Antifungal activity against planktonic and biofilm Candida albicans in an experimental model of foreign-body infection

Elena Maryka Maiolo a,b, Alessandra Oliva b, Ulrika Furustrand Tafin b, Nancy Perrotet c, Olivier Borens b, Andrej Trampuz a,*

a Charité – University Medicine Berlin, Center for Musculoskeletal Surgery, Berlin, Germany
b Septic Surgical Unit, Department of Surgery and Anesthesiology, Lausanne University Hospital, Lausanne, Switzerland
c Department of Hospital Pharmacy, Lausanne University Hospital, Lausanne, Switzerland

Accepted 11 December 2015
Available online

KEYWORDS
Candida spp.; Biofilm; Foreign-body infection; Animal model; Antifungals

Summary  Objectives: The treatment of Candida implant-associated infections remains challenging. We investigated the antifungal activity against planktonic and biofilm Candida albicans in a foreign-body infection model.

Methods: Teflon cages were subcutaneously implanted in guinea pigs, infected with C. albicans (ATCC 90028). Animals were treated intraperitoneally 12 h after infection for 4 days once daily with saline, fluconazole (16 mg/kg), amphotericin B (2.5 mg/kg), caspofungin (2.5 mg/kg) or anidulafungin (20 mg/kg). Planktonic Candida was quantified, the clearance rate and cure rate determined.

Results: In untreated animals, planktonic Candida was cleared from cage fluid in 25% (infected with 4.5 \times 10^3 CFU/cage), 8% (infected with 4.8 \times 10^4 CFU/cage) and 0% (infected with 6.2 \times 10^5 CFU/cage). Candida biofilm persisted on all explanted cages. Compared to untreated controls, caspofungin reduced the number of planktonic C. albicans to 0.22 and 0.0 CFU/ml, respectively, and anidulafungin to 0.11 and 0.13 CFU/ml, respectively. Fluconazole cured 2/12 cages (17%), amphotericin B and anidulafungin 1/12 cages (8%) and caspofungin 3/12 cages (25%).

Conclusion: Echinocandins showed superior activity against planktonic C. albicans. Caspofungin showed the highest cure rate of C. albicans biofilm. However, no antifungal exceeded 25% cure rate, demonstrating the difficulty of eradicating Candida biofilms from implants.

© 2015 The British Infection Association. Published by Elsevier Ltd. All rights reserved.
Introduction

*Candida* spp. rarely causes prosthetic joint infections (PJI), causing about 1–3% of infections. However, *Candida* is considered a difficult-to-treat microorganism in implant-associated infection and a two-stage exchange of the prosthesis with a long interval between explantation and implantation of a new prosthesis (usually 6–10 weeks) is recommended in order to eradicate the infection. Little is known about the optimal antifungal treatment of *Candida* PJI. In vitro experiments suggest that microorganisms are considerably more resistant to antifungals than their planktonic counterparts. In a recent in vitro study, fluconazole activity against biofilm *Candida* was reduced by >1000-fold compared to planktonic counterparts, whereas echinocandins and amphotericin B mainly preserved their activity in biofilm.

In previous studies, antifungals have been evaluated in animal models with different materials implanted subcutaneously or intraperitoneally in mice, rats or guinea pigs. In this study we investigated the activity of antifungal agents (fluconazole, amphotericin B, caspofungin and anidulafungin) against planktonic and biofilm *Candida albicans* in an established animal model of foreign-body infection, the guinea-pig tissue-cage infection model. This model has been validated for testing the activity of antimicrobial agents against bacterial implant-associated infections.

To our knowledge, this is the first evaluation of antifungal treatment against *C. albicans* in this foreign-body infection model.

Materials and methods

Study organism

*C. albicans* (ATCC 90028) was used for in vivo antifungal testing. The strain was stored at −80 °C by use of a cryovial bead preservation system (Roth, Karlsruhe, Germany). *C. albicans* was cultured on Sabouraud dextrose agar (SDA) for 24 h at 37 °C. The inoculum was prepared by McFarland and the exact quantity of organisms was determined by performing quantitative cultures.

Antifungal agents

Fluconazole was obtained in liquid form (2000 mg/l, Teva Pharma, Aesch, Switzerland). Amphotericin B (Sigma, St Louis, MO, USA) and caspofungin (Merck & Co., Inc. Whitehouse station, NJ, USA) were obtained in powder form and dissolved in sterile water. Anidulafungin was kindly provided in powder form by Pfizer Pharma AG (Zurich, Switzerland) and dissolved according to the manufacture instructions.

In vitro antifungal susceptibility

The minimal inhibitory concentration (MIC) was determined by microbroth dilution. Antifungal susceptibility of planktonic *C. albicans* was determined by microbroth dilution method according to the EUCAST guidelines. 100 µl of a final concentration of 1–5 × 10² CFU/ml in RPMI-1640 (Roswell Park Memorial Institute) were added to 100 µl of a serial two-fold dilutions of each antifungal previously prepared. Plates were subsequently incubated at 37 °C for 24 h and read by spectrophotometer at 530 nm. The MIC was defined as the lowest antifungal concentration inhibiting 50% of growth, except for amphotericin B, where growth inhibition of ≥90% was considered. Experiments were performed in triplicates.

Animal model

A foreign-body infection model in guinea pig was used, as described previously. In brief, male albino guinea pigs (Charles River, Sulzfeld, Germany) were kept in the Animal Facility of the University of Lausanne, Switzerland. The experiments were performed according to the regulations of Swiss veterinary law. Guinea pigs were weighed every week to ensure their well-being. Animals were anesthetized with a subcutaneous injection of ketamine and xylazine. Four sterile polytetrafluorethylene (Teflon) cages (32 × 10 mm) with 130 regularly spaced perforations of 1 mm diameter (Angst-Pfister, Zürich, Switzerland) were subcutaneously implanted in flanks of the guinea pigs (450–550 g) under aseptic conditions. Two weeks after implantation, cage fluid was aspirated to confirm sterility. Contaminated cages were excluded from further studies.

Infection profile

Cages were infected by percutaneous inoculation of 200 µl of *C. albicans* containing 4.5 × 10³ CFU/cage (low inoculum), 4.8 × 10⁵ CFU/cage (intermediate inoculum) and 6.2 × 10⁶ CFU/cage (high inoculum) in order to determine the optimal infection inoculum. The infection was confirmed by aspiration of the cage fluid and quantification of the culture on SDA plates. Planktonic *Candida* was quantified in aspirated cage fluid on day 1, 2, 3 and 6 (in CFU/ml), and clearance rate (in %) in cage fluid was determined. On day 6, the animals were sacrificed and the cages were aseptically removed and cultured in 5 ml Sabouraud dextrose broth (SDB) for 48 h to determine the spontaneous cure rate of *Candida* biofilm (in %). Aliquots of 100 µl were spread on a Sabouraud plates and incubated at 37 °C for additional 48 h to evaluate the biofilm presence.

Pharmacokinetic studies

Cage fluid was aspirated in uninfected animals during 48 h (1, 2, 4, 8, 24 and 48 h) following intraperitoneal administration of a single dose of fluconazole (8 and 16 mg/kg), caspofungin (1 and 2.5 mg/kg) and anidulafungin (6 and 12 mg/kg). For each antifungal dose three guinea pigs were used (i.e. 12 cages). At each time point, 150 µl aliquots of cage fluid were aspirated from one cage from each animal (three replicates per time point and drug dose). Contaminated cages were excluded from further studies. The collected fluid was centrifuged (4500 rpm for 5 min at 4 °C) and the supernatant was stored at −20 °C until further analysis.
Pharmacokinetic analysis was performed using a liquid chromatography tandem mass spectrometry assay (LC tandem MS assay).

Pharmacokinetic parameters were calculated for each animal: \( C_{\text{max}} \) was defined as the maximum concentration observed, \( T_{\text{max}} \) was defined as the time needed to achieve the maximum concentration, \( C_{\text{min}24} \) was defined as the concentration measured at 24 h, \( C_{\text{min}48} \) was defined as the concentration measured at 48 h, \( \text{AUC}_{0-24} \) and \( \text{AUC}_{0-48} \) (area under the curve) were estimated by trapezoidal method after 24 h and 48 h, respectively. Variability of PK parameters was expressed as mean ± standard deviation. Antifungals concentration profiles were plotted using mean of each sampling time per group, with errors bars representing standard deviation (SD).

**Antifungal treatment in animals**

For treatment studies animals were infected with \( 4 \times 10^3 \) CFU/cage. Antifungal treatment started 12 h after infection. Cage fluids were aspirated and plated for quantitative analysis, followed by the antifungal treatment. Three animals, each animal holding 4 cages (i.e., 12 cages/treatment regimen), received one of the following treatment regimens based on pharmacokinetic studies: control group (no antifungal treatment); fluconazole (16 mg/kg); amphotericin B and caspofungin (2.5 mg/kg) and anidulafungin (20 mg/kg). All antifungals were administered intraperitoneally every 24 h for 4 days. The antifungal dose was chosen based on pharmacokinetic studies performed in previously reported studies on rats, mice, guinea pigs and humans.

To determine the activity of antifungals against *C. albicans*, cage fluid was aspirated before (to confirm the presence of infection), during and 10 days after treatment. The fungal counts were expressed as \( \log_{10} \) CFU/ml cage fluid. To determine the activity against *C. albicans* biofilm, animals were sacrificed 10 days after treatment and the cages were explanted under aseptic conditions and incubated for 48 h in 5 ml of SDB after vigorous vortexing. After 48 h, tubes were vigorously vortexed again and 100 \( \mu l \) were spread on a blood agar plate and incubated at 37°C for additional 48 h and assessed for fungal growth.

**Statistical analysis**

Comparisons were performed by using the Mann–Whitney U test for continuous variables. For all test differences were considered significant when \( P \) values were <0.05. Figures were plotted with GraphPad Prism (version 6.01) software (GraphPad Software, La Jolla, CA).

**Results**

**In vitro antifungal susceptibility**

The MIC values of *C. albicans* obtained by microbroth dilution were 0.25 \( \mu g/ml \) for fluconazole, 0.25 \( \mu g/ml \) for caspofungin, 0.03 \( \mu g/ml \) for anidulafungin and 1 \( \mu g/ml \) for amphotericin B.

**Infection profile**

Fig. 1 represent planktonic *C. albicans* in cage fluid after infection with \( \approx 10^3 \) CFU/cage (A), \( \approx 10^5 \) CFU/cage (B) and \( \approx 10^6 \) CFU/cage (C). A spontaneous progressive reduction of the planktonic counts of *Candida* from tissue cage fluid was observed during 6 days with all inocula. On day 6 (just before explantation), *C. albicans* was cleared from 3 of 12 cage fluids (25%) with low inoculum of \( \approx 10^3 \) CFU/cage and from 1 of 12 (8%) cage fluids with intermediate inoculum of \( \approx 10^5 \) CFU/cage. No clearance was observed.
with the high inoculum of \(\approx 10^6\) CFU/cage. After explanta-
tion of the cages one week after infection, no spontaneous
cure of the cage infection was observed for independent
of inoculum size, i.e. all cages were culture-positive for
Candida indirectly proving the presence of biofilm. No
sign of skin inflammation or perforation of the cage was
seen during the infection profile.

Pharmacokinetic studies

Fig. 2 shows the concentration—time profile in cage fluid
after the administration of a single intraperitoneal dose
in non-infected animals. Table 1 summarizes the calcu-
lated pharmacokinetic parameters. The \(C_{\text{max}}\) of flucona-
zole after the administration of a single intraperitoneal
dose of 8 mg/kg and 16 mg/kg were 3.64 mg/l and

**Table 1**

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>MIC (mg/l)</th>
<th>MHIC (mg/l)</th>
<th>Dose (mg/kg)</th>
<th>(C_{\text{max}}) (mg/l)</th>
<th>(C_{\text{min}}) (mg/l)</th>
<th>(T_{\text{max}}) (h)</th>
<th>AUC(_{0\rightarrow24}) (h * mg/l)</th>
<th>AUC(_{0\rightarrow48}) (h * mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole</td>
<td>0.25</td>
<td>256</td>
<td>8</td>
<td>3.64 ± 0.10</td>
<td>0.01 ± 0.01</td>
<td>1.14 ± 0.05</td>
<td>6.67 ± 0.07</td>
<td>6.67 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.20</td>
<td>16</td>
<td>9.07 ± 0.10</td>
<td>0.02 ± 0.01</td>
<td>0.23 ± 0.01</td>
<td>16.16 ± 0.07</td>
<td>16.16 ± 0.07</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>0.25</td>
<td>2</td>
<td>1</td>
<td>0.32 ± 0.00</td>
<td>0.05 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>24 ± 0.00</td>
<td>24 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.15</td>
<td>2.5</td>
<td>1.41 ± 0.05</td>
<td>0.22 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>11.31 ± 0.00</td>
<td>11.31 ± 0.00</td>
</tr>
<tr>
<td>Anidulafungin</td>
<td>0.03</td>
<td>0.04</td>
<td>5</td>
<td>0.15 ± 0.05</td>
<td>0.04 ± 0.00</td>
<td>0.10 ± 0.00</td>
<td>6 ± 0.00</td>
<td>6 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.02</td>
<td>12</td>
<td>1.07 ± 0.01</td>
<td>0.04 ± 0.00</td>
<td>0.06 ± 0.01</td>
<td>2.64 ± 0.01</td>
<td>2.64 ± 0.01</td>
</tr>
</tbody>
</table>

a Values are means ± SDs from three animals.

*Please cite this article in press as: Maiolo EM, et al., Antifungal activity against planktonic and biofilm Candida albicans in an experimental model of foreign-body infection, J Infect (2015), http://dx.doi.org/10.1016/j.jinf.2015.12.008*
9.07 mg/l, respectively, which were achieved at 6.7 h after dosing. At 8 mg/kg, the fluconazole maximum concentration in the cage fluid reached 14X the MIC of the tested organism (0.25 mg/l), whereas at 16 mg/kg it reached 36X the MIC. The fluconazole concentrations remained above the MIC for 24 h (Cmin24, 1.14 and 2.90 mg/L for doses of 8 and 16 mg/kg, respectively) and decreased below the MIC at 48 h at 8 mg/kg (Cmin48, 0.20 mg/l). Nevertheless, the fluconazole concentrations were below the MHICb.

The Cmax of caspofungin after the administration of a single intraperitoneal dose of 1 and 2.5 mg/kg were 0.32 mg/l and 1.41 mg/l, respectively, which were achieved at 24 h and 16 h, respectively. The maximum concentration in the cage fluid was above the MIC of C. albicans (0.25 mg/l) but below the MHICb at both doses. The maximum concentration reached 1X and 2.8X the MIC at 24 h (Cmin24, 0.32 and 1.40 mg/l for doses of 1 and 2.5 mg/kg, respectively). Concentrations were below the MIC at 48 h at 1 mg/kg (Cmin48, 0.15 mg/l).

The Cmax of anidulafungin after the administration of a single intraperitoneal dose of 6 and 12 mg/kg were 0.15 mg/l and 0.22 mg/l respectively, which were achieved at 6.0 h and 13.3 h, respectively. At 6 mg/kg, the maximum concentration in the cage fluid reached 5X the MIC (0.03 mg/l). The maximum concentration reached 7X the MIC at 12 mg/kg. The anidulafungin concentrations remained above the MIC for 48 h at 1 mg/kg (Cmin48, 0.04 and 0.06 mg/l for doses of 6 and 12 mg/kg, respectively) but below the MHICb.

**Antifungal treatment in animals**

Cage fluid sterility was confirmed prior to infection. At 12 h after infection, the median (±SD) concentration of the yeast in the cage fluid was 10^3 CFU/ml (2.73 ± 0.68 log10 CFU/ml) in control animals receiving no drug, fungal counts in cage fluid were 2.22 ± 0.8 log10 and 0.70 ± 1.17 log10 CFU/ml after 4 and 14 days, respectively, which correspond to decrease of 0.51 and 2.03 log10 CFU/ml, respectively. No spontaneous cure of the cage-associated infection occurred in the untreated animals. Compared to untreated control, fluconazole and amphotericin B did not reduce planktonic C. albicans during and after treatment, whereas caspofungin reduced the numbers to 0.22 ± 0.51 and 0 CFU/ml and anidulafungin to 0.11 ± 0.38 and 0.13 ± 0.46 CFU/ml cage fluid (Fig. 3). No spontaneous cure of the cage infection occurred in the untreated controls (Fig. 4), whereas fluconazole cured 2 of 12 cages (17%), amphotericin B and anidulafungin 1 of 12 cages (8%) and caspofungin 3 of 12 cages (25%). No statistically significant differences were observed between treatment regimens.

**Discussion**

The biofilm formation in Candida spp. is increasingly recognized as a significant clinical problem, especially in transplant, oncology and intensive care medicine.20 Implant-associated infections caused by yeasts are characterized by high complexity and treatment challenges often due to concomitant antifungal resistance and limited therapeutic options against Candida biofilms. Data on optimal
antimicrobial and surgical management of implant-associated infections caused by Candida spp. are limited.\textsuperscript{20} In most of the cases, explanation of the device is performed, followed by long-term antifungal treatment. However, the outcome is often characterized with relapses, persistent infection and need of multiple radical surgical interventions.\textsuperscript{3}

Azoles, the most studied antifungal agents, especially fluconazole, demonstrates low activity against Candida biofilms.\textsuperscript{21–24} In an in-vitro study sessile yeast cells could grow, proliferate and form biofilms after 1 h of adherence despite the presence of high concentrations of fluconazole up to 1024 mg/L.\textsuperscript{25} In a time-kill study, fluconazole showed lacking ability to eradicate Candida biofilm, whereas caspofungin and amphotericin B deoxycholate showed good activity over 48 h.\textsuperscript{26} In several studies echinocandins showed superior in vitro activity against Candida biofilms than azoles,\textsuperscript{27,28} as was also showed in a recent study using a microcalorimetry assay.\textsuperscript{5}

In this study we evaluated the in vivo activity of antifungals in an established foreign-body model using guinea pigs, both against planktonic and biofilm C. albicans. Several interesting observations were made. During the study of the infection profile of C. albicans in untreated animals, a spontaneous decrease of the number of inoculated planktonic C. albicans in the cage fluid was observed. When using a low inoculum, planktonic Candida was completely cleared from the cage fluid in 25% of the cages. However, when infected cages were explanted, Candida was detected in all cage cultures independently on the inoculum size (low, intermediate or high) used. This observation supports the hypothesis that Candida switch from planktonic into biofilm form when a foreign body is present in order to persist on the surface of the cages. Since there was no spontaneous eradication of Candida biofilms, this model is suitable to test the activity of individual antifungals against biofilms. Imaging studies could be performed in the future in order to investigate the fungal biofilm architecture in the in-vivo setting, however, these analyses were out of the scope of this study focusing on antifungal treatment.

The pharmacokinetics of tested antifungals was characterized to determine the appropriate dosing and administration intervals. After 24 h of administration, the concentration of all four tested antifungals in the cage fluid was above the MIC of the test organism, whereas after 48 h with fluconazole at 8 mg/kg and caspofungin at 1 mg/kg the concentrations in cage fluid were below the MIC. Therefore, the once-daily doing was chosen for further experiments. Furthermore, we observed that the antifungal concentrations found in the cage fluid at 24 and 48 h were under the MHIC\textsubscript{B}, which could partially explain our results. A limitation of the study was that the pharmacokinetics of amphotericin B could not be performed due to absence of an analytic assay at our institution. However, the dose of amphotericin B used was based on literature and on previous studies performed in our laboratory.

In treatment studies, fluconazole and amphotericin B did not reduce planktonic C. albicans in the cage fluid during and after treatment and showed limited anti-biofilm activity with cure rates from 8% to 17%. In contrast, caspofungin and anidulafungin had a superior activity against planktonic Candida in the cage fluid. Against C. albicans biofilm, anidulafungin exhibited similar activity than amphotericin B (cure rate 8%), whereas caspofungin showed superior activity against C. albicans biofilm (cure rate 25%).

The observed antifungal activity is in general lower than the one of antibacterial substances against Staphylococcus aureus, Escherichia coli, Enterococcus faecalis or Propionibacterium acnes\textsuperscript{13–16} using the same foreign-body infection model. This fact underlines that Candida remains a difficult-to-treat organisms and removal of a device with staged concept of re-implantation seems to be the rational treatment strategy. Other treatment strategies, such as novel antifungals, combination therapies or mechanical (e.g. sonication), biological (e.g. phages) or chemical (e.g. enzymes) biofilm removal strategies may improve the treatment outcome and make the retention and salvage of an infected prosthesis possible.

In conclusion, caspofungin and anidulafungin showed superior activity against planktonic C. albicans compared to amphotericin B and fluconazole at physiological doses. No antifungal drug administered alone achieved cure rates above 25%, demonstrating the difficulty of eradicating Candida biofilms from implants. In further studies, higher doses of the antifungals, their combinations or addition of a non-pharmacological approach that may improve the treatment outcome should be evaluated.

Funding

This study was supported by an educational grant from Pfizer Pharma GmbH (Nr. W178378), from the Berlin Institute of Health (BIH) and the PRO-IMPLANT Foundation.

Conflict of interest

None.

Acknowledgments

We thank Bertrand Betrisey for helping with the animal experiments and Laurent Decosterd and his team for the pharmacokinetic analysis. Part of the results of this study was presented at the 23\textsuperscript{rd} European Congress of Clinical Microbiology and Infectious Diseases, Berlin, Germany, 27 to 30 April 2013 [E. Maiolo, U. Furustrand Tafin, A. Trampuz, abstr. P-1097] and at the 32\textsuperscript{nd} meeting of the European Bone and Joint Infection Society, Prague, Czech Republic, 12 to 14 September 2013 [E. Maiolo, U. Furustrand Tafin, O. Borens, A. Trampuz, oral presentation].

References

3. Kuiper JW, van den Bekerom MP, van der Steffen J, Holte PA, Colen S. 2-stage revision recommended for treatment of fungal
Antifungal activity against planktonic and biofilm Candida


Please cite this article in press as: Maiolo EM, et al., Antifungal activity against planktonic and biofilm *Candida albicans* in an experimental model of foreign-body infection, *J Infect* (2015), http://dx.doi.org/10.1016/j.jinf.2015.12.008