Activities of Systemically Administered Echinocandins against *In Vivo* Mature *Candida albicans* Biofilms Developed in a Rat Subcutaneous Model

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This study addresses the effects of micafungin, caspofungin, and anidulafungin against *Candida albicans* biofilms developed in a subcutaneous catheter rat model system. Doses of 5, 10, and 30 mg/kg (of body weight)/day (the last only for micafungin) were given intravenously for 5, 7, and 10 days. All three echinocandins caused a significant reduction of the *Candida* cell numbers on the implanted catheters and are thus promising for the treatment of biofilm-related infections.

The formation of *Candida* biofilms makes the removal and/or replacement of infected devices often compulsory (1). Nevertheless, the potent activity of the currently licensed echinocandins on mature biofilms has been demonstrated *in vitro* (2, 3). Moreover, caspofungin could be particularly useful against catheter-related infections when used as a lock solution alone or in combination with systemic administration (4, 5). Recently, we demonstrated that anidulafungin is also active *in vivo* against mature *C. albicans* biofilms when administered intraperitoneally in an avascular subcutaneous rat model (6). In contrast, micafungin was found to be fairly ineffective against *C. albicans* biofilms developed in a denture stomatitis model in rodents (7). As a direct comparison of the three echinocandins in the same *in vivo* model, taking into account pharmacokinetic differences, is currently lacking, we examined in detail the activities of caspofungin, micafungin, and anidulafungin against mature *C. albicans* biofilms developed in a subcutaneous rat biofilm model and evaluated the effects of different doses, i.e., 5 and 10 mg/kg of body weight for all echinocandins, and additionally 30 mg/kg for micafungin, and different durations of exposure, i.e., 5, 7, and 10 days.

C. albicans SC5314 was used for all experiments (8). Pure substances of micafungin (Astellas, The Netherlands), caspofungin (Merck, Sharp and Dohme, Belgium), and anidulafungin (Pfizer, Belgium) were dissolved in sterile water and subsequently diluted in normal saline (0.9% NaCl) before use. The MICs for planktonic cells were determined according to the CLSI M27-A3 protocol (9). *In vitro* biofilm drug susceptibility assays were performed using 1-cm pieces of serum-coated polyurethane catheters (Arrow International Reading, United States) according to the method of Říčicová et al. (10). These experiments were performed three times independently; 2 catheter fragments were tested for each concentration. Mature (24-h) *in vitro* biofilms were treated for 24 h with echinocandins (0.0625 µg/ml to 32 µg/ml). The MICs were determined as the minimal drug concentration that caused ~50% reduction in the amount of CFU compared to controls (untreated samples).

In vivo biofilms were formed on polyurethane catheter pieces implanted subcutaneously in immunosuppressed female Sprague-Dawley rats as described by Říčicová et al. (10). Up to nine fragments were implanted subcutaneously in the back of each animal. Biofilms were allowed to mature for 48 h prior to antifungal treat-

ment. All echinocandins or normal saline (control animals) were administered intravenously for 5, 7, and 10 days at a dosage of 5 mg/kg or 10 mg/kg once daily. In total, three independent experiments were performed, always using two rats per treatment regimen. Additionally, micafungin was administered intravenously at a higher dose (30 mg/kg) in two independent experiments (2 animals each time) for 5, 7, and 10 days. Animal experiments were approved by the ethical committee of the KU Leuven (project number 125/2011).

Catheters retrieved from *in vitro* and *in vivo* experiments were washed and sonicated before biofilm quantification by counting of CFU on yeast extract-peptone-dextrose (YPD) plates. Statistical analyses were carried out using the Mann-Whitney *U* test (Analyze-it software). Statistical significance was considered when *P* values were ≤0.05 and ≤0.01.

The *in vitro* biofilm MICs for micafungin and caspofungin were 0.125 µg/ml, whereas the MIC for anidulafungin was 0.5 µg/ml. The numbers of *Candida* cells recovered from the explanted catheters (CFU from three independent experiments) from rats treated with 5 mg/kg/day or 10 mg/kg/day of micafungin, caspofungin, or anidulafungin are shown in Fig. 1. Results of the individual experiments with the different dosages and treatment regimens are presented in Fig. S1 and S2 in the supplemental material.

Treatment of the animals with caspofungin at 5 mg/kg for 5, 7, and 10 days significantly reduced the number of CFU recovered from the explanted catheters (1.97 ± 0.99 , 1.55 ± 1.29 , and $0.87 \pm$

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FIG 1 Effects of systemic administration of micafungin (MFG), caspofungin (CSP), and anidulafungin (AND) on mature *Candida albicans* biofilms developed in a subcutaneous rat model. Animals were treated with two different doses of each drug, 5 mg/kg (A) and 10 mg/kg (B), for 5, 7, or 10 days. Open circles represent the log₁₀ numbers of CFU of *C. albicans* cells retrieved from each catheter. The horizontal lines indicate the median values for log₁₀ numbers of CFU obtained per catheter. Differences between the echinocandin-treated group and the control (C) group were considered statistically significant at *P* values of ≤0.05 (*) and ≤0.01 (**).

1.19 log₁₀ CFU/catheter, respectively) compared to the ones retrieved from the control animals (2.90 ± 0.57, 2.98 ± 0.57, and 2.46 ± 0.73 log₁₀ CFU/catheter, respectively; *P* < 0.01). It is noteworthy that after treatment with caspofungin (5 mg/kg) for 7 and

10 days, 34% and 57% of the catheters, respectively, were sterile. The polyurethane fragments retrieved from the animals treated with 10 mg/kg of caspofungin for 5, 7, and 10 days contained also significantly smaller numbers of CFU (1.77 ± 1.23, 1.66 ± 1.16,

and $1.54 \pm 1.12 \log_{10}$ CFU/catheter, respectively) compared to ones from the nontreated animals ($P < 0.01$). Treatment of *C. albicans* biofilms with 5 mg/kg of anidulafungin or micafungin for 10 days did not cause a significant reduction in biofilm cells compared to controls, although 6% of catheters retrieved from the anidulafungin-treated group were sterile. On the other hand, using a dosage of 10 mg/kg of anidulafungin for 5, 7, and 10 days (2.21 ± 0.91 , 1.51 ± 1.21 , and $1.18 \pm 1.01 \log_{10}$ CFU/catheter, respectively) resulted in significantly reduced CFU compared to those in animals exposed to normal saline treatment (2.91 ± 0.60 , 2.73 ± 0.58 , and $2.93 \pm 0.73 \log_{10}$ CFU/catheter, respectively; $P < 0.01$). It is noteworthy that 6%, 27%, and 39%, respectively, of the catheters treated with this regimen were sterile.

The systemic treatment of the animals with 10 mg/kg of micafungin for 5, 7, and 10 days failed to decrease mature biofilm development compared to the controls considering the P value cutoff of <0.01 for statistical significance ($P = 0.012$, $P = 0.030$, and $P = 0.056$, respectively). As it was shown that micafungin is metabolized faster in rats (see below), we also tested a higher dosage of micafungin (30 mg/kg/day) in proportion to its rate of metabolism in rats compared to that of caspofungin. The administration of this dose for 5 and 7 days did not affect the number of viable cells within a biofilm compared to the normal saline control condition ($P = 0.333$ and $P = 0.454$, respectively; see Fig. S3 in the supplemental material). However, prolonged treatment (up to 10 days) with 30 mg/kg of micafungin resulted in significantly decreased biofilm burden in comparison with that in control animals ($P < 0.01$; see Fig. S3).

Here we demonstrated that intravenous administration of caspofungin at a dosage of 5 or 10 mg/kg/day, and anidulafungin only at a dosage of 10 mg/kg/day, in rats significantly reduced *C. albicans* cells living within biofilms *in vivo*. In contrast, a higher dose of micafungin (30 mg/kg/day) and longer treatment (10 days) were needed to achieve a significant ($P < 0.01$) reduction of the number of cells in mature *C. albicans* biofilms.

The differences in echinocandin activity might be explained by important differences in their pharmacokinetics in rats versus humans. The clearance of all three echinocandins is about 5- to 6-fold higher in rats versus humans (11–14). The higher clearance for caspofungin and anidulafungin in rats is explained by a 2- to 3-fold-higher volume of distribution compared to that in humans, potentially explaining the potent efficacy for both agents in our rat model, as probably distribution in the interstitial inflammatory fluid surrounding the implanted catheter fragment is the driving pharmacokinetic parameter correlating with efficacy (11, 13, 14). For micafungin, however, clearance in rats is also 6-fold higher and the half-life is 3-fold shorter than values reported for humans, but, as opposed to the case with caspofungin and anidulafungin, the volume of distribution is only slightly higher than that in humans (12). Therefore, it is speculated that the higher clearance of micafungin in rats versus humans is mainly due to a higher elimination rate in the liver (12), potentially leading to suboptimal interstitial concentrations and poor effect on fungal CFU. The ineffectiveness of micafungin tested at 5 mg/kg/day or 10 mg/kg/day (considering a P value cutoff of <0.01 for statistical significance) is in agreement with the findings of Nett et al. (7); i.e., topical and intraperitoneal administration (5 mg/kg) of micafungin is ineffective against mature *C. albicans* biofilms developed in a rodent denture stomatitis model.

For this reason, we increased the dosage to 30 mg/kg/day in an

attempt to augment the distribution in the surrounding interstitial fluid and to compensate for the lower distribution of micafungin than of caspofungin and anidulafungin. This dosage of micafungin proved to be effective in reducing the number of viable cells in the biofilm. The fact that anidulafungin resulted in a significant reduction in CFU only when administered at 10 mg/kg/day, as opposed to the potent activity of caspofungin already when used at 5 mg/kg/day, can also be attributed to differences in the agents' underlying pharmacokinetics (15). These findings are in agreement with the differences we see in humans in whom higher (2- to 3-fold) doses of anidulafungin are used compared to caspofungin. The pharmacokinetic differences in different animal species versus humans, as demonstrated by Hajdu et al. (11) and described above, clearly indicate the need for further research in other animal species and eventually in patients.

Besides pharmacokinetic differences between animal models and humans, the choice of the biofilm model may also have an important effect on the outcome of the experiment. For example, each host niche (subcutaneous, bloodstream, or mouth) is characterized by differences in accessibility for the drug and by different immune responses. In the central venous system, biofilms are subjected to constant blood flow; biofilms developing in catheters implanted under the skin of rats are not exposed to a physiological flow. Additionally, the access to nutrients will also differ and will be greater in the blood. The subcutaneous model is somewhat more related to biofilm infections that develop in joint prostheses and voice prostheses, for example, and may reflect better these host infection sites, in term of environmental conditions and nutrient supplies.

In conclusion, micafungin, caspofungin, and anidulafungin displayed potent activities, when used in appropriate doses and treatment durations, for the treatment of *C. albicans* mature biofilms in a subcutaneous rat model. These results should be confirmed in further studies in order to explore the applicability in clinical practice.

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