Activity of systemically administered echinocandins against *in vivo* mature *Candida albicans* biofilms developed in a rat subcutaneous model

Running title: Echinocandin activity against *in vivo* biofilms

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Abstract

This study addresses the effect of micafungin, caspofungin and anidulafungin against Candida albicans biofilms developed in a subcutaneous catheter rat model system. Five, 10 and 30 mg/kg/day (latter one only for micafungin) were given intravenously for 5, 7 or 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.

Key words: Candida albicans, in vivo biofilm, echinocandins, micafungin, caspofungin, anidulafungin
The formation of *Candida* biofilms makes the removal and/or replacement of the infected device 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 activity of caspofungin, micafungin and anidulafungin against mature *C. albicans* biofilms developed in a subcutaneous rat biofilm model and evaluated the effect of different dosages, i.e. 5 mg/kg and 10 mg/kg for all echinocandins, and additionally 30 mg/kg for micafungin, and different duration of exposure, i.e. 5, 7 or 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 minimal inhibitory concentration (MIC) values 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, USA) according to 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 hours 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 Colony Forming Units (CFUs) 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 Říčcová 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 treatment. 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 (each time 2 animals) during 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 colony-forming units (CFUs) on YPD plates. Statistical analyses were carried out using the Mann-Whitney U test (Analyse-it Software). Statistical significance was considered when \( p \leq 0.05 \) (*) and \( p \leq 0.01 \) (**).

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 (CFUs 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 Figure 1 A and B. Results of the individual experiments with the different dosages and treatment regimens are presented in Supplementary figures 1 and 2.

Treatment of the animals with caspofungin at 5 mg/kg for 5, 7 and 10 days significantly reduced the number of CFUs recovered from the explanted catheters (1.97 ± 0.99; 1.55 ± 1.29 and 0.87 ± 1.19 log_{10} 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 log_{10} CFU/catheter, respectively, \(p<0.01\)). Noteworthy, after treatment with caspofungin (5 mg/kg) for 7 or 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 days and 10 days contained also significantly lower amount of CFUs (1.77 ± 1.23; 1.66 ± 1.16 and 1.54 ± 1.12 log_{10} CFU/catheter, respectively) compared to ones from the non-treated animals (\(p<0.01\)). Treatment of \textit{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 during 5, 7 and 10 days (2.21 ± 0.91; 1.51 ± 1.21 and 1.18 ± 1.01 log_{10} CFU/catheter, respectively) resulted in significantly reduced CFUs compared to the ones exposed to normal saline treatment (2.91 ± 0.60; 2.73 ± 0.58 and 2.93 ± 0.73 log_{10} CFU/catheter, respectively, \(p<0.01\)). Noteworthy, 6%, 27% and 39%, respectively of the catheters treated with this regimen were sterile.

The systemic treatment of the animals with 10 mg/kg micafungin for 5, 7 and 10 days failed to decrease mature biofilm development compared to the controls considering the \(p<0.01\) cutoff 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 further), we also tested a higher dose 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 when compared to the normal saline control condition (\(p=0.333\) and \(p=0.454\), respectively, Supplementary figure 3). However, prolonged treatment (up to 10 days) with 30 mg/kg of micafungin resulted in significantly decreased biofilm burden in comparison with control animals (\(p<0.01\), Supplementary figure 3).
Here we demonstrated that intravenous administration of caspofungin at a dose of 5 or 10 mg/kg/day, and anidulafungin, only at a dose 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 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 vs. humans. The clearance of all three echinocandins is about 5 to 6-fold higher in rats vs. humans (11-14). The higher clearance for caspofungin and anidulafungin in rats is explained by a 2- to 3-fold higher volume of distribution when compared to 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 compared to values reported in humans, but, as opposed to 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 vs. humans is mainly due to a higher elimination rate in the liver (12), potentially leading to suboptimal interstitial concentrations and poor effect on fungal CFUs. The ineffectiveness of micafungin tested at 5 mg/kg/day or 10 mg/kg/day (considering *p*<0.01 cutoff 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.

Because of this reason, we increased the dose 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 when compared to 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 CFUs 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 both agents’ underlying pharmacokinetics (15). These findings are in agreement with the differences we see in humans in whom higher (two- to threefold) doses of anidulafungin are used compared to caspofungin. The pharmacokinetic differences in different animal spp. vs. 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.

Beside 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, blood stream 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 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 activity, when used in appropriate doses and treatment duration, 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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References


Figure 1. Effect of systemic administration of micafungin (MFG), caspofungin (CSP) and anidulafungin (AND) on mature Candida albicans biofilms developed in subcutaneous rat model. Animals were treated with two different dosages of each drug, 5mg/kg (A) and 10 mg/kg (B) for 5, 7 or 10 days. Open circles represent the log_{10} numbers of colony forming units (CFUs) of C. albicans cells retrieved from each catheter. The horizontal lines indicate the median values for log_{10} numbers of CFUs obtained per catheter. Statistical significant difference between the echinocandin treated group and the control group p≤0.05 (*) and p≤0.01 (**).