Poor *in vivo* efficacy of caspofungin, micafungin and amphotericin B against wild-type *Candida krusei* clinical isolates does not correlate with *in vitro* susceptibility results

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Abstract

We determined micafungin, caspofungin and amphotericin B (AMB) MICs and killing rates in RPMI-1640 ~~with~~ and in RPMI-1640 with~~out~~ 50% serum against three *C. krusei* bloodstream isolates. MIC ranges in RPMI-1640 were 0.125-0.25, 0.25 and 0.125-0.5

mg/L, ~~respectively;~~ in RPMI-1640 with 50% serum~~with serum~~, MICs were 64-128-, 8- and 4-16-fold higher, respectively. In RPMI-1640 micafungin and caspofungin at 1, 4, 16 and 32 mg/L as well as AMB at 2 mg/L were fungicidal against all isolates in ≤3.96, ≤4.42 and 14.96 hours, respectively. In RPMI-1640 with 50% serum, caspofungin was fungicidal for all ~~three~~ isolates only at 32 mg/L, micafungin and AMB were fungistatic. In ~~a~~ neutropenic ~~murine model~~mice 5 mg/kg caspofungin and 1 mg/kg AMB were ineffective against two of the three isolates. Thus, *in vivo* efficacy of echinocandins and AMB is weak or absent against *C. krusei*. Prescribers treating *C. krusei* infections with echinocandins should ~~be on the~~ watch out for clinical resistance and therapeutic failure.

1. Introduction

The primarily fluconazole resistant *Candida krusei* is one of the most important non-*albicans Candida* species causing life-threatening infections among severely ill patients. Haematological malignancies, neutropenia, solid tumors and recent gastrointestinal surgery are well-known risk factors for invasive infections caused by *C. krusei*, it is a major pathogen in breakthrough fungemia in patients with fluconazole chemoprophylaxis.[1,2] For many decades, amphotericin B was the only systematically used antifungal agent for the treatment of invasive *C. krusei* infections; however, *in vitro* and *in vivo* data suggest that efficacy of amphotericin B is strongly questionable against *C. krusei*.[3-6] As echinocandins (anidulafungin, caspofungin and micafungin) show relatively low MIC values and concentration-dependent fungicidal activity against *C. krusei in vitro*, currently echinocandins are among the preferred antifungals against *C. krusei*, besides amphotericin B and voriconazole.[7,8] However, mortality rate due to invasive infections by *C. krusei* among intensive care unit patients is still unacceptably high (50-70%) even with the widely used echinocandin therapy.[9-11]

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We cultured 43 *C. krusei* bloodstream isolates from thirteen patients between 2011 and 2016 in our laboratory serving an 1665-bed tertiary care center. After initiation of micafungin or caspofungin therapy, *C. krusei* repeatedly cultured from the bloodstream of four echinocandin-treated patients for up to 18 days (persistent candidemia) suggesting insufficient eradication of this fungus by echinocandins. Notably, all isolates were fully susceptible to echinocandins and amphotericin B by the routinely used Etest interpreted using the CLSI breakpoints.[12,13]

As echinocandins are highly protein-bound antifungal drugs, the free (thus active) drug concentration may be low, which may, at least partly, be responsible for the poor sterilizing ability of echinocandins.[7,13,14] This inspired this study in which we determined the *in vivo* efficacy of micafungin, caspofungin and amphotericin B in a neutropenic murine model against *C. krusei* clinical isolates and compared it to *in vitro* activity using killing rates in RPMI-1640 and RPMI-1640 plus 50% serum to model protein binding.

2. Materials and methods

2.1. Strains

We used three *C. krusei* isolates, all three isolated prior to antifungal administration from three different intensive care unit patients from different units and years. The first patient (isolate 22910) with acute pancreatitis was treated with caspofungin (Cancidas ®) followed by micafungin (Mycamine ®) for 28 days, and died on day 29 from the first isolation of *C. krusei* from his bloodstream. After the initial isolation of *C. krusei*, positive follow-up cultures were found on four different days within the first 15 days. The total number of the positive blood cultures was eight. The second patient (isolate 26513) with ileus and colon resection was treated with caspofungin (Cancidas ®) for 31 days and survived. His follow-

up blood cultures in the next 18 days were positive on five different days. The total number of the positive blood cultures was eight. The third patient (isolate 25193) was neutropenic, suffering from acute myelogeneous leukaemia, and she died on the day the blood was collected and received no antifungals. The first and second patients were not neutropenic at the time of blood culture obtained and they were given standard doses of micafungin or caspofungin (100 mg per day and 70 mg on the first day followed by 50 mg daily, respectively). Isolates were identified with conventional methods (Micronaut-Candida and API ID32C) and MALDI Biotyper (Bruker, Bremen, Germany).[15]

2.2 Susceptibility testing

Micafungin pure powder was kindly provided by Astellas while caspofungin and amphotericin B pure powders were purchased from Sigma (Budapest, Hungary). MICs in RPMI-1640 and RPMI-1640 plus 50% serum (serum from a human male, type AB, Sigma, Budapest, Hungary) were determined using the standard broth macrodilution method at 35 °C.[16-20] For caspofungin and micafungin in RPMI-1640 and in RPMI-1640 plus 50% serum, drug concentrations ranged between 0.015-8 and 0.5-32 mg/L, respectively. Amphotericin B concentrations were 0.125-8 mg/L for both media. MIC values were read visually after 24 h.[12] For micafungin and caspofungin we used the partial inhibition criterion (the lowest concentration that produced a prominent decrease in turbidity compared to the drug-free control). In case of amphotericin B the total inhibition criterion was used. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 strains were used as quality control strains.[12]

2.3. Time-kill studies

Activity of micafungin and caspofungin against *C. krusei* clinical isolates were determined in RPMI-1640 with and without 50% human serum at 1, 4, 16 and 32 mg/L concentrations using a starting inoculum of ~10^5 cells/ml in a final volume of 10 ml.[17-20] Activity of amphotericin B was determined at 0.5, 1 and 2 mg/L in both media as well.[3,21] Aliquots of 100 µl were removed after 0, 4, 8, 12, 24 and 48 hours of incubation, tenfold serial dilutions were prepared, and samples of dilutions (4x30 µl) were plated onto a single Sabouraud dextrose agar (SDA) plate and incubated at 35 °C for 48 hours.[16-20] All experiments were performed twice in both media.

2.4. Analysis of in vitro *data*

Antifungal activity was defined as fungicidal when at least 99.9% reduction in viable cell count was observed as compared to the starting inoculum.[6]

Killing kinetics was analysed in both media (RPMI-1640 and RPMI-1640 plus 50% serum), as described previously.[6,18,19] Briefly, an exponential equation was fitted to the mean data at each time point: $N_t = N_0 \times e^{-kt}$, where N_t is the number of viable yeasts at time t, N_0 is the number of viable yeasts in the initial inoculum, k is the killing rate, and t is the incubation time. Negative and positive k values indicate growth and killing, respectively. The goodness of fit for each isolate was assessed by the r^2 value ($r^2 > \pm 0.8$). The mean times to achieve the fungicidal endpoint ($T99.9 = 3/k$) were calculated from the k values for each isolate and concentrations in both media.[6,18,19]

Killing kinetics for different isolates was compared using one-way ANOVA with Tukey's post-testing in RPMI-1640 or RPMI-1640 plus 50% serum. The effect of the same drug concentration in RPMI-1640 and RPMI-1640 plus 50% serum was analyzed using paired *T* tests (with Welch's correction, where appropriate).[18,19]

2.5. In vivo studies

Groups of seven to nine male BALB/c mice (23-25 g) were immunosuppressed with four doses of cyclophosphamide, i.e. 4 days before infection (150 mg/kg), 1 day before infection (100 mg/kg), 2 days postinfection (100 mg/kg) and 5 days postinfection (100 mg/kg).[17-19] The Guidelines for the Care and Use of Laboratory Animals was strictly followed during maintenance of the animals; experiments were approved by the Animal Care Committee of the University of Debrecen (permission no. 12/2014). Mice were inoculated intravenously through the lateral tail vein with an infectious dose of 4-4.5x10^6 CFU/mouse. Inoculum density was confirmed by plating serial dilutions onto SDA plates. At the beginning of the therapy (day 1) fungal kidney burden was determined after dissection of 4-5 untreated mice in case of each isolate (day 1 control burden).[19]

Five-day intraperitoneal treatment with daily 5, 10 and 1 mg/kg caspofungin (Cancidas®), micafungin (Mycamine®) and amphotericin B (Fungizone), respectively, against wild-type clinical isolates were started after 24 hours postinfection. Five and 10 mg/kg caspofungin and micafungin correspond to 70 mg and 200-300 mg daily doses in humans, respectively, which are higher than the currently recommended daily doses.[22-29]

On day six after infection, all mice were sacrificed; both kidneys were removed, weighed and homogenized aseptically. Homogenates were diluted tenfold; aliquots of 0.1 ml of the undiluted and diluted (1:10) homogenates were plated onto SDA plates and incubated at 35 °C for 48 h. The lower limit of detection was 50 CFU/g of tissue. Statistical analysis of the kidneys tissue burden was performed using the Kruskal-Wallis test with Dunn's post-test for multiple comparisons.[17-19]

3. Results

3.1. In vitro studies

3.1.1. MIC results in RPMI-1640 and RPMI-1640+50% serum

In RPMI-1640 MIC values for the quality control strains were within the published acceptable ranges.[12] MICs for clinical isolates are presented in Table 1. In RPMI-1640 all three isolates were susceptible to micafungin and caspofungin (susceptibility breakpoints for *C. krusei* are ≤0.25 mg/L for both drugs).[13] In RPMI-1640 plus 50% serum, micafungin and caspofungin MICs were 64-128 and 8 times higher than in RPMI-1640, respectively.

In RPMI-1640, MIC ranges for amphotericin B were 0.12-0.5 mg/L which is lower than the suggested susceptibility break-point (1 mg/L).[8,12] In RPMI-1640 plus 50% serum, amphotericin B MIC values were increased 4-16-fold (Table 1).

3.1.2. Killing activity of micafungin in RPMI-1640 and RPMI-1640+50% serum

Growth rate (i.e. the times to achieve 1 log increase in CFU compared to the starting inoculum) for the control isolates in RPMI-1640 and RPMI-1640 plus 50% serum was similar ($P>0.05$). The mean times to achieve 99.9% (T99.9) growth reduction from the starting inocula at different antifungal concentrations are shown in Table 2.

In RPMI-1640, at 1, 4, 16 and 32 mg/L micafungin was rapidly fungicidal against clinical isolates (all three were killed ≤3.96 hours) (Table 2). Killing activity of micafungin was concentration independent in cases of isolates 26513 and 25193 ($p>0.05$). Numerically, the highest *k* value (1.536 1/h) was found in case of isolate 22910 at 16 mg/L (Fig. 1).

In RPMI-1640 plus 50% serum, growth curves were similar to controls at 1 mg/L. At 4 and 16 mg/L micafungin produced only transient CFU decreases, but the mean *k* values were always negative. Positive *k* values were noticed only at 32 mg/L (CFU decreases were -1.48-2.78 CFU/mL) (Fig. 1).

3.1.3 Killing activity of caspofungin in RPMI-1640 and RPMI-1640+50% serum

Caspofungin in RPMI-1640 was fungicidal within 4.42 hours against clinical isolates (Table 2). Killing activity of caspofungin against isolate 22910 was concentration independent. For isolates 25193 and 26513 the highest *k* values were measured at 1 and 4 (1.23 1/h for both concentrations) and 4 mg/L (1.30 1/h), respectively. In RPMI-1640 plus 50% serum, with the exception of 1 mg/L, killing rates were positive and increased with concentrations (concentration-dependent killing). Killing activity of caspofungin in RPMI-1640 plus 50% serum decreased at 4, but increased at 32 mg/l compared to RPMI-1640 for all clinical isolates.

3.1.4. Killing activity of amphotericin B in RPMI-1640 and RPMI-1640+50% serum

In RPMI-1640, 0.5 mg/L amphotericin B produced positive *k* values for isolates 22910 and 25193. Though *k* values at 1 mg/L were positive (0.07-0.14 1/h) the CFU decreases were weak (1.06-1.48 CFU/mL). At 2 mg/L, amphotericin B was fungicidal within 14.69 hours against all three isolates (Table 2, Fig. 1).

In RPMI-1640 plus 50% serum, at 1 mg/L positive *k* value was observed only in case of isolate 25193 (0.014 1/h), while at 2 mg/L *k* values for all three isolates were positive (0.109-0.168 1/h). However, the *k* values are much lower than in case of echinocandins. In RPMI-1640, amphotericin B killing activity at 2 mg/L was significantly higher than in RPMI-1640 plus 50% serum (Table 2, Fig. 1).

3.2. *In vivo* experiments

All three isolates showed weak replication ability in the untreated neutropenic mice. In cases of isolates 22910 and 26513, the mean fungal tissue burdens decreased on day 6 as

compared to day 1; the decreases were higher than one log in case of isolate 26513. In case of isolate 25193, the mean fungal tissue burdens on day 6 increased only slightly (less than 1 log) comparing to day 1 (Fig 2).

Caspofungin and amphotericin B were effective against isolate 25193 ($p<0.05$); the CFU decreases were lower than 1 log compared to untreated controls (on day 6) and were similar to day 1 control burden. Micafungin did not show any activity in this model system. Against isolates 22910 and 26513 none of the tested drugs showed activity.

4. Discussion

Theoretically, the *in vitro* fungicidal activity of echinocandins and amphotericin B against *Candida* species is considered advantageous as they expected to eradicate fungal cells rapidly from infected tissues and the bloodstream.[3,6-8,13,14,30] This drives their increasing preference for treating severe fungal infections. However, the high protein binding decreases the unbound, thus active drug concentration in the blood and tissues, leading to lower drug exposure and potentially to therapeutic failure.[7,13,14,21,30,31] Previous *in vitro* data support this hypothesis, because in the protein-free RPMI-1640 medium used in standard susceptibility testing, echinocandins as well as amphotericin B are more frequently fungicidal at low drug concentrations than in RPMI-1640 plus 50% serum, indicating the clinical relevance of the protein binding.[5,16-20,30,31] Moreover, in a preclinical study with micafungin, *in vivo* efficacy showed poorer correlation with MICs by standard RPMI-1640 than by serum-based susceptibility tests.[32]

It was previously suggested that determination of MIC alone in case of caspofungin is misleading and may misclassify wild-type *C. krusei* or *C. glabrata* clinical isolates as non-wild-type or resistant.[33] Therefore, currently micafungin or anidulafungin MICs in RPMI-1640 are recommended as best predictors for the clinical efficacy of all three echinocandins against *Candida* species, not excepting *C. krusei*. However, in our experiments standard MICs and killing studies with micafungin in RPMI-1640 yielded misleading results, falsely suggesting efficacy against *C. krusei in vivo*.

In our study adding 50% serum to RPMI-1640 did not influence the growth rate of the three *C. krusei* isolates, indicating that 50% serum did not inhibit *C. krusei*. In contrast, other authors noticed very poor growth of three *C. krusei* isolates (as well as of *C. glabrata* and *C. lusitaniae*) in RPMI-1640 plus 50% serum even after seven days.[34] The explanation for this difference is unknown. However, the cited study used the CLSI broth microdilution

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method for MIC determination, and the smaller final volume (0.2 mL) in their experiment may have influenced the growth of fungi.

In this study we have found significant differences in the killing rates of echinocandins in RPMI-1640 and RPMI-1640 plus 50% serum against *C. krusei*. RPMI-1640 plus 50% serum more profoundly decreased killing activity of micafungin than that of caspofungin as compared to RPMI-1640 (Fig. 1., Table 2). The difference is probably due to the difference between the protein binding for micafungin (99.9%) and caspofungin (97.5%).[8,13,14] Our *in vitro* results in RPMI-1640 plus 50% serum (negative *k* values at ≤16 mg/L) proved to be a good predictor for the lack of *in vivo* efficacy even of elevated daily micafungin dose, as micafungin never decreased the fungal tissue burden against any of the tested *C. krusei* isolates. This result is concordant to our previous *in vitro* findings with different *C. krusei* bloodstream isolates.[16]

In RPMI-1640 plus 50% serum, *in vitro* activity of caspofungin decreased less markedly as positive *k* values were noticed even at 4 mg/L which is the trough concentration using the standard or elevated daily doses.[22-24] However, the efficacy of caspofungin was found to be unreliable against *C. krusei in vivo* in the neutropenic murine model (i.e. only one of three isolates responded to the 5 mg/kg daily dose). A previous study of our group with two different isolates showed a statistically demonstrable efficacy of caspofungin 5 mg/kg daily in a similar model system.[18] However, in the present study the two isolates derived from persistent candidemia showed weak *in vivo* replication ability (as indicated by comparison of fungal tissue burdens on day 1 and 6), and weak efficacy of echinocandins against slowly replicating cells is a well known phenomenon.[7,13,14] Moreover, other factors (i.e. neutropenia, slow drug penetration into inflamed tissues, etc.) may strongly influence therapeutic outcome.[8,35]

Amphotericin B used at clinically attainable concentration showed weak *in vitro* killing activity against *C. krusei* regardless of test media, which was confirmed *in vivo* as well.[3-6] Our results correlate with previous *in vitro* and *in vivo* findings that amphotericin B is not superior to echinocandins for the treatment of invasive infections caused by *C. krusei*.[8] However, currently there is no alternative in echinocandin resistant cases.[3,10,13]

In conclusion, standard RPMI-1640-based susceptibility tests did not provide reliable information on the *in vivo* efficacy of micafungin or caspofungin against wild-type *C. krusei* clinical isolates. Serum based susceptibility testing methods were good predictors of the *in vivo* efficacy of micafungin, while neither the standard not the serum based method were good predictors in case of caspofungin. Our *in vivo* results strongly correlate with the currently reported experience that efficacy of echinocandins may be poor against *C. krusei* in some clinical situations. This does not mean echinocandin resistance, but reflects the lower activity of the cell-wall active echinocandins on a fungal species slowly replicating *in vivo*. Our results suggest that prescribers treating *C. krusei* infections with echinocandins should be on the watchout for clinical resistance and therapeutic failure.

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Figure legends

Figure 1

Killing rates of micafungin (MICA), caspofungin (CAS) and amphotericin B (AMB) and the corresponding adjusted regression lines (dashed lines) against three *Candida krusei* bloodstream isolates in RPMI-1640 (RPMI) and RPMI-1640 plus 50% serum (Serum). Positive and negative *k* values indicate the decreases and increases, respectively, in viable cell numbers.

Figure 2

Kidney tissue burden of severely neutropenic BALB/c mice infected intravenously with three *C. krusei* isolates. Intraperitoneal caspofungin (CAS), micafungin (MICA) and amphotericin B (AMB) (5, 10 and 1 mg/kg, respectively) daily treatments were started 24 hours after the infection. The bars represent the medians. Level of statistical significance compared to the control population (day six) is indicated at $P<0.05$ (*).

Table 1. *Candida krusei* isolates and MICs of micafungin (MICA), caspofungin (CAS) and amphotericin B (AMB) in RPMI-1640 and RPMI-1640 plus 50 % serum (50% serum)

Isolates	Media	MIC (mg/L)		
		MICA	CAS	AMB
22910	RPMI-1640	0.25	0.25	0.5
	50% serum	16	2	2
26513	RPMI-1640	0.12	0.25	0.5
	50% serum	16	2	2
25193	RPMI-1640	0.25	0.25	0.12
	50% serum	16	2	2

Table 2. Time (h) to reach 99.9% (T99.9) growth reduction from the starting inocula at different micafungin, caspofungin and amphotericin B (AMB) concentrations (mg/L) in RPMI-1640 (RPMI) and RPMI-1640 plus 50 % serum (serum). NR means that fungicidal effect (99.9% growth reduction) was not reached.

Isolate	Media	Time (hours)										
		Micafungin (mg/L)				Caspofungin (mg/L)				AMB (mg/L)		
		1	4	16	32	1	4	16	32	0.5	1	2
22910	RPMI	3.04	3.03	1.95	3.96	2.94	2.59	2.59	3.04	NR	NR	8.67
	Serum	NR	NR	NR	NR	NR	NR	2.71	2.71	NR	NR	NR
26513	RPMI	2.72	2.76	2.85	2.84	4.25	2.31	2.74	3.81	NR	NR	9.18
	Serum	NR	NR	NR	NR	NR	NR	3.22	3.12	NR	NR	NR
25193	RPMI	3.38	3.91	3.07	2.95	2.43	2.43	4.42	3.04	NR	NR	14.69
	Serum	NR	NR	NR	NR	NR	NR	NR	3.04	NR	NR	NR