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

Investigation of the *in vitro* efficacy of caspofungin, micafungin and nikkomycin Z using micro- and macrodilution methods against the major *Candida* species

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Debrecen, 2012

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Members of the Examination Committee: Miklós Vecsernyés, Ph.D.
Edit Urbán, Ph.D.

The Examination takes place at the Lecture Hall of the 1st Department of Internal Medicine, Medical and Health Science Center, University of Debrecen, 2. October 2012. at 11:00 am.

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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, 2. October 2012. at 1:00 pm.

INTRODUCTION

Candida species are responsible for approximately 90% of all severe fungal infections. As part of the normal flora, they are found in healthy organisms; however, as a result of opportunistic infections or different medical treatments, their numbers can increase significantly. *Candida* species are considered the fourth most common cause of nosocomial sepsis among all pathogens (8-10%). The most frequently isolated pathogen, which is also considered the most virulent one, is the *Candida albicans*. However, from the 1990's, the number of infections caused by non-albicans *Candida* species has also increased.

In vitro antifungal sensitivity assays play an important role in choosing appropriate drugs for therapies, experiments targeting drug-development and tracking the evolution of antifungal resistance.

During our investigations, we used antifungal sensitivity methods that were established by the CLSI (Clinical and Laboratory Standards Institute). The document was first released in 1992, and then it was modified several times until it reached its current form with version M27-A3.

The time-kill method used in our experiments is an indispensable tool of ascertaining the activity of antimicrobial agents. It provides proper information on the magnitude of the effect and on the pharmacodynamic properties (e.g. concentration, relationship between drug mechanism and the postantibiotic effect) of the drugs and during the combined application of different medications, contingent antagonistic or synergistic effects can be examined.

The latest milestone of research in this field is the introduction of echinocandins that block the fungal cell wall synthesis. These drugs can be used with high safety and efficiency in cases of invasive candidiasis and aspergillosis; moreover, they have favorable properties in terms of pharmacokinetics and

pharmacodynamics. Their effect is based on the non-competitive inhibition of the enzyme1,3-β-D glucan synthase, which is responsible for the biosynthesis of the main glucan component of the fungal cell wall, 1,3-β-D glucan. The drug changes the integrity of the cell wall, its thickness decreases thus losing most of its mechanical strength and as a result, the cell will not be able to resist the intracellular osmotic pressure, which in turn, leads to the lysis of the cell.

Echinocandins have a broad spectrum among *Candida* and *Aspergillus* species, while against *Zygomycetes* species and *C. neoformans* they proved ineffective as their cell wall contains 1,3-α and 1,6-α glucan. All three echinocandins are fungicide both *in vitro* and *in vivo* against most *Candida* species, including ones resistant against azoles (*C. krusei*, *C. glabrata*) as well as amphotericin B (AMB) (*C. lusitaniae*).

Echiocandins typically have a high protein-binding ability in human serum. This value is the lowest in the case of caspofungin (CAS) (96,5%), followed by anidulafungin (ANI) with 98-99% and the highest is micafungin (MICA) with 99,8%. With respect to this property, it is hypothesized that serum proteins can have a role in altering the effect of antimicrobial agents both under *in vitro* and *in vivo* conditions when serum is present. The decreased activity of echinocandins in the presence of serum leads to the conclusion that their protein-binding capabilities have a direct effect on the drug, which most probably manifests in altering their capability to inhibit glucan synthases.

According to the free drug hypothesis, only 1% of ANI, 3,5% of CAS and 0,2% of MICA is pharmacologically active in the presence of serum. As a consequence, their MIC values would have to rise 200-500 times, which does not concur with the results obtained during the above mentioned experiments. Thus, it is plausible that a fraction of the protein-bound drug maintains its

antifungal activity. This hypothesis has been confuted in the case of MICA, as according to observations, the drug does bind serum proteins, but the binding is weak and reversible.

As a consequence of being less toxic, the dose of echinocandins can be tripled during therapy. During *in vitro* experiments, however, there is a recurring phenomenon; after administering higher concentrations of echinocandins, the activity of the drug decreases against the given pathogen. In certain cases echinocandins exert their killing effect at low concentrations; however, at higher concentrations the pathogens grow at the same rate as in the case of control samples. This is called paradoxical growth, which, among antifungal agents, has only been observed in the case of echinocandins.

There is a remarkable fact that the *in vitro* paradoxical growth appears at drug concentrations that can be reached in human serum by the application of regular dosing strategies.

By the combined application of these drugs, many advantages can be obtained, which include increased efficiency, reduced toxicity or side effects due to lower doses. The infrequent emergence of drug-resistant variants and the broader spectrum all indicate that different combinations can lead to increased therapeutic success. Therefore, during our experiments, we not only used CAS alone, but also used it in combination with nikkomycin Z (NIK), another inhibitor of a cell wall component.

AIMS OF OUR STUDY

We examined the *in vitro* activity of caspofungin, micafungin and the chitin synthesis inhibitor nikkomycin Z against the clinically relevant *Candida* species which were isolated at the UD MHSC Institute of Medical Microbiology using micro- and macrodilution methods.

Our aim was:

- to determine the MIC values of micafungin and compare it in RPMI-1640 with or without the addition of 50% human serum against the different *Candida* species and also, to investigate the effectiveness of the drug in the two types of medium using time-kill experiments
- to determine the MIC values of caspofungin and nikkomycin Z against the clinically relevant *Candida* species then examine and compare the killing activity of caspofungin and nikkomycin Z separately and jointly using time-kill methods in the two types of medium
- to observe the occurrence and alteration of paradoxical growth which takes place in the presence of high concentration of caspofungin adding nikkomycin Z and human serum to the medium

MATERIALS AND METHODS

Source of the tested fungal species

The 24 wild-type clinical isolates and the 5 American Type Culture Collection (ATCC) strains used in the study of MICA. All *C. albicans, C. tropicalis, C. glabrata, C. parapsilosis, C. krusei* and 1 of 3 *C. inconspicua* isolates were derived from bloodstream. Isolates were identified using CHROMagar Candida and API ID32C; species identify of *C. parapsilosis, C. orthopsilosis, C. metapsilosis* and *C. inconspicua* was confirmed by molecular biological methods. All clinical isolates were first isolate (1 patient per 1 isolate).

In the study of CAS 15 isolates each of *C. albicans, C. glabrata, C. tropicalis* and *C. parapsilosis* and 4 isolates each of *C. dubliniensis, C. orthopsilosis* and *C. metapsilosis* were screened for paradoxical growth using the CLSI broth microdilution and the time-kill methodology in RPMI-1640. Each of these strains, 2-5 isolates were used for further experiments. Identification of isolates were carried out the previously explained way; species identify of *C. parapsilosis, C. orthopsilosis, C. metapsilosis* and *C. dubliniensis* was confirmed by molecular biological methods.

C. metapsilosis and *C. orthopsilosis* isolates were made available by Tavanti and colleagues.

Determination of susceptibility using microdilution methods

The broth microdilution method was executed as recommended by the CLSI M27-A3 reference document. Each investigation was executed at least two times.

For preparing the fungal suspensions 24h cultures were used. The density of the suspensions were 0,5 McFarland and they were diluted in 0,9%saline. RPMI-

1640 was used for the dilution of the inoculum. Each ELISA plate contained a drug-free control and a medium control.

The drugs were dissolved in sterile distilled water; final concentration ranged between 0.015– 8 mg/l in RPMI-1640. In case of MICA using RPMI-1640 plus 50% human serum medium the final contrentration ranged between 0,06-32 mg/l. As the mean attainable concentration of NIK was found not to be higher than 8 mg/l in humans, the highest NIK concentration tested was 8 mg/l both in microdilution and time-kill tests.

Plates were incubated at 35°C for 24h in case of CAS and MICA and for 48h in case of NIK. After incubation of the microdilution plates the contents of each well were homogenized by pipetting and read visually based on the prominent inhibition criteria, i.e. the endpoint was the lowest concentration of the drug that produced prominent (50%) decrease in turbidity compared to the drug-free control.

Investigation of antifungal effect using time-kill methods

In time-kill experiments the method described by Klepser and colleagues was used. All tests were repeated at least twice.

The final concentration range in case of MICA 0,5-16x MIC in RPMI-1640 and in RPMI-1640 supplemented with 50% human serum. The highest concentration of the drug was 64 mg/l.

In case of CAS and NIK the final concentration range 0,125-16 mg/l and 0,125-8 mg/l in RPMI-1640, respectively. In case of the combination of the two drugs isolates were tested at all (0,125-16 mg/l) CAS concentration, NIK concetrations were selected based on the NIK MIC of the isolates to be tested. Strains with ≥8

mg/l NIK MICs were tested only at 8 mg/l NIK, while for other isolates, NIK concetrations ranged between 1 and 8 mg/l.

Two-two isolates of *C. albicans* (10920 and 17433), *C. tropicalis* (555 and 375), *C. glabrata* (6605 and 14545*), C. parapsilosis* (9150 and CP117), *C. orthopsilosis* CP85, and *C. metapsilosis* CP5 were further studied in RPMI-1640 plus human serum. In killing studies, 50–50% RPMI-1640 and human serum were used with CAS concentrations of 32, 16, 1, and 0.12 mg/ml. Finally, the effect of 8 mg/l NIK on the tested CAS concentrations (16, 1, and 0.12 mg/l) was also examined in RPMI-1640 plus serum.

The starting inoculum was 10^5 CFU/ml (Colony Forming Unit/ml). Test tubes were incubated with agitation in the dark at 35°C. At predetermined time points (0, 4, 8, 12, 24, 48h) samples (100µl) were removed and serially diluted 10-fold in sterile saline.

4x30 µl aliquots were subsequently plated onto Sabouraud dextrose agar. The plates were let to dry at room temperature for 15-20 minutes. Colony counts were determined after incubation of the plates at 35°C for 48h. Time-kill curves were prepared the knowledge of the dilution data.

The drug was defined fungicidal if there was a 99,9% (3-$\log_{10}$) reduction in viable CFU/ml of the starting inoculum. If the reduction of the starting inoculum was lower than this, the efficacy of the drug was defined as fungistatic.

Paradoxical growth was defined in time-kill tests, as fungicidal activity (99.9% reduction of the starting inoculum) observed at at least two supra- MIC concentrations, but lack of fungicidal effect at higher concentrations.

In the combination studies, synergy and antagonism were defined, respectively, as a >100-fold increase or decrease in killing compared with the killing of the

most active single agent. If the change was less than 100-fold, the interaction was considered indifferent.

RESULTS

Determination of micafungin susceptibility by standard broth microdilution method

In RPMI-1640, all tested *C. albicans, C. tropicalis, C. glabrata, C. krusei* and *C. parapsilosis* isolates were susceptible according to the revised species-specific CLSI breakpoints. All *Candida* species grew well in RPMI-1640 supplemented with 50% human serum, the ratios of MICs were from 4 to 128 higher as compared to those in RPMI-1640. Four-fold growth was observed in the case of two *C. metapsilosis* isolates, whereas the highest value (128x) was merely in case of one *C. inconspicua*. The 64-fold growth occurred in most case. (11 of 29 isolates)

The results of time-kill investigation in case of micafungin

In RPMI-1640, micafungin showed fungistatic activity against all *C. albicans* and C. *tropicalis* isolates, at even 16x MIC. Against all other species, micafungin showed fungicidal activity at ≥2-8x MIC micafungin concentrations after 48 h.

Addition of serum to the test medium diminished micafungin activity for all isolates. The drug was fungistatic in the case of *C. albicans, C. tropicalis, C. parapsilosis, C. metapsilosis* and *C. orthopsilosis*, whereas the fungicidal activity occurred on notable higher concentrations against all other species compared with RPMI-1640.

In presence of human serum the MICA proved fungistatic at 1-2x MIC values against all *C. albicans* isolates, whereas ranges of colony-forming unit reduced at the highest drug concentration between –log 1,54 and –log 2,78 CFU/ml after 48 h.

MICA was fungistatic at 1 mg/l (1x MIC) against all *C. tropicalis* and C. *glabrata* isolates when tested in RPMI-1640 supplemented with 50 % human serum. The drug produces fungistatic effect the highest concentration against *C. tropicalis*, whereas in the case of *C. glabrata* proved fungicide at ≥2x MIC after 24 h.

In the case of *C. krusei* MICA was fungistatic after 24 h at 1x MIC value, but this effect was break off the next 24 h. Micafungin was fungistatic and fungicide at 16 mg/l concentration, whereas all isolates were killed at 32 mg/l after 48h.

Micafungin possessed fungistatic activity against C. inconspicua isolates at 1-2 mg/l. Fungicidal activity was observed on the higher concentrations (≥2-4x MIC) after 12 h.

MICA with high concentration possesses potent fungistatic activity in the case of *C. metapsilosis*. Contrarily, MICA was fungistatic at 8 mg/l against *C. parapsilosis* and *C. orthopsilosis* ATCC strains, whereas low fungistatic effect was observed on 32-64 mg/l at clinical isolates.

The MICs values of caspofungin and nikkomycin Z against *Candida* species

The MIC values of CAS varied between 0,015-0,06 mg/l in the case of *C. albicans, C. dubliniensis, C. tropicalis, C. glabrata*. Higher values were got on *C. orthopsilosis* (0,12 mg/l) and *C. metapsilosis* (0,25 mg/l) isolates, whereas between 0,5-1 mg/l values for *C. parapsilosis* were observed, but this value remained within revised susceptibility breakpoint.

Significant reduction was experienced neither *C. tropicalis* nor *C. glabrata* isolates compared to control against NIK. In case of NIK the other species MICs values varied between 1-8 mg/l except the *C. albicans* 17433 where inhibition was observed by 0,25 mg/l yet.

Antifungal activity of caspofungin in time-kill studies

C. albicans isolates can show paradoxical growth on high drug concentration. This phenomenon occurs at 8-16 mg/l concentrations in case of 4780 17433 isolates, whereas at other three strains (10920, 19627, 19888) emerged between 4-16 mg/l. At lower concentration (0,12-2 mg/l) the drug was fungicide.

In the case of *C. tropicalis* isolates the paradoxical growth noticeable as well. On *C. tropicalis* 3404 and 375 isolates at 8-16 mg/l, whilst at the other three strains (555, 5093, 8640) at 16 mg/l concentration.

CAS was fungistatic three strains of *C. tropicalis* isolates examined (2712, 14545, 27510) after 48 h, whilst the medicine was fungicide against 69 and 6605 strains, although paradoxical growth was observed at 8-16 mg/l after 24 h.

C. parapsilosis 9150 and CP117 isolates showed paradoxical growth in presence of 8-16 mg/l CAS concentration. The phenomenon cannot be observed in case of other two isolates examined (CP120, CP121) and CAS was fungistatic on all drug concentrations.

Fungistatic effect was noticed against *C. dubliniensis* isolates at all CAS concentrations. Fungicidal activity was observed in the case of *C. metapsilosis* CP5 at ≥1 mg/l and CP86 at ≥8 mg/l, respectively.

Paradoxical growth was showed the *C. orthopsilosis* CP85 isolate by 8-16 CAS concentrations, whereas the drug was fungicide at 16 mg/l against CP125.

Antifungal activity of nikkomycin Z in time-kill studies

NIK showed fungistatic effect against those isolates where the MICs values varied between 0,25-4 mg/l. Growth curves for all tested isolates (*C. albicans* 10920, *C. parapsilosis* 9150, *C. orthopsilosis* CP85 and all *C. tropicalis* and *C. glabrata* strains) with NIK MICs ≥8 mg/l were similar to the controls.

The results of time-kill studies in the case of caspofungin + nikkomycin Z combination

Antagonism between CAS and NIK was never observed. Synergy was experienced in case of *C. albicans* 10920, *C. tropicalis* 555, *C. glabrata* 69 and 6605 as well as *C. parapsilosis* 9150 and CP121, which was observed at relatively high CAS concentration (≥4 mg/l) and this phenomenon appeared at sub-MIC NIK concentrations. Remaining isolates as well as other species always showed indifferent effect, which coupled with in the most case fungicidal activity, excepting the *C. dubliniensis* isolates and *C. glabrata* strains, which cannot be show synergistic effect. Paradoxical growth was always eliminated by NIK.

Fungicidal effect was noticed at each *Candida* isolates at all drug concentrations after 48 h (in the case of the CAS + NIK combination showed synergistic effect) nevertheless the ranges of colony-forming units were not reduced in monotherapy by NIK. The paradoxical growth was eliminated always by combination, although fungistatic effect was showed at *C. tropicalis* 555 isolate at 8 and 12 h by two highest concentrations, whilst this phenomenon was marked at 24h by 16 mg/l CAS + 8 mg/l NIK drug concentration. The lower doses proved fungicide.

In case of *C. parapsilosis* CP117 (it did not show synergistic effect) observed fungicidal activity just at 4-8-16 mg/l CAS + 4 mg/l NIK. The drug was fungistatic at lower concentrations after 48h, whereas the 0,12mg/l CAS + 4 mg/l NIK were similar to the controls.

Results of time-kill studies in RPMI-1640 medium supplemented with 50% human serum

CAS MIC values in RPMI-1640 medium supplemented with 50% human serum for *C. albicans, C. dubliniensis* and *C. tropicalis* were 0,12 mg/l, which means 2-8-fold increase compared to RPMI-1640. *C. glabrata* (MIC= 0,5 mg/l), *C. parapsilosis* (MIC= 8 mg/l), and *C. orthopsilosis* (MIC= 1mg/l) showed 8-16-fold rising, whilst the MICs values of all *C. metapsilosis* isolates increased 4-fold (1 mg/l) in presence of human serum.

None of strains occurred paradoxical growth in presence of 50% human serum. In case of all strains examined the 0,12 mg/l drug concentration was ineffective so fungi showed powerful growth. The fungi growth was not inhibited by 1 mg/l concentration at *C. parapsilosis sensu lato*, whilst in case of *C. parapsilosis* 9150 the highest concentration was inefficient. Fungicidal activity was observed *C. albicans, C. tropicalis* and *C. glabrata* 6605 by 16 mg/l drug concentration.

On the basis of the time-kill curves of *C. albicans* 17433 and *C. glabrata* 6605 isolates beside the only NIK, the 8 mg/l NIK combined with the lowest CAS doses proved ineffective after 48h. The combination of 1 and 16 mg/l CAS + 8 mg/l NIK evolved fungicidal activity after 48 h.

The efficacy cannot be observed two monotherapeutic doses by 8 mg/l NIK and 0,12 mg/l CAS in case of *C. tropicalis* 555. The other drug concentrations predominated heavy fungistatic (0,12 mg/l CAS + 8 mg/l NIK) and fungicide (1 and 16 mg/l CAS + 8 mg/l NIK) activity after 48 h.

The time-kill curves of *C. parapsilosis* 9150 showed that merely the combination with the highest concentration (16 mg/l CAS + 8 mg/l NIK) reduced the fungi colonies after 48h. Low fungistatic effect was observed similar to 16 mg/l CAS. The other drug concentrations were not effective.

Synergy between CAS and NIK was observed *C. tropicalis* 555 and *C. albicans* 17433 by 0,12 mg/l CAS, whereas 1 and 0,12 mg/l concentrations showed synergistic activity in case of *C. albicans* 10920. Remaining isolates always showed indifferent considering the two drugs combination. In case of *C. parapsilosis* where CAS proved ineffective, considerable colony count reduction cannot be produce by CAS+NIK combination.

DISCUSSION

Invasive candidiasis is a common public health care issue. Despite the fact that antifungal therapeutic approaches have been constantly developed recently, its prevalence and mortality has not changed over the last decade. Prevalence of yeast infections in immunodeficient patients or in patients hospitalised with serious underlying diseases has been reported to increase since the beginning of the 1980s.

Introduction of echinocandins that inhibit cell wall synthesis into clinical practice has been a major breakthrough owing to their favourable pharmacokinetic and pharmacodynamic characteristics. The MIC value of *Candida* species for echinocandins is generally low as determined during *in vitro* experiments, while there are some species, i.e. *C. parapsilosis* and *C. guilliermondii*, whose MIC values are ≥2 mg/l.

Besides that, clinical isolates of *C. albicans*, *C. glabrata*, *C. krusei* and *C. tropicalis* with high (>2 mg/l) MIC values have also been described, which might be a consequence of amino acid substitutions in HS1 and HS2 regions of the Fks1 subunit of the glucan synthase enzyme.

The previously mentioned resistance brought about by amino acid changes is generally considered to be different from yeast growth in the presence of high concentrations of echinocandin, which is sometimes higher than the actual MIC value. This paradox is a stress reaction brought about by the fact that β-1,3-glucan is replaced by chitin in the cell wall of surviving cells. In fact, high concentrations of CAS reduced the amount of β-1,3-glucan in the cell wall of *C. albicans* by 81%, while resulted in a 6-fold elevation in chitin levels.

Echinocandins have a high protein binding affinity in the presence of human serum; therefore, to establish a milieu that best mimics *in vivo* conditions,

medium supplemented with 50% human serum was applied during *in vitro* experiments. Although the nature of the effect of human serum on echinocandins is not clear yet, it is not likely that paradoxical growth might be involved; not even in case of a single, high-dose echinocandin therapy as human serum and other serum components halt paradoxical growth at frequently used drug concentrations.

In spite of these data, echinocandin resistance brought about by enhanced chitin synthesis has recently been described in clinical practice. Following empirical treatment of a 51-year-old patient with severe liver failure with CAS (70 mg loading dose, followed by 35 mg daily dose), a rare *C. albicans* variant with FKS mutation (Ser residue at position 645 was changed to Pro) as well as four-fold chitin content compared to wild type was isolated from their blood sample. Apparently, the risk of compensatory reactions (i.e., elevated chitin levels) and subsequent inefficiency of drug therapy should be considered during *in vivo* applications.

In case of MICA, MIC values were found to be distributed within the modified sensitivity thresholds in RPMI-1640 medium, and a 4- to 128-fold increase was detected in the presence of human serum.

MICA, in the presence of 50% human serum, proved to be effective at ≤4 mg/l concentration against all *C. albicans, C. tropicalis, C. glabrata* and *C. inconspicua* isolates investigated in this study.

Application of the clinically accepted daily dose of 100 mg/kg MICA is probably insufficient to reach the abovementioned concentration in blood serum and tissues, but the elevated daily dose of 150 mg/kg may provide effective MICA levels in blood and other tissues.

During *in vitro* experiments, *C. krusei* was found to be less sensitive to MICA as fungistatic effects were only reported at ≥16 mg/l concentration, which implicates that the daily dose of 150 mg/kg is not sufficient for the treatment of candidiasis caused by *C. krusei* infection.

Current and previous results also imply that echinocandins are not considered as first line antifungal drugs for the treatment of invasive candidiasis brought about by *C. parapsilosis*; however, the elevated daily dose may cause a sufficiently high peak serum drug concentration (C_{max}).

Furthermore, the decreased virulence of this particular species in comparison with other *Candida* species may also be an important factor in the observed efficiency of echinocandins. In contrast, a close correlation between treatment with echinocandins and relapse of candidaemia in case of *C. parapsilosis* infection has been reported.

C. orthopsilosis exhibited similar characteristics to *C. parapsilosis*; in contrast, fungistatic effects could be observed in case of *C. metapsilosis* isolates at concentrations as low as 1–8 mg/l, despite the fact that all three members of the *C. parapsilosis sensu lato* group possess the same amino acid substitution in the FKS subunit.

During our studies with CAS, time–kill experiments proved to be a useful tool to investigate paradoxical growth in the presence of NIK and human serum. By using RPMI-1640 as growth medium, paradoxical growth of *C. albicans, C. tropicalis*, two *C. parapsilosis* and one *C. orthopsilosis* isolates were detected at drug concentrations (with the exception of the three *C. tropicalis* isolates) that are also detectable in the human serum (12.1 mg/l) in case of normal dosage strategies.

When we performed experiments aimed at determining the MIC value for NIK, it did not cause prominent inhibition for *C. tropicalis* and *C. glabrata* isolates even at the highest (8 mg/l) concentration; however, MIC values were distributed over a wide concentration range (0.25–8 mg/l) for other species investigated.

Application of CAS + NIK in combination totally abolished paradoxical growth, which could often be observed at NIK concentrations slightly below its MIC value. Furthermore, NIK enhanced the activity of CAS in case of isolates that did not exhibit paradoxical growth.

Paradoxical growth halted in medium supplemented with 50% human serum; nonetheless, it must be noted that the efficiency of CAS considerably decreased for all *Candida* species investigated. The observed change in the activity of the drug can probably be accounted for its 96.5% protein binding affinity.

In RPMI-1640 medium supplemented with serum, strong fungistatic or fungicide effects were observed against *C. albicans, C. glabrata, C. tropicalis* and *C. dubliniensis* species at a concentration as low as 1 mg/l of CAS. For *C. orthopsilosis* and *C. metapsilosis* isolates, only 16–32 mg/l of CAS proved to be effective, while in case of *C. parapsilosis*, it only exhibited a weak fungistatic effect. The CAS + NIK combination only exerted synergistic effects against *C. albicans* 10920 and 17433, as well as *C. tropicalis* 555 isolates, compared to lower CAS concentrations. In case of other isolates, application of the combination treatment in RPMI-1640 medium supplemented with 50% serum did not result in a significant change in cell numbers.

Although sensitivity assays performed in media supplemented with serum better mimic processes of the human body; moreover, it is easier to distinguish

between FKS mutants and wild type cells; still, only a few MIC values have been determined by using human or bovine serum so far.

As it is evident from the MIC values, the sensitivity of fungi directly depends on the presence or absence of serum; however, the amount of serum to be used during experiments is still not clear.

The outcome of invasive fungal infections is predominantly determined by the virulence of the pathogen, the nature of the underlying disease, and the number of white blood cells of the patient. *In vitro* pharmacodynamical experiments play an important role in growth inhibition or elimination of the pathogen with the given MIC value by a particular antifungal agent. By knowing the pharmacokinetics of a particular antifungal drug (based on human or experimental animal models), it will be possible to improve planning of drug therapies, thereby contributing to ever more successful treatment regimes of invasive fungal infections.

In spite of all these efforts, there may be large differences between the treatment of a pathogen that proved to be sensitive *in vitro* and the outcome of the infection. In fact, the high number of unsuccessful therapies in the last couple of years has led to the need to introduce species-specific threshold values.

Our results gained by using human serum may further improve the chances of these patients to recover from their disease since in case of the two echinocandins investigated, antifungal effects were found to be decreased in the presence of human serum. This inevitably implies that higher echinocandin doses would probably result in improved therapeutic effects; however, this strategy requires more investment. Our results concerning the presence of serum during antifungal therapies seem to be important for a possible revision of current threshold values.

SUMMARY

During the tests we studied the *in vitro* activity of two echinocandins (micafungin and caspofungin) against the clinically relevant *Candida* species in RPMI-1640 and in 50% human serum supplemented RPMI-1640 medium with help of micro- and macrodilution methods. The effects of nikkomycin Z and human serum were investigated in case of using caspofungin against those isolates which showed or not paradoxical growth.

Reduced micafungin and caspofungin efficacy were observed in presence of 50% human serum in terms of results against the clinically relevant *Candida* species. In the case of micafungin the MIC values closed or raised above the clinical breakpoints in presence of serum. In base of our studies fungi which can be inhibited by high concentration of micafungin may required higher daily dosage therapy such as *C. parapsilosis*, *C. orthopsilosis* or *C. krusei.*

During our studies of caspofungin we convinced first paradoxical growth is eliminated by nikkomycin Z and 50% human serum against several *Candida* species using time-kill curves. However the paradoxical growth seems to be only an *in vitro* phenomenon, our results of nikkomycin Z on the one hand may help a clearer understanding the echinocandin induced stress adaptation pathways. On the other hand nikkomycin Z is currently in clinical trial for its antifungal activity, the combination of NIK with other drugs may success in the treatment of mycoses. The combination of caspofungin with nikkomycin Z showed synergistic effect against several *Candida* species in our investigation.

Our results gained by using human serum may further improve the chances of these patients to recover from their disease since in case of the two echinocandins investigated, antifungal effects were found to be decreased in the presence of serum. Our results should have to confirm by animal attempts.

Register Number: DEENKÉTK/267/2012.
Item Number:
Subject: Ph.D. List of Publications

Candidate: Richárd Földi

Neptun ID: A6IM32

Doctoral School: Doctoral School of Pharmaceutical Sciences

List of publications related to the dissertation

1. **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
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2. Szilágyi, J., **Földi, R.**, Sedigh, B., Kardos, G., Majoros, L.: Effect of nikkomycin Z and 50% human serum on the killing activity of high-concentration caspofungin against Candida species using time-kill methodology.
J. Chemother. 24 (1), 18-25, 2012.
DOI: http://dx.doi.org/10.1179/1120009X12Z.0000000005
IF:1.084 (2011)

List of other publications

3. **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. Epub ahead of print (2012)
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IF:1.654 (2011)

4. 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.
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J. Chemother. 23 (2), 107-109, 2011.
IF:1.084

Total IF: 8.166

Total IF (publications related to the dissertation): 3.612

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.

04 September, 2012

DEBRECENI EGYETEM KENÉZY ÉLETTUDOMÁNYI KÖNYVTÁRA EGYETEMI ÉS NEMZETI KÖNYVTÁR

Posters and presentations at conferences

Varga I, Sóczó G, Kardos G, Majoros L., **Foldi R**. A Caspofungin ölő hatásának összehasonlító vizsgálata RPMI-1640 és antibiotikum medium 3 közegben Candida dubliniensis törzsek esetén. DEOEC TDK Konferencia. 2008. Debrecen (oral presentation)

J. Szilágyi, **R. Foldi**, S. Bayegan, G. Kardos, Á. Kemény-Beke, R. Gesztelyi, B. Juhász, A. Adnan, L. Majoros. Evaluation of the Etest method for determining micafungin MICs for 360 clinical isolates of ten *Candida* species. MMT konferencia. 2010. Keszthely (oral presentation)

Richárd Földi, Judit Szilágyi, Gábor Kardos, Réka Berényi, Renátó Kovács, László Majoros. Effect of 50% human serum on the killing activity of micafungin against eight Candida species using time-kill methodology. 2nd Joint Workshop of ÖGMM, ÖGACH, ÖGIT & ÖGHMP. 2011. Vienna (poster presentation)

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 Joint Workshop of ÖGMM, ÖGACH, ÖGIT & ÖGHMP. 2011. Vienna (poster presentation)

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The work of R. Földi was supported by the TÁMOP 4.2.1./B-09/I/KONV-2010-0007 project.