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A comparative study of the post-antifungal effect (PAFE) of amphotericin B, triazoles and echinocandins on *Aspergillus fumigatus* and *Candida albicans*

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***Objectives*: To study the post-antifungal effect (PAFE) of antifungal drugs on *Aspergillus fumigatus* by a radiometric assay and compare the results with those obtained for *Candida albicans*.**

***Methods*: *A. fumigatus* cultures pregrown for 48 h in 96-well microtitre plate were exposed to various concentrations of the antifungal drug for 2 h. The drug-treated mycelia were washed, incubated in RPMI 1640 containing ^{14}C-labelled amino acids and the accumulation of radioactivity in the mycelia at different time intervals was determined. The PAFE was determined by plotting the amount of radioactivity associated with the mycelia against post-treatment incubation time. The PAFE of antifungal drug on *C. albicans* was examined by determining the multiplication (cfu/mL) of drug-pretreated cells at different time intervals for 24 h in drug-free medium.**

***Results*: Amphotericin B produced a prolonged PAFE (7.5 ± 0.70 h) against *A. fumigatus* whereas itraconazole (0.5 ± 0.0 h), voriconazole (0.5 ± 0.0 h), posaconazole (0.75 ± 0.35 h), ravuconazole (0.38 ± 0.17 h) and the echinocandins caspofungin (≤0.5 h) and micafungin (≤0.5 h) produced short PAFE. Short exposure (1 h) of *C. albicans* to low concentrations (0.125–1 mg/L) of amphotericin B (5.3 ± 1.15 h), caspofungin (5.6 ± 0.57 h) and micafungin (5 ± 1.0 h) produced prolonged PAFE whereas the triazoles produced a short (≤0.5 h) PAFE.**

***Conclusions*: Determination of ^{14}C-labelled amino acid accumulation in antifungal drug-pretreated mycelia is a suitable method for studying PAFE in *A. fumigatus*. Antifungal drugs with fungicidal activity tend to possess longer PAFE compared to fungistatic drugs.**

Keywords: caspofungin, micafungin, azole antifungals, susceptibility testing, antifungal drugs

Introduction

The post-antifungal effect (PAFE) is commonly defined as the time required for the fungal cell to recover from the transient crippling injury sustained by the organism as a result of a brief exposure to the antifungal drug. Although the PAFEs of polyenes, azoles, echinocandins and 5-fluorocytosine against pathogenic yeasts have been previously investigated,[1–4] very little is known about PAFE on filamentous fungi mainly because of the lack of a suitable method(s) for studying post-drug exposure effect in filamentous fungi. In unicellular organisms such as bacteria and pathogenic yeasts, PAFE is usually evaluated by the ability of the cells to grow and multiply following short exposure to the antimicrobial drug as determined by the number of cfu/mL of culture. Since no reliable method is currently available for the determination of cfu/unit volume of culture of a filamentous fungus, other suitable methods are needed. Recently, Vitale *et al.*[5] investigated the PAFE of amphotericin B and itraconazole in five *Aspergillus* species using ungerminated conidia. These investigators reported that amphotericin B produced a prolonged PAFE against *Aspergillus fumigatus* whereas three other *Aspergillus* species, namely, *Aspergillus terreus, Aspergillus nidulans* and *Aspergillus ustus* showed significantly shorter PAFEs or no PAFE at all. We therefore investigated the PAFE of amphotericin B, triazoles and echinocandins against *A. fumigatus* using actively growing hyphae by a radiometric assay where fungal growth was assessed by measuring ^{14}C-labelled amino acid accumulation and incorporation in mycelia.

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Materials and methods

Antifungal drugs

Voriconazole, itraconazole, posaconazole and ravuconazole were obtained from Pfizer Pharmaceuticals (New York, NY, USA), Janssen Pharmaceutica (Beerse, Belgium), Schering-Plough Research Institute (Kenilworth, NJ, USA) and Bristol-Myers Squibb Institute for Medical Research (Princeton, NJ, USA), respectively. Caspofungin and micafungin were obtained from Merck and Company (Rahway, NJ, USA) and Fujisawa Pharmaceuticals (Osaka, Japan), respectively. Amphotericin B was purchased from Sigma Chemical Company (St. Louis, MO, USA). The triazoles and amphotericin B were dissolved in dimethylsulphoxide (DMSO) to obtain a stock solution of 1 g/L and stored as 0.25 mL aliquots at –20°C. Caspofungin and micafungin were dissolved in sterile double distilled water to obtain a concentration of 10 g/L and stored as 0.25 mL aliquots at –70°C. The frozen stocks of the antifungal agents were thawed at room temperature and used within 24 h.

Organism and cultures

A. fumigatus clinical isolates F55064 (ATCC 208995), W73355 (ATCC 208996), H27023 (ATCC 208997), T53454 and W43719 used in this study were obtained from the Microbiology Laboratory of the Detroit Medical Center (Detroit, MI, USA). The original cultures obtained on Sabouraud Dextrose (SD) agar slants were subcultured on the same medium to check for purity and viability. Working cultures were maintained on SD agar slants at 4°C. Long-term preservation of the cultures was done in 25% glycerol as conidial suspension at –80°C.

MIC determination

The MICs of antifungal agents for various *A. fumigatus* isolates were determined by the broth microdilution technique M38-A recommended by NCCLS[6] except that the MIC was defined as the lowest concentration of the drug that provided 100% growth inhibition (MIC-0). Drug concentrations ranging from either 0.015 to 16 mg/L (voriconazole, itraconazole, posaconazole, ravuconazole and amphotericin B) or 0.25 to 256 mg/L (caspofungin and micafungin) were used for MIC determinations. Where applicable, comparable concentrations of DMSO were used as control. The MIC determinations of all antifungal drugs for all isolates were done a minimum of two times and the results were within ±1 serial dilution of the drug.

The MICs of various antifungal agents for *Candida albicans* 90028 were determined by the broth microdilution technique M27-A recommended by the NCCLS.[7] Drug concentrations ranging from 0.015 to 16 mg/L were used for MIC determinations. Where applicable, comparable concentrations of DMSO were used as control. The MIC determination of all antifungal drugs was repeated at least once and the results were within ±1 serial dilution of the drug. MICs for *C. albicans* were also determined using an inoculum of 10^6 cfu/mL and were within one dilution of those obtained using 10^3 cfu/mL.

Determination of PAFE

A. fumigatus. PAFEs of antifungal drugs on *A. fumigatus* were determined by a radiometric assay. Briefly, cultures of *A. fumigatus* isolates were grown in 0.2 mL RPMI 1640 in 96-well microtitre plates from a conidial suspension (1×10^4 conidia/mL) at 35°C for 48 h. The growth medium from each well was removed by a multichannel pipette and the mycelia were incubated with 0.2 mL RPMI 1640 containing various concentrations of antifungal drugs (amphotericin B and triazoles, 1–16 mg/L; echinocandins, 8–128 mg/L) for 2 h at 35°C. The antifungal agent was removed by washing the mycelia three times with RPMI 1640 using a multichannel pipette and the washed mycelia were incubated with 0.2 mL fresh RPMI 1640 containing 200 000 cpm/mL [^{14}C(U)]L-amino acid mixture (NEN Life Science Products, Inc., Boston, MA, USA). At specified time intervals, the RPMI 1640 medium containing ^{14}C-amino acids was removed, the mycelia were washed three times with 0.2 mL ice-cold 10 mM Tris–HCl (pH 7.0) and the radioactivity associated with the mycelia was determined by scintillation counting as described previously.[8] Where applicable, mycelia in the control wells were treated with DMSO (≤2%, v/v) and processed identically. The amount of radioactivity (cpm) accumulated in the mycelia was plotted against time of incubation. An increase in the radioactivity associated with the mycelia above the background level signifies increased accumulation and incorporation of ^{14}C-amino acids, and hence resumption of metabolic activity and mycelial growth. The duration required for the antifungal drug treated mycelia to recover from the inhibitory effect of the drug as indicated by the intramycelial accumulation of ^{14}C-labelled amino acids was defined as the PAFE.

C. albicans. The PAFE of antifungal drugs on *C. albicans* was evaluated by determining the duration required for the drug-treated cells to multiply after the removal of the drug. Briefly, 1×10^6 *C. albicans* 90028 cells were incubated in 1 mL RPMI 1640 for 1 h in the presence of various concentrations (amphotericin B and triazoles, 1–16 mg/L; echinocandins, 0.25–16 mg/L) of the antifungal drugs at 35°C. The drug-treated cells were washed, resuspended in fresh growth medium and incubated at 35°C for 24 h. At various time intervals, 0.1 mL aliquots of the cell suspension were removed, diluted 10–1000 fold and 0.1 mL aliquots were spread on SD agar. The SD agar plates were incubated at 35°C for 48 h and the number of cfu/mL of culture was determined. Where applicable, the *C. albicans* cells were treated with DMSO (≤2%, v/v) and processed identically. The number of cfu/mL obtained at specified time intervals was plotted against time of incubation to determine PAFE. An increase in the cfu/mL of culture signifies growth and multiplication of *C. albicans* cells. The duration required for the antifungal drug-treated cells to recover from the inhibitory effect of the drug as indicated by an increase in cfu/mL of culture was defined as the PAFE.

Results and discussion

In vitro susceptibility study

A. fumigatus F55064, W73355, H27023, T53454 and W43719 were highly susceptible to amphotericin B (MIC-0 ranged from 0.5 to 1 mg/L) and the triazoles, itraconazole, voriconazole, posaconazole and ravuconazole (MIC-0 ranged from 0.06 to 1 mg/L). Although both caspofungin and micafungin exhibited prominent growth inhibition at low concentrations (0.015–0.125 mg/L), the MIC-0s of these antifungal drugs for *A. fumigatus* isolates ranged from 64 mg/L (caspofungin) to >256 mg/L (micafungin). On the other hand, *C. albicans* 90028 was highly susceptible to amphotericin B (MIC-0 = 0.125 mg/L), triazoles [MIC-1 (80% inhibition of growth compared to drug-free growth control) ranged from 0.06 to 0.25 mg/L] and the echinocandins caspofungin and micafungin (MIC-0 ranged from 0.03 to 0.125 mg/L).

PAFE

Figure 1(a–c) shows representative plots of ^{14}C-labelled amino acid incorporation by amphotericin B- (a), voriconazole- (b) and caspofungin (c)-treated *A. fumigatus* mycelia as a function of incubation time after the removal of the drug. Amphotericin B-treated mycelia accumulated radioactivity in a drug concentration-dependent manner. The higher the concentration of the drug used to treat the mycelia, the lower the amount of radioactivity accumulated in the mycelia. Similarly, the duration required for amphotericin B-treated mycelia from

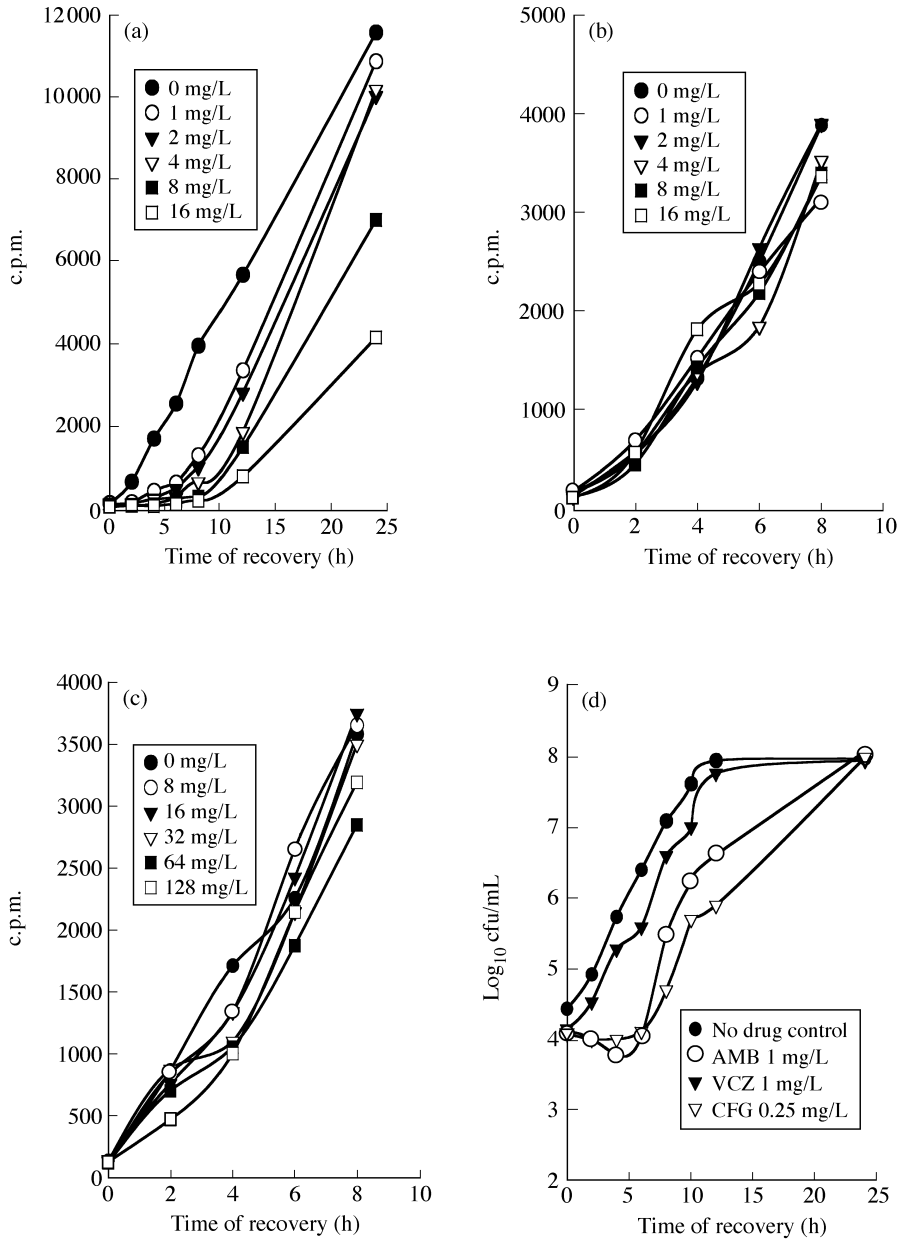

Figure 1. (a–c) Representative plots of ^{14}C-labelled amino acid accumulation (cpm) in amphotericin B- (a), voriconazole- (b) or caspofungin- (c) pretreated *A. fumigatus* mycelia as a function of post-pretreatment incubation time (time of recovery) used for the determination of PAFE of various antifungal drugs against *A. fumigatus*. Each point on the curve represents the mean cpm (standard deviation ≤ 15% of the mean) obtained for five *A. fumigatus* isolates. (d) Representative plots of cfu/mL of culture obtained for amphotericin B-, voriconazole- or caspofungin-pretreated *C. albicans* cells as a function of post-pretreatment incubation time used for the determination of the PAFE of various antifungal against *C. albicans*. Each point on the curve represents the mean cfu/mL (standard deviation ≤ 10% of the mean) obtained for duplicate determination.

the time of removal of the antifungal drug to the accumulation of a substantial amount of radioactivity varied from 4 to 8 h for drug concentrations ranging from 1 to 16 mg/L. In general, the higher the concentration of the drug used to treat the mycelia, the longer the delay for the accumulation and incorporation of ^{14}C-labelled amino acids in the mycelia suggesting that the PAFE of amphotericin B is concentration-dependent. In contrast to the delayed accumulation of ^{14}C-labelled amino acids by amphotericin B-treated mycelia, neither voriconazole- (1–16 mg/L) nor caspofungin- (8–128 mg/L) treated mycelia showed delayed accumulation of radioactivity in the mycelia compared to that of drug-untreated mycelia (control) suggesting that voriconazole and caspofungin possess no prolonged PAFE. A summary of the data obtained for seven antifungal agents belonging to three different classes of the drug is shown in Table 1. All five *A. fumigatus* clinical isolates provided similar PAFE and the reproducibility between two independent experiments was very good.

Figure 1(d) shows representative graphs illustrating the effect of a 1 h exposure of *C. albicans* 90028 cells to amphotericin B, voriconazole and caspofungin on their growth and subsequent multiplication. Both amphotericin B (1 mg/L) and caspofungin (0.25 mg/L) signifi-

Table 1. PAFE ± S.D. of amphotericin B, triazoles and echinocandins on *A. fumigatus* and *C. albicans*

	PAFE ± S.D. (h)	
Antifungal drug[a]	*A. fumigatus* ($n = 5$)[b]	*C. albicans* 90028[c]
Amphotericin B	7.5 ± 0.70	5.3 ± 1.15
Itraconazole	0.5 ± 0.0	≤0.5
Voriconazole	0.5 ± 0.0	≤0.5
Posaconazole	0.75 ± 0.35	≤0.5
Ravuconazole	0.38 ± 0.17	≤0.5
Caspofungin	≤0.5	5.6 ± 0.57
Micafungin	≤0.5	5.0 ± 1.0

[a]The drug concentrations used were: for *A. fumigatus*: amphotericin B, itraconazole, voriconazole, posaconazole and ravuconazole 16 mg/L, caspofungin and micafungin 128 mg/L; for *C. albicans*, amphotericin B, itraconazole, voriconazole, posaconazole and ravuconazole 1 mg/L, caspofungin and micafungin 0.25 mg/L.
[b]Each value represents the mean of two independent experiments.
[c]Each value represents the mean of three independent experiments.

cantly delayed the growth and multiplication of *C. albicans* cells whereas cells exposed to voriconazole (1 mg/L) showed no substantial delay of growth and multiplication suggesting that amphotericin B and caspofungin had prolonged PAFE on *C. albicans* but not voriconazole. A summary of the data obtained for multiple experiments for amphotericin B, four triazoles and two echinocandins is shown in Table 1.

Among various antifungal drugs we examined for their PAFE against *A. fumigatus*, amphotericin B is known to be fungicidal against filamentous fungi, including *A. fumigatus*. Amphotericin B acts on ergosterol of fungal cytoplasmic membrane and create pores through which essential nutrients and ions are leaked out of the cell. The rapid loss of essential nutrients and ions leads to the death of the fungal cell. Since amphotericin B is a rapidly acting fungicidal agent, even a brief exposure of fungal cells to it produces a prolonged PAFE. On the other hand, the echinocandins, known to be fungistatic agents against *A. fumigatus*, do not elicit permanent injury to the fungal cell. As soon as the drug is removed, the cells recover immediately and resume growth and multiplication. Thus, fungistatic agents are expected to produce short PAFE as opposed to fungicidal drugs. Our results on the PAFE of caspofungin and micafungin on *A. fumigatus* confirm this assumption.

In contrast to the fungistatic activity of triazoles against pathogenic yeasts such as *Candida* species, these agents are fungicidal against *Aspergillus* species, including *A. fumigatus*.[9,10] Our results show that all the triazoles we used possessed short PAFE. This apparent contradiction of the fungicidal activity and the short PAFE of triazoles against *Aspergillus* species may be related to the mode of action of these compounds. In the case of amphotericin B, the effect on the fungal cell is direct by physically creating 'pores' in the cytoplasmic membrane rendering it biologically non-functional. Since it is a physical injury, the effect is rapid and requires only short exposure (e.g. 2 h) to inflict a prolonged crippling effect on the cell. On the other hand, the fungicidal effect of triazoles on *A. fumigatus* is slow and it takes 12–24 h to obtain ≥90% killing of the cells.[9] This is mainly because of the prolonged time required for the depletion of lanosterol in the fungal cell by inhibiting cytochrome P450-dependent lanosterol demethylase. A time period equivalent to several generation times is required for the near-complete depletion of ergosterol in the membrane, and the lack of the required amount of the sterol interferes with the function of the membrane. The PAFE is commonly defined on the basis of short exposure of the cells to the drug instead of long exposure. Therefore, we treated the pregrown *A. fumigatus* mycelia for 2 h to study PAFE and this short period may not be sufficient for the triazoles to induce a prolonged PAFE by these compounds.

In conclusion, our results indicate that measurement of ^{14}C-labelled amino acid accumulation and incorporation in antifungal drug-pretreated mycelia is a suitable radiometric method for studying PAFE in *A. fumigatus*. Antifungal drugs showing fungicidal activity tend to possess longer PAFE compared to fungistatic drugs.

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