

SHORT THESIS FOR DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

**The paradoxical growth of *Candida albicans*, *C. dubliniensis*, *C. krusei* and
C. tropicalis strains in the presence of high concentration caspofungin**

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Doctoral School of Pharmaceutical Sciences**

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INTRODUCTION

The epidemiology of infections caused by Candida species

Several antifungal agents have been introduced into clinical practice recently, such as new generation azoles and echinocandins. These antifungals may produce good clinical results even in the case of invasive fungal infections, when the conventional antimicrobial therapy proved to be ineffective. The invasive candidiasis often accompany serious systemic diseases, and frequently lead to lethal complications. The most important predisposing factors are the immunosuppressive therapy, diabetes mellitus, malignant processes, too old or too young age, surgical procedures and the AIDS.

Newer antifungal agents were needed because significant alterations occurred in the epidemiology of the *Candida* species. Although the most important pathogen is *C. albicans*, the significance of other species such as *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* is continuously increasing, as well. It is also important that the *non-C. albicans* infections are often related to haematological malignant processes, furthermore the occurrence of certain species differs depending on the age. Consequently, the incidence of *C. parapsilosis* is gradually decreasing, while the ratio of invasive mycoses caused by *C. glabrata* is increasing with age.

Several investigations have recently proved the presence of *non-C. albicans* species in the oral cavity. Out of these, one of the most often examined oral pathogen is the *C. dubliniensis*, a species phenotypically similar to *C. albicans*, which was first isolated from the oral lesion of HIV infected patients. Some authors described the severe extraoral symptoms (candidemia, meningitis) with *C. dubliniensis* infections.

According to the literature the number of diseases caused by *non-C. albicans* species has greatly increased both in the superficial and in the systemic fungal infections and in these cases the patients' chances for survival proved to be worse. High mortality may be reduced by the earlier diagnosis of the mycosis on the one hand, and the more exact investigation of the effects of the applied antifungal substances on the other hand.

Determining the antifungal susceptibility in the case of yeast

The reference process for the determination of the antifungal susceptibility is the standard dilution method. The standard dilution method can be divided into two large groups. One of them is the standard macrodilution method, in which we use 10 mL end volume (test medium+antifungal agent+yeast) to determine the minimal inhibitory concentration (MIC) value.

By definition MIC is the smallest concentration of the antifungal substance, which already inhibits the increase of the visible growth of microorganisms compared to the control which does not contain the substance.

The protocol of the *microdilution method* was modified by the National Committee for Clinical Laboratory Standards (NCCLS), and the modifications were summarized in the M27-A2 document. We used this method in the case of echinocandins developed in the last ten years. Due to the established standards the method became easily reproducible.

In the microdilution method we apply the RPMI-1640 test medium, whose exact composition is included in the M27-A2. The starting inoculum is usually 10^3 CFU/mL, and ELISA plate is used, as well as we determine the MIC value after 24-48 hour incubation time.

A possible alternative of the test medium applied at the standard microdilution method is the antibiotic medium 3 (AM3), which may make the reading of the MIC value easier and may also influence the fungicid activity of the antifungal substance.

When determining MIC value, the trailing effect may be recognized, which means a reduced but continuous growth in the wells above the MIC value.

Determination of the minimal fungicid concentration

The minimal fungicid concentration (MFC) by definition is the smallest concentration of the antifungal substance, which kills 99,9 % of the fungi within 24 or 48 hours compared to the starting inoculum.

MFC characterizes the *in vitro* killing activity of the drug. Unfortunately the method is not standardized either in relation to the different medicines or the various *Candida* species. In addition, in the previous studies the authors could only measure a maximum of 90-99 % killing rate, when they sampled 10-100 μ L onto agar plates from those wells of the tray where increase was not traced.

It is caused by the fact that the determination the MFC value is usually preceded by the determination of the MIC value, and in the standard method (NCCLS M27-A2) the starting inoculum size can only be $0,5 \times 10^3$ or $2,5 \times 10^3$ CFU/mL. However the diagnostic criterium of the MFC (99,9% reduction) cannot be fulfilled even if entire volume were sampled.

Recently, Canton et al. proposed a modification of the procedure used to determine MFC in the case of AMB. To fulfil the diagnostic criterium they used a larger inoculum of 10^4 CFU/mL and larger sampling volume (200 μ L). Antifungal carryover was avoided by subculturing onto two Sabouraud dextrose agar plates. Each 100 μ L aliquot was spotted onto the plate and allowed to soak into the agar. After the plate was dry, it was streaked uniformly to separate the cells and remove them from the drug source.

It was found that the use of the higher inoculum did not significantly increase the MICs thus the determination of the fungicid activity by definition became possible.

Time-kill studies (The determination of time-kill curves)

Besides the determination of MFC values, time-kill curves can be used to characterize the fungicid activity of a certain substance. The interaction between the antifungal substance and the germ can be described more dynamically by using this method, and it may have a greater clinical utility, too. In general, the curves can be used to describe the fungicid or fungistatic effect of a given substance against the germs, but the antagonism or synergy among two or more antifungal substances can also be examined.

Standardization of the investigation method was introduced by Klepser at al., by taking the criteria described in the M27-A2 document into account (10^3 CFU/mL, RPMI-1640, 48 hour incubation time). The concentration of the starting inoculum was increased (10^5 CFU/mL) so that the 99.9% fungicid effect could be detectable. In order to avoid sampling errors and the antifungal carryover, the sampling volume was defined as $4 \times 30 \mu$ L. This „standardized” time-kill method is easily reproducible (90-93%). Although the method suggests the use of RPMI-1640 test medium for the investigations, several authors agree that the fungicid concentration also depends on the type of the chosen medium to a great extent. This difference related to the test media can be observed in the case of the echinocandins, too (RPMI-1640/AM3).

Resistance and paradoxical growth in the case of echinocandins

The β -1,3-D-glucan synthase is a multi-subunit-enzyme complex responsible for maintaining the integrity of the fungal cell walls. Echinocandins affect the Fks1 subunit of the glucan synthase, and the mutation of this enzyme may result in resistance to different extents. MIC value in the case of *C. parapsilosis* and *C. guilliermondii* may be 100 times as much as the MIC values in the case of *C. albicans* strains. Clinical isolates of *C. albicans*, *C. glabrata*, *C. krusei* and *C. tropicalis* with high MIC values (> 2 mg/L) and therapeutic failure were reported by several authors. In some cases amino-acid alteration was detected on the 1st and 2nd „hot spot” regions of the Fks1 subunit of the glucan synthase enzyme.

Inhibition of the glucan synthase may result in a rise in the compensatory chitin level as an adaptive stress response. This process may be responsible for the so-called paradoxical growth of the *C. albicans* in the presence of high concentration caspofungin.

Paradoxical growth (PG) by definition means that there is growth inhibition in at least 2 wells above the MIC value, however growth can be observed in the wells of the plate at higher concentrations. The paradoxical effect is a quadriphasic process depending on the concentrations. The phases are the following: subinhibitory phase; inhibition of growth above the minimum inhibitory concentration; attenuation of activity at higher concentration; inhibition of growth at the highest drug concentrations. It implies that the higher the drug concentration is, the less the efficacy of the drug may be. The *in vitro* results question whether the efficacy of caspofungin (and the other echinocandins) grows at higher drug concentrations.

At the beginning of my research there was relatively scarce data in the international literature concerning the investigation of the paradoxical growth in the presence of caspofungin. Therefore I carried out the *in vitro* susceptibility examination of the yeast isolated in the Medical Microbiological Institute of the University of Debrecen, Medical and Health Science Center (DEOEC). I also investigated the possible ways of detecting paradoxical growth. Finally, I also examined the clinical utility of the *in vitro* results.

OBJECTIVES

1. To examine the prevalence of paradoxical growth at high concentrations of caspofungin in the case of *C. albicans*, *C. krusei*, *C. dubliniensis* and *C. tropicalis* strains by means of determining the minimum inhibitory concentration values in RPMI-1640 and antibiotic medium 3.
2. To determine the accurate occurrence of paradoxical growth in RPMI-1640 and antibiotic medium 3 by the comparative investigation of the time-kill curves and the fungicidal concentration values.

MATERIALS AND METHODS

The origin of the yeast

In our experiments we selected 45 isolates (15 of *C. albicans*, 15 of *C. krusei*, 15 of *C. tropicalis*) out of the *Candida* strains isolated in the Medical Microbiological Institute of the University of Debrecen, Medical and Health Science Center (DEOEC). The test strains of the American Type Culture Collection (ATCC®) were used as reference in the case of all strains (*C. albicans* ATCC 14053, *C. krusei* ATCC 6258, *C. tropicalis* ATCC 750). Six *C. dubliniensis* isolates were obtained from Ferenc Somogyvári, which originate from his previous work. CG36 strains were used as reference in the case of the *C. dubliniensis*.

The strains were isolated from blood samples (8 of *C. albicans*, 4 of *C. krusei*, 5 of *C. tropicalis*), as well as from wounds, body cavities, oral cavities and mucous membrane.

Identification of the yeasts

The identification of the strains is based on the detection of the germ tube production, which implies the incubation of the cultures grown on Sabouraud dextrose agar (SDA) into fetal calf serum for 2 hours. The test was positive in the case of *C. albicans*, while it was negative in the case of the *C. tropicalis* and *C. krusei* strains.

CHROMagar Candida (Becton Dickinson) medium was used for presumptive species identification and for controlling the purity of the cultures.

For further identification we used API ID32 panel (BioMérieux), the results of which were read after 48 hour incubation time. Identification of the 6 *C. dubliniensis* isolates by means of molecular biological methods had already been done previously.

Determination of the minimal inhibitory concentration

Determination of the MIC value was executed as recommended by the NCCLS M27-A2 document. MIC value was determined with standard 10^3 CFU/mL and with elevated 10^5 CFU/mL inoculum both in RPMI-1640 (Sigma) and AM3 (Fluka) media as described above.

CAS substance (Merck Research Laboratories) was dissolved in sterile distilled water as prescribed by the standard NCCLS method. CAS concentration in the wells of the plate was 0.024-12.5 mg/L in the case of the *C. tropicalis*, while 0.015-8 mg/L in the case of the other strains. The same plates were used for determining the MFC, which were stored at -20 °C. When preparing the fungal suspensions 0.5 McFarland density solution was made using a densitometer. The suspensions were diluted in 0.85% saline. Each plate contained a drug-free control (growth control) and a medium control (negative control). Each investigation was conducted at least twice.

Determination of minimum fungicidal concentration

MFC was determined after the microdilution plates were incubated at 35 °C for 48 hours in the case of all isolates. The starting inoculum was 10^5 CFU/mL both in RPMI-1640 and AM3 media. In the case of *C. albicans*, *C. krusei* and *C. dubliniensis* strains after determining the 48 h MIC values, the entire contents (200 µL) of the visually clear wells were homogenized by pipetting. Aliquots were placed on 2 – 2 SDA plates (100-100 µL) and after drying, the fungal cells were dispersed by streaking. The colonies were counted after incubation at 35 °C for 48 hours.

In the case of *C. tropicalis* the MFC value was determined after 24 hours with elevated inoculum size (10^5 CFU/mL), as well.

In the case of *C. dubliniensis* and *C. tropicalis* the prevalence of trailing growth (continuous growth in concentrations above MIC) in RPMI-1640 and AM3 media was also examined.

Determination of time-kill curves

Time-kill studies were performed according to the method described by Klepser et al. Before the experiments we examined how the starting inoculum size is influenced by the various concentrations of the CAS (0.5 – 16xMIC) (antifungal carryover).

The starting inoculum was 500 CFU/mL. After preparing the fungal suspension, we immediately placed 4x30 µL on SDA plates. The colonies were counted after 48 h incubation. Antifungal carryover is observed if the number of the colonies is by more than 25% less than the colonies in the control test tubes in the case of the different drug concentrations.

The starting inoculum was 10^5 CFU/mL in all cases, which was checked before the tests. The values of the CAS concentration were 0.024-12.5 mg/L in the case of *C. tropicalis*, while 0.06-16 mg/L in the case of *C. albicans*, *C. krusei* and *C. dubliniensis* strains.

Test tubes were incubated with agitation in the dark at 35 °C. At predetermined time points, samples (100 µL) were removed and serially diluted in sterile saline (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}).

If the colony number was expected to be less than 1000 CFU/mL (more significant killing effect), undiluted samples were plated onto SDA.

On the basis of our preliminary results with the test strains, the sampling points were defined as follows: 0, 2, 4, 8, 12, 24 and 48 hours in the case of *C. tropicalis* and *C. dubliniensis*, and 0, 4, 8, 12, 24 and 48 hours in the case of *C. albicans* and *C. krusei*.

Four 30 µL were subsequently plated onto SDA. The samples were let to dry at room temperature, and they were incubated at 35 °C for 48 hours. Colonies were counted and the number of the living fungal cells was determined by taking the dilutions into account.

In the case of *C. tropicalis* we also investigated how adding 1 mg/L amphotericin B (AMB), 1 mg/L fluconazole (FLU) and 1 mg/L 5-fluorocytosine (5-FC) to the test tubes containing high concentrations of CAS (6-12,5 mg/L) influences the paradoxical growth, or if it is able to suspend PG. Each investigation was conducted at least twice and the average of the results was calculated.

Evaluation of the results

In our investigations MIC values were determined after 24 hours in the case of all four strains according to the recommendations of NCCLS M27-A2. MICs were read using the partial inhibition criterion (MIC_{PI}, the lowest concentration of drug that produced a prominent decrease in turbidity compared to the drug-free control).

Additionally, after 48 h MIC values were also evaluated in the case of *C. albicans* and *C. krusei* strains using the total inhibition criterion (MIC_{TI}, the lowest concentration of drug that yielded no visible growth).

According to our definition of PG, after the MIC_{PI} value at least two clear wells should be observed between wells showing growth. It means that growth occurs at low and high, but not at immediate concentrations.

RESULTS

Results found through minimal inhibitory concentration

MIC_{PI} for *C. tropicalis* was 0.024mg/L in both media (RPMI-1640 and AM3) when using normal (10^3 CFU/mL) as well as increased (10^5 CFU/mL) starting inocula.

MIC_{PI} values read for *C. dubliniensis* were 0.06-0.25 mg/L in RPMI-1640 in normal starting inocula, with the exception of isolate number 4 where the MIC_{PI} read 8 mg/L. In increased starting inocula (10^5 CFU/mL) the MIC_{PI} value rose slightly (0.12-0.25 mg/L) but for isolate number 4 the MIC_{PI} value remained unchanged (8 mg/L). In AM3, MIC_{PI} values were uniformly 0.03mg/L regardless of the starting inoculum.

For the *C. albicans* the MICs were read after 24h (using partial inhibition criterion (MIC_{PI})) and after 48h MICs were also evaluated (using total inhibition criterion (MIC_{TI})). MIC_{PI} ranges read after 24h for the *C. albicans* isolates grew 0.015-0.06 mg/L in RPMI-1640 and somewhat lower (0.015-0.03 mg/L) in AM3. MIC_{TI} ranges read after 48h for the *C. albicans* isolates were 0.25-1 mg/L in RPMI-1640 but in AM3 the numbers were smaller (0.015-0.06 mg/L).

MIC ranges read after 24h with the partial inhibition concentration for the *C. krusei* isolates were 0.12-0.25mg/L in RPMI-1640, while in AM3 it remained at 0.12 mg/L. MIC_{TI} ranges read after 48h were 2mg/L in RPMI-1640 and 0.25mg/L in AM3.

Studying paradoxical growth on the basis of the minimal inhibitory concentration results

The *C. albicans* 14057 strain showed PG in both test medium.

For the *C. krusei* only the reference strain (ATCC 6258) showed PG and only in AM3 medium.

Two *C. tropicalis* isolates (isolate 4 and 15) showed PG in RPMI-1640 at 12.5 mg/L concentration at 10^3 CFU/mL starting inocula. All *C. tropicalis* clinical isolates showed trailing growth when increased starting inoculum was used, up to the highest concentration value (12.5 mg/L).

PG was not observed for *C. dubliniensis* in RPMI-1640 when using increased inocula. In AM3, PG was detected in two isolates using elevated inocula after 48h. In AM3 trailing

growth was not detected regardless of the starting inoculum and incubation time. In contrast, in RPMI-1640, trailing growth was detected in all wells after 48h.

Studying paradoxical growth on the basis of minimal fungicidal concentration results

MFC ranges read after 48h for *C. albicans* and *C. krusei* were 0.5-1, 1-2 mg/L in RPMI-1640 and 0.03 mg/L, 0.25-0.5 mg/L in AM3. During the MFC tests, one *C. albicans* isolate (14057) and one *C. krusei* isolate (4363) showed PG in RPMI-1640.

MFC values in RPMI-1640 were > 8mg/L for all *C. dubliniensis* strains. In AM3, all MFCs were \leq 0.12 mg/L and all but isolate number 4 showed PG.

By the MFC tests for *C. tropicalis* in RPMI-1640 the total number of 6 isolates grew at 6.25 mg/L CAS concentration after 24h and this number increased to 7 in 48h. All isolates except number 7 grew at 12.5 mg/L after 24 and 48h of incubation. PG was detected in 15 out of 16 isolates. In AM3, the MFC concentrations were \leq 0.09 mg/L, which was \leq 4xMIC (growth only took place at low concentrations). In other words, in AM3, CAS showed increased kill curves, PG was only observed in one case (isolate number 6).

Results of the investigation of time-kill curves

Antifungal carryover was not observed during the experiments.

The killing patterns obtained using the time kill method for *C. tropicalis* reinforced the results of the MFC tests, after both 24 and 48h. At low CAS concentrations fungistatic effects were observed, at moderate concentrations (\leq 3.12 mg/L) fungicidal activity took place, but at high CAS concentrations (6.25-12.5 mg/L) fungistatic activity was observed again. When the strains growing at 12.5 mg/L were retested, the same kill curves were obtained. The killing patterns obtained using the MFC method correlated with the killing patterns obtained through the time-kill method, with the exception of isolate number 4 and the ATCC 750 strain in RPMI-1640. *C. tropicalis* reference strain grew at all CAS concentrations tested in MFC tests, after both 24 and 48h. In the time-kill test, this strain portrayed fungicidal effect at 256 times the MIC after 24h, but growth was observed at 512 times the MIC. A similar discrepancy, although smaller, was found for isolate number 4.

Adding FLU (1mg/L) to the starting inoculum resulted in the elimination of PG for all clinical isolates, regardless of the medium used, even after 24h. (Neither the same concentration AMB, nor 5-FC had the same effect.) In contrast, in the case of the *C. tropicalis* reference strain PG was eliminated by adding 1mg/L AMB. (In this case neither the FLU nor the 5-FC was effective, in other words PG was still present.)

When studying *C. dubliniensis* two types of killing curves were obtained for the 6 clinical isolates and the CD 36 reference strain. In RPMI-1640 fungistatic effect was observed but in AM3 the same clinical isolates and the reference strain showed typical PG. Fungicidal effect was observed at 0.12-4 mg/L as concentration (4-128 times MIC) within 12h but only fungistatic effect was noted at lower concentrations. 8-16 mg/L (256-512 times MIC) CAS concentration correlated to fungistatic activity.

Isolate number 4, however behaved in a different way. In this case the MIC was 8 mg/L. Fungicidal effect was observed at 8-16 mg/L (1-2 times MIC) CAS concentrations and it was fungistatic at lower concentrations in RPMI-1640. In AM3 CAS was fungicidal at all concentrations in 24h. PG, in this case, was not observed.

When studying *C. albicans* different time-kill curves were observed in different media.

The ATCC 14053 reference strain showed PG in both media. Fungicidal effect was observed even at 0.12 mg/L CAS concentration and at concentrations higher than this fungistatic effect was detected at 1-16 and 8-16 mg/L concentrations in RPMI-1640 and AM3, respectively.

Two clinical isolates (7111 and 10598) behaved in a similar way, in RPMI-1640 after 24h fungistatic effect was observed regardless of the concentration. Both isolates were killed after 48h incubation at 16 mg/L CAS concentration (fungicidal effect). At lower concentrations fungistatic effect was observed.

In AM3 CAS proved to be fungicidal against isolate number 7111 after 12h at 0.12-8 mg/L concentrations. After 24h it was fungicidal at 16 mg/L (higher concentrations resulted in slower kill time). Isolate number 10598 showed PG in 24h at 16 mg/L concentrations, but at lower CAS concentrations fungicidal effect was observed and in 48h > 0.5mg/L concentration showed fungicidal effect.

Isolates 8812 and 21186 showed a fungistatic effect after 24h in RPMI-1640 but gave typical PG curves after 48h. We observed fungicidal activity at 0.5-2 mg/L and fungistatic activity at 4-16 mg/L and 0.12 mg/L CAS concentrations.

In AM3, isolate number 8812 proved to be fungicidal at 0.12-16 mg/L and showed fungistatic activity at 16 mg/L in 24h, and then in 48h at > 12 mg/L CAS concentrations this strain was killed fully (fungicidal effect).

Isolate number 14057 showed fungistatic killing curves in RPMI-1640 at all CAS concentrations. In AM3 this isolate showed typical PG. In this case in 24h caspofungin proved to be fungistatic (4-16 mg/L), at ≤ 2 mg/L it was undoubtedly fungicidal. In 48h the killing activity was increased, at ≤ 8 mg/L it was fungicidal, while at 16 mg/L we observed fungistatic effect.

For the three *C. krusei* clinical isolates, fungicidal activity was observed in RPMI-1640 after 24h and 48h at ≥ 2 mg/L CAS concentrations. In AM3, the killing activity of CAS was increased. It was fungicidal at 0.12-0.5 mg/L concentrations in 24h but this fungicidal effect was observed at lower concentrations in 48h at ≥ 0.12 - 0.25 mg/L CAS concentrations.

When studying the ATCC 6258 strain fungicidal activity was observed in 24h at ≥ 0.5 mg/L CAS concentrations in RPMI 1640. In 48h the fungicidal effect was observed at ≥ 0.25 mg/L. Similar results were obtained in AM3 as well. During the study of *C. krusei* paradoxical growth was not detected.

DISCUSSION

The introduction of the clinical use of echinocandins in the beginning of the new Millennium was joyous as mortality rates caused by intensive fungi infection were still high for both neutropenic and non-neutropenic patients despite the fact that FLU had already been widely used for 10 years. Although the echinocandins are effective against some *Aspergillus* strains, their major role is fighting intensive *Candida* infections.

With regards to echinocandins, there are three major, deeply connected questions which arise during research and clinical use: what break-point should be chosen to detect resistant isolates, what is the optimal dosage and can PG play a role in treatment.

The break-point of echinocandins is 2 mg/L at present. This break-point has been calculated from establishing MIC values for several thousand clinical isolates, the distribution of the MIC values, as well as the MIC value and the likelihood of clinical success. It is well-known that the majority of *Candida* species have low MIC₉₀ values against all echinocandins and that the MIC₉₀ values of *C. parapsilosis* and *C. guilliermondii* isolates are relatively high (1-2 mg/L). In the case of the “psilosis” group this is caused by the fact that the β-1.3-D-glucan synthase enzyme can be observed change proline to alanine in the 660th amino acid. For the *C. guilliermondii* the 642nd amino acid exchange (methionine-leucine) seems to be the most important. *In vivo* animal experiments have proven that both *C. parapsilosis* and *C. guilliermondii* isolates have reduced susceptibility against echinocandins. *C. parapsilosis* isolates show less virulence than the *C. albicans* for example, which may be the key for the clinically effective echinocandin therapy against this strain.

The routine, clinical isolate *Candida* species have ≤ 0.25 mg/L MIC₉₀ value except for *C. guilliermondii* and *C. parapsilosis*. Therefore it seems logical to separate the natural lower sensitivity of *C. parapsilosis* and *C. guilliermondii* against echinocandin from the real resistance to echinocandin, which develops during therapy in a secondary way. The first step for this is exact species identification. In other words, if the species in question are *C. parapsilosis* or *C. guilliermondii*, then the echinocandins should not be the first choice of antifungal substances used for treatment. Secondary resistance usually occurs in *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. krusei*, which have low MIC₉₀ values naturally. If the MIC values for these species appear to be relatively high (≥ 0.5 mg/L) after the exact species determination, then it is more than likely that we are facing gained resistance.

The next important question is optimal dosage. At present this is 1-2 mg/kg depending on the echinocandin. The efficiency of these doses is also supported by pharmacokinetics and pharmacodynamics *in vivo* data. Patients tolerate these doses well, but unfortunately the mortality rate has not decreased significantly compared to previous years. This is why the question of increased dosage arose as a possible solution to improve clinical success. Pappas et al. has proved that in the case of micafungin the daily 8 mg/kg dosage for a minimum of 7 days is safe and the side-effects are not significantly more frequent than for the 100 mg daily dosage.

Similar results were found by Betts et al. during his studies where they compared the daily standard 50 mg CAS therapy to the three times the amount, daily 150 mg CAS therapy. The increased dosage increased the therapeutic success for patients infected with the *C. albicans* and the *C. parapsilosis*, however there was more therapeutic failure in the case of *C. tropicalis* and *C. glabrata*.

As I have mentioned in the introduction, PG is an *in vitro* phenomenon and its clinical importance is not clear. It can influence the MIC readings during the MIC determination carried out by microdilution method, especially if the readers are not experienced. At the same time, in addition to PG, the MIC values can also be affected by the trailing effect. By definition, trailing means visible growth in the wells, which are over the MIC value, but both the time-kill curves and *in vivo* results show that these isolates respond to antifungal therapy. The trailing effect can also be seen at the echinocandins. Moreover, initially the total inhibition was the final stage for determining MIC, so compared to the partial inhibition, the MIC values could even be 4-5 dilution degrees higher. It was also noted that the growth taking place after the partial inhibition is not real growth but deceased yeast fragment without living cells. Therefore the partial inhibition became the final criterion for the MIC readings.

During our study we observed a small percentage of PG for all four species at low (10^3 CFU/mL) inoculum during the MIC tests regardless of the starting medium. Increasing the starting inocula should have increased the number of yeast, which indicates PG, but only the trailing effect was increased in RPMI-1640. In AM3 trailing was reduced and therefore it was easier to read the MIC values.

In the case of *C. tropicalis* isolates we were able to detect the decreased killing at lower and higher concentrations after both 24h and 48h with the MFC tests regardless of the medium. In AM3, killing took place at lower concentrations, which reinforced the findings of the researchers mentioned previously, that in AM3 the antifungal substances kill the yeast faster than in RPMI-1640. During the MFC tests CAS proved to be fungicidal in RPMI-1640 with the exception of *C. dubliniensis* isolates. In AM3, the MFC test seems useful for detecting PG, which was extremely noticeable in the case of *C. dubliniensis* isolates ($\geq 8\text{mg/L}$ MFC in RPMI-1640 while in AM3 there was typical PG after 48h except for one isolate).

The time-kill curves reinforced and refined the results of the MFC experiments. In our opinion, PG has to be determined with the help of time-kill curves for some *Candida* strains. This method shows us how the killing takes place in time. After shorter incubation time (24h) fungistatic or possibly PG effect was observed, while after long incubation time (48h) fungicidal, or in some cases PG effect was detected. In other words, after longer incubation time, the fungistatic effect can change into PG (killing increases at lower concentrations) while PG can change into full fungicidal effect (killing takes place at higher concentrations). This tendency was best observed in the case of *C. albicans* and its close relative *C. dubliniensis*. This phenomenon was heavily influenced by the medium for both strains (CAS killed faster in AM3). Moreover, for one *C. dubliniensis* isolate MIC was significantly lower in AM3 than in RPMI-1640 (8 mg/L) and fungicidal effect was observed at medium concentrations. For the same isolates PG was still present at the highest concentrations even in AM3. Unfortunately we were not able to study the molecular background of this phenomenon.

The *in vivo* effect of echinocandins depends on the volume of the medicine concentration where the infection takes place. The most recent study to determine the 24h echinocandin levels in the plasma for all three echinocandins at 5, 20 and 80 mg/kg doses was carried out by Andres et al. using an animal model. It is notable that even a 5 mg/kg caspofungin dosage administered only once results in a 18 mg/L peak plasma concentration. Taken into account that in the serum the bound to the proteins is 97-99% in the echinocandins, it is likely that the free and active echinocandin concentration will not be larger than 0.1-0.2 mg/L.

It is worth making a distinction between the echinocandin levels in the serum after one dose and after continuous large doses. In theory it is possible that the fraction of the echinocandin which is not attached to the protein grows in the serum and reaches a level, where the killing effect against the causative agent does not prevail. Unfortunately, the serum echinocandin levels have not yet been studied for therapies where the echinocandin was used permanently

in large doses. Another important factor is that the fungi can not only be present in the blood but it can also trigger the invasion of any of our internal organs, from where its extinction can be more difficult. The echinocandins enter up to the tissue from the blood and then from there they return to the blood as if they are returning from a depot. This typical pharmacokinetics makes the daily one dose of echinocandin possible. We do not know (there is no literature on it) the amount of the echinocandin levels or the degree of bound to the proteins in case of permanently larger daily doses. In theory it is possible that a free echinocandin concentration can develop in the tissues, where the causative agent survives. Therefore, in our opinion the importance of PG can not be excluded from the list of reasons of therapeutic failures.

Long term echinocandin therapies which use a higher dose of the echinocandin on a permanent basis did not increase the survival rates of patients suffering from invasive candidiasis, meanwhile the already expensive daily cost of the therapy was increased two, three-fold. In addition, in the case of some strains the role of PG can not be excluded in unsuccessful therapies. For this reason it appears that the permanently higher daily dosage echinocandin therapy has more disadvantages than advantages. Therefore it appears to be more appropriate to chose normal dosage for echinocandins and maybe combine them with AMB or some kind of triazole in order to reduce mortality rates. The AMB, FLU, 5-FC and posakonazole do not show antagonism effect with the echinocandins, on the contrary even our results show that these medicines extinguish the *in vitro* PG. In the future, combination therapy might play a more important role in antifungal therapy.

SUMMARY

Caspofungin, which is a member of the echinocandin antifungal class, is a new antifungal drug that can be used to treat both oropharyngeal and systemic *Candida* infections. During the *in vitro* susceptibility testing many researchers detected growth in higher echinocandin concentrations (Eagle effect), but the exact frequency of the occurrence of this phenomenon was not known.

During our research we examined the paradoxical growth that appears when using large dosage of caspofungin in case of *Candida* species which are important from oropharyngeal point of view (*C. albicans*, *C. dubliniensis*, *C. krusei* and *C. tropicalis*). We also examined this phenomenon through establishing the minimal inhibitory concentration and also with the help of the killing triggered by caspofungin using minimal fungicidal concentration and time-kill curves in two media (RPMI-1640, AM3).

During our research we observed the following:

1. In AM3 the minimal inhibitory concentration results were lower and the trailing effect, which usually perplexed the exact readings, was not present for any of the four species.
2. With the help of minimal fungicidal concentrations and time-kill curves we undoubtedly proved that for *C. tropicalis* the paradoxical growth, like reduced killing ability can be observed in high caspofungin concentrations, regardless of the media.
3. We observed fungistatic effect for *C. albicans* and *C. dubliniensis* isolates in RPMI-1640 but the killing effect was increased after 24h and more so after 48h in AM3 which appeared in the form of paradoxical growth and fungicidal effect.
4. The time-kill curves proved that for *C. krusei* paradoxical growth does not take place in either media.
5. In our work, we used minimal fungicidal concentration and time-kill curves for the first time to study paradoxical growth.

Our results decisively confirmed and refined the result found by other researchers with regards to paradoxical growth. In order to achieve safe echinocandin therapy, preclinical and clinical studies need to be carried out to clarify the *in vivo* significance of the *in vitro* paradoxical growth at high caspofungin concentrations.

PUBLICATIONS

Publications used in thesis

1. Sóczó G., Kardos G., **Varga I.**, Kelentey B., Gesztelyi R., Majoros L. 2007. In vitro studies with *C. tropicalis* isolates exhibiting paradoxical growth in the presence of high concentrations of caspofungin. *Antimicrob. Agents Chemother.* 51(12): 4474-4476. **IF: 4.39**
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3. **Varga I.**, Sóczó G., Kardos G., Kemény-Beke A., Kelentey B., Márton I., Majoros L. 2009. Differences in killing activity of caspofungin and paradoxical growth between *C. albicans* and *C. krusei* clinical isolates in different media. *J. Chemother.* 21(1): 36-41. **IF: 1.166**

Other publications

1. **Varga I.**, Sóczó G., Kardos G., Borbély A., Szabó Z., Kemény-Beke A., Majoros L. 2008. Comparison of killing activity of caspofungin against *Candida parapsilosis*, *Candida orthopsilosis* and *Candida metapsilosis*. *J. Antimicrob. Chemother.* 62: 1466-1468. **IF:4.328**
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List of publications related to the dissertation

1. **Varga, I.**, Sóczó, G., Kardos, G., Kemény-Beke, Á., Kelentey, B., Márton, I., Majoros, L.: Differences in killing activity of caspofungin and paradoxical growth between *Candida albicans* and *C. krusei* clinical isolates in different media.
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The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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