17818	512 (256-512)	>300	8 (4-8)	75 (4.7-75)
	10252	512 (256- >512)	>300	8 (4-8)	37.5 (9.4-37.5)
	9613	256 (256-512)	>300	8	37.5 (18.75-75)
	ATCC 22019	1 (1-2)	>300	0.5	75 (37.5-300)

Table 3 *In vitro* interactions by FIC indices (FICI) and MacSynergy II analysis of caspofungin and micafungin in combination with farnesol against *C. parapsilosis* planktonic cells.

Drug	Isolate	FICI		MacSynergy II analysis	
		Median (range) of FICI	Interaction	Synergy/antagonism (μM^2%)	Interaction
CAS	16641	0.53 (0.28-0.563)	Indifferent	2.31/-2.97	Synergy for most combinations
	17432	0.375	Synergy	34.69/-2.85	Synergy for most combinations
	17818	0.515 (0.375-0.530)	Indifferent	8.1/-1.25	Synergy for most combinations
	10252	0.530	Indifferent	28.5/-2.73	Synergy for most combinations
	9613	0.53 (0.53-0.56)	Indifferent	2.98/-2.28	Synergy for most combinations
	ATCC 22019	0.75 (0.625-1)	Indifferent	12.48/-10.54	Synergy for most combinations
MICA	16641	0.31 (0.12-0.5)	Synergy	20.09/-0.21	Synergy for most combinations
	17432	0.131 (0.016-0.5)	Synergy	51.12/0	Synergy
	17818	0.31 (0.28-0.56)	Synergy	9/-0.6	Synergy for most combinations
	10252	0.5 (0.375-0-53)	Synergy	3.83/0	Synergy
	9613	0.5 (0.5-0.56)	Synergy	8.52/0	Synergy
	ATCC 22019	0.56 (0.56-0.62)	Indifferent	78.21/-19.47	Synergy for most combinations

Table 4 *In vitro* interactions by FIC indices (FICI) and MacSynergy II analysis of caspofungin and micafungin in combination with farnesol against *C. parapsilosis* biofilms.

Drug	Isolate	FICI		MacSynergy II analysis	
		Median (range) of FICI	Interaction	Synergy/antagonism (μM^2%)	Interaction
CAS	16641	0.5 (0.375-0.5)	Synergy	80.54/0	Synergy
	17432	0.28 (0.185-0.28)	Synergy	131.13/0	Synergy
	17818	0.155 (0.141-0.155)	Synergy	126.03/0	Synergy
	10252	0.155 (0.155-0.28)	Synergy	155.01/0	Synergy
	9613	0.25 (0.156-0.25)	Synergy	273.08/0	Synergy
	ATCC 22019	0.502 (0.5-0.531)	Indifferent	38.71/-3.39	Synergy for most combinations
MICA	16641	0.5 (0.375-0.5)	Synergy	66.38/0	Synergy
	17432	0.188 (0.157-0.281)	Synergy	81.28/0	Synergy
	17818	0.14 (0.125-0.156)	Synergy	297.4/0	Synergy
	10252	0.093 (0.077-0.1925)	Synergy	337.93/0	Synergy
	9613	0.188 (0.155-0.188)	Synergy	250.53/0	Synergy
	ATCC 22019	0.625 (0.563-1)	Indifferent	15.98/-13.74	Synergy for most combinations