

## *In vitro* susceptibilities of Zygomycota to polyenes

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The *in vitro* activity of polyenes was determined for 36 isolates of Zygomycota including *Rhizopus* spp. (15), *Absidia corymbifera* (10), *Mucor* spp. (6), *Rhizomucor* spp. (3), *Cunninghamella bertholletiae* (1) and *Apophysomyces elegans* (1). All isolates were tested with amphotericin B, heated amphotericin B and nystatin by a broth microdilution test. There were no significant differences between heated and unheated solutions of amphotericin B in terms of their antifungal activities. The MICs of amphotericin B for most of the strains were <2 mg/L. For all isolates, nystatin was significantly less active than amphotericin B ( $P < 0.001$ ). The one *C. bertholletiae* and one *A. elegans* isolates were less susceptible to amphotericin B (MICs 2 mg/L) and were also less susceptible to nystatin.

### Introduction

Zygomycosis is a severe infection occurring mostly in immunocompromised patients. The most frequent predisposing factors are diabetic ketoacidosis, neutropenia and corticosteroid therapy.<sup>1</sup> Members of the genera *Rhizopus*, *Mucor* and *Absidia* are the most commonly isolated organisms from patients who have zygomycosis. *Rhizomucor*, *Cunninghamella*, *Apophysomyces* and *Saksenea* are other Zygomycota that have been implicated in causing human diseases.<sup>1</sup>

Intravenous amphotericin B is the treatment of choice for zygomycosis. Nevertheless, the use of conventional amphotericin B is limited by its severe side effects, and clearly new therapeutics are needed. It has been shown recently that mild heating of amphotericin B could greatly reduce its toxicity to mammalian cells *in vitro*.<sup>2</sup> Moreover, it has been demonstrated that heated amphotericin B is less toxic *in vivo* in mice, allowing the use of higher drug doses than those of conventional amphotericin B.<sup>3</sup> Heated amphotericin B maintained *in vitro* antifungal activity against *Candida albicans*<sup>2,4</sup> and *Cryptococcus neoformans*,<sup>3</sup> and showed *in vivo* therapeutic activity in different models of candidiasis and crypto-

coccosis.<sup>3,4</sup> Nevertheless, the efficacy of heated amphotericin B *in vitro* and *in vivo* is unknown in filamentous fungi.

Like amphotericin B, nystatin is a polyene antibiotic that is active against a broad spectrum of fungi. However, toxicity associated with parenteral administration has not allowed its use for intravenous treatment. Recently, a liposomal formulation of nystatin, less toxic than the free drug, has been developed. Liposomal nystatin has previously been reported to be active *in vitro* against medically important yeasts and filamentous fungi,<sup>5,6</sup> as well as *in vivo* in animal models of fungal infections.<sup>7–9</sup>

The aims of this study were (i) to compare the activity of unheated and heated amphotericin B, and (ii) to test the activity of nystatin against strains belonging to different genera of Zygomycota.

### Materials and methods

#### Test isolates

A total of 36 isolates from our private collection, mostly of clinical origin, were tested. These comprised 15 *Rhizopus* spp. (eight *R. oryzae* and seven *R. microsporus*), 10 *Absidia corymbifera*, six *Mucor* spp. (three *M. hiemalis*, one *M. circinelloides*, one *M. racemosus* and one *M. rouxii*),

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three *Rhizomucor* spp. (two *R. pusillus* and one *R. miehei*), one *Cunninghamella bertholletiae* and one *Apophysomyces elegans*.<sup>10</sup>

All isolates were cultured from frozen stock on Sabouraud dextrose agar (Difco Laboratories, Detroit, MI, USA) supplemented with 0.02% chloramphenicol for 7 days at 35°C to ensure purity and viability. Two reference strains, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were included to ensure quality control.

#### Antifungal agents

Amphotericin B (Bristol-Myers Squibb, Woerden, The Netherlands) and nystatin (Gist-Brocades, Delft, The Netherlands) were obtained as standard powder and dissolved in dimethyl sulphoxide to produce stock solutions of 5 and 3.2 mg/mL, respectively.

Stock heated amphotericin B was prepared as described by Gaboriau *et al.*<sup>2</sup> by heating the solution of amphotericin B for 20 min at 70°C.

#### Susceptibility testing

Isolates were grown on Sabouraud dextrose agar for 7 days at 30°C and stock spore suspensions were prepared by washing over the surface of the slants with 2 mL of sterile saline containing 0.05% Tween 80. For *A. elegans*, sporulation was obtained by culturing the mycelium in sterile distilled water supplemented with 0.1% yeast extract (Difco) for 10 days at 37°C. Spore suspensions were counted with a haemocytometer and then diluted into RPMI 1640 to a concentration of  $2 \times 10^4$  spores/mL (two times final concentration). Inoculum sizes were checked by quantitative colony counts on Sabouraud dextrose agar. MICs were determined by a microdilution method based on the National Committee for Clinical Laboratory Standards (NCCLS) M38P document.<sup>11</sup> Each well of the microdilution plates containing 100 µL of the drug concentrations (at twice their final concentration) was inoculated with 100 µL of the inoculum suspension (also at twice the final concentration). The final concentrations of the antifungal agents were 0.015–16 mg/L and the final inoculum concentration was  $10^4$  spores/mL. Microplates were incubated at 35°C for 24 h, then read visually with the aid of a reading mirror. The MICs of all drugs were defined as the lowest concentration showing complete growth inhibition. MIC determination was carried out in duplicate and results were within two dilutions in 97% of the cases.

#### Data analysis

MICs for 50% (MIC<sub>50</sub>) and 90% (MIC<sub>90</sub>) of the isolates tested were determined for genera for which  $\geq 5$  and  $\geq 10$  isolates were available, respectively. For calculation, the high off-scale MICs of  $>16$  mg/L were converted to the next

highest concentration of 32 mg/L. The difference in the distributions of MICs was determined with a one-way analysis of variance after transformation of MICs to log<sub>2</sub> values. Statistical significance was defined as  $P \leq 0.05$ .

#### Results

The MIC of heated and non-heated amphotericin B was 1 mg/L for the two NCCLS quality control isolates *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019. This is within the expected range of MICs for the pure substance.<sup>11</sup> Nystatin MIC was 4 mg/L for the *C. krusei* strain and 2 mg/L for the *C. parapsilosis*.

No statistically significant differences were found between the antifungal activities of heated and non-heated amphotericin B against the zygomycete isolates. Amphotericin B MICs obtained without and after heating were within  $\pm 1$  log<sub>2</sub> dilution step in 100% of the strains.

Table 1 summarizes the *in vitro* activity of the three antifungal agents tested. Amphotericin B had MIC values of  $<2$  mg/L for most of the strains. *Rhizopus* spp. were significantly less susceptible to amphotericin B than *Mucor* spp. ( $P < 0.001$ ) and *A. corymbifera* ( $P < 0.001$ ). For two strains (one *C. bertholletiae* and one *A. elegans*), amphotericin B showed high MICs of 2 mg/L. Nystatin was active against most of the strains, with an overall geometric mean MIC of 1.59 mg/L. Nevertheless, nystatin was significantly less active than amphotericin B ( $P < 0.001$ ) and this difference was noted for all the genera. The two strains with elevated amphotericin B MICs were also less susceptible to nystatin.

#### Discussion

The results of this study show that heating of amphotericin B at 70°C for 20 min does not alter its *in vitro* antifungal activity against Zygomycota. To our knowledge this is the first study to test the influence of heating of amphotericin B on its antifungal activity against filamentous fungi. Our data are consistent with previous results obtained in yeasts, where heating did not reduce the fungistatic and fungicidal activity of amphotericin B against *C. albicans*<sup>2,4</sup> or *C. neoformans*.<sup>3</sup> Previous studies have demonstrated that thermal treatment of amphotericin B-deoxycholate changed the aggregation state of the drug and reduced its toxicity to mammalian cells *in vitro*. Heating of amphotericin B reduced both the permeabilizing effect and the cytotoxicity of the drug against human red blood cells, as measured by K<sup>+</sup> leakage and haemolysis efficiency.<sup>2,4</sup> The heated formulation of amphotericin B was also less toxic to a human cell line in culture than the unheated solution.<sup>2</sup> Efficacy with reduced toxicity has been demonstrated for heated amphotericin B in animal models of severe candidiasis in leucopenic mice<sup>4</sup> and in models of disseminated candidiasis, cryptococcal pneumonia and

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**Table 1.** *In vitro* susceptibility of 36 zygomycete strains to amphotericin B, heated amphotericin B and nystatin

Strain (no. of isolates) and antifungal drug	MIC (mg/L)			
	range	MIC <sub>50</sub>	MIC <sub>90</sub>	GM
<i>Rhizopus</i> spp. (15)				
AMB	0.25–1	0.5	1	0.5
heated AMB	0.25–1	0.5	1	0.55
nystatin	1–8	4	8	3.82
<i>Absidia corymbifera</i> (10)				
AMB	0.06–0.25	0.125	0.25	0.14
heated AMB	0.06–0.25	0.125	0.25	0.16
nystatin	0.5–1	0.5	1	0.71
<i>Mucor</i> spp. (6)				
AMB	0.06–0.125	0.125	ND	0.11
heated AMB	0.06–0.125	0.125	ND	0.1
nystatin	0.5–1	0.5	ND	0.56
<i>Rhizomucor</i> spp. (3)				
AMB	0.06–0.125	ND	ND	0.1
heated AMB	0.06–0.125	ND	ND	0.08
nystatin	0.5–1	ND	ND	0.79
<i>Cunninghamella bertholletiae</i> (1)				
AMB	2	ND	ND	ND
heated AMB	2	ND	ND	ND
nystatin	8	ND	ND	ND
<i>Apophysomyces elegans</i> (1)				
AMB	2	ND	ND	ND
heated AMB	1	ND	ND	ND
nystatin	8	ND	ND	ND
All isolates (36)				
AMB	0.06–2	0.25	1	0.26
heated AMB	0.06–2	0.25	1	0.26
nystatin	0.5–8	1	8	1.59

AMB, amphotericin B; GM, geometric mean; ND, not done.

cryptococcal meningoencephalitis in immunocompetent mice.<sup>3</sup> Further experiments are needed to compare the antifungal activity of heated and unheated amphotericin B in animal models of infection with filamentous fungi.

Entrapment of polyenes into liposomes is another way to reduce their toxicity and to increase their therapeutic index. Recently, a liposomal formulation of nystatin has been developed. Liposomal nystatin was shown to be less toxic than free nystatin and to retain activity *in vitro* against medically important yeasts<sup>5</sup> and *Aspergillus fumigatus*, including itraconazole-resistant isolates.<sup>5,6</sup> The *in vivo* efficacy of liposomal nystatin was also demonstrated against experimental disseminated and pulmonary *Aspergillus* infections in animals,<sup>7–9</sup> including infection with an isolate of *A. fumigatus* that had reduced susceptibility to amphotericin B.<sup>9</sup>

Published reports of the *in vitro* activity of liposomal nystatin against other opportunistic filamentous fungi are very scarce: it had a poor *in vitro* activity against *Scedosporium*

*apiospermum* and *Scedosporium prolificans*. In this study, we have tested nystatin against a broad range of Zygomycota. The results show that although nystatin exhibits higher MICs than amphotericin B, it is active against most of the strains.

The high amphotericin B MICs for one *C. bertholletiae* and one *A. elegans* are in accordance with previous *in vitro* studies and clinical reports. *Cunninghamella* infections mostly develop in immunocompromised patients and are often fatal with an overall mortality of 79%. These infections seem to be unresponsive to amphotericin B therapy.<sup>1</sup> Although infections with *A. elegans* occur predominantly in immunocompetent patients, response to amphotericin B therapy is variable<sup>10</sup> and many of the patients required surgical interventions. Further studies of these rare but severe infections in animal models are needed to evaluate the beneficial effect of amphotericin B therapy and the potential of new antifungal drugs.

In conclusion, our data show the following. (i) Mild heating of amphotericin B did not alter the *in vitro* antifungal activity of the drug against Zygomycota. Further animal experiments are needed to assess the potential of heated amphotericin B for the treatment of zygomycosis. (ii) Nystatin was active against most of the strains. The new liposomal formulation of nystatin should be evaluated for use in the treatment of zygomycosis.

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