Efficacy of Combination Antifungal Therapy with Intraperitoneally Administered Micafungin and Aerosolized Liposomal Amphotericin B against Murine Invasive Pulmonary Aspergillosis

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Targeted intrapulmonary delivery of drugs may reduce systemic toxicity and improve treatment efficacy. In the current study, we evaluated the effects of a combination treatment consisting of inhalation of aerosolized liposomal amphotericin B (L-AMB) with intraperitoneal administration of micafungin (MCFG) against murine invasive pulmonary aspergillosis. The combination of aerosolized L-AMB with intraperitoneal MCFG significantly improved the survival rate, and the fungal burdens and histopathology findings after this treatment were superior to those of the control and both monotherapy groups.

Invasive pulmonary aspergillosis (IPA) results in significant morbidity and mortality in severely immunocompromised patients (6). Targeted intrapulmonary delivery of antifungals has the potential to reduce systemic toxicity and improve treatment efficacy as well as prophylaxis (1, 8) and may be used as an optional route in combination with other systemic antifungals. In the current study, we evaluated the efficacy of aerosolized liposomal amphotericin B (L-AMB) both singly and in combination with intraperitoneally administered micafungin (MCFG) in a murine model of IPA.

Aspergillus fumigatus MF13 was clinically obtained from a patient admitted to the Nagasaki University Hospital. The minimum effective concentration of MCFG (Astellas Pharmaceuticals Inc., Tokyo, Japan) and the MIC of AMB (Sigma, St. Louis, MO) were determined using the microdilution method in accordance with Clinical Laboratory Standards Institute document M38-A2 (2). Drug interactions were assessed using the checkerboard titration broth microdilution-based method (3), and the fractional inhibitory concentration index was determined as previously described (5).

Six-week-old female ICR mice (Charles River Breeding Laboratories, Shiga, Japan) were immunosuppressed and then challenged on day 0 with 5 × 10⁶ conidia of A. fumigatus MF13 intratracheally for monitoring of survival, as previously described (7, 11). Eight-week-old female ICR mice were used to determine fungal burdens and for histopathological examination. Mice were immunosuppressed by subcutaneous injection of cortisone acetate (Sigma, Tokyo, Japan) at 250 mg/kg of body weight and intraperitoneally administered cyclophosphamide (Sigma) at 200 mg/kg on days −2 and 0 for the survival study. Only cortisone acetate (200 mg/kg) was used on days −1, 0, and 1 for fungal-burden analysis and histopathological examination. Mice were assigned into the following groups: (i) control mice, (ii) mice receiving MCFG intraperitoneally, (iii) mice receiving aerosolized L-AMB, and (iv) mice receiving a combination treatment of intraperitoneally administered MCFG and aerosolized L-AMB. Each group consisted of 11 and 10 mice for survival and fungal-burden analyses, respectively. MCFG was administered intraperitoneally once daily at 1 mg/kg/day. L-AMB was administered once daily in an 8-ml

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FIG. 1. Survival curves for mice with IPA (Kaplan-Meier plot). Groups of 11 mice were treated with a combination of intraperitoneal administration of MCFG (1 mg/kg/day) and inhalation of aerosolized L-AMB (8 ml at 1.2 mg/ml [open squares]), inhalation of aerosolized L-AMB (8 ml at 1.2 mg/ml [filled triangles]), intraperitoneal administration of MCFG (1 mg/kg/day [open triangles]), and no therapy (control [filled circles]). *P < 0.05 versus the control; **P < 0.05 versus the control group, intraperitoneal-MCFG group, or aerosolized-L-AMB group (log rank test). The survival times for all treatment groups were longer than that for controls (P < 0.05). The survival time for the combination treatment group was significantly longer than those of the intraperitoneal-MCFG group and the aerosolized-L-AMB group (P < 0.05).
suspension (at 1.2 mg/ml) per inhalation. Antifungals were initiated 16 h after inoculation and continued for 5 and 3 days for survival and fungal-burden analyses, respectively. The L-AMB solution was aerosolized using a nebulizer (Muromachi Kikai Co., Ltd., Tokyo, Japan), and mice were exposed to aerosol treatment for 60 min as previously described (9). Control mice were treated with sterile saline. Survival was observed until 11 days following the challenge. For fungal-burden and histopathological examinations, mice were sacrificed 4 h after the treatment on day 3. Numbers of CFU per lung tissue were calculated, and removed lungs were fixed and stained with Grocott’s methenamine silver nitrate and hematoxylin-eosin as previously described (11). Survival and fungal burden data are presented from a combination of two sets of experiments. The concentration in blood and the pharmacokinetics of aerosolized L-AMB were evaluated. Uninfected mice were also exposed to several concentrations of aerosolized L-AMB for 5 days, and blood samples and lungs were collected. AMB concentration was quantified as previously described (10). Survival curves were generated using the Kaplan and Meier method, and statistical differences were evaluated by the log rank test. To assess fungal burden in lung tissue, geometric means of numbers of CFU per organ were compared by Student’s t test. Statistical significance was defined as a P of <0.05.

The MIC of AMB against *A. fumigatus* MF-13 was 1.0 g/ml, and the minimum effective concentration of MCFG was 0.0315 g/ml. The fractional inhibitory concentration index of AMB and MCFG was 1.5, and drug interaction was classified as indifferent (5).

Survival periods of monotherapy groups, in which mice either were treated with intraperitoneally administered MCFG or inhaled aerosolized L-AMB were significantly longer than...
that of the control group (MCFG alone versus the control, \( P = 0.006 \), L-AMB versus the control, \( P < 0.001 \)) (Fig. 1). The combination treatment group showed significantly longer survival than the intraperitoneal-MCFG (\( P < 0.001 \)), aerosolized-L-AMB (\( P = 0.037 \)), and control (\( P < 0.001 \)) groups. Numbers of CFU in the lungs of mice in the combination treatment group were significantly reduced compared to those in each of the intraperitoneal-MCFG (\( P < 0.001 \)), aerosolized-L-AMB (\( P = 0.027 \)), and control (\( P < 0.001 \)) groups (Fig. 2). The lungs of aerosolized-L-AMB-administered and combination treatment mice showed obviously smaller numbers of hyphae and fewer foci of inflammation than the intraperitoneal-MCFG and control groups (Fig. 3). The mean AMB concentrations in the lung tissue following L-AMB inhalation at 1.2, 2.6, and 4.0 \( \mu \text{g/ml} \) were 35.5, 73.2, and 94.2 \( \mu \text{g/ml} \), respectively. Recorded levels in sera were 0.02, 0.06, and 0.06 \( \mu \text{g/ml} \) when inhaled-L-AMB suspensions were administered at 1.2, 2.6, and 4.0 \( \mu \text{g/ml} \), respectively.

The current study demonstrated the efficacy of monotherapy of aerosolized L-AMB in a murine IPA model. The AMB concentrations in lung tissue in our study were relatively higher but extremely lower in serum than those from another report of a murine model of intravenously administered L-AMB, although experimental conditions were not the same (10). These results suggested that systemic toxicity generally caused by AMB treatment may be reduced by L-AMB inhalation therapy.

The effect of combined intraperitoneal-MCFG and aerosolized-L-AMB treatment was an enhanced survival rate, even though this drug interaction was classified as indifferent in vitro. Since 78% of all control mice died in first 3 days in a survival analysis, we changed the experimental conditions for analysis of fungal burden and histopathological examination. In this model, no mice died before euthanasia, a prerequisite for the organ CFU assay. Both fungal-burden data and histopathological findings supported the survival data in our study.

Unlike in our study, Graybill et al. previously reported that combination therapy demonstrated a lack of synergistic effects following intravenous-L-AMB and intraperitoneal-MCFG treatment in a model of murine IPA (4). These discrepancies are likely due to differences between our model and Graybill et al.’s model, including (i) the route of infection, (ii) the status of immunosuppression, and (iii) the administration route of antifungal drugs. These differences also suggest that targeted intrapulmonary delivery of drugs by inhalation raises the drug concentration at the active site of infection in the lungs, thus contributing to the efficacy of combination therapy. Further comparative efficacy studies in a clinical setting are warranted.

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