MYCOLOGY

Amphotericin B resistance of *Aspergillus terreus* in a murine model of disseminated aspergillosis

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The in-vivo activity of amphotericin B and itraconazole against a clinical isolate of *Aspergillus terreus* was determined in a murine model of disseminated aspergillosis. MICs of amphotericin B and itraconazole for the strain, determined by an NCCLS-based technique, were 2 µg/ml and 1 µg/ml, respectively. Mice infected intravenously were treated with either itraconazole (50 or 100 mg/kg/day) or amphotericin B 4.5 mg/kg/day for 10 days. Treatment with both doses of itraconazole significantly prolonged the survival rates compared with those for untreated mice. In comparison, mortality rate and median survival time were identical for mice treated with amphotericin B and for mice given no therapy, indicating that the strain was highly resistant to amphotericin B in this model. Analysis of sterol composition showed that the major sterol was ergosterol. This suggests that amphotericin B resistance was not related to a modified sterol profile.

Introduction

The frequency of aspergillus infections has increased in recent years, particularly in patients being treated for haematological malignancies. Among the major species of *Aspergillus* involved in invasive infections in man, *A. fumigatus* is the most common, but other species such as *A. terreus* are emerging [1, 2]. Amphotericin B remains the most established antifungal agent used for the treatment of invasive aspergillosis, even if the overall success rate is low [3]. The antifungal action of amphotericin B originates from its binding to ergosterol in the membrane of fungi, altering permeability and causing leakage of cell components [4].

Antifungal susceptibility testing for filamentous fungi is not yet standardised, but different reports have suggested that *A. terreus* is less susceptible to amphotericin B than *A. fumigatus* *in vitro* [5, 6] and *in vivo* [2]. Nevertheless, in-vitro detection of amphotericin B resistance of *Aspergillus* spp. is difficult and correlation of in-vitro results with clinical outcome data is poor [7, 8].

Resistance to amphotericin B has often been associated with qualitative or quantitative alterations of membrane lipids, especially sterols [9].

In the present study, a clinical isolate of *A. terreus* from a patient with invasive pulmonary aspergillosis was tested against amphotericin B and itraconazole *in vitro* and *in vivo* in a murine model of aspergillosis and a biochemical study of the sterol composition of this strain was performed.

Materials and methods

Strain and patient history

A. terreus strain AT 81 was isolated from a broncho-alveolar lavage (BAL) fluid from a 54-year-old man with invasive pulmonary aspergillosis who was hospitalised with acute myeloblastic leukaemia. Five weeks after admission, he developed antibiotic-resistant fever and cough, after 30 days of granulocytopenia. Computer tomography scan of his chest showed bilateral nodules with a halo sign. A BAL fluid sample showed hyphae and culture was positive for *A. terreus*. Circulating *Aspergillus* antigen (Platelia® *Aspergillus*, Sanofi Diagnostics Pasteur, Marnes La Coquette, France) was detected in several serum samples. The diagnosis of highly probable pulmonary aspergillosis was established and treatment was started with intravenous amphotericin B at 1.3 mg/kg/day and oral

itraconazole at 600 mg/day. Amphotericin B therapy was given for 7 weeks and itraconazole for 6 months. Itraconazole concentrations in serum were $>1 \mu\text{g/ml}$, measured by high-performance liquid chromatography (HPLC). One month after therapy was begun, clinical symptoms improved and the lesions remained stable radiographically. The patient died 5 months later from a *Stenotrophomonas maltophilia* septicaemia.

The *A. terreus* strain was stored at -80°C in glycerol 10% until used.

In-vitro susceptibility testing

Susceptibility testing was performed by an NCCLS-based broth microdilution technique [10, 11]. RPMI 1640 (Gibco BRL, Uxbridge) with L-glutamine and without sodium bicarbonate, buffered at pH 7.0 with 0.165 M morpholinepropanesulphonic acid (MOPS) was used as the test medium. Amphotericin B (Sigma) and itraconazole (Janssen Pharmaceutica, Beerse, Belgium) were provided by the manufacturers as powders. Both drugs were dissolved in dimethylsulphoxide (DMSO; Sigma) to a concentration of $1600 \mu\text{g/ml}$ and stored frozen in small volumes at -80°C as stock solutions. The drug dilutions were prepared by following the standard additive two-fold drug dilution scheme described in the NCCLS reference method for yeasts [12], with medium used as the diluent. The final drug concentrations were 0.03– $16 \mu\text{g/ml}$ for amphotericin B and itraconazole. The strain was cultured on Malt Extract Agar (MEA) slants (Sanofi Diagnostics Pasteur) at 35°C for 5 days. The inoculum was prepared by washing the surface of the agar slants with 1 ml of sterile NaCl 0.9% containing Tween 80 0.05%. The resulting conidial suspension was counted with a haemocytometer. Sterile 96-well U-shaped microtitration plates were used. The conidia were diluted in RPMI and each well of rows 2–12 was inoculated. The final inoculum concentration was 1×10^4 conidia/ml. Row 1 was used as sterility control and row 12 as growth control. The MICs were run in duplicate. Microtitration plates were incubated at 35°C for 48 h and the growth in each well was compared with that of the growth control with the aid of a microtitre reading mirror. Each well was then given a numerical score [10]: 4, no reduction in growth; 3, growth reduction of 25%; 2, growth reduction of 50%; 1, growth reduction of $\geq 75\%$; and 0, absence of growth (optically clear). MIC endpoints were defined as the lowest drug concentration which had a score of 1 for itraconazole and the lowest concentration that had a score of 0 for amphotericin B.

Mice

Female OF-1 outbred mice (IFFA CREDO, l'Arbresle, France), 5–7 weeks old, weighing 20–22 g were used throughout the experiments. Mice were housed in groups of 10 and were given food and water *ad*

libitum. Animal studies were conducted in accordance with the recommendations of the European Communities (Directive no. 86/609/EEC, 24 November 1986).

Infection

The inoculum was prepared by culturing the strain on MEA for 5 days at 35°C . Spores were harvested by washing the agar slant with sterile NaCl 0.9% containing Tween 80 0.05% and the spore suspension was counted in a haemocytometer. Viability was determined by plating serial 10-fold dilutions prepared in NaCl 0.9% with Tween 80 0.05%. Plates were incubated at 35°C and the numbers of cfu were counted at 24 h and 48 h. On the day of infection, the spore suspension was adjusted to the required concentration in NaCl 0.9%. A preliminary study was performed to determine the LD₉₀ (90% lethal dose) for the strain by testing three inoculum sizes (1×10^5 , 1×10^6 and 1×10^7 cfu/mouse). Mice were infected by injection of 0.1 ml of the conidial suspension into a lateral tail vein. After infection, mice were randomised to the different treatment groups.

Drugs and therapy

Amphotericin B desoxycholate (Fungizone[®]; Bristol-Myers Squibb, Paris, France) was given in glucose 5% intraperitoneally (i.p.) and itraconazole (Sporanox[®] oral solution; Janssen Pharmaceutica, Beerse, Belgium) was diluted in sterile water and given by gavage. Treatment with both drugs was begun 2 h after infection and was continued for 10 days. Five groups of 10 mice were used. One group was treated with amphotericin B 4.5 mg/kg/day by once-daily injection and two groups were treated with itraconazole 100 mg/kg/day and 50 mg/kg/day, respectively, administered in two daily doses. Control mice were infected, but received only glucose 5%, one group by intraperitoneal injection and one group by gavage. Animals were checked twice daily for mortality. The mice were observed for 12 days after the end of treatment.

Organ cultures

Surviving mice on day 22 after infection were killed by cervical dislocation and quantitative cultures of brain and kidney were performed. Organs were removed and homogenised with 3 ml of NaCl 0.9% in a tissue grinder. Two 10-fold serial dilutions were made and 0.1 ml of each dilution (including the neat dilution) was plated on to Sabouraud's agar. Plates were incubated at 35°C and colonies were counted daily for 3 days for control groups and 5 days for treated groups. Qualitative organ cultures were performed on all mice that died before the end of the experiment.

Itraconazole assay

Two groups of six uninfected mice were given itraconazole by gavage at 50 mg/kg/day and 100 mg/kg/day, respectively. In each group, three mice were killed after 4 days of treatment and three after 6 days of treatment, 6 h after the last dose was administered. Blood was obtained by cardiac puncture and organs (kidney and brain) were homogenised in a tissue grinder with 3 ml of NaCl 0.9%. Serum and tissue levels of itraconazole were determined by HPLC [13, 14].

Sterol analysis

For sterol analysis, the strain was cultured on MEA for 5 days at 35°C. Spores were harvested and 100 ml of Sabouraud liquid medium (Sanofi Diagnostics Pasteur) were inoculated with 5×10^5 conidia/ml and the strain was grown for 24 h at 35°C with agitation. Mycelium (500 mg) was saponified at 80°C for 2 h with 3 ml of methanol, 2 ml of pyrogallol 0.5% in methanol, and 2 ml of aqueous KOH 60%. The non-saponifiable lipids (sterols) were then extracted three times with 5 ml of hexane. After the hexane had been evaporated under nitrogen, samples were stored at -20°C before use. Sterols were analysed under trimethylsilyl ether derivatives by gas chromatography-mass spectrometry on an HP 5970 apparatus. The capillary column (30 m $\times$ 0.25 mm) was coated with DB-XLB (J. W. Scientific, Folsom, CA, USA). Injection (1–2 μ l) was *via* a splitless injector at 280°C and the carrier gas was helium at 100 kPa. The oven temperature was set at 200°C for 1 min, then raised from 200 to 260°C at 8°C/min and from 260 to 320°C at 2°C/min. Electronic impact spectra were obtained at 70 eV. The sterols were quantified by electronic integration of chromatogram peaks, and results are expressed as percent area. Identification was made by comparison of their fragmentation data with controls and published data [15–17].

Data analysis

Data for the two control groups were pooled for the analysis. Mortality and quantitative culture data were compared by the Kruskal-Wallis test ($p < 0.05$ was considered significant). Organs from mice that died before the end of the experiment were assigned quantitative values equal to the highest counts in the organs of any surviving mouse, assuming that death represented a worse outcome than survival with any fungal organ burden. Qualitative organ cultures were compared by the Fisher's exact test ($p < 0.01$ was considered significant).

Results

In-vitro susceptibility testing was performed three times with reproducible results. The MICs of the *A.*

terreus strain obtained from the patient BAL, determined by an NCCLS-based technique, were 2 μ g/ml for amphotericin B and 1 μ g/ml for itraconazole.

For the animal model, a preliminary experiment was performed to determine the LD₉₀ of the strain at day 11 (which corresponded to the end of the treatment period in the treatment experiment). Survival rates of mice infected with three different inoculum sizes were determined. Mortality rates were 10, 40 and 80% for 1×10^5 , 1×10^6 and 1×10^7 cfu/mouse, respectively. The LD₉₀ was estimated at 2×10^7 cfu/mouse and this inoculum size was used for the treatment experiment.

Fig. 1 shows the survival curve for the treatment experiment and median survival time are shown in Table 1. In the control groups, 100% of mice receiving glucose i.p. died and 90% died in the group receiving glucose by gavage. Treatment with itraconazole at 50 mg/kg/day and 100 mg/kg/day resulted in a 70% and 90% survival rate, respectively; significantly better than for mice receiving no treatment ($p < 0.001$). In comparison, mice treated with amphotericin B died within 12 days, which was not significantly different from control groups ($p > 0.05$).

Concentrations of itraconazole in serum and in kidney and brain are presented in Table 2. In serum, itraconazole levels were 1.7–2.5-fold higher in the group treated with 100 mg/kg/day than in the group treated with 50 mg/kg/day. Serum levels were in same range at day 4 and day 6. Concentrations of itraconazole in organs (kidney and brain) were determined only in mice receiving 100 mg/kg/day. In kidney, the level of itraconazole was *c.* 60% that of the level in plasma at day 4 and was 2.6 times higher at day 6. In brain, levels were in the same range at day 4 and day 6, but were lower than those detected in kidney and serum.

Qualitative culture results of kidney and brain for all mice are presented in Table 3 and quantitative fungal burdens are shown in Table 4. All control mice had positive kidney and brain cultures. Identical results were found in the amphotericin B group. Itraconazole therapy at dosages of both 50 and 100 mg/kg/day did not reduce kidney infections. Brain cultures were positive in 80% of the cases in the group given itraconazole at 100 mg/kg/day and in 67% in the group given itraconazole at 50 mg/kg/day. As shown in Table 4, amphotericin B did not reduce fungal burdens in kidney and brain compared with controls. Itraconazole therapy at 50 and 100 mg/kg/day significantly reduced colony counts in kidney and brain and appeared to act in a dose-dependent manner.

The relative sterol composition of the strain is shown in Table 5. Ergosterol was the major sterol detected, representing >85% of the total amount of sterols. 24-

Fig. 1. Cumulative mortality for mice infected with *A. terreus* AT 81 in treated and control groups. ■, itraconazole 100 mg/kg/day; ◆, itraconazole 50 mg/kg/day; □, amphotericin B 4.5 mg/kg/day; ▲, 5% glucose control (by gavage); △, 5% glucose control (i.p.).

Table 1. Survival times for mice infected with *A. terreus* AT 81 and treated with amphotericin B (4.5 mg/kg/day) or itraconazole (100 or 50 mg/kg/day)

Group	Number of survivors/number of mice in group	Median survival time (days) and range
Controls	1/20	5 (3–23)
Amphotericin B	0/10	5 (3–12)
Itraconazole 100 mg	9/10	23* (20–23)
Itraconazole 50 mg	7/10	23* (17–23)

* $p < 0.001$ (compared with controls).

Table 2. Serum and tissue levels of itraconazole (native plus hydroxylated itraconazole) in mice after treatment for 4 days and 6 days with itraconazole at 100 or 50 mg/kg/day

Group	Mean (SD) level in *		
	serum ($\mu\text{g/ml}$)	kidney ($\mu\text{g/g}$)	brain ($\mu\text{g/g}$)
Day 4			
Itraconazole 100 mg	7.09 (1.52)	4.55 (1.38)	1.33 (0.40)
Itraconazole 50 mg	4.05 (0.53)	ND	ND
Day 6			
Itraconazole 100 mg	8.24 (0.51)	11.82 (1.20)	1.81 (0.69)
Itraconazole 50 mg	3.32 (0.93)	ND	ND

ND, not determined.

*Three mice in each group.

Table 3. Qualitative culture results for kidney and brain for all mice

Group	Number of mice positive/number of mice in group	
	kidneys	brains
Controls	20/20	20/20
Amphotericin B	10/10	10/10
Itraconazole 100 mg	10/10	8/10
Itraconazole 50 mg	10/10	6/9*

*Brain from one mouse was not cultured.

Ethyl-cholesta-5,7,22-trienol, previously characterised in zygomycetes [18], was also detected (*c.* 10% of the total amount of sterols). Each fraction of other biosynthetic intermediates was $<2\%$.

Discussion

The present study demonstrated amphotericin B resistance in a strain of *A. terreus* in an animal model of aspergillosis.

Table 4. Quantitative culture results for kidney and brain

Strain and treatment group	Number of mice in group	Number of survivors	Mean (SD) cfu $\times 10^2$ /g in	
			kidney	brain
Controls	20	1	11609 (0)	33.9 (7.2)
Amphotericin B	10	0	11609 (0)	35.5 (0)
Itraconazole 100 mg	10	9	1219* (3652)	10.8 [†] (16)
Itraconazole 50 mg	10	7	3894 [‡] (5423)	12.1 [‡] (16.4)

* $p < 0.001$; [†] $p < 0.01$; [‡] $p < 0.05$ (compared with controls).

Table 5. Relative sterol composition of *A. terreus* AT 81 mycelium grown for 24 h without antifungal agents

Sterol	Percentage of total sterol
Ergosterol	85.2
24-Ethyl-cholesta-5,7,22-trienol	10.1
Episterol	1.5
Ergosta 5,8,22,triol	1.4
Unknown sterols	1.8

Technical procedures for the in-vitro susceptibility testing of *Aspergillus* spp. are currently not standardised and numerous methods have been applied, with very different results [19]. Nevertheless, large-scale collaborative inter-laboratory studies have shown that adaptation of the NCCLS standard method for susceptibility testing of yeasts [12] is reproducible and reliable for the in-vitro determination of the antifungal susceptibility of filamentous fungi [10, 11]. The present study used an NCCLS-based technique to determine MICs of itraconazole and amphotericin B. The MIC of the *A. terreus* AT 81 was $\leq 2 \mu\text{g/ml}$ against both drugs. Nevertheless, amphotericin B resistance of *Aspergillus* spp. is difficult to detect *in vitro* and correlation between MIC and outcome of treatment with amphotericin B is poor [7, 8]. For this reason, an animal model of aspergillosis was used to test the amphotericin B resistance of *A. terreus*. This model of disseminated aspergillosis in immunocompetent mice has been used previously to confirm in-vitro resistance to itraconazole in *A. fumigatus* strains [20]. In the present study, the strain of *A. terreus* was shown to be highly resistant to amphotericin B. The low MIC of the strain against itraconazole and the good activity of itraconazole therapy in prolonging survival and reducing colony counts in both brain and kidney of infected mice demonstrate that there was no cross-resistance between amphotericin B and itraconazole. In the case reported here, itraconazole was given concurrently with amphotericin B and the patient showed a clinical improvement, which is consistent with a previous report [21].

The antifungal action of amphotericin B originates from its binding to ergosterol, leading to the formation of transmembrane channels, which induce altered permeability and cause leakage of cell components [4]. Moreover, it has been shown that oxidative damage plays a role in the mechanism of fungal cell killing by

amphotericin B [22]. The analysis of sterol composition of strain AT 81 showed an ergosterol content of 85%. A similar ergosterol content has been described in a strain of *A. fumigatus* which is susceptible to amphotericin B *in vivo* [23, 24]. Polyene antifungal resistance has previously been attributed to lesions in the ergosterol biosynthetic pathway in clinical isolates of *Candida albicans* [22, 25, 26] and *Cryptococcus neoformans* [27] and in laboratory mutants of *C. albicans* [22, 28], *A. fennelliae* [29] and of a protozoan, *Leishmania donovani* [30]. Nevertheless, amphotericin B resistance has also been reported in *C. albicans* [31], *Cr. neoformans* [32, 33] and *Ustilago maydis* [34] without significant alterations in sterol biosynthesis. In these cases, the underlying mechanism of such resistance remains unclear.

In conclusion, this study demonstrated the in-vivo resistance of a clinical isolate of *A. terreus* to amphotericin B. Biochemical studies suggest that the resistance is not related to a lack of ergosterol. Further studies are needed to confirm these results in other strains of *A. terreus* and to define the mechanism of amphotericin B resistance.

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