Introduction

Zygomycetes are opportunistic pathogens capable of causing acute infections in humans. Among them, some of the predominant organisms causing disease are Rhizopus spp., Mucor spp. and Absidia spp. Despite the introduction of several new antifungal drugs over the past decade, options for the treatment of zygomycosis remain limited. Amphotericin B is the drug of choice and has been the only effective agent. It is associated with improved outcome in human and animal zygomycosis,1–3 although morbidity and mortality remain high.

The use of azole antifungal agents for the treatment of zygomycosis is not supported by clinical data and has barely been evaluated in in vivo models. In vitro, this group of antifungals is inefficacious with most, but not all, zygomycetes,2,4–6 and in vivo against Rhizopus spp.2,7,8

Compared with some zygomycetes, Absidia spp. have been shown to be much more susceptible to azoles in vitro;4–6 however, no data in vivo are available. Absidia spp. cause some cases of human zygomycosis, generally in immunocompromised hosts, and they are the most frequent agents of zygomycosis among lower animals. In this study we tested and compared in vitro and in vivo susceptibility data for amphotericin B and itraconazole for the first time against Absidia corymbifera murine infection.

Materials and methods

Two A. corymbifera strains were studied in vitro and used in a new murine model of disseminated disease. Isolate CM-537 was obtained from a bone biopsy of a 41-year-old male and isolate CM-1074 was from the nasal biopsy of a 47-year-old male diabetic who had received a kidney transplant.

Male CD-1 mice, 4–5 weeks old and weighing between 18 and 20 g were purchased from Charles River UK Ltd (Margate, UK) and randomized into groups of 10. Animals were immunosuppressed with cyclophosphamide (Sigma–Aldrich, Poole, UK) administered intravenously via the lateral tail vein to all animals at a dose of 200 mg/kg. A state of profound neutropenia was achieved 3 days after administration and lasted for 4 days.

The isolates were grown on Sabouraud dextrose agar (Oxoid, Basingstoke, UK) plus chloramphenicol (Sigma) (SAB/H11001C) for 10 days. The spores were harvested in 25 mL of sterile phosphate-buffered saline–0.05% Tween 80 (Sigma) (PBS–Tween), with the flask shaken gently, stored at 4°C and used within 3 days. On the day of infection, the stock solution was adjusted to an inoculum that would give an LD90 (4.5 × 10^4 and 1.1 × 10^5 cfu/mL for CM-537 and CM-1074, respectively), according to preliminary studies based on viability counts. Three days after immuno-suppression all animals were infected with 0.15 mL of the LD90 suspension, via the tail vein (day 0). The inoculum was rechecked from the remaining conidial suspension after the animals were infected.

Deoxycholate amphotericin B and itraconazole were dissolved in 5% dextrose and an aqueous solution of 2-hydroxypropyl-β-cyclodextrin, respectively, as described previously.3 Doses employed were 5 and 0.5 mg/kg for amphotericin B and 75 and 25 mg/kg for itraconazole.

© 2001 The British Society for Antimicrobial Chemotherapy
Amphotericin B was administered via ip injection (0.15 mL) od at 24, 48 and 96 h and 7 days post-infection. Itraconazole was given by gavage (0.15 mL) tds on days 1 and 2 and bd on days 3–7.

A separate group of four uninfected, cyclophosphamide pre-treated mice, was treated with the 75 and 25 mg/kg itraconazole doses and then serum was collected and analysed on day 4, 3 h after the morning dosage, by bioassay, as described previously.

On day 11 of the experiment, all surviving mice were humanely killed. The lungs, brain, liver and kidneys were removed and transferred into 2 mL of PBS–Tween and homogenized in a tissue grinder (Polytron, Kinematica AG, Luzern, Switzerland) for 15–30 s and then diluted 10-fold. One hundred microlitres of the neat and diluted suspensions were then transferred to SAB + C plates and the liquid spread over the surface of the plates. Histology was not done. Plates were incubated at 37°C and colony counts were recorded from all plates that showed growth in 3 days.

Mortality and culture data were analysed by the Mann–Whitney U-test or the Kruskall–Wallis test if all values in one group were identical. Two-sided P values are given. Mice that died before day 10 were assumed to have organ counts at least as high as the highest counts in surviving mice in the calculation of culture result statistics. All data analysis was carried out with the computer package Arcus Quik Stat (Addison Wesley Longman Ltd).

In vitro susceptibility testing was carried out with a methodology derived from published work from our laboratory that validated in vivo a method of detecting itraconazole resistance in Aspergillus fumigatus but failed to do so for amphotericin B. Two final inocula were compared. The main differences from the NCCLS recommendations were that: itraconazole was dissolved at a stock concentration of 3200 mg/L in acetone and hydrochloric acid and then diluted down in medium; doubling dilutions instead of batch dilutions of the drug were used; spores were harvested by shaking the inoculated flask gently; the inoculum suspension densities were counted with an improved Neubauer haemocytometer; the final inoculum in the wells was 5 × 10² or 5 × 10³ cfu/mL; microdilution plates were incubated at 37°C; the MICs were read visually and were defined as the lowest drug concentration with no growth.

Results

Mortality in each in vivo experiment is shown in the Figure and the Table. Both strains caused a lethal infection in mice in the inoculum-finding studies. The mortality rates in the control groups were high (80–100%). The infection was rapidly fatal. At the end of the experimental period of 11 days, only liver and kidney were still infected in surviving animals, according to organ colony counts. The serum concentration of itraconazole in the 75 mg/kg group was 26.2 mg/L and a mean of 6.5 mg/L in the 25 mg/kg group by bioassay, which overestimates concentrations compared with more precise high-pressure liquid chromatography (HPLC) measurements of itraconazole and hydroxyitraconazole.

Amphotericin B

In our murine model, both A. corymbifera strains were responsive to amphotericin B. Mortality was 0% and 10% at 5 and 0.5 mg/kg, respectively, with both strains (P ≈ 0.0004 versus controls). CM-537 was inhibited by MICs of 0.12 and 0.25 mg/L at 5 × 10³ and 5 × 10² cfu/mL, respectively, and CM-1074 by 0.25 mg/L with both inocula. The highest control counts were from the liver, and were quite low from kidneys. For both strains counts were significantly lower than in the controls with both amphotericin B treatments for liver (CM-537 P < 0.0133; CM-1074 P < 0.0008).

Itraconazole

Itraconazole was less effective than the lower amphotericin B dose in the in vivo experiments with both strains. A dose effect of itraconazole was observed. Mortality was 40% at
Amphotericin B and itraconazole versus *A. corymbifera*

**Table.** Organ culture results for *A. corymbifera* CM-537 and CM-1074

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survivors/ no. in group (%)</th>
<th>Geometric mean cfu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sterilized lungs</td>
</tr>
<tr>
<td>CM-537 (itraconazole MIC 2–4 mg/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Itraconazole 75 mg/kg</td>
<td>4/10 (40)</td>
<td>0</td>
</tr>
<tr>
<td>Itraconazole 25 mg/kg</td>
<td>0/10 (0)</td>
<td>0</td>
</tr>
<tr>
<td>Amphotericin B 5 mg/kg</td>
<td>10/10 (100)</td>
<td>6</td>
</tr>
<tr>
<td>Amphotericin B 0.5 mg/kg</td>
<td>9/10 (90)</td>
<td>1</td>
</tr>
<tr>
<td>Glucose</td>
<td>0/10 (0)</td>
<td>0</td>
</tr>
<tr>
<td>Cyclodextrin</td>
<td>2/10 (20)</td>
<td>0</td>
</tr>
<tr>
<td>CM-1074 (itraconazole MIC 1–2 mg/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Itraconazole 75 mg/kg</td>
<td>6/10 (60)</td>
<td>0</td>
</tr>
<tr>
<td>Itraconazole 25 mg/kg</td>
<td>4/10 (40)</td>
<td>1</td>
</tr>
<tr>
<td>Amphotericin B 5 mg/kg</td>
<td>10/10 (100)</td>
<td>3</td>
</tr>
<tr>
<td>Amphotericin B 0.5 mg/kg</td>
<td>9/10 (90)</td>
<td>0</td>
</tr>
<tr>
<td>Glucose</td>
<td>0/10 (0)</td>
<td>0</td>
</tr>
<tr>
<td>Cyclodextrin</td>
<td>1/10 (10)</td>
<td>0</td>
</tr>
</tbody>
</table>

**Note:**

- Control amphotericin B.
- Control.

75 mg/kg and 60% at 25 mg/kg with isolate CM-1074 (itraconazole MIC 1 mg/L) (*P* =0.0353 versus controls), and 60% and 100% with isolate CM-537 (MIC 2 mg/L) (not significant versus controls), respectively. Itraconazole MICs varied somewhat depending on the inoculum employed. The higher the inoculum used, the higher the MICs. MICs for CM-537 were 0.5 and 2 mg/L and for CM-1074 were 0.25 and 1 mg/L, at $5 \times 10^3$ and $5 \times 10^5$ cfu/mL of final inocula, respectively.

For both strains, counts were not significantly lower than in the controls in any itraconazole treatment groups for any organ except liver with itraconazole at 25 mg/kg in the CM-1074 model (*P* = 0.0436). For both liver and kidneys in the CM-1074 model counts were higher with the higher itraconazole dose.

**Discussion**

To our knowledge this is the first study on the *in vivo* efficacy of amphotericin B or anyazole against experimental *A. corymbifera* infections. Colony forming units may under-represent disease, if data from human infections are considered. Other *in vivo* efficacy data for any of both antifungals on zygomycetes are only available for *Rhizopus* spp. These were neutropenic and non-neutropenic mice, guinea pigs or rabbits, disseminated or pulmonary infection models. *In vitro* susceptibility testing has been little used and no methodologies have been validated with either amphotericin B or itraconazole, to detect resistance in zygomycetes.

Amphotericin B is an effective drug against *A. corymbifera* infection in this model. It has *in vitro* activity against *Absidia* spp. We obtained low amphotericin B MICs for both strains (0.12–0.25 mg/L). This is consistent with our *in vivo* results. In other mice models of *Rhizopus* spp. infection, amphotericin B was also effective, although to a lesser extent with lower doses.

Itraconazole was not efficacious against *Rhizopus* infection in guinea pig and murine models, and neither were other azaoles, ketoconazole, fluconazole or saperconazole in a guinea pig model. These data are consistent with the high MICs determined *in vitro* for that species with itraconazole, ketoconazole, miconazole and saperconazole. Nevertheless,azole MICs were low for *Absidia* spp. in the same studies. Goldani & Sugar reported the triazole SCH 42427 to be active in a murine model of pulmonary *Rhizopus oryzae* infection. In our animal model of *A. corymbifera* infection, itraconazole treatment was inferior to amphotericin B, although the response was better than with the control group. This correlates with the intermediate response observed *in vitro*. The highest MICs were associated with the less responsive strain, although differences in MICs were small and overall efficacy mediocre.

**Acknowledgements**

This work was supported by the European Commission Training and Mobility of Researchers grant FMRX-CT970145 Eurofung and the Fungal Research Trust.
References


Received 2 January 2001; returned 6 April 2001; revised 29 May 2001; accepted 5 July 2001