Post-antifungal effect of amphotericin B and voriconazole against germinated Aspergillus fumigatus conidia

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Objectives: The post-antifungal effect (PAFE) of amphotericin B and voriconazole on germinated Aspergillus fumigatus conidia was studied using the BacT/Alert detection system based on fungal CO2 production.

Methods: Germinated conidia of A. fumigatus were exposed to 1–10 \times \text{MIC} of amphotericin B for 1 and 4 h and to 2.5–40 \times \text{MIC} of voriconazole for 4 and 24 h. After removal of the drug by washing, similar numbers of exposed and control germlings were inoculated into Pedi-BacT culture bottles. CO2 production was automatically monitored until the bottles signalled positive. The difference in time for positive signals in drug-exposed and control bottles was used to calculate the PAFE.

Results: The killing rate of amphotericin B against germlings was both concentration- and time-dependent, as has been previously found for actively growing hyphae. Similarly, voriconazole showed fungicidal effect after 24 h of exposure, but not after 4 h. Amphotericin B induced a long concentration- and time-dependent PAFE, whereas voriconazole resulted in a short and dose-independent PAFE that was significantly longer after 24 h than after 4 h of exposure.

Conclusions: An automated method is presented for the determination of PAFE on filamentous fungi using quantifiable numbers of germinated conidia. In contrast to previous results obtained from conidia, this method could demonstrate a PAFE of amphotericin B on Aspergillus that shared characteristics similar to that on Candida spp.

Keywords: azoles, filamentous fungi, polyenes

Introduction

The germination of Aspergillus conidia is the first step in the establishment of infectious disease in the body. Conidial germination is a morphological transition process that includes the activation of resting conidia, isotropic growth involving water uptake that leads to swollen conidia and growth in a polarized manner resulting in a germ tube. This so-called germling continues to grow and converts eventually into an elongating and branching mycelium.1,2

Post-antifungal effect (PAFE) is defined as the delay of fungal regrowth persisting after exposure to an antifungal drug. Although the PAFE of different antifungals against Candida has been extensively studied,3 data regarding Aspergillus are still limited. There are studies on PAFE against Aspergillus fumigatus either on ungerminated conidia or on mycelia by the use of turbidometric or radiometric assays.4,5 We have recently developed and evaluated an automated method to measure the PAFE of amphotericin B and voriconazole against Candida spp. yeast cells and A. fumigatus conidia based on the monitoring of CO2 production by the BacT/Alert culture system.6,7

The aim of the present study was to apply our automated method for the determination of PAFE of amphotericin B and voriconazole against germinated A. fumigatus conidia and to analyse the relationship between PAFE and drug concentrations and exposure times.

Materials and methods

Aspergillus strain, antifungal agents and susceptibility testing

A. fumigatus CBS 13361 strain was used. Amphotericin B (Fungizone®, Bristol-Myers Squibb, Bromma, Sweden) and voriconazole (Pfizer Pharmaceuticals, New York, NY, USA) were stored

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at −70 °C as stock solutions of 5 g/L until required. The MIC determined by the CLSI (formerly NCCLS) method was 0.5 mg/L for both amphotericin B and voriconazole.

Germination of Aspergillus conidia
In pre-experiments, antibiotic medium 3 (AM3) (BBL, Becton Dickinson Microbiology Systems, Sparks, MD, USA) was chosen for germination, giving the highest germination rate. After incubation for 2–3 days on potato dextrose agar (PDA) at 37 °C, the conidia were suspended in AM3 broth supplemented with Tween 20. The conidia were counted in a haemocytometer and the concentration was adjusted to a range of 2.6–4.1 × 10⁶ cfu/mL. The conidal suspension (60 mL) in an Erlenmayer flask was incubated with gentle agitation at 37 °C for 6 h. The average germination rate was determined by measuring the size of 50 ungerminated and germinated conidia (>3 μm long) per experiment in a microscope equipped with a micrometer. The viability of the germlings was confirmed by colony counts on PDA plates.

Determination of PAFE and survival of germlings after exposure to amphotericin B and voriconazole
The PAFE of amphotericin B and voriconazole at concentrations of 1.0, 2.5, 5.0 and 10 and 2.5, 10 and 40 times the MIC, respectively, was determined as described previously.7 Germling cultures were agitated at 37 °C for 1, 2 or 4 h for amphotericin B and 4 or 24 h for voriconazole experiments. For kill-curve experiments, viable counts were made on PDA plates in duplicate from the exposed and control cultures. The controls were diluted in PBS in order to obtain an inoculum concentration as close as possible to the viable count of cultures exposed to antifungal drugs. Aliquots (2 mL) of each culture were injected into Pedi-BacT bottles (bioMérieux, Göteborg, Sweden) in triplicate. Bottles were incubated in the BacT/Alert Microbial Detection System until they signalled positive.

The duration of PAFE was calculated by using the formula PAFE = T − C, where T was the time required for the antifungal-exposed germlings and C was the time required for the diluted controls to produce CO₂ and signal positive. Exact values of C were obtained from curves of averaged viable counts (log₁₀ cfu/mL) versus time (h) for a positive signal for a series of diluted control bottles. Experiments were repeated on three separate occasions.

Statistics
A repeated measures ANOVA was used in the statistical calculations (STATISTICA software, StatSoft, Inc., Tulsa, OK, USA). Values are presented as means ± SEM.

Results
The germination rate of A. fumigatus was calculated for all PAFE experiments and it was on average 94.4 ± 0.84% after 6 h incubation in the A3 broth. The mean length of germlings after incubation was 6.2 ± 0.1 μm.

The exposure of A. fumigatus germlings to amphotericin B resulted in a substantial killing activity of up to 2.5 log that was both concentration- (P < 0.001) and time-dependent (P < 0.01). The exposure to voriconazole led to killing that was dependent on time (P < 0.001) with a 2 log killing after 24 h, whereas no significant killing was seen after 4 h. Although less marked than for amphotericin B, there was a slight concentration effect of voriconazole (P < 0.05).

Amphotericin B-induced PAFE in A. fumigatus at concentrations of 1–10 × MIC is shown in Figure 1 and ranged from 2.6 to 15.3 h on average. The PAFE of amphotericin B was both time- (P < 0.001) and concentration-dependent (P < 0.001). At all levels, doubling of time led to a more pronounced PAFE than doubling of the amphotericin B concentration.

Voriconazole at concentrations of 2.5–40 × MIC induced a short and dose-independent PAFE, which was significantly longer after 24 h than after 4 h of exposure, 2.73 ± 0.60 and 0.53 ± 0.17 h, respectively (P < 0.05).

Discussion
The determination of PAFE in filamentous fungi is difficult, suitable methods are lacking and the effect demonstrated on conidia is not necessarily transferable to hyphae. Turbidometric methods have been used, but alteration in the frequency of branching may affect the optical density and necessitates laborious microscopic examination. The method described by Manavathu et al., using labelled amino acid incorporation is interesting and has the advantage of studying actively growing hyphae, irrespective of the degree of branching. However, with this method, it is not the PAFE per se that is analysed but the combined result of antifungal and PAFEs. To avoid the effect of killing, the amount of fungi should be the same in the control as in the antifungal-exposed sample at the start of the post-antifungal period.

Our method using germinated conidia offers an alternative for the determination of PAFE. Germinated conidia have grown and synthesized a new cell wall that is not protected by the complex conidial wall. Most likely, the cell wall composition of germlings has great similarities to that of actively growing hyphae, and, therefore, it is reasonable to believe that pharmacodynamic results will be transferable to those of hyphae. In contrast to hyphae, germlings have the advantage of being easily quantified, which allows the selection of a suitable control with a similar number of germlings as that in the drug-exposed sample.
Post-antifungal effect against germinated conidia

We have previously shown that the exposure of ungerminated *Aspergillus fumigatus* conidia to amphotericin B for up to 8 h resulted in a limited concentration-dependent killing and that exposure to voriconazole for the same time period even at high concentrations did not result in any killing at all. In the present study, the time–kill curves demonstrated a rapid concentration- and time-dependent fungicidal effect of amphotericin B on *A. fumigatus* germlings, similar to that previously reported against Candida yeast cells. Also, for voriconazole, a slightly concentration-dependent fungicidal activity was found, but after 24 h of exposure. These results are in agreement with the data by Krishnan *et al.* who showed that actively growing hyphae are needed to demonstrate the fungicidal activity of voriconazole after 24 and 48 h of exposure. Thus, the killing data of the present study indicate that the cell wall of germlings in the pharmacodynamic aspect is different from that of conidia and has more similarities to that of actively growing hyphae and yeast cells.

In the present study, the PAFE induced by amphotericin B against germinated *A. fumigatus* conidia increased markedly at higher concentrations over a range of 1–10× MIC. In our earlier study on PAFE induced by amphotericin B against *A. fumigatus* conidia, there was no concentration dependency at levels above the MIC. Also, from these observations, it can be concluded that the PAFE results for amphotericin B on *Aspergillus* conidia cannot be extrapolated to germinated conidia. Moreover, compared with the results for conidia, the duration of PAFE was substantially longer against *A. fumigatus* germlings, 0.1–7.2 versus 1.2–16.4 h for the same concentrations. For both germinated and ungerminated conidia, the PAFE of amphotericin B increased at longer exposure times. The lack of a concentration-dependent effect on conidia indicates that the PAFE against conidia is not caused by an effect on germinating conidia but by a direct effect on ungerminated conidia.

The combined dependency of concentration and exposure time implies that the PAFE of amphotericin B on germlings is dependent on the AUC. The cell wall of *Aspergillus* conidia and hyphae is very different from that of yeast cells, whereas the cell membrane of both *Aspergillus* hyphae and Candida cells contains ergosterol that makes up the receptor to which amphotericin B binds before it exerts its fungicidal effect. Therefore, it is not surprising that the pharmacodynamic effect of amphotericin B on fungal killing and PAFE are similar for germinated conidia with the growing hyphae and budding yeast cells.

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Transparency declarations

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References


