Delay of antifungal therapy influences the outcome of invasive aspergillosis in experimental models of infection

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Objectives: The aim of the present study was to evaluate the effects of delayed antifungal therapy on the outcome of invasive aspergillosis due to Aspergillus fumigatus in experimental models of infection.

Methods: A clinical isolate of A. fumigatus susceptible to amphotericin B (MIC 0.5 mg/L) and micafungin [minimum effective concentration (MEC) 0.03 mg/L] was used in all experiments. Two models of infection were investigated in immunosuppressed mice: disseminated infection and pulmonary infection. Twenty-four hours (early therapy) and 48 h (delayed therapy) post-infection, the mice were given vehicle, liposomal amphotericin B, micafungin or liposomal amphotericin B plus micafungin (combination). Drug efficacy was assessed by either survival or tissue burden experiments.

Results: In disseminated infection, any drug regimen given early significantly prolonged survival. When therapy was delayed, only micafungin and the combination were effective. In pulmonary infection, although there was a trend towards a prolongation of survival of mice treated early with liposomal amphotericin B, only the combination was effective. Similarly, when therapy was delayed, only the combination was effective. In disseminated infection, any drug regimen given early was effective at reducing the cfu in kidney tissue. In pulmonary infection, only liposomal amphotericin B and the combination given early were effective at reducing the cfu in lung tissue. Conversely, when therapy was delayed, no regimen was effective at reducing the tissue burden, regardless of the type of infection.

Conclusions: Our data indicate that delayed initiation of antifungal therapy is deleterious in experimental models of invasive aspergillosis. A combination regimen seems to have some advantages over a single-drug approach when the therapy is started late.

Introduction

Invasive aspergillosis (IA) is a life-threatening fungal infection associated with significant mortality.1,2 Both voriconazole and liposomal amphotericin B are shown to be effective in the treatment of IA.3,4 To further improve the outcome of IA, several antifungal combination approaches have been investigated.5,6 For its unique mode of activity, an echinocandin derivative would represent an interesting drug partner in combination regimens.

The difficulty in establishing an early diagnosis of IA can delay antifungal treatment, resulting in an increased risk of mortality. We hypothesized that a drug combination approach might be more effective than monotherapy in infections in which antifungal treatment is started late. Therefore, we investigated the effects of liposomal amphotericin B and micafungin, administered alone and in combination, in experimental models of IA in which therapy was initiated either soon or late after the infection.

Materials and methods

Isolate characterization

A clinical isolate of Aspergillus fumigatus (AF 119701) cultured from a respiratory secretion of a patient diagnosed with proven aspergillosis was used in all the experiments. Antifungal susceptibility testing, following the CLSI recommendations, found that the isolate was susceptible in vitro to both amphotericin B (median MIC 0.5 mg/L) and micafungin (median minimum effective concentration (MEC) 0.03 mg/L).7
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Drugs

Liposomal amphotericin B (AmBisome, Gilead Sciences) was reconstituted according to the manufacturer’s instructions and diluted in sterile 5% dextrose solution. Micafungin (Mycamine, Astellas) was rehydrated in sterile water and diluted in 0.9% sodium chloride solution.

Experimental infections

CD1 female mice (20 g, 8 weeks old; Charles River Laboratories, Calco, Italy) were used in all studies. Two models of infection were investigated: disseminated infection, performed by giving the conidia intravenously (~1.6 × 10⁷ conidia/mouse in 0.2 mL of saline solution); and pulmonary infection, performed by giving the conidia intranasally (~1.0 × 10⁷ conidia/mouse in 0.04 mL of saline solution). The mice were rendered neutropenic by intraperitoneal administration of cyclophosphamide (200 mg/kg of body weight) on days −4, +1 and +4 and every 3 days thereafter. Additionally, in the pulmonary infection model, the mice were given subcutaneously a single 300 mg/kg dose of cortisone acetate suspension on day −1. Experiments were conducted with the approval of the University of Ancona Ethics Committee, and animals were cared for in accordance with national regulations.

Twenty-four hours (early therapy) and 48 h (delayed therapy) post-infection, the mice were given vehicle, liposomal amphotericin B, micafungin or liposomal amphotericin B plus micafungin (combination). Drugs were used at low (3 mg/kg/day) or at high (10 mg/kg/day) dosages. Liposomal amphotericin B was given intravenously, whereas micafungin was given intraperitoneally. Both drugs were administered in a final volume of 0.2 mL. Therapy was given for three consecutive days.

Drug efficacy was assessed either by survival analysis (15 days) or tissue burden experiments (via kidney in disseminated infection; via lung in pulmonary infection). In the latter experiments, the mice were euthanized 24 h after the last dose of the drug was given, and the quantity of cfu per organ per mouse was determined. There were 10 to 25 animals for each treated group in survival studies and seven animals for each treated group in tissue burden experiments. In order to monitor the progression of infection, additional groups of untreated mice were killed at 24, 48 and 72 h after the initial infection. Kidneys (disseminated infection; a–d) and lungs (pulmonary infection, performed by giving the conidia intranasally) were removed, fixed in 10% neutral buffered formalin solution, embedded in paraffin and stained with Grocott–Gomori’s methenamine silver nitrate from mice infected with A. fumigatus. The mice were killed either 24 h (a, b, e and f) or 48 h (c, d, g and h) post-infection. Arrows indicate focal areas of hyphal formation. Magnifications ×20 (a, c, e and g) and ×150 (b, d, f and h).

Statistical analysis

Survival results were plotted as Kaplan–Meier curves and analysed by log rank (due to multiple comparisons, a P value < 0.008 was considered statistically significant). The cfu per organ per mouse values were analysed by one-way analysis of variance followed by the Tukey’s test corrected for multiple comparison. A P value < 0.05 was considered statistically significant.

Results

The histopathological appearances showed a disease progression that slightly differed based on the type of infection (Figure 1). Particularly, in disseminated infection, renal structure appeared normal at 24 h post-inoculation, whereas focal areas of hyphal formation began to appear at 48 h (Figure 1a–d). In pulmonary infection, lung fungal infiltrates were already evident at 24 h post-inoculation, and a progressive hyphal extension was seen at 48 h (Figure 1e–h). At 72 h post-inoculation, a further progression of tissue invasion was seen in both types of infection (data not shown).

Figure 1. Representative results for histopathological sections of kidney (disseminated infection; a–d) and lung (pulmonary infection; e–h) tissues stained with Grocott–Gomori’s methenamine silver nitrate from mice infected with A. fumigatus. The mice were killed either 24 h (a, b, e and f) or 48 h (c, d, g and h) post-infection. Arrows indicate focal areas of hyphal formation. Magnifications ×20 (a, c, e and g) and ×150 (b, d, f and h).

Survival results of disseminated infection are shown in Figure 2(a–d). Median survival time in early therapy was 3, 7, 6 and 9 days for mice treated with vehicle, liposomal amphotericin B at 3 mg/kg, micafungin at 3 mg/kg and the combination, respectively (Figure 2a). Either liposomal amphotericin B (P = 0.0024) or micafungin (P = 0.0031) were effective at prolonging the survival against the controls. The combination was more effective than control (P < 0.0001) and more effective than liposomal amphotericin B (P = 0.0005), but not more effective than micafungin alone (P = 0.02). Median survival times in delayed therapy were 3, 5, 5 and 7 days for mice treated with vehicle, liposomal amphotericin B at 3 mg/kg, micafungin at 3 mg/kg and the combination, respectively (Figure 2b). The only regimen which prolonged the survival was the combination (P = 0.005). When drug doses, singly and combined, were increased to 10 mg/kg, median survival time in early therapy was 4, 12, 11.5 and > 15 days for mice treated with vehicle, liposomal amphotericin B, micafungin and the combination, respectively (Figure 2c). All regimens were effective against the controls (P < 0.0001). When therapy was delayed (Figure 2d), the median survival times were 4, 7, 8.5 and 8 days for mice treated with vehicle, liposomal amphotericin B, micafungin and...
the combination, respectively, and only micafungin \((P=0.001)\) and the combination \((P=0.0026)\) were effective.

Survival results of pulmonary infection are shown in Figure 2(e and f). Pulmonary infection experiments were conducted with drugs given at 3 mg/kg. The median survival times in early therapy were 7, 9, 9 and >15 days for mice treated with vehicle, liposomal amphotericin B, micafungin and the combination, respectively (Figure 2e). Although there was a trend towards a prolongation of survival of mice treated with liposomal amphotericin B, the results of this regimen did not reach statistical significance \((P=0.025)\). The only regimen that significantly prolonged the survival compared with the control was the combination \((P=0.007)\). Median survival time in delayed therapy was 7, 9, 9 and >15 days for mice treated with vehicle, liposomal amphotericin B, micafungin and the combination, respectively (Figure 2f). Only the combination approach was effective \((P=0.004)\).

Tissue burden experiments were performed by giving the drugs at 3 mg/kg. In early therapy of disseminated infection, all regimens

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**Figure 2.** Survival curves of mice infected intravenously (a–d) or intranasally (e and f) with \(\sim 1.6 \times 10^4\) (a–d) or \(\sim 1.0 \times 10^7\) (e and f) A. fumigatus conidia per mouse and treated with vehicle (circles), liposomal amphotericin B (squares), micafungin (triangles) or the combination of liposomal amphotericin B plus micafungin (upside-down triangles). Both drugs, alone or in combination, were given at 3 mg/kg/day (a, b, e and f) or 10 mg/kg/day (c and d) for three consecutive days. Therapy was started 24 h (early therapy; a, c and e) or 48 h (delayed therapy; b, d and f) post-infection.
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were effective at reducing the cfu/kidney with respect to controls being the mean cfu/organ ± standard deviation (SD) of 398 ± 154, 110 ± 60, 196 ± 115 and 25 ± 15 for mice treated with vehicle, liposomal amphotericin B, micafungin and the combination, respectively. Additionally, the combination was more effective than micafungin but not more effective than liposomal amphotericin B. In early therapy of pulmonary infection, only liposomal amphotericin B and the combination were effective at reducing the cfu/lung with respect to controls; the mean cfu/organ ± SD were 8057 ± 1201, 498 ± 380, 4429 ± 2719 and 488 ± 303 for mice treated with vehicle, liposomal amphotericin B, micafungin and the combination, respectively. In delayed therapy, no regimen was effective, regardless of the type of infection (data not shown).

Discussion

Our data showed that the delayed onset of antifungal therapy is deleterious to the treatment of experimental IA. An earlier study conducted in a neutropenic rabbit model of IA showed that amphotericin B deoxycholate therapy given at 1 mg/kg and initiated ≥48 h post-infection was associated with suboptimal therapeutic outcome. Here, we showed that liposomal amphotericin B given at doses as high as 10 mg/kg was not effective when initiated 48 h post-infection. Although one can hypothesize that the highest polyene dose used in this study can be toxic in mice (and therefore not effective), its effectiveness when the same dose is initiated earlier eliminates this possibility. Doses of both drugs selected in this study were either based on previously published murine aspergillosis models showing a clear dose-dependent effectiveness or based on their clinical relevance.3,9–13 Here, we showed that an extensive hyphal invasion, as seen in both models at 48 h post-infection, makes delayed therapy ineffective. It is interesting to note that the combination approach was often the only effective regimen when therapy was delayed. This phenomenon was observed at both low and high doses of both drugs. It is likely that a mutual potentiation of drugs acting against different fungal targets still persists at a later stage of hyphal growth when any monotherapy lacks efficacy. Data from the literature investigating the effects of liposomal amphotericin B plus an echinocandin in experimental models of infections have produced varying results.10,11 In A. fumigatus infections, the combination was generally as effective as the polyene alone either in terms of survival or tissue burden results.12 In Aspergillus flavus infections, concomitant therapies produced significantly enhanced survival and reduction in fungal burden, while antagonism was seen in some sequential regimens.13 Although it is difficult to generalize our results into clinical practice, our data suggest that a combination regimen may have an advantage over monotherapy when treatment is started late. Further studies involving other drug combinations, multiple strains and species of Aspergillus, and additional schemes of experimental treatment initiation are warranted to corroborate these findings.

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Transparency declarations

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References