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

Alternative therapeutic approaches against *Candida* biofilms

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UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES

DEBRECEN, 2021

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The PhD Defense takes place at the Lecture Hall of In Vitro Diagnostics Center Faculty of Medicine, University of Debrecen, 1th of September 2021 at 13:00.

Introduction

Nowadays, it has been estimated that there are 2.2 to 3.8 million fungal species worldwide; however, approximately 300 species have been described to cause human disease. Fungi cause a broad spectrum of disorders ranging from superficial to invasive infections, affecting 1.7 billion individuals worldwide and invasive fungal infections killing 1.5 million humans per year.

A significant proportion of human pathogenic fungal species are opportunistic, infecting hosts with impaired response secondary to HIV infection, degenerative or malignant diseases, as well as patients submitted to invasive medical procedures, organ transplantations, use of immunosuppressants and broad spectrum antibacterials.

The *Candida* genus is the most frequently recovered from human fungal infection. Among the *Candida* species, *Candida albicans* is the most frequently isolated species, which responsible for fungal mucosal and systemic infections of 70% around the world. *Candida* species can colonize several anatomically distinct sites including the skin, gastrointestinal and genital tract. Due to the epidemiological change in the past decades, the prevalence and incidence of non-*albicans* species, such as *C. parapsilosis, C. glabrata, C tropicalis, C. krusei, C. auris* have increased significantly. In recent years, the usage of various implanted medical devices in clinical practice is associated with the increase of the incidence of biofilm-associated infections. Biofilms formed inside or on the surface of the central venous may be the source of systemic infections, thus these medical devices are one of the most significant risk factors for candidaemia. Candidaemia episodes are associated with unacceptably high overall mortality rate exceeding 40-50% (depending on species) among patients in the intensive care unit.

Biofilms provide an excellent protection for fungal cells thanks to the their compact structure against antifungal drugs and host immune defenses. Hence, therapy of biofilm-associated infections is a remarkable clinical challenge nowadays too. The number of available antifungals is limited, moreover not all of systemic antifungal drugs can reach therapeutic concentration in matrix of biofilms. Only amphotericin B (AMB) and the echinocandins have species-dependent activity against biofilms uut of the traditional antifungal agents.

Given the increasing incidence of biofilm-associated infections and the emergence of multiresistant *Candida* species with limited efficacy to currently available antifungal agents, new approaches are absolutely needed.

It is not necessarily mean the synthesis, testing and introduction of new drugs into the clinical practice, but recent drug repurposing endeavors as alternative methods against fungal pathogen control. The combination therapies may serve as further alternative therapeutic approach against invasive fungal infections including against biofilms. Previous studies revealed the potential synergizing effect of nucleoside-analogue nikkomycin Z (NIKZ) in combination with traditional antifungal agents against *Candida* species; however, these works examined exclusively the anti-planktonic effect of these combinations.

In the future, one of the most promising anti-biofilm treatment may be a therapeutic approaches based on disruption of quorum-sensing (QS), and the usage of antifungal agents in combination with certain quorum-sensing molecules produced by *Candida* species.

QS is a special form of communication between individual microbial cells, which synchronise several physiological processes at population level, increasing the survival chances of the microbial community. The two major QS molecules secreted by *Candida* species are farnesol (FAR) and tyrosol (TYR), which have a remarkable effect on the regulation of morphogenesis, virulence and biofilm formation.

Previous studies have shown that certain quorum-sensing molecules at supraphysiological concentration inhibit growing fungal cells within the biofilm; furthermore, *in vitro* synergy between traditional antifungal agents (AMB, azoles, echinocandins) and FAR or TYR against planktonic and sessile cells have been reported for different *Candida* species. The abovementioned combination-based therapies may be superior against infections caused by Candida species with decreased susceptibility to traditional antifungal drugs. Therefore, we hope that our results will contribute to the development of new alternative treatment strategies to combat more effectively against biofilm-associated infections.

Aims

To extend our knowledge about the *in vitro* anti-biofilm activity of caspofungin (CAS) and micafungin (MICA) their activity was compared in RPMI-1640 and RPMI-1640 with 50% human serum against *C. albicans* and *C. parapsilosis* biofilms to mimic better the *in vivo* conditions. In our further experiments, the aim of our study to determine new antifungal strategies, which help to increase the activity of echinocandins enabling more effective treatment against biofilm-associated infections caused by *C. albicans* and *C. parapsilosis*.

The aim of our study was:

- To determine the minimal inhibitory concentrations (MICs) of CAS and MICA against planktonic and sessile *C. albicans* and *C. parapsilosis* isolates in the presence of 50% human serum.
- To examine the effect of human serum during the *in vitro* biofilm development.
- To examine the metabolic activity of *C. albicans* and *C. parapsilosis* isolates after CAS and MICA exposure in the presence of 50% human serum.
- To determine the minimal inhibitory concentrations of CAS, MICA and NIKZ against planktonic and sessile *C. albicans* and *C. parapsilosis* isolates.
- To examine the *in vitro* interaction between CAS, MICA and NIKZ against sessile cells formed by *C. albicans* and *C. parapsilosis* clinical isolates.
- To determine the minimal inhibitory concentrations of CAS, MICA and TYR against planktonic and sessile *C. parapsilosis* cells.
- To examine the *in vitro* effect of CAS, MICA and TYR in combination against planktonic and sessile clinical isolates of *C. parpasilosis.*

Materials and methods

Isolates

We examined 15 *C. albicans* [1822, 2009, 456, 10431, 8568, 1544, 10072, 37181, 10763, 25784, 10781, 10431, 19954, 31401, DPL18 (echinocandin-resistant strain) and 17 *C. parapsilosis sensu stricto* (26977, 8190, 19680, 17820, 4133, 6999, 29042, 16895, 18154, 22913, 16879, 16977, 22482, 27001, 10252, 17432, 9613) clinical isolates and three reference strains (*C. albicans* SC5314, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258). All tested clinical isolates originated from blood culture. The *C. albicans* and *C. parapsilosis* clinical isolates were identified by MALDI-TOF [(Matrix-assisted laser desorption/ionization time of flight), (Microflex, Bruker Daltonics, Bremen, Germany)].

Susceptibility testing of planktonic cells

MICs of CAS (Sigma, Budapest, Hungary), MICA (Sigma, Budapest, Hungary) NIKZ (Sigma, Budapest, Hungary) and TYR (Sigma, Budapest, Hungary) were determined using the broth microdilution method in RPMI-1640 with L-glutamine and without bicarbonate (pH 7.0) with MOPS [3-(Nmorpholino)propanesulfonic acid] (Sigma) according to Clinical and Laboratory Standards Institute (CLSI) standard M27-A3 protocol.

In part of our experiments, MICs of CAS and MICA against 10-10 *C. albicans* and *C. parapsilosis* clinical isolates were determined in RPMI-1640 with or without of 50% human serum (human male type AB, Sigma, Budapest, Hungary). Final drug concentrations ranged from 0.015 to 8 mg/l for CAS and MICA and between 0.06-32 mg/l for NIKZ in the case of all *Candida* isolates. The examined TYR concentrations ranged from 3.9 to 1000 μM in case of *C. parapsilosis* strains. The working concentrations of the antifungal agents and TYR were prepared in RPMI-1640. The inoculum was 0.5–2.5 $\times 10^3$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 $100\% \times (A_{well} - A_{background})/(A_{drug\text{-}free\ well} - A_{background})$. The background was measured from the fungus-free well. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 reference strains were used as quality controls for *in vitro* susceptibility testing of planktonic cells, according to CLSI.

Biofilm formation

Isolates were subcultured on Sabouraud dextrose agar (SDA) for two consecutive days and then the renewed strains were plated onto 1-1 SDA 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 Burker chamber of each isolates. The final density of the suspension was 1×10^6 CFU/ml in RPMI-1640. A total of 100 µl of the *C. albicans* and *C. parapsilosis* suspension was pipetted into polystyrene flat-bottom 96-well microtitre plates (TPP, Trasadingen, Switzerland) and incubated statically for 24 h at 37 °C. Negative control wells contained 100 µl of RPMI-1640.

Biofilm mass determination

To determine the biofilm mass one-day-old biofilms were washed three times with sterile physiological saline. In brief, 125 µl 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 physiological saline to remove excess crystal violet completely. Afterwards 125 µl of a 33% acetic acid solution was added to each well. After 15 min incubation at room temperature, 100 µl supernatant was transferred to a new 96-well plate and read spectrophotometrically at 540 nm. Blank wells contained 100 µl of 33% acetic acid.

The effect of human serum on *in vitro* biofilm development and antifungal susceptibility testing of *Candida* biofilms in presence of 50% human serum

To investigate the effect of various concentrations of human serum on adherence of *Candida* cells, the standard above-mentioned cell suspensions were supplemented with 1%, 5%, 10%, 30%, or 50% human serum. After, 2, 4, 8, 12, and 24 hours, the corresponding wells were washed three times with sterile physiological saline and the metabolic activity was measured

using XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)-assay. Following the washing process, 100 µl aliquot of XTT/menadione solution (0.5 g/l XTT solution supplemented with 1 µM menadione) was added to the wells and plates were incubated in darkness at 37◦C for 2 hours. After the incubation period, 80 µl of supernatant was aspirated from each well and measured spectrophotometrically at 492 nm. Curves were prepared from the obtained relative values of metabolic activity using GraphPad Prism 6.05 (GraphPad Software Inc. La Jolla, CA, USA). At given time point, the measured values in RPMI-1640 without human serum was considered as 100% metabolic activity. Fifty percent human serum concentration was chosen for further analysis, which mimics better the *in vivo* conditions.

During the examine of sessile *Candida* cells susceptibility against antifungals agents, the preformed biofilms were washed three times with sterile physiological saline. Afterward, MIC determination was performed in RPMI-1640 with and without 50% human serum for CAS and MICA using XTT-assay as described above. In case of *C. albicans*, the examined final concentrations of MIC determinations ranged from 0.015 to 32 mg/l for both tested echinocandins. For *C. parapsilosis*, the tested concentrations ranged from 0.015 to 512 mg/l for CAS as well as MICA. MICs of sessile cells was defined as the lowest drug concentration resulting at least 50% metabolic activity decrease compared to growth control cells for all antifungals.
Susceptibility pattern of biofilms against antifungals was evaluated using metabolic activity-based assay. Activity was calculated using the following formula: metabolic activity reduction (%)=[1-(absorbance of experimental cells/absorbance of control wells]*100.

Antifungal susceptibility testing of *Candida* biofilms

The examined echinocandin concentrations for MIC determination ranged from 0.015 to 1 mg/l, from 0.25 to 16 mg/l and from 8 to 512 mg/l for echinocandin-susceptible *C. albicans* isolates, echinocandin-resistant *C. albicans* strain (DPL18) and *C. parapsilosis* isolates respectively. Final drug concentrations ranged between 0.03 and 8 mg/l for NIKZ in case of both *Candida* species. The examined TYR concentrations ranged from 3.9 to 1000 µM in case of *C. parapsilosis* strains. One-day-old biofilms were washed three times with sterile physiological saline. The working concentrations of the antifungal agents and TYR were prepared in RPMI-1640, from which 100 µl was measured in wells of microtiter plate. The drug-treated biofilms were incubated at 37 °C for 24 h. After the incubation time, the plates were washed three times with sterile physiological saline. Afterwards, MIC determination was performed in RPMI-1640

using the XTT-assay (492 nm) as described previously. MICs of biofilms were defined as the lowest drug concentration resulting in at least 50% metabolic activity reduction compared to growth control cells. The percentage change in metabolic activity was calculated on the basis of absorbance (A) as 100% * (A_{well} - $A_{background}$)/($A_{drug\text{-}freewell}$ - $A_{background}$).

Interactions between antifungals and tyrosol

Interactions between echinocandins, NIKZ and TYR were assessed using two-dimensional broth microdilution checkerboard assay for planktonic and sessile *Candida* cells. Based on the preliminary results, the examined concentration ranges were the same as mentioned above for planktonic and sessile MIC determination. Interactions were then analysed using Fractional Inhibitory Concentration Index (FICI) determination, which was calculated using the following formula: $\Sigma FIC = FIC_A + FIC_B = MIC_A^{comb} / MIC_A^{alone} + MIC_B^{comb} / MIC_B^{alone}$, where MIC_A^{alone} and MIC_B^{alone} stand for MICs of drugs A and B when used alone, and MIC_A^{comb} and MIC_B^{comb} represent the MIC values of drugs A and B in combination at isoeffective combinations respectively. In case of all isoeffective combinations were determined FIC value and FICI was defined as the lowest ΣFIC. The interactions were considered as synergistic when FICI was ≤ 0.5 no interaction when FICI was between > 0.5 and 4 and antagonism when FICI was > 4.

***Candida* biofilm viability assay**

Susceptibility of *C. albicans* and *C. parapsilosis* biofilms were examined in the presence or absence of 50% human serum. Both *Candida* species had similar parameters in metabolic activity and biofilm mass results, thus one representative *C. albicans* (1544) and one *C. parapsilosis* isolate (26977) were chosen randomly for viability assays. Biofilms were grown on the surface of 8 well Permanex slide for 24 h (Lab-Tek, Chamber SlideTM System, VWR, Debrecen, Hungary) in RPMI-1640 with or without 50% human serum. The slides were incubated statically for 24 h at 37 °C. The ratio of viable and dead cells was measured in fluorescent microscopic analysis using a LIVE/DEAD BacLight ^{TM} viability kit (ThermoFisher Scientific, USA). One-day old biofilms were washed three times with sterile physiological saline, and biofilms were stained for 15 minin darkness at 37°C using Syto9 (3.34 mmol/l solution DMSO) and propidium iodide (20 mmol/l solution in DMSO) to examine viable and dead *Candida* cells respectively. Based on staining process, live cells showed green

fluorescence and dead cells showed red fluorescence. Fluorescent cells were examined with a Zeiss Ax-ioScope A1 microscope (Jena, Germany) coupled with a Zeiss AxioCam ICm1 camera (Jena, Germany). The analysis of images was performed using ZEN lite 2012 (Jena, Germany).

During the *in vitro* efficacy of CAS and MICA and combination with NIKZ was studied both *Candida* species, viability assay was used in order to confirm our results. Sessile *Candida* cells were treated the following drug concentrations: *C. albicans* (10431): 8 mg/l NIKZ, 0.25 mg/l CAS, 0.25 mg/l MICA, 0.25 mg/l CAS + 8 mg/l NIKZ, 0.25 mg/ MICA + 8 mg/l NIKZ; *C. parapsilosis* (17820): 8 mg/l NIKZ, 128 mg/l CAS, 128 mg/l MICA, 128 mg/l CAS + 8 mg/l NIKZ, 128 mg/l MICA + 8 mg/l NIKZ. These concentrations were chosen based on the checkerboard results.

Scanning electron microscopy analysis

The biofilm structure of one representative *C. parapsilosis* (16977) isolate was examined after CAS and MICA treatment in the presence and absence of TYR.

One-day-old biofilms were created on 10-mm diameter circular coverslips and were incubated at 37 °C for 24 h. After 24 h of growth, the discs were washed three times with sterile physiological saline, and various drug concentrations were added to the samples (256 mg/l CAS, 256 mg/l MICA, 1 mM TYR, 256 mg/l CAS + 1 mM TYR, 256 mg/l MICA + 1 mM TYR. After 24 h of antifungal treatment, biofilms were washed with sterile physiological saline and placed in 2% glutaraldehyde fixative solution. Samples were then dehydrated in a sequence of ethanol and dried in a desiccator. Eventually, they were coated with gold prior toobservations (Hitachi S-4300).

Statistical analysis

In case of each methods, all isolates were tested in three independent experiments, and the median of these three values wasused in the analysis.

The effect of various serum concentrations on metabolic activity was compared to control (metabolic activity of biofilms without serum) using one-way analysis of variance (ANOVA) with Dunnett's post-testing. Wilcoxon matched-pairs test was used to analyse the differences in metabolic activity change between in RPMI-1640 and in RPMI-1640 with 50% human serum caused by caspofungin and micafungin. Data were analyzed using GraphPad Prism 6.05 software. The results were considered significant if the P value was <.05.

Results

MIC results in RPMI-1640 with and without 50% human serum for planktonic and sessile *Candida* cells

Planktonic *C. albicans* and *C. parapsilosis* clinical isolates and reference strains were susceptible to CAS and MICA according to CLSI breakpoints in RPMI-1640. The observed median MIC values of clinical *C. albicans* clinical isolates and reference strains in 50% serum were 4-to 8-fold and 8-to 64-fold higher in case of CAS and MICA, respectively.

In RPMI-1640 without serum the median CAS and MICA MIC values against sessile *C. albicans* were 0.25 and 0.03 mg/l. The median MIC values showed: 0.125 mg/l (CAS) and 0.015 mg/l (MICA) against *C. albicans* SC5314 reference strain. The median MICs for CAS and MICA against sessile cells formed by clinical *C. parapsilosis* isolates showed higher value (384 mg/l and 256 mg/l) to that of planktonic form. In case of *C. parapsilosis* ATCC 22019 reference strain the median MIC values were 1 mg/l and 2 mg/l, for CAS and MICA, respectively.

In comparison, the median CAS and MICA MICs against *C. albicans* clinical isolates in the presence of 50% human serum were 0.5 mg/l and 2 mg/l respectively. In case of *C. albicans* SC5314 reference strain the median MIC values were 0.25 mg/l and 4 mg/l for CAS and MICA. Interestingly, the median MICs for CAS and MICA against sessile cells formed by clinical *C. parapsilosis* isolates and reference strain (ATCC 22019) showed 0.015 mg/l.

Effect of human serum on biofilm formation

One percent serum in RPMI-1640 had no effect on the metabolic activity of *C. albicans* and *C. parapsilosis* cells. However, serum concentrations ranging from 5% to 50% influence the metabolic activity of biofilms in a dose-dependent manner for both tested species. In case of *C.albicans*, significant differences were observed only at 8 hours ($P<.05–.001$) and at 12 hours ($P<.01–.001$) compared to control at concentrations ranging from 5 to 30% and from 5 to 10%, respectively. At 24 hours, the metabolic activity of *C. albicans* biofilms at serum concentrations from 5% to 50% was statistically comparable to values observed in RPMI-1640. In contrast, the metabolic activity of *C. parapsilosis* biofilms was significantly inhibited by serum concentrations from 5% to 50% between 2 and 24 hours compared to values obtained in RPMI-1640 ($P<0.05–0.001$).

Susceptibility pattern of *Candida albicans* and *Candida parapsilosis* biofilms in the presence and absence of 50% human serum

In case of *C. albicans*, significantly higher metabolic activity reduction was observed in RPMI-1640 compared to serum-containing medium at concentrations ranging from 0.015 mg/l to 1 mg/l CAS concentrations for all isolates ($P<0.05$). At least 50% metabolic activity reduction was found from 0.06 mg/l in RPMI-1640 and from 1 mg/l in RPMI-1640 with serum. MICA showed high antifungal efficacy (>50% metabolic activity reduction) at concentrations from 0.015 mg/l in RPMI-1640 and from 2 mg/l in the presence of 50% human serum against *C. albicans* biofilms. At MICA concentrations above 1 mg/l, there was no significant differences in metabolic activity reduction between serum-exposed and serum-free biofilms ($P>0.05$).

The tested echinocandins showed high activity against *C. parapsilosis* biofilms in the presence of 50% serum, where significant metabolic activity reduction was observed at ≥0.015 mg/l compared to values observed in RPMI-1640 ($P<0.01–0.05$).

Biofilm structure and viability in the presence and absence of 50% human serum

The structural and viability properties of *C. albicans* and *C. parapsilosis* biofilms with and without 50% human serum were demonstrated with fluorescent microscopy. There was no apparent damage in either *C. albicans* or *C. parapsilosis* biofilms in RPMI-1640 after 24 hours incubation period. The ratio of viable cells was high in RPMI-1640 for both species. Serum exposure caused a remarkable adverse effect in terms of biofilm structure in case of both species. Serum-exposed biofilms of *C. albicans* were very sparse compared to RPMI-1640, additionally, exclusively yeast cells were observed. Nevertheless, the ratio of viable cells was similarly high compared to the biofilm in RPMI-1640 (96.83% vs. 3.17%). In images of serum exposed *C. parapsilosis* biofilms, the ratio of nonviable cells significantly increased, and the biofilm show disorganised structure with lots of nonviable cells.

Susceptibility testing of planktonic *Candida albicans* and *Candida parapsilosis* cells against echinocandins and nikkomycin Z

Under planktonic conditions five out of six *C. albicans* isolates were susceptible to the tested echinocandins (10431, 19954, 31401, 10781, 1544) based on revised CLSI breakpoints. As expected, isolate DPL18 was resistant both to CAS and MICA (MIC: 2 mg/l), due to F641S mutation.

For susceptible *C. albicans* clinical isolates, the planktonic MICs ranged from 0.015 to 0.03 mg/l, from 0.015 to 0.06 mg/l and from 4 to 8 mg/l for CAS, MICA and NIKZ respectively. Four out of five planktonic clinical *C. parapsilosis* isolates were susceptible to both echinocandins (17820, 9613, 10252, 27001) with a narrow range of MIC values (between 1 and 2 mg/l), while isolate 17432 showed intermediate susceptibility (MIC of 4 mg/l) both for CAS and MICA. The examined NIKZ concentrations resulted prominent inhibitory effect from 8 mg/l concentration against planktonic *C. parapsilos* isisolates.

Susceptibility testing of sessile *Candida albicans* cells against echinocandins and nikkomycin Z, furthermore interactions between echinocandins and nikkomycin Z

In case of echinocandin-susceptible *C. albicans* isolates, NIKZ resulted in 2- to 16-fold and 16- to 128-fold decrease of the median MICs for CAS and MICA, respectively. Moreover, NIKZ median MIC values showed 8- to 512-fold decrease in combination with CAS or MICA against susceptible *C. albicans* sessile cells. NIKZ exerted 64-fold and 4-fold MIC value decrease against isolate DPL18 for CAS and MICA respectively.

By FICI, synergy between NIKZ and CAS or MICA was observed against the majority of *C. albicans* isolates (10431, 10781, 1544, DPL18) . An indifferent effect was noticed exclusively for isolate 19954 and 31401 but these biofilms had lower MIC values against CAS or MICA without NIKZ.

Susceptibility testing of sessile *Candida parapsilosis* cells against echinocandins and nikkomycin Z, furthermore interactions between echinocandins and nikkomycin Z

In case of *C. parapsilosis* biofilms, the median MIC values observed for CAS and MICA in combination with NIKZ demonstrated 2- to 4-fold and 2- to 64-fold decrease for CAS and MICA respectively. The median MIC values of NIKZ in combination with echinocandins

showed 2- to 512-fold reduction for *C. parapsilosis* biofilms. The combination of CAS and NIKZ yielded an indifferent interaction against all five *C. parapsilosis* isolates (FICI: 0.502-1). Of note, FICIs of three out of five isolates were very close to threshold indicating synergy (FICI 0.502–0.508). In contrast, based on FICI values, a striking synergistic interaction was observed for four out of five *C. parapsilosis* isolates after MICA with NIKZ exposure (FICI: 0.017–0.5).

Candida albicans and *Candida parapsilosis* biofilms structure and viability

To LIVE/DEAD viability staining, the number of cultivable sessile cells was determined by quantitative culturing of one-one representative isolates from *C. albicans* (10431) and *C. parapsilosis* (17820), and the ratio of viable and dead cells was measured after echinocandin with NIKZ exposure. In case of untreated *Candida* biofilms, the ratio of viable cells was the highest for both species. LIVE/DEAD viability staining revealed that echinocandin-exposed *C. albicans* and *C. parapsilosis* biofilms exhibited increased cell death in the presence of NIKZ compared to untreated biofilms, echinocandin-exposed sessile populations or NIKZ treated biofilms.

Susceptibility testing of planktonic *Candida parapsilosis* cells against echinocandins and tyrosol, furthermore interactions between echinocandins and tyrosol

Three out of five planktonic clinical isolates and ATCC 22019 were susceptible to CAS while isolates 16977 and 17820 showed intermediate susceptibility based on revised CLSI breakpoints, while isolate 16977 had intermediate susceptibility for MICA. The tested TYR concentrations had no inhibitory effect on against planktonic isolates, and exclusively indifferent effect was noticed between echinocandins and TYR.

Susceptibility testing of sessile *Candida parapsilosis* cells against echinocandins and tyrosol, furthermore interactions between echinocandins and tyrosol

The median MICs for CAS and MICA against sessile cells formed by clinical *C. parapsilosis* isolates showed higher value (128 - ≥512 mg/l) to that of planktonic form (2-4 mg/l). Tyrosol caused 2–16-fold and 2–32-fold decrease in median CAS and MICA MIC values respectively. Furthermore, median TYR MICs showed 2- to 8-fold reduction when combined with echinocandins in case of biofilms. By FICI, synergy was observed in isolates 27001 (FICI: 0.312) and 17820 (FICI: 0.298) for CAS and 27001 (FICI:0.193) for MICA.

Scanning electron microscopy

Isolate 16977 was randomly chosen to evaluate with SEM as the representative isolate.

The untreated control biofilm displayed yeast cells with normal morphology and few pseudohyphae. TYR-exposed biofilm (1 mM/l) displayed denser structure as compared with untreated biofilm. After exposure to 256 mg/l echinocandin enlarged round blastospores with wrinkled surface were observed but appeared unaffected cells as well. Combined treatment with echinocandins and TYR (256 mg/l echinocandin + 1 mM TYR) resulted in more significant cell damage, what confirmed by quantitative culturing. Numerous collapsed yeast cells and blastospores with abnormal morphology were detected both for CAS+TYR and MICA+TYR.

Discussion

Based on epidemiological data, over two million bloodstream infections episodes are registered in Europe each year with fatality rate of 13–20%. Annually, there are approximately 400.000 bloodstream infections caused by *Candida* species globally, with mortality rates exceeding 40-50% among adults and it can reach 30% among children (depending on species). The incidence and prevalence of candidemia are higher among immunocompromised and/or patients hospitalized with serious underlying disease in intensive care units. Although *C. albicans* remains the most prevalent species causing invasive candidiasis, the total proportion of infections attribut able to *C. albicans* has decreased from 57.4 to 46.4% over the 20-year surveillance period. In the last decades, epidemiological data have shown a marked increase in the number of invasive infections caused by non-*albicans* species (*C. parapsilosis C. glabrata, C. tropicalis, C. krusei*). Biofilm production in *Candida* isolates is associated with the high mortality rate of candidaemia. Based on previous studies, the hospital mortality was 51.2% in the biofilm-forming *Candida* bloodstream infection group (CBSI) compared with 31.7% in the non-biofilm-forming CBSI group. In addition to biofilm-formation, the metabolic activity of biofilms can also influence for mortality. Vitalis *et al*. reported that biofilm-former strains with intermediate or high metabolic activity resulted in significantly higher 30-day mortality rates for bloodstream infection compared to *Candida* biofilms with low metabolic activity.

Candida species are responsible for approximately 8% of catheter-associated infections. In addition to *C. albicans, C. parapsilosis* is the second most important biofilm-forming species, colonizing catheters and other invasive medical devices. Echinocandins are reccomended as first-line treatment for invasive candidiasis according to current IDSA (Infectious Diseases Society of America) guidelines. Based on previous *in vitro* and *in vivo* studies, the different AMB formulations (amphotericin B lipid complex, liposomal amphotericin B) and echinocandins can penetrate into the biofilms, although this efficacy may be significantly different in case of different *Candida* species.

The majority of studies focusing on antifungal susceptibility testing against biofilms were performed in RPMI-1640, which simulates well the glucose and amino acid concentration in human serum; however, it completely ignores the effect of serum proteins. In spite of the well documented susceptibilty of planktonic *Candida* cells against antifungal agents in presence of 50% human serum; the best of our knowledge, there is no comparative data about susceptibility of biofilms against the most frequently administered antifungal agents in the presence of human serum.

Based on our studies, both tested echinocandins (CAS and MICA) produced remarkable anti-biofilm activity in RPMI-1640 against sessile *C. albicans* cells. *C. parapsilosis* biofilms showed lower susceptibility at clinically relevant CAS and MICA concentrations (peak serum concentrations are 7.64 mg/l and 8.8 mg/l for 50 mg CAS and 100 mg MICA standard dose, respectively). Based on our fluorescent images, lower cell density was observed in serum-treated *C. albicans* biofilms compared to non-treated sessile cells and in images of serum exposed *C. parapsilosis* biofilms; furthermore, the ratio of nonviable cells significantly increased. The activity of examined antifungal agents showed remarkable changes in the presence of 50% human serum both against *C. albicans* and *C. parapsilosis* isolates compared to results obtained in RPMI-1640. In case of *C. albicans*, the tested echinocandins showed significantly lower antifungal effect in the presence of serum at lower drug concentrations (0.015- 1 mg/l). In contrast, echinocandins showed higher activity against *C. parapsilosis* biofilms, in RPMI-1640 with human serum.

To reduce the high mortality rate of invasive fungal infections and to prevent the spread of isolate resistant to the traditional antifungal agents, there is an urgent need to develop new and innovative antifungal and anti-biofilm therapeutic approaches. One the most promising options is the usage of the highest clinicaly possible doses of given antifungal agents, antifungal "lock" therapy (high-dose antimycotics are used in the inrtavenous catheter to eradicate intraluminal biofilm) and combination-based therapies.

Previous studies revealed the potential synergizing effect of NIKZ in combination with traditional antifungal agents against *Candida* species, however, these works examined exclusively the anti-planktonic effect of these combinations and recently there is no information about susceptibility of biofilms formed by *C. albicans* or non-*albicans* species. In our studies, synergistic interaction was observed between echinocandins (CAS, MICA) and NIKZ against *C. albicans* and *C. parapsilosis* biofilms. Based on our fluorescent images, echinocandin-exposed *C. albicans* and *C. parapsilosis* biofilms exhibited increased cell death in the presence of NIKZ compared to untreated biofilms.

A particularly novel and innovative therapeutic approach may be the disturb of QS system in microorganisms. Previous studies have shown, that *Candida*-secreted quorum-sensing molecules at supraphysiological concentration can negatively influence the „communication" between individual cells within biofilms, furthermore inhibits growing fungal cells within the sessile population.

In vitro synergy between traditional antifungal agents (AMB, fluconazole, echinocandins) and

FAR against planktonic and sessile cells have been reported for different *Candida* species. However, our information on interactions between echinocandins and tyrosol and the number of studies focusing on *C. parapsilosis* are very limited.

In our experiments, we have found no inhibitory effect of tyrosol against *C. parapsilosis* planktonic cells. Furthermore, there is no synergistic interaction between echinocandins (CAS, MICA) and TYR against planktonic *C. parapsilosis* cells. Based on FICI described in this study, in case of echinocandin with TYR exposed *C. parapsilosis* biofilms, synergism was observed in two out of six and one out of six sessile isolates for CAS (mean FICI 0.298–0.312) and MICA (mean FICI: 0.193) respectively. In our SEM experiments, combined treatment with echinocandins and TYR (256 mg/l echinocandin + 1 mM TYR) resulted in more significant cell damage, which was confirmed by quantitative culturing.

In summary, NIKZ and TYR may be a potential adjuvant in certain alternative therapies (e.g. antifungal lock therapy) in the catheter-associated infections caused by *C. albicans* and *C. parapsilosis*. Nevertheless, further *in vivo* catheter-modell studies are needed in the future to confirm these promising *in vitro* results.

Summary

The therapeutic difficulties of biofilm-associated infections highlight the importance of the development of innovative treatment strategies to eradicate effectively these sessile population. The anti-biofilm activity of caspofungin and micafungin against *Candida albicans* and *C. parapsilosis* biofilms was investigated in RPMI-1640 with 50% human serum. In the case of *C. albicans* biofilms, reduced echinocandin activity was observed in the presence of serum at concentrations ranging from 0.015 to 1 mg/l ($P<0.05$). In contrast, echinocandins showed increased activity against *C. parapsilosis* biofilms in the presence of serum from the lowest drug concentration tested (0.015 mg/l). In further experiments, the susceptibility of *C. albicans* and *C. parapsilosis* biofilms to caspofungin, micafungin, nikkomycin Z and tyrosol alone or in combination was investigated using two-dimensional "checkerboard" microdilution. The nature of the *in vitro* interactions was evaluated using fractional inhibitory concentration index (FICI) determination. Nikkomycin Z resulted in 2- to 16-fold and 2- to 128- fold decrease of the median median minimum inhibitory concentration values (MICs) for caspofungin and micafungin respectively. When echinocandins and tyrosol were combined, tyrosol caused 1–16- fold and 2–32-fold decrease in median caspofungin and micafungin MICs respectively. The combination of caspofungin and nikkomycin Z yielded an indifferent interaction against all *C. parapsilosis* isolates (FICI: 0.502-1). Based on FICI values, a striking synergistic interaction was observed for four out of five *C. parapsilosis* isolates after micafungin with nikkomycin Z exposure (FICI: 0.017–0.5). Synergistic interaction was observed for most of the concomitant applications of echinocandins and nikkomycin Z (FICI: 0.037-0.245) against *C. albicans*. Synergism was observed in a strain-dependent manner upon exposure to echinocandin and tyrosol (FICI: 0.193-0.312), which was confirmed by scanning electron microscopy too. In summary, our results suggest that biofilm susceptibility studies in the presence of human serum can mimick the *in vivo* environment and therefore its application in pharmacodynamic studies are absolutely justified. Based on our combination-based experiments, nikkomycin Z and tyrosol could be a potential adjuvant in the alternative therapy of biofilm-associated infections caused by *C. albicans* and *C. parapsilosis*; nevertheless, further *in vivo* studies are absolutely needed to confirm these *in vitro* results.

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Registry number: DEENK/357/2021.PL
Subject: PhD Publication List

Candidate: Fruzsina Nagy
Doctoral School: Doctoral School of Pharmacy
MTMT ID: 10063834

List of publications related to the dissertation

1. **Nagy, F.**, Tóth, Z., Bozó, A., Czeglédi, A., Rebenku, I., Majoros, L., Kovács, R. L.: Fluconazole is not inferior than caspofungin, micafungin or amphotericin B in the presence of 50% human serum against Candida albicans and Candida parapsilosis biofilms.
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IF: 4.307

Total IF of journals (all publications): 73,757
Total IF of journals (publications related to the dissertation): 7,155

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

09 June, 2021

DEBRECENI EGYETEM Egyetemi és Nemzeti Könyvtár