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

Efficacy of caspofungin dose escalation against *Candida albicans* and *Candida glabrata* clinical isolates

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DEBRECEN, 2016

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The Examination takes place at the Library of Department of Pharmacology, Faculty of Medicine, University of Debrecen, 28th of June 2016 at 11:00

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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, 28th of June 2016 at 13:00

INTRODUCTION

Fungal infections are increasingly recognized as a major global health problem. There are more than 300 million people afflicted by a serious fungal infection resulting in nearly 1.4 million deaths annually. The majority of these infections caused by *Candida*, *Aspergillus* and *Cryptococcus* species.

Several risk factor contribute to invasive candidiasis, such as use of broad-spectrum antibiotics and chemotherapy-induced neutropenia (>500 neutrophil granulocyte/µl). According to a study which include many countries the mortality rate due to candidaemia in all hospital ward was 29%, however in the intensive care units 60-70% mortality rates have been reported.

Although the prevalence of different *Candida* species have changed over the last decades, 90% of invasive infections are attributed to five species: *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*. The most frequently isolated species is still *C. albicans*. However the therapeutic and prophlytactic FLU administration resulted an epidemiological shift towards non-albicans species with primer fluconazole (FLU) resistance (*C. krusei*) or reduced FLU susceptibility (*C. glabrata*).

Due to the problems with earlier antifungal agents (toxicity and resistance) the milestone of the antifungal chemotherapy was the introduction of echinocandins which inhibit glucan synthase responsible for fungal cell wall biosynthesis. Caspofungin (CAS) was the first echinocandin approved for the treatment of invasive candidiasis and invasive aspergillosis. CAS shows fungicide *in vitro* activity against *Candida* spp., have favourable pharmacokinetics and pharmacodynamics. Therefore CAS is recommended as first-line agent for empirical treatment of invasive candidiasis.

The echinocandins show concentration-dependent killing. Their efficacy best correlate with C_{max}/MIC (peak concentration in serum/minimal

inhibitory concentration) and AUC/MIC (area under plasma the concentration-time curve) pharmacodynamics parameters. The approved dosing strategy of CAS is 70 mg loading dose than 50 mg daily dose. Because of concentration-dependent activity the higher echinocandin doses may hypothetically produce better clinical outcomes. The daily 150 mg CAS dose can be used for the treatment of invasive candidiasis without causing any severe side effects. Nevertheless clinical studies have not already confirm that the increased doses may lead to better outcome compared to the standard daily doses.

Echinocandins at drug concentration above the MIC show fungistatic effect in spite of the fact that these agents at low concentrations exert fungicide activity against certain *Candida* species (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. dubliniensis*). This paradoxical growth could appear at concentrations achievable in human serum, but the clinical relevance of this phenomenon is questionable.

We examined the CAS *in vitro* activity against *C. albicans* and *C. glabrata* isolates with time-killing methodology. This method provide remarkable data concerning the killing activity of antifungal agents as well as their pharmacodynamics properties. As echinocandins are highly protein-bound antifungals (96.5-99.8%) serum or tissue proteins may alter their activity.

The aim of our study was to test the *in vivo* activity of CAS dose escalation in a neutropenic murine model against *C. albicans* and *C. glabrata* clinical isolates. In order to compare *in vivo* activity to *in vitro* efficacy, CAS killing rate in RPMI-1640 and RPMI-1640 plus 50% serum were determined.

AIMS

We examined the efficacy of dose escalation against the two most frequently isolated *Candida* species in Hungary.

The aim of our study was:

- To determine the caspofungin minimal inhibitory concentration using standard microdilution against *C. albicans* and *C. glabrata* isolates and to compare the values to the results of Etest method in case of *C. albicans* isolates.
- To examine the *in vitro* efficacy of caspofungin against *C. albicans* and *C. glabrata* isolates in RPMI-1640 and RPMI-1640+50% human serum using time-kill methodology.
- To determine killing rates exerted by caspofungin against *C. albicans* and *C. glabrata* isolates.
- To determine the *in vivo* efficacy of caspofungin using daily 1, 2, 3, 5 and 15 mg/kg doses against *C. albicans*, as well as daily 1, 2, 3, 5 and 20 mg/kg doses against *C. glabrata* in neutropenic murine model.

MATERIALS AND METHODS

Isolates

We studied six *C. albicans* (14171, 18799, 35035, 5265, 10781, 34350), four *C. glabrata* (11900, 9098, 18910, 15242) clinical isolates, four ATCC (American Type Culture Collection) reference strains (10231, 90030, 6258, 22019), and two echinocandin resistant *C. albicans* (DPL18, DPL20) and *C. glabrata* (DPL27, DPL245) strains. All clinical isolates originated from blood samples and were first isolates (isolated prior antifungal administration). The *C. albicans* and *C. glabrata* isolates were identified by API ID32C and Matrix-assisted laser desorption/ionization time of flight in the Department of Medical Microbiology, University of Debrecen. The echinocandin resistant DPL18 (F641S mutation), DPL20 (F645P mutation), DPL27 (S663P mutation) and DPL245 (S629P mutation) isolates were originated from David S. Perlin laboratory (Public Health Research Institute, New Jersey Medical School-Ruthgers, Newark, New Jersey, USA).

In vitro susceptibility testing

Two test media were used, RPMI-1640 (referred to as RPMI) as recommended by CLSI and RPMI-1640 supplemented with 50% human serum from a human male, type AB, Sigma, Budapest, Hungary (referred to as 50% serum). CAS MICs in RPMI and in 50% serum were determined simultaneously using the standard CLSI broth microdilution method. CAS final concentration ranges were 0.015-32 mg/L. ATCC 6258 *C. krusei* and ATCC 22019 *C. parapsilosis* were used as reference strains. For the fungal suspension prepared with 0.5 McFarland density we used 24 h old colonies cultured on Sabouraud agar. We used RPMI for the adjustment of starting inoculum approximately 10^3 CFU (Colony Forming Unit)/ml. The 96-well ELISA plate also contained yeast control (without antifungal drug) and

medium control (without yeast). After 24 h incubation at 35°C MICs were determined after 24 h according to the partial inhibition criteria.

MIC values were also determined using Etest method (AB Biodisk, Sweden) for *C. albicans* isolates. Etest was carried out using freshly prepared RPMI agar supplemented with 2% glucose with and without 50% human serum. Etest was carried out using 0.5 McFarland density fungal suspensions prepared with 24 h old cultures. These suspensions were evenly spreaded on the surface of the RPMI agar plates with sterile swabs. After the plates were dried we placed the test stripes impregnated with different concentrations of CAS onto them. Results were read after 24 hours of incubation at 35°C.

Time-kill studies

Time-kill studies were performed according to the standardized method of Klepser and his colleagues. The *in vitro* activity of CAS was investigated in RPMI-1640 with and without 50% human serum. The examined CAS ranges were 0.25-32 mg/L in a final volume of 10 mL. For the echinocandin resistant isolates, only the concentrations 8, 16 and 32 mg/L were used. The highest CAS concentration was 32 mg/L, because according to clinical data the current maximal administrable daily dose (150-200 mg) produce 30.4-40.6 mg/L geometric peak concentrations in humans.

We prepared ~10^5 CFU/mL starting inoculum from each tested isolates using densitometer. Aliquots of 100 μL were removed at 0, 4, 8, 12, 24 and 48 hours from test tubes containing different CAS concentrations, serially diluted 10-fold, plated (4x30 μL) onto a single Sabouraud dextrose agar plate and incubated at 35°C for 48 hours. We counted the grown colonies and determined the CFU according to the degree of dilutions. Time-kill curves were prepared using the computer curve-fitting software GraphPad Prism 4.03 for Windows.

Analysis of *in vitro* data

CAS activity was defined as fungicidal when at least 99.9% reduction in viable cell count was observed as compared to the starting inocula. Killing kinetics at the tested concentrations were analysed in both media. An exponential equation was fitted to the mean data at each time point: $N_t=N_0 \times e^{-kt}$, where N_t is the number of viable yeasts at time *t*, N_0 is the number of viable yeasts in the initial inoculum, *k* is the killing rate, and *t* is the incubation time. Thus killing rate represents the overall killing capability of the drug, taking into account of killing at each tested concentrations. Negative *k* values indicate growth and positive *k* values indicate killing. The goodness of fit for each isolate was assessed by the r^2 value ($r^2>\pm0.8$). The mean times to achieve the fungicidal endpoint ($T_{99.9}=3/k$) were calculated from the *k* values for each isolate and concentrations in both media. Killing kinetics among isolates was compared using one-way ANOVA with Tukey's post-testing in either RPMI-1640 or 50% serum. The effect of the same drug concentration in RPMI-1640 and 50% serum was analysed using T test. The result was significant if $p<0.05$.

***In vivo* studies**

Groups of seven to eight female BALB/c mice (20-22 g) were immunosuppressed with four doses of cyclophosphamide, i.e. 4 days before infection (150 mg/kg), 1 day before infection (100 mg/kg), 2 days postinfection (100 mg/kg) and 5 days postinfection (100 mg/kg). The Guidelines for the Care and Use of Laboratory Animals was strictly followed during maintenance of the animals. Experiments were approved by the Animal Care Committee of the University of Debrecen (permission number 12/2014). Each group consisted of 7-8 animals.

Preparation of the infectious doses

For the infective doses we plated the isolates onto Sabouraud agar plates on two consecutive days and then the renewed strains were plated onto 3-4 Sabouraud agar plates again. The grown isolates were taken from the surface of the agar plates with sterile swab and suspended in sterile saline. The suspensions were centrifugated for 4 times per 10 minutes at 3000 g. We removed the supernatant from the cells after each centrifugation and added 20-25 mL fresh, sterile saline again to them. After the last centrifugation we removed the supernatant again and added 8 mL of sterile saline to the fungal cells. From this cell suspension we preparated a 10-fold dilution in two steps and adjusted the required cell count of the infective dose with Burker chamber. The punctuality of the infective dose's cell count was checked by quantitative inoculation.

Mice were infected with the fungal suspension intravenously (0.2 mL/mouse) through the lateral tail vein on the fourth day of the immunosuppression. The infectious doses of *C. albicans* and *C. glabrata* was $7.5x10^4$ CFU/mouse and $6.6\text{-}8.4x10^7$ CFU/mouse, respectively. For the echinocandin-resistant strains, these doses lead to 100% mortality within five days, thus the experiments with the resistant strains were repeated with a lower inoculum of $2.5x10^7$ CFU/mouse, which did not cause mortality during the experiments.

Antifungal therapy

Five-day intraperitoneal treatment with daily 1, 2, 3, 5 and 15 mg/kg CAS (Cancidas) for *C. albicans* and daily 1, 2, 3, 5, 20 mg/kg CAS for *C. glabrata* was started after 24 hours. This dosing strategy was based on previous pharmacokinetic studies and on previous result of our study group. On day six after infection all mice were sacrificed. The kidneys removed (both) weighed and homogenized aseptically. Homogenates were diluted

10-fold, aliquots of 0.1 mL of the undiluted and diluted (1:10) homogenates were plated onto Sabouraud agar plates and incubated at 35°C for 48 h. The lower limit of detection was 50 CFU/g of tissue. Statistical analysis of the kidney burden was performed using the Kruskal-Wallis test with Dunn's post-test for multiple comparisons. The result was significant if $p<0.05$.

RESULTS

MIC results with standard microdilution and Etest

Clinical isolates as well as the ATCC type strains were susceptible to CAS according to the revised CLSI breakpoints in RPMI. Confirming the result from the preliminary experiments, isolates 10781 and 34350 showed PG in RPMI.

Etest MICs were 2-≥8 times higher than MICs observed in the broth microdilution. PG was not observed, the inhibition zone was clear for all C. albicans clinical isolates. *C. albicans* and *C. parapsilosis* ATCC strains were susceptible to CAS, while *C. krusei* ATCC strain was intermediate susceptible to CAS. MIC values for the two echinocandin resistant isolates were 2 and 32 mg/L.

MIC values in both methods were 2-16-fold higher in 50% serum when compared to the MICs obtained in RPMI without serum for *C. albicans*. Although we used low number of wild-type isolates for MIC testing, it is notable that the MIC range was narrower than in RPMI. We also observed higher MIC values (4-8-fold) in 50% serum for *C. glabrata* isolates. PG was never observed regardless of the method used. The DPL18, DPL20, DPL27 and DPL245 isolates were CAS resistant regardless of the medium used.

Time-kill results in RPMI-1640 and RPMI-1640+50% human serum against *Candida albicans*

All isolates grew similarly well in both media. The mean time ranges of controls to growth 1 log in RPMI and 50% serum were 8.66-9.77 and 8.9-10.89 hours, respectively. In RPMI CAS against 14171 and 35035 isolates produced fungistatic effects, however, at lower (0.25 and 1 mg/L) concentration CFU decreased were higher than at 16 and 32 mg/L concentrations. Against isolate 18799, CAS was fungicidal at ≥4 mg/L. In cases of 5265, 10781 and 34350 isolates typical PG was observed,

fungicidal activity of CAS at lower concentrations and fungistatic effects at 16 and 32 mg/L. The T99.9 values for these three isolates at 1-8 mg/L were short (<12 hours). CAS in 50% serum produced fungistatic effect against isolates 14171, 18799 and 35035. CAS killing activity in 50% serum was increased in case of all three isolates which showed PG in RMPI-1640, all of these were killed at 4-32 mg/L within 10 hours.

Caspofungin killing rates against *Candida albicans* isolates

CAS killing rates (*k*) were isolate and concentration dependent. There was a trend producing higher *k* values at lower (0.25 or 1 mg/L) concentrations, while *k* values were very low at 32 mg/L. For all isolates *k* values were significantly higher at 1 than at 16 and 32 mg/L ($p<0.001$). This paradoxical effect was the most prominent in cases of 35035 and 5265 isolates where *k* values at 1 and 32 mg/L were 0.314 and 0.295 1/h, and 0.021 and 0.011 1/h, respectively. Numerically, the highest *k* value was observed in case of 10781 isolate, at 1 mg/L (0.961 1/h). In 50% serum killing rate values at 0.25 mg/L were negative in cases of isolates 14171, 35035 and 10781, indicating that growth occurred. For the remaining three isolates *k* value ranges at 0.25 mg/L were 0.093-0.398 1/h. Killing rates for 18799, 35035, 5265, 10781 and 34350 isolates were concentration independent at 1-32 mg/L. The lowest *k* value range was noticed in cases of isolates 18799 (0.085-0.109 1/h), while the highest range was found in case of isolate 10781 (0.882-0.985 1/h). Isolate 14171 behaved differently. Killing rate values at 1, 4, 8 and 16 mg/L (*k* value range was 0.241-0.271 1/h) were significantly higher that at 32 mg/L (*k* value was 0.126 1/h) ($p<0.05$-0.001). Killing rates at 0.25 mg/L were higher in RPMI than in 50% serum for all isolates ($p<0.05$-0.001). In cases of isolates 14171 and 18799 *k* values were significantly higher at all tested concentrations in RPMI than in 50% serum, with the exception of 16 mg/L in case of 14171 and 32 mg/L in case of 18799 isolate ($p<0.05$-0.001). CAS killing activity at 4-32 mg/L against the

remaining isolates increased in 50% serum when compared to RPMI (p<0.05-0.001) with the exception of the concentration of 8 mg/L in case of 5265, and of 4 and 32 mg/L in case of 34350 isolates.

***In vivo* efficacy of caspofungin against *Candida albicans* isolates**

All CAS doses decreased the fungal tissue burden for all tested clinical isolates. One mg/kg daily CAS did not decreased significantly the fungal tissue burden in cases of isolates 18799, 10781 and 34350, moreover 2 mg/kg also proved to be ineffective in case of isolates 10781. However, CAS doses of 3, 5 and 15 mg/kg proved to be effective for all isolates (p<0.05-0.001). The largest CAS dose produced the highest mean fungal tissue burden decreases in cases of isolates 18799, 5265 and 10781. Numerically, CAS doses of 2, 3 and 2 mg/kg produced the lowest mean fungal tissue burden in cases of isolates 14171, 35035 and 34350, respectively. However statistically significant differences between the effective doses were never observed.

Time-kill results in RPMI-1640 and RPMI-1640+50% human serum against *Candida glabrata*

All isolates grew significantly better in RPMI-1640 than in 50% serum. Without drug, the mean time to achieve 1 log increase in CFU in RPMI-1640 and 50% serum was 8.14 and 11.17 hours, respectively, for the clinical isolates and 8.57-8.95 and 14.76-14.86 hours, respectively, for the resistant isolates. In RPMI-1640, CAS killing activity was better at 1 and 4 mg/L than at 16 and 32 mg/L against all clinical isolates. The mean times to achieve 99.9% growth reduction from the starting inoculum were shorter than 7 hours for isolates 9098, 18910 and 15242 and for the ATCC type strain. CAS at 16 and 32 mg/L was fungistatic against strain DPL27, but for strain DPL245, a weak and transient fungistatic effect was observed at 32 mg/L during the first 8 hours in the killing study. In 50% serum the time to

reach the fungicidal effect was significantly shorter (≤3.32 hours) than in RPMI-1640. The killing activity of CAS in 50% serum was better at 32 mg/L than in RPMI-1640, even against the resistant strain DPL27.

Caspofungin killing rates against *Candida glabrata* isolates

The CAS killing rates for clinical isolates 11900, 18910, 9098 and the ATCC strain were paradoxically higher at 0.25 and 1 mg/L than 16 and 32 mg/L (p<0.05-0.001). In case of isolate 15242, the k values were significantly higher at 0.25, 1 and 32 mg/L than 4, 8 and 16 mg/L, but did not differ significantly from each other. Numerically, the highest k values were observed with isolate 9098 at 1 and 4 mg/L (1.058 1/h). The lowest k value was noted with isolate 11900 at 32 mg/L (0.177 1/h). For the two echinocandin resistant strains, the k values were always negative (indicating growth) with the exception of the DPL27 isolate at 32 mg/L.

In 50% serum the killing rate values at 0.25 mg/L showed a wide range, the k value was negative (indicating growth) for isolate 11900 (-0.059 1/h), but positive (indicating killing) for isolates 9098 (0.916 1/h), 18910 (0.006 1/h) and 15242 (0.265 1/h) and for the ATCC strain (0.031 1/h). The killing rates at 4-32 mg/L for isolates 11900, 18910, 9098 and 15242 were concentration independent (p>0.05 for all comparisons). For the ATCC strain, the highest k value was detected at 16 mg/L (1.426 1/h), the k values at 1, 4, 8 and 32 mg/L (range 1.022-1.085 1/h) did not differ significantly. The k values for the DPL245 strain were uniformly negative at 8-32 mg/L, but for the DPL27 isolate, the k values at 16 and 32 mg/L were 0.065 and 0.354 1/h, respectively.

The k values at 0.25 mg/L with the exception of isolate 9098 were higher in RPMI-1640 than in 50% serum for all isolates (p<0.05-0.001). The killing activity of CAS in 50% serum was significantly higher at 4-32 mg/L than in RPMI-1640 for isolates 9098, 18910 and 11900 and for the ATCC strain (p<0.05-0.001). For isolate 15242 at 4, 8 and 16 mg/L the k values were

higher in 50% serum than in RPMI-1640 ($p<0.05$-0.001), but at 32 mg/L, a significant difference was not observed. For DPL27, the killing rates at 16 and 32 mg/L (0.07 and 0.36 1/h, respectively) were significantly higher in 50% serum than in RPMI-1640.

In vivo efficacy of caspofungin against *Candida glabrata* isolates

The initial inoculum used ($7x10^7$ cell/mL) killed all mice before the pre-determined endpoint, dead animals showed consistent increase in fungal burden over time [$6.8x10^7$ and $7.2x10^6$ cells/ kidney tissue (g) at day 2 p.i. to $4.3x10^8$ and $6.8x10^7$ cells/ kidney tissue (g) at day 5 p.i. for DPL27 and DPL245, respectively]. In contrast, all mice infected with susceptible isolates survived, with a mean fungal burden of between 10^7 and 10^8 cells/ kidney tissue (g) at day 6 p.i. in untreated controls. For this reason resistant isolates were tested at a lower infectious dose to harmonize the endpoint for all tested isolates.

At the beginning of therapy (day 1), the mean fungal tissue burden ranges for clinical isolates and resistance strains (DPL27 and DPL245) in untreated control mice were $4.4x10^6$–$6.7x10^6$ and $9.7x10^6$–$1.4x10^7$ cells/ kidney tissue (g) per mouse, respectively. All mice in all groups survived. All tested isolates, except isolate 11900, grew <1 mean log unit in untreated control mice as determined at day 6. All caspofungin doses decreased the fungal tissue burden significantly for all tested clinical isolates compared with the controls. However, the mean fungal tissue burdens never fell below 10^5 cells/g, and statistically significant differences between the five treatment regimens were not observed. Paradoxically decreased activity of caspofungin by 20 mg/kg was also not observed. Against the echinocandin-resistant strains, caspofungin was ineffective regardless of the elevated doses. However, for isolate DPL27 but not for DPL245, growth inhibition was observed compared with the control at day 1 with all three treatment regimens.

DISCUSSION

Mean mortality rate is species dependent, the highest for *C. krusei* and *C. glabrata* (50-70%) and the lowest in case of *C. parapsilosis* (20-30%). The trend in the mortality did not change radically in the last decade, despite echinocandins were introduced into the antifungal armamentarium.

In our study we used two types of serum-based susceptibility methods (broth microdilution and Etest) to determine the MIC values for wild-type and echinocandin resistant strains. In order to detect PG by Etest, we used RPMI with and without 50% serum. The serum-based Etest showed good correlation with serum-based broth microdilution, hence, it may be applicable to determine caspofungin MICs both in case of susceptible and resistant isolates of *C. albicans*. As serum-based susceptibility methods have not yet been standardized, they are not recommended currently for routine susceptibility testing of fungi. The issues to be solved in the future include the optimal concentration of serum used in the tests, the origin (human or animal source), as well as the high cost of serum. However, serum from animals (i.e. bovine serum) may replace human serum in the laboratories, decreasing the cost of serum-based MIC determinations. Testing higher number of clinical *Candida* isolates with serum may provide a sufficient database to determine new epidemiological cutoff values and possible new clinical breakpoints for caspofungin against *Candida* species.

In our study we used six clinical isolates which showed diverse behavior in killing studies using RPMI as test medium; caspofungin was fungicidal against one isolate, was fungistatic against two isolates and three isolates showed PG. Decreased activity of caspofungin at higher (16-32 mg/L) concentrations was confirmed by the lower k values for all isolates in RPMI. In agreement with other studies CAS showed excellent in vitro activity against WT clinical isolates as well as against the ATCC 90030

type strain. However, in RPMI-1640, caspofungin killing activity decreased at 16–32 mg/L when compared with the killing at 0.25–1 mg/L.

In 50% serum we observed 2-16-fold higher MIC values for *C. albicans* isolates and 4-8-fold higher MICs for *C. glabrata* isolates than in RPMI-1640. Paradoxically decreased activity of higher concentration of CAS was eliminated by 50% serum both in MIC and in time-kill tests. Fifty percent serum restored the killing activity of CAS at higher concentrations as revealed by *k* values. On the contrary, in case of isolate 14171 the killing rate was higher at 32 mg/L in RPMI than in 50% serum.

In 50% serum the killing rate exerted by CAS showed concentration independent activity at the effective concentrations (where CFU decreases were observed) so after we achieved the first effective concentration, concentration enhancement did not produce significant changes in the killing activity of CAS. This can be associated with the protein-binding characteristic of echinocandins, therefore the pharmacological active drug concentration did not increase such extent that it would enhance the killing activity. CAS activity was concentration independent at 1-32 mg/L (except 14171) for *C. albicans* and at 4-32 mg/L for *C. glabrata*. Nevertheless *k* values showed a great variability among isolates (0,085-0,985 1/h; and 0,480-1,536 1/h).

We expected that isolates showing higher *k* values of caspofungin either in RPMI or in 50% serum will produce better efficacy in a severely neutropenic murine model, especially at the largest dose. The utilized doses namely 1 mg/kg/day is equivalent with 35 mg/day for humans, 2 mg/kg/day is equivalent with 50 mg/day for humans. Based on AUC values 3 and 5 mg/kg/day are equivalent with 70 mg once and 50 mg/day as well as 70 mg/day for humans.

Caspofungin produced concentration-dependent *in vivo* efficacy against the clinical isolates and the ATCC type strain, i.e. concentrations 3, 5 and 15

mg/kg, but not 1 and 2 mg/kg were uniformly effective against all tested strains. Statistically significant differences between the effective doses was not observed, thus dose escalation did not produce better clinical outcomes. Moreover, the largest (15 mg/kg) caspofungin dose not only did not produce significantly better fungal tissue burden decrease than the lower effective doses 3 and 5 mg/kg, but sometimes the highest numerical decrease was found in case of lower doses. These in vivo results also confirm that PG has no effect on the *in vivo* efficacy of caspofungin against *C. albicans*. The comparable efficacy of the safely effective doses is in line with the killing rate results in 50% serum.

Contrast with *C. albicans*, wild-type *C. glabrata* clinical isolates were highly susceptible in vivo to caspofungin even at 1 mg/kg in mice. However all treatment arms were unable to reduce the mean fungal burden below 10^5 CFU/ g kidney tissue. This suggests that, even at higher doses, only suppression of fungal growth can be achieved in severely neutropenic mice and thus higher doses do not have a therapeutic benefit compared with lower doses.

Despite the *in vitro* fungicidal effects, the *in vivo* activity of caspofungin was only fungistatic, in line with previous results. Although immediate or early caspofungin treatment may lead to decreased fungal tissue burdens, treatment started 24 h after challenge did not. The delay in treatment allowed the establishment of high tissue fungal burden in the deeply neutropenic host, modelling the most probable clinical situation; in some cases, the unfavourable therapeutic response may be the result of delayed antifungal treatment.

Moreover, the randomly selected bloodstream isolates used in the present study showed weak replicating ability, as demonstrated both in 50% serum and *in vivo*. The decreased growth of our wild-type *C. glabrata* isolates in 50% serum is consistent with a previous report, and the low *in vivo* growth

rate suggests a relatively low virulence. Considering this, the extremely weak elimination capacity of the treatments was even more notable. A potential explanation may lie in the activity of echinocandins; i.e. the slower growth in tissues may lead to slower cell wall synthesis, which may, in turn, weaken the in vivo killing activity of caspofungin, similarly to what is seen with antibacterial agents inhibiting cell wall synthesis. Certainly, other factors (e.g. neutropenia, poor drug penetration into the inflamed tissues) influence therapeutic outcome. Whether the relatively low *in vivo* fitness found in this study is common among *C. glabrata* bloodstream isolates, as well as its clinical importance, remain to be determined in further studies.

Although resistance of *Candida spp*. to echinocandins is low worldwide, secondary resistance to caspofungin at some medical centres is more substantial. Our preliminary experiments revealed that the virulence of the caspofungin-resistant isolates was higher than that of wild-type clinical isolates. Moreover, resistant isolates produced slightly higher fungal burdens at day 6 p.i. at markedly lower challenge doses. However, the susceptible and the resistant isolates were not isogenic. Even higher doses were inefficient against isolates with prominent FKS-mediated resistance.

Our studies confirmed the clinical experience that the efficacy of echinocandins is not enhanced by higher CAS doses against echinocandin-susceptible *C. albicans* and *C. glabrata* clinical isolates. The *in vitro* data showed good correlation with the *in vivo* results. The PG does not influence the *in vivo* activity of CAS. The invasive candidiasis caused by prominent FKS mutant strain cannot be treated with high dose CAS in a neutropenic host.

SUMMARY

During our investigations killing rate exerted by caspofungin was determined in RPMI-1640 with and without 50% human serum against *C. albicans* and *C. glabrata* clinical and echinocandin resistant isolates. Moreover the activity of different daily caspofungin doses were investigated in neutropenic mouse model in case of the previously mentioned species.

Caspofungin killing rate (*k*) in RPMI-1640 at 1 mg/l was higher than at 16 and 32 mg/L for all *C. albicans* isolates. In the same medium caspofungin at 1 and 4 mg/L showed fungicidal effect within 7 hours against three *C. glabrata* isolates but was only fungistatic at higher concentrations (16 and 32 mg/L) (paradoxically decreased killing activity). Caspofungin is highly protein-bound antifungal agent (96.5%), therefore it possess reduced activity in medium supplemented with serum. However adding 50% serum eliminated paradoxical growth. In the presence of 50% serum killing rates for *C. albicans* isolates were concentration independent between 1-32 mg/L (except one isolate). Similarly concentration independent killing was observed at 4-32 mg/L against *C. glabrata* isolates. Nevertheless *k* values showed a great variability among isolates (0,085-0,985 1/h; and 0,480-1,536 1/h). Daily 3, 5 and 15 mg/kg caspofungin was effective in a neutropenic murine model against all *C. albicans* isolates, without significant differences between the effective doses. All caspofungin doses (1, 2, 3, 5 and 20 mg/kg/day) decreased the fungal tissue burdens significantly in the case of *C. glabrata* isolates without statistical differences between doses, but the mean fungal tissue burdens never fell below 10^5 cells/g tissue. The echinocandin-resistant *C. glabrata* strains were highly virulent and all doses were ineffective. According to our *in vitro* and *in vivo* results caspofungin dose escalation does not improve efficacy against *C. albicans* and *C. glabrata* isolates. In addition paradoxical growth does not affect the *in vivo* efficacy of caspofungin.

Registry number: DEENK/84/2016.PL
Subject: Ph.D. List of Publications

Candidate: Marianna Domán
Neptun ID: I441RU
Doctoral School: Doctoral School of Pharmaceutical Sciences

List of publications related to the dissertation

1. **Domán, M.**, Kovács, R., Perlin, D.S., Kardos, G., Gesztelyi, R., Juhász, B., Bozó, A., Majoros, L.: Dose escalation studies with caspofungin against Candida glabrata.
J. Med. Microbiol. 64 (9), 998-1007, 2015.
DOI: http://dx.doi.org/10.1099/jmm.0.000116
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2. **Domán, M.**, Kovács, R., 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.
Current Drug Del. 12, 1-10, 2015.
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IF:1.478 (2014)

List of other publications

3. Kovács, R., Bozó, A., Gesztelyi, R., **Domán, M.**, Kardos, G., Nagy, F., Tóth, Z., Majoros, L.: Effect of caspofungin and micafungin in combination with farnesol against Candida parapsilosis biofilms.
Int. J. Antimicrob. Agents. Epub ahead of print (2016)
DOI: http://dx.doi.org/10.1016/j.ijantimicag.2016.01.007
IF:4.296 (2014)

4. Kovács, R., Gesztelyi, R., Perlin, D.S., Kardos, G., **Domán, M.**, Berényi, R., Majoros, L.: Killing rates for caspofungin against Candida albicans after brief and continuous caspofungin exposure in the presence and absence of serum.
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5. Kovács, R., Gesztelyi, R., Berényi, R., **Domán, M.**, Kardos, G., Juhász, B., Majoros, L.: Killing rates exerted by caspofungin in 50 % serum and its correlation with in vivo efficacy in a neutropenic murine model against Candida krusei and Candida inconspicua.
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6. Berényi, R., Kovács, R., **Domán, M.**, Gesztelyi, R., Kardos, G., Juhász, B., Perlin, D., Majoros, L.: Efficacy of single large doses of caspofungin in a neutropenic murine model against the "psilosis" group.
New Microbiol. 37 (3), 355-362, 2014.
IF:1.784

Total IF of journals (all publications): 13,582
Total IF of journals (publications related to the dissertation): 3,726

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

01 April, 2016

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List of major presentations and posters

R. Kovács, R. Gesztelyi, R. Berényi, **M. Domán**, G. Kardos, B. Juhász, L. Majoros. Should echinocandin doses be increased against *Candida* species? An *in vitro* and *in vivo* study of caspofungin against *Candida albicans*, *C. krusei* and *C. inconspicua*. 6th Trends in Medical Mycology, 11-14 October 2013. Coppenhagen, Denmark (P011) Mycoses

L. Majoros, R. Kovács, R. Berényi, **M. Domán**, C. Miszti and G. Kardos. Effect of 50% human serum on the killing activity of micafungin against *C. dubliniensis*, *C. lusitaniae*, *C. guilliermondii* and *C. kefyr* using time-kill methodology. 6th Trends in Medical Mycology, 11-14 October 2013. Coppenhagen, Denmark (P021) Mycoses

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

Marianna Domán, Renátó Kovács, Réka Berényi, Gábor Kardos, László Majoros. Efficacy of caspofungin in vitro and in vivo against Candida albicans isolates showing and not showing paradoxical growth. Congress of Hungarian society for microbiology. Oct. 15-17. 2014, Keszthely.

Kovács Renátó, Berényi Réka, **Domán Marianna**, Majoros László. In vitro efficacy of caspofungin against C. krusei, C. inconspicua and C. albicans clinical isolates. Spring Wind Conference. May 31-Jun 2, 2013, Sopron, Hungary.

Kovács Renátó, Gesztelyi Rudolf, Kardos Gábor, **Domán Marianna**, Berényi Réka, Majoros László. Comparative exanimation of caspofungin in vitro pharmacodynamics in RPMI-1640 and RPMI-1640+50% human serum against C. albicans isolates. Oct. 15-17. 2014, Keszthely.