In vitro activity of miltefosine as a single agent and in combination with voriconazole or posaconazole against uncommon filamentous fungal pathogens

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Objectives: Antifungal treatment of uncommon filamentous fungal infections is problematic. This study determined the in vitro susceptibility of miltefosine, as a single agent and in combination with posaconazole or voriconazole, against these pathogens.

Methods: Susceptibility to miltefosine of 34 uncommon filamentous fungi was tested using CLSI broth microdilution M38-A2 methodology. Twenty isolates were studied for potential synergy using miltefosine/posaconazole and miltefosine/voriconazole combinations and the chequerboard microdilution assay.

Results: MICs of miltefosine were high (in general, >8 mg/L) for most isolates compared with amphotericin B, echinocandins and the azoles. Miltefosine had greatest activity against Scedosporium spp., Lichtheimia corymbifera and Rhizomucor sp. (MICs ≤ 4 mg/L). Miltefosine in combination either with posaconazole or voriconazole demonstrated synergy [fractional inhibitory concentration index (FICI) ≤ 0.5] in 12 instances (11 isolates): miltefosine/posaconazole combinations were synergistic against 3 of 4 Fusarium oxysporum strains (FICI range 0.37–0.5) and 5 of 10 mucormycete strains (FICI range 0.06–0.5). The combination of voriconazole with miltefosine showed synergy against one Scedosporium prolificans isolate and three mucormycetes—a single strain each of L. corymbifera, Rhizopus oryzae and Rhizomucor sp. No antagonism was observed.

Conclusions: Miltefosine demonstrated synergy in 8/20 (40%) and 4/20 (20%) instances when combined with posaconazole and voriconazole, respectively. Synergy was most often observed against F. oxysporum and the mucormycetes. Study of miltefosine/azole combinations as a novel antifungal approach is indicated.

Keywords: susceptibility, synergy, mucormycetes

Introduction

Infections due to uncommon but emerging filamentous fungal pathogens are difficult to treat. Many are resistant/less susceptible to current antifungals.1 Novel antifungal treatment approaches remain central to improving therapeutic outcomes. Combination antifungal therapy with different, including new, drug classes based on the premise of synergistic effects is one approach used with good clinical response.2–4 However, in vitro data identifying effective combinations of antifungals against uncommon filamentous fungi are relatively few.

The antileishmanial agent miltefosine (hexadecylphosphocholine) has fungicidal activity against Scedosporium, Fusarium and the mucormycetes (previously zygomycetes).5 Here, we investigated the (i) in vitro susceptibility of uncommon filamentous fungi to miltefosine and (ii) potential synergy/additive effects of voriconazole and posaconazole in combination with miltefosine. Voriconazole has activity against certain Fusarium and Scedosporium species, whilst posaconazole is used to treat mucormycoses.5,6,7

Materials and methods

Thirty-four filamentous fungal isolates were studied: Aspergillus flavus ATCC 204304, Aspergillus fumigatus ATCC 204305 and 32 clinical isolates (8 Aspergillus, 8 Fusarium, 6 Pseudallescheria/Scedosporium species and 10

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mucormycetes) obtained from the Centre for Infectious Diseases and Microbiology, Westmead Hospital, New South Wales, Australia. All isolates were identified using phenotypic methods and sequencing of the fungal rRNA gene (internal transcribed spacer region and/or 28S ribosomal subunit) or elongation factor 1α gene. A. flavus ATCC 204304 and A. fumigatus ATCC 204305 were the quality control strains in each test run. Inoculum suspensions were prepared according to the CLSI M38-A2 protocol and adjusted to an optical density (at 530 nm) of 0.09–0.13 for A. flavus and 0.15–0.17 for all other isolates.

**Sensititre testing**

MICs of voriconazole and posaconazole (and the other antifungal drugs in this commercial assay) were determined using Sensititre YeastOne plates (YO10; Trek Diagnostic System, ThermoScientific, Australia). The drug combinations studied were miltefosine/voriconazole and miltefosine/posaconazole. Based on the respective drug MICs for each isolate, the final drug concentration ranges were 0.06–16 mg/L for miltefosine, 0.06–64 mg/L (0.14–157 μM) for voriconazole and posaconazole (0.08–22.8 μg/mL) for anidulafungin and caspofungin.

**Miltefosine synergy testing**

Miltefosine (final concentration range 0.5–256 mg/L) MICs were determined according to the CLSI M38-A2 protocol. MICs were read visually after 48–72 h. The MIC was the lowest concentration demonstrating 100% growth inhibition. Minimum fungicidal concentration (MFC) was determined using the equation (MIC drug A in combination/MIC drug A alone) and (MIC drug B in combination/MIC drug B alone). FICI values of 0.5 were indicative of antagonism and values of >0.5–4 were indicative of no interaction.

**Antifungal synergy testing**

Twenty isolates (10 mucormycete strains, 8 Fusarium spp. and 2 Scedosporium spp.) were tested using the CLSI chequerboard broth microdilution method. The drug combinations studied were miltefosine/voriconazole and miltefosine/posaconazole. Based on the respective drug MICs for each isolate, the final drug concentration ranges were 0.06–16 mg/L for posaconazole (0.08–22.8 μM) and voriconazole (0.17–45.8 μM), and 0.06–64 mg/L (0.14–157 μM) for miltefosine (data not shown). Serial 2-fold dilutions of miltefosine and voriconazole or posaconazole were placed into 96-well plates in a vertical and horizontal direction, respectively. Fifty microliters of each dilution of the respective drugs were dispensed; 100 μL of inoculum containing 0.4–5 × 10³ spores/mL was dispensed into each well. The fractional inhibitory concentration index (FICI) was calculated using the equation (MIC drug A in combination/MIC drug A alone) + (MIC drug B in combination/MIC drug B alone). FICI values of ≤0.5 were indicative of synergy, values of >4 were indicative of antagonism and values of >0.5–4 were indicative of no interaction. The assay was performed twice for each isolate.

**Results**

**Single drug susceptibilities**

The geometric mean (GM) MICs and MIC ranges of miltefosine, amphotericin B, posaconazole, voriconazole, itraconazole, caspofungin and anidulafungin for 34 clinical isolates are shown in Table 1. The miltefosine MICs for Aspergillus species were ~4-fold higher than those of the other test drugs (GM MIC 5.28 mg/L; Table 1). Of 8 Fusarium isolates, Fusarium oxysporum strains had a higher miltefosine GM MIC than Fusarium solani (13.45 versus 8 mg/L; Table 1). For Scedosporium apiospermum strains, the GM MIC of miltefosine was similar to that of amphotericin B, but higher than those of voriconazole and posaconazole (Table 1). Miltefosine had a lower

**Table 1. GMIC (MIC range) (in mg/L) of miltefosine and other antifungal agents for 34 clinical isolates of filamentous fungi**

<table>
<thead>
<tr>
<th>Strains (no. of isolates)</th>
<th>Miltefosine</th>
<th>Amphotericin B</th>
<th>Anidulafungin</th>
<th>Caspofungin</th>
<th>Voriconazole</th>
<th>Itraconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus spp. (8)</td>
<td>5.17 (2–16)</td>
<td>1.25 (0.25–4)</td>
<td>3.17 (0.25–8)</td>
<td>3.17 (0.25–8)</td>
<td>0.12 (0.013–0.12)</td>
<td>0.12 (0.006–0.12)</td>
</tr>
<tr>
<td>Fusarium spp. (8)</td>
<td>10.37 (2–16)</td>
<td>1.51 (0.25–4)</td>
<td>3.17 (0.25–8)</td>
<td>3.17 (0.25–8)</td>
<td>0.12 (0.006–0.12)</td>
<td>0.12 (0.006–0.12)</td>
</tr>
<tr>
<td>Fusarium oxysporum (4)</td>
<td>1.51 (0.25–4)</td>
<td>3.17 (0.25–8)</td>
<td>3.17 (0.25–8)</td>
<td>3.17 (0.25–8)</td>
<td>0.12 (0.006–0.12)</td>
<td>0.12 (0.006–0.12)</td>
</tr>
<tr>
<td>Scedosporium spp. (2)</td>
<td>1.51 (0.25–4)</td>
<td>3.17 (0.25–8)</td>
<td>3.17 (0.25–8)</td>
<td>3.17 (0.25–8)</td>
<td>0.12 (0.006–0.12)</td>
<td>0.12 (0.006–0.12)</td>
</tr>
<tr>
<td>Rhizopus spp. (1)</td>
<td>10.37 (2–16)</td>
<td>1.51 (0.25–4)</td>
<td>3.17 (0.25–8)</td>
<td>3.17 (0.25–8)</td>
<td>0.12 (0.006–0.12)</td>
<td>0.12 (0.006–0.12)</td>
</tr>
<tr>
<td>Rhizomucor spp. (1)</td>
<td>15.17 (2–16)</td>
<td>1.51 (0.25–4)</td>
<td>3.17 (0.25–8)</td>
<td>3.17 (0.25–8)</td>
<td>0.12 (0.006–0.12)</td>
<td>0.12 (0.006–0.12)</td>
</tr>
</tbody>
</table>

**ND, not determined. For Aspergillus species were ~4-fold higher than those of the other test drugs (GM MIC 5.28 mg/L; Table 1). Of 8 Fusarium isolates, Fusarium oxysporum strains had a higher miltefosine GM MIC than Fusarium solani (13.45 versus 8 mg/L; Table 1). For Scedosporium apiospermum strains, the GM MIC of miltefosine was similar to that of amphotericin B, but higher than those of voriconazole and posaconazole (Table 1). Miltefosine had a lower
MIC for one *Scedosporium prolificans* strain compared with amphotericin B (8 to >8 mg/L) and posaconazole (8 mg/L).

Among mucormycetes, overall, miltefosine MICs were ≥4 mg/L, higher than those of amphotericin B and posaconazole; susceptibility varied with genus. *Rhizopus* spp. had the highest MICs, with GM values 5–60-fold greater than those of posaconazole and voriconazole (25.39 versus 0.39 and 5.65 mg/L, respectively). Miltefosine MICs were lowest for *Lichtheimia corymbifera* and *Rhizomucor* sp. (Table 1).

For all isolates, the MFCs of miltefosine were the same as the corresponding MICs (data not shown).

### Synergy testing

Twenty strains (Table 2) with relatively high MICs of miltefosine, posaconazole and voriconazole (Table 1) were chosen for synergy studies. *Aspergillus* spp. were not further studied as the voriconazole and posaconazole MICs were low.

Synergy of miltefosine with posaconazole was observed for three *F. oxysporum* strains (isolates CB-9, CB-11 and CB-12; FICIs of 0.37, 0.5 and 0.5, respectively; Table 2). The MICs of posaconazole fell 10–100-fold (from 0.5 to 0.06 mg/L) for strain CB-9 and 4-fold (16 to 4 mg/L) for strains CB-11 and CB-12. Miltefosine and voriconazole were synergistic against one strain of *S. prolificans*; indifferent effects (FICIs of 1–2) were generally observed using the miltefosine/azole combinations (Table 2).

Miltefosine/posaconazole combinations demonstrated synergy against an *L. corymbifera* strain, two *Rhizopus oryzae* isolates and two *Mucor* isolates (5 of 10 mucormycetes; Table 2). For both *R. oryzae* isolates, the miltefosine MICs were reduced 8–100-fold and those of posaconazole were reduced 5–10-fold. When miltefosine was tested with voriconazole, isolate *R. oryzae* CB-30 demonstrated synergy, as did *L. corymbifera* CB-23 and *Rhizomucor* sp. CB-32.

No other synergistic combinations were observed and no antagonism was demonstrated for any isolate with either drug combination.

### Discussion

Despite the lack of robust supportive data, combination antifungal therapy, which in theory enables each drug to be effective at lower drug concentrations, is increasingly used to treat invasive fungal infections. Here, we show that miltefosine has *in vitro* activity against *Scedosporium* spp. and certain mucormycetes. A key finding was the *in vitro* synergy against 55% of 20 isolates tested, particularly amongst *F. oxysporum* and mucormycetes, when miltefosine was combined with posaconazole (eight instances) or voriconazole (four instances). The MICs of individual drugs were lowered substantially.

*Fusarium* spp. are resistant to most antifungals, with reports showing relatively lower MICs of amphotericin B, voriconazole and posaconazole, though susceptibility varies with species. We observed amphotericin B to be the most active agent against both *F. oxysporum* and *F. solani*. MICs of 8–16 mg/L (Table 1) suggest

### Table 2. Synergy of miltefosine/posaconazole and miltefosine/voriconazole combinations against 20 filamentous fungal pathogens

<table>
<thead>
<tr>
<th>Isolates (identification no.)</th>
<th>MIL MIC (mg/L)</th>
<th>POS MIC (mg/L)</th>
<th>MIL MIC (mg/L)</th>
<th>VRC MIC (mg/L)</th>
<th>MIL + POS</th>
<th>MIL</th>
<th>VRC</th>
<th>MIL + VRC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>alone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>combined&lt;sup&gt;b&lt;/sup&gt;</td>
<td>alone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>combined&lt;sup&gt;b&lt;/sup&gt;</td>
<td>FICI</td>
<td>alone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>combined&lt;sup&gt;b&lt;/sup&gt;</td>
<td>alone&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>F. oxysporum</em> (CB-9)</td>
<td>16</td>
<td>4</td>
<td>0.5</td>
<td>0.06</td>
<td><strong>0.37</strong></td>
<td>16</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td><em>F. oxysporum</em> (CB-10)</td>
<td>16</td>
<td>8</td>
<td>2</td>
<td>0.25</td>
<td><strong>0.62</strong></td>
<td>16</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td><em>F. oxysporum</em> (CB-11)</td>
<td>8</td>
<td>2</td>
<td>16</td>
<td>4</td>
<td><strong>0.5</strong></td>
<td>8</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td><em>F. oxysporum</em> (CB-12)</td>
<td>16</td>
<td>4</td>
<td>16</td>
<td>4</td>
<td><strong>0.5</strong></td>
<td>16</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td><em>F. solani</em> (CB-13)</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>0.06</td>
<td>1</td>
<td>8</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td><em>F. solani</em> (CB-14)</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>0.06</td>
<td>1</td>
<td>8</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td><em>F. solani</em> (CB-15)</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>0.06</td>
<td>1</td>
<td>8</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td><em>F. solani</em> (CB-16)</td>
<td>8</td>
<td>8</td>
<td>16</td>
<td>0.06</td>
<td>1</td>
<td>8</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td><em>S. prolificans</em> (CB-21)</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>0.06</td>
<td>1</td>
<td>8</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td><em>S. prolificans</em> (CB-22)</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>0.06</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td><em>L. corymbifera</em> (CB-23)</td>
<td>4</td>
<td>2</td>
<td>0.25</td>
<td>0.06</td>
<td>0.74</td>
<td>4</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td><em>L. corymbifera</em> (CB-24)</td>
<td>4</td>
<td>2</td>
<td>16</td>
<td>0.06</td>
<td><strong>0.50</strong></td>
<td>4</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td><em>Mucor circinelloides</em> (CB-25)</td>
<td>8</td>
<td>8</td>
<td>1</td>
<td>0.06</td>
<td>1</td>
<td>8</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td><em>Mucor indicus</em> (CB-26)</td>
<td>8</td>
<td>4</td>
<td>16</td>
<td>0.06</td>
<td><strong>0.50</strong></td>
<td>8</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td><em>Mucor racemosus</em> (CB-27)</td>
<td>8</td>
<td>0.06</td>
<td>1</td>
<td>0.06</td>
<td><strong>0.06</strong></td>
<td>8</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td><em>Mucor ramossissimus</em> (CB-28)</td>
<td>8</td>
<td>8</td>
<td>1</td>
<td>0.06</td>
<td>1</td>
<td>8</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td><em>Rhizopus microsporus</em> (CB-29)</td>
<td>8</td>
<td>4</td>
<td>0.25</td>
<td>0.06</td>
<td>0.36</td>
<td>32</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td><em>R. oryzae</em> (CB-30)</td>
<td>32</td>
<td>4</td>
<td>0.25</td>
<td>0.06</td>
<td><strong>0.36</strong></td>
<td>32</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td><em>R. oryzae</em> (CB-31)</td>
<td>64</td>
<td>0.06</td>
<td>1</td>
<td>0.125</td>
<td><strong>0.13</strong></td>
<td>64</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td><em>Rhizomucor</em> sp. (CB-32)</td>
<td>2</td>
<td>2</td>
<td>0.12</td>
<td>0.25</td>
<td>3.08</td>
<td>2</td>
<td>0.25</td>
<td>4</td>
</tr>
</tbody>
</table>

MIL, miltefosine; POS, posaconazole; VRC, voriconazole.

<sup>a</sup>Single drug MICs.

<sup>b</sup>Individual drug MICs obtained in combination in checkerboard assays.

<sup>c</sup>FICI values in bold indicate synergy.
that miltefosine is an unlikely antifusarial candidate. Against Fusarium spp., data are conflicting regarding synergy between combinations of marketed antifungals, e.g. the azoles and amphotericin B, but may reflect species differences. We observed synergy between miltefosine and posaconazole against three of four F. oxysporum isolates, but not against F. solani. The data suggest that miltefosine/posaconazole combinations have potential for study as a novel treatment for F. oxysporum infections.

S. prolificans is resistant to all licensed antifungal agents, although voriconazole is often incorporated into the antifungal regimen to treat such infections. In the present study, miltefosine MICs for S. prolificans were 2–4-fold lower than those of posaconazole, voriconazole and itraconazole. We also observed synergy between miltefosine and voriconazole against a single S. prolificans isolate. The combination of voriconazole and terbinafine, which often demonstrate synergy in vitro, has been used successfully to treat S. prolificans infections. Anecdotal reports of success using miltefosine to treat such infections in combination with other antifungals are supported by our finding that certain strains may exhibit synergy with voriconazole.

In this study, amphotericin B was the most potent drug against mucormycetes (MICs < 0.5 mg/L for most strains), while posaconazole had the greatest activity amongst the azoles. Amphotericin B formulations are preferred for intensive initial treatment of mucormycosis, with a switch to posaconazole suppressive therapy after an initial clinical response. Although miltefosine MICs were high for most mucormycete isolates, L. corymbifera and a Rhizomucor were more susceptible. Further, azole/miltefosine synergy was observed in seven instances (Table 2), with 5 of 10 isolates exhibiting voriconazole/miltefosine synergistic interactions. Interestingly, synergy was seen with the posaconazole/miltefosine combination against both R. oryzae isolates, which demonstrated the highest miltefosine MICs. Given the current uncertainty concerning the in vivo activity of miltefosine, it is important for the in vitro synergy to be replicated by animal and clinical data in order to use the posaconazole/miltefosine combination as an alternative to polyene therapy. This combination approach may alleviate difficulties in achieving target posaconazole blood levels due to variable bioavailability by enabling posaconazole to be effective at lower concentrations.

The mechanism by which miltefosine could synergize with the azoles is uncertain. While the triazoles, posaconazole and voriconazole, inhibit fungal lanosterol 14α-demethylase, miltefosine, a membrane phospholipid (phosphatidylcholine) analogue, enhances fungal membrane permeability (C. Biswas, unpublished results) and targets fungal phospholipase B1 enzymes and the yeast mitochondrial subunit COX9. The different mechanistic effects of miltefosine and the triazoles on the fungal cell membrane could be exploited in developing future therapeutic strategies. In conclusion, antifungal combinations of posaconazole or voriconazole with the novel antifungal agent miltefosine are synergistic against some strains of drug-resistant fungi, most notably the mucormycetes. These combinations may be of value in clinical therapy.

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References


