The effects of antifungal agents to conidial and hyphal forms of Aspergillus fumigatus

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Susceptibility testing for Aspergillus fumigatus is usually performed using a fungal conidial suspension. However, assessment of the susceptibility of fungal hyphae may be more relevant in attempting to mimic the fungal status in infected tissues. In the present study of 12 A. fumigatus clinical isolates and 1 ATCC strain, the antifungal susceptibilities of conidial suspensions, suspensions of hyphal fragments and of hyphal clumps were determined by the XTT-based broth susceptibility assay measuring decrease in fungal metabolic activity. Amphotericin B inhibited A. fumigatus conidia and hyphal fragments in a sharp concentration-dependent manner, with inhibitory concentrations (ICs) of 1 μg/ml for both fungal structures, whereas, hyphal clumps were inhibited at 8 μg/ml. Conidia and hyphal fragments were inhibited by the azoles itraconazole and voriconazole in a more gradual concentration-dependent manner, with ICs of 0.5 μg/ml for both structures with both agents. Hyphal clumps were not inhibited by the azoles at all. Caspofungin inhibited A. fumigatus in a moderate, neither sharp nor gradual, concentration-dependent manner. ICs for conidia were 128 μg/ml and inhibition in metabolic activity was not obtained for both hyphal growth forms. Antifungal susceptibility of conidia was also determined using the E-test in which it was found that the XTT assay gave comparable ICs for amphotericin B, itraconazole and voriconazole but not for caspofungin.

Keywords Aspergillus fumigatus, antifungal susceptibility, hyphae, XTT

Introduction

Aspergillus fumigatus is an opportunistic pathogen responsible for life-threatening infections in immunocompromised patients [1,2]. The mortality rate of invasive aspergillosis remains high despite recent advances in chemotherapy [3]. For filamentous fungi such as A. fumigatus the predictive in vivo significance of in vitro antifungal susceptibility testing remains unclear. Several in vitro susceptibility tests have been used with A. fumigatus, including the CLSI M38-A method and the E-test [4–6]. In these assays a conidial suspension is used as fungal inoculum. It can be questioned to what extent these susceptibility data predict the therapeutic potential of the agent, since they may only give insight into the potential of the antifungal agent for prophylactic use. Susceptibility testing of a hyphal suspension mimicking the fungal status in infected tissues might better reflect the therapeutic potential of the agent. A standardized susceptibility assay using hyphae as inoculum is not yet available. Few studies have been published on this topic [7–11]. In some of them the inhibitory concentrations (IC) for conidia and hyphae to the antifungal agents appeared similar [7], whereas other studies showed increased ICs for hyphae compared to conidia [8,9]. One of the studies even considered hyphal clumps similar to fungal biofilms [11]. The nature of the hyphal suspension was different in each study, which complicates the interpretation of the data. No study has been published in which the different hyphal inocula were compared to each other or to conidia.

Unbiased and standardized assessment of the in vitro activity of antifungal agents against hyphae by visual
Materials and methods

A. fumigatus strains

A total of 12 clinical strains of A. fumigatus isolated from different patients and reference strain ATCC 204305 were investigated in this study. The clinical strains were isolated from the lower airways of patients with invasive pulmonary aspergillosis (IPA) seen on different wards of the Erasmus University Medical Center, Rotterdam, The Netherlands in 2005. The strains were maintained on sabouraud maltose agar (SMA; Difco Laboratories, Paris, France).

Antifungal agents

For the XTT assay, itraconazole was obtained from Janssen Pharmaceutical Products (Beerse, Belgium), voriconazole from Pfizer BV (Capelle aan de IJsel, The Netherlands) and amphotericin B from Bristol-Myers Squibb (Woerden, The Netherlands). Caspofungin was obtained from Merck and Company (Rahway, NJ, USA). All antifungal dilutions were prepared in DMSO except for caspofungin which was dissolved in water. When DMSO was used, the final concentration DMSO per inoculum was as described by the CLSI criteria [4].

In vitro antifungal susceptibility testing

ICs were determined independently in triplicate with the broth microdilution method described by the CLSI [4]. For preparation of the inocula, A. fumigatus was cultured for 4 days at 37°C on SMA. The conidia of each isolate were harvested in PBS with 0.01% Tween-20. A conidial inoculum was prepared in RPMI-1640 medium supplemented with 0.165 M MOPS (Sigma-Aldrich, Zwijndrecht, The Netherlands) to achieve a final concentration of 5 × 10^4 CFU/ml, and then transferred to a microwell tray (Costar, Cambridge, MA, USA). For preparation of the hyphal fragments, the harvested conidia were suspended in RPMI 1640 medium, and incubated for 24 h at 37°C. At this time point conidia had formed hyphal clumps. For preparation of the hyphal fragments, the suspension was homogenized by sonication (20 s at 28-μm maximum power; soniprep, Beun de Ronde, The Netherlands) [13] and adjusted to a transmission of 80% at 660 nm (Novaspec II, Pharmacia Biotech). This transmission results in the same amount of hyphae present in the hyphal clumps. The susceptibility assays of the three different fungal inoculum forms were performed in microwell trays. All antifungal dilutions were prepared in DMSO or water and added to the fungal cells. Final drug concentrations ranged from 0.03–16 μg/ml for amphotericin B, itraconazole and voriconazole and from 0.06–128 μg/ml for caspofungin. The cells were incubated for 48 h before the inhibition of fungal metabolic activity was determined.

To facilitate endpoint reading using the XTT assay the substrate 2,3-bis(2-methoxy-4-nitro-5-[(sulphenylamino)carbonyl]-2H-tetrazolium-hydroxide (XTT), menadione was added to each well resulting in final concentrations of 250 μg/ml XTT and 15 μM menadione. After incubation for 2 h at 37°C, 50 μl DMSO was added in order to further release the coloured formazan product. Although XTT is secreted into the culture medium, adding DMSO results in the release of XTT still present in the fungal cells. The supernatant was removed and measured spectrophotometrically at 450 nm. The IC endpoints for each antifungal agent were defined as the first concentration at which 80% or more spectrophotometric reduction of mitochondrial dehydrogenase activity was achieved [13].

E-test

MICs were determined according to the manufacturer’s descriptions (AB Biodisk, Goes, The Netherlands). In short, conidia were harvested as described above and diluted to 0.5 McFarland. Conidia were spread onto RPMI agar plates and the E-test strip was applied. After 48 h incubation at 37°C, MICs for amphotericin B, itraconazole and voriconazole were determined as the lowest drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip. For caspofungin, instead of the MIC the minimal effective concentration (MEC) was determined as the lowest drug concentration at which the border of the elliptical inhibition zone with alternative growth intercepted the scale on the antifungal strip.
Results

The effect of the polyene amphotericin B on A. fumigatus

As shown for the conidial suspension of A. fumigatus ATCC 305204 in Fig. 1, amphotericin B inhibited the fungal metabolic activity in a sharp concentration-dependent manner, which was also documented for the 12 A. fumigatus clinical isolates. Exposure to concentrations of at least 1 μg/ml lowered the relative extinction to less than 20%, resulting in a growth reduction of 80%. The sharp concentration-dependent inhibition was also noticed when hyphal fragments were exposed to amphotericin B, with an IC of 1 μg/ml, equal to that found for conidia. A high concentration of 8 μg/ml amphotericin B was needed to inhibit hyphal clumps. This was a three step dilution increase in the IC when compared to hyphal fragments or conidia.

The ICs for the 12 A. fumigatus clinical isolates tested were comparable to the ICs found for the ATCC strain, as shown in Fig. 2. For all isolates ICs for hyphal fragments were comparable to those found for conidia. ICs observed for hyphal clumps were generally three dilution steps higher.

The effect of the azole itraconazole on A. fumigatus

As shown in Fig. 1, itraconazole inhibited the metabolic activity of A. fumigatus ATCC 305204 conidia also in a sharp concentration-dependent way, with the IC of 0.25 μg/ml. However, the 12 A. fumigatus clinical isolates were...
inhibited in a more gradual concentration-dependent manner. Again as demonstrated in Fig. 1, hyphal fragments also exhibited an apparent gradual decrease in metabolic activity when exposed to itraconazole. The IC for itraconazole obtained for hyphal fragments is equal to the MIC obtained for conidia, but itraconazole was not able to inhibit the metabolic activity of the hyphal clumps, with only a slight decrease in metabolic activity noted. At a concentration of 16 μg/ml itraconazole, 70% of the cells remained metabolically active.

For all 12 A. fumigatus clinical isolates the ICs were comparable to those obtained for the ATCC strain (Fig. 2). ICs found for hyphal fragments were equal to or one dilution step higher than the ICs obtained for conidia. Hyphal clumps were not inhibited in metabolic activity for all strains tested.

The effect of the azole voriconazole on A. fumigatus

Voriconazole inhibited A. fumigatus conidia and hyphal fragments in the same gradual concentration-dependent manner as found for itraconazole (Fig. 1). The ICs found for conidia (0.25 μg/ml), hyphal fragments (0.5 μg/ml) and hyphal clumps (16 μg/ml) for the ATCC strain were comparable to those found for the 12 A. fumigatus clinical isolates (Fig. 2).

The effect of the echinocandin caspofungin on A. fumigatus

Caspofungin inhibited the metabolic activity of A. fumigatus in a moderate manner, since neither a sharp nor a gradual concentration-dependent inhibitory effect was observed (Fig. 1). For conidial suspensions of each isolate a concentration-dependent decrease of metabolic activity was observed with a minimal metabolic activity (MMC) at 1 μg/ml. However, a further increase in concentration did not result in a further decrease in metabolic activity but rather an increase, with a maximum at 8 μg/ml. Only at the high concentration of 128 μg/ml did the metabolism dropped to non-detectable levels (Fig. 1). This MMC was only seen for conidia and for hyphal fragments with some A. fumigatus strains, but never for hyphal clumps. ICs obtained for caspofungin for all A. fumigatus clinical isolates tested are shown in Fig. 2. It appeared that the metabolic activity was only inhibited when conidia were exposed to very high concentrations of caspofungin. For all strains, neither hyphal fragments nor hyphal clumps were completely inhibited in metabolic activity.
Reproducibility of the XTT assay with conidia or hyphae

The reproducibility of the XTT assay used at the different inoculum forms was determined by calculating the percentage agreement of the triplicate values tested for each condition. A test was considered reproducible if there was no or only a single dilution step difference between all three independent measurements compared to the median of the three measurements. As shown in Table 1 very high percentages of agreement were found for all four antifungal agents tested with the XTT assay, with percentages ranging from 77–100%.

E-test compared to the XTT assay

The E-tests which were performed for A. fumigatus conidia exposed to amphotericin B, itraconazole, voriconazole and caspofungin are presented in Fig. 1. Clear inhibitory ellipses were obtained for amphotericin B and both azoles. For caspofungin, instead of complete inhibition of fungal growth, ‘alternative growth’ was noticed in the inhibitory ellipse, and the inhibitory concentration obtained for caspofungin was therefore specified as the minimal effective concentration (MEC). The alternative growth observed in the E-test could explain the unique metabolic inhibition pattern found in the XTT assay. In addition, the E-test and the XTT assay did not give the same ICs for the antifungal agents, although 85% of the ICs for amphotericin B, 92% of the ICs for itraconazole, and 69% of the ICs for voriconazole were within two twofold dilution differences. The results obtained for caspofungin exceeded by far this dilution difference, with no agreement at all between the different tests (Table 2).

Discussion

There have been only a few studies on in vitro susceptibility testing involving the use of fungal hyphae and they have provided variable results. This can be explained in part by the use of non-standardized hyphal inoculum suspensions. Some investigators chose to prepare homogeneous suspensions of hyphae [7,8], while others used heterogeneous inoculum suspensions [9]. To our knowledge no papers have been published on the susceptibility of fungal conidia as compared to hyphal fragments and hyphal clumps. We here present the in vitro antifungal susceptibility of conidia versus hyphal fragments versus hyphal clumps, which employed standardized inoculums prepared from a total of 13 A. fumigatus strains (12 clinical isolates and 1 ATCC strain). The number of hyphae present in the suspension of hyphae fragments is comparable to the number present in a hyphal clump. The methods used allowed us to determine if the low susceptibilities found with hyphal clumps was due to the fact that hyphae were present instead of conidia or due to the fact that hyphal clumps are less accessible for antifungal agents. This is the reason for the use of two hyphal forms. The hyphal fragments were prepared by using sonication, a process often used to disrupt fungal cells or to extract fungal cell components whereby the high sonication power and the long duration of the sonication results in fungal killing. In the present investigation, we employed a mild sonication method which had previously been described for the fungus Madurella mycetomatis [13–15]. When using this mild method, Aspergillus fumigatus remained viable and reproducible results were obtained. Still, one should take into consideration that this method could cause the release of fungal cell wall components and mitochondria into the culture medium which could be of influence on the assay.

It was found that conidia and hyphal fragments showed equal susceptibilities to amphotericin B and the azoles as the ICs were basically identical. This was in agreement with the results obtained by Pujol et al. [8] who found that when comparing a hyphal suspension with a conidial suspension there was 83% agreement in their susceptibility to amphotericin B and 75% with itraconazole [8]. In contrast, Guarro et al. noted only 33% agreement in the susceptibilities with amphotericin B and 8% for itraconazole [7]. In both studies, as well as in the present investigation, it was found that in cases when the ICs differed, the hyphal suspensions showed higher ICs than the conidial suspensions.

Amphotericin B appeared to be the only agent active against hyphal clumps, although, relatively high concentrations of amphotericin B were needed to completely

Table 1 The reproducibility of determination of inhibitory concentrations (ICs) obtained with the XTT assay

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>Reproducibility1 of the XTT assay with conidia (%)</th>
<th>Reproducibility of the XTT assay with hyphal fragments (%)</th>
<th>Reproducibility of the XTT assay with hyphal clumps (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMB</td>
<td>100</td>
<td>85</td>
<td>100</td>
</tr>
<tr>
<td>ITZ</td>
<td>92</td>
<td>77</td>
<td>100</td>
</tr>
<tr>
<td>VCZ</td>
<td>100</td>
<td>77</td>
<td>100</td>
</tr>
<tr>
<td>CAS</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

1Reproducibility is calculated as the percentage of ICs obtained by three independent assays that were within 1 dilution step difference compared to the median of the ICs obtained for that strain.
The observation in the present study that the metabolic activity of conidia and hyphal fragments were both inhibited by the azoles, whereas hyphal clumps were not, is in agreement with the data of Lass-Flörl et al. and Mowat et al. [11,19]. The latter used different conidial seeding densities to produce hyphal biofilms. It appeared that the conidial seeding density played an important role in the overall structural integrity of the biofilms. Relatively thin biofilms were obtained at high conidial densities, whereas thicker biofilms were obtained when less conidia were seeded [11]. We used an inoculum of 5 x 10^4 CFU/ml, which according to Mowat et al. should result in a thick biofilm-like structure. The ICs found for these biofilms were comparable with the ICs found in our study. The biofilms were only inhibited by high concentrations of amphotericin B, whereas the azoles itraconazole and voriconazole and the echinocandin caspofungin did not inhibit the biofilms at all. Lass-Flörl reported that itraconazole and voriconazole were less able to kill hyphal suspensions than conidial suspensions [19]. In their study, the viability of the hyphae was determined by using the dye FUN-1. It was found that when hyphae were exposed to high concentrations of the azoles, most of the hyphae died, but some of them still remained alive. These data again suggest that shielding of the hyphae in clumps results in fungal survival at concentrations at which growth of hyphal fragments are completely inhibited.

While amphotericin B and the azoles both act on the fungal plasma membrane, caspofungin has a totally different antifungal mechanism in that it acts on the 1,3-D-glucan synthesis [18]. The E-test results obtained for

### Table 2: Inhibitory concentrations (ICs) for 12 *Aspergillus fumigatus* clinical strains and the ATCC 204305 strain after exposure of conidia using the XTT assay or the E-test

<table>
<thead>
<tr>
<th>Strain</th>
<th>AMB</th>
<th>ITZ</th>
<th>VCZ</th>
<th>CAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC</td>
<td>0.25</td>
<td>1</td>
<td>0.125</td>
<td>0.25</td>
</tr>
<tr>
<td>Af26</td>
<td>0.19</td>
<td>2</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>Af38</td>
<td>&lt;0.002</td>
<td>2</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Af39</td>
<td>0.5</td>
<td>1</td>
<td>&gt;32</td>
<td>2</td>
</tr>
<tr>
<td>Af40</td>
<td>0.75</td>
<td>1</td>
<td>0.75</td>
<td>0.5</td>
</tr>
<tr>
<td>Af41</td>
<td>0.75</td>
<td>2</td>
<td>0.75</td>
<td>0.25</td>
</tr>
<tr>
<td>Af42</td>
<td>0.125</td>
<td>1</td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>Af44</td>
<td>0.75</td>
<td>1</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>Af45</td>
<td>0.5</td>
<td>1</td>
<td>0.38</td>
<td>0.5</td>
</tr>
<tr>
<td>Af46</td>
<td>0.5</td>
<td>1</td>
<td>0.75</td>
<td>0.5</td>
</tr>
<tr>
<td>Af47</td>
<td>0.5</td>
<td>1</td>
<td>0.75</td>
<td>0.5</td>
</tr>
<tr>
<td>Af48</td>
<td>1</td>
<td>1</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Af49</td>
<td>0.8</td>
<td>1</td>
<td>1.487</td>
<td>0.5</td>
</tr>
</tbody>
</table>

1MIC determined with the E-test.
2IC determined with the XTT assay and representing T80% decrease in fungal metabolic activity.
3IC determined with the XTT assay and representing the minimal metabolic activity (MMC).
caspofungin were different compared to the other antifungals, in that rather than obtain clear inhibition of growth we found an alteration of growth, as has been reported by others [5]. Alteration of growth (MEC) was confirmed with the XTT assay. In this assay, the growth of A. fumigatus was inhibited only at very high concentrations of 128 µg/ml caspofungin. At lower concentrations of the drug, growth inhibition did not occur, but rather microscopic morphology of the mycelia was altered. These high ICs were also reported by others [20–22]. When using the antifungal broth susceptibility assay described by the CLSI, the MEC is usually defined microscopically as the lowest concentration at which abnormal hyphae growth is visible [23]. Unfortunately, this microscopic assay is time-consuming. When using the XTT assay, Antachopoulos et al. recently reported they found that at a certain concentration, called the minimal metabolic concentration (MMC), usually one to two dilutions higher than the MEC, a decline in metabolic activity could be found [24]. In the present study we noticed this MMC only when conidia were exposed to caspofungin, not with hyphae. After comparing the MMC found in the XTT assay with the MEC found in the E-test it appeared that they did not correspond to each other.

The E-test as an antifungal susceptibility test for filamentous fungi has been employed since 2000 [25]. The results obtained with the E-test and the CLSI M 38-A assay have been extensively compared for A. fumigatus strains. Good to excellent concordances have been reported for amphotericin B, itraconazole and voriconazole [6,26–28]. This is confirmed by the present study as we also observed relatively high percentages of agreement for the same antifungal agents in both assays, i.e., 85% for amphotericin B, 69% for voriconazole and 92% for itraconazole. With respect to caspofungin in some studies good agreements were also reported when the E-test and the MEC results in M3 medium were compared [5]. This was in contrast with the data of the present study in that we found that there was no agreement when we compared the E-test results with the MMC obtained in the XTT assay in RPMI medium. Since the E-test is also being performed on RPMI agar, the discrepancy in results between both assays was therefore not related to differences in nutrients. Until now, the CLSI has not described the correct conditions to be applied in the determination of the antifungal susceptibility of A. fumigatus against caspofungin.

In conclusion, conidia and hyphal fragments are equally susceptible to amphotericin B and the azoles, whereas hyphal clumps were not susceptible to the azoles. Amphotericin B, although at relatively high concentrations, was the only antifungal agent able to inhibit metabolic activity of the hyphal clumps.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References


This paper was first published online on Early Online on 18 February 2009.