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

Examination of pharmacodynamics for caspofungin against *Candida* albicans, *C. krusei* and *C. inconspicua* isolates

by Renátó László Kovács

Supervisor: László Majoros, PhD

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES

DEBRECEN, 2014

Examination of pharmacodynamics for caspofungin against *Candida albicans*, *C. krusei* and *C. inconspicua* isolates

By Renátó László Kovács, MSc

Supervisor: László Majoros, PhD

Doctoral School of Pharmaceutical Sciences, University of Debrecen

Head of the **Examination Committee**:	László Maródi, DSc
Members of the Examination Committee:	Attila Bácsi, PhD
	Edit Urbán, PhD

The examination takes place at the Department of Infectious and Pediatric Immunology, Faculty of Medicine, University of Debrecen, 11:00, 07. 10. 2014.

Head of the **Defense Committee**:	László Maródi, DSc
Reviewers:	Ágnes Belák, PhD
	Judit Zsuga, PhD
Members of the Defense Committee:	Attila Bácsi, PhD
	Edit Urbán, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 13:00, 07. 10. 2014

INTRODUCTION

Approximately 15% of health-care associated infections are caused by fungi. The different *Candida* species account for 70-90% of all invasive fungal infections and *Aspergillus* for 10-20%

The last few decades prevalence of nosocomial fungal infections increased dramatically. The reason of increasing are many predisposing factors including persistent neutropenia (<500 neutrophil granulocyte/µl), the increasing of prevalence of several tumor types, administration of broad-spectrum antibiotics, immunosuppressive chemotherapy, surgery interventions, burns, prolonged length of stay (especially in intensive care units), aging populations in countries with advanced medical technologies.

Candida species are the fourth most common cause of bloodstream infections in United States of America (USA) but in Europe *Candida* spp. is usually the 6-10th most frequently isolated genus.

The increasing tendency illustrates, that between 1995 and 2002 the frequency of *Candida* bloodstream infections in USA hospitals rose from 8% to 12%.

The mortality of caused by invasive *Candida* infections is 30-60% that depends on *Candida* species, geographical locations as well as underlying diseases.

Although *C. albicans* is the most frequent isolated *Candida* species (40-50%), so-called non-*albicans* species with primer fluconazol (FLU) resistance or reduced FLU susceptibility (*C. glabrata*, *C. krusei*, *C. inconspicua*) are identified more frequently due to the therapeutic and prophylactic FLU administration.

One of the newest milestones of the antifungal chemotherapy was the introduction of echinocandins (caspofungin (CAS), micafungin (MICA), anidulafungin (ANI). Owing to their favourable pharmacodynamics and pharmacokinetics properties, invasive candidiasis and aspergillosis can be used for treatment.

The echinocandins are cyclic lipopeptide molecules which are non-competitive inhibitors of β-1,3-D-glucan synthase enzyme complex. Due to this mechanism the cell wall integrity of fungi disturbs which leads to osmotic instability then the death of fungal cell.

The echinocandins demonstrate fungicide effect against most of *Candida* species and fungistatic effect against *Aspergillus* species. In addition, it has activity against *Pneumocystis jirovecii*. The echinocandins are ineffective against *Zygomycetes* genus, *Cryptococcus neoformans* as well as *Fusarium* species at clinical relevant concentrations.

The echinocandins show concentration-dependent killing, with efficacy that is best correlated C_{max}/MIC (maximal concentration/minimum inhibitory concentration) and AUC/MIC (AUC: area under curve, area under the plasma concentration-time curve)

pharmacodynamics parameters.

CAS - the first member of echinocandins – was approved in 2001 for the treatment of invasive candidiasis. Similar to other echinocandins CAS is the first-line therapy for empirical treatment of invasive *Candida* infections. The approved dosing strategy of CAS is 70 mg loading dose then 50 mg daily.

On the basis of previous studies, the multiple dose of currently approved daily dose (50 mg) does not trigger seriously side effects. Even so clinical studies have not already confirmed that the therapeutic outcome would be better in case of increased dose compared with the approved standard daily dose.

Other remarkable property of echinocandins including CAS is a relative prolonged postantifungal effect (PAFE) in RPMI-1640. PAFE is the continued suppression of fungal growth after exposure of the fungi to an antifungal agent and removal of this agent from the environment. In previous studies, the time of PAFE by CAS was >12 hours in RPMI-1640 above the MIC concentrations.

On the basis of several published studies the echinocandin resistance is relatively rare in case of *Candida* species (2,9-3,1%). The most frequent reason

of CAS resistance is a mutation of FKS1 gene that leads to amino acid substitution in two so-called hot-spot regions (HS) of Fks1p subunit.

The echinocandins have high serum protein binding. Under the "free-drug hypothesis" solely free drug molecules possess with pharmacological activity. The degree of protein binding at CAS is 96,5%. Serum increased CAS MICs an average of 2-fold, increasing, an average of 16-fold for ANI and an average of 64-fold for MICA.

The precise knowledge of the pharmacodynamics/pharmacokinetics properties of antifungal drugs is crucial for the development of adequate treatment. The introduction of the first standardized pharmacodynamics parameter (MIC value) that was essential in terms of therapy had to be waited for the 1990s.

In vitro, *in vivo* as well as clinical pharmacodynamics studies which were published in the last two decades are contribute to successful therapy of bloodstream infections caused by *Candida* species. In addition, they could help the determination of clinical break-points which are important in terms of interpretation of MIC values.

Time-kill investigations provide remarkable data concerning the killing activity of antifungal agents as well as their pharmacodynamics properties (postantifungal effect, dose-effect). These data could help to understand the dynamic relationship between fungi and drugs.

During our investigations, the aim of our study was the *in vitro* results combine and harmonize with *in vivo* findings. Therefore more effective CAS-based therapy will be elaborated not only against *C. albicans* but also against clinical relevant *C. krusei* and *C. inconspicua* isolates.

AIMS

During our experiments *in vitro* and *in vivo* efficacy of caspofungin was investigated against three clinical relevant *Candida* species

We aimed in our study:

- Investigation of *in vitro* efficacy of caspofungin against *C. albicans*, *C. krusei* and *C. inconspicua* using time-kill methodology in normal RPMI-1640 with and without 50% human serum.
- Investigation of postantifungal effect of caspofungin against *C. albicans* isolates in normal RPMI-1640 medium with and without 50% human serum.
- Determination of killing rates exerted by caspofungin against *C. albicans*, *C. krusei* and *C. inconspicua* isolates.
- Knowing of killing rate, calculation of the times to achieve 50, 90, 99 and 99,9% reductions in CFU compared with the starting inoculum size in case of *C. krusei* and *C. inconspicua* isolates.
- Determination of *in vivo* efficacy of caspofungin using 1, 2, 3, 5, 15 mg/kg daily caspofungin doses in neutropenic mouse model against *C. krusei* and *C. inconspicua*.

MATERIAL AND METHODS

Isolates

Ten clinical isolates of three *Candida* species (*C. albicans*, *C. krusei*, *C. inconspicua*) were used to determine the *in vitro* efficacy of CAS. In addition, two ATCC (American Type Culture Collection) type strains and two echinocandin resistant isolates (*C. albicans*, DPL20; *C. krusei* DPL45) were investigated. All *C. albicans* (183, 3666, 10920, 12132), all *C. krusei* (4363, 5029, 27393) and one of three *C. inconspicua* clinical strain (20114) originated from blood samples. The remaining two *C. inconspicua* isolates were isolated from peritoneal (22027) and wound samples (12060). Two echinocandin resistant isolates (DPL45, FKS F645F/C; DPL20, FKS F645P) were originated from David S. Perlin's laboratory (Public Health Research Institute, New Jersey Medical School-Ruthgers, Newark, New Jersey, USA).

C. albicans and *C. krusei* isolates were identified by APID32C and Matrix-assisted laser desorption/ionization time of flight, while *C. inconspicua* isolates were identified using molecular biological methods.

Determination of minimum inhibitory concentration (MIC) of caspofungin

CAS MICs were determined using the CLSI standard macrodilution method (M27-A3) in RPMI-1640, as well as in RPMI-1640 supplemented by 50% human serum (from a human male, blood type AB; Sigma), at least twice.

CAS final concentration ranged between 0,015-32 mg/L. MICs were read after 24 hours using the partial inhibition criterion. It means at least 50% growth reduction compared with the growth control.

Time-kill studies

In killing studies *in vitro* activity of CAS was investigated in normal RPMI-1640 with and without 50% human serum. The lowest examined concentration was 0,5 x MIC in case of all isolates, while the highest concentration was 32

mg/L. Higher MIC values were experienced in 50% serum in case of all investigated species. Therefore we plotted the data where measurable activity of CAS was detected in both media. These values were in the case of *C. albicans* 0,25, 1, 4, 8, 16 and 32 mg/L, while in case of *C. krusei* and *C. inconspicua* were 1, 2, 4, 8, 16, 32 mg/L CAS concentrations in a final volume of 10 mL. The highest CAS concentration used during *in vitro* studies was 32 mg/L (regardless of MIC), because according to clinical data the current maximum administrable daily doses of CAS (150-200 mg) produce 30,4-40,6 mg/L geometric peak concentrations in humans.

During our investigation the starting inoculum was 0,5 McFarland (McF) suspension. This value is equivalent in case of fungal cells approximately 10^5 CFU/mL.

Under investigations in case of *C. krusei* and *C. inconspicua* samples (100 µL) were removed at 0, 4, 8, 12, 24 and 48 hours, serially diluted tenfold, plated (4 x 30 µL) onto Sabouraud dextrose agar (SDA) and incubated at 35 °C for 48 hours.

The change of fungal cells was examined to 24 hours in the case of *C. albicans* strains.

Time-kill curves were prepared using the computer curve-fitting software GraphPad Prism 4.03 for Windows.

Investigation of postantifungal effect

In our preliminary attempts 5, 10 and 30 minutes exposures (0,5-2 mg/L) to CAS did not produce measurable PAFEs in 50% serum. Therefore we used CAS at 4, 16 and 32 mg/L concentrations with a 60 minutes exposure time in RPMI-1640 with and without 50% serum.

The starting inocula in PAFE experiments were approximately 10^5 cells/mL. After 1 hour incubation at 35 °C cells were collected by centrifugation at 1500 g for 10 minutes and were washed three times with sterile saline, resuspended in

10 mL drug-free warm RPMI-1640 with and without 50% human serum. Samples (100 μL) were removed at 0, 4, 8, 12 and 24 hours, serially diluted tenfold, plated (4 x 30 μL) onto SDA and incubated at 35 °C for 48 hours.

Data analysis

Fungicidal activity of CAS was defined as a reduction of 99,9% or higher in viable cell count compared with the starting inoculum.

The killing kinetics of the fungicidal activity of CAS in RPMI-1640 with and without 50% human serum were analysed by fitting the mean data at each time point to an exponential equation: $N_t=N_0 \times e^{-kt}$, where N_t is the number of viable cells at time *t*, N_0 is the number of viable cells at the beginning of the experiment, *k* is the killing rate and *t* is the incubation time. Positive *k* values indicate growth and negative *k* values indicate killing. The goodness of fit for each isolate was assessed using the r^2 value (>0,8).

The times (h) to achieve 50, 90, 99, 99.9% reductions in CFU compared with the starting inoculum size were calculated from *k* value (T_{50}=0,30103/k, T_{90}=1/k, T_{99}=2/k, $T_{99,9}$=3/k) for each CAS concentration and each strain.

One-way ANOVA with Tukey's post-testing was used to determine significant differences in killing kinetics among isolates and concentrations in either RPMI-1640 or 50% serum. T-test was used for the same CAS concentrations in RPMI-1640 and 50% serum to determine significant differences in killing kinetics between the different media.

***In vivo* experiments**

Animals

In our experiments we used female BALB/c mice weighing 21-23 g, which were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals. Each group consisted of 7-9 animals. The number of the *in vivo* experiment's permission: 12/2008 DE MÁB.

Mice were immunosuppressed 4 days before infection (150 mg/kg), 1 day before infection (100 mg/kg) and 2 and 5 days post-infection (100 mg/kg).

Infection of mice

For the infective doses preparation we plated the isolates of *C. krusei* (5029, 27393) and *C. inconspicua* (20114, 22027) as well as the echinocandin resistant *C. krusei* isolate (DPL45) onto SDA plates on two consecutive days and then the renewed strains were plated onto three or four Sabouraud agar plates again. After 24 hours incubation the grown isolates were taken from the surface of the agar plates with sterile swab and suspended in sterile saline. These suspensions were centrifuged for four times (10 minutes) at 3000g. We removed the supernatant from the cells after each centrifugation and added 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 fungi cells. From this cell suspension we prepared 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 through the lateral tail vein (0,2 mL suspension/mouse). On the basis of our preliminary experiment the infectious doses of *C. krusei* and *C. inconspicua* $4x10^6$ and $2x10^7$ CFU per mouse, respectively. These infectious doses did not cause mortality.

Antifungal therapy

Five days of intraperitoneal treatment daily 1, 2, 3, 5 and 15 mg/kg CAS was started after 24 hours post-infection. On day 6 post-infection, all mice were sacrificed by cervical dislocation then dissected them. Owing to determination of cell count, both kidneys of each mouse removed, weighed and homogenized by sterile pistillus aseptically. Then 1 mL sterile physiological saline was added to undiluted and diluted (1:10) homogenates. 100 µL from each dilution plated

onto SDA plates and incubated at 35 °C for 48 hours. Values for CFU were determined after 48 hours. The lower limit of detection was 50 cells/tissue (g).

Statistical analysis

Kidney burden was analysed using Kruskal-Wallis test (GraphPad Prism 4.03, Windows). The result was significant if $P<0,05$.

RESULTS

Susceptibility

All of investigated *C. albicans*, *C. krusei* and *C. inconspicua* type strain and clinical isolates were susceptible in normal RPMI-1640 according to the revised CLSI break-points. DPL20 and DPL45 isolates were resistant to CAS.

All of examined *Candida* isolates MIC values were increased in 50% serum. This increasing was 4-16-fold, 4-8-fold and 4-fold for *C. albicans*, *C. krusei* and *C. inconspicua*, respectively.

The results time-kill and postantifungal effect experiments in RPMI-1640 against *Candida albicans* isolates

In time-kill experiments CAS was fungistatic ≥1-2 x MIC (0,03 mg/L) concentrations in normal RPMI-1640 against *C. albicans* clinical isolates as well as against the 10231 ATCC reference strain. It means <99,9% reduction in viable cell count compared to the starting inoculum. Against the resistant strain (DPL20), CAS produced weak fungistatic effect in first 4 hours (8-32 mg/L).

On the basis of PAFE investigations in RPMI-1640 clinical isolates and the ATCC 10231 strain showed inhibition of re-growth at 4, 16 and 32 mg/L for 4,89- >19,34; 13,84- >19,88 and 7,65- >19,88 hours, respectively.

The results time-kill and postantifungal effect experiments in RPMI-1640 with 50% human serum against *Candida albicans* isolates

Generally, *in vitro* activity of CAS was markedly decreased by serum both in time-kill and PAFE experiments against *C. albicans* isolates. CAS was fungistatic ≥1-2 x MIC against the clinical isolates and against the ATCC strain. In 50% serum, the killing curves were generally similar to control against DPL20, at 32 mg/L a negligible reduction was observed after 4 hours, but later the killing curves again became similar to control.

PAFE in 50% serum decreased markedly at 4, 16 and 32 mg/L CAS concentrations. At the lowest CAS concentration (4 mg/L) PAFE was not experienced in 50% serum in case of 3666 and 183 isolates. In RPMI-1640 with 50% serum clinical isolates and the ATCC 10231 reference strain demonstrated inhibition of re-growth at 4, 16 and 32 mg/L for 0-2,27; 0,24-10,14; 1,01->18,79 hours, respectively. In case of DPL20 isolate PAFE was not observed in serum similar to in RPMI-1640.

Most isolates showed growth in 50% serum, colony count decreases occurred only in cases of isolates 183 at 16 and 32 mg/L and 3666 at 32 mg/L and only after 4 hours and were negligible. It is noteworthy that, growth of control cells was not decreased by serum.

Comparison of colony count changes in time-kill and postantifungal effect experiment in case of *Candida albicans*

Comparing the two different media at the same concentrations in killing experiments, the CFU decrease was generally higher in 50% serum than in RPMI-1640 especially at 32 mg/L.

Contrastingly, the CFU decrease in PAFE experiments was significantly higher in RPMI-1640 than in 50% serum with all isolates and concentrations ($P<0,05$-$0,001$).

Comparing the colony counts reductions at the same concentrations in PAFE and time-kill experiments, we observed similar or sometimes higher reductions (10920, 3666) in PAFEs than that seen with continuous 24 hours exposure in RPMI-1640.

It is noteworthy that, 50% serum significantly reduced the PAFE killing for all investigated strains ($P<0,01$-$0,001$).

Killing rates exerted by caspofungin against *Candida albicans* isolates

In time-kill experiments killing activity of CAS was significantly weaker at 16-32 mg/L than at 0,25; 1; 4 and 8 mg/L ($P<0,05$-$0,001$) in RPMI-1640. This

phenomenon can be explained by mini-paradoxical effect. However, killing rates at 1-32 mg/L were concentration independent in 50% serum against the susceptible strains. In case of DPL20 *k* values were negative that refers growth regardless of media.

In PAFE investigations killing rates for clinical isolates and the ATCC strain in RPMI-1640 were isolate- and concentration dependent (*k* values from -0,111 to +1,019 1/h), while in 50% serum the *k* values demonstrated narrower range (from -0,017 to -0,185 1/h).

Determination of *in vitro* activity for caspofungin using time-kill experiments in RPMI-1640 against *Candida krusei* and *Candida inconspicua* isolates

In RPMI-1640 without serum, CAS was fungistatic at ≥0,25 mg/L against *C. krusei* clinical isolates after 24 hours, but regrowth was observed in the case of isolate 4363 at 0,25-4 mg/L concentrations. All three isolates were killed at 16 and 32 mg/L CAS concentrations after 3,75±0,71 and 4,64±0,33 hours, respectively. *C. krusei* reference strain (ATCC 6258) was killed by ≥1 mg/mL CAS after 12-24 hours. CAS was fungistatic against the resistant strain at ≥4 mg/L in the first 12 hours, but growth was experienced after 24-48 hours. However, 32 mg/L CAS proved to be fungicidal.

In case of *C. inconspicua* CAS was fungistatic at 0,06 mg/L concentration in RPMI-1640. In addition, CAS proved to be fungicidal after 24 hours ≥0,5 mg/L concentrations against all of three *C. inconspicua* isolates.

Determination of *in vitro* activity for caspofungin using time-kill experiments in RPMI-1640 + 50% human serum against *Candida krusei* and *Candida inconspicua* isolates

In 50 % serum at 1 mg/L all three *C. krusei* isolates were inhibited by CAS in the first 4 hours, but after 12–24 hours killing curves became similar to the control curves. CAS at 2–4 mg/L was fungistatic against *C. krusei* isolates, but

regrowth was noticed after 24 hours. *C. krusei* clinical isolates was fungicide after 6.47±3.48 and 7.42±5.91 hours at 16 and 32 mg/L CAS, respectively. CAS demonstrated a fungistatic effect against *C. krusei* DPL45, reaching the maximum cell count decreases at 16–32 mg/L after 12 hours, but regrowth occurred after 24–48 hours and the curves became similar to the control as opposed to the data with RPMI-1640 without serum.

In the presence of 50% serum, in first 24 hours cell counts of all *C. inconspicua* strains decreased under the fungicidal endpoint. The fungicidal endpoint was reached significantly faster at 1-32 mg/L CAS concentrations in 50% serum than in RPMI-1640.

Investigation of killing rates exerted by caspofungin against *Candida krusei* and *Candida inconspicua* isolates

In case of *C. krusei* clinical isolates, the highest killing rate was noticed at 4 mg/L in RPMI-1640. It was significantly higher than at 2 and 32 mg/L ($P<0,05$).

In 50% serum *k* values (0,42-0,57 1/h) at effective concentrations (4-32 mg/L) did not differ significantly ($P>0,05$). The killing rates at 1, 2 and 4 mg/L CAS were significantly higher in RPMI-1640 comparing to in the presence of 50% serum ($P<0,01$-$0,005$).

In the case of ATCC 6258 reference isolate the measured *k* values in RPMI-1640 varied between 0,46 and 0,96 1/h at 2-32 mg/L CAS concentrations. Contrastingly, in the presence of 50% human serum the killing rate increased to 0,87-1,37 1/h. The killing rates at 8 and 16 mg/L CAS were significantly higher in 50% serum than in RPMI-1640 ($P<0,01$).

In the case of the CAS resistant DPL45 strain, with the exception of 32 mg/L CAS in RPMI-1640 (1,12 1/h) killing was observed in neither RPMI-1640 nor 50% serum. 50% serum significantly enhanced the activity of CAS at all tested concentrations against *C. inconspicua* ($P<0,0003$).

In vivo efficacy of caspofungin against *Candida krusei* isolates.

In our *in vivo* experiments two *C. krusei* isolates were investigated (5029, 27393) as well as one echinocandin resistant strain (DPL45). Although all of tested doses reduced the fungal tissue burden comparing to control group, significant CFU reduction was resulted just 3, 5 and 15 mg/kg daily CAS doses (P<0,05-0,001). One and 2 mg/kg/day were ineffective. It is noteworthy that there were no significant differences between effective doses.

Only the highest CAS dose (15 mg/kg) was effective against the *C. krusei* strain DPL45 (P<0,01).

In vivo efficacy of caspofungin against *Candida inconspicua* isolates.

In the case of *C. inconspicua* (20114, 22027) all treatment arms decreased the fungal tissue burden in the kidneys at least with two log degrees, which corresponded to a significantly lower tissue burden than seen in the untreated control (P<0,05-0,001). In case of *C. inconspicua* 20114 isolate the fungal tissue burden decreases were higher than four log reductions in mice treated with 5 and 15 mg/kg/day. Significant differences were not observed among the effective doses (P>0,05).

CONCLUSION

In past three decades, infections caused by different fungi pose one of the biggest health-care challenges among immunosuppressed as well as hospitalized patents.

Although *C. albicans* is the most frequent isolated human pathogen fungi, so-called non-*albicans* species with primer FLU resistance or reduced FLU susceptibility (*C. glabrata*, *C. krusei*, *C. inconspicua*) are identified more frequently due to the therapeutic and prophylactic FLU administration.

In early 2000s a new family of antifungals, echinocandins, was introduced for therapy of systemic *Candida* infections.

Echinocandins including CAS are the first-line therapy for critical ill, clinical instable patients and after FLU prophylactic. In addition, it is effective against *Candida* infections where the pathogen possess with reduced FLU susceptibility. Clinical efficacy from dose escalation of echinocandins including CAS was influenced by highly serum protein bound. In our *in vitro* experiments 50% human serum was utilized in order to demonstrate this phenomenon. It is noteworthy that these examinations could demonstrate better the *in vivo* circumstances.

On the basis of published literature, *in vivo* and clinical efficacy of CAS was attributed to two major factors. One of these the relative long PAFE in normal RPMI-1640 while the other one is the persistence of CAS in tissue. The importance of PAFE was highlighted by a published study from Clancy and his co-workers. According to this work PAFE is responsible for remarkable part of killing because in RPMI-1640 the killing was similar 100% in case of both the short CAS exposure (PAFE experiments) and continuous CAS exposure (time-kill experiments). In addition, the regrowth of examined *C. albicans* isolates was inhibited at least 24 hours.

On the basis of these facts, during our experiments killing rates of exerted by short (1 hour) and continuous (24 hours) CAS exposure was investigated

against the most frequent *Candida* species, *C. albicans* in normal RPMI-1640 with and without 50% human serum. Time-kill and PAFE experiments against *C. albicans* the fungal cell count reduction were similar in RPMI-1640. It is important to emphasize that the degree of killing rate reduced at 16 and 32 mg/L CAS concentrations in normal RPMI-1640. Presumably, this phenomenon thanks to compensatory chitin synthesis, which can be observed at high CAS concentrations in isolates dependent way (paradoxical growth).

During our PAFE experiments in the presence of 50% serum the killing rate reduced significantly at 4, 16 and 32 mg/L CAS concentrations comparing to values of RPMI-1640. In our time-kill studies high, concentration independent killing rate was detected at ≥1 mg/L concentrations.

As in our preliminary experiments 1 hour CAS exposure to 0,5-2 mg/L CAS did not produce measurable PAFEs in 50% serum in contrast with the results experienced in RPMI-1640.

Based of Louie and his co-workers, tissues serve as drug reservoir from which occurs slowly, continuous drug release into blood.

Our *in vitro* findings strongly suggest that PAFE is lost in the presence of 50% serum, even though marked PAFE is detected in RPMI-1640 after five minutes CAS exposure. The negligible PAFEs found in 50% serum indicate that prolonged *in vitro* PAFEs (in RPMI-1640), frequently interpreted as a contributor to better clinical efficacy, may be less important *in vivo* at least against *C. albicans*.

Next, *in vitro* susceptibility of two clinical relevant *Candida* species with primer FLU resistance (*C. krusei*, *C. inconspicua*) was compared in RPMI-1640 with and without 50% human serum. In our experiments we reached the fungicidal endpoint at 32 mg/L drug concentration 4,64 hours in case of *C. krusei* without serum. Adding 50% serum to the medium increased MIC values 4-8-fold against examined *C. krusei* isolates. The killing rate at effective CAS concentrations (4-32 mg/L) was concentration independent against *C. krusei*

clinical isolates, since no statistically significant differences were noted among the different CAS concentrations tested. In the presence of 50% serum the fungicidal endpoint at 32 mg/L increased to 7,42 hours.

Comparing to clinical isolates the reference strain behaved slightly differently, this can be attributed to different origin of this strain. The ATCC 6258 type strain was isolated from sputum as opposed to clinical isolates, which originated from blood.

These results were also confirmed in the neutropenic murine model. The utilized doses namely 1 mg/kg per day is equivalent with 35 mg per day for humans, 2 mg/kg/day is equivalent with 50 mg/day for humans. Based on AUC values the 3 and 5 mg/kg per day are equivalent with 70 mg once then 50 mg/day as well as 70 mg per day for humans. Both *in vitro* and *in vivo* result suggest that a very high dose of CAS does not offer much therapeutic benefit against *C. krusei*. However dose escalation of CAS to 15 mg/kg/day proved to be effective against the heterozygous *C. krusei* mutant strain *in vivo*. This dose is equivalent with 150 mg/day human dose according to AUC values. Therefore, dose escalation may be effective against echinocandin resistant *C. krusei* isolates.

Interestingly, *C. inconspicua* behaved differently from *C. krusei*. Although the MICs were increased slightly by 50% serum, CAS at 1-32 mg/L concentrations significantly increased killing rates in 50% serum against *C. inconspicua* when compared with RPMI-1640. Similar result have been reported Chiller *et* al. (2000). They used only 0,05-5% serum at 0,1 and 0,05 CAS concentrations. The utilized dose enhanced the activity of CAS against *A. fumigatus* in the presence of serum. Experiments of Wiederhold *et* al. CAS activity were enhanced by using of 5 and 50% serum against *C. glabrata* isolates.

The killing activity of CAS against *C. inconspicua*, similarly to *C. krusei*, was concentration independent, but started at concentrations as low as 1 mg/L CAS. Comparing to *C. krusei*, the fungicidal endpoint of *C. inconspicua* was shorter

at 1-32 mg/L CAS concentrations (2,09-2,67 hours). In our *in vivo* studies, all treatment arms significantly decreased the kidneys fungal burden compared with control mice in the case of *C. inconspicua* isolates.

This was the first study in which the killing rate produced by an echinocandin drug has been compares head to head in RPMI-1640 and 50% serum against three clinical relevant *Candida* species. Continuous but not brief CAS exposure produced measurable killing rates against *C. albicans* clinical isolates in killing studies in the presence of 50% serum. Therefore, PAFE after brief exposure to CAS, even when marked, may play a limited role in the excellent clinical efficacy of echinocandins.

In the second section of our experiments CAS MICs of examined *C. krusei* and *C. inconspicua* isolates markedly increased in the presence of 50% serum. In 50% serum the killing rate at effective CAS concentrations was concentration independent against *C. krusei* clinical isolates. In the case of *C. inconspicua* the killing rate was concentration independent and significantly higher compared with the values measured in RPMI-1640. On the basis of our *in vitro* and *in vivo* results the clinical benefit of dose escalation is questionable comparing to the approved standard dosing strategy. In addition, it may result unnecessary cost growth against two *Candida* species with FLU resistance (*C. krusei*, *C. inconspicua*).

Despite using of antifungal agents introduced at the beginning of the new millennium, the mortality of *Candida* species is 30-60%, which depends on species. *In vitro* experiments supplemented by serum as well as investigations of *in vivo* pharmacodynamics may help that a more effective echinocandin-based therapy can be worked out in the future against the different *Candida* species. Hopefully, reducing one of the most dangerous public health problems of the modern age.

SUMMARY

During our investigations killing rate exerted by caspofungin was determined as well as the extent of postantifungal effect in RPMI-1640 with and without 50% human serum against *C. albicans* isolates. Thereafter killing rate exerted by caspofungin was determined mentioned above two type of medium against *C. krusei* and *C. inconspicua* isolates. Finally, *in vivo* efficacy of caspofungin was investigated using different doses (1, 2, 3, 5, 15 mg/kg/day) against *C. krusei* and *C. inconspicua* strains in neutropenic mouse model.

Caspofungin possess with reduced activity in medium supplemented with serum due to the high degree of protein binding. However, fungistatic activity was observed in both media in case of *C. albicans* clinical isolates and the reference strain (≥0,03 mg/L in RPMI-1640, ≥0,25-0,5 mg/L in presence of 50% serum). In contrast, fungicid activity was detected during the examination of *C. krusei* and *C. inconspicua* (≥0,5-8 in RPMI-1640, ≥0,5-16 mg/L in presence of 50% serum). One-hour caspofungin exposure did not cause killing in the presence of serum against *C. albicans* isolates. During the investigation of killing rate, continuous but not brief caspofungin exposure produced measurable killing rates against *C. albicans* clinical isolates in killing studies in the presence of 50% serum, consequently postantifungal effect may play a limited role in the excellent clinical efficacy of caspofungin. The killing rate at effective caspofungin concentrations (4-32 mg/L) was concentration independent in serum against *C. krusei* clinical isolates.

Significantly higher and concentration independent killing was observed at 1-32 mg/L concentrations in the presence of 50% human serum against *C. inconspicua* strains in comparison with values in RPMI-1640. On the basis of our *in vitro* and *in vivo* findings the clinical relevance of caspofungin dose escalation against *C. krusei* and *C. inconspicua* is questionable.

Register number: DEENKÉTK/190/2014.
Item number:
Subject: Ph.D. List of Publications

Candidate: Renátó László Kovács
Neptun ID: HEOGB8
Doctoral School: Doctoral School of Pharmaceutical Sciences
Mtmt ID: 10036819

List of publications related to the dissertation

1. **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.
Mycopathologia. "accepted by publisher" (2014)
DOI: http://dx.doi.org/10.1007/s11046-014-9799-4
IF:1.545 (2013)

2. **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.
J. Med. Microbiol. 63 (2), 186-194, 2014.
DOI: http://dx.doi.org/10.1099/jmm.0.066381-0
IF:2.266 (2013)

List of other publications

3. 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:1.603 (2013)

4. **Kovács, R.**, Czudar, A., Horváth, L., Szakács, L., Majoros, L., Kónya, J.: Serum interleukin-6 levels in murine models of Candida albicans infection.
Acta Microbiol. Immunol. Hung. 61 (1), 61-69, 2014.
DOI: http://dx.doi.org/10.1556/AMicr.61.2014.1.6.
IF:0.78 (2013)

5. Földi, R., **Kovács, R.**, Gesztelyi, R., Kardos, G., Berényi, R., Juhász, B., Szilágyi, J., Mózes, J., Majoros, L.: Comparison of In Vitro and Vivo Efficacy of Caspofungin Against Candida parapsilosis, C. orthopsilosis, C. metapsilosis and C. albicans.
Mycopathologia. 174 (4), 311-318, 2012.
DOI: http://dx.doi.org/10.1007/s11046-012-9554-7
IF:1.489

6. Földi, R., Szilágyi, J., Kardos, G., Berényi, R., **Kovács, R.**, Majoros, L.: Effect of 50% human serum on the killing activity of micafungin against eight Candida species using time-kill methodology.
Diagn. Microbiol. Infect. Dis. 73 (4), 338-342, 2012.
DOI: http://dx.doi.org/10.1016/j.diagmicrobio.2012.05.011
IF:2.26

7. Bayegan, S., Majoros, L., Kardos, G., Kemény-Beke, Á., Miszti, C., **Kovács, R.**, Gesztelyi, R.: In vivo studies with a Candida tropicalis isolate exhibiting paradoxical growth in vitro in the presence of high concentration of caspofungin.
J. Microbiol. 48 (2), 170-173, 2010.
DOI: http://dx.doi.org/10.1007/s12275-010-9221-y
IF:1.266

Total IF of journals (all publications): 11.209
Total IF of journals (publications related to the dissertation): 3.811

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.

13 August, 2014

List of major 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 ont he 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

Berényi Réka, Földi Richárd, Szilágyi Judit, Kardos Gábor, **Kovács Renátó**, Majoros László: Effect of 50% human serum on the killing activity of micafungin against eight *Candida* species using time-kill methodology. 5th Hungarian Mycological Conference May 23-25, 2012, Budapest

R. Földi, J. Szilágyi, **R. Kovács**, R. Berényi, G. Kardos, L. Majoros Effect of 50% human serum on the killing activity of micafungin against eight Candida species using time-kill methodology. 2nd workshop Medical Mycology: From basix science to clinical needs. December 8-10. 2011, Vienna, Austria (poster: PP-13)

L. Majoros, **R. Kovács**, R. Berényi, J. Szilágyi, R.Földi, R. Gesztelyi, G. Kardos, B. Juhász In vitro and in vivo efficacy of caspofungin against Candida parapsilosis, C. orthopsilosis, C. metapsilosis and C. albicans. 2nd workshop Medical Mycology: From basix science to clinical needs. December 8-10. 2011, Vienna, Austria (poster: S6-02)

Bayegan S, Szilágyi J, Gesztelyi R, Kardos G, Mózes J, Kemény-Beke Á, Szabó Z, **Kovács R**, Majoros L. Correlation between postantifungal effect and the efficacy of the single 5 and 10 mg/kg caspofungin doses for treatment of disseminated candidiasis caused by *Candida krusei* in a neutropenic mouse model. 2nd Central Europen Forum for Microbiology (CEFORM). October 7-9. 2009, Keszthely, Hungary (poster: MP 19).

List of major presentations

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

Kovács Renátó, Berényi Réka, Földi Richárd, Gesztelyi Rudolf, Kardos Gábor, Juhász Béla, Majoros László
Postantifungal effect of caspofungin in 50% serum and efficacy of single high dose of caspofungin against the "psilosis" group
Congress of Hungarian Sociaty for Microbiology, October 24-26, 2012, Keszthely, Hungary

Berényi Réka Renáta, **Kovács Renátó**, Földi Richárd, Gesztelyi Rudolf, Kardos Gábor, Juhász Béla, Majoros László
In vitro and In vivo efficacy of caspofungin against fluconazole resistant *Candida krusei* and *C. inconspicua* clinical isolate
Congress of Hungarian Sociaty for Microbiology, october 24-26, 2012, Keszthely

Kovács Renátó, Majoros László, Berényi Réka, Szilágyi Judit, Földi Richárd, Gesztelyi Rudolf, Kardos Gábor és Juhász Béla: In vivo and in vitro efficacy of caspofungin against *Candida parapsilosis*, *C. orthopsilosis*, *C. metapsilosis* and *C. albicans*
5th Hungarian Mycological Conference May 23-25, 2012, Budapest

László Majoros, **Renátó Kovács**, Réka Berényi, Judit Szilágyi, Richárd Földi, Rudolf Gesztelyi, Gábor Kardos, Béla Juhász: *In vitro* and *vivo* efficacy of caspofungin against *Candida parapsilosis*, *C. orthopsilosis*, *C. metapsilosis* and *C. albicans*
2nd workshop Medical Mycology December 8-10. 2011, Vienna, Austria

The research was supported by the **TÁMOP 4.2.4 A/2-11-1-2012-0001** 'National Excellence Program – Elaborating and Operating an Inland Student and Researcher Personal Support System'. The project was subsidized by the European Union and co-financed by the European Social Fund.