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

Study of the Pharmacodynamics of Micafungin against Clinically Important *Candida* Species

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Introduction

In the last thirty years, the importance of invasive infections caused by various fungi has increased. This is due in part to the increasing frequency of invasive and immunosuppressive interventions and to the growing number of patients with serious underlying diseases who are more susceptible to fungal infections. Worldwide, it is estimated that more than 1 billion fungal infections occur each year, but only a fraction of these cases are acute invasive or severe chronic (4.5-5 million). Yet the estimated global death toll of more than 1.6 million per year is a telling figure and an indication of the importance of these typically opportunistic eukaryotic pathogens for human health. If we were to compare this mortality figure with other infectious diseases, we would find that it is equivalent to the estimated mortality caused by tuberculosis and about three times that caused by malaria. It is important to note, however, that this high mortality is spread over a number of diseases caused by several different fungal species and even genera. The human pathogens considered to be the most important are the various *Candida*, *Aspergillus* and *Cryptococcus* species.

The number of cases of invasive candidiasis (IC) globally is estimated at ~700,000 per year, of which 23,000-25,000 occur in the United States. The associated health care expenditure is estimated by Benedict et al. to be USD 1.4 billion, which is roughly equivalent to the amount of additional health care costs due to AMR (antimicrobial resistance). In Hungary, it is estimated that around 1100 cases of candidaemia and a few hundred other invasive, e.g. post-operative peritoneal candidiasis, occur annually.

For the treatment of invasive Candida infections, we have a relatively small number of active substances, which can be divided into three groups: polyenes, azoles and echinocandins. The introduction of the latter group in the 2000s had high hopes as safer and more effective antifungal agents with outstanding *in vitro* activity, but the promises associated with them have only been partially fulfilled. Although indeed a very safe class of agents and clinically somewhat more effective than previous antifungal drugs in invasive *Candida* infections, the mortality associated with candidaemia remains unacceptably high, reaching up to 70% in critically ill patients for some species. An increasingly frequent explanation for the persistently high mortality rate is the inappropriate, too low daily dose of echinocandins and the consequent failure to reach the target PK/PD concentration, both in the bloodstream and at other infected foci. One reason for this may be that the dosing regimen was based on data from healthy volunteers, but it is now known that pharmacokinetic parameters for different echinocandin regimens vary significantly among critically ill patients, generally in the direction of lower

exposure. A further problem is that the dosing of echinocandins has not changed since their introduction. At the time when the currently used doses were established, a pharmacometric approach that takes into account the slightly different echinocandin sensitivities of different *Candida* species and the pharmacokinetic variability in different patient populations and anatomical landscapes was not common, today highlighting possible inadequacies in dosing.

Compared to bloodstream infections caused by *Candida*, infections in other anatomical areas have been less in the focus of science and only in the last 7-8 years, for example, pharmacokinetic data on MCF peritoneal and pleural fluid have started to emerge, allowing the pharmacodynamic activity of relevant drug concentrations in these areas to be investigated. Interestingly, for *C. auris*, described in 2009, there is still a paucity of pharmacodynamic data, for example, only sensitivity data for MCF are available, although the knowledge of clinically available concentrations allows to investigate whether the current doses are sufficient to eradicate the pathogens from the anatomical site.

In our studies, we investigated whether the pharmacodynamic activity of MCF, the second echinocandin to be introduced into the clinic, is adequate based on the available pharmacokinetic data in inflammatory diseases of the pleura and peritoneum caused by different *Candida* species, and whether MCF has activity at clinically relevant concentrations in the currently prevalent *C. auris* isolates belonging to different clades at the concentrations available in blood plasma. In our experiments, we considered the relevant protein concentrations at each anatomical site, thus approximating the pharmacodynamic conditions experienced in vivo. We hope that our results may contribute to a more efficient clinical application of MCF.

Aim of our studies

Our experiments have investigated several aspects of the *in vitro* activity of micafungin against clinically relevant *Candida* species, which we hope will contribute to a more effective clinical use of micafungin and other echinocandin.

Our research covered the following narrow topics:

- *In vitro* activity of micafungin against *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei* and *Candida kefyr* isolates from pleural and peritoneal infections at micafungin concentrations (0.25-2 mg/L) in pleural and peritoneal fluids in standard RPMI-1640 and RPMI-1640 medium supplemented with 10% and 30% human serum using time-kill method

- *In vitro* activity testing of micafungin and two clinically marketed (caspofungin, anidulafungin) and clinically investigational rezafungin against *Candida auris* isolates by time-kill method at clinically relevant concentrations (0.25-32 mg/L)

Material and methods

Isolates tested in experiments

In pleural and peritoneal pharmacodynamic studies, clinical isolates (n=3 per species) from the anatomical sites mentioned (*C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. krusei* and *C. kefyr*) and reference strains (*C. albicans* ATCC 10231, *C. glabrata* ATCC 90030, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258) were tested. All isolates, except the reference strains and *C. auris* isolates, were identified at the Department of Medical Microbiology, University of Debrecen between 2010 and 2017 using MALDI-ToF (Matrix Assisted Laser Desorption/Ionization Time of Flight) mass spectrometry (Bruker Daltonics, Bremen, Germany) and API-32 IDC (BioMérieux, Marcy l'Etoile, France) biochemical automata. Isolates stored at -70 C° were re-identified by MALDI-ToF using the latest available database before starting the experiments. The *C. auris* strains were obtained from the NCPF (National Collection of Pathogenic Fungi, Bristol, UK) strain collection and was kindly provided by Dr. Andrew Borman**.**

The antifungal agents used in the experiments and the human serum

For the MCF activity tests against pleural and peritoneal *Candida* isolates, MCF was purchased from AbMole (AbMole Europe, Brussels, Belgium), while for the experiments involving *C. auris*, ANF, CSF and MCF were purchased from MolCan (MolCan, Toronto, Canada), and RZF was provided by Cidara (Cidara Therapeutics, San Diego, USA). Human serum (pooled male blood group AB) and RPMI-1640 (2% glucose, L-glutamine free) medium were supplied by Sigma-Aldrich (Sigma, Budapest, Hungary).

Susceptibility testing of the isolates to micafungin and to other echinocandins

To investigate the pleural and peritoneal pharmacodynamics of MCF, the sensitivity of the isolates to MCF was determined by macrodilution in each culture medium (RPMI-1640 and RPMI-1640 supplemented with 10 and 30% human serum) according to the CLSI-M27-A3 protocol in the range 0.015-8 mg/L, as well as *C. auris* isolates to MCF and its comparators, the two media tested being standard RPMI-1640 and RPMI-1640 supplemented with 50% human serum for the latter. MIC values were read visually after 24 h, and the sensitivity of the isolates to echinocandin was defined based on the internationally accepted partial (50%) inhibition criteria.

In vitro pharmacodynamics of micafungin against pleural and peritoneal *Candida* isolates

Twenty-four hours before the start of the experiments, the isolates to be tested were cultured as described above. At the start of the experiment, a suspension of 0.5 McF in physiological saline was prepared from each isolate and 0.5 ml of this suspension was added to media containing different concentrations of MCF (normal RPMI-1640 and RPMI-1640 supplemented with 10% or 30% human serum). The final volume was 5 ml and the MCF concentrations tested were 0.25 mg/l, 0.5 mg/l, 1 mg/l and 2 mg/l. The different serum concentrations were used to simulate the protein environment in pleural and peritoneal fluid in cases of normal and inflammatory conditions, respectively. In the case of *C. parapsilosis*, due to the higher echinocandin MIC values typical for this species, the concentration of 0.25 mg/L was not tested in any of the media. The assembled tubes were incubated at 37 °C for 24 h and 100 µL samples were taken at the indicated time points (0, 4, 8, 12 and 24 h) after homogenisation. A tenfold serial dilution was prepared from the samples and 4x30 µl of the respective dilutions were inoculated onto SDA (Sabouraud Dextrose Agar). Each medium was incubated at 37 °C for 48 h and the number of fungal colonies grown was counted.

Comparative study of the *in vitro* pharmacodynamics of micafungin against *C. auris* isolates belonging to different clades

The same methodology was used to study the *in vitro* pharmacodynamics of MCF and its comparators (RZF, ANF, CSF) against *C. auris* isolates as was used to study the activity of MCF against pleural and peritoneal isolates, but the echinocandin concentrations and culture media differed slightly. The two culture media used were normal RPMI-1640 and RPMI-1640 supplemented with 50% human serum, the latter simulating the protein concentration in the bloodstream. The concentrations of echinocandin (MCF, CSF, ANF, RZF) were 0.25, 0.5, 1, 2, 4, 8, 16, 32 mg/L in normal RPMI-1640 and 1, 2, 4, 8, 16, 32 mg/L in serum-supplemented RPMI-1640.

Study of aggregation *C. auris* isolates exposed to echinocandins

To investigate the morphological changes induced by echinocandins in the two media studied, phase-contrast microscopy was performed at echinocandin concentrations of 1 and 16 mg/L for each clade using 2 isolates (12, 27, 15, 12372, 204, 2, 13108 and I-172) in both media after 24 h incubation with MCF, RZF, ANF and CSF. The total volume examined was 10 µL.

Images were acquired on a Zeiss Axioskop 2 mot microscope using an AxioCam HRc camera. Images were analysed using Zeiss Axiovison 4.8.2 software.

Statistical analysis

In the experiments, the number of colonies grown at each time point was used to calculate a so-called killing rate, using the equation Nt=N0*e-kt, where Nt is the number of cells alive at time t, N0 is the initial number of living cells, t is the elapsed time, e is Euler's number and k is the killing rate. The equation shows that k is positive if there are fewer living cells in the system at a given time compared to the initial number of cells, and negative if there is multiplication. The 50, 90, 99 and 99,9% decrease in the number of live cells compared to the initial germination rate was calculated as follows: T50=0,30103, T90=1/k, T99=2/k, T99,9=3/k. The goodness of fit was checked with the r2 test. Comparison of killing kinetics for each isolate and echinocandin concentration was performed by one-tailed analysis of variance with Tukey's post-hoc test for both studies comparing calculated killing rate (k) values. The comparison of the effect of the same echinocandin concentrations in different culture media was performed using Welch's T-test. Results were considered significantly different when $p<0.05$. Statistical analysis was performed using GraphPad Prism 6.05 software (GraphPad Software, La Jolla California USA).

Results

In vitro activity of micafungin against pleural and peritoneal *Candida* isolates

Determination of the micafungin sensitivity of the isolates used in the experiments

In the MCF sensitivity determination, MIC values in standard RPMI-1640 medium for QC (quality control) strains were all within the internationally accepted range (CLSI 2008). The clinical strains were found to be sensitive to MCF based on currently accepted breakpoints. For *C. kefyr* isolates, there are no currently accepted species-specific breakpoints, but the MIC of the isolates was lower than the reported ECOFF (Epidemiological Cut-Off Value) for the species ($\leq$ 0.12 mg/L).

In the presence of serum, the MIC of the *Candida* species tested was significantly increased. In the culture medium containing 10% serum, a 2-64-fold increase was observed compared to the normal RPMI-1640. Interestingly, in the presence of 30% human serum, the MCF MIC values were also only 8-64-fold higher than those observed in serum-free culture medium. And between the two serum-containing media, only a 2-4-fold increase was detected. MIC values for *C. albicans* and *C. glabrata* isolates were less than 2 mg/L in both serum-free media.

Time required for a 50% reduction in CFU for different micafungin concentrations in each media tested against different Candida isolates in time-kill experiments and the killing-rate values for the clinical isolates of Candida species tested

In the experiments, the activity of MCF was at most fungistatic (no 99.9% CFU (Colony Forming Unit) reduction) against the tested *C. albicans*, *C. tropicalis* and *C. parapsilosis* isolates in all tested media and concentrations, therefore only the T50 values are presented (Table 2). In the serum-free RPMI-1640 medium, a 50% reduction in CFU for *C. albicans* isolates was achieved in a short time. In the presence of 10% human serum, MCF activity was significantly reduced against all *C. albicans* isolates except isolate 20261. In culture media containing 30% serum, only MCF concentrations of 2 mg/L were effective against ATCC 10231, in contrast to clinical isolates, where a CFU reduction of at least 50% was observed in the presence of 30% serum over the concentration range of 0.5-2 mg/L. Activity against clinical *C. albicans* isolates was concentration independent over this concentration range.

Against the reference strain of *C. glabrata* (ATCC 90030), MCF showed similarly good activity in all media and concentrations tested. The activity was both medium and concentration independent. In contrast, against clinical isolates, MCF activity was significantly affected by

the presence of serum. A concentration of 0.25 mg/L MCF proved to be ineffective in the presence of 10% serum, and in the presence of 30% serum, even a concentration of 0.5 mg/L did not reduce the number of viable *C. glabrata* cells in case of clinical isolates. The MCF concentration of 1 mg/L, although causing a reduction in the number of cells in the presence of 30% serum, was significantly less active than that observed in RPMI-1640.

MCF showed fungistatic activity against both the reference strain and clinical *C. tropicalis* isolates in all the media and the concentrations tested. Against the reference strain ATCC 750, a reduction of at least 50% in CFU was observed at all MCF concentrations except 0.25 mg/L in the presence of 30% serum, with no significant difference in activity between concentrations and media except for this combination. In contrast, *C. tropicalis* clinical isolates were significantly more resistant to MCF exposure. For isolates 34017 and 3947, 10% serum showed a significant reduction in MCF activity compared to standard RPMI-1640 media, and for 30% serum, only isolate 19739 showed a slight reduction in viable cell numbers at MCF concentrations of 1 and 2 mg/L (k 0.00003-0.01).

In the case of *C. parapsilosis*, CFU reduction was only observed in serum-free culture medium for isolates 17820 and 26977 at MCF concentrations of 2 mg/L. For all other concentrations and media, the kill rate values remained in the negative range.

In serum-free RPMI-1640 medium, all concentrations of MCF showed rapid fungicidal activity against the reference strain of *C. krusei* (ATCC 6258) and clinical isolates. In contrast, activity was significantly reduced even in the presence of 10% serum and fungistatic effects were observed only at concentrations of 1 and 2 mg/L. In the culture medium supplemented with 30% serum, killing-rate values remained negative at all concentrations tested.

MCF in serum-free media also showed rapid fungicidal activity against *C. kefyr* isolates. However, in the presence of 10% serum, MCF was fungicidal against C. kefyr isolate 31709 only at concentrations ≥ 1 mg/L and against isolate 38001 only at 2 mg/L. In the culture medium supplemented with 30% serum, only concentrations of 1 and 2 mg/L caused a reduction in CFU against *C. kefyr* isolates, and only at 2 mg/L was fungicidal activity observed against isolates 31709 and 38001. For isolate 24700, MCF activity was significantly lower than for the other two clinical isolates, with only weak fungistatic activity observed at the 2 mg/L concentration.

The presence of serum did not significantly affect the growth of individual isolates and reference strains, with the exception of *C. glabrata* clinical isolates, which grew significantly slower in the presence of 10% and 30% serum ($p < 0.05$)

The *in vitro* activity of micafungin and comparator echinocandins (caspofungin, anidulafungin, rezafungin) against *C. auris* isolates

Determination of echinocandin sensitivity of C. auris isolates used in the experiments

Species-specific breakpoints for *C. auris* isolates are currently not available, therefore, we categorised the susceptibility of *C. auris* isolates to echinocandins according to the tentative breakpoints (S<4 mg/L ANF, S<2 mg/L CSF, S<4 mg/L MCF, S=susceptible) published by the Centers for Disease Control and Prevention (CDC). For RZF, there are currently no agreed breakpoints at all. Based on the results of the susceptibility testing, all *C. auris* isolates tested were found to be susceptible to the currently used echinocandins (CDC 2021). In the presence of serum, the MIC values of the different echinocandins were increased by a factor of 2 to 24 compared to the normal RPMI-1640. The largest increase was observed for ANF, while the smallest increase was observed for CAS. This may be due to differential binding to serum proteins. A smaller increase was observed for RZF compared to ANF.

Time required for a 50% CFU reduction of the C. auris isolates tested and the observed killing-rates

All the echinocandins tested showed fungistatic activity against the tested isolates (99.9% reduction in CFU was not achieved), irrespective of the *C. auris* clade and culture medium. Interestingly, this fungistatic effect was often only present during the first 8-12 hours of incubation, followed by a significant regrowth.

For the South Asian clade, the mean killing-rate values were concentration-dependent between 0.25 and 32 mg/L for ANF, RZF and MCF in RPMI-1640 medium, but only RZF (4-32 mg/L) and ANF (16-32 mg/L) reduced the number of living cells in case of all isolates. Isolate 27 showed a so-called mini-paradox phenomenon in the presence of RZF, as the killing-rate values obtained in the concentration range 0.25-2 mg/L were higher than those observed at 4-32 mg/L. CSF did not reduce the number of living cells at any concentration, although it was able to slow the multiplication rate somewhat. In the presence of serum, the activity of RZF, ANF and MCF was concentration-dependent between 4 and 32 mg/L, with no regrowth in the presence of serum for these echinocandins and the isolates tested. In serum-supplemented media, CSF showed slightly better activity than in normal RPMI-1640, but only at the 32 mg/L concentration, reducing the number of living cells against all three isolates belonging to the South Asian clade.

Against the reference strain of the East Asian clade (NCPF 13029/CBS 10913), RZF, ANF and CSF showed concentration-dependent killing activity ranging from 0.25-32 mg/L,

with a nearly fungicidal effect (2.6-2.9 log CFU reduction) at the highest concentration in RPMI-1640 medium. MCF, on the other hand, showed the best activity at 0.25-2 mg/L (k values were 0.21-0.25 1/h), with a mini-paradox phenomenon also observed at higher concentrations (k: 0.11-0.14 1/h). The activity of echinocandins against clinical isolates was below that observed for the reference strain, with mean killing-rate values not always positive at concentrations of 0.25-4 mg/L. Only RZF and ANF showed positive k values against both isolates in the concentration range 8-32 mg/L. In the presence of serum, RZF and CSF showed concentration-dependent killing activity against the reference strain between 1 and 32 mg/L, whereas ANF was only able to reduce the number of live cells at concentrations ≥ 2 mg/L and MCF at ≥ 4 mg/L. The killing activity of the echinocandins against the clinical isolates started at the same concentrations as for the reference strain, but interestingly, in an concentration-independent manner.

The South African isolates were found to be exceptionally resistant to echinocandins in RPMI-1640 medium. Although the growth rate was significantly reduced at all echinocandin concentrations tested, no significant CFU reduction was observed even at 256-512 x MIC concentrations. The highest activity (0.4 log CFU reduction) was shown against isolate 185 at ANF concentration of 1 mg/L. Slightly better killing activity was observed in the presence of serum, but only RZF exposure resulted in positive k values against all three isolates over the concentration range of 8-32 mg/L (mean k values 0.07-0.09 1/h).

In the case of the South American clade, invasive isolates from Israel and hospital environmental isolates from Colombia responded differently to echinocandin exposure. In serum-free RPMI-1640 culture medium, the behaviour of the Israeli isolates was very similar to that of the South African isolates, with no killing observed for any of the echinocandin at any of the concentrations tested. In contrast, a CFU decrease was achieved for the Colombian hospital isolates and a significant mini-paradox phenomenon was observed, as all echinocandin showed significantly better killing activity at lower concentrations (mean k values at 32 mg/L echinocandin concentration was 0.0975, while the mean k value at 0.5 mg/L was 0.26). In the presence of serum, RZF showed killing activity against Israeli isolates between 8 and 32 mg/L (mean k values 0.03-0.08 1/h), and ANF and MCF at concentrations of 32 mg/L (MCF k 0.06, ANF k 0.04 1/h). None of the CSF concentrations reduced the number of living fungal cells. RZF, ANF and MCF had a concentration-dependent killing effect against Colombian isolates, whereas the effect of CSF was concentration-independent. It is worth pointing out that RZF was able to reduce the number of living cells against these two isolates at concentrations as low as 1 mg/L.

Overall, CSF showed the weakest activity in the studies, while the activity of the other three echinocandin was broadly similar, with RZF proving to be at least as effective as ANF and MCF against all isolates.

Morphological changes induced by echinocandins in C. auris isolates belonging to different clades

Large aggregates, up to 100 cells in size, were frequently observed in normal RPMI-1640 medium after exposure to echinocandin, irrespective of the *C. auris* clade and isolate tested, as well as the type of echinocandin and its concentration. In the presence of serum, the size of aggregates was significantly smaller than in serum-free medium and their number was also lower. Interestingly, the presence of serum alone, without echinocandin, was able to induce the formation of small aggregates, but their size was smaller than that induced by echinocandin exposure.

Discussion

There is extensive knowledge available about the pharmacokinetic parameters and pharmacodynamic activity of MCF in the bloodstream, whereas much less is known about other invasive *Candida* infections such as those involving the pleural and peritoneal sites. Based on current recommendations, all three currently used groups of antifungals (AMB, triazoles, echinocandins) are suitable for the therapy of such infections. However, due to the frequent primary triazole resistance or reduced sensitivity of potential pathogens (e.g. *C. glabrata*, *C. krusei*) and the significant toxicity to AMB, echinocandins are currently the first choice agents for the therapy of *Candida* infections in these anatomical sites.

Based on our current limited knowledge, MCF appears to be present in both pleural and peritoneal fluid at significantly lower concentrations than in the plasma. Using standard doses of 100 mg, the C_{max} is ≤ 2 mg/L and the $C_{through}$ ≤ 1 mg/L in peritoneal fluid. It is also worth pointing out that, based on population pharmacokinetic simulations, concentrations are below 1 mg/L in a significant proportion of cases. Echinocandins, including MCF, bind to serum proteins to a significant extent, and thus their pharmacodynamic activity may be significantly reduced during infections of these anatomical sites. Protein concentrations in pleural or peritoneal fluid without inflammatory processes range from 20 to 30 g/L, which is lower than the protein concentration in serum, but during infection it can reach protein concentrations of up to 50 mg/L. In our studies, we therefore used RPMI-1640 supplemented with 10% and 30% serum, the former corresponding to physiological and the latter to inflammatory protein concentrations.

Our results showed that even 10% human serum significantly affected the killing activity of MCF against the species tested, with the exception of *C. albicans* clinical isolates. In their case, we observed a significant reduction in living fungal cells at all MCF concentrations tested (0.25-2 mg/L), although the killing efficiency was significantly ($p<0.05$) lower at 0.25 mg/L MCF concentration in 30% serum compared to the 10% serum medium. Based on recent epidemiological data, non-albicans species are the causative agent in more than 35-48% of empyema thoracis cases caused by *Candida* species, and they are responsible for almost half of IAC cases. However, insufficient killing activity was observed against the non-albicans species studied at the clinically relevant MCF concentration of 1 mg/L in 30% serum, which raises the question of the inadequacy of the currently used MCF dosage in the treatment of pleural and peritoneal *Candida* infections caused by non-albicans species. The low MIC values in RPMI-1640 media and the significant killing activity, in contrast to that observed in

the presence of serum, confirm the results that the activity of echinocandins, including MCF, in serum-free media is significantly overestimated and may not be relevant for the activity in vivo, especially against non-albicans species.

A further consequence of the weak killing activity against the species studied is the potential development of echinocandin resistance during therapy. Sub-optimal exposure has been associated with breakthrough infections caused by echinocandin-resistant isolates during echinocandin therapy according to several studies. In this regard, abdominal *Candida* infections are of particular relevance. Although pleural infections caused by *Candida* species are rare clinical manifestations, several cases of echinocandin-resistant *Candida* isolate-associated breakthrough infections in this anatomical site have been described.

The presence of serum proteins also strongly influenced the anti-*C. auris* activity of MCF, but interestingly, it increased it at concentrations of 4 mg/L and above, in contrast to the other non-*albicans* species tested. Significant differences were observed against different clades in RPMI-1640 media. Although the average killing-rate values against the East Asian isolates were significantly lower than those in case of ANF and RZF, the killing activity of MCF in serum was similar to the other two echinocandin mentioned. Against isolates belonging to the South American clade from South Africa and Israel, MCF produced negative killing-rate values similar to the other echinocandins at all concentrations tested in serum-free culture medium, but the presence of serum also enhanced the activity against these isolates. Killing activity was also observed against South Asian and Colombian isolates in RPMI-1640, as well as a mini-paradox phenomenon; in the presence of serum, the latter was abolished and the killing-rate values were similar for isolates from two different geographical locations. It is important to note, however, that the increased activity also proved to have only a weak fungistatic activity, as no fungicidal effect (99.9% reduction in live cell count) was observed against any of the isolates tested. At present, only one other study is available that investigated the *in vitro* pharmacodynamic activity of echinocandins, which only involved ANF and CSF. Dudiuk et al. reported similarly poor static activity in their studies with the two mentioned drugs against South American isolates. It is not yet clear what underlies this weak activity compared to other clinically relevant *Candida* species. It is known that reduced susceptibility to antifungal agents is also common among species phylogenetically related to *C. auris*, but this alone does not provide a satisfactory explanation for the poor activity. It is known and confirmed that some *C. auris* isolates form large aggregates in the absence of negative external conditions, while others form large aggregates in response to cell wall and cell membrane-active antifungal drugs, which may partly explain the poor activity, as aggregated cells are thought to provide protection

against echinocandins and other antifungal agents. However, this is still not a sufficient explanation for the improved activity in serum. Since *C. auris* also has high levels of chitin in the cell wall compared to other *Candida* species, and *Candida* species are known to increase chitin synthesis proportionally in response to echinocandin exposure, it is possible that the low free echinocandin concentrations in the presence of serum proteins are the reason for the improved activity observed. This is somewhat contradicted by the fact that no mini-paradox phenomenon was observed in the presence of serum at the maximum echinocandin concentrations tested. The picture is further clouded by the fact that *in vivo* animal model results investigating the efficacy of MCF and RZF reported good efficacy against *C. auris*. This may be due to the much better pharmacokinetic parameters of RZF (higher C_{max} and $C_{through}$) and the fact that the survival and reproduction of *C. auris* under *in vivo* conditions, combined with RZF exposure, may exceed its adaptive capacity.

Our results suggest that the currently used standard doses of MCF may not achieve therapeutic concentrations in pleural and peritoneal infections caused by non-*albicans Candida* species, nor in serum for the treatment of bloodstream infections caused by *C. auris*. Since echinocandins, including MCF, have a wide therapeutic window, using higher doses than currently used would probably increase the therapeutic success rate in these pathologies.

Summary

Our knowledge on pharmakokinetics and pharmacodynamics of echinocandins, including micafungin have increased substancially in the past decades, yet data on activity on body sites other than the bloodstream, and also against the highly transmissable often drug resistant *C. auris* is scarce. Based on pharmacokinetic data obtained by others, micafungin supposed to have sufficient activity in case of pleural and peritoneal infections and also against *C. auris* bloodstream infections. Pharmacokinetic data however does not take into account the highly protein bound nature of echinocandins, which may partly explain the high rate of therapeutic failures despite adequate drug concentrations seemingly achieved. In our experiments we examined the role of serum proteins on pharmacodynamic activity of micafungin using time-kill methodology *in vitro* on clinically attainable concentrations in case of pleural and peritoneal infections (0.25-2 mg/L) against a panel of clinical isolates and reference strains of most prevalent *Candida* species in RPMI-1640 medium, and to simulate the high protein binding of micafungin, human serum (10 and 30 %) was also added resulting in protein concentrations equal to normal and inflammatory pleural and peritoneal fluids. The impact of presence of serum proteins on activity of micafungin against geographically distinct clades of *C. auris* was also assessed and was compared to already approved echinocandins (anidulafungin and caspofungin) and rezafungin at clinically relevant concentrations in the bloodstream (0.25-32 mg/L in absence and 1-32 mg/L in presence of 50% human serum).

For pleural and peritoneal isolates the activity of micafungin was satisfactory against *C. albicans* clinical isolates resulting in significant decrease in living fungal cells at all tested concentrations even in presence of 30% human serum for clinical isolates, however against non-*albicans Candida* species the results are less appealing, only 2 mg/L was uniformly effective against them, with the exemption of *C. krusei* and *C. parapsilosis* both of them was able to grow at the highest concentration tested in presence of 30% serum. Interestingly, against *C. auris* the activity of all echinocandins was better in presence of serum at higher concentrations (4-32 mg/L) compared to the normal RPMI-1640, however the effect was merely fungistatic in both media. In conclusion our data suggests that the recently used micafungin dosages may result in inefficient drug exposures unable to eradicate fungal cells of non-*albicans Candida* species from the pleura and the peritoneum and a contiuous 4 mg/L concentration should be achieved in the bloodstream against *C. auris*, which can only be met with increased doses.

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Registry number: DEENK/228/2022.PL
Subject: PhD Publication List

Candidate: Zoltán Tóth
Doctoral School: Doctoral School of Pharmacy
MTMT ID: 10062962

List of publications related to the dissertation

1. Kovács, R. L., **Tóth, Z.**, Locke, J. B., Forgács, L., Kardos, G., Nagy, F., Borman, A. M., Majoros, L.: Comparison of In Vitro Killing Activity of Rezafungin, Anidulafungin, Caspofungin, and Micafungin against Four Candida auris Clades in RPMI-1640 in the Absence and Presence of Human Serum.
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