Effects of amphotericin B on Aspergillus flavus clinical isolates with variable susceptibilities to the polyene in an experimental model of systemic aspergillosis

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Received 2 April 2013; returned 22 April 2013; revised 6 May 2013; accepted 17 May 2013

Objectives: The aim of the present study was to evaluate the effects of amphotericin B (AMB) on clinical isolates of Aspergillus flavus.

Methods: MICs of both standard AMB and liposomal AMB (L-AMB) were determined using a broth dilution method for seven isolates of A. flavus. AMB MICs were also determined using the Etest. The activity of the polyene was then investigated in a murine model of systemic aspergillosis in which animals were infected intravenously, treated intravenously with several doses of the polyene (1–10 mg/kg/day) and observed for survival.

Results: Broth dilution AMB, broth dilution L-AMB and Etest AMB MICs ranged from 0.5 to 2.0 mg/L, 0.06 to 0.16 mg/L and 1.0 to 3.2 mg/L, respectively. There were two isolates for which all doses were effective at prolonging the survival. Their AMB MICs were ≤ 1.0 mg/L, regardless of the method/drug formulation utilized for testing. There were four isolates for which no regimen was effective. Their broth dilution AMB, broth dilution L-AMB and Etest AMB MICs ranged from 1.0 to 2.0 mg/L, 0.06 to >16 mg/L and 2.0 to >32 mg/L, respectively. There was one isolate for which only L-AMB given at 10 mg/kg/day was effective; broth dilution MICs of AMB and L-AMB were 0.5 mg/L, while the Etest MIC of AMB was 2.0 mg/L.

Conclusions: Our data indicate that not all isolates of A. flavus should be considered resistant to AMB. The Etest represented the in vitro method that best correlated with the experimental infection. Finally, a clinical isolate showing an MIC ≥2.0 mg/L may be reasonably considered resistant in vivo to any dose/formulation of the polyene.

Keywords: antifungal susceptibility testing, antifungal resistance, in vitro–in vivo correlation

Introduction

In addition to Aspergillus fumigatus, other Aspergillus species, such as Aspergillus flavus, Aspergillus terreus and Aspergillus niger, have increasingly become the causative agents of severe opportunistic infections, mainly in immunocompromised patients.1 It has been reported that some Aspergillus species other than A. fumigatus might be less susceptible to currently used antifungal agents. Indeed, a reduced susceptibility to amphotericin B (AMB) has been described among isolates of A. flavus and A. terreus.2–6 In addition, the former species has been reported to be consistently more virulent than A. fumigatus.7–9 These two characteristics (i.e. reduced drug susceptibility along with enhanced virulence traits) makes A. flavus a species more difficult to manage than A. fumigatus.10,11

In this study, we investigated the effects of AMB on the outcome of experimental invasive aspergillosis caused by clinical isolates of A. flavus with variable susceptibilities to the polyene.

Methods

Isolates

Seven clinical isolates of A. flavus were utilized in this study (see Table 1). They were cultured from several specimens of patients suffering from haematological malignancies. Each strain represented a unique isolate.
from a patient. Identification to the species level was first made by conventional methods and then confirmed by molecular analysis, as described elsewhere. The isolates were maintained in slopes of nutrient broth containing 10% glycerol in liquid nitrogen for subculturing. Before testing, each isolate was subcultured on Sabouraud dextrose agar to ensure viability and purity.

**Drugs**

AMB was used as pure powder (Sigma–Aldrich, Milan, Italy) dissolved in DMSO for in vitro studies and as a commercial preparation (Fungizone; Bristol-Myers Squibb, Latina, Italy) dissolved in 5% dextrose water for in vivo studies. Liposomal AMB (L-AMB; Gilead Sciences, Milan, Italy) was used as a commercial preparation for both in vitro and in vivo studies.

**In vitro studies**

Antifungal susceptibility testing was performed by a broth dilution method following the recommendations of CLSI document M38-A2. The final range of drug concentrations tested was 0.03–16 mg/L. Following incubation, MIC endpoints were determined as the lowest drug concentration that resulted in a 100% reduction in growth compared with that of the drug-free controls. As specified in the CLSI methodology, quality control was ensured by testing the following strains: Aspergillus fumigatus ATCC MYA-3626; A. flavus ATCC MYA-3631; Candida parapsilosis ATCC 22019; and Candida krusei ATCC 6258. Additionally, the AMB Etest (AB BIODISK, Solna, Sweden) was performed on RPMI 1640 agar plates with 2% glucose (Sigma–Aldrich) according to the manufacturer’s instructions, with inoculum suspensions prepared in the same way as for the CLSI method. After 48 h of incubation, the Etest MIC was defined as the lowest drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip. Each isolate was tested in triplicate by broth dilution and the Etest.

**In vivo studies**

CD1 female mice (20 g, 8 weeks old; Charles River Laboratories, Calco, Italy) were utilized in all in vivo studies. Mice were rendered neutropenic by intraperitoneal administration of cyclophosphamide (200 mg/kg of body weight) on days −6, +1 and +4 and every 3 days thereafter. Experiments were conducted with the approval of the University of Ancona Ethics Committee and animals were cared for in accordance with national regulations. A murine model of systemic aspergillosis was established by intravenous injection of ~1 × 10⁴ (range 0.5–3.4 × 10⁴) conidia of each A. flavus isolate. Drugs were administered intravenously in a 0.2 mL final volume. AMB was given at 1 mg/kg/day. L-AMB was given at 1, 3 and 10 mg/kg/day. All drugs were initiated 6 h post-infection and were continued to day 2 post-infection (i.e., three daily doses on days 0, 1 and 2 post-infection). The mice were observed twice daily until day 15 post-infection. There were 8–10 mice in each group.

**Statistical analysis**

The survival studies were analysed by the log-rank test and plotted using Kaplan–Meier curves. All P values <0.05 were considered significant.

**Results**

AMB MICs for control strains were within the expected ranges: 0.5 mg/L for A. fumigatus ATCC MYA-3626, 2.0 mg/L for A. flavus ATCC MYA-3631, 1.0 mg/L for C. parapsilosis ATCC 22019 and 1.0 mg/L for C. krusei ATCC 6258. AMB MICs for seven clinical isolates of A. flavus ranged from 0.5 to 2.0 mg/L, while L-AMB MICs ranged from 0.06 to >16 mg/L. AMB MICs obtained by the Etest ranged from 1.0 to >32 mg/L (Table 1). Figure 1 shows survival studies for seven isolates of A. flavus. There were two isolates (# 163 and # 235) for which only L-AMB given at 10 mg/kg/day was effective at prolonging the survival compared with the controls. For these isolates, the AMB MIC was ≤1.0 mg/L, regardless of the method/drug formulation utilized for testing. There were four isolates (# 175, # 225, # 243 and # 264) for which no regimen was effective. Three of these isolates showed AMB MICs ranging from 1.0 to 2.0 mg/L, while L-AMB MICs ranged from 16 to >16 mg/L. AMB MICs obtained by the Etest for the four isolates were all ≥20 mg/L. Since isolate # 225 showed the lowest AMB MIC (0.06 mg/L) among all the isolates tested, we repeated a survival study and found identical results (Figure 1). For this isolate, the AMB MIC obtained by the Etest was 2.0 mg/L. There was one isolate (# 218) for which only L-AMB given at 1 mg/kg/day was effective at prolonging survival. Broth dilution yielded AMB/L-AMB MICs of 0.5 mg/L, while the Etest yielded an AMB MIC of 2.0 mg/L.

**Discussion**

To our knowledge, this is the first study in which AMB has been tested in vivo against a considerable number of clinical isolates of A. flavus. Our study highlights several features.

First, not all clinical isolates of A. flavus should be considered resistant to AMB. Indeed, we found a wide range of MICs (0.06 to >32 mg/L). The in vitro results were greatly affected by the methodology utilized for testing. As repeatedly described in the literature, CLSI methodology (i.e. AMB tested by broth dilution) produced the narrower MIC range (0.5–2.0 mg/L). These data reinforce the notion that this method is not able to differentiate isolates with variable susceptibility to the polyene. This fact has been demonstrated for both yeasts and mould isolates.

To overcome this inconvenience the use of antibiotic medium 3 has been proposed, with the caveat that batch-to-batch variability of the medium should be monitored. Highly controversial and
debated are the data concerning AMB MICs determined using lipid derivatives of the polyene, since these formulations after reconstitution and filtering might not be exact and could result in substantial error in the concentration of drug present in the assay, as could the release of amphotericin B from the liposomes. Some studies have shown that the use of liposomal formulations for in vitro testing yielded a broadening of MIC ranges with respect to those obtained with the pure powder form of AMB.3,22–25 Our results confirm this concept, having identified an L-AMB MIC range of 0.06 to >16 mg/L. Similarly, the Etest revealed variable susceptibility among the clinical isolates, with an MIC range of 1.0 to >32 mg/L. In addition, the latter method has proved to correlate better with the outcome of experimental infection. It is interesting to note that animals infected with isolates

Figure 1. Survival of mice infected intravenously with ~1 × 10⁴ conidia per mouse of seven isolates of A. flavus. All studies were conducted by initiating intravenous therapy 6 h post-infection (day 0) and were continued through day 2 post-infection (three consecutive daily doses). There were 8–10 mice in each group. Asterisks indicate groups with prolonged survival over controls (P < 0.05).
showing Etest AMB MICs of 1.0 mg/L responded to all doses. By contrast, animals infected with isolates showing Etest AMB MICs ≥2.0 mg/L did not respond to any dose/formulation of the polyene. The only exception was represented by the animals infected with isolate # 218, which responded, with limited statistical significance, only to the highest dose of L-AMB.

The most significant discrepancy between in vitro and in vivo data was observed for L-AMB/isolate # 225. The L-AMB MIC for this isolate was 0.06 mg/L. This value, confirmed by multiple experiments, represented the lowest MIC obtained for this series of clinical isolates. By contrast, the AMB MICs obtained by broth dilution and the Etest were 1.0 and 2.0 mg/L, respectively. This discrepancy led us to repeat the in vivo experiment, which confirmed the total inefficacy of the various regimens, including those employing high doses of the liposomal derivative. This further emphasizes that an MIC value of 2.0 mg/L characterizes an unresponsive isolate, at least in an experimental context.

Although no definitive breakpoints for AMB have been validated, several documents and case reports indicate a value of 2.0 mg/L as identifying fungal strains (yeasts or moulds) that are less susceptible to AMB therapy. Although limited to seven clinical isolates, our experimental data seem to indicate that this breakpoint is also suitable for characterizing the susceptibility/resistance of A. flavus.

In conclusion, our data indicate that not all clinical isolates of A. flavus are resistant to AMB. The in vitro method that we correlate with the experimental infection is the Etest. Finally, a clinical isolate showing an MIC ≥2.0 mg/L may be reasonably considered resistant in vivo to any dose/formulation of the polyene. Clearly, further studies are needed to corroborate these findings.

Funding
This project was supported by internal funding from Ricerca di Ateneo to F. B.

Transparency declarations
None to declare.

References
Amphotericin B against *Aspergillus flavus*


