Decreased killing activity of micafungin against *Candida guilliermondii*, *C. lusitaniae* and *C. kefyr* in the presence of human serum

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Abstract

Currently, echinocandins are first-line drugs for treatment of invasive candidiasis. However, data on how serum influences killing activity of echinocandins against uncommon *Candida* species are limited. Therefore, the killing activity of micafungin in RPMI-1640 and in 50% serum was compared against *C. guilliermondii*, *C. lusitaniae* and *C. kefyr*. MIC ranges in RPMI-1640 were 0.5-1, 0.12-0.25 and 0.06-0.12 mg/L, respectively. In 50% serum, MICs increased 32-256-fold. In RPMI-1640 ≥0.25, ≥4 and 32 mg/L micafungin was fungicidal against all four *C. kefyr* (≤4.04 hours), two of three *C. lusitaniae* (≤16.10 hours) and two of three *C. guilliermondii* (≤12.30 hours) respectively. In 50% serum, all three species grew at ≤4 mg/L. Micafungin at 16-32 mg/L was fungicidal against all *C. kefyr* isolates (≤3.03 hours) and at 32 mg/L was fungistatic against one of three *C. lusitaniae* isolates. Two *C. lusitaniae* isolates and all three *C. guilliermondii* grew at all tested concentrations. Adding human serum to susceptibility test media drew attention to loss of fungicidal or fungistatic activity of micafungin in the presence of serum proteins, which is not predicted by MICs in case of *C. kefyr* and *C. lusitaniae* in RPMI-1640. Our results strongly suggest that micafungin and probably other echinocandins should be used with caution against rare *Candida* species.

Introduction

Though five *Candida* species (*C. albicans*, *C. glabrata*, *C. parapsilosis sensu stricto*, *C. tropicalis* and *C. krusei*) account for ≥95 % of all candidemia, less common other species (*C. guilliermondii*, *C. lusitaniae*, and *C. kefyr*), may cause problems in the treatment of candidemias or other forms of invasive candidiasis, especially among cancer and leukaemia patients.[1-4] Optimal treatment against these species is not well defined as they often show decreased susceptibility to different antifungal classes including echinocandins.[1,5,6]

Currently, echinocandins such as micafungin are first-line drugs for treatment of invasive candidiasis.[7] Echinocandins are highly protein-bound with 0.2-3% free drug, i.e. the serum and tissues concentrations may be low.[8] Decreased killing activity of echinocandins in 50% serum against medically important *Candida* species was confirmed.[9-12] However, data on how serum influences killing activity of echinocandins against uncommon *Candida* species are limited.[9-11,13] In order to extend our knowledge about the *in vitro* activity of echinocandins against uncommon *Candida* species, the killing activity of micafungin in RPMI-1640 and in RPMI-1640 plus 50% serum (50% serum) was compared against *C. guilliermondii*, *C. lusitaniae* and *C. kefyr*.

Materials and methods

Bloodstream-derived clinical isolates (Table 1) were identified using Micronaut-Candida and API ID32C methods.[14] All strains were isolated prior to antifungal administration. Micafungin pure powder was kindly provided by Astellas. MICs in RPMI-1640 and in 50% serum (serum from a human male, type AB, Sigma, Budapest, Hungary) were determined using the standard broth macrodilution method. Final

concentration ranged between 0.03-32 mg/L. MIC values were read visually after 24 h using the partial inhibition criterion.[15]

Time kill studies were performed in both media at 1, 4, 16 and 32 mg/L at least twice. In case of *C. kefyr* 0.25 mg/L micafungin was also tested. The starting inocula were 3.5-5 x10^5 CFU/mL. Aliquots of 100 μl were removed at 0, 4, 8, 12, 24 and 48 hours, serially diluted tenfold, plated onto Sabouraud agar and incubated at 35 °C for 48 hours. The same method was used to test growth kinetics.[9-12,16,17]

Fungicidal activity of micafungin was defined as 99.9% or higher reduction in viable cell count.[9-13,16,17] Killing kinetics were analysed in both media (RPMI-1640 with and without serum) as described previously.[11,12] 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; reducing time points of each killing curve to one value (k). Positive k values indicate killing, negative values indicate growth. The goodness of fit was assessed by the r^2 value (r^2>0.8). The mean times to achieve the fungicidal endpoint (T99.9=3/k) were calculated from the k values for each isolate and concentration in both media.[11,12]

Killing kinetics among isolates and concentrations in either RPMI-1640 or 50% serum were compared using one-way ANOVA with Tukey's post-testing; growth rates and the efficacy of the same micafungin concentrations in different media were compared by *T* test (with Welch's correction, where appropriate).[11,12]

Results

C. guilliermondii isolates were susceptible to micafungin in RPMI-1640 (Table 1.).[18] No breakpoint is available for *C. kefyr* and *C. lusitaniae.* However, MICs of isolates of these two species were not higher than the epidemiological cut off values (0.12 and 0.5 mg/L for *C. kefyr* and *C. lusitaniae*, respectively).[19] In 50% serum, MICs increased 32-256-fold. All *C. kefyr* isolates and *C. guilliermondii* isolate 5540 grew significantly better in RPMI-1640 than RPMI-1640 plus serum ($P<0.05$).

Representative time-kill plots are shown in Fig. 1. In RPMI-1640, killing exerted by micafungin was rapid against *C. kefyr*; mean times to achieve 99.9% growth reduction were shorter than 4.04 hours for all isolates (Table 2.). Contrastingly, 0.25, 1 and 4 mg/L micafungin did not produce any killing against *C. kefyr* isolates in serum (*k* values were negative and did not differ significantly from those of the drug free controls) (Fig. 2, Table 3.). Higher concentrations (16 and 32 mg/L), however, produced fast (≤3.03 hours) killing in 50% serum as well.

Micafungin at 4, 16 and 32 mg/L was fungicidal against two of three *C. lusitaniae* isolates in RPMI-1640 (Table 2. and 3.); the killing activity of micafungin was strain dependent but concentration independent (i.e. *k* values did not differ significantly) (Fig. 3). In 50% serum, micafungin produced only transient CFU decreases even at 16-32 mg/L against *C. lusitaniae* isolates (except isolate 3834 at 32 mg/L) (Table 3.). Positive *k* value (0.2369 1/h, indicating killing) was noticed only at 32 mg/L in case of isolate 3834 (Fig. 3.).

Though micafungin was fungicidal at 32 mg/L against *C. guilliermondii* isolates 5465 and 21060 in RPMI-1640, the inhibitory effect was only transient (re-growth occurred) at lower concentrations (1, 4 and 16 mg/L) (Table 3.). In RPMI-1640, *k* values at 16 and

32 mg/L were strain-dependent but concentration-independent (Fig. 3). In 50% serum, killing curves for *C. guilliermondii* at any concentrations were similar to controls (*k* values were always negative, indicating growth) (Fig. 1. and 3.).

Discussion

It was previously shown that *C. guilliermondii* is intrinsically less susceptible to echinocandins, while wild-type *C. kefyr* and *C. lusitaniae* MIC values are close to those obtained with innately echinocandin susceptible *C. albicans* or *C. glabrata*.[6,19] However, mortality rates caused by *C. guilliermondii*, *C. lusitaniae* and *C. kefyr* among seriously ill patients are extremely high (53-83%), suggesting that currently used therapeutic approaches, including echinocandin therapy, are insufficient.[1-4] Furthermore, infections caused by *C. lusitaniae* and *C. kefyr* have became increasingly prevalent in parallel with the continuous increase in echinocandin use.[1,6,20]

The CLSI introduced clinical break-points for the 5 most important *Candida* species (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*) but not for uncommon (rare) *Candida* species (with the exception of *C. guilliermondii*).[15,18] However, it is not taken into account that RPMI-1640 is a serum-free medium, thus in case of the highly protein bound echinocandins, their *in vitro* potency against these *Candida* species may be overestimated in spite of the very low MIC values in standard susceptibility tests.[6,18] Epidemiological cutoff values for the species against which official breakpoints has not yet been published are also based on the standard susceptibility testing using RPMI-1640.[18,19]

Serum based testing of echinocandin susceptibility, which mimics the *in vivo* conditions occurring in the blood or tissues more closely, have some advantages.[25] First, *in vivo*

efficacy of micafungin showed better correlation with MICs by serum-based than by standard RPMI-1640 susceptibility tests.[26] Second, 50% serum may help to differentiate between wild-type isolates and isolates harboring resistance mutations.[6,25] Third, serum eliminates the paradoxically decreased killing activity at high concentration of echinocandins in RPMI-1640.[12,18,25,27]

Killing activities in this study were found to be markedly lower than against *C. albicans*[9] in the presence of serum, i.e. no killing by 4 mg/L micafungin; even higher concentrations proved to be consistently fungicidal only against *C. kefyr*. Consequently, reported median peak and trough plasma levels (15.15 and 4.85 mg/L, respectively) produced by the elevated 150 mg micafungin daily doses,[21] predict inadequate eradication and increased risk of breakthrough infections[1] by these three species. Concordantly, only weak eradication capacity of echinocandins was reported in neutropenic animal models against these species even after 7-10 days echinocandin therapy (i.e. internal organs were not sterilized by echinocandins).[22-24] In addition, inadequate serum echinocandin levels may facilitate development of acquired resistance to echinocandins.[6]

In summary, adding human serum to susceptibility test media drew attention to loss of killing activity in the presence of serum proteins, which is not predicted by standard MICs in case of *C. kefyr* and *C. lusitaniae* in RPMI-1640. Even elevated therapeutic micafungin doses (150 mg/day) do not produce serum drug levels sufficient for reliable fungicidal or fungistatic activity against any of the three studied species, which may explain the observed high probability of therapeutic failure in infections with these species. This issue is especially important in case of patients with neutropenia or other immunosuppression. Although serum-based susceptibility testing has not yet been

standardized, our results strongly suggest that micafungin and probably other echinocandins should be used with caution against rare *Candida* species.

Conflict of interests

No competing financial interests exist.

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Figure legends

Figure 1

Representative time-kill curves against *C. kefyr, C. lusitaniae* and *C. guilliermondii* in RPMI-1640 with (MIC_{SERUM}) and without 50 % serum (MIC_{RPMI})

Figure 2

Killing rates of micafungin and the corresponding adjusted regression lines (dashed lines) against four *Candida kefyr* bloodstream isolates in RPMI-1640 with (serum) and without 50 % serum (RPMI). Positive and negative *k* values indicate the decrease and increase, respectively, in viable cell numbers.

Figure 3

Killing rates of micafungin and the corresponding adjusted regression lines (dashed lines) against three-three *Candida lusitaniae* (left) and *Candida guilliermondii* (right) bloodstream isolates in RPMI-1640 (RPMI) and RPMI-1640 plus 50% serum (serum). Positive and negative *k* values indicate the decrease and increase, respectively, in viable cell numbers.