A comparative study of fungicidal activities of voriconazole and amphotericin B against hyphae of *Aspergillus fumigatus*

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Objectives: To study the in vitro fungicidal activity of voriconazole against hyphae of *Aspergillus fumigatus* and compare the results with those obtained for the known fungicidal drug amphotericin B.

Methods: *A. fumigatus* mycelia were grown on Sabouraud dextrose agar and in peptone yeast extract glucose broth until the cultures reached a mid-logarithmic growth phase. The fungicidal activities of voriconazole and amphotericin B against actively growing hyphae of *A. fumigatus* were examined by a kill-curve experiment and a fungal cell viability test. For the kill-curve study, the drug-treated hyphae were washed, homogenized and resuspended in 1 mL of sterile water, diluted 10–1000 fold and aliquots of 0.1 mL were spread on Sabouraud dextrose agar and allowed to grow for 48 h at 35°C. The cfu were determined and plotted against drug concentrations for each time of exposure to obtain the kill curve. The viability of drug-treated *A. fumigatus* hyphae was determined by their ability to reduce tetrazolium compound 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide.

Results: Exposure of *A. fumigatus* hyphae to several concentrations (1–16 mg/L) of voriconazole or amphotericin B for various time intervals killed the hyphae in a time- and drug concentration-dependent manner. Voriconazole at 1 mg/L killed >95% of the hyphae grown on Sabouraud dextrose agar after 48 h of exposure, whereas amphotericin B at the same concentration killed ~70% of the hyphae after exposure for the same duration. Approximately 99% killing of hyphae grown in peptone yeast extract glucose broth was obtained for voriconazole at 1 mg/L after 48 h of exposure, whereas amphotericin B at 1 mg/L yielded ~82% killing after 48 h. The fungal cell viability test by tetrazolium reduction assay showed that mycelia exposed to >1 mg/L (Sabouraud dextrose agar blocks) and >2 mg/L (broth cultures) of voriconazole for 48 h completely failed to reduce 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide. At low concentrations (1–2 mg/L) amphotericin B had no detectable effect on 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide reduction by drug-treated mycelia, whereas mycelia treated with 16 mg/L for 48 h showed ~50% inhibition of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide reduction compared with the control.

Conclusions: Voriconazole possesses excellent fungicidal activity against actively growing hyphae of *A. fumigatus*. A comparison of results with those obtained for the known fungicidal drug amphotericin B shows that, in peptone yeast extract glucose broth, voriconazole has superior fungicidal activity against *A. fumigatus* hyphae compared with that of amphotericin B.

Keywords: voriconazole, fungicidal activity, *Aspergillus fumigatus*, fungal hyphae, kill curve, viability test

Introduction

Voriconazole is a second-generation triazole antifungal drug exhibiting excellent in vitro and in vivo activity against a wide variety of pathogenic yeasts, \(^1\)–\(^7\) and dimorphic \(^8\) and filamentous fungi \(^10\)–\(^28\) including *Aspergillus* species. Recently, it has been approved by the Food and Drug Administration of the USA for primary use against infection caused by pathogenic filamentous fungi, in particular by *Aspergillus* species. \(^29\) Members of the azole group of antifungal drugs in general are known to have...
fungistatic qualities, effective in pathogenic yeasts such as Candida species, resulting in a significant amount of growth inhibition in the presence of the drug. Removal of the antifungal drug from exposed fungal cells by washing would release them from the inhibitory effect of the drug and then they could resume normal growth under favourable conditions. Thus, prolonged exposure of yeast cells to azoles, including voriconazole, does not kill the cells but arrests their growth. The lack of fungicidal activity of azoles against the pathogenic yeast Candida albicans has been previously demonstrated in time–kill experiments. On the other hand, voriconazole possesses excellent fungicidal activity against Aspergillus species when germinated and ungerminated conidia are used. Although the inhaled conidia are the primary source of infection in man, such conidia must germinate producing hyphae for subsequent entry into the lung alveoli to establish disease. Conidia are generally absent in infected tissues. Death occurs with hyphal proliferation within the alveoli, and vascular invasion and dissemination producing pulmonary and distant infarctions. Thus, it is important that the antifungal drug treatment should kill the actively growing hyphae for a successful outcome in invasive aspergillosis.

None of the previous studies on the fungicidal activity of voriconazole against A. fumigatus included actively growing hyphae, the biological form of aspergillus found in infected tissues. Therefore, in this study we investigated the in vitro fungicidal activity of voriconazole against actively growing hyphae of 12 clinical isolates of A. fumigatus and the results were compared with those obtained for the known fungicidal drug amphotericin B.

Materials and methods

Antifungal drugs

Voriconazole and amphotericin B were obtained as pure powders from Pfizer Pharmaceuticals, New York, NY, USA and Squibb Institute for Medical Research, Princeton, NJ, USA, respectively. The stock solutions were prepared by dissolving the powders in dimethyl sulphoxide at a concentration of 1 mg/mL and were stored as 0.25 mL aliquots at −20°C. The frozen stocks were thawed at room temperature and vortexed gently several times to ensure that any remaining crystals present were completely dissolved before use. Since amphotericin B is light-sensitive, the stock solution and the culture tubes containing amphotericin B were covered with aluminum foil to prevent light exposure. For both antifungal drugs, a concentration range of 1–16 mg/L was used for fungicidal testing.

Fungal isolates

A. fumigatus F55064, W73355, H27023, X60141, N31647, H36754, H50246, F48686, X25421, S66182, W18870 and T45830 (MIC range: amphotericin B, 0.25–1 mg/L; voriconazole, 0.062–0.25 mg/L) isolated from immunocompromised patients were obtained from the Microbiology Laboratory of the Detroit Medical Center, Detroit, MI, USA. The original cultures obtained on Sabouraud dextrose agar slants were subcultured on the same medium to check the purity and viability of the cultures. For long-term preservation, isolates were stored as conidial suspensions in 25% glycerol at −70°C.

Kill-curve experiment

The fungicidal activity of voriconazole against A. fumigatus hyphae was examined by kill-curve experiments using mycelia grown on Sabouraud dextrose agar (VWR Scientific Products, West Chester, PA, USA) and in peptone yeast extract glucose (Difco Laboratories, Detroit, MI, USA), yeast extract 1 g (Sigma Chemical Company, St Louis, MO, USA) and glucose 3 g/L of distilled water broth. To obtain hyphae grown on solid medium, a single colony of A. fumigatus was grown on Sabouraud dextrose agar at 35°C for 3–4 days from a point source of inoculum. From the translucent outer zone of the fungal colony consisting of actively growing non-conidiating hyphae, 5 mm diameter agar blocks carrying thousands of actively growing hyphae were aseptically excised using a sterile cork borer (Figure 1). These agar blocks were incubated at 35°C in 6 mL polystyrene culture tubes in 1 mL of peptone

![Figure 1](https://academic.oup.com/jac/article-abstract/55/6/914/725451/725451)
yeast extract glucose broth (four blocks/tube) containing various concentrations (1–16 mg/L) of voriconazole or amphotericin B. Peptone yeast extract glucose broth with the required amount of dimethyl sulphoxide was used as a drug-free growth control. After 24 h of incubation, two of the agar blocks were removed from each drug concentration and washed with 20 mL of sterile distilled water in a Petri dish for 30 min at room temperature without agitation. The washed agar blocks containing drug-treated hyphae were homogenized individually for 30 s in distilled water (1 mL/agar block) using a tissue homogenizer fitted with a sterile miniprobe. The homogenate was diluted 10–1000-fold and 0.1 mL aliquots of the diluted suspension were spread in duplicate on Sabouraud dextrose agar. The agar plates were incubated for 48 h at 35°C and the number of cfu per agar block was determined. Agar blocks exposed to peptone yeast extract glucose broth without the drug were used as a control. The same procedure was repeated for the remaining two agar blocks in the culture tube after 48 h of drug exposure and the number of cfu determined. The mean cfu per agar block for each drug concentration was plotted for 24 and 48 h time intervals to obtain time–kill curves.

To examine the fungicidal activity of voriconazole and amphotericin B against *A. fumigatus* hyphae grown in liquid medium by kill-curve experiment, 1 mL cultures of *A. fumigatus* isolates were grown in peptone yeast extract glucose broth for 24 h at 35°C from conidia (1 × 10⁶ conidia/mL as determined by haemocytometry) in 6 mL polystyrene tubes. To these pre-grown mycelial cultures voriconazole was added to obtain final concentrations of 1–16 mg/L and the cultures were incubated for an additional 24–48 h at 35°C for drug treatment. Peptone yeast extract glucose broth containing the required amounts of dimethyl sulphoxide was used as a control. At the end of the drug treatment period, 3 mL of fresh peptone yeast extract glucose broth was added to each tube (total volume 4 mL), the mycelial suspension was mixed well, diluted 10–1000-fold by serial dilution in sterile distilled water and 0.1 mL aliquots of the diluted mycelial suspension was spread on Sabouraud dextrose agar. The plates were incubated for 48 h at 35°C and the number of cfu per mL of cultures was determined and plotted against various drug concentrations for each time interval to obtain kill curves. The minimum fungicidal concentration (MFC) was defined as the lowest concentration of the antifungal drug that provided ≤ 5 cfu/mL of cultures. The highest possible amount of drug carried over from the culture tube after the dilution was estimated to be 0.0025–0.04 mg/L that had no effect on the growth of *A. fumigatus* mycelia on Sabouraud dextrose agar.

**Fungal cell viability test**

The viability of *A. fumigatus* hyphae treated with voriconazole or amphotericin B was evaluated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide reduction assay⁹⁸,³⁷ using mycelia grown on Sabouraud dextrose agar and peptone yeast extract glucose broth. To examine the effect of voriconazole and amphotericin B on the viability of hyphae grown on solid medium, Sabouraud dextrose agar blocks containing *A. fumigatus* hyphae were prepared and treated with various concentrations of the antifungal drugs as described earlier. After 24–48 h of incubation, drug-treated agar blocks were removed and washed with distilled water. The washed agar blocks (one agar block/well) containing either voriconazole- or amphotericin B-treated hyphae were incubated in fresh peptone yeast extract glucose broth (0.2 mL/well) containing 100 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide and 0.2 mM menadione for 3 h at 35°C for the reduction of the tetrazolium compound. The MFC was defined as the lowest concentration of the drug that completely prevented the development of blue colour.

For mycelia grown in liquid medium, cultures of *A. fumigatus* were grown in 96-well microtitre plates (0.1 mL/well) in peptone yeast extract glucose medium for 24 h at 35°C from a conidial suspension (1 × 10⁶ conidia/mL). Under these conditions, the conidia germinate and produce uniform mycelial growth and the hyphae adhere tightly to the microtitre plate rendering it highly suitable for further manipulation with minimal loss of mycelia. After 24 h of growth, the spent growth medium was removed with a multichannel pipette and replaced with fresh peptone yeast extract glucose broth (0.2 mL/well) containing various concentrations (1–16 mg/L) of either voriconazole or amphotericin B. The first two vertical columns of the plate (16 wells) were drug-free controls. There were eight replicate wells for each drug concentration. The plates were then incubated for either 24 or 48 h, and at the end of the incubation period the growth medium containing the drug solution was removed and the fungal cells washed twice with 0.2 mL of sterile distilled water. Then to each well, 0.1 mL of 100 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide solution containing 0.2 mM menadione prepared in peptone yeast extract glucose broth (b). The data shown here were obtained for *A. fumigatus* clinical isolate W73355, and each point represents the mean ± SD (vertical bar) of two independent time–kill studies.
broth was added and the plates incubated at 35°C for 3 h for reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide. The MFC was defined as the lowest concentration of the drug that completely prevented the development of blue colour.

**Results**

**Time–kill study**

The fungicidal activities of voriconazole and amphotericin B against hyphae of a representative *A. fumigatus* isolate (W73355) grown on Sabouraud dextrose agar and peptone yeast extract glucose broth are shown in Figure 2. Exposure of actively growing hyphae to various concentrations of voriconazole significantly decreased their ability to produce fungal colonies in a time- and drug concentration-dependent manner. For instance, voriconazole at 4 mg/L showed 96 and 100% killing of hyphae grown on Sabouraud dextrose agar after 24 and 48 h of incubation, respectively, whereas amphotericin B at 4 mg/L showed 50 and 85% killing after 24 and 48 h of exposure, respectively, compared with the drug-free control (Figure 2a). Similar results were obtained for hyphae grown in peptone yeast extract glucose broth, except that almost complete killing was achieved at 1 mg/L for voriconazole after 48 h of incubation (Figure 2b). In contrast, amphotericin B at 1 mg/L showed very poor killing activity (~62%) against hyphae grown in peptone yeast extract glucose broth even after 48 h of exposure to the drug.

A summary of the results of the fungicidal activities of voriconazole and amphotericin B against 12 randomly selected *A. fumigatus* clinical isolates from our culture collection is shown in Table 1. The mean percentage killing of hyphae grown on Sabouraud dextrose agar and peptone yeast extract glucose broth for 24 and 48 h exposures was significantly higher than that obtained for amphotericin B.

**Viability test**

In addition to kill-curve experiments, we examined the fungicidal activities of voriconazole and amphotericin B against actively growing *A. fumigatus* hyphae by a previously described fungal cell viability test based on the ability of viable fungal hyphae to reduce the tetrazolium compound 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide. As the fungal mitochondrial dehydrogenase reduces 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, an insoluble blue formazan salt will accumulate in the mycelia. The rate of reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide resulting in the formation of formazan salt is directly proportional to the amount of viable hyphae, suggesting that the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide reduction assay can be adapted for qualitative as well as quantitative measurement of the viability of *A. fumigatus* hyphae. Figure 3 shows the effect of voriconazole and amphotericin B on *A. fumigatus* mycelia for their ability to reduce the tetrazolium compound 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide. *A. fumigatus* hyphae grown on Sabouraud dextrose agar (Figure 3a) and in peptone yeast extract glucose broth (Figure 3b) were completely killed by voriconazole at 1 and 2 mg/L, respectively, after 48 h of incubation. On the other hand, amphotericin B at concentrations in the range 1–16 mg/L showed very poor killing even after 48 h of exposure to the drug, as evaluated by the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide.

**Table 1.** Fungicidal activity of voriconazole and amphotericin B against *A. fumigatus* hyphae grown on solid and liquid growth media

<table>
<thead>
<tr>
<th>Antifungal drug (mg/L)</th>
<th>Percentage killing of hyphae grown on</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sabouraud dextrose agar (<em>n</em> = 12)<em>a</em></td>
<td>Peptone yeast extract glucose broth (<em>n</em> = 5)<em>b</em></td>
</tr>
<tr>
<td></td>
<td>24 h exposure</td>
<td>48 h exposure</td>
</tr>
<tr>
<td><strong>Voriconazole</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>1</td>
<td>86.3 ± 6.4</td>
<td>95.1 ± 5.4</td>
</tr>
<tr>
<td>2</td>
<td>90.5 ± 2.9</td>
<td>96.3 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>96.5 ± 6.7</td>
<td>99.7 ± 1.1</td>
</tr>
<tr>
<td>8</td>
<td>98.0 ± 2.1</td>
<td>99.9 ± 0.1</td>
</tr>
<tr>
<td>16</td>
<td>98.8 ± 1.2</td>
<td>99.9 ± 0</td>
</tr>
<tr>
<td><strong>Amphotericin B</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>1</td>
<td>49.3 ± 9.7</td>
<td>67.4 ± 2.5</td>
</tr>
<tr>
<td>2</td>
<td>35.5 ± 1.3</td>
<td>81.1 ± 0.8</td>
</tr>
<tr>
<td>4</td>
<td>39.8 ± 6.0</td>
<td>84.7 ± 1.2</td>
</tr>
<tr>
<td>8</td>
<td>72.9 ± 3.4</td>
<td>87.9 ± 0.6</td>
</tr>
<tr>
<td>16</td>
<td>78.7 ± 1.7</td>
<td>88.2 ± 0.5</td>
</tr>
</tbody>
</table>

*a*Each value represents the mean of two independent determinations for 12 *A. fumigatus* clinical isolates. *P* values (Student’s t-test) for pairwise comparisons of the percentage killing obtained for various concentrations of amphotericin B and voriconazole for 24 and 48 h drug exposures were in the range 0.0179–0.00017.

*b*Each value represents the mean of two independent determinations each for five *A. fumigatus* clinical isolates. *P* values (Student’s t-test) were in the range 0.0287–0.0016.
Aspergillus vegetative growth phase, filamentous fungi including various glucose broth and is superior to amphotericin B in its activity. We were able to examine the fungicidal effect of voriconazole and amphotericin B on the actively growing hyphal filaments using a novel method, we investigated the fungicidal activity of voriconazole on actively growing A. fumigatus hyphae by kill-curve experiments, and the results thus obtained were compared with those obtained for amphotericin B, a known fungicidal agent. Our results show that voriconazole is a highly effective fungicidal drug against A. fumigatus hyphae in peptone yeast extract glucose broth and is superior to amphotericin B in its activity.

Unlike unicellular organisms such as bacteria and yeasts that show uniform growth throughout the cell surface during vegetative growth phase, filamentous fungi including various Aspergillus species show polar growth by extending the apices of each hyphal filament. Thus, the metabolic and biochemical activity of the fungal cell governing vegetative growth is primarily restricted to the tips of the hyphae. Therefore it is generally believed that the fungicidal activity of antifungal drugs is directed to this region of the hyphae and older fungal cultures are less susceptible to the fungicidal activity of the drug. In this study, we were able to examine the fungicidal effect of voriconazole and amphotericin B on the actively growing hyphal filaments using Sabouraud dextrose agar blocks containing growing tips of A. fumigatus hyphae. The results obtained for the agar block experiments were compared with those obtained for the mycelia grown for 24 h in peptone yeast extract glucose broth cultures consisting of actively growing and non-growing hyphal elements. The hyphal tips on the agar blocks and the mycelia grown in liquid cultures were killed by voriconazole with almost identical effectiveness, suggesting that A. fumigatus cultures containing growing and non-growing hyphal elements are equally susceptible to voriconazole as are the actively growing tips of the hyphae.

Previous studies have shown that the fungicidal activity of amphotericin B against ungerminated and germinated conidia was superior to that of triazoles, including voriconazole. For instance, amphotericin B at 1 mg/L killed >99.9% of the conidia within 6 h of exposure to the drug, whereas almost 24 h of exposure was required to obtain the same amount of killing with voriconazole at 1 mg/L. The rapid and efficient fungicidal action of amphotericin B is thought to be due its ability to form channels or pores in the cytoplasmic membrane through which essential nutrients and ions are leaked out of the cell, and the loss of nutrients and essential ions are thought to be the primary reason for the lethal effect of amphotericin B. Voriconazole is a slow-acting fungicidal agent against A. fumigatus, and for voriconazole to be effective as a fungicidal agent, fungal cell growth equal to several generation times should occur. Therefore, an extended period of growth in the presence of voriconazole is required to deplete ergosterol content completely to bring about a lethal effect on A. fumigatus cells.

The intriguing question is why voriconazole is highly effective against A. fumigatus hyphae as a fungicidal agent compared with amphotericin B, whereas the reverse is true for ungerminated and germinated conidia of A. fumigatus. It is possible that a key component(s) that is directly or indirectly involved in the vegetative growth phase of A. fumigatus is absent or sparsely present in the conidia and germinating conidia. The function of this key component is more effectively inhibited by voriconazole, but not by amphotericin B, which results in eventual fungal cell death. Our recent investigation (E. K. Manavathu and P. H. Chandrasekar, 2004, unpublished) on the effect of voriconazole on the gene expression profile of actively growing A. fumigatus mycelia by partial microarray experiments suggests several possible candidates, including hyp1 and chsE genes, whose functions are known to be important for the vegetative and reproductive growth phases of Aspergillus species.

In summary, in contrast to its fungistatic effect against pathogenic yeasts such as Candida species, voriconazole shows excellent fungicidal activity against A. fumigatus hyphae as determined by kill-curve experiments and a fungal cell viability test by the reduction of a tetrazolium compound. A comparison of the results obtained for the time-kill study and the tetrazolium reduction assay showed that in peptone yeast extract glucose broth voriconazole possesses superior fungicidal activity against A. fumigatus hyphae as compared with that of amphotericin B.

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Part of the data described in this manuscript was presented previously at the Forty-third Interscience Conference on Antimicrobial Agents and Chemotherapy, 14–17 September 2003, Chicago, USA, Abstract M-1250.

References


35. Espinel-Ingroff A, Chaturvedi V, Fothergill A et al. Optimal testing conditions for determining MICs and minimum fungicidal

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