

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Efficacy of antifungal agents in combination with farnesol against *Candida albicans* and *Candida parapsilosis* biofilms

by Aliz Bozó

Supervisor: László Majoros, PhD



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By Aliz Bozó, biology MSc

Supervisor: László Majoros, MD, PhD

Doctoral School of Pharmaceutical Sciences, University of Debrecen

Head of the **Examination Committee:**

Árpád Tósaki, PD, PhD, DSc

Members of the Examination Committee:

Ferenc Rozgonyi, MD, PhD, DSc

Tamás Emri, PhD

The Examination takes place at the Library of Department of Pharmacology, Faculty of Medicine, University of Debrecen, 18th of June 2018 at 11:00

Head of the **Defense Committee:**

Árpád Tósaki, PD, PhD, DSc

Reviewers:

Eszter Ostorházi, MD, PhD

Ádám Kemény-Beke, MD, PhD

Members of the Defense Committee:

Ferenc Rozgonyi, MD, PhD, DSc

Tamás Emri, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 18th of June 2018 at 13:00

INTRODUCTION

The incidence and prevalence of invasive fungal infections have increased since the 1980s, especially in the population of immunocompromised patients and those who are hospitalized for long periods. Approximately 80% of invasive fungal infections are due to *Candida* species and 0.3–19% to *Aspergillus* species.

Candida species belong to the normal microbiota of an individual's mucosal oral cavity, gastrointestinal tract and vagina. Although this commensal organism normally colonizes mucosal surfaces in an asymptomatic manner, it can become one of the most significant causes of lethal infection. In the past decades, *Candida* species emerged as major causes of nosocomial infections, which are the third-fourth most common cause of bloodstream infections.

The widespread use of intravascular devices (such as cannulas, catheters, prosthetic valves, endotracheal tubes, replacement joints) in the past has paralleled the increasing incidence of catheter-associated infections. *Candida* species are the third most common cause of catheter-associated bloodstream infections. In these infections the most frequently isolated species are *C. parapsilosis* complex (42.8%), and *C. albicans* (34.7%).

Fungal biofilms complicate treatment because they increase resistance to antifungal therapies. The biofilm structure is intrinsically resistant to limited conventional antimicrobial drugs and host immune responses. Fungal biofilm formation inside or on the surface of the catheters can be a major source of invasive candidiasis. Currently IDSA (Infectious Diseases Society of America) guidelines support removal, and/or replacement of catheters to prevent the formation of biofilm on surfaces of these devices; however, this practice is not always feasible due to the patient's condition.

An alternative solution can be the use of special therapies, such as antifungal lock therapy; however there is no officially approved antifungal lock therapy to treat *Candida* biofilm. Out of the traditional antifungal agents currently in clinical use only amphotericin B (AmB) and the echinocandins have consistent activity against biofilms, but on their own they are not always effective. A new, innovative solution could be to combat biofilms using antifungal agents in combination with special communication quorum-sensing molecules produced by *Candida* species. During *Candida* biofilm formation extracellular signalling molecules accumulate in the extracellular matrix, that coordinate their metabolic activities and gene expression promote their survival. Previous studies have shown that certain quorum-sensing molecules in supraphysiological concentration inhibits growing fungal cells within the

biofilm. Based on our presumption supraphysiological/high concentrations of this compound confuses the communication between the *Candida* cells, thereby weakening the compact structure of the biofilms and allowing penetration of traditional antifungals inside the biofilm. It is important in the case of *C. parapsilosis*, which is innately less susceptible to antifungal drugs which have antifungal effect, like echinocandins. The aim of our study was to reveal the expected *in vitro* synergizing effect of quorum-sensing molecules derived from *Candida* species and to illuminate the potential therapeutic advantage of these communication molecules. Our findings support the development of new alternative therapeutic strategies which are more effective against *Candida* species biofilms.

AIMS

The primary aim of our study was to determine new antifungal strategies which help eradicate the biofilm-associated infections caused by *C. albicans* and *C. parapsilosis*. In our experiments we combined traditional antifungal agents with quorum-sensing molecules - farnesol - derived from fungi, to increase this component's *in vitro* and *in vivo* efficacy.

The aim of our study was:

- ❖ To determine the minimal inhibitory concentrations of caspofungin, micafungin and farnesol against planktonic and sessile *C. parapsilosis* clinical isolates
- ❖ To examine the interaction between caspofungin, micafungin and farnesol against sessile cells formed by *C. parapsilosis* clinical isolates by a two-dimensional broth microdilution assay.
- ❖ To examine the reduction of metabolic activity over time by caspofungin, micafungin and farnesol against sessile cells formed by *C. parapsilosis* clinical isolates.
- ❖ To determine the minimal inhibitory concentrations of fluconazole and farnesol against planktonic and sessile cells formed by fluconazole-sensitive and fluconazole-resistant clinical isolates of *C. albicans*.
- ❖ To examine the *in vitro* effect of fluconazole and farnesol in combination against fluconazole-sensitive and fluconazole-resistant planktonic and sessile clinical isolates of *C. albicans*.
- ❖ To evaluate the *in vivo* effects of fluconazole and farnesol alone and combination on vaginal fungal burden in a murine vulvovaginitis model.

MATERIALS AND METHODS

Isolates

We examined five *C. parapsilosis sensu stricto* (16641, 17432, 17818, 10252, 9613), two fluconazole (FLU)-sensitive (1216, 10431) and two FLU-resistant (21616, 27700) *C. albicans* clinical isolates and two reference strains [*C. parapsilosis* ATCC 22019 (American Type Culture Collection), (*C. albicans* SC5314)]. All tested *C. parapsilosis* clinical isolates originated from blood culture, and *C. albicans* clinical isolates were isolated from vulvovaginal candidiasis. The *C. parapsilosis* and *C. albicans* clinical isolates were identified by API® ID32C (bioMérieux, Marcy-l'Étoile, French) and MALDI-TOF [(Matrix-assisted laser desorption/ionization time of flight), (Microflex, Bruker Daltonics, Bremen, Germany)]. Biofilm production of the examined *C. parapsilosis*, *C. albicans* clinical isolates, and reference strains was verified using a crystal violet assay by Marcos-Zambrano et al.

Susceptibility testing of planktonic cells

Minimum inhibitory concentrations (MICs) of caspofungin (CAS) (pure powder; Sigma, Budapest, Hungary), micafungin (MICA) (pure powder; Astellas Pharma Inc., Tokyo, Japan) FLU (Sigma) and farnesol (Sigma) were determined using the broth microdilution method in RPMI-1640 with L-glutamine and without bicarbonate (pH 7.0) with MOPS [3-(N-morpholino) propanesulfonic acid] (Sigma) according to Clinical and Laboratory Standards Institute (CLSI) standard M27-A3 protocol. We used *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 as quality controls.

The isolates of both species' final drug concentrations ranged between 0.06 and 4 mg/L for CAS, MICA and FLU in the case of susceptible isolates, between 2-128 mg/L for FLU in the case of resistant isolates, and between 1.17-300 µM for farnesol in the case of all isolates.

The working concentrations of the drugs were prepared in RPMI-1640 (Sigma). Each drug-free control well contained 1% (v/v) methanol. The inoculum was $0.5\text{--}2.5 \times 10^3$ cells/mL. Susceptibility testing for planktonic cells was carried out in 96-well plates at 37°C for 24 h. MICs were defined based on turbidity (492 nm) as being at least 50% growth reduction compared with the drug-free control. Percentage change in turbidity was calculated on the basis of absorbance (A) as $100\% \times (A_{\text{well}} - A_{\text{background}})/(A_{\text{drug-free well}} - A_{\text{background}})$. The background was measured from the fungus-free well. All isolates were tested in three independent experiments and the median of the three values was used in the analysis.

Biofilm formation

Biofilms were prepared as described by Pierce et al. Isolates were subcultured on Sabouraud dextrose agar for two consecutive days and then the renewed strains were plated onto 1-1 Sabouraud agar plates again per isolates. The grown isolates were taken from the surface of the agar plates with a sterile swab and suspended in 25 mL sterile physiological saline. The suspensions were centrifugated for 10 minutes at 3000 g. We removed the supernatant from the cells after the centrifugation and added 25 mL fresh, sterile physiological saline to them again and repeat the last step. After the last centrifugation we removed the supernatant again and added 5-6 mL of sterile saline to the fungal cells. We then counted the suspension using a Burkler chamber of each isolates. The final density of the suspension was 1×10^6 CFU/mL in RPMI-1640 as also confirmed by quantitative culture. A total of 100 μ L of the *C. parapsilosis* and *C. albicans* suspension was pipetted into polystyrene flat-bottom 96-well microtitre plates (TPP, Trasadingen, Switzerland) and was then sealed with Parafilm and incubated statically for 24 h at 37 °C. After the incubation time, the medium was removed and the plates were washed three times. Negative control wells contained 100 μ L of RPMI-1640.

Biofilm mass determination

To determine the biofilm mass one-day-old biofilms were washed three times with 200 μ L of sterile physiological saline. In brief, 125 μ L of a 0.1% crystal violet solution was added to each well containing prewashed biofilms and incubated for 15 min at room temperature. The solution was then removed and plates were washed three times with 200 μ L physiological saline to remove excess crystal violet completely. Afterwards 125 μ L of a 30% acetic acid solution was added to each well to solubilize the biofilm bound crystal violet. After 15 min incubation at room temperature, 100 μ L supernatant was transferred to a new 96-well plate and read spectrophotometrically at 540 nm. Blank wells contained 100 μ L of 30% acetic acid.

Susceptibility testing of biofilms

The examined concentrations for MIC determination in *C. parapsilosis* biofilms were 4–256 mg/L, 2–512 mg/L and 1.17–300 μ M for CAS, MICA and farnesol, respectively. CAS and MICA concentrations ranged between 0.5–32 mg/L for the sessile *C. parapsilosis* ATCC 22019 reference strain. The concentrations tested in MIC determination in *C. albicans* biofilms were 8–512 mg/L and 1.17– 300 μ M for FLU and farnesol, respectively. To determine the 24-h biofilm MICs, the one-day-old biofilms were first washed three times with 200 μ l sterile physiological saline. All wells, including the blank ones, were filled with 100 μ l of 0.5 g/L XTT/1 μ M menadione solution. The plates were covered with aluminium foil and incubated at 37°C for 2 h. After incubation, 80 μ l of the supernatant was removed and transferred into a new 96-well plate in order to measure the absorbance spectrophotometrically at 492 nm. MICs were defined as the lowest concentration that produced at least 50% reduction in metabolic activity of fungal biofilms. The percentage change in metabolic activity was calculated in the same way as described for the measurement of turbidity for planktonic cells. All isolates were tested in three independent experiments and the median of the three values was used in the analysis.

Interactions between caspofungin, micafungin, fluconazole and farnesol

Drug interactions were measured by a two-dimensional broth microdilution checkerboard assay both for planktonic and sessile cells. The concentration ranges were same as described above for MIC determination against planktonic cells and biofilms. A fractional inhibitory concentration index (FICI) was used to assess the drug interactions between farnesol and echinocandins, as follows: $\Sigma\text{FIC} = \text{FIC}_A + \text{FIC}_B = \text{MIC}_A^{\text{comb}}/\text{MIC}_A^{\text{alone}} + \text{MIC}_B^{\text{comb}}/\text{MIC}_B^{\text{alone}}$, where $\text{MIC}_A^{\text{alone}}$ and $\text{MIC}_B^{\text{alone}}$ are the MIC values of agents A and B when used alone, and $\text{MIC}_A^{\text{comb}}$ and $\text{MIC}_B^{\text{comb}}$ are the MICs of agents A and B when acting in combination, respectively. The FICI was defined as the lowest ΣFIC . 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 or at least 50% reduction of metabolic activity for sessile cells compared to the untreated controls. The interaction between the drugs was interpreted as synergistic when FICI was ≤ 0.5 , as indifferent interaction when FICI was between > 0.5 and 4 and as antagonistic when FICI was > 4 .

Examination the changes of metabolic activity over time

The findings of fractional inhibitory concentration index were confirmed by other method, thus we investigated the changes of metabolic activity over time for sessile *C. parapsilosis* and *C. albicans* cells. Using this method provide more accurate information about the efficacy and dynamic of drugs alone and combination. The *in vitro* efficacy of CAS and MICA and combination with farnesol was studied on *C. parapsilosis* and the FLU alone and in combination with farnesol against *C. albicans* sessile cells. Based on the results of the XTT assay, three CAS and MICA concentrations were chosen (4, 8 and 16 mg/L) and their antibiofilm effect alone and in combination with 75 μ M farnesol was examined. The drug concentrations tested in the experiments over time were 0.5 mg/L, 8 mg/L, 64 mg/L, and 512 mg/L FLU with and without 75 μ M farnesol against the FLU-sensitive strains and against the reference strain, as well as 64 mg/L, 128 mg/L, 256 mg/L, and 512 mg/L FLU with and without 75 μ M farnesol against the two resistant strains.

After the biofilms were prepared, one-day-old biofilms were washed three times with 200 μ L of physiological saline. Predetermined wells were assigned to endpoints of 3, 6, 9, 12, and 24 hours, then the different drug concentrations were added in RPMI-1640 to all wells at time 0. After 3, 6, 9, 12, and 24 hours, the corresponding pre-assigned wells were washed in both plates and the biofilm mass as well as the metabolic activity of biofilms was measured as described above. Baseline biofilm mass and metabolic activity was measured from wells assigned as such prior to adding the drug. All isolates were tested in three independent experiments. Baseline metabolic activity was measured without adding the drug. Experimental results are presented in diagrams which were prepared from the measured metabolic activity values using GraphPad Prism 6.05.

Data analysis

One-way ANOVA with Dunnett's post-testing was used to analyse the biofilm mass and the reduction in metabolic activity caused by the drug alone and in combinations compared with the drug-free control. Concentrations with and without farnesol were compared with each other using one-way ANOVA with Sidak's post-testing. Significance was defined as $p < 0.05$.

***In vivo* experiments**

Animals

In our experiments we used BALB/c immunocompetent female mice (22-23 g) (Charles River), which were maintained in accordance with the *Guidelines for the Care and Use of Laboratory Animals*. Each group consisted of ten animals. The permission number of the *in vivo* experiment: 12/2014 DEMÁB.

Infection of the mice

Mice were administered 50 µl subcutaneous estradiol-valerat (10 mg/ml in sesame seed oil) three days prior to vaginal infection. For the infectious doses we plated the isolates of *C. albicans* onto Sabouraud agar plates on two consecutive days and then the renewed strains were plated onto 3-4 Sabouraud agar plates again. After 24 hours incubation at 35 °C the grown isolates were taken from the surface of the agar plates with a sterile swab and suspended in sterile saline. The suspensions were centrifugated for 10 minutes at 3000 g then we removed the supernatant from the cells. After the centrifugation we added 25 mL fresh, sterile physiological saline to them and centrifugated again. The suspensions were washed four times and after the last centrifugation we removed the supernatant and added 8 mL of sterile saline to the fungal cells. From this cell suspension we prepared 10-fold dilution in two steps and adjusted the required cell count of the infectious dose with Burker chamber. Inoculum density was confirmed by plating serial dilutions on Sabouraud dextrose agar. In accordance with our preliminary experiments, animals were infected intravaginally with $1-1.2 \times 10^7$ CFU in 25 µl physiological saline.

Antifungal therapy

All regimens were started 24 h postinfection: i) control; ii) 5 mg/kg/day; iii) 35 mg/kg fluconazole in a single dose; iv) 150 µM/day farnesol; v) 300 µM/day farnesol; 5 mg/kg/day fluconazole combined vi) with 150 µM/day farnesol and vii) with 300 µM/day farnesol.

All regimens were started 24 h postinfection. FLU doses were administered intraperitoneally at a volume of 0.5 ml, while farnesol was given intravaginally at a volume of 25 µl one hour after the FLU treatment. Farnesol was diluted in 0.25% Tween 80 solution. Control mice were given 0.5 ml physiological saline intraperitoneally as well as 25 µl 0.25% Tween 80 solution without farnesol intravaginally. At 4 days postinfection, mice were euthanized and their

vaginae were excised, weighed and homogenized aseptically. Homogenates were diluted 10-fold, aliquots of 100 μ L of the diluted and undiluted (1:10) homogenates were plated onto Sabouraud agar plates and incubated at 35 °C for 48 hours. Vaginal burden was analyzed using the Kruskal-Wallis test with Dunn's post-test (GraphPad Prism 6.05.). Significance was defined as $p < 0.05$.

RESULTS

Susceptibility testing of planktonic *Candida parapsilosis* isolates to caspofungin and farnesol using broth microdilution method

One of the tested *C. parapsilosis* clinical isolates (9613) and the reference strain (ATCC 22019) were susceptible, but isolates 16641, 17432, 10252 and 17818 were intermediate to caspofungin according to CLSI breakpoints.

The median MICs observed for caspofungin in combination with farnesol showed a 2-64-fold decrease. Farnesol MIC was reduced 4-32-fold in combination with farnesol. In the case of reference strain ATCC 22019 a 2-8-fold MIC decrease was detected in combination.

Susceptibility testing of sessile *Candida parapsilosis* isolates to caspofungin and farnesol using broth microdilution method

The median MICs for CAS against sessile cells formed by clinical isolates showed higher value (32-256 mg/L) to that of planktonic form. In combination with farnesol MICs values decreased these rates to 4 mg/L against all tested clinical isolates. This reduction in median MICs was equal 8-64-fold reduction but the median MICs for farnesol was changed 4-32-fold reduction to that of the planktonic form. The measured median MICs in the case of reference strain ATCC 22019 showed only one dilution degree. The median MICs for CAS against the planktonic strain *C. parapsilosis* ATCC 22019 for CAS was equal to the sessile form (2 mg/L), probably due to its poorer biofilm production.

Examination of the *in vitro* interactions between caspofungin and farnesol against *Candida parapsilosis* clinical isolates

For the combination of CAS and farnesol we observed indifferent interaction in all tested planktonic clinical isolates except for 17432. Synergy between farnesol and CAS was observed for all clinical isolates when grown in biofilm ($FICI_{\text{median}}$: 0.155-0.5). Antagonism was never observed (all $FICIs \leq 4$). In case of the ATCC reference strain we observed indifferent interaction against both planktonic and sessile cells.

The changes of the metabolic activity in sessile cells formed by *Candida parapsilosis* by caspofungin and farnesol

Simultaneously to the above mentioned method the changes of the metabolic activity of treated cells were compared to the changes of the metabolic activity of untreated sessile cells. The chosen CAS concentrations (4 mg/L, 8 mg/L, 16 mg/L) alone did not significantly inhibit the metabolic activity of sessile clinical isolates in the first 3 hours compared with control biofilms. However, a significant reduction was observed between 6-24 hours in a concentration dependent manner. In combination with 75 μ M farnesol(4 mg/L+75 μ M, 8 mg/L+ 75 μ M, 16 mg/L+ 75 μ M), a significant reduction in metabolic activity was observed for all sessile clinical *C. parapsilosis* isolates at each tested time point ($p<0.05$ – 0.001) compared with control biofilms. Nevertheless, after the first twelve hours where the marked reduction in metabolic activity was inherent, we observed slow growing, meaning that the combination was the most effective in the first twelve hours. Farnesol in combination with CAS (8 and 16 mg/L) was significantly better/more effective compared with CAS alone between 3-24 hours ($p<0.001$ - 0.05). CAS (4 mg/L) in combination with farnesol was significantly better just in the first 9 hours compared CAS without farnesol ($p<0.001$ - 0.05).

Susceptibility testing of planktonic *Candida parapsilosis* isolates to micafungin and farnesol using broth microdilution method

The tested *C. parapsilosis* isolates (16641, 17818, 10252) and the reference strain (ATCC 22019) were susceptible and intermediate to MICA according to CLSI breakpoints. In the cases of clinical isolates MICA in combination with farnesol caused a 2-64-fold decrease compared with the median MICs of MICA alone. For clinical isolates, farnesol in combination with MICA farnesol MICs was reduced 16-256-fold. In the case of planktonic ATCC 22019 reference strain 8-16-fold MIC decrease was detected in combination.

Susceptibility testing of sessile *Candida parapsilosis* isolates to micafungin and farnesol using broth microdilution method

Upon examination of the sessile cells formed by clinical isolates the measured median MICs were higher (16-512 mg/L) compared with planktonic cells. Median MICs for MICA in combination with farnesol decreased their rates 4-8 mg/L. Median MICs for farnesol showed

an 8-16-fold decrease in combination. In the case of ATCC 22019 a 2-fold MIC reduction was observed. The measured median MIC for MICA against the sessile sessile strain ATCC 22019 was equal to that of the planktonic form (1 mg/L).

Examination of the *in vitro* interactions between micafungin and farnesol against *Candida parapsilosis* clinical isolates

The FICI values were under 4 all times, antagonism was never observed. Synergy between MICA and farnesol was observed for all planktonic and sessile clinical isolates. In case of the sessile cells the synergistic interaction was proved by $FICI \leq 0.5$ values. We observed ≥ 0.5 values in several tested planktonic clinical isolates (17818, 10252, 9613). In the case of ATCC 22019 we observed indifferent interaction against planktonic and sessile cells.

The changes of metabolic activity for micafungin and farnesol against sessile cells formed by *Candida parapsilosis*

We can see in the dynamics of metabolic activity changes that MICA like CAS had a concentration-dependent effect on the metabolic activity of biofilms was observed for: 4 and 8 mg/L MICA in the first 9 hours ($p < 0,05$ - $0,001$), but 16 mg/L MICA in the first 12 hours decreased significantly the metabolic activity compared with the control ($p < 0,001$). At 24 hours, we did not detect significantly difference compared to the untreated cells, the metabolic activity of fungal cells increased steadily. A significant reduction in metabolic activity was observed for all three tested MICA concentration combined with farnesol (4 mg/L+75 μ M, 8 mg/L+75 μ M, 16 mg/L+75 μ M) in the first 12 hours ($p < 0,001$).

At 24 hours metabolic activity was similar levels to controls, therefore the inhibition of the metabolic activity was partially observed. Unlike the CAS, MICA triggered a marked reduction in metabolic activity in the first 9 hours in combinations. However, after the 9 hours the metabolic activity of fungal cells increased steadily, reaching similar levels to controls. Despite this increase, the metabolic activity of sessile fungal cells was significantly lower for all of three combinations between 3 and 12 hours compared with the control ($p < 0,001$).

It is noteworthy that paradoxical growth was observed for several clinical isolates. Paradoxical growth was observed exclusively in the sessile cells. This effect was revealed between 32–128 mg/L and 64–512 mg/L concentrations for CAS and MICA, respectively which was eliminated by addition of farnesol.

Susceptibility testing of planktonic *Candida albicans* isolates to fluconazole and farnesol using broth microdilution method

Two out of four tested planktonic clinical isolates and the reference strain were susceptible (SC5314, 1216, 10431), while isolates 27700 and 21616 were resistant to FLU according to CLSI breakpoints. Farnesol had no effect alone regardless of the FLU susceptibility of planktonic isolates. The MICs of farnesol in combinations decreased, however it was primarily the effect of FLU that dominated in these combinations.

Susceptibility testing of sessile *Candida albicans* isolates to fluconazole and farnesol using broth microdilution method

In combination (FLU and farnesol) farnesol caused a 2- to 64-fold decrease in the median FLU MICs against biofilms. Furthermore, median farnesol MIC values exhibited a 2- to 4-fold reduction in combination with FLU for sessile cells.

Examination of the *in vitro* interactions between fluconazole and farnesol against *Candida albicans* clinical isolates

With the combination of FLU and farnesol only indifferent interactions were detected in the case of planktonic isolates. For biofilms, synergy between FLU and farnesol was observed only against the reference strain SC5314. Against other sessile clinical isolates, the interaction was indifferent, similar to the findings with planktonic forms. Antagonism was never observed (FICIs < 4).

The changes in metabolic activity for fluconazole and farnesol against sessile cells formed by *Candida albicans*

Concentration- and time-dependent action of the drugs on the metabolic activity of biofilms was observed for all three FLU-sensitive *C. albicans* isolates. Significant reduction was observed for all tested FLU concentrations (alone and in combination) at 24 hours compared with the control curve ($p < 0.01$ – 0.001). The highest tested FLU concentration (512 mg/L) with farnesol (75 μ M) was effective even at 6 hours ($p < 0.05$). There were no significant differences between the given concentrations alone and the corresponding combinations (0.5 mg/L vs. 0.5 mg/L+75 μ M, etc.) ($p > 0.05$). In the case of resistant isolates, a marked

reduction in metabolic activity was observed at 6 hours for all tested FLU concentrations ($p < 0.001$); however, metabolic activity showed transitional increase between 6 and 12 hours. Yet, at 24 hours significantly reduced metabolic activity was observed for treated cells compared to the control biofilm for all concentrations ($p < 0.001$). At 24 hours, only the 256 mg/L + 75 μ M combination caused a significantly higher reduction in metabolic activity compared to the corresponding concentration alone (256 mg/L) ($p > 0.001$).

Examination of the *in vivo* efficacy of fluconazole using mouse vulvovaginitis model

In our *in vivo* experiments we used two FLU-susceptible and two FLU-resistant isolates. Against both FLU-susceptible *C. albicans* clinical isolates (1216, 10431), 5 mg/kg/day and a single dose of 35 mg/kg proved to be able to decrease the vaginal fungal burden significantly ($p < 0.05$ – 0.001).

Furthermore, the regimens of 5 mg/kg/day with 150 μ M/day and 300 μ M/day farnesol were also effective compared to control mice ($p < 0.05$ – 0.001). Neither farnesol dose was effective alone compared to untreated mice ($p > 0.05$). Against the FLU-resistant isolate 21616, the regimen of 5 mg/kg/day of FLU was ineffective, but when combined with farnesol it reduced vaginal burdens significantly, regardless of farnesol dose ($p < 0.05$ – 0.01). The higher FLU dose [(35 mg/kg mouse dose, approximately corresponding to the normal human dose of 150 mg based on 24h- AUC (area under curve))] was effective alone. Nevertheless, in multiple comparisons (5 mg/kg/day + 150 μ M/day farnesol; 5 mg/kg/day + 300 μ M/day farnesol) none of the combinations were superior to the single dose of 35 mg/kg FLU ($p > 0.05$). Against the FLU-resistant 27700 *C. albicans* isolate, none of the regimens were effective ($p > 0.05$).

DISCUSSION

The past decades have witnessed a dramatic increase in invasive fungal infections caused by different *Candida* species, especially in immunocompromised individuals with a prolonged hospital stay. Nowadays the most frequent isolated species is the *C. albicans* but the latest epidemiological data have shown the invasive infections caused by non-albicans species (*C. parapsilosis*, *C. glabrata*, *C. tropicalis*, *C. krusei*) increasing continuously. Considering the biofilm forming ability on biotic (host tissues) and abiotic surfaces (implanted medical devices), relatively few antifungal drugs can penetrate the biofilms associated with high mortality rate. With the antifungal drugs used in systemic infections only different AmB formulations (amphotericin B lipid complex, liposomal amphotericin B) and echinocandins can penetrate the biofilms. Echinocandins are not the primary treatment used in the case of *C. parapsilosis*, because this species has reduced echinocandin susceptibility. This is naturally associated with mutations in the conserved regions of FKS. In the course of catheter-associated infections caused by fungi, catheter removal is recommended in addition to the systemic antifungal therapy. This procedure does not always have better outcome, because biofilm can reproduce (24-48 hours) quickly on the surface of the indwelling devices, moreover the primer adherence forms after the first 90 minutes. Removal and replacement of devices is a painful surgical procedure for the patients and costly.

The prevention of biofilm formation – and catheter-associated infections – can be achieved by coating or impregnating them with antimicrobial drugs.

Using the impregnated catheters is a costly method in the treatment of catheter-associated infections, but a more innovative and cheaper technique is antifungal lock therapy, which is employed in the case of antibiotics in clinical practice. Antifungal lock therapy utilizes prolonged instillation of a solution containing high concentrations of antimicrobial agents in an infected intravascular catheter then rinsing and opening it.

Antifungal lock therapy has recently been recommended by current IDSA guidelines as a first-line option for the management of catheter-related bloodstream infections caused by coagulase-negative staphylococci and gram-negative bacteria; however, in the case of fungi there are no officially accepted guidelines. Based on previous reports, quorum-sensing molecules produced by *C. albicans* (farnesol) can increase the activity of certain antifungal drugs and may be potential adjuvants in the treatment of infections caused by different *Candida* species. The research examined only the effect against *C. albicans*. Our research

group examined the *in vitro* potential adjuvant activities of farnesol combined with caspofungin and micafungin against five different *C. parapsilosis* biofilms.

Based on our hypothesis, the exogenous farnesol in supraphysiological concentration disrupted the communication and metabolic activity of *Candida* cells in the biofilms (ergosterol synthesis pathway) and made them more susceptible to farnesol combined with traditional antifungal agents.

Therefore, the generic lower susceptibility of *C. parapsilosis* against echinocandins (which is markedly increased in biofilms) can be decreased.

In our experiments against planktonic *C. parapsilosis* cells the median MICs observed for CAS in combination with farnesol showed a significant decrease (2-64 fold; 4-32 fold) compared with MICs of CAS and farnesol alone. MICA in combination with farnesol caused a similar degree of reduction (2-64 fold; 4-32 fold). For the combination of CAS and farnesol we observed indifferent interaction in all tested planktonic clinical isolates except for 17432; however our calculated median FICI values were very close to the synergy threshold in most isolates (median FICI: 0.375-0.75).

We observed synergistic interactions for MICA and farnesol against all tested *C. parapsilosis* clinical isolates (median FICI: 0.131-0.5).

The biofilm-based results demonstrated consistent synergistic interactions both for CAS+farnesol and MICA+farnesol combinations against all tested *C. parapsilosis* clinical isolates. CAS combined with farnesol caused 8-64-fold reduction, MICA and farnesol reduced the MICs 4-32-fold compared to the median MICs alone. Based on FICIs for sessile clinical isolates, synergism was observed for CAS (range of median FICIs, 0.155–0.5) and MICA (range of median FICIs, 0.093–0.5).

Based on FICI values the presence of synergy for biofilm also confirmed changes in metabolic activity investigations. A significant reduction in metabolic activity was detected with all tested combinations at all time points for CAS, while significant inhibition in metabolism was observed only in the first 12 hours for MICA.

It is noteworthy that indifferent interactions were observed for planktonic and sessile cells of the *C. parapsilosis* ATCC 22019 strain probably due to its poorer biofilm production and its ability to form thinner biofilm layer. Farnesol consistently enhanced the activity of CAS and MICA against 1-day-old *C. parapsilosis* biofilms, as concordantly shown in two independent experimental settings.

In treatment of the biofilm-associated infections the azol-type antifungals does not suggested primarily, but exogenous quorum-sensing molecules are able to enhance the activity of

various traditional antifungal drugs against *Candida* biofilms. Perhaps may be utilize the augmentation of the antimicrobial efficacy of farnesol in the therapy of mucosal infection.

Previous studies described that farnesol shows *in vitro* synergistic interaction with different azoles against *C. albicans* which was confirmed by using immunocompetent murine vulvovaginitis model. *Candida* biofilm can form in the host cells and tissues of the oral cavity and respiratory, gastrointestinal and urinary tracts. Among women biofilm most frequently forms in the mucosal vaginal cavity, which leads to recurrent vulvovaginal candidiasis. Based on previous epidemiological studies vulvovaginitis caused by *Candida* species pose a major threat to public health, thus discovery of new compounds with antibiofilm activity has become more important due to the emergence resistance to most azol-type antifungal agents.

Cornerstone of antifungal drug therapy can be utilize quorum-sensing molecules as potential adjuvants in case of various alternative treatment strategies for disruption the biofilms on the surface of the vaginal epithelium.

Our *in vitro* research demonstrates indifferent interaction for all tested planktonic isolates, including the reference strain SC5314 in case of FLU combined with farnesol. The present study reports *in vitro* synergistic interaction against sessile SC5314 as shown by FICI. Notably, this was never observed against biofilms formed by clinical isolates, where only indifferent interactions were detected.

Despite the uniform behavior of the clinical isolates was observed unusual physiological properties in some cases. In the case of sessile azole-resistant isolates, checkerboard microdilution revealed paradoxically enhanced metabolic activity with high FLU and farnesol concentrations as compared to control wells. It was reported previously that exogenous farnesol exposure may up-regulate genes of the central carbon-metabolic pathways including glycolysis, gluconeogenesis, acetyl-CoA pathway, nitrogen metabolism and amino acid biosynthesis, which may explain the observed paradoxical enhancement of metabolic activity. Presumably, this phenomenon occurred in experiments over time with the resistant isolates (21616, 27700) accounting for the transitionally increased metabolic activity observed between 6–12 hours. Based on our *in vivo* findings neither 150 μ M nor 300 μ M farnesol alone had a protective effect against vaginal *Candida* infection. However, against the FLU-resistant isolate 21616, both farnesol regimens administered enhanced the activity of 5 mg/kg daily FLU significantly as compared to the untreated control mice ($p < 0.05$ - 0.01).

In our *in vitro* experiments we observed indifferent interaction in all tested planktonic isolates; however the only FLU-susceptible wild-type isolate against which farnesol enhances the *in vitro* activity of FLU consistently is the reference strain SC5314.

Nevertheless, the clinical importance of farnesol as a potential adjuvant remains questionable (21616 was the only isolate in this study where farnesol was able to enhance the *in vivo* activity of FLU and the effect was relatively small) and seems to be confined to FLU-resistant isolates.

In summary, this is the first study to our knowledge that examines the effect of farnesol alone and in combination with FLU *in vivo*. This work documented that vaginal administration of farnesol alone did not have protective effect in murine vulvovaginitis and it did not consistently enhance the effect of FLU either *in vitro* or *in vivo* against *C.albicans* clinical isolates. This indicates that *in vitro* findings are not directly applicable for predicting *in vivo* utility of farnesol as adjuvant; further *in vivo* experiments are needed to clarify the effect of farnesol in different types of *Candida* infection or against different species.

SUMMARY

Biofilms may be a potential source of invasive candidiasis and mucosal infections. The primary aim of this study was to examine a potential adjuvant which would enhance the *in vitro* and *in vivo* activity of antifungal agents used in the treatment. Previous *in vitro* data suggest that certain quorum-sensing molecules (e.g. farnesol) may potentiate the activity of antifungal agents, hence they may be used as an adjuvant in alternative treatment strategies. During the first part of our investigations *in vitro* interactions were examined between caspofungin, micafungin and farnesol using two-dimensional broth microdilution assays then followed the changes of metabolic activity against *C. parapsilosis* biofilms. Then *in vitro* and *in vivo* activity of fluconazole was studied alone and in combination with farnesol against *C. albicans* biofilms using classical broth microdilution „checkerboard” assay and a murine vulvovaginitis model. The median sessile MICs ranged between 32-256 mg/L and 16-512 mg/L for caspofungin and micafungin, respectively. Median MICs for caspofungin and micafungin in combination with farnesol decreased these rates to 4-8 mg/L. Based on FICIs for sessile clinical isolates, synergism was observed for caspofungin (range of median FICIs: 0.155-0.5) and micafungin (range of median FICIs: 0.093-0.5). The metabolic activity of fungal cells was inhibited by caspofungin+farnesol in all three tested combinations between 3 and 24 hours compared to the control ($p<0.05$ -0.001). Significant inhibition was observed for micafungin+farnesol in the first 12 hours ($p<0.001$). In the case of sessile *C. albicans* cells farnesol caused a 2-64-fold MIC decrease in median MICs. For biofilms, synergy between fluconazole and farnesol was observed only against the reference strain (FICIs: 0.16-0.27). *In vivo* findings revealed that farnesol alone was not protective in a murine vulvovaginitis model, but in combination it can enhance the effect of fluconazole against fluconazole-resistant isolates of *C. albicans*. Our results suggest that farnesol may be a potential adjuvant in special therapies (e.g. antifungal lock therapy) in the catheter-associated infections caused by *C. parapsilosis*, but further *in vivo* studies are needed to confirm its anti-biofilm effect in mucosal biofilms.



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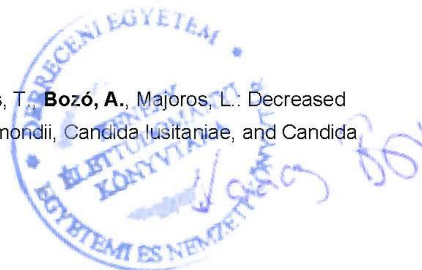
Candidate: Aliz Bozó
Neptun ID: MEOPYO
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List of publications related to the dissertation

1. Kovács, R. L., **Bozó, A.**, Gesztelyi, R., Domán, M., Kardos, G., Nagy, F., Tóth, Z., Majoros, L.:
Effect of caspofungin and micafungin in combination with farnesol against *Candida*
parapsilosis biofilms.
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DOI: <http://dx.doi.org/10.1016/j.ijantimicag.2016.01.007>
IF: 4.307
2. **Bozó, A.**, Domán, M., Majoros, L., Kardos, G., Varga, I., Kovács, R. L.: The in vitro and in vivo
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List of other publications

3. Kovács, R. L., Tóth, Z., Nagy, F., Daróczy, L., **Bozó, A.**, Majoros, L.: Activity of exogenous tyrosol
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4. Saleh, Q., Kovács, R. L., Kardos, G., Gesztelyi, R., Kardos, T., **Bozó, A.**, Majoros, L.: Decreased
Killing Activity of Micafungin Against *Candida guilliermondii*, *Candida lusitanae*, and *Candida*
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DOI: <http://dx.doi.org/10.1099/jmm.0.000116>
IF: 2.269

Total IF of journals (all publications): 21,076

Total IF of journals (publications related to the dissertation): 6,231

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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List of major presentations and posters

Renátó Kovács, **Aliz Bozó**, Marianna Domán, Fruzsina Nagy, Zoltán Tóth, László Majoros. Effect of caspofungin and micafungin in combination with farnesol against *Candida parapsilosis* biofilms. 7th Trends in Medical Mycology, 9-12 October 2015. Lisbon, Portugal (P073)

Bozó Aliz, Nagy Fruzsina, Tóth Zoltán, Kardos Tamás, Majoros László, Kovács Renátó. A caspofungin, a micafungin és a farnesol kombinációjának *in vitro* vizsgálata *Candida parapsilosis* biofilmek ellen. Magyar Mikrobiológiai Társaság Nagygyűlése és a XII. Fermentációs Kollokvium 2016. október 19-21. Keszthely.

Kovács Renátó, **Bozó Aliz**, Nagy Fruzsina, Tóth Zoltán, Majoros László. A flukonazol és a farnesol kombinációjának *in vitro-in vivo* vizsgálata *Candida albicans* biofilmek ellen. Magyar Mikrobiológiai Társaság Nagygyűlése és a XII. Fermentációs Kollokvium 2016. október 19-21. Keszthely.

Bozó Aliz, Nagy Fruzsina, Tóth Zoltán, Daróczi Lajos, Majoros László, Kovács Renátó. A caspofungin, micafungin és a tyrosol kombinációjának *in vitro* vizsgálata *Candida parapsilosis* biofilmek ellen. VI. Magyar Mikológiai Konferencia 2017. július 3-5. Szeged.

Kovács Renátó, Nagy Fruzsina, **Bozó Aliz**, Tóth Zoltán, Daróczi Lajos, Majoros László. A fluconazol, amphotericin b, caspofungin és a micafungin *in vitro* hatékonyságának vizsgálata *Candida kefyr* planktonikus és szesszilis sejtek ellen. VI. Magyar Mikológiai Konferencia 2017. július 3-5. Szeged.

Kardos Tamás, Saleh Qasem, Kovács Renátó, Gesztelyi Rudolf, Kardos Gábor, **Bozó Aliz**, Tóth Zoltán, Majoros László. A micafungin posztantifungális hatásának vizsgálata a *Candida albicans* komplex fajai ellen. VI. Magyar Mikológiai Konferencia 2017. július 3-5. Szeged.