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In vitro studies on the activity of amphotericin B and lipid-based amphotericin B formulations against *Aspergillus* conidia and hyphae

In vitro-Empfindlichkeit von *Aspergillus*-Konidien und -Hyphen gegenüber Amphotericin B und Amphotericin B-Lipidpräparationen

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Schlüsselwörter. *Aspergillus*, Amphotericin B, liposomale Formulierung, kolloidale Dispersion, Empfindlichkeitsprüfung.

Summary. The minimum inhibitory concentrations (MICs) of amphotericin B and lipid-based amphotericin B formulations against isolates of *Aspergillus* spp. were tested using a broth microdilution method. Twelve isolates of *Aspergillus fumigatus*, eight of *Aspergillus flavus*, six of *Aspergillus niger* and seven of *Aspergillus terreus* were examined. In addition, an assay for hyphae of *Aspergillus* spp. was performed since the invasive form is manifested by the appearance of hyphal structures. MICs of hyphae against lipid-based amphotericin B formulations were within three dilutions higher than those against conidia for almost all isolates of *Aspergillus* spp. ($P < 0.01$). In contrast, the differences in the *in vitro* efficacies of amphotericin B were the lowest for *Aspergillus* spp. This study demonstrates the importance of the type of inoculum used to test antifungal susceptibilities of *Aspergillus* spp. The significance of these results for *in vivo* outcome needs to be determined.

Zusammenfassung. Die minimalen Hemmkonzentrationen (MHK) von Amphotericin B und Amphotericin B-Lipidpräparationen gegenüber *Aspergillus*-Isolaten wurden mittels Mikrodilutionsmethode getestet. Zwölf Isolate von *Aspergillus fumigatus*, acht von *Aspergillus flavus*, sechs von *Aspergillus niger* and sieben von *Aspergillus terreus* wurden untersucht. Zuzüglich wurde ein Empfindlichkeitstest mit Hyphen durchgeführt, da diese die eigentliche invasive Form der Aspergillose darstellen. Die MHKs der Lipidpräparationen für Hyphen waren im Vergleich zu Konidien immer signifikant erhöht ($P < 0.01$). Für Amphotericin B wurden geringere Unterschiede für alle *Aspergillus*-Isolate festgestellt. Unsere Untersuchungen zeigen signifikante Unterschiede der MHKs in Abhängigkeit von Hyphen oder Konidien als Inokula auf. Die klinische Signifikanz der Ergebnisse muß evaluiert werden.

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Introduction

Fungal pathogens are recognized as a major and increasing source of infection in immunocompromised hosts [1, 2], with *Aspergillus* species, *Fusarium* species, zygomycetes, dematiaceous fungi, and other opportunistic fungi being the main pathogens [3, 4]. Infection with *Aspergillus* spp. is a common cause of nosocomial pneumonia and is associated with an extremely high mortality rate of 40% [5, 6]. The most common species causing disease in patients are *Aspergillus fumigatus* (83%), *Aspergillus flavus* (9%), *Aspergillus niger* (5%) and *Aspergillus terreus* (3%) [4, 7].

Over the last 30 years, amphotericin B has remained the drug of choice for invasive aspergillosis in immunosuppressed hosts despite its toxicity

and low response rates of 30–55% [4]. In the mean time, less toxic antifungal drugs have become available for treatment, and antifungal susceptibility testing with these opportunistic pathogens is important in the clinical laboratory. However, reliable antifungal susceptibility testing is still poorly developed, especially for filamentous fungi. Broth dilution methods are the most common techniques for antifungal susceptibility testing.

In a previous study using viability staining we found that the minimum fungicidal concentrations (MFCs) for azoles against hyphae were always higher in comparison to those against conidia [8]. In the present study we investigated the minimum inhibitory concentrations (MICs) of amphotericin B and lipid-based amphotericin B formulations against the hyphae of *Aspergillus* spp. since the conidial form is under-represented in invasive aspergillosis. We used a microbroth dilution assay and compared the results to those obtained with conidial inocula.

Materials and methods

Strains

The *in vitro* tests were performed on clinical isolates of *Aspergillus* spp. comprising *A. fumigatus* ($n = 12$), *A. flavus* ($n = 8$), *A. niger* ($n = 6$) and *A. terreus* ($n = 7$). All strains were grown on Sabouraud glucose agar (Merck, Darmstadt, Germany) and incubated at 37 °C for 7 days.

Antifungal agents

Stock solutions of amphotericin B (Squibb, Middlesex, UK), liposomal amphotericin B (Ambisome, Nexstar Pharmaceuticals, Leavosan, Linz, Austria) and colloidal amphotericin B dispersion (Amphocil, ABCD, Sequus Pharmaceuticals, Torrex, Vienna, Austria) were dissolved in water for injection (Fresenius, Linz, Austria) and diluted as described [9, 10].

Broth microdilution assay for conidia

Fungi were tested using a modification of the broth microdilution method for antifungal susceptibility testing of conidia-forming filamentous fungi [9]. The conidial suspension was harvested by flooding each colony with 2 ml sterile 0.85% saline. Turbidity was measured with a spectrophotometer at 530 nm (Beckman, DU-64 spectrophotometer, Foulerton, MN, USA) and transmission was adjusted with sterile water to 78–82% for *A. flavus* and *A. niger* and to 80–82% for *A. fumigatus* and *A. terreus*. The suspension was further diluted 1 : 50 in RPMI-1640 medium (BioWhittaker, Vienna, Austria) to obtain 1×10^4–5×10^4 colony-forming units (CFU) ml^{-1}. Drug dilutions were prepared and final concentrations between 5 and 0.07 μg ml^{-1} for amphotericin B and between 50 and 0.09 μg ml^{-1} for the lipid-based amphotericin B were used. For the tests, 100 μl of the drug solutions was inoculated with 100 μl of the fungal suspensions and incubated at 37 °C for 48 h. The tests were performed twice and in duplicate. The MIC end-point criterion was the lowest drug concentration showing no visible growth.

Broth microdilution assay for hyphae

Fungi were tested using a modification of the procedure as previously described [8]. The conidial stock solutions were prepared as described above and diluted 1 : 50 in RPMI-1640 (BioWhittaker) containing 10 mM HEPES (Sigma, St Louis, MO, USA) to obtain the desired inoculum size of 1×10^4–5×10^4 CFU ml^{-1}. Then, 100 μl of these solutions was added onto 94-well plates (Costar, Vienna, Austria) and incubated at 30 °C for 16–22 h to allow the formation of hyphae. This allowed outgrowth of more than 95% of conidia, with hyphal length varying from 50 to 70 μm, as determined by an inverted microscope. Wells were washed and refilled with 100 μl RPMI-1640 and the antifungal agents were added and incubated at 37 °C for 48 h. All tests were performed twice and in duplicate, MIC was determined as described above.

Statistics

Analysis of variance for repeated measures was employed for statistical comparison of the MICs for antifungal drugs, *Aspergillus* spp., conidia and hyphae. P values <0.05 were considered statistically significant.

Results

Table 1 summarizes the *in vitro* activities of amphotericin B and lipid-based amphotericin B formulations. The data are presented as MIC ranges and as the drug concentrations required to inhibit 50% or 90% of the isolates (MIC_{50}, MIC_{90}, respectively).

The *in vitro* efficacy of the lipid-based amphotericin B formulations did not differ significantly within the isolates tested (MIC, $P = 0.18$). MICs for hyphae were significantly higher ($P < 0.01$) than those for conidia for almost all isolates of *Aspergillus* spp.

Table 1. Comparision of MICs (μg ml^{-1}) obtained with conidial and hyphal inocula of *Aspergillus* spp.

Fungi (no. tested)		Amphotericin B		Liposomal Amphotericin B		Colloidal Amphotericin B	
		Conidia	Hyphae	Conidia	Hyphae	Conidia	Hyphae
A. fumigatus	MIC range	0.39–1.25	0.63–1.25	0.39–0.78	0.78–3.13	0.19–0.78	0.39–3.13
($n = 12$)	MIC_{50}	0.39	0.63	0.78	1.56	0.39	0.39
	MIC_{90}	1.25	1.25	0.78	3.13	0.78	3.13
A. flavus	MIC range	0.63–1.25	1.25–2.5	0.78–3.13	0.78–6.25	0.19–1.56	0.39–3.13
($n = 8$)	MIC_{50}	0.63	1.25	0.78	1.56	0.39	1.56
	MIC_{90}	1.25	2.5	1.56	3.13	0.39	3.13
A. niger	MIC range	0.31–1.25	0.31–1.25	0.1–0.78	0.78–3.13	0.19–0.39	0.39–0.39
($n = 6$)	MIC_{50}	0.31	0.31	0.39	0.78	0.19	0.39
	MIC_{90}	0.63	1.25	0.78	3.13	0.19	0.39
A. terreus	MIC range	0.63–1.25	1.25–1.25	0.1–0.39	1.56–3.13	0.39–1.56	0.39–3.13
($n = 7$)	MIC_{50}	0.63	1.25	0.39	1.56	0.39	1.56
	MIC_{90}	1.25	1.25	0.39	3.13	0.78	3.13

The differences in MICs of amphotericin B were slightly higher I hyphae than in conidia, representing a statistical significance of $P < 0.05$.

Discussion

In vitro testing using conidial suspension as inoculum yielded narrow ranges for MIC ranges, MIC_{50} and MIC_{90} against amphotericin B and lipid-based amphotericin B formulations (see Table 1); similar results were obtained by others [10–12]. However, only a few data document an agreement of MICs and clinical outcome, because both low- and high-level amphotericin B-resistant *Aspergillus* spp. isolates are associated with clinical failure [6, 13].

Aspergilli are respiratory pathogens, and pulmonary infections are usually acquired through inhalation of conidia [14], whereas the invasive form is dominated by the appearance of hyphae. So far, the main target of antifungal agents has been the hyphal structure, and impairment of hyphae must be guaranteed by a therapy in order for it to be successful.

Our study demonstrates that in order to inhibit the hyphae of *Aspergillus* spp. *in vitro* the antifungal agents must be applied at higher concentrations (up to fourfold) than those required to inhibit conidia ($P < 0.01$). Different composition of the fungal membrane, an endogenous catalase [15], or the quantity of hyphal biomass in comparison to that of conidia [16] could account for the higher resistance of hyphae.

However, for amphotericin B the differences in MICs between conidia and hyphae were lower ($P < 0.05$). Martin *et al.* [17] compared inocula of conidia and of germinating conidia and observed that MFCs of amphotericin B and itraconazole were higher for the germinated forms. Similar data were found in a previous study using viability staining of hyphae exposed to azoles [11]. Manavathu *et al.* [16] however, reported that MICs and MFCs of various antifungal agents for germinated conidia were identical to those for ungerminated conidia of *A. fumigatus*. Since our study observed that higher doses were required to inhibit hyphae, we deduce that the maximal tolerated dose of antimycotics should be administered to patients with diagnosed or probable fungal infections in order to overcome infection. Whether antifungal drug concentrations which inhibit hyphae are achievable in infected tissue is not known. The average tissue concentrations of lipid formulations of amphotericin B were twofold higher than those of conventional amphotericin B. Hence, the overall value of treatment of infections remains controversial [18].

In conclusion, our data demonstrate the importance of the type of inoculum used to test the antifungal susceptibilities of *Aspergillus* spp. The use of hyphae possibly reflects the *in vivo* situation better than the use of conidia and partly explains the difficulty of providing successful therapy. More studies are needed to implement a standard assay for testing fungal hyphae and to compare *in vitro* findings with *in vivo* outcome.

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