In vitro antifungal susceptibility of filamentous fungi causing rare infections: synergy testing of amphotericin B, posaconazole and anidulafungin in pairs

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Objectives: Mucormycetes (formerly known as zygomycetes of the order Mucorales) and hyaline moulds such as those of the genus Fusarium or Paecilomyces are emerging as significant human pathogens. The aim of the study was to determine the in vitro antifungal susceptibility of these fungi to older and newer antifungals and to investigate the antifungal activity of amphotericin B, posaconazole and anidulafungin in dual combinations.

Methods: Twenty-one clinical isolates of mucormycetes and 16 of rare hyaline moulds were tested. MICs were determined by EUCAST methodology for conidia-forming moulds and Etesting. For antifungal combinations a chequerboard method based on EUCAST methodology was used.

Results: Against mucormycetes, amphotericin B exhibited the lowest MICs, followed by posaconazole. Ravuconazole was active against eight of the Rhizopus isolates (MIC 1 mg/L). Resistance to amphotericin B (MIC ≥ 2 mg/L) and posaconazole (MICs ≥ 4 mg/L) was observed in five and three Rhizopus isolates, respectively. Among Fusarium species variable susceptibility patterns were detected. Amphotericin B exhibited the lowest MICs, followed by voriconazole. Etesting for amphotericin B and posaconazole had excellent agreement with EUCAST methodology (78.6%–100%). Synergy between amphotericin B and anidulafungin was observed against two isolates (one Mucor circinelloides and one Fusarium proliferatum). Synergy or antagonism was not detected in any other combination.

Conclusions: The study showed that mucormycetes and other rare hyaline moulds exhibit variable susceptibilities to antifungals, and hence antifungal testing is valuable. The fact that the combination of amphotericin B with anidulafungin was found synergistic in some cases merits further investigation.

Keywords: mucormycetes, zygomycetes, Mucorales, Fusarium, hyaline moulds, chequerboard

Introduction

Recently developed antifungal drugs offer the potential to improve management and therapeutic outcomes of rare emerging invasive mycoses. Combination therapy with antifungals belonging to different classes could have a synergistic effect and has been used for difficult-to-treat infections. In vitro testing is helpful in defining the activity spectrum of an antifungal agent. However, data about the in vitro combination of antifungals against mucormycetes or other rare moulds are scarce.

The aim of this study was to investigate: (i) the in vitro susceptibility of rare moulds to different classes of antifungals; (ii) the potential synergy or antagonism of amphotericin B, posaconazole and anidulafungin when combined in pairs; and (iii) the performance of a user-friendlier susceptibility method, such as the Etest, compared with the standard broth microdilution method.

Materials and methods

The material comprised 37 clinical isolates: 21 mucormycetes and 16 hyalohyphomycetes. The isolates were recovered during the period

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2005–09, from an equal number of patients from collaborating hospitals of the broader area of Athens. Mucormycosis cases were classified as proven or probable according to the revised European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (‘EORTC/MSG’) criteria. For the study of hyalohyphomycetes, isolates from superficial infections such as keratitis were also included.

Molecular identification was carried out at the Mycology Reference Centre, National Centre for Microbiology, Madrid, Spain, in the framework of the European Confederation of Medical Mycology (ECMM) working group collaboration for the registry of zygomycosis in Europe. Fusarium spp. isolates recovered after 2008 were molecularly identified at the Università degli Studi di Milano, Milan, Italy, in the framework of the ECMM survey of fusariosis in Europe. Part of the molecular identification of mucormycetes was carried out at Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, by one of us (M. D.-A.), and three of the strains were deposited at this collection under accession numbers: Lichtheimia ramosa CBS 124667, Rhizopus arhizus CBS 124668 and Rhizopus microsporus CBS 124669.

**Broth microdilution antifungal testing**

The EUCAST methodology was applied. For amphotericin B and the azoles the MIC was determined, whereas for the echinocandins (anidulafungin, micafungin and caspofungin) the minimum effective concentration (MEC) was evaluated microscopically, as described previously.

**Antifungal synergy testing**

Antifungal synergy was tested for 24 of the strains (14 Mucorales, 7 Fusarium spp., 1 Paecilomyces lilacinus, 1 Paecilomyces variotii and 1 Trichoderma longibrachiatum) using the EUCAST broth microdilution method in a checkerboard fashion: serial 2-fold dilutions of drugs A and B were dispensed into a 96-well microtitre plate, in the vertical and horizontal direction, respectively. The volume of dilutions of each drug dispensed was 50 μL, to yield 100 μL per well. Each well was inoculated with 100 μL of the inoculum suspension containing 2 × 10^5–5 × 10^6 cfu/mL. Final drug dilutions ranged from 0.03 to 8 mg/L. MICs of individual drugs alone were determined on the same plate (last row and column). The combinations studied were: amphotericin B/posaconazole, amphotericin B/anidulafungin and anidulafungin/posaconazole. For the combinations with anidulafungin the MEC was evaluated. Tests were performed at least twice. Aspergillus fumigatus ATCC 204305 and Aspergillus flavus ATCC 204304 were used as controls. Synergy was interpreted by calculating the fractional inhibitory concentration indexes (FICIs) using the equation: FICI = (Ac/Aa) + (Bc/Ba) where Ac and Bc are the MICs of drugs A and B in combination, and Aa and Ba are the MICs of drugs alone. FICI values ≤0.5 are considered to indicate synergism, FICI values >4 indicate antagonism and values 0.5–4 are indicative of no interaction.

**Etest**

The Etest was applied for amphotericin B and posaconazole against 14 Mucorales and 8 hyalohyphomycetes using RPMI-agar plates buffered with MOPS and 2% glucose as described previously. Etest strips for voriconazole, posaconazole, anidulafungin and micafungin were kindly donated by the manufacturers. All others were purchased from AB Biodisk, Sweden. Plates were read after 48–72 h, depending on fungal growth. Agreement was defined as the percentage of strains showing difference within two consecutive dilutions when compared with the EUCAST method. For the purpose of calculation, values >4 mg/L were converted to 8 mg/L.

**Results**

**Identification**

The species distribution of the Mucorales was: 13 R. arrhizus (formerly known as Rhizopus oryzae), 3 R. microsporus, 1 Rhizopus stolonifer, 2 Mucor circinelloides and 2 L. ramoss. The hyalohyphomycetes were: 10 Fusarium spp. (4 Fusarium oxysporum, 2 Fusarium solani, 2 Fusarium verticillioides, 1 Fusarium proliferatum and 1 Fusarium sp.), 4 Paecilomyces spp. (3 P. lilacinus and 1 P. variotii) and 2 T. longibrachiatum.

**Antifungal susceptibility testing**

Against the mucormycetes, the geometric mean of amphotericin B MICs was 0.52 mg/L and the median value was 0.25 mg/L (Table 1). The geometric mean and median values of posaconazole MICs were slightly higher (0.75 and 0.5 mg/L, respectively). Five of the 17 Rhizopus strains were resistant to amphotericin B (MIC ≥2 mg/L). Posaconazole MICs were ≥4 mg/L for three strains, of which one was also resistant to amphotericin B.

Against the Fusarium strains, amphotericin B had the lowest MICs, but values varied between species, the lowest being observed against two F. solani strains and 1 F. verticillioides strain (0.25, 0.50 and 0.25 mg/L, respectively). The second most potent drug was voriconazole. Intracconazole, ravuconazole, terbinafine and the echinocandins had no effect on any of the Fusarium strains. Anidulafungin results shown in Table 1 are representative of all three echinocandins tested.

Of the Paecilomyces strains, only P. variotii was susceptible to amphotericin B (MIC 0.06 mg/L). The P. lilacinus isolates were susceptible to the azoles voriconazole, posaconazole and ravuconazole, and terbinafine. Against the Trichoderma isolates only voriconazole and the echinocandins exhibited low MICs.

**Synergy testing**

Synergy of amphotericin B with anidulafungin was observed against one M. cirrhelloides and one F. proliferatum strain (FICI = 0.31 and 0.27, respectively; Table 1). No other combination showed synergy against any of the strains, and in no case was antagonism observed.

**Etest**

Against 11 of 14 mucormycetes tested, amphotericin B MICs were within two 2-fold dilutions when compared with the broth microdilution test (78.6% agreement) (Table 2). For posaconazole, agreement was 100%. When testing Fusarium species, agreement was 100% for both drugs.

**Discussion**

In this study amphotericin B was the most potent drug against mucormycetes, with MICs lower than 0.5 mg/L for most of the strains tested. However, variability was observed, as five Rhizopus strains showed resistance.

Posaconazole is considered the second most potent drug against mucormycetes. In our study posaconazole had the highest activity among the azoles, with a median MIC value of...
0.5 mg/L. The true meaning of this MIC in clinical practice is unknown.

Itraconazole exhibits reportedly some antifungal activity against certain genera of mucormycetes. In our study one Lichtheimia and one R. arrhizus strain were susceptible to itraconazole.

As expected, all of our mucormycetes were resistant to voriconazole, terbinafine and the echinocandins. Ravuconazole had MICs ≤1 mg/L for some strains.

Fusarium species are usually resistant to most antifungals; in vitro studies have shown lower MICs of amphotericin B, nystatin, ketoconazole, voriconazole and posaconazole for these fungi. Moreover, variable susceptibility among the different species has been reported. Two of our F. solani strains were resistant to all azoles, but susceptible to amphotericin B. The F. verticillioides strains displayed variable susceptibility to the azoles, with lower MICs of voriconazole and posaconazole. The F. oxysporum strains were more resistant.

### Table 1. Susceptibility testing (MICs in mg/L) and synergy of antifungals against mucormycetes and rare hyaline moulds

<table>
<thead>
<tr>
<th>Organism</th>
<th>AMB</th>
<th>VRC</th>
<th>ITC</th>
<th>POS</th>
<th>RAV</th>
<th>TBF</th>
<th>AND</th>
<th>FICI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhizopus spp. (n=17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>median</td>
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<td>&gt;8</td>
<td>0.5</td>
<td>1</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>2a</td>
</tr>
<tr>
<td>range</td>
<td>0.25–0.29</td>
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<td>0.5–0.25</td>
<td>&gt;8</td>
<td>0.5–0.25</td>
<td>&gt;8</td>
<td>1–&gt;8</td>
<td>1.5–3</td>
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<tr>
<td>L. ramosa</td>
<td>0.25</td>
<td>6</td>
<td>&gt;8</td>
<td>0.5</td>
<td>&gt;8</td>
<td>8</td>
<td>&gt;8</td>
<td>2</td>
</tr>
<tr>
<td>L. ramosa</td>
<td>0.25</td>
<td>3</td>
<td>0.38</td>
<td>ND</td>
<td>ND</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>2</td>
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<td>0.50</td>
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<td>&gt;8</td>
<td>&gt;8</td>
<td>0.5</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>2</td>
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<tr>
<td>Fusarium spp. (n=9)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>1.5</td>
<td>2</td>
<td>&gt;8</td>
<td>4</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>2b</td>
</tr>
<tr>
<td>range</td>
<td>0.25–0.25</td>
<td>2–&gt;8</td>
<td>0.25–0.25</td>
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<td>&gt;8</td>
<td>&gt;8</td>
<td>1.5–2.5</td>
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<td>F. proliferatum</td>
<td>&gt;8</td>
<td>2</td>
<td>&gt;8</td>
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<td>P. lilacinus (n=3)</td>
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<td>median</td>
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<td>&gt;8</td>
<td>0.25</td>
<td>0.37</td>
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<td>1–&gt;8</td>
<td>0.12–0.5</td>
<td>0.25–0.5</td>
<td>0.5</td>
<td>&gt;8</td>
<td>1.5c</td>
</tr>
<tr>
<td>P. variotii</td>
<td>0.06</td>
<td>3</td>
<td>0.02</td>
<td>0.5</td>
<td>&gt;8</td>
<td>4</td>
<td>&gt;8</td>
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<tr>
<td>T. longibranchiatum</td>
<td>1</td>
<td>1</td>
<td>&gt;8</td>
<td>2</td>
<td>&gt;8</td>
<td>1</td>
<td>0.03</td>
<td>ND</td>
</tr>
<tr>
<td>T. longibranchiatum</td>
<td>2</td>
<td>0.38</td>
<td>&gt;8</td>
<td>&gt;4</td>
<td>8</td>
<td>2</td>
<td>&gt;8</td>
<td>0.52</td>
</tr>
</tbody>
</table>

AMB, amphotericin B; VRC, voriconazole; ITC, itraconazole; POS, posaconazole; RAV, ravuconazole; TBF, terbinafine; AND, anidulafungin; ND, not determined.

*Numbers in bold indicate synergy.

*aResults from 10 Rhizopus strains tested.

*bResults from six Fusarium strains tested.

*cResults from one strain tested.

### Table 2. Comparison between broth microdilution assay and Etest (number of isolates per dilution difference); agreement was defined as the percentage of strains showing a difference within two consecutive dilutions when compared with the EUCAST method

<table>
<thead>
<tr>
<th>Species</th>
<th>Amphotericin B dilution differences</th>
<th>Posaconazole dilution differences</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Mucorales (n=14)</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Fusarium spp. (n=7)</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>
Our P. lilacinus strains were resistant to amphotericin B, itraconazole and the echinocandins, but susceptible to voriconazole, posaconazole, ravuconazole and terbinafine. The P. variotii strain had a completely different profile, with very low MICs of amphotericin B and itraconazole and higher MICs of the other azoles.

Our combination experiments showed synergy against one Mucorales and one *Fusarium* strain when amphotericin B and anidulafungin were combined. The significance of these results is unknown, as only clinical studies can show a potential benefit. No other combination gave synergy, in agreement with others when testing *R. arrhizus* strains. Against *Fusarium* spp. the data are conflicting. Philip et al. found no synergy when combinations of anidulafungin, itraconazole, voriconazole and amphotericin B were tested against seven *Fusarium* isolates, whereas Arikan et al. observed synergy against at least three of six strains using the combination amphotericin B/caspofungin. It is not known whether these differences were due to species variation or the different echinocandins used.

Traditionally, echinocandins have been considered to have no effect on mucormycetes in vitro. However, the combination polyene/caspofungin has shown promising results in treating rhino-orbital-cerebral mucormycosis. The mechanism by which echinocandins could synergize with amphotericin B against mucormycetes is not known. Echinocandins act on the synthesis of (1,3)-β-D-glucan, a fungal cell-wall compound lacking in mucormycetes. However, the genetic mechanism for the synthesis of β-D-glucan apparently exists in Mucorales, as *R. arrhizus* was found to possess a genetic homologue of FKS, the molecular target of echinocandins and a subunit of (1,3)-β-D-glucan synthase. In the case of synergy of amphotericin B with anidulafungin, we can speculate that the structural changes of the fungal membrane caused by amphotericin B could unmask/expose the target molecules for the echinocandin, thus leading to enhancement of its action. Similar mechanisms have been speculated by Guembe et al. on the synergistic effect of the combination posaconazole/caspofungin that they observed against clinical mucormycetes.

In conclusion, our results show that among rare moulds, susceptibility to antifungals is variable. Identification to species level cannot always predict susceptibility, therefore antifungal testing is meaningful, and Etest is an easy to perform and reliable method for the clinical laboratory, at least for amphotericin B and posaconazole. Antifungal combinations may have a synergistic effect against some strains. As in other studies, no case of antagonism was observed, and this is encouraging for deciding to use combination therapy in difficult-to-treat cases.

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**Transparency declarations**

None to declare.

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