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

In vitro activity of micafungin against rare *Candida* species

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The examination takes place at the Library of Department of Pharmacology and Pharmacodynamics, Faculty of Pharmacy, at 11:00 am, 31st of October, 2017

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 1:00 pm, 31st of October, 2017

Abbreviations

AMB	Amphotericin B
AUC	Area under the concentration curve
ATCC	American Type Culture Collection
CAS	Caspofungin
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
C_{max}	Peak concentration of the drug
C_{min}	Lowest concentration of the drug
ECOFF	Epidemiological cutoff value
FLU	Flukonazole
IDSA	Infectious Diseases Society of America
MIC	Minimal inhibitory concentration
MICA	Micafungin
RPMI-1640	Roswell Park Memorial Institute Medium
T50/T90	Time needed to reduce the CFU to 99,9% and 50%
50% serum	RPMI-1640+50% human serum

Introduction

The epidemiology of infections caused by Candida species

Invasive life threatening fungal infections increased worldwide in the last three decades. The most important fungal pathogens are various *Candida* (70-80%) and *Aspergillus* species (20-30%). Mortality rate caused by *Candida* species is high despite the introduction of new diagnostic methods and antifungal class (echinocandins) into the clinic. Patients with invasive candidasis have generally severe basic illness and the mortality rate, depending on the species may reach 20-70%. Though five *Candida* species (*C. albicans, C. glabrata, C. parapsilosis sensu stricto, C. tropicalis* and *C. krusei*) account for ≥95 % of all candidemia, less common other species (*C. guilliermondii, C. lusitaniae, C. famata* and *C. kefyr*), may cause problems in the treatment of candidemias or other forms of invasive candidiasis, especially among cancer and leukaemia patients. Optimal treatment against these species is not well defined as they often show decreased susceptibility to different antifungal classes including echinocandins. Virulence of these uncommon *Candida* species is significantly lower than *C. albicans*, but the mortality rate is higher than 50%.

Currently, echinocandins such as micafungin are first-line drugs for treatment of invasive candidiasis. Echinocandins are highly protein-bound with 0.2-3% free drug, i.e. the serum and tissue concentrations may be low. Decreased killing activity of echinocandins in 50% serum against medically important *Candida* species was confirmed. However, data on how serum influences killing activity of echinocandins against uncommon *Candida* species are limited. In order to extend our knowledge about the *in vitro* activity of echinocandins against uncommon *Candida* species, the killing activity of micafungin in RPMI-1640 and in RPMI-1640 plus 50% serum (50% serum) was compared against *C. dubliniensis, C. africana, C. guilliermondii, C. lusitaniae* and *C. kefyr* clinical isolates and ATCC strains.

Pathogenesis of Candida infections

Invasive bacterial and fungal infections are the most important causes of mortality. As reported by Wisplinghoff et al. in the 1960s and 1970s, Gram-negative bacterial pathogens (*Escherichia coli*, *Klebsiella* species, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*) were most frequently isolated from patients with nosocomial bloodstream infections. Later, Gram-positive pathogens ((*Staphylococcus aureus*, coagulase-negative *Staphylococci* and *Enterococcus* species) have become increasingly frequent. The mortality rate decreased because of the introduction of newer drug classes (third and fourth generation

cephalosporins, fluoroquinolons) and better treatment in the intensive care units. However, colonization by different *Candida* species has been noticed more frequently among patients with long term hospitalization, radical surgery and neutropenia due to chemotherapy. Colonization is easily explained due to the various virulence factors for *Candida* species (agglutinin-like sequence proteins, hypha-associated GPI-linked protein, lipases, phospholipases and secreted aspartic proteases). It is well known that *C. albicans* and *C. dubliniensis* are produce hyphae which help in the invasion.

All factors which damage the integrity of the skin and mucosal surfaces and decrease the number of the neutrophil granulocytes lead to colonization with *Candida* species and invasion of the sterile body sites. Catheter-associated infections are related by the ability of *Candida* species producing biofilm. Using broad spectrum antibiotics, especially with anti-anaerobic spectra (amoxicillin plus clavulanic acid or metronidazole), too low or too old age are important predisposing factors for invasive *Candida* infections.

Rare Candida species causing invasive infections

Despite the fact that *C. albicans* remained the most common species worldwide (overall, 50-60% of all *Candida* species), from many countries have noted a decreasing trend in the isolation of *C. albicans.* The isolation rates of rare species such as *C. dubliniensis*, *C. guilliermondii*, *C. kefyr*, *C. rugosa*, and *C. famata* increased significantly in the last two decades.

C. dubliniensis is one of the most interesting *Candida* species and genetically is very close to *C. albicans*. This species is the important pathogen among HIV infected patients in the mouth, and during the fluconazole (FLU) therapy/prophylaxis more easily develop resistance to fluconazole. *In vitro,* in the presence of serum and neutrophile granulocytes its hypha production is weak or absent. *In vivo*, in contrast to *C. albicans* never produces hypha. These *in vitro* results explain the fact why produces *C. dubliniensis* infections on the mucosal surfaces. However, if enter into the bloodstream the mortality rate is similar to *C. albicans* (around 40%). Over the last two decades several research groups have described atypical *C. albicans* isolates, mainly from vulvovaginous clinical specimens (from Madagascar and Angola). These isolates, similarly to *C. albicans* and *C. dubliniensis*, had a germ tube in serum but did not form chlamydospore on rice-agar medium. They did not assimilate trehalose, they do not have an N-acetylgalactosidase enzyme and they formed hypha slower than the other two species. Molecular biology studies have shown that these atypical *C. albicans* isolates belong to a distinct species within the *C. albicans sensu lato* and are

classified as a new species called ***C. africana***. *C. africana* is predominantly derived from vulvovaginogenic clinical specimens, but can also cause invasive infections - based on literature data. It can be found probably worldwide but only few references are available on its exact epidemiology. Its susceptibility to antifungal agents is similar to *C. albicans* and *C. dubliniensis*. Symptomatic vulvovaginitis has always been cured of azol-type (clotrimazole or FLU) antifungal agents.

Similar to the two previous species (*C. dubliniensis* and *C. africana*), ***C. guilliermondii*** develops less than 1% of invasive *Candida* infections, but may also be responsible for 3-5% of candidami in South America. It may have reduced sensitivity to FLU and a natural, decreased susceptibility to echinocandins. Its clinical significance is that in the case of invasive infections, concomitant immunosuppression is common, which limits the number of therapeutically-administered antifungal agents in leukemic patients due to possible decreased anti-fungal sensitivity.

C. lusitaniae is also one of the few isolated pathogens and capable for formation of invasive infection. It can be isolated mainly from the bloodstream, but it can be isolated from the abdominal or the urinary tract, as well. This pathogen got to the focus of interest because some of the species isolated from the patients showed a decreased susceptibility to amphotericin B (AMB) and during AMB therapy, secondary resistance often developed. Most often, it can be isolated from patients suffering from cancer, and those of had surgical intervention. The patients have not received antifungal therapy or steroid before. Their MIC values for FLU are generally low. According to North American data, the mortality rate they cause is generally below 30%, but in malignant hematologic patients who received echinocandin treatment, the mortality is over 50%.

C. kefyr was first isolated from kefir in 1909. In the PATH Alliance Registry in 2004-2008 in North America, it was isolated only in 11 (0.2%) of 5526 invasive *Candida* infections. Based on the latest data, in patients with haematological cancer it is a common colonizing fungus, and it is more frequent in bloodstream infections as well. Colonization is more frequent in the summer, which may be related to the fact that the *C. kefyr* appears in the case of incorrect cooling of yogurt and other dairy products. *C. kefyr* is responsible for the deterioration of dairy products, so inappropriate cooling can help its colonisation in malignant hematologic patients. According to some observations, the more frequent detection of *C. kefyr* is related to the increasing use of (micafungin) MICA instead of CAS. According to well-documented observation, echinocandin resistance may develop during 10 to 14 days during echinocandin therapy.

Aims

According to the current recommendations, echinocandins are the first choice antifungal drugs in case of invasive *Candida* infections. Despite the therapeutic role of echinocandins, the mortality caused by invasive *Candida* infections, particularly the less isolated *Candida* species, is still unacceptably high. Since echinocandins are highly bound to the various proteins in serum, this binding decreases the pharmacologically active echinocandin concentration. Therefore, with the help of the minimal inhibiting concentration and the time-killing curves, we have compared the in vitro activity of micafungin with clinically important but less isolated *Candida* species such as *C. dubliniensis, C africana, C. guilliermondii, C. lusitaniae* and *C. kefyr* in RPMI-1640 és RPMI-1640+50% serum broth. During our project, we were trying to find answer whether micafungin is suitable for treatment of invasive infections caused by less frequently isolated *Candida* species.

Materials and methods

The origin and identification of fungi

Sabouraud agar medium containing chloramphenicol was used for the cultivation of fungi. The chromogene substrate containing CHROMagar Candida (Becton Dickinson) was used to identify the species in advance and to verify the purity of the cultures. Clinical isolates of *C. albicans* (3 isolates), *C. guilliermondii* (3 isolates), *C. lusitaniae* (3 isolates) and C. *kefyr* (4 isolates) were isolated from the blood of patients with candidiasis. For species-level identification, API ID 32C (BioMérieux, Marcy l'Etoile, France) and MICRONAUT-Candida System (Merlin Diagnostics Gmbh, Bornheim, Germany) were used. *C. dubliniensis* (4 isolates) and *C. africana* (2 isolates) isolates were derived from previous studies and identified by molecular biological methods. For the *C. albicans* complex assay, one ATCC test strain was also studied.

Determination of minimum inhibitory concentration of micafungin

The MICA stock solution was prepared on the basis of the CLSI recommendation (M27-A4, 2012). Two media were used. The first was RPMI-1640, which is the standard medium for antifungal sensitivity determination. As we wanted to model physiological conditions for MICA with high protein binding, RPMI-1640 medium supplemented with human serum (Sigma, Budapest) was also used (50% serum). Since in case of 50% serum, it is difficult to read MIC with the naked eye, the MICA MIC values were determined using the macro broth dilution method (final volume of 1 ml). The highest tested MICA concentration was 32 mg / L. In the physiological saline solution, we used fungi cultures cultured in 24 h Sabouraud agar, and they were used for suspensions of 0.5 McFarland density. After that, a RPMI-1640 liquid medium was used for the appropriate number of germs (~ 10^3 CFU/mL). In all cases, fungal control (containing no antifungal drugs) as well as broth control (containing no budding fungi) cavities were set.

The tubes were incubated at 35 ° C and read visually after 24 hours. The MICA MIC value was the concentration that showed a decrease of at least 50% in the control (prominent inhibition).

Time-kill curves

Using a densitometer, ~ 10^5 CFU/mL starting yeast fungal suspension was prepared in RPMI-1640 medium. The tested concentrations were 1, 4, 16 and 32 mg/L, but in case of the *C. albicans complex*, a concentration of 0.25 mg/L was also investigated. Tubes containing media, fungi suspension and various concentrations of antifungal agents were continuously shaken (10 mL final volume) in a 35 ° C dark thermostat for 48 hours. 100-100 μL of the tubes taken at 0, 4, 8, 12, 24 and 48 hours were diluted in a 1:10 scale in physiological saline; then, from the dilutions to the surface of Sabouraud Agar, 4×30 μL dilution were dropped. In case that the number of colonies was expected to be <1000 CFU/mL, the fungi were extinguished without dilution. The lowest limit of detection is 50 CFU/mL. Forty-eight hours later, the deposited colonies were counted and the number of live fungal cells calculated respecting the dilution degrees. The resulting germ numbers were graphically depicted as a function of time. All time-kill experiments were performed at least twice, and the results were averaged. The computer curve-fitting software (GraphPad Prism 4.03 Windows version) was used to produce time-kill curves. The drug was considered to be fungicidal if it reduced the number of viable cells at least ≥ 99.9% (≥3 log) compared to the initial cell count. The antifungal agents causing a lower decrease in the number of infections (<99.9%; <3 log) were considered fungistatic.

Detection of the killing rate

Using data obtained from time-kill experiments, we compared the killing kinetics of MICA in RPMI-1640 and in 50% human serum. Killing kinetics were calculated based on the relation $N_t = N_0 \times e^{-kt}$ where N_t is the number of living cells at a given time, N_0 is the living cell count at the beginning of the experiment, *k* is the killing rate, and t is the incubation time. A positive *k* value is the killing of fungal cells, while the negative k value means the growth of fungal cells. The goodness of fit was assessed by the r^2 value (r^2>0.8). The mean times to achieve the fungicidal endpoint (T99.9=3/*k*) were calculated from the *k* values for each isolate and concentration in both media.

Differences between the different isolates and concentrations were studied in both media using single-way ANOVA test completed with Tukey's post-testing. Growth rates and the efficacy of the same micafungin concentrations in different media were compared by *T* test. The results were considered significant in case of $p < 0.05$.

In vivo experiments

In our *in vitro* experiments very low proliferation rate was detected in 50% serum for the *C. africana* ATCC test strain and the clinical isolate, therefore, the *in vivo* proliferation ability of two separate isolates of *C. albicans* was analysed in neutropenic mouse model as well. In the experiments, BALB/c mice were used (Charles River Laboratories) and they were kept according to the guidelines included into the "Care and housing of laboratory animals". The certificate number of the *in vivo* experiments was 12/2014 DE MÁB. 6-6 female mice with a mass of 21–23 g were used for each species.

For the *in vivo* experiments, a randomly selected clinical isolate of the *C. albicans* and the *C. dubliniensis* as well as the clinical isolate 97-135 of the *C. africana* were used. The mice were infected via lateral tail vein injection (0.2 ml fungi suspension/mouse). In case of the *C. albicans*, an infective dose of $6x10^4$ CFU/ml, in case of *C. dubliniensis* that of 10^5 CFU/ml, while in case of *C. africana* that of 10^6 CFU/ml were used. The $2x10^6$ CFU/ml infective dose in case of the isolate 97-135 of the *C. africana* triggered almost 100% mortality within 4 hours.

On the sixth day after infection, the mice were killed by cervical dislocation, their kidneys were removed, weighted and homogenised in a sterile mortar. Out of the different dilutions, 100 µl was inoculated onto Sabouraud-agar substrate and after 48 hours of incubation at 35°C, the grown colonies were counted. For the statistical analysis of the fungi grown on the substrate, Kruskal-Wallis test was applied (GraphPad Prism 4.03, Windows). The values at $p<0.05$ were considered to be significant.

Results

MIC values of the isolates

In *RPMI-1640*, clinical isolates of *C. albicans* and *C. guilliermondii* as well as the *C. albicans* ATCC test strain proved to be sensitive to MICA. The MIC values obtained for *C. dubliniensis, C. lusitaniae* and *C. kefyr* were not higher than the ECOFF values, therefore, these isolate are considered as "wild", i.e. no acquired resistance may be counted upon. Neither the clinical limit value nor the ECOFF values are available for *C. africana*, although the MIC values were similar to the ones measured for the *C. albicans* and the *C. dubliniensis*.
In 50% serum, all isolates showed growth. In case of the *C. albicans* complex, the MIC values increased 16-64 fold compared to the MIC values measured in the RPMI-1640. The highest MIC value increase was found in case of the *C. kefyr* and the *C. lusitaniae* isolates (64-256 fold MIC value growth). When quantified, the highest MIC value in the 50% serum was measured for the *C. guilliermondii* (32 mg/L).

Results of the time-killing curves in case of the **Candida albicans** *complex*

In RPMI-16400, MICA showed fungicide effect only in case of the *C. albicans* ATCC test strain and the isolate 5265, however, only at the two highest concentrations.
Against the third *C. albicans* isolate and the other two species, MICA showed fungistatic effect. In case of the *C. dubliniensis*, the fungistatic effect was weak and after reaching the maximum germ count decrease, the isolates began growing again in several cases.
The dynamics of killing was analysed on the basis of the **killing rate (*k*)** values. The correlation between the killing rate and the analysed MICA concentrations were linear for the analysed species in both substrates.
In RPMI-1640, for the *C. albicans* ATCC test strain and the clinical isolate 183, the killing effect of MICA was increased by the increase in the concentration. In case of the *C. albians*, the highest *k* value was observed for the isolate 183 at the concentrations 16 and 32 mg/L MICA (0.58 l/h), which was significantly higher than the *k* values measured at lower concentrations. MICA showed a killing effect against the isolate 5265 independent of the concentration at the analysed concentration values ($P>0.05$).
In case of the *C. africana*, the highest *k* value was obtained for the isolate 97-135 at the concentration of 32 mg/L (0.13 l/h). The killing effect of MICA was very weak in case of the

C. dubliniensis, since at the concentrations 0.25, 1 and 4 mg/L, the *k* values were negative for the isolates CBS 8500, 1081 and 2953, i.e. growth was observed.

In 50% serum, in case of the control isolates, the mean times for a growth of one order of magnitude (1 log) were very similar for the *C. albicans* and the *C. dubliniensis* in the two substrates ($P>0.05$). However, in case of the *C. africana* ATCC test strain and the clinical isolate, the mean time for a growth of one order of magnitude was significantly higher in 50% serum ($P<0.01$), which shows the low proliferation of the species in the 50% serum.

In 50% serum, the 0.25 and the 1 mg/L MICA were without any effects for all three species, since always growth was observed in all cases compared to the initial germ count, and the killing rate (*k*) values were always negative. The killing triggered by the 4, the 16 and the 32 mg/L MICA was dependent upon the species, the isolate and the concentration.

MICA had fungistatic effect on the *C. albicans* at the concentrations ≥4 mg/L. In case of the ATTC test strain, the *k* values measured at the concentrations 4, 16 and 32 mg/L in two different substrates were not significantly different from each other. In case of the *C. albicans* isolates 183 and 5265, the killing activity of MICA in 50% serum at the concentrations 4, 16 and 32 mg/L significantly decreased compared to the killing experienced in RPMI-1640. However, the *k* values did not increase significantly in 50% serum by the increase of the concentration (concentration-independent killing).

MICA showed concentration-dependent killing effect against the *C. africana* ATCC test strain and the clinical isolate, i.e. the killing effect increased by the increase in the concentration.

In case of the *C. dubliniensis*, the 50% serum aggravated the killing effect of MICA at the concentrations 4, 16 and 32 mg/L in each case except the type strain no. CD36 ($P<0.05$ – 0.001); upon reaching the maximum germ count decrease rate, regrowth occurred in much less cases.

Findings of the *in vivo* experiments

In case of the *C. albicans* isolate 183 and the *C. dubliniensis* isolate 1081, the count of the living fungus cells cultured from kidneys were similar contrary to that the quantity of the fungi incorporated was lower in case of the *C. albicans*. Although, mice received the highest count of fungi in case of the *C. africana* isolate 97-135, the kidneys of 4 mice were sterile after 6 days and living fungi cells were able to be cultured only in case of two mice. On the basis of our findings, the *C. albicans* is the most virulent, while the *C. africana* is the least virulent (*C. albicans*>*C. dubliniensis*>*C. africana*).

The findings of the time-killing curves in case of *Candida kefyr, Candida lusitaniae* és *Candida guilliermondii*

In the RPMI-1640, MICA had fungicidal effect at all analysed concentrations within ≤4.04 hours against all four *C. kefyr* isolates. The killing activity of MICA was independent of the concentration against the four *C. kefyr* isolates (P>0.05).

In RPMI-1640, MICA showed fungicidal effect at concentrations ≥4 mg/L within 16.10 hours against the *C. lusitaniae* isolates 3834 and 7849, while in case of the isolate 582, at concentrations ≥1 mg/L the effect was fungistatic. In case of all three isolates the killing effect of MICA was independent of the concentration (P>0.05).

In RPMI-1640, MICA showed fungicidal effect only at the concentration of 32 mg/L and only against two *C. guilliermomndii* isolates. In case of all three isolates, upon reaching the maximum germ count decrease rate, re-growth was observed at several concentration values. The killing effect of MICA was independent of the concentration in this case as well.

In 50% serum, MICA did not show growth inhibition effect against the *C. kefyr* at concentrations 0.25, 1 and 4 mg/L and the *k* values were also negative in all cases. At the two higher concentration values, killing was rapid (≤3.03 hours) and the killing rate values did not show significant changes compared to the values measured in RPMI-1640.

The killing activity of MICA against the *C. lusitaniae* significantly decreased (at the concentration of 32 mg/L) except in case of the isolate 3834. Even in case of the MICA concentrations 16 and 32 mg/L the germ count decrease was only temporary for the other two isolates. The killing rate values were always negative, except the isolate 3834 at 32 mg/L.

In 50% serum, the *k* values of MICA against *C. guilliermondii* isolates were always negative. Temporary and minimal germ count decrease (-0.05 log CFU/ml) occurred only in case of the isolate 5540.

Discussion

The use of echinocandins started in 2001 with the introduction of CAS and since then they have become the primary choice of antifungal drugs in treatment of invasive candidiasis. By the 2016 recommendation of IDSA except for infections of the central nervous system, urinary tract or eyes, basically these are the first choice of drugs, including treatment of candidemia (in case of neutropenic and non-neutropenic patients), chronic disseminated candidiasis and endocarditis and infections of abdominal cavity and joints caused by *Candida* species. The 2016 recommendation of IDSA does not discriminate among echinocandins, so in theory any of the three echocandins can be administered in case of proper indication.

In case of invasive *Candida* infections widespread use of echinocandins became a routine procedure in Northern America, Western Europe and Japan, while AMB (including lipid associated versions) significantly decreases in therapeutic application. The use of FLU has not changed significantly. Widespread use of echinocandins has been significantly influenced by the high daily price. In Hungary 5-6 years ago the daily treatment cost was well above 100 thousand forints in case of any echinocandin. However, this class of medicine could have played an important role in our country as between the period of 1999 and 2009 average mortality caused by candidemia was 60% at the clinics of the University of Debrecen (unpublished data by László Majoros MD.). Luckily, in the past 3-4 years the price of daily echinocandin administration has decreased to the one fourth of the original price, this way echinocandins belong to the easily available antifungal drugs in Hungary nowadays.

In case of invasive Candida infections, the majority of information is about *Candida* infection in the bloodstream in literature. It must be noted that pathogens can get into other organs as well from blood (liver, spleen, kidneys ect.) generating a more serious disseminated candidiasis (Kontoyiannis et al, 2000). What is more, such organs can be infected where antifungal drug penetration is poor (eyes, joints, pleural and peritoneal cavities) making antifungal treatment difficult. As protein concentration can be significant in the bloodstream and other regions, in case of antifungal drugs with strong protein binding (AMB, echinocandins, posaconazole) depending on body regions not only the level of drug can be low but depending on the protein level of the given region, free, that is pharmacologically active drug level can be low as well, which leads to unsuccessful therapy. However, proteins in serum will not only influence clinical efficiency of echinocandins in a negative way. This theory claims that albumin echinocandin serves as a reservoir from where they can maintain the proper drug concentration by dissociation, in case of decreased free

echinocandin concentration. In case of hipoalbuminemia because of decreased colloid osmotic pressure echinocandin diffusion into tissues can be decreased, which together with multi-organ insufficinecy, can result in higher serum echinocandin level, while in the inner organs the echinocandin concentration will be lower. Obesity can also significantly influence echinocandin pharmacokinetics because of higher cardiac output and blood volume. Echinocandin concentration in serum will be smaller as hydrophilic echinocandins penetrate into fat tissues, which contain roughly 30 % of water.

In case of patients in critical conditions pathophysiological changes (sepsis, hipoalbuminemia, changed capillary premeability, liver and kidney failure, fluid balance disorder) can have basic effect on the pharmacokinetics of antibacterial and antifungal drugs. In cases of both CAS and MICA it was stated that in case of patients at intensive therapy units AUC_{0-24}, C_{max} and C_{min} values are significantly lower compared to healthy controls or patients at non-intensive therapy units. These pharmacokinetic changes are not surprising if we consider that some patients are hemodynamically unstable, which means they need vasopressor therapy because of low cardiac output. This has a close connection to organ hypoperfusion, which leads to organ damage, this means that drug distribution volume and elimination will also change in patinets with serious conditions. This explains that echinocandin concentration in serum in patients at surgical intensive therapy wards can be higher compared to healthy controls because of decreased distribution volume and/or decreased elimination of echinocandins.

Experiments with MICA served a more precise understanding of the efficiency echinocandins against rarely appearing *Candida* species. Our examinations may help understand the limitations of echinocandin therapy against species without echinocandin resistance. In our workgroup's previous experiments mainly CAS plays the role as this type of echinocandin was first introduced into medicine and was available in Hungary. In the past 5-6 years MICA was used at the clinics of University of Debrecen, that is why our workgroup started to pay attention to it.

Previous experiments about the 99,8% protein bound MICA proved that in 50% serum MIC values are 4-128 times higher in clinically significant *Candida* species than MIC values in RPMI-1640 In 50% serum against all *C. albicans, C. tropicalis, C. glabrata* és *C. inconspicua* isolates ≤4 mg/L MICA had fungistatic or fungicidal effects (Földi et al 2012.). This serum concentration (C_{min}) can be easily achieved by administering 100 mg MICA daily. Against *C. krusei* and „psilosis" only 8-32 mg/L MICA proved to have fungistatic or fungicidal effective: these concentrations can be achieved by higher peak concentration

(C_{max}) of daily dose (150-300 mg). Considering the fact that peak concentrations are in serum for only 1-2 hours against *C. krusei* and „psilosis" group neither MICA nor probably the other two echinocandin are totally effective.

In vitro antifungal sensitivity test is a basic requirement nowadays in case of fungal infections. Methods used in case of bacteria were adapted in the 1990s and a certain medium was found (RPMI-1640), that helped to make MIC assays from all over the world compatible with each other. Clinical limit values and ECOFF values of antifungal drugs have been specified on the basis of MIC values in RPMI-1640 medium. This gives a reliable base for the use of the most effective antifungal drug. Later, in case of high protein binding echinocardins it turned out that correlation between in vitro results in RPMI 1640 and in vivo efficiency cannot always be found. Maki et al (2008.) revealed in neutropenic mouse models that in vivo efficiency of MICA in case of *C. albicans* shows a closer connection with MIC values in a 50% serum than MIC values in RPIM 1640. Our workgroup has confirmed this result earlier during a comparitive study between the efficiency of CAS invitro and in vivo in case of *C. albicans, C. krusei* és *C. inconspicua* species.

Although in our present study we have not confirmed our in vitro findings with *in vivo* examinations, but the results give a strong basis to give one possible explanation for therapeutic failure in case of rare *Candida* species. In RPMI-1640 MIC results of the 6 *Candida* species examined by us were similar to the data in international literature, that is they were either sensitive to MICA (*C. albicans* and *C. guilliermondii*) or MIC values were not higher than ECOFF values (*C. dubliniensis, C. kefyr* and *C. lusitaniae*). Taking the time-killing curve on the easily attainable concentration level in the serum MICA showed fungicidal or fungistatic effects at 0.25 mg/L against all three members of C. albicans complex and *C. kefyr*. The weakest result was experienced against *C. dubliniensis* (regrowth and negative killing rate results on several concentrates). Similar results were reported in RPMI-1640 by Gil-Alonso et al (2015.) in case of *C. albicans* complex and (Cantón et al (2013.) in case of *C. lusitaniae*. From *C. guilliermondii* isolates, which have natural, reduced sensitivity against echinocandins, in cases of 5465 and 21060 positive *k* result (killing) was experienced only at 16 and 32 mg/L; at 1 and 4 mg/L both isolates showed growth similar to the control.

In 50% serum neither 32 mg/L MICA could stop the growth of *C. guilliermondii* isolate (in both cases we got negative *k* results). *C. guilliermondii* behaved similarly to *C. parapsilosis* sensu stricto mentioned in a previous study, in which the 32 mg/L MICA showed a fairly weak fungistatic effect against *C. parapsilosis* sensu stricto isolates that show decreased

sensitivity against echinocandins. The extent of the decrease of killing by MICA was more surprising in case of *C. kefyr* (only in case of 16 and 32 mg/L had MICA positive *k* result) and *C. lusitaniae* (only in case of 3834 isolate and at 32 mg/L was positive *k* result). In practice it means that at C_{min} level (4,85 mg/L) that can be achived by a daily 150 mg MICA level, these concentrates are a lot higher, which means that MICA is not effective against *C. kefyr, C. lusitaniae* and *C. guilliermondii*. Although with a daily 150 mg MICA therapy C_{max} values can be around 20-30 mg/L, short term drug exposion in serum has no colony count reducing effect, as we have already proved it in our earlier study in case of CAS. Our results correlate well with data in literature, where echinocandins can not sterilize the kidneys of neutropenic mice after a 7-10-day higher dose of echinocandin therapy against the three species. Although the virulence of the three species based on neutropenic mice is significantly lower than *C. albicans*'s virulence, its inefficiency in MICA 50% serum can explain the large number of mortality in long-term neutropenic patients. At the same time we should consider that because of low virulence, persistent *Candida* in patients due to low echinocandin concentration can easily become resistant, making therapeutic options even more difficult.

A 50% serum changed MICA killing ability in a less drastic way against *C. albicans* isolates and ATCC ttype strain (≥4 mg/L MICA fungistatic effect has remained). On the contrary, against *C. dubliniensis* in RPMI-1640 experienced weak MICA activity significantly encreased in 50% serum: at 4, 16 and 32 mg/L we experienced positive *k* results (killing). MICA killing effect in RPMI-1640 against *C. dubliniensis* was so weak that only T50 results could be counted on most concentrations.

As the efficiency of echinocandins is clinically proved against *C. dubliniensis* relatively weak MICA effect in RPMI-1640 can be misleading in traditional (RPMI-1640) sensitivity definitions. At the same time, because of th high number of mortality at intensive therapy wards the efficiency of MICA (and other two echinocandins) against *C. albicans* and *C. dubliniensis* is not obvious. Clinical resistance can be explained with the earlier mentioned low level $AUC_{0\text{-}24h}$/MIC, C_{max} and C_{min} or weak penetration of echinocandins to infected areas. Grau et al (2015.) presented important data about the latter, who determined MICA concentration from peritoneal fluid of 10 patients who had abdominal operation, were treated with peritonitis at the intensive ward of surgical department and were in critical condition. From the ten patients in four cases *C. albicans* was cultured from the peritonela fluid (MICA MIC values were ≤0,016 mg/L in every case). During a daily 100 mg MICA therapy on the first and third days in plasma MICA C_{max} values' medians were 5,7 and 4 mg/L. At the same

time peritoneal fluid MICA C_{max} results' medians were 0.9 and 1.2 mg/L. On the first and third days the plasma/peritoneal fluid AUC_{0-24h} ratio was 0.3. A daily 100 mg MICA provided proper serum level, but because of weaker penetration in peritoneal fluid, significantly lower concentration of MICA concentration was present. Yamada et al (2011.) measured similar MICA concentration in pleural fluid (0.56-0.58 mg/L) and in peritoneal fluid (1.02 mg/L) by daily administartion is 150 mg MICA. Considering that in case of peritonitis (or pleuritis) protein content can be fairly high (>25 g/L), killing can be slow or may not happen at all. Due to this fact there is an increased risk that in case of abdominal infections (abscess, peritonitis, cholecystitis) a secondary resistance can develop because of the lower level of echinocandin level, especially in case of *C. glabrata* but also in case of *C. albicans*.

The results obtained with *C. africana* are interesting, though, that only one ATCC type strain and a clinical isolate were available for the experiments. The results obtained in RPMI-1640 (MIC and time-kill curve assays) did not differ significantly with the results obtained with *C. albicans* and *C. dubliniensis* isolates. However, a slow increase in 50% serum warned that the very rare isolation of this pathogen from sterile sites could be related to the serum's pathogenic effect. Our neutropenic mouse model confirmed that *C. africana in vivo*, unlike *C. albicans* and *C. dubliniensis*, is very poorly replicated. Despite the fact that in *C. africana*, mice received at least one order of magnitude more infectious doses, most of the kidney kidney was completely sterile. The species probably lack the virulence factors responsible for translocation from the mucous membranes to the sterile body sites. Our results correspond to Borman et al. (2013) who investigated the virulence of the 3 species with larvae of the large wax moth (*Galleria mellonella*). However, according to our results, the sensitivity of *C. albicans*, *C. dubliniensis* and *C. africana* to MICA is very similar, so in those diagnostic laboratories where the conditions are not met, the therapeutic probability of separating the 3 species from each other is unlikely to be significant. Precise separation of the 3 species from an epidemiological point of view may continue to be important.

In our work, the *in vitro* efficacy of MICA was compared to the less frequent *Candida* species in the conventional RPMI-1640 and in 50% serum-containing serum in vivo. This comparative study is important because in the case of invasive *Candida* infections, during the detection of the growth of sprouting fungus from the hemoculture bottle, there is no information available regarding the fungus species and its antifungal susceptibility. The current recommendation of IDSA (2016) for "blind" therapy clearly suggests the use of echinocandins. Therapeutic experiences with echinocandins are mainly related to the 5 most

common *Candida* species, but it is entirely logical that echinocandins with favorable pharmacokinetics and few side-effects are also preferred against rare *Candida* species. Based on our results for the less isolated species (*C. kefyr, C. lusitaniae*), although the MICA MIC values obtained in RPMI may be very close to the MIC values of *C. albicans, C. glabrata* or *C. tropicalis* highly susceptible to echinocandins. MIC assay and killing curve in serum-based medium may exhibit much lower efficacy than *C. albicans, C. glabrata* or *C. tropicalis*. The decrease in MICA's effect was less surprising in the case of *C. guilliermondii*, since the species has a natural, decreased susceptibility to echinocandins. In the light of all this the best solution seems to be to decide on further therapy after the precise identification of the species, taking into account the patient's clinical condition. If the invasive *Candida* infection caused by the rare species is clinically and microbiologically improved, echinocandin therapy may be continued. If the condition of the patient gets worse, AMB may also be discussed (lipid-associated versions). If the pathogen has decreased susceptibility to other antifungal agents, increasing the daily dose of echinocandin or combination therapy may help improve the condition of the patient.

Summary

According to the current recommendation, in case of invasive *Candida* infections, echinocandins (anidulafungin, caspofungin and micafungin) are the first antifungal agents to be chosen. Contrary to the therapeutic introduction of the echinocandins, the mortality triggered by the invasive *Candida* infections, especially in case of the less frequently isolated *Candida* species, is still unacceptably high. In my work, by using the minimal inhibition concentration (MIC) and the time-killing curves, the *in vitro* activity of the micafungin was compared against *C. albicans, C. dubliniensis, C. africana, C. guilliermondii, C. lusitaniae* and *C. kefyr* isolates in RPMI-1640 and 50% serum substrates.

In RPMI-1640, the *C. albicans* (MIC≤0.03 mg/L) and the *C. guilliermondii* (MIC ≤1 mg/L) isolates proved to be sensitive to micafungin. In case of *C. dubliniensis* (MIC≤0.03 mg/L), *C. lusitaniae* (MIC≤0.25 mg/L) and *C. kefyr* (MIC≤0.12 mg/L), the MIC values were not higher than the epidemiological limit values. In case of the *C. africana* MIC values of 0.015 mg/L were obtained. The micafungin proved to be of fungicidal or fungistatic effect at values close to the MIC against the species analysed.

In 50% serum, the MIC values increased to 16-64 fold in case of *C. albicans*, *C. dubliniensis* and *C. africana* compared to the MIC values measured in RPMI-1640 and at the clinically easily achievable concentrations of ≥4 mg/L, micafungin proved to be of fungistatic effect. The *C. africana* had very low proliferation rate *in vivo*. The MIC values against *C. kefyr, C. lusitanaie* and *C. guilliermondii* increased to 64-256, 64-128 and 32-64 fold, respectively compared to the MIC values measured in RPMI-1640. The killing rate values were positive for *C. kefyr* only at concentrations of 16 and 32 mg/L, while those for one of the *C. lusitaniae* isolates were positive only at the concentration of 32 mg/L. In case of the *C. guilliermondii*, the killing rates were negative in each case, even at the concentration of 32 mg/L.

On the basis of our findings, the species of the less frequently isolated species (*C. kefyr* and *C. lusitaniae*), although the micafungin MIC values obtained in RPMI-1640 may be very close to the MIC values of the species sensitive to the echinocandins, the MIC determination performed in the serum-based substrate and the establishment of the killing curve may exhibit a much more intense response loss than in case of the *Candida* species sensitive to the echiocandins. Therefore, echinocandins shall be used with due care against the rare *Candida* species. On the basis of the three species genetically close to each other (*C. albicans, C. dubliniensis* and *C. africana*), the most virulent is the *C. albicans,* while the less virulent is the *C. africana*.

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List of publications related to the dissertation

1. **Saleh, Q.**, Kovács, R. L., Kardos, G., Gesztelyi, R., Kardos, T., Bozó, A., Majoros, L.: Decreased Killing Activity of Micafungin Against Candida guilliermondii, Candida lusitaniae, and Candida kefyr in the Presence of Human Serum.
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2. Kovács, R. L., **Saleh, Q.**, Bozó, A., Tóth, Z., Gesztelyi, R., Kardos, T., Kardos, G., Takács, I., Majoros, L.: Killing Activity of Micafungin Against Candida albicans, C. dubliniensis and Candida africana in the Presence of Human Serum.
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List of other publications

3. Domán, M., Kovács, R. L., Kardos, G., Gesztelyi, R., Juhász, B., Bozó, A., Kardos, T., **Saleh, Q.**, Majoros, L.: Killing rates of caspofungin in 50 percent serum correlate with caspofungin efficacy against Candida albicans in a neutropenic murine model.
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Bozó A, Domán M, Kovács R, Perlin DS, Kardos G, Kardos T, Tóth Z, **Saleh Q**, Majoros L: Dose escalation studies with caspofungin against *Candida glabrata*. 17TH INTERNATIONAL CONGRESS OF THE HUNGARIAN SOCIETY FOR MICROBIOLOGY. Acta Microbiologica et Immunologica Hungarica 2015, **62:**MPP-2