Susceptibility profile of amphotericin B and posaconazole against clinically relevant Mucorales in hypoxia

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The effect of hypoxia on the in vitro efficacy of amphotericin B and posaconazole against Mucorales was evaluated by defining minimal inhibitory concentrations (MIC) with Etest® and microbroth dilution, and minimal fungicidal concentrations (MFCs). With Etest® oxygen dependent changes were detected, while MIC and MFC determined with microbroth dilution remained unaltered with reduced oxygen. Observed differences depended on the method used.

Mucormycosis has increased within the last decades in patients at risk and are related with high mortality rates (1-3). Therapy of mucormycosis is complicated because Mucorales are resistant to most antifungal drugs (4) and clinical breakpoints are not available yet. Treatment with lipid formulations of amphotericin B in combination with surgery, if possible, is the first choice, but also adjunctive therapeutic options, such as hyperbaric oxygen therapy have been explored (5-7). At the sites of infection, microenvironmental factors influence fungal growth and most likely also the efficacy of antifungal drugs (8). Hypoxia is one factor occurring during pulmonary fungal infections in vivo (9), and tissue oxygen concentrations can decrease to ≤1% (10, 11).

Simulating host environment in in vitro testing will contribute to a better understanding of how conditions, such as hypoxia, might influence susceptibility to antifungal drugs. Previous studies demonstrated superior end point reading for caspofungin, micafungin (12) and anidulafungin (13) for Aspergillus species in hypoxia. Additionally, amphotericin B MICs were reduced in hypoxia, whereas no changes were detected for itraconazole and micafungin (12). This study is the first focusing on hypoxia-driven changes in susceptibility patterns of Mucorales.
Therefore, the in vitro activity of amphotericin B (Bristol Meyer Squibb, Austria) and posaconazole (Schering-Plough, Kenilworth, NJ) in normoxic and hypoxic conditions were compared for clinically relevant Mucorales by standard methods. All clinical isolates tested (n=56) were identified by ITS sequencing, according to White et al. (14). The strain set comprised: Lichtheimia (L.) corymbifera (n=20), L. ramosa (n=6), Rhizomucor (Rh.) pusillus (n=8), Rhizopus (R.) arrhizus (n=7), R. microsporus (n=12) and Mucor (M.) circinelloides (n=3). Hypoxic conditions were set to 1% O₂, 5% CO₂, 94% N₂ and controlled throughout the duration of experiments (Biospherix C-Chamber & Pro-Ox, Pro-CO₂ controller USA). All experiments were done in parallel in normoxia, which were considered general atmospheric levels (~21% O₂). MICs were determined by agar diffusion method (Etest®, Biomerieux, France) and microbroth dilution according to EUCAST guidelines 9.2 (15) at 24h. For better comparison, Etest® MICs were raised to the next corresponding EUCAST concentration. These methods were chosen to verify the different impact of hypoxia on surface (exposure to actual 1% oxygen) or liquid cultures, where oxygen concentration might vary also in the normoxic cultures. MFCs were determined as described in (12) and defined as lowest drug concentration resulting in 99.9% killing. To check for normal distribution of MICs, the D’Agostino & Pearson omnibus normality test was performed. The Kruskal-Wallis test was applied, as the data were not normally distributed. P values of ≤0.05 were regarded as statistically significant. With Etest®, influence of hypoxia on susceptibility profile occurred in a species- and drug-dependent manner (Table 1). For Lichtheimia spp. differences due to hypoxia were detected, with a shift to lower amphotericin B MICs, which was shown to be statistically significant. Same was observed for M. circinelloides and R. microsporus isolates. Rhizomucor pusillus and R. arrhizus isolates exhibited no significant
changes in antifungal susceptibility to amphotericin B. Statistical comparison of MIC
distributions of posaconazole resulted in no significant differences for all Mucorales.
Reduction of amphotericin B MICs can be partly explained by impaired growth, as
species with decreased MICs showed reduced growth ability in hypoxia, especially
*Lichtheimia* spp. (25–40% growth reduction in colony diameter) and *M. circoelloides*
isolates (35–40% growth reduction in colony diameter). This, in addition to the fact
that ergosterol biosynthesis is oxygen dependent (16), leads to the hypothesis that
Mucorales are confronted with two stressors – antifungal exposure and the
maintenance of cell membrane components - and may further explain the shift to
lower MICs in hypoxia. Additionally to forming pores in fungal membranes,
amphotericin B was previously shown to induce reactive oxygen species in
amphotericin B-susceptible species (17, 18). This effect on mitochondrial respiration
might further contribute to the observed shift to lower MICs for amphotericin B in
hypoxia.

In microbroth dilution assays, MICs of amphotericin B and posaconazole were not
altered in hypoxia for all Mucorales tested (Table 2), and consequently, no significant
differences in MIC distributions were detected. Similarly, no significant oxygen-
dependent differences in MFCs for both antifungal agents were observed (Table 2).
Amphotericin B was shown to be fungicidal in both oxygen conditions, with a close
correlation of MICs and MFCs. The only exception was *L. ramosa*, for which no
MFCs could be determined even though the MIC$_{50}$ was 0.25 µg/ml. Regarding
posaconazole, MFCs exceeded the highest concentration tested (16 µg/ml), except
for *L. corymbifera* and *Rh. pusillus*, for which median MFC was 4 µg/ml or 8 µg/ml,
respectively. Contrary to amphotericin B, no correlation between MICs and MFCs
were observed for posaconazole against all Mucorales.
Various *in vitro* factors such as media, inoculum and temperature are affecting MICs significantly (8, 19). Additionally, hypoxia was shown to have a significant impact on targets of antifungal drugs such as ergosterol biosynthesis and on β-glucan in *A. fumigatus* (16, 20), so one would expect severe hypoxia-driven effects on antifungal activity. Here, in the first study investigating the effect of hypoxia on clinically relevant Mucorales, changes in MICs are in part due to the growth ability of fungal species in hypoxia, and are further influenced by the method of choice. The minor differences in MICs or MFCs in hypoxia closely correlate with own data and already published data of *Aspergillus* spp. and *Candida* spp. (12, 13). Generally, available data on MFCs of Mucorales are limited, therefore, data presented herein, contribute to a better overview of antifungal activity of amphotericin B and posaconazole. The major finding of this study was the discrepancy between posaconazole MICs and MFCs found for all Mucorales species, resulting in the *in vitro* classification of posaconazole as a fungistatic compound against Mucorales *in vitro*.

In conclusion, hypoxia only has a marginal influence on *in vitro* antifungal susceptibility pattern of Mucorales. The minor differences observed, where most pronounced using the Etest® method, which is not the method of choice for MIC determination of Mucorales due to difficulties in MIC reading caused by cottony overgrowth of these fungi.

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All other authors have no conflicts of interest to declare.
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   through cell wall modulation. Microbes Infect 15:259-269.
TABLE 1: *In vitro* susceptibility of amphotericin B and posaconazole against Mucorales species determined by Etest® under normoxic and hypoxic growth conditions.

<table>
<thead>
<tr>
<th>species</th>
<th>antifungal agent</th>
<th>normoxia MIC (µg/ml)</th>
<th>hypoxia MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MIC range</td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td><em>L. corymbifera</em> (n=20)</td>
<td>AMB</td>
<td>0.03-2</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>POS</td>
<td>0.125-32</td>
<td>0.25</td>
</tr>
<tr>
<td><em>L. ramosa</em> (n=6)</td>
<td>AMB</td>
<td>0.25-2</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>POS</td>
<td>0.125-1</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Rh. pusillus</em> (n=8)</td>
<td>AMB</td>
<td>0.03-0.25</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>POS</td>
<td>0.25-0.5</td>
<td>0.5</td>
</tr>
<tr>
<td><em>R. microsporus</em> (n=12)</td>
<td>AMB</td>
<td>0.25-32</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>POS</td>
<td>0.5-32</td>
<td>2</td>
</tr>
<tr>
<td><em>R. arrhizus</em> (n=7)</td>
<td>AMB</td>
<td>0.25-16</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>POS</td>
<td>0.25-2</td>
<td>1</td>
</tr>
<tr>
<td><em>M. circinelloides</em> (n=3)</td>
<td>AMB</td>
<td>0.03-0.06</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>POS</td>
<td>32-32</td>
<td>32</td>
</tr>
</tbody>
</table>

MIC<sub>50</sub>/MIC<sub>90</sub>: MIC causing complete growth inhibition in 50% or in 90% of tested isolates

AMB: amphotericin B
POS: posaconazole
<table>
<thead>
<tr>
<th>species</th>
<th>Antifungal drug</th>
<th>normoxia MIC (µg/ml)</th>
<th>hypoxia MIC (µg/ml)</th>
<th>normoxia MFC</th>
<th>hypoxia MFC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MIC range</td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>MIC range</td>
</tr>
<tr>
<td>L. corymbifera</td>
<td>AMB</td>
<td>0.25-1</td>
<td>0.5</td>
<td>0.5</td>
<td>1-16</td>
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<tr>
<td>(n=20)</td>
<td>POS</td>
<td>0.5-4</td>
<td>1</td>
<td>1</td>
<td>1-16</td>
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<tr>
<td>L. ramosa</td>
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<td>0.25-0.5</td>
<td>0.25</td>
<td>0.25</td>
<td>0.5-4-16</td>
</tr>
<tr>
<td>(n=6)</td>
<td>POS</td>
<td>1-2</td>
<td>1</td>
<td>2</td>
<td>16-16</td>
</tr>
<tr>
<td>Rh. pusillus</td>
<td>AMB</td>
<td>0.25-1</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5-16</td>
</tr>
<tr>
<td>(n=6)</td>
<td>POS</td>
<td>1-16</td>
<td>1</td>
<td>16</td>
<td>1-16</td>
</tr>
<tr>
<td>R. microsporus</td>
<td>AMB</td>
<td>0.25-0.5</td>
<td>0.5</td>
<td>1</td>
<td>0.25-1</td>
</tr>
<tr>
<td>(n=12)</td>
<td>POS</td>
<td>1-2</td>
<td>1</td>
<td>2</td>
<td>16-16</td>
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<tr>
<td>R. arrhizus</td>
<td>AMB</td>
<td>0.25-1</td>
<td>0.5</td>
<td>1</td>
<td>0.5-1</td>
</tr>
<tr>
<td>(n=7)</td>
<td>POS</td>
<td>1-1</td>
<td>1</td>
<td>1</td>
<td>16-16</td>
</tr>
<tr>
<td>M. circinelloidies</td>
<td>AMB</td>
<td>0.125-0.5</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5-1</td>
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<tr>
<td>(n=3)</td>
<td>POS</td>
<td>2-2</td>
<td>2</td>
<td>2</td>
<td>16-16</td>
</tr>
</tbody>
</table>

MIC<sub>50</sub>/MIC<sub>90</sub>, MIC causing complete growth inhibition in 50% or in 90% of tested isolates
MFC, median of the minimum fungicidal concentration
AMB, amphotericin B
POS, posaconazole