Combination therapy with micafungin and amphotericin B for invasive pulmonary aspergillosis in an immunocompromised mouse model

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Objectives: Antifungal monotherapy with polyenes, azoles or echinocandins is not always effective for invasive pulmonary aspergillosis (IPA). The main purpose of this study was to evaluate the efficacy of a combination of micafungin and amphotericin B for the primary treatment of IPA in an immunocompromised mouse model.

Methods: Female ICR mice were used in all experiments. An immunosuppressive state was induced in mice by an intraperitoneal injection of cyclophosphamide. Mice were intratracheally inoculated with Aspergillus fumigatus conidia, treated with micafungin, amphotericin B or both for 7 days, and were tested for their survival 20 days after the Aspergillus inoculation. Fungal burden in lungs, serum galactomannan index (GMI) and histopathology of lungs, spleen and kidneys were also evaluated.

Results: Combination therapy with micafungin and amphotericin B gave excellent survival of infected mice compared with monotherapy with micafungin or amphotericin B alone. Combined therapy reduced the fungal burden in the lungs and the serum GM levels compared with monotherapy or untreated controls, resulting in a significant histological improvement with disappearance of fungi in the lungs.

Conclusions: These findings suggest that combination therapy with micafungin and amphotericin B is more effective compared with monotherapy with either of them alone for IPA treatment.

Keywords: primary therapy, histopathology, fungal burden, galactomannan index

Introduction

Invasive pulmonary aspergillosis (IPA) is a life-threatening infection that frequently occurs in patients who are heavily immunosuppressed after intensive chemotherapy or long-term immunosuppressive treatment. The clinical efficacy of polyenes, azoles or echinocandins alone is still limited and is associated with substantial mortality.1

Micafungin is an echinocandin class compound, which inhibits 1,3-β-D-glucan synthesis in the cell wall of fungi.2 Echinocandins mainly destroy hyphal cells at the advancing tips, but other hyphal cell structures may remain viable after drug withdrawal. Amphotericin B, a polyene macrolide, has long been used as the first-line agent for systemic fungal infection because of its broad spectrum. Amphotericin B displays a fungicidal effect through producing aqueous pores in the fungal membrane. However, clinical use of amphotericin B is occasionally hampered, for example by its nephrotoxicity.3

Philip et al.4 reported, by using a checkerboard method, that a combination of echinocandin and polyene has synergistic effects on the growth of Aspergillus species in vitro. Recent studies also showed that this combination is effective in human
IPA even for salvage from unsuccessful initial monotherapy with echinocandin. Here, by utilizing an immunocompromised mouse model, we demonstrate formal evidence that a combination of micafungin and amphotericin B exerts synergic effects in the treatment of primary IPA in vivo.

Materials and methods

Mice, induction of an immunocompromised state and intratracheal inoculation

Female ICR mice (4–5 weeks old) weighing 23–28 g were purchased from Charles River Breeding Laboratory Japan (Yokohama, Japan).

An immunocompromised state was induced by two intraperitoneal injections (on days −3 and 0) of cyclophosphamide (Shionogi, Osaka, Japan) at a dose of 200 mg/kg. The presence of neutropenia was confirmed and the duration of neutropenia (<100 per mm$^3$) was at least 6 days.

Mice were anaesthetized with intraperitoneal injections of ketamine hydrochloride (1 mL/kg). A small incision was made in the skin of the anterior region of the neck. We inserted a catheter via the oropharynx and the tip of the catheter was fixed just above the skin of the anterior region of the neck. We inserted a catheter via the skin incision window. An Aspergillus fumigatus (ASM-7) conidial suspension of $1.1\times10^5$/mouse was inoculated through the catheter. All these procedures were performed according to the protocol approved by the Institutional Animal Care and Use Committee at Kyushu University in Japan.

Treatment with antifungal agents and assessment of survival

A concentration of 1 mg/kg micafungin (Astellas Pharmaceutical, Tokyo, Japan) and 0.5 mg/kg amphotericin B (Bristol-Myers Squibb, Tokyo, Japan) showed similar efficacies when the 50% effective dose (ED$_{50}$) was assessed (data not shown). These agents, at the same dosage, alone and in combination, were intravenously administered to infected mice via the tail vein. The treatment was initiated 3 h after fungal inoculation at day 0 and continued for 7 consecutive days. The survival rate was determined at the end of the observation period (day 20) by the percentages of surviving/total mice receiving fungal inoculations.

Quantitative measurement of fungal burden in lungs

Enumeration of A. fumigatus cfu in the tissue homogenates of lungs was carried out on day 4.

Galactomannan assay

Serum galactomannan concentrations in blood samples at day 4 were determined by the Platelia Aspergillus enzyme immunoassay (Bio-Rad Laboratories, Tokyo, Japan).

Histopathology of the lungs, spleen and kidneys

Routine histological techniques were processed and sections were then stained with haematoxylin and eosin (H&E) and Grocott–Gomori methenamine silver (GMS).

Statistical analysis

Kaplan–Meier analysis was carried out using the STATVIEW software package in order to compare survival rates among four groups of mice: (i) receiving micafungin alone; (ii) receiving amphotericin B alone; (iii) receiving both micafungin and amphotericin B; or (iv) controls. Statistical evaluation of survival was performed by using a log rank test. Comparison of A. fumigatus cfu and serum galactomannan (GM) index levels was assessed by Mann–Whitney U-test. P values of <0.05 were considered significant in these analyses.

Results

Combination of micafungin and amphotericin B improves survival of infected mice

The cumulative survival of infected mice treated with micafungin or amphotericin B alone and with a combination of micafungin plus amphotericin B is shown in Figure 1(a). All untreated mice died by day 6. The survival rate on day 20 was low in mice receiving either micafungin or amphotericin B alone (16% and 22%, respectively). The combination therapy significantly improved survival up to 61% on day 20 compared with survival of mice treated with monotherapy or controls ($P<0.05$).

Combination therapy significantly reduces the fungal burden

Organ clearance of fungi was measured by determining the fungal burden in lungs of mice on day 4 (Figure 1b). Compared with untreated controls, mice treated with amphotericin B alone had a decreased fungal burden in the lungs ($P<0.05$), whereas mice treated with micafungin alone did not. However, in combination with amphotericin B, micafungin further decreased the fungal burden in the lungs as compared with micafungin alone ($P<0.05$), amphotericin B alone ($P<0.05$) and untreated controls ($P<0.05$). We then evaluated serum GM levels. As shown in Figure 1(c), serum samples from untreated controls contained high levels of GM on day 4. Amphotericin B treatment decreased GM levels significantly ($P<0.05$), whereas micafungin treatment did not. GM levels were further decreased in mice treated with a combination of micafungin and amphotericin B ($P<0.05$).

Combination therapy inhibits hyphae formation in the infected lungs

Histopathological examination was performed in the lungs from infected mice on day 4. Hyphae were clearly observed in the lungs, while they were not seen in the spleen and the kidneys (data not shown). Infiltration of inflammatory cells was rarely seen in the lungs, reflecting the severe neutropenic status. In the lungs from untreated mice, a number of proliferating hyphae (Figure 2a) and coagulation necrosis were noted in the blood vessels, septa, bronchioli and alveoli. In the lungs from mice treated with micafungin, hyphae were frequently observed in the bronchioli and the alveoli. However, invasion of hyphae into blood vessels was not observed (Figure 2b). In mice treated with amphotericin B, hyphae were seen only in the bronchioli (Figure 2c). In contrast, in mice treated with the combination treatment, hyphae were hardly seen (not shown).
Successful combined antifungal therapy for IPA

Figure 1. (a) Survival of immunocompromised mice inoculated intratracheally with \(1.1 \times 10^6\) conidia of \(A. fumigatus\). A total of 18 mice was used per group for the experiments. The treatment continued with seven consecutive daily injections. The combination therapy of micafungin and amphotericin B increased the survival rate (\(P<0.05\) by the log rank test) significantly compared with that in the group treated with monotherapy and in untreated controls (Kaplan–Meier test). (b) Fungal burden in the lungs. Quantitative measurement of the fungal burden was performed by enumeration of \(A. fumigatus\) cfu per lungs from 5–7 mice per group. The fungal burden in the lungs from mice treated with micafungin alone was not lower than that of untreated controls, whereas the burden from mice treated with amphotericin B alone was lower. Asterisks indicate statistically significant differences (\(P<0.05\)). (c) Serum GM concentrations in blood samples at day 4. GMI was evaluated in infected mice following combination therapy with micafungin and amphotericin B or monotherapy. As compared with untreated controls, amphotericin B significantly decreased the GMI, whereas micafungin did not. There was a marked decrease in serum GM (\(P<0.05\)) levels in combined therapy with micafungin and amphotericin B compared with micafungin alone, amphotericin B alone and untreated controls. *\(P<0.05\). M, micafungin; A, amphotericin B; MA, micafungin plus amphotericin B; C, controls; NS, not significant.

Figure 2. Histological findings in the lungs with lower (H&E staining; \(\times20\)) and higher (H&E staining and GMS; \(\times100\)) magnification. (a) In untreated control mice, there were widespread hyphal lesions in the lungs and hyphae invading the blood vessels were especially conspicuous. (b) In mice treated with micafungin, hyphae were observed in the septa and the bronchioli, but not in the blood vessels. (c) In mice treated with amphotericin B, hyphae remained in the bronchioli. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
Discussion

This study provides evidence that the simultaneous administration of micafungin and amphotericin B is effective in vivo for primary treatment of IPA in an immunocompromised mouse model. The combination therapy of micafungin and amphotericin B yielded excellent survival and reduction of the fungal burden of infected mice, in comparison with monotherapy with micafungin or amphotericin B alone. Given that this combination is effective in salvage therapy for recalcitrant IPA, our results suggest the potential usefulness of this combination as primary therapy for IPA.

Although intravenous infection models have long been used to study the pathogenesis of IPA, we infected mice via the intratracheal route in order to mimic the authentic infection route of IPA in humans. The fungal burden in the lungs after intravenous administration of fungus is <1%, compared with that after intranasal administration. Thus, our experimental method might have an advantage over the conventional intravenous administration strategy to recapitulate the pathogenesis of IPA in mouse models.

The Platelia Aspergillus enzyme immunoassay method that can quantify a fungal cell wall component, galactomannan, has recently been used for the diagnosis of IPA. Favourable responses to antifungal therapies in patients, and in experimental neutropenic animals, are associated with decreased GM levels. We, however, found that micafungin improves the survival of infected mice without affecting the level of serum GM.

Histopathological analysis of the lungs of untreated controls showed that proliferating hyphae and coagulation necrosis frequently exist in the blood vessels, septa, bronchioli and alveoli, and these changes appear to be reminiscent of pulmonary haemorrhage and infarction. On the other hand, in mice treated with micafungin alone, hyphae invaded the septa and bronchioli, but not the blood vessels. In addition, swelling of the tips of hyphae was only seen in mice treated with micafungin, not in the controls (data not shown). Petraitis et al. suggest that the efficacy of micafungin might be dependent upon its action of damaging the tip of hyphae, thereby reducing the angioinvasive and tissue-injurious properties of Aspergillus. This unique action of micafungin may contribute to prolonged survival of mice treated with this agent.

In contrast to mice treated with micafungin, most invading hyphae in mice treated with amphotericin B were observed only in the bronchioli, but not in the alveoli or blood vessels. Moreover, the remaining hyphae looked shorter and rarely branched, which may be due to the fungicidal action of amphotericin B. Our histological findings are consistent with this fungicidal action. Finally, amphotericin B not only critically damages the tips of hyphae but also disrupts the total membrane integrity of A. fumigatus. Increased efficacy of the combination therapy against A. fumigatus is probably due to the distinct mechanisms of antifungal action of the components; micafungin inhibits the synthesis of 1,3-β-D-glucan in the cell wall, whereas amphotericin B directly damages intact fungal cell membranes.

In summary, this study shows that the combination of micafungin and amphotericin B is useful in the primary treatment of IPA in a strictly controlled animal model. Our data strongly support the clinical data regarding synergistic effects of echinocandin and polyene in a salvage setting, but also suggest the clinical efficacy of this combination as a primary treatment for human IPA in future studies.

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Transparency declarations

None to declare.

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