

Evaluation of the post-antifungal effect (PAFE) of amphotericin B and nystatin against 30 zygomycetes using two different media

Roxana G. Vitale^{1,2}, Jacques F. G. M. Meis³, Johan W. Mouton³ and Paul E. Verweij^{1,2*}

¹Department of Medical Microbiology, University Medical Center Nijmegen, PO Box 9101, 6500 HB Nijmegen;

²Nijmegen University Center for Infectious Diseases, Nijmegen; ³Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands

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The post-antifungal effect (PAFE) of amphotericin B and nystatin against 30 clinical zygomycetes was evaluated using two different media. PAFE is a suppression of fungal growth after limited drug exposure. The MICs of both drugs were determined using NCCLS M38-P guidelines. A spectrophotometric method was used to determine PAFE *in vitro*. Spores were exposed to amphotericin B and nystatin in RPMI-1640 or AM3 at concentrations of 4 × and 1 × MIC for 4 h for *Absidia* sp. and at 1 × and 0.5 × MIC for 1 h for the other strains. Drugs were eliminated by washing. Exposed and control spores were cultured in microtitre wells and incubated for 48 h. PAFE was calculated as $T - C(\Delta t)$ between the control and the exposure fungi. The first increase in optical density (OD₀) was used to calculate PAFE and was considered significant when the value of the lower 95%CI of the exposed strain was greater than the upper 95%CI of the control. MIC ranges in RPMI-1640 were: 0.06–4 mg/L for amphotericin B and 0.5–8 mg/L for nystatin; MIC ranges in AM3 were: 0.06–2 mg/L for amphotericin B and 0.5–4 mg/L for nystatin. Killing was not observed at the concentration and exposure time used. In RPMI-1640, for amphotericin B the rank order for PAFE was *Absidia corymbifera* (5.6 h) > *Rhizopus oryzae* (5.2 h) > *Mucor* spp. (3.5 h) > *Rhizopus microsporus* (3 h), and for nystatin the rank order was *Mucor* spp. (5.8 h) > *R. oryzae* (3.3 h) > *A. corymbifera* (2.9 h) > *R. microsporus* (1.7 h). PAFE was not induced in *Rhizomucor* spp. PAFE was dependent on drug concentration.

Keywords: PAFE, zygomycetes, amphotericin B, nystatin

Introduction

Organisms of the class zygomycetes were first noted to cause disease in humans in publications from the 19th century. The first description of a case of disseminated disease was in a cancer patient and was caused by *Absidia corymbifera*.¹ The main clinical syndromes in human disease are sinusitis, and rhinocerebral, pulmonary, cutaneous, subcutaneous, gastrointestinal and disseminated zygomycosis. Other disease states occur with a much lower frequency. Risk factors for developing invasive zygomycosis include diabetes with ketoacidosis, neutropenia, transplant or patients with leukaemia who receive treatment with corticosteroids. The most common causative organisms are *Rhizopus* and *Absidia* followed by *Mucor* and *Rhizomucor*.^{2,3}

Treatment of zygomycosis requires surgical intervention, antifungal therapy and resolution of the underlying immunocompromised condition. Amphotericin B remains the first-line choice of treatment for infections due to zygomycetes. However, the clinical response is poor, especially in patients with disseminated disease.² Triazoles and

allylamines exhibit some *in vitro* activity against this class of fungi,⁴ but these agents are not used clinically. Nystatin exhibits good *in vitro* activity, but no clinical data are available to confirm *in vivo* activity.

Post-drug exposure effects are important to understand and optimize drug efficacy *in vivo*. We have developed an *in vitro* model that enables the study of post-antifungal effects (PAFEs) in filamentous fungi.⁵ The aim of the present study was to evaluate the PAFE induced by the polyenes amphotericin B and nystatin against zygomycetes and to compare the PAFE in two different media.

Materials and methods

Isolates

Thirty strains from our private collection were evaluated: *A. corymbifera* (AZN 24, AZN 319, AZN 911, AZN 1184, AZN 2134, AZN 2543, AZN 3113, AZN 3114, AZN 4095, AZN 6429); *Rhizopus oryzae* (AZN 593, AZN 1523, AZN 3440, AZN 5618, AZN 6142, AZN 6373, AZN 1925); *Rhizopus microsporus* (AZN 23, AZN 190, AZN 410, AZN 1185, AZN

*Corresponding author. Tel: +31-24-361-4356; Fax: +31-24-354-0216; E-mail: p.verweij@mmb.umcn.nl

5005, AZN 5816, AZN 8894); *Rhizomucor miehei* AZN 4839; *Rhizomucor pusillus* AZN 22; *Mucor hiemalis* (AZN 21, AZN 175, AZN 1379) and *Mucor rouxii* AZN 1183.

Antifungal agents

Amphotericin B (Bristol-Myers Squibb, Woerden, The Netherlands) and Nystatin (Gist-Brocades, Delft, The Netherlands) were utilized for MIC determinations and PAFE studies. The drugs were dissolved in dimethyl sulphoxide (DMSO) and aliquots of the stock solution were stored at -70°C until use. Then they were diluted in RPMI-1640 medium (with L-glutamine, without bicarbonate) (Gibco BRL, Life Technologies, Woerden, The Netherlands) and Antibiotic Medium 3 (AM3) (BBL/Becton Dickinson, Cockeysville, MD, USA), both buffered to pH 7.0 with 0.165 M MOPS (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

Antifungal susceptibility testing

The isolates were passaged twice at an interval of 5–7 days at 28°C by subculturing onto Sabouraud glucose agar (SGA) to obtain adequate sporulation. Spores were collected with a cotton swab and suspended in saline with 0.01% Tween 80. The resulting spore suspensions were counted with a haemocytometer and diluted in RPMI-1640 or AM3 1:100 in order to obtain a final inoculum of $1-5 \times 10^4$ spores per mL. The viability was confirmed by plating serial dilutions onto SGA plates.

Amphotericin B and nystatin were dissolved in DMSO at concentrations of 3200 mg/L. Two-fold serial dilutions of the drugs were made in RPMI-1640 and AM3 medium in order to obtain final concentrations of both drugs that ranged from 0.015 to 16 mg/L. A drug-free well containing 0.01% DMSO in the medium served as the growth control. The tests were carried out in 96-well flat-bottomed microtitration plates (Corning), which were kept at -70°C until the day of testing. After the inoculation, the microtitration plates were incubated at 35°C for 48 h. The MICs were read by spectrophotometric reader (Rosys Anthos ht3, Anthos Labtex Instruments GmbH, Salzburg, Austria). Background optical density (OD) was measured spectrophotometrically in non-inoculated wells processed in the same way as the inoculated wells. The relative ODs for each well based on measurements at 405 nm were calculated (as percentages) based on the following equation: $[(\text{OD of drug-containing well} - \text{background OD})/(\text{OD of drug-free well} - \text{background OD})] \times 100\%$. The MIC of both drugs was defined as the lowest concentration of the drug that showed at least 95% reduction of growth compared with that of the growth control (MIC-0).

PAFE assay

The method used to determine PAFE was recently described for *Aspergillus* spp.⁵ Amphotericin B and nystatin were dissolved in DMSO at initial concentrations of 400 mg/L and aliquots of the stock solution were stored at -70°C until use. Then they were diluted 50 times in RPMI-1640 (with L-glutamine without bicarbonate) or AM3 medium buffered to pH 7.0 with 0.165 M MOPS. Serial dilutions of the drugs were made in both media in order to obtain final concentrations of 4, 1 and 0.5 times the corresponding MIC. Control spore suspensions were made in RPMI-1640 and AM3 without drug. The isolates were passaged twice at an interval of 5–7 days at 28°C by subculturing onto SGA to obtain adequate sporulation. Spores were collected with a cotton swab and suspended in saline with 0.01% Tween 80. After the heavy particles had been allowed to settle, the supernatant was transferred to another tube, vortexed for 10 s, and 10 and 100 times dilutions were made. The concentration of spores was established microscopically using haemocytometer Burker Turk chambers. Then, the concentration was adjusted to obtain 4×10^5 spores/mL. One millilitre of this suspension was added to tubes containing 9 mL

of RPMI-1640 or AM3 alone (control) or with amphotericin B or nystatin in concentrations mentioned above resulting in a final volume of 10 mL. The final inoculum therefore was 4×10^4 cfu/mL. Following this procedure, each strain was incubated for 4 or 1 h with continuous shaking at 37°C .

After incubation, the spores were washed with saline plus 0.01% Tween 80 and centrifuged at 3500g for 15 min. After three wash cycles, 98% of the supernatant was completely decanted and the pellets were resuspended in a final volume of 10 mL of RPMI-1640 or AM3 with 0.01% Tween 80. Following this step, 100 μL of sample was diluted 10-fold in sterile water and 30 μL aliquots were plated onto SGA plates for colony count determination, and incubated at 37°C for 24 h. The concentration of viable cfu/mL for exposed spores was determined in order to verify the concentration of viable spores post-drug exposure and to allow adjustment of the inoculum, if necessary, to match that of controls. From the resuspended suspension, 200 μL was placed in microtitration plates and incubated at 37°C in a computerized spectrophotometric reader (Rosys Anthos ht3). Any growth was automatically monitored in terms of change in turbidity at 405 nm, at 10 min intervals for 48 h. All assays were carried out in duplicate.

Data analysis

The repetitive OD measurements for each well resulted in the growth curve. PAFE was determined by comparing the growth curve of the exposed spores with that of the controls. The first increase in OD (OD_0) was used to calculate PAFE as previously described,⁵ by using the formula $\text{PAFE} = T - C$, where T was the time of the first significant increase in OD_0 of the exposed spores after removal of the drug and C was the time of the first significant increase in OD_0 of the control. Thus, PAFE was defined as the difference in time (Δt) between exposed and controls to reach the defined point in the growth curve and was expressed in hours. The time to reach this chosen point, OD_0 , of at least eight controls for each species was calculated and the mean, range, upper 95% confidence interval (CI) and the coefficient of variance were calculated in order to determine the reproducibility of the control curves at that point and to establish the reproducibility of the experiments. For each species, the upper 95% CI of the controls was chosen as the cut-off level that distinguished between the presence or absence of PAFE. When re-growth of the exposed isolates occurred within the upper 95% CI time-frame of the controls, PAFE was considered to be absent. Alternatively, if re-growth was delayed following drug exposure and the lower 95% CI of the exposed isolates was delayed until beyond the upper 95% CI of the controls, PAFE was considered to be present. Growth curves of each exposed isolate were compared only with pooled controls from that same isolate.

Comparisons of PAFE between both media and both drugs were analysed by analysis of variance for repeated observations followed by Bonferroni's Multiple Comparison Tests. P values of <0.05 were considered statistically significant.

Results

The MIC ranges determined in RPMI-1640 were: 0.063–4 mg/L for amphotericin B (*A. corymbifera* 0.125–0.25 mg/L; *R. oryzae* 0.5–4 mg/L; *R. microsporus* 0.5–2 mg/L; *R. miehei* 0.063 mg/L; *R. pusillus* 0.125 mg/L; *M. hiemalis* 0.063–0.25 mg/L and *M. rouxii* 0.063 mg/L) and 0.5–8 mg/L for nystatin (*A. corymbifera* 0.5–1 mg/L; *R. oryzae* 2–8 mg/L; *R. microsporus* 2–8 mg/L; *R. miehei* 0.5 mg/L; *R. pusillus* 1 mg/L; *M. hiemalis* 0.5 mg/L and *M. rouxii* 0.5 mg/L).

For AM3 the MICs of amphotericin B ranged from 0.063 to 2 mg/L (*A. corymbifera* 0.125–1 mg/L; *R. oryzae* 0.5–2 mg/L; *R. microsporus* 0.25–2 mg/L; *R. miehei* 0.063 mg/L; *R. pusillus* 0.125 mg/L; *M. hiemalis* 0.25–0.5 mg/L and *M. rouxii* 0.063 mg/L) and for

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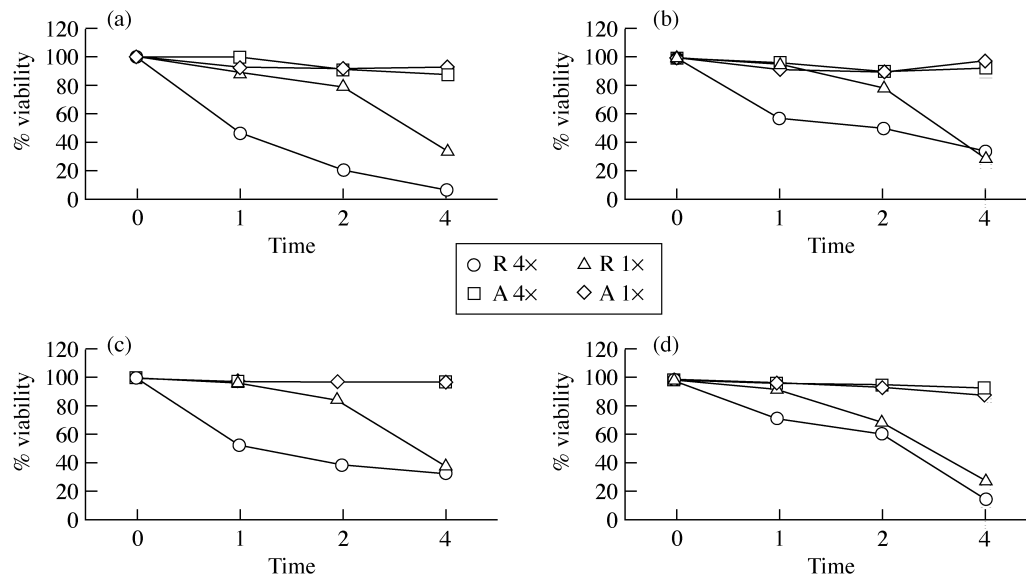


Figure 1. Effect of drug exposure over time (h) on the viability of *A. corymbifera* (A) and *R. oryzae* (R) spores at 4× and 1× MIC. (a) Amphotericin B (RPMI-1640); (b) nystatin (RPMI-1640); (c) nystatin (AM3); (d) amphotericin B (AM3).

nystatin from 0.5 to 4 mg/L (*A. corymbifera* 0.5–2 mg/L; *R. oryzae* 1–4 mg/L; *R. microsporus* 1–2 mg/L; *R. miehei* 0.5 mg/L; *R. pusillus* 1 mg/L; *M. hiemalis* 0.5–1 mg/L and *M. rouxii* 0.5 mg/L).

Viability of the spores

Exposure of spores for 1 to 4 h to the concentrations of amphotericin B and nystatin used had no effect on the viability of the *A. corymbifera* isolates, but for the other genera tested both drugs induced fungicidal activity after 4 or 1 h of incubation at a concentration of four times the MIC (Figure 1). Therefore, PAFE was determined for 1 h exposure at 1 and 0.5 times the corresponding MIC for these genera. Under these conditions, killing was not observed for the concentration and exposure time chosen based on subculture of serial two-fold dilutions (data not shown).

Inoculum

By using a haemocytometer chamber, a limited range of 2 to 4×10^4 viable cfu/mL was achieved.

PAFE assay

All growth curves were reproducible among the replicates. For the same species, the shape of the growth curve of the control was identical to that of the exposed strains. When PAFE was present the growth curve of the exposed spores was shifted to the right compared with that of the control. Examples of growth curves for *A. corymbifera* and *R. oryzae* are shown in Figure 2.

Microscopic morphology

Microscopic examination of the spores before and after drug exposure showed that germination did not occur within the maximal exposure period of 4 h for *Absidia* and 1 h for the other species in both media. In RPMI-1640 even after 5 h of incubation germination had not occurred for control or exposed spores for any of the genera. However, in AM3, spores of *Rhizopus* started to germinate after 3 h, and after 5 h 100% of spores had germinated. For *Absidia* 80% of

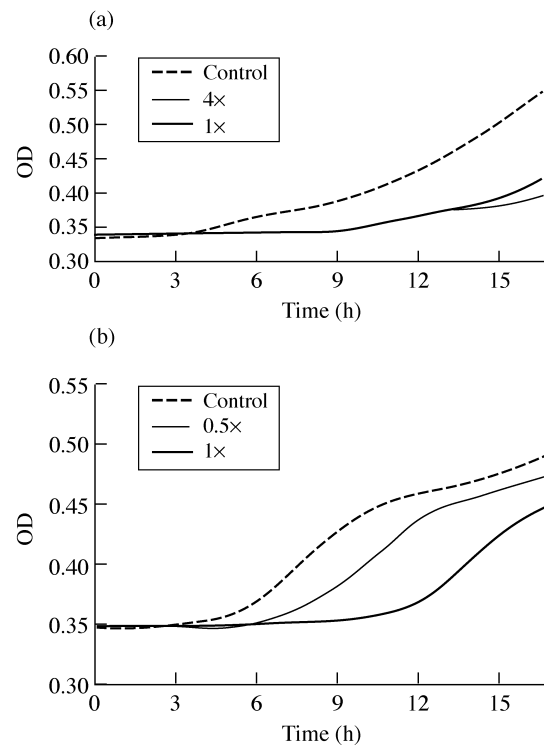


Figure 2. PAFE for amphotericin B in AM3 medium for *A. corymbifera* and *R. oryzae* after 4 and 1 h incubation, respectively. (a) *A. corymbifera* at 4× and 1× MIC; (b) *R. oryzae* at 1× and 0.5× MIC.

spores had germinated after 5 h of incubation. Germination of controls and drug exposed spores occurred at the same time and to the same extent in those strains where PAFE was not present. When PAFE was present, germination of spores was further delayed.

Microscopic examination of the moulds at different time intervals post-drug exposure revealed that increase in OD correlated with the development of hyphae as described previously.⁶ No differences in morphology were noted between exposed and non-exposed fungi.

Table 1. PAFEs induced by exposure of different zygomycetes to amphotericin B (AMB) and nystatin (Nys) in RPMI-1640 and AM3

Strain	Cc ^a	Exposure ^b	Drug	PAFE in RPMI-1640			PAFE in AM3		
				mean ^c	range	N/TN ^d	mean	range	N/TN ^d
<i>A. corymbifera</i> (<i>n</i> = 10)	4×	4	AMB	5.6	1.9–9.1	10/10	5.9	5.2–6.9	10/10
	1×	4	AMB	4.2	1.4–5.5	10/10	5.3	4.3–6	10/10
	4×	4	Nys	2.9	2.5–4	9/10	5.5	4.2–6.9	10/10
	1×	4	Nys	0.8	0.1–1.2	0/10	4.9	3.5–6	10/10
<i>R. oryzae</i> (<i>n</i> = 7)	1×	1	AMB	5.2	3.1–7.5	7/7	4.3	2.4–5.9	7/7
	0.5×	1	AMB	3.9	2–6.1	7/7	2.2	1–3.7	7/7
	1×	1	Nys	3.3	2.4–4.6	7/7	2.6	1.6–3.7	5/7
	0.5×	1	Nys	2.7	2–3.5	7/7	1.5	0.5–2.2	3/7
<i>R. microsporus</i> (<i>n</i> = 7)	1×	1	AMB	3	0.8–8.5	6/7	2.9	–0.1–9.4	3/7
	0.5×	1	AMB	1	0.3–3.4	3/7	1	–0.2–3.9	3/7
	1×	1	Nys	1.7	0.7–5.4	3/7	0.2	–0.35–0.9	0/7
	0.5×	1	Nys	0.7	0.2–1.2	1/7	0.1	–0.2–0.3	0/7
<i>Mucor</i> spp. (<i>n</i> = 4)	1×	1	AMB	3.5	2.1–6.9	3/4	2.2	0.9–3.4	3/4
	0.5×	1	AMB	2.1	1.1–3.6	2/4	1	–0.1–1.7	0/4
	1×	1	Nys	5.8	4–8.3	3/4	4.8	2.9–6.4	3/4
	0.5×	1	Nys	2.9	2.1–3.8	2/4	3	2.2–3.9	2/4
<i>Rhizomucor</i> spp. (<i>n</i> = 2)	1×	1	AMB	0.5	–0.1–1	0/2	0.2	0.1–0.2	0/2
	0.5×	1	AMB	–0.7	–0.2–1.2	0/2	0	0–0.1	0/2
	1×	1	Nys	1	0.7–1.4	0/2	0	0	0/2
	0.5×	1	Nys	–0.4	–1.2–0.5	0/2	–0.2	–0.3–0	0/2

^aConcentration of the drug expressed in 4 ×, 1 × and 0.5 × MIC.^bExposure period in hours.^cPAFE is expressed in hours.^dN/TN, number of strains that displayed significant PAFE/total number of strains tested.

The variability in OD among the control and exposed isolates, expressed as coefficient of variation (CV), was <13%.

PAFE was induced by both drugs in both media for all strains tested with the exception of *Rhizomucor* spp. (Table 1). PAFE was induced by amphotericin B after 4 or 1 h of exposure for the 10 *A. corymbifera* and for the seven *R. oryzae* strains for all the conditions tested (Table 1).

Overall, higher concentrations of drug induced longer PAFEs. However, the extent of PAFE differed between amphotericin B and nystatin for the various species.

In general, amphotericin B induced longer PAFE compared with nystatin: 4.6 h for amphotericin B versus 2.9 h for nystatin in RPMI-1640 ($P < 0.05$), and 4.3 h versus 3 h for amphotericin B and nystatin, respectively, in AM3 ($P < 0.05$). No statistically significant difference was observed for amphotericin B and nystatin between both media ($P > 0.05$), with the exception of low concentrations of nystatin (Table 2).

Comparison between different genera

All *A. corymbifera* strains displayed PAFE for both drugs at 4 × and 1 × MIC after 4 h exposure in the same medium. However, for nystatin, longer PAFEs in AM3 were seen for both concentrations compared with RPMI-1640 (Table 1). PAFE was not induced by amphotericin B or nystatin after 1 h exposure (data not shown). For *Rhizopus* spp. no differences in PAFE values were seen when both drugs were compared. Both drugs tended to induce longer PAFE in *R. oryzae* compared with *R. microsporus* (Table 1). For *Mucor* spp. nystatin tended to induce longer PAFE values than with amphotericin

B in the same medium. This observation was only found for this genus. Both amphotericin B and nystatin failed to induce PAFE in *Rhizomucor* strains.

Sub-MIC exposure

A. corymbifera did not display any PAFE in contrast to the other genera where most showed significant PAFE values. For amphotericin B, *Rhizopus* spp. and *Mucor* spp. displayed PAFE in RPMI-1640, but in AM3 only *Mucor* spp. did not show the effect.

For nystatin, *Rhizopus* spp. and *Mucor* spp. displayed PAFE in RPMI-1640, but in AM3 only *R. microsporus* did not show the effect.

Discussion

The method to quantify post-antifungal drug exposure in zygomycetes was based on the method recently described for *Aspergillus* species using a spectrophotometric procedure and OD₀ (first increase in OD) as criterion to calculate PAFE.⁵ The reliability of measurement of fungal growth using this spectrophotometric system has been described previously and even small changes in morphology can be detected. Furthermore, the system was validated previously for *Rhizopus*.⁶

The exposure of *Absidia* spores to amphotericin B or nystatin did not affect the viability of the spores at any of the concentrations or incubation periods tested. This finding was similar to that observed for *Aspergillus* species, where viability was not affected by exposure of the conidia to amphotericin B.⁵ However, exposure of the other genera of zygomycetes at concentrations above the MIC resulted in a

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Table 2. PAFEs for all the strains ($n = 30$) comparing amphotericin B (AMB) versus nystatin (Nys) and RPMI-1640 versus AM3

Concentration	Drug	PAFE in RPMI-1640		PAFE in AM3		Statistical analysis ^c
		mean	range	mean	range	
Hc ^a	AMB	4.6	(-0.1–9.1)	4.3	(-0.1–9.4)	$P > 0.05$
Hc	Nys	2.9	(0.7–8.3)	3.0	(0–6.9)	$P > 0.05$
Lc ^b	AMB	2.7	(-0.2–6.1)	2.4	(0–6)	$P > 0.05$
Lc	Nys	1	(-1.2–3.8)	2.2	(-0.2–6)	$P < 0.05$

^aHigher concentration evaluated corresponded to $4 \times \text{MIC}$ for *A. corymbifera* and $1 \times \text{MIC}$ for the other genera.

^bLower concentration evaluated corresponded to $1 \times \text{MIC}$ for *A. corymbifera* and $0.5 \times \text{MIC}$ for the other genera.

^cStatistical analysis of variance for repeated observations followed by Bonferroni's Multiple Comparison.

significant decrease in viability indicating rapid fungicidal activity. Rapid fungicidal activity was observed previously for *Candida* at concentrations of amphotericin B above the MIC.⁷

Post-antibiotic effects have been found to be dependent on several factors such as the concentration of the antibiotic, exposure time, media used and pH.⁸ In zygomycetes, PAFE appears also to depend on the class of drugs, concentration and duration of exposure, as was previously described for *Candida* and *Aspergillus* species.^{5,9}

The nutrient medium is a major factor that influences the results of *in vitro* susceptibility tests.¹⁰ RPMI-1640 has been evaluated extensively for *in vitro* susceptibility testing of yeasts and moulds and has been shown to give reproducible results.^{11–13} AM3 has been shown to discriminate better between *Candida* strains susceptible and resistant to amphotericin B,¹⁴ although there are no data that show that this is also the case for zygomycetes. Since batch-to-batch variation was described for the *in vitro* testing of antifungal agents with AM3, a single batch was used in the present study. In our evaluation PAFE was observed for both media, indicating that the effect was not dependent on the media used.

In general, PAFE was similar in RPMI-1640 and AM3 for both drugs with the exception of *A. corymbifera* for which nystatin tended to induce longer PAFE in AM3 than in RPMI-1640.

Amphotericin B and nystatin belong to the polyenes, which have a broad fungicidal spectrum *in vitro*. As a result of problems of solubilization and toxicity, parenteral administration of nystatin is not used for systemic treatment.¹⁵ However, the incorporation of this drug in liposomes reduced toxicity and preserved antifungal activity.¹⁶ Intravenous liposomal nystatin studied in rabbits displayed non-linear pharmacokinetics, potentially therapeutic peak plasma concentrations and substantial penetration in tissues. After multiple dosing over 15 days, the maximum concentrations in $\mu\text{g/g}$ (mean) were: in lung (72.84); liver (41.26); spleen (46.57); kidney (22.85) with a plasma concentration of 34.74 $\mu\text{g/L}$.¹⁷

When studying the relationship between drug concentration and antimicrobial effect, the time course of antifungal activity of polyenes is characterized by concentration-dependent killing, i.e. enhanced microbial killing by increasing drug levels, and long PAFE.¹⁸ Although this relationship was studied with conventional amphotericin B, it has been demonstrated with antibacterial drugs that specific pharmacodynamic parameters predictive of activity vary for different drug classes but not for drugs within a class.¹⁸ This was confirmed in the present study where amphotericin B and

nystatin, both polyenes, showed comparable PAFE characteristics against zygomycetes. It can be assumed that the same is true for lipid-formulations of amphotericin B since the active compound is amphotericin B.

The interpretation of our results can only be meaningful if drug levels that induce PAFE *in vitro* are within the range achievable in humans. Although serum concentrations of conventional amphotericin B are generally below 2 mg/L, higher levels have been found at the site of infection.^{19,20} The association between dosing of conventional amphotericin B and treatment effect is best described by the pharmacodynamic parameter peak level/MIC, indicating that the peak concentration achieved in the tissues is a major factor in relation to efficacy.^{18,21} The drug levels of the lipid formulations of amphotericin B are higher than those of conventional amphotericin B, although the pharmacodynamic properties of the individual lipid formulations differ significantly.²² Also, for some isolates even sub-MIC concentrations induced PAFE.

The strains tested in this study had a maximum MIC of 8 mg/L for nystatin and 4 mg/L for amphotericin B, indicating that when testing the PAFE by using $4 \times \text{MIC}$, the maximum concentration is 32 or 16 mg/L respectively, values that are achievable in tissues.

For both drugs, PAFE was observed, however amphotericin B displayed longer values compared with nystatin.

PAFE displayed by amphotericin B and nystatin were concentration-dependent. This is consistent with previous studies with *Aspergillus* and *Candida* where longer PAFEs were found following exposure to amphotericin B or nystatin at higher concentrations.^{5,7,9}

The mean PAFE values found in our study for all zygomycetes were similar to those described for *Aspergillus* species previously tested although amphotericin B induced longer PAFE against *A. fumigatus* (mean PAFE = 9.94 h).⁵ For nystatin, similar PAFEs were found for *Candida albicans* (mean = 6.85 h) whereas *Candida non-albicans* species presented longer PAFE.²³

In conclusion, amphotericin B and nystatin induced PAFE in zygomycetes but lower PAFE values were observed with nystatin. In general, the media used did not significantly influence the effect. Determination of PAFE could be useful as an *in vitro* tool together with *in vitro* susceptibility testing to give a better understanding of the activity of antifungal agents. There are no data available for *in vivo* studies of PAFE for zygomycetes that could support any theory about the importance of this effect as a predictor of therapy failure. For this reason, further studies are warranted, including *in vivo* exper-

iments, to study the impact of PAFE in zygomycetes on dosing regimens of amphotericin B or nystatin and the usefulness of this assay to assist in predicting clinical outcome.

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