In vivo activity of micafungin in a persistently neutropenic murine model of disseminated infection caused by Candida tropicalis

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Micafungin is a new echinocandin with broad-spectrum in vitro and in vivo antifungal activity against both Aspergillus and Candida species. We compared the activity of micafungin with that of amphotericin B and fluconazole in a persistently immunocompromised murine model of disseminated candidiasis against a strain of Candida tropicalis that was resistant to amphotericin B and fluconazole in vitro. Mice were rendered persistently neutropenic with multiple doses of cyclophosphamide and infected intravenously with C. tropicalis. Mice were treated with either intraperitoneal amphotericin B (0.5–5 mg/kg per dose), oral fluconazole (50 mg/kg twice a day), intravenous micafungin (1–10 mg/kg per dose) or solvent control for 7 days. Mice were killed at 11 days post-infection and kidneys, lungs, brain and liver removed for quantitative culture. Overall mortality in the model was low, with rates varying between 10% and 25% in treatment groups. Micafungin at doses between 2 and 10 mg/kg were the only regimes able to reduce cfu below the level of detection of tissues infected with C. tropicalis. Micafungin was well tolerated by the mice and was much more effective than amphotericin B or fluconazole against an amphotericin B- and fluconazole-resistant C. tropicalis.

Keywords: micafungin, murine, Aspergillus

Introduction

Invasive disease caused by Candida has increased dramatically over the past three decades and is now a commonplace complication of many surgical and medical therapies. Candidaemia accounts for 0.5% of all medical and surgical discharges. Recently there has been a substantial increase in the frequency of candidaemia caused by species other than Candida albicans.1–4 Increases in Candida tropicalis (4–24% of all candidaemias) have been noted particularly from blood cultures from leukaemic, oncology and intensive care unit patients.

Options for treatment of invasive fungal disease have been limited until recently to the polyenes and azoles, with the possible addition of flucytosine. Use of polyenes is limited by drug toxicity (particularly nephrotoxicity with amphotericin B) even though this has been reduced with the introduction of lipid preparations of the drug.

Increased resistance or tolerance to antifungal agents amongst pathogenic yeasts is being recognized worldwide in theazole group of drugs5 with higher attributable mortality in isolates that are resistant to antifungal agents.6 Echinocandins with their novel mode of activity and lack of cross-resistance offer the possibility of additional options in the treatment of fungal disease.

In this study, we examined micafungin (FK463) in a persistently immunocompromised murine model of disseminated candidiasis against an amphotericin B- and fluconazole-resistant C. tropicalis and compared its efficacy with that of fluconazole and amphotericin B.

Materials and methods

Test strain

C. tropicalis MY1012 was provided by J. Rex (Division of Infectious Diseases, University of Texas-Houston Medical
In vitro susceptibility testing

Microdilution susceptibility tests of amphotericin B and fluconazole were carried out in vitro as recommended by the NCCLS to determine the MIC (using RPMI 1640 for fluconazole and antibiotic medium 3 for amphotericin B). Minimum fungicidal concentrations (MFCs) were determined by culturing 100 µL from each well in the microdilution plate that had no visible growth; the MFC was taken as the first well with <5 cfu. The susceptibility tests for micafungin were also performed as described in NCCLS M27-A; in brief the final blastoconidia inoculum in the test wells was 0.5 × 10^3 cfu/mL, the culture medium was RPMI plus 2% glucose buffered with MOPS. Microdilution plates were incubated for 48 h at 37°C and the MIC was recorded as the first well to have an 80% reduction in optical density measured at 490 nm when compared to a drug-free control well.

Animals

All mice included in this study were part of ongoing studies carried out under UK Home Office project licence PPL/40/1523 entitled Invasive Fungal Infections. Data are combined from two separate (but identical) experiments.

Male CD1 mice, weighing between 22 and 25 g (Charles River UK Ltd, Margate, UK) were virus-free and were allowed free access to food and water. Mice were randomized into groups of 10. A total of 20 mice were used in each treatment group and 40 mice in the controls (10 mice per treatment group and 20 controls per experiment).

Immunosuppression

Cyclophosphamide (Sigma-Aldrich) was administered intravenously (iv) at 200 mg/kg 3 days before infection. Four days and 8 days after the initial dose the mice were re-immunosuppressed with 200 mg/kg: profound neutropenia occurred 3 days after initial administration and continued for the entire experimental procedure.

Preparation of inoculum

For each experiment, the isolate was thawed and then incubated for 2 days on Sabouraud dextrose agar. One colony was transferred into 25 mL of Sabouraud dextrose broth and incubated on an orbital mixer for 8 h at 37°C, washed twice in saline, then finally resuspended in saline and the density adjusted by spectrophotometry at 490 nm.

Infection of mice

Before the experiment, inoculum finding studies were carried out using iv injections of 0.2 mL of a range of fungal densities. The dose selected was the highest dose of organisms that could be administered without rapid death caused by toxic shock. The inoculum for this isolate producing severe but non-fatal disease was 6 × 10^6 cfu/mL. The inoculum was administered 6 h before the start of treatment.

Antifungal therapy

Micafungin (Fujisawa, Osaka, Japan) in a 25 mg vial was dissolved in 5% glucose (1.67 mg/mL) and kept in the dark at all times. This was further diluted to 0.83, 0.33 and 0.17 mg/mL (stored in the dark at all times). The 1.67 mg/mL solution was stored at 4°C for up to 96 h before use. Micafungin (10, 5, or 1 mg/kg per day) was administered iv (0.15 mL) once daily.

Fungizone (ER Squibb & Sons Ltd, Hounslow, UK) 50 mg amphotericin B was reconstituted in 5% glucose (stored at 4°C for up to 7 days before use). This was further diluted with 5% glucose for use. Amphotericin B (5 or 0.5 mg/kg per dose) was given via intraperitoneal injection (0.15 mL) on days 1, 2, 4 and 7.

Fluconazole (Pfizer Ltd, Sandwich, UK) was dissolved in sterile saline plus 0.03% Noble agar (Oxoid) to provide a 50 mg/kg dose. Fluconazole was prepared daily immediately before use and administered by gavage (0.15 mL) once daily.

Control mice were infected but received no active treatment. Groups received either 5% glucose iv or saline plus 0.03% agar by gavage.

Experimental endpoints

Mice were examined four times daily. Any infected animals with severely reduced mobility, unable to reach the drinker or otherwise in substantial distress, were humanely terminated. Attention was paid to postural changes, torticollis and staggering or staining of the anal region, as these were indicators of imminent deterioration. Mice had their temperatures taken by an infra-red gun at least twice daily and were culled if their temperature dropped below 33.3°C. On day 11 of the experiment, all surviving mice were humanely terminated and frozen at −20°C.

Organ culture

Immediately before dissection, mice were thawed. The kidneys, liver, brain and lungs were removed aseptically and transferred into 2 mL of sterile PBS (BDH, Poole, UK). The organs were homogenized in a tissue grinder (Polytron; Kinematica AG, Lucerne, Switzerland) and colony counts determined using serial 10-fold dilutions plated on the surface of the plates. The plates were incubated at 37°C and examined.
daily for 5 days. This method detected *C. tropicalis* at >30 cfu/organ.

**Statistical analysis**

Culture data were analysed with the Kruskal–Wallis test (pairwise comparison Conover-Inman) using the computer package StatsDirect (Ashwell, UK).

**Results**

**In vitro susceptibility data**

Both the MIC and MFC of amphotericin B were >16 mg/L. Both the MIC and MFC of fluconazole were >128 mg/L. The MIC of micafungin was 0.03 mg/L and the MFC 0.125 mg/L.

**In vivo response**

Mice became ill ∼48 h after infection but in most cases the disease did not develop beyond moderate illness. Mice that either died or were humanely terminated showed reduced mobility and a hunched appearance. After this initial period of moderate morbidity the surviving mice gradually improved and most mice showed few signs of severe disease by day 11. Control mice had 20% mortality, demonstrating the low overall mortality of this model. In the treatment arms of the study, the overall mortality was also low, with rates varying between 10% and 25%. Mice treated with micafungin (particularly the higher doses) tended to improve most rapidly and regained weight lost at an earlier stage of infection.

**Organ counts**

Geometric mean colony counts of the lungs, kidneys, brain and liver are shown in Table 1. The only regimes to eradicate *C. tropicalis* below detectable levels from all organs were micafungin 2–10 mg/kg, which cleared up to 25% of the mice.

The lung, kidney and liver burden after micafungin treatment showed a dose-dependent pattern of reduction. Only in the kidneys did this reach statistical significance with the 10 and 5 mg/kg doses being significantly superior to 1 mg/kg (*P* = 0.002).

All doses of micafungin were superior to amphotericin B in all organs. Micafungin 10 mg/kg was superior to fluconazole in all organs. Other doses of micafungin tended to be superior to fluconazole but all did not reach statistical significance.

**Discussion**

Micafungin was well tolerated with no obvious side effects. When administered at doses of 1 mg/kg or higher, micafungin was highly effective (in a dose-dependent fashion) at reducing organ burden in disseminated sepsis caused by *C. tropicalis* in this persistently neutropenic mouse model. Furthermore, higher doses of micafungin (2–10 mg/kg) were the only treatment regimes able to reduce *C. tropicalis* cfu in organs below detectable levels and were much more effective than amphotericin B or fluconazole. As the mice were persistently neutropenic throughout the course, and the isolate was resistant to multiple antifungal agents, this is an important result demonstrating the efficacy of micafungin in the absence of a white blood cell response. This isolate has now been shown to be resistant to amphotericin B and fluconazole both in vitro and in vivo.

Increasingly there are reports of resistance to flucytosine and the azole antifungals (including the second generation triazoles, which are not yet licensed for clinical use), along with issues of toxicity associated with the polyenes. These

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**Table 1. Organ burdens—mean organ counts and number of animals with cfu below detectable levels in all organs post-*C. tropicalis* MY1012 infection and treatment**

<table>
<thead>
<tr>
<th>Geometric mean counts</th>
<th>Micafungin (mg/kg)</th>
<th>Amphotericin B (mg/kg)</th>
<th>Fluconazole 50 mg/kg</th>
<th>Control regimes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>lungs</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;1</td>
<td>17</td>
</tr>
<tr>
<td>liver</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8</td>
</tr>
<tr>
<td>kidneys</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5212</td>
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<tr>
<td>brain</td>
<td>189&lt;sup&gt;d&lt;/sup&gt;</td>
<td>357&lt;sup&gt;d&lt;/sup&gt;</td>
<td>263&lt;sup&gt;d&lt;/sup&gt;</td>
<td>378</td>
</tr>
<tr>
<td>Survival to day 11 (%)</td>
<td>17/20 (85)</td>
<td>17/20 (85)</td>
<td>18/20 (90)</td>
<td>16/20 (80)</td>
</tr>
<tr>
<td>No cfu (%)</td>
<td>5/17 (30)</td>
<td>3/17 (18)</td>
<td>6/18 (33)</td>
<td>0/16 (0)</td>
</tr>
</tbody>
</table>

<sup>a</sup>*P* < 0.0001 versus amphotericin B 0.5 mg/kg.

<sup>b</sup>*P* < 0.001 versus amphotericin B (both doses).

<sup>c</sup>*P* < 0.001 versus micafungin 1 mg/kg, amphotericin B (both doses), fluconazole and controls.

<sup>d</sup>*P* < 0.0001 versus amphotericin B and controls, and *P* < 0.01 versus fluconazole.
factors make the development of new antifungal drugs essential and the echinocandins offer a new target for treatment of fungal infections. Resistance to echinocandins in clinical isolates of Candida has not yet been identified, with the possible exception of Candida guilliermondii and Candida parapsilosis.9,10 Echinocandin-resistant laboratory isolates have been generated but these isolates are either of reduced virulence or are still treatable in in vivo models.10 Tolerance, however, may be an issue, although it requires validation in animal models or in clinical settings in which it could be important, such as Candida endocarditis.10

The data presented here again demonstrate the efficacy of micafungin in the treatment of invasive murine candidiasis and warrant further investigation of this agent.

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References


