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

The *in vitro* and *in vivo* antifungal susceptibility testing of *Candida parapsilosis sensu stricto, Candida orthopsilosis* and *Candida metapsilosis* isolates

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	Miklós Vecsernyés, Ph.D.

The Examination takes place at the Library of the Department of Pharmacology and Pharmacodynamics, Medical and Health Science Center, University of Debrecen. 11th of January 2013 at 11:00

Head of the **Defense Committee**:	Árpád Tósaki, Ph.D., D.Sc.
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The Ph.D. Defense takes place at the Lecture Hall of the 1st Department of Internal Medicine, Medical and Health Science Center, University of Debrecen. 11th of January 2013 at 13:00

INTRODUCTION

Despite the increasing number of antifungal agents the successful treatment of *Candida* infections remains a major challenge. This contradiction can be explained by the increasing frequency of the species, epidemiological alterations, poor clinical outcomes and the difficult diagnosis. During the last decades the invasive human infections caused by yeast species have become more frequent. The most frequent *Candida* species causing human infections are the *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei*. Among these species the incidence of *C. parapsilosis* has risen the most since 1990.

Due to the modern molecular biological studies *C. orthopsilosis* and *C. metapsilosis* have been separated from the *C. parapsilosis sensu stricto* less then ten years ago. Since then several studies have pointed out and still pointing out the clinical relevance of *C. orthopsilosis* and *C. metapsilosis* which account for approximately 1 % of the *Candida* invasive infections.

The *C. orthopsilosis* and *C. metapsilosis* are phenotypically identical but genotypically different from the *C. parapsilosis sensu stricto*.

In vitro studies showed that the antifungal susceptibility of *C. orthopsilosis* and *C. metapsilosis* are not identical with the *C. parapsilosis sensu stricto*'s susceptibility. *C. orthopsilosis* and *C. metapsilosis* show relatively high minimum inhibitory concentrations (MICs) for fluconazole (FLU) but lower MICs for amphotericin B (AMB) compared to *C. parapsilosis sensu stricto.* Besides all of the three species have reduced *in vitro* echinocandin susceptibility. However echinocandin MICs for *C. orthopsilosis* and *C. metapsilosis* are lower compared to *C. parapsilosis sensu stricto* thus the newly separated two species are more susceptible against these antifungal agents.

Successful infection management can only be achieved by a properly applied and well-timed antifungal agent. As treatment guideline only exsists for the infections caused by the *C. parapsilosis sensu stricto* it is likely that new management strategy is needed to be established for the two newly separated species.

The information about antifungal susceptibility of *C. orthopsilosis* and *C. metapsilosis* is scant in the literature which confirms the importance of our studies. Furthermore collecting data on echinocandin susceptibility of the less frequent *Candida* species is necessary as caspofungin (CAS) is increasingly used in treatments of invasive *Candida* infections.

The verification of *in vitro* results by *in vivo* methods is essential. Although the *in vitro* results characterize a certain degree of antifungal activity it can differ from the actual effect at the site of infection due to the host-drug inetractions. Based on these thoughts we compared the *in vitro* activity of five antifungal agents (AMB, FLU, voriconazole (VOR), CAS, 5-fluorocytosine (5-FC)) against the *Candida parapsilosis sensu stricto*, *C. orthopsilosis* and *C. metapsilosis* species then the *in vivo* comparison was also performed with three out of five (AMB, FLU, CAS) selected drugs. Finally we wanted to know if the current treatment guideline of the *C. parapsilosis sensu stricto* infections could be applied for the *C. orthopsilosis* and *C. metapsilosis* species.

AIMS

As in the current literature not too much information is available about the *in vitro* and mainly about the *in vivo* antifungal susceptibility of the recently separated *Candida orthopsilosis* and *Candida metapsilosis* species, furthermore therapeutic guidelines do not exist for the infections caused by these fungi we aimed in our study:

- to determine the minimal inhibitory concentrations of amphotericin-B, 5-fluorocytosine, fluconazole, voriconazole, posaconazole and caspofungin (*in vitro* microdilution method) against the *Candida parapsilosis sensu stricto*, *Candida orthopsilosis* and *Candida metapsilosis* species and in view of this information,
- to study and compare the *in vitro* pharmacodynamics of amphotericin-B, 5-fluorocytosine, fluconazole, voriconazole and posaconazole by time-kill curves (*in vitro* macrodilution method) within the *„psilosis”* group,
- furthermore to investigate and compare the *in vivo* activity of amphotericin-B, fluconazole and caspofungin against the *Candida parapsilosis sensu stricto*, *Candida orthopsilosis* and *Candida metapsilosis* species in neutropenic murine models,
- finally based on the results we wanted to determine the applicability of the *Candida parapsilosis sensu stricto*'s international guideline for the therapies of infections caused by the *Candida orthopsilosis* and *Candida metapsilosis* species

MATERIALS AND METHODS

Origin of the yeast isolates

The six *Candida parapsilosis sensu stricto,* three *Candida orthopsilosis* and four *Candida metapsilosis* clinical isolates tested in our work were previously identified by molecular biological methods. The italian strains (*C. parapsilosis sensu stricto* CP120, CP117; *C. orthopsilosis* CP85, CP25, CP125; *C. metapsilosis* CP5, CP92, CP86) were provided by Arianna Tavanti (Universitá di Pisa, Pisa, Italy). Four in six *C. parapsilosis sensu stricto* (9150, 509, 2845, 896/1) and one in four *C. metapsilosis* (12821) isolates were identified in the Department of Medical Microbiology's Diagnostic Laboratory, University of Debrecen. In our experiments we also tested three ATCC reference strains (*C. parapsilosis* ATCC 22019, *C. orthopsilosis* ATCC 96139, *C. metapsilosis* ATCC 96144).

In vitro antifungal susceptibility testing

Determination of the minimal inhibitory concentracion

The minimal inhibitory concentrations (MIC) of AMB, 5-FC, FLU, VOR, POS and CAS were performed minimum two times in accordance with the CLSI's (Clinical and Laboratory Standards Institute) M27-A3 document. The FLU, 5-FC and CAS antifungal agents were dissolved in sterile distilled water while the other drugs were dissolved in 100 % DMSO. AMB, VOR, POS and CAS were tested at 0,015-8 µg/mL, 5-FC and FLU were tested at 0,12-64 µg/mL concentrations. For the fungi suspensions prepared in 0,85 % saline with 0,5 McFarland density we used 24 hours old colonies, cultured on Sabouraud agar plate. We used RPMI-1640 for the appropriate cell count adjustment which was

10^4 CFU/mL in case of the AMB and 10^3 CFU/mL in case of the other tested drugs.

The 96-well Elisa-plates used for the MIC determination contained fungi control (antifungal drug free) and media control (yeast free) wells. After 48 (AMB, 5-FC, FLU, VOR, POS) and 24 (CAS) hours of incubation at 35 °C the contents of the wells were suspended with pipette and the results were read visually.

Data evaluation

The MIC of the AMB is the lowest concentration of the antifungal drug which does not cause visible growth (total inhibition) compared to the control while the MIC of the other antifungal agents cause 50 % decrease (prominent inhibition) compared to the control.

E-test

As E-test is considered the best method for amphotericin B resistance detection in *Candida* strains, amphotericin B MICs were also determined by this method. E-test was carried out using 0,5 McFarland density fungal suspensions prepared with 24 hours old cultures in 0,85 % saline and RPMI-1640 agar supplemented with 2 % glucose. These suspensions were evenly spreaded on the surface of the RPMI agar plates with steril swabs, after the plates were dried we placed the test stripes impregnated with AMB onto them. Results were read visually after 24 and 48 hours of incubation at 35 ºC.

Time-kill curves

Time-kill studies were performed according to the standardised method of Klepser and his colleagues. We prepared 10^5 CFU/mL starting inoculum from each tested isolates in RPMI-1640 liquid media using densitometer. Antimycotics (5-FC, FLU, VOR, POS) were tested at 0,5-16×MIC

concentrations. As the maximal attainable free amphotericin B concentration in the serum is less than 1 μg/mL, the highest tested amphotericin B concentration was only 4 μg/mL, regardless of the actual MIC. Test tubes containing the media, the fungi suspension and the antifungal agents at different concentrations were incubated for 48 hours with agitation in the dark at 35 °C. At 0, 4, 8, 12, 24 and 48 hours 100-100 μL samples were removed from the tubes and serially diluted 10-fold in sterile saline; four 30-μL aliquots were subsequently plated onto Sabouraud agar plates. In those cases when colony counts were suspected to be less than 1000 CFU/mL undiluted samples were plated as above. The limit of quantification is 50 CFU/mL. Agar plates were incubated after the inoculated liquid was dried for 15-20 minutes at room temperature, 48 hours later we counted the grown colonies and determined the CFU (colony forming unit) according to the degree of the dilutions. Finally the CFU results versus time were plotted. All of the time-kill experiments were performed minimum twice and the results were averaged. Time-kill curves were prepared using the computer curve-fitting software (GraphPad Prism 4.03 for Windows). Fungicidal activity was defined as a ≥99,9% (≥3 $\log_{10}$) reduction in viable CFU/mL of the starting inoculum. Fungistatic activity was defined as detectable colony number decrease, which do not reach 99,9% (<3 $\log_{10}$) compared to the starting inoculum.

In vivo antifungal susceptibility testing

Animals

In our experiments we used female BALB/c mice weighing 26-28 g which were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals. Each group consisted of 7-11 animals. The number of the *in vivo* experiment's permission: 12/2008 DE MÁB.

Immunosuppression of the BALB/c mice

Mice were immunosuppressed using a single 200 mg/kg intraperitoneal cyclophosphamide dose three days prior to infection. In order to prevent emerging Gram-negative bacterial infections all murine recieved 5 mg/kg subcutan ceftazidime from the day of the infection until the end of the experiment.

Preparation of the infectious doses

For the infective doses preparation we plated the isolates of the three species (*C. parapsilosis sensu stricto* 9150, 896/1, *C. orthopsilosis* CP25, CP125, CP85, *C. metapsilosis* CP5, CP92, CP86) onto Sabouraud agar plates on two consecutive days and then the renewed strains were plated onto three or four Sabouraud agar plates again. The grown isolates were taken from the surface of the agar plates with sterile swab and suspended in sterile saline. These suspensions were centrifugated for four times/10 minutes at 30000g. We removed the supernatant from the cells after each centrifugation and added 20-25 mL fresh, sterile saline again to the them. After the last centrifugation we removed the supernatant again and added 3 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.

Infection of mice

Mice were infected with the fungi suspension intravenously (0,2 mL/mouse) through the lateral tail vein on the fourth day of the immunosuppression.

The infective dose of the *C. metapsilosis* isolates was 5×10^7 CFU/mouse, and slightly lower, 2×10^7 CFU/mouse in the case of the *C. orthopsilosis* and *C.*

parapsilosis sensu stricto strains. These doses did not cause lethality in the control groups.

Antifungal therapy

The intraperitoneal AMB (1 mg/kg/day), FLU (1, 5, 10, 20 mg/kg/day) and CAS (1, 2, 5 mg/kg/day) treatments of the mice were started 24 hours after infection and lasted for five days. All drug doses were given in a 0,5 ml bolus. In the experiments we applied the currently suggested doses for the human therapies. The control groups were given 0,5 mL sterile saline.

Organ culture

For the quantitative CFU determination mice still alive on the 7^{th} day were dissected after cervical dislocation, the kidney pairs were removed and homogenised aseptically. After one mL sterile saline was added to the homogenate an aliquot was serially diluted 10-fold. Aliquots of 100 μL of the appropriate dilutions were plated on Sabouraud agar plates. The numbers of the colonies grown on the agar plates were determined after 48 hours of dark incubation at 35°C.

For the statistical analysis we used the Kruskal-Wallis test in case of the fungi cultured on the agar from the kidneys. P values of $<0,05$ were regarded as significant.

RESULTS

Results of the *in vitro* antifungal susceptibility testing

Minimal inhibitory concentrations

MIC values of the ATCC quality control strains were in the acceptable CLSI ranges. The amphotericin B MICs obtained with the CLSI and Etest methods agreed within ±1 dilution and ranged between 0,25-2 µg/mL. In the case of the E-test method 1 µg/mL was the highest read concentration. Using the CLSI method the *C. orthopsilosis* CP25 isolate was the most susceptible against this polyen antifungal agent while using the E-test the *C. metapsilosis* ATCC 96144 and CP86 strains proved to be the most susceptible. Against the triazole antifungal agents all of the tested isolates were susceptible. In the case of FLU the *C. parapsilosis sensu stricto* isolates showed greater susceptibility (MIC: 0,5-2 µg/mL) than the *C. orthopsilosis* and *C. metapsilosis* species (the highest MIC: 8 µg/mL). Applying the 24 hours incubation the MICs of the isolates were 1-4 dilutions lower than in the case of the 48 hours incubation. We observed 0,015-0,12 µg/mL MIC ranges for VOR and POS. The *C. parapsilosis sensu stricto* 2845 and CP120 strains were the most susceptible to VOR (0,015 µg/mL). The *C. metapsilosis* CP5 (0,015 µg/mL) and CP92 (0,015 µg/mL) were the most susceptible strains to POS. The 5-FC MICs were ≤0,12 µg/mL in case of all of the tested isolates. After 24 hours incubation the CAS MIC values of the two newly separated *C. orthopsilosis* and *C. metapsilosis* species were lower (0,12-1 µg/mL) than the MICs of the *C. parapsilosis sensu stricto* isolates (0,5-2 µg/mL).

Results of the time-kill studies

Amphotericin B

In the time–kill studies, AMB was fungicidal at 1 to 2 μg/mL (0,5–2× MIC) after 24 and 48 h against the ***C. parapsilosis sensu stricto*** ATCC 22019 strain. However against the 896/1 and also against the CP120 isolates slightly higher 1 to 4 (1–4×MIC) and 2 to 4 (2–4×MIC) μg/mL AMB concentrations were fungicidal, respectively. The remaining *C. parapsilosis sensu stricto* clinical isolates (9150, 509, 2845, CP117) were also killed at 4 μg/mL AMB after 48 h (≥99,9 % CFU reduction).

After 24 h of incubation AMB at 2-4 (2-16×MIC) μg/mL concentrations was fungicid against all of the tested ***C. orthopsilosis*** strains. The CP125 isolate was most readily killed by AMB; killing was observed even at 1 μg/mL (1×MIC) AMB concentration after 24 h. After 48 h, 1 to 4 μg/mL of AMB were fungicidal (≥99,9 % CFU decrease) against 3 of 5 ***C. metapsilosis*** isolates (CP92, CP86 and 12821). In the case of the remaining CP5 and *C. metapsilosis* ATCC 96144 strains we observed fungicidal killing at slightly higher, 2 to 4 μg/mL (2–4×MIC) AMB concentrations also after 48 h. Against the *C. metapsilosis* CP86 isolate AMB was fugicid even after 12 h at 4-8×MIC concentrations.

5-fluorocytosine

5-FC caused less than 99,9 % reduction in viable CFU/mL of the starting inoculum, thus proved to be fungistatic against all of the members of the „*psilosis*” group. None of the tested 5-FC concentrations were fungicidal against the ***C. parapsilosis*** ATCC 22019 isolate. Some, but not all ***C. orthopsilosis*** (CP85, CP125) and ***C. metapsilosis*** (CP92, 12821) isolates required higher 2–4×MIC (0,24-0,48 μg/mL) 5-FC concentrations for greater effective inhibition

(<99,9 % CFU decrease). Similarly to the other members of the „*psilosis*" group we did not observe the 5-FC's fungicidal effect against the 12821 *C. metapsilosis* isolate.

Fluconazole

Triazoles were fungistatic against all three *Candida* spp. (less than 99,9 % reduction in viable CFU/mL of the starting inoculum). **FLU** was fungistatic even at ≥1×MIC against all ***C. parapsilosis sensu stricto*** isolates. The lower 0,5×MIC FLU concentration more moderately inhibited the growth of the CP120 *C. parapsilosis sensu stricto* isolate than the higher 8-16×MIC values. ***C. orthopsilosis*** CP25 was inhibited at 1×MIC (8 µg/mL). *C. orthopsilosis* CP85, CP125 and ATCC 96139 were fungistatically inhibited at higher, 2-16 times FLU MICs (16–128 µg/mL). The majority of the ***C. metapsilosis*** strains (ATCC 96144, CP5, CP86, 12821) were inhibited fungistatically at 1×MIC (4 µg/mL), whereas isolate CP92 was only inhibited by higher 2×MIC (16 µg/mL) FLU.

Voriconazole

While testing the ***C. parapsilosis sensu stricto*** isolates VOR caused growth inhibition (<99,9 % CFU reduction) at low concentrations ≥1×MICs (0,015-0,12 µg/mL). The ***C. orthopsilosis*** ATCC 96139, CP85 and CP125 isolates were inhibited at 0,5 µg/mL or only higher (4–8×MIC values) VOR while isolate CP25 was effectively inhibited at lower, 1×MIC (0,12 µg/mL). While ***C. metapsilosis*** ATCC 96144 strain was inhibited even by 2×MIC (0,25 µg/mL), all *C. metapsilosis* clinical isolates were inhibited at VOR concentrations of 4-8×MICs (0,25-0,5 µg/mL) and no significant fungistatic inhibition was observed at concentrations that were twice the MICs.

Posaconazole

POS caused growth inhibition (<99,9 % CFU reduction) in the case of the ***C. orthopsilosis*** and ***C. metapsilosis*** ATCC strains and all clinical isolates at 0,03–0,06 and 0,015–0,03 μg/mL concentrations, respectively. At 0,25-0,5 μg/mL (4-8×MIC) POS concentrations more than 1 lo_{g10} colony number decreas was detected for the CP85 and CP25 *C. orthopsilosis* and the CP92 *C. metapsilosis* clinical isolates (1,2–1,48 log_{10} and 2,22 log_{10} CFU/mL, respectively).

We did not test the *C. parapsilosis* isolates against POS, and all of the other members of the group against CAS as our team has published these results previously.

Results of the *in vivo* antifungal susceptibility testing

***C. parapsilosis sensu stricto* isolates**

While 1 mg/kg/day FLU was ineffective (P>0,05) against the *C. parapsilosis sensu stricto* **9150** strain in neutropenic mice 5, 10 (P<0,05) and 20 mg/kg (P<0,01) FLU daily doses significantly reduced the tissue fungal burden. CAS was only effective at 5 mg/kg daily dose (P<0,05). AMB at 1 mg/kg daily dose also significantly reduced the tissue fungal burden (P<0,001). Similarly to the 9150 isolate all of the tested FLU doses (5 mg/kg-P<0,05; 10 mg/kg-P<0,01; 20 mg/kg-P<0,001) proved to be effective against the **896/1** strain except of the 1 mg/kg dose. Against this isolate 2 mg/kg daily CAS was also effective (P<0,05) in addition to the daily 5 mg/kg CAS and 1 mg/kg AMB (P<0,01).

***C. orthopsilosis* isolates**

In the case of the *C. orthopsilosis* **CP25** isolate the daily 10 and 20 mg/kg FLU (P<0,05), 2 and 5 mg/kg CAS (P<0,01) and 1 mg/kg (P<0,01) AMB were effective. While the daily 1 and 5 mg/kg FLU were ineffective (P>0,05) when

we were testing the **CP85** strain 10 and 20 mg/kg daily doses proved to be effective (P<0,05). All of the daily doses of CAS (1, 2, 5 mg/kg-P<0,05) and 1 mg/kg AMB (P<0,001) significantly reduced the tissue fungal burden. One mg/kg daily FLU was ineffective (P>0,05) but 10 and 20 mg/kg significantly reduced the tissue fungal burden in the neutropenic mice infected with the **CP125** strain. Unlike the two previous strains (CP25, CP85) the higher 5 mg/kg daily FLU proved to be effective against the CP125 isolate. CAS at 2 and 5 mg/kg daily doses and as well as 1 mg/kg AMB were effective against this strain.

***C. metapsilosis* isolates**

The results of the *C. metapsilosis* **CP5** isolate best correlate with the CP25 strain's results. Namely in both experiments the lower FLU (1 and 5 mg/kg/day) doses were ineffective while the higher doses (10 and 20 mg/kg) were effective (P<0,05; P<0,01). Against this isolate 2 and 5 mg/kg CAS (P<0,05; P<0,001) and 1 mg/kg AMB (P<0,001) daily doses caused significant tissue fungal burden reduction compared to the control mice. In the case of the **CP86** strain 5, 10 and 20 mg/kg FLU (P<0,05; P<0,01), 2 and 5 mg/kg CAS (P<0,05) and 1 mg/kg AMB (P<0,05) daily doses caused significant tissue fungal burden reduction. In the **CP92** infected neutropenic mice model all of the tested antifungal agenst were effective except of the 1 mg/kg daily FLU dose.

DISCUSSION

Before an antifungal agent's introduction into the clinical practice *in vitro* and *in vivo* studies have to be performed which more or less predict it's effect against the pathogens.

The formerly known *C. parapsilosis sensu lato* species has been recently separated into three: *C. parapsilosis sensu stricto*, *C. orthopsilosis* and *C. metapsilosis*. Since 2004 the frequency of *C. orthopsilosis* has increased from 2,3 % to 9 % and the prevalence of *C. metapsilosis* from 0,9 % to 6,9 %. According to the epidemiological surveys the most frequent species of the „*psilosis*" group causing human infections is the *C. parapsilosis sensu stricto*.

Compared to the *C. albicans* the virulence of the *C. parapsilosis sensu stricto* is lower. The virulence is even different within the „*psilosis*" group, the most virulent is the *C. parapsilosis sensu stricto*, the less virulent is the *C. metapsilosis*.

In order to increase the therapeutical efficiency the CLSI recently defined new species-specific breakpoints for the five most frequent *Candida* species including the *C. parapsilosis sensu stricto.* These new values can be three (FLU) or four dilutions lower (echinocandin) compared to the old ones.

According to the former *in vitro* studies the antifungal susceptibility of the *„psilosis"* group is not uniform. Namely the *C. parapsilosis sensu stricto* is less susceptible against AMB, echinocandins and FLU than the *C. metapsilosis* or *C. orthopsilosis* species. Based on this the susceptibility breakpoints of the *C. parapsilosis sensu stricto* may not be successfully used in the case of the *C. orthopsilosis* and *C. metapsilosis* species. In our *in vitro* studies the AMB MIC

values of the tested *C. orthopsilosis* and *C. metapsilosis* clinical isolates were lower than the *C. parapsilosis sensu stricto'*s.

None of the isolates we tested had amphotericin B MICs greater than 2 µg/mL. After 24 and 48 h 1-4 µg/mL AMB (0,5-16×MIC) were fungicidal against the „*psilosis*" group.

AMB efficacy is best predicted by the Cmax/MIC (maximum concentration of AMB in serum per MIC) pharmacodynamic parameter. In our *in vivo* experiments AMB at 1 mg/kg dose was effective against all of the tested isolates and even though the 1 mg/kg produced slightly higher Cmax in mice than in humans, taking into account the high protein binding of AMB, the free drug levels are comparable.

In contrast to the 48-hour reading of the FLU MIC the 24-hour endpoint were found to be lower by 1-4 two-step dilutions for all three species in the *in vitro* experiments. The MIC values of the *C. orthopsilosis* and *C. metapsilosis* were equal to or lower than the suggested new breakpoint of the *C. parapsilosis sensu stricto* (2 mg/L).

The CLSI decreased the old 8 µg/mL FLU susceptible breakpoint by two-step dilutions to 2 µg/mL, thus the tested *C. orthopsilosis* and *C. metapsilosis* isolates are considered susceptible based on the 24 h endpoint but according to the 48 h MIC reading they are not.

Our time-kill studies and previous observations showed that the management of the *C. orthopsilosis* and *C. metapsilosis* infections required higher FLU doses (4-8 µg/mL; ≥2×MIC). In contrast, the *C. parapsilosis sensu stricto* isolates were *in vitro* inhibited by FLU concentrations close to the MIC (0,5-2 µg/mL; ≥1×MIC).

The AUC/MIC (area under the concentration curve per MIC) is the pharmacodynamic parameter that best predicts the efficacy of FLU. *In vivo* all tested isolates were inhibited even by 10 and 20 mg/kg FLU daily doses in our work. As 25 mg/kg FLU in mice and 100 mg/kg FLU in humans produced essentially the same 24-hour AUC (90 mg·h/l), our results suggest that FLU is highly effective even at the minimum therapeutic doses. This can be explained by the fact that FLU shows long, concentration-independent postantifungal effect *in vivo*. Based on our results the 24 hour FLU MIC values showed good correlation with the *in vivo* effect.

The VOR MIC values necessary for growth inhibiton of the three species were lower than the VOR concentrations attainable in the serum. In the *in vitro* time-kill studies VOR proved to be fungistatic (<99,9% CFU decrease), for this effect *C. parapsilosis sensu stricto* isolates needed lower (≥1×MIC; 0,015-0,12 µg/mL) while *C. orthopsilosis* and *C. metapsilosis* strains needed higher concentrations (4-8×MIC; 0,25-0,5 µg/mL). These time-kill studies may indicate the poor *in vivo* efficacy of VOR which is caused by it's fast metabolism in mice.

POS was also fungistatic at relatively low concentrations *in vitro* against the *C. orthopsilosis* and *C. metapsilosis* species. Similar results were observed in the case of *C. parapsilosis sensu stricto* where POS was not even fungicidal at the higher 32-64×MIC concentrations. The POS's applicability in the treatments of infections caused by the „*psilosis*" group has to be also proved *in vivo* which is limited by the fact that POS only available in oral formulations.

We observed the 5-FC MIC values at ≤0,12 µg/mL in the case of all tested isolates. This antifungal agent caused excellent fungistatic effect at low concentrations which in combination with other agents can be effective in treatments of infections caused by the „*psilosis*" group.

CAS can be characterised with concentration-dependent activity. It's *in vivo* activity is significantly defined by it's concentration at the site of the infection.

The *in vitro* CAS MIC values of *C. orthopsilosis* and *C. metapsilosis* were lower (0,12-0,5 µg/mL) than the *C. parapsilosis sensu stricto*'s (0,5-2 µg/mL).

The CLSI preserved the earlier 2 µg/mL echinocandin susceptibility breakpoint for the *C. parapsilosis sensu stricto* species. Echinocandins can not be considered as first-line agents in the infections caused by *C. parapsilosis*. During CAS therapy breakthrought infections and even emerging CAS resistance can be observed.

Furthermore significant correlation can be detected between the agent's increased use and the greater frequency of the *C. parapsilosis sensu stricto* candidaemia.

The primarily decreased echinocandin susceptibility of the „*psilosis*" group is explained by the fact that in the glucan synthase enzyme's *Fks1* „hot spot" second region alanin substitutes prolin at amino acid position 660.

In previous *in vitro* time-kill studies *C. orthopsilosis* behaved similarly to *C. parapsilosis sensu stricto* namely both showed fungistatic activity or paradox growth to CAS. In our previous work CAS was fungicidal only against one *C. parapsilosis sensu stricto* and *C. orthopsilosis* after 48 hours of incubation.

CAS efficacy as in the case of FLU is linked to the AUC/MIC ratio. This can be explained by it's long persistance in the kidney and the long, concentration-dependent post-antifungal effect. In our previous work CAS at 1, 2 and 4 mg/kg produced 59, 118 and 164 mg·h/l mean AUC, respectively in mice; the corresponding data for humans are 35, 50 and 70 mg/kg daily dose of CAS with 55, 100 and 114 mg·h/l mean AUC, respectively.

Against the two newly separated species CAS was even effective at 2 mg/kg daily dose which corresponds to the human 50 mg daily dose.

In contrast to this only the higher 5 mg/kg daily CAS treatment was able to reduce the tissue fungal burden against the tested *C. parapsilosis sensu stricto* isolates. The 5 mg/kg daily dose of mice corresponds to the human's 70 mg daily dose.

The high CAS dose proved to be effective against the *C. parapsilosis sensu stricto* isolates in our models. Therefore we should not be affraid of the unsuccessful empirical CAS treatment of the infections caused by this species.

Our results indicate that all of the three antifungal agents have excellent *in vivo* activity against all of the members of the „*psilosis*" group.

As the members of the „*psilosis*" group cause infections mainly in non-neutropenic patients FLU could be the first therapeutic choice in the case of clinically stable patients with candidaemia. The echinocandin therapy could remain if the patient has recieved an echinocandin during the empirical treatment and it's condition is improving. In case of clinically instable patients unequivocally AMB can be recommended.

Further preclinical and clinical studies have to confirm the correctness of our ideas.

SUMMARY

The *C. parapsilosis sensu lato* species was separated into *C. parapsilosis sensu stricto, C. orthopsilosis* and *C. metapsilosis* using molecular biological methods. According to the literature the *in vitro* antifungal susceptibility of the the latter two species differ from the *C. parapsilosis sensu stricto's*, however treatment guidelines only known for the *C. parapsilosis sensu stricto*.

First we performed time-kill studies with amphotericin B, fluconazole, voriconazole, posaconazole and 5-fluorocytosine. Against the „*psilosis*" group amphotericin B was fungicidal at 1-4 µg/mL and fluconazole proved to be fungistatic at ≤8 µg/mL concentrations. Voriconazole, posaconazole and 5-fluorocytosine showed excellent fungistatic activity at concentrations still attainable in the blood.

In case of the temporarly neutropenic mice 1 mg/kg daily amphotericin B, 10 and 20 mg/kg daily fluconazole significantly reduced the tissue fungal burden against the *„psilosis"* group, thus both drugs proved to be efficacious. Although the *„psilosis"* group has reduced echinocandin susceptibility the 5 mg/kg daily CAS was effective.

The basis of a successful treatment is the *in vitro* susceptibility testing which together with the recent susceptibility breakpoints can help to effectively cure the infections caused by the less known species as the routine diagnostic methods are currently not capable of the accurate separation of the *„psilosis"* group. Our *in vitro* and *in vivo* results show that the treatment of the *C. parapsilosis sensu stricto, C. orthopsilosis* and *C. metapsilosis* infections do not differ basically, thus depending on the clinical status of the patients fluconazole, amphotericin B and also caspofungin are considered as applicable therapeutic alternatives.

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Candidate: Judit Szilágyi

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List of publications related to the dissertation

1. **Szilágyi, J.**, Földi, R., Gesztelyi, R., Bayegan, S., Kardos, G., Juhász, B., Majoros, L.: Comparison of the kidney fungal burden in experimental disseminated candidiasis by species of the Candida parapsilosis complex treated with fluconazole, amphotericin B and caspofungin in a temporarily neutropenic murine model.
Chemotherapy. 58 (2), 159-164, 2012.
DOI: http://dx.doi.org/10.1159/000337088
IF:1.816 (2011)

2. Szabó, Z., **Szilágyi, J.**, Tavanti, A., Kardos, G., Rozgonyi, F., Bayegan, S., Majoros, L.: In vitro efficacy of 5 antifungal agents against Candida parapsilosis, Candida orthopsilosis, and Candida metapsilosis as determined by time-kill methodology.
Diagn. Microbiol. Infect. Dis. 64 (3), 283-288, 2009.
DOI: http://dx.doi.org/10.1016/j.diagmicrobio.2009.03.011
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List of other publications

3. 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.
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4. 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.
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J. Chemother. 24 (1), 18-25, 2012.
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IF:1.084 (2011)

6. Bayegan, S., **Szilágyi, J.**, Kemény-Beke, Á., Földi, R., Kardos, G., Gesztelyi, R., Juhász, B., Adnan, A., Majoros, L.: Efficacy of a single 6 mg/kg versus two 3 mg/kg caspofungin doses for treatment of disseminated candidiasis caused by Candida albicans in a neutropenic mouse model.
J. Chemother. 23 (2), 107-109, 2011.
IF:1.084

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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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List of major presentations

J. Szilágyi, S. Bayegan, R. Gesztelyi, G. Kardos, Á. Kemény-Beke, Z. Szabó, L. Majoros. *In vivo* efficacy of fluconazole, voriconazole and caspofungin against *Candida orthopsilosis* in a neutropenic mouse model. 2ND Central European Forum for Microbiology, Keszthely, 2009.

J. Szilágyi, S. Bayegan, R. Gesztelyi, G. Kardos, Á. Kemény-Beke, Z. Szabó, L. Majoros, *In vivo* efficacy of fluconazole and caspofungin against *Candida parapsilosis* and *C. orthopsilosis* in temporarily and deeply neutropenic mouse models. Annual Meeting of the Hungarian Society for Microbiology, Keszthely, 2010.

List of posters related to the thesis

S. Bayegan, **J. Szilágyi**, R. Gesztelyi, G. Kardos, J. Mózes, Á. Kemény-Beke, Zs. Szabó, R. Kovács, L. Majoros. Correlation between postantifungal effect and the efficacy of single 5 and 10 mg/kg caspofungin doses for treatment of disseminated candidiasis caused by *Candida krusei* in a neutropenic mouse model. 2ND Central European Forum for Microbiology, Keszthely, 2009.

L. Majoros, **J. Szilágyi**, S. Bayegan, A. Tavanti, Á. Kemény-Beke, G. Kardos,A. Adnan, R. Gesztelyi. *In vivo* efficacy of amphotericin B, fluconazole, voriconazole and caspofungin against *Candida orthopsilosis* in a neutropenic mouse model. 4th Trends in Medical Microbiology. Athene, 2009.

L. Majoros, R. Kovács, R. Berényi, **J. Szilágyi**, R. Földi, R. Gesztelyi, G. Kardos, B. Juhász. *In vitro* and *vivo* efficacy of caspofungin against *Candida parapsilosis, C. orthopsilosis, C. metapsilosis* and *C. albicans.* 2nd Joint Workshop of ÖGMM, ÖGACH, ÖGIT & ÖGHMP, Vienna, 2011.