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Efficacy of combination antifungal therapy of intraperitoneal micafungin with aerosolized liposomal amphotericin B in murine invasive pulmonary aspergillosis

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ABSTRACT

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

Invasive pulmonary aspergillosis (IPA) results in significant morbidity and mortality in severe immunocompromised patients (6). Targeted intrapulmonary delivery of antifungals has potential to reduce systemic toxicity, improve treatment efficacy as well as prophylaxis (1, 8), and may be used as an optional route in combination use with other systemic antifungals. In the current study, we evaluated the efficacy of aerosolized liposomal amphotericin B (L-AMB) either in single and 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 (MEC) of MCFG (Astellas Pharmaceuticals Inc., Tokyo, Japan) and the MIC of AMB (SIGMA, St Louis, MO, USA) were determined using the microdilution method in accordance to the Clinical Laboratory Standards Institute M38-A2 (2). Drug interactions were assessed using the checkerboard titration broth microdilution-based method (3) and the fractional inhibitory concentration (FIC) 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 zero with 5×10^6 conidia of *A. fumigatus* MF13 intratracheally for monitoring survival as previously described (7, 11). Eight-week old female ICR mice were used for fungal burdens and

histopathological examination. Mice were immunosuppressed by subcutaneous injection of cortisone acetate (Sigma, Tokyo, Japan) at 250 mg/kg and intraperitoneal cyclophosphamide (Sigma) at 200 mg/kg on days -2, 0 for survival study. Whereas, 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, 1) control, 2) intraperitoneal MCFG, 3) aerosolized L-AMB and 4) combination of intraperitoneal MCFG and aerosolized L-AMB treatment. Each group is consisted of 11 and 10 mice for survival and fungal burden analysis, respectively. MCFG was administered intraperitoneally once daily at 1 mg/kg/day. L-AMB was administered once daily at a dose of 8 ml of 1.2 mg/ml per inhalation. Antifungals were initiated 16 hours after inoculation and continued for 5 and 3 days for survival and fungal burden analysis, 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 hisotpathologcal examinations, mice were sacrificed 4 hours after treatment of day 3. 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 in combined of two sets of experiments. The blood concentration 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 CFU per organ were compared by Student's *t* test. Statistical significance was defined as $P < 0.05$.

The MIC of AMB against *A. fumigatus* MF-13 was 1.0 μg/ml and the MEC of MCFG was 0.0315 μg/ml. FIC index of AMB and MCFG was 1.5 and drug interaction was classified as indifferent (5).

Survival periods of monotherapy groups, in which mice were treated with either intraperitoneal MCFG or aerosolized L-AMB inhalation were significantly longer than that of the control group (MCFG alone vs. control, $P = 0.006$; L-AMB vs. control, $P < 0.001$) (Figure 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. Lung CFU of combination-treated group was significantly reduced compared to each of the intraperitoneal MCFG ($P < 0.001$),

aerosolized L-AMB ($P = 0.027$) and control ($P < 0.001$) groups (Figure 2). The lungs of aerosolized L-AMB- and combination-treated mice showed obviously smaller number of hyphae and less foci of inflammation compared to intraperitoneal MCFG and control groups (Figure 3). The mean AMB concentrations in the lung tissue following L-AMB inhalation at 1.2, 2.6 and 4.0 mg/ml were 35.5, 73.2 and 94.2 μg/g, respectively. Recorded levels in the serum were 0.02, 0.06 and 0.06 μg/ml when inhaled L-AMB suspensions were administered at 1.2, 2.6 and 4.0 mg/ml, respectively.

The current study demonstrated the efficacy of monotherapy of aerosolized L-AMB in murine IPA model. AMB concentration in lung tissue in our study was relatively higher with extremely lower in serum compared to data from another report of murine intravenous L-AMB administered model, although experimental conditions were not 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 demonstrated an enhanced survival rate, even though this drug interaction was classified as indifferent *in vitro*. Since 78% of all control mice died in first three days in 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 organ CFU assay. Both fungal burden data and histopathological findings supported the survival data in our study.

In contrast to 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 murine IPA (4). These discrepancies are likely due to differences between our model and Graybill's model including 1) the route of infection, 2) status of immunosuppression and 3) 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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FIGURE LEGENDS

Figure 1

Survival curves for mice with IPA (Kaplan-Meier plot). Groups of 11 mice were treated with a combination of intraperitoneal MCFG (1 mg/kg/day) and aerosolized L-AMB inhalation (8ml of 1.2 mg/ml, open squares), aerosolized L-AMB (8ml of 1.2 mg/ml, filled triangles), intraperitoneal MCFG (1 mg/kg/day, open triangles), and control (no therapy, filled circles). * $P < 0.05$ vs. control. ** $P < 0.05$ vs. control, intraperitoneal MCFG group or aerosolized L-AMB group (Log rank test). The survival time for all treatment groups was longer than that of 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 ($P < 0.05$).

Figure 2

CFU from homogenized lung tissues of invasive pulmonary aspergilosis mice. Groups of 10 mice were treated once per day with a combination of intraperitoneal MCFG (1 mg/kg/day) and aerosolized L-AMB (8 ml of 1.2 mg/ml), aerosolized L-AMB (8 ml of 1.2 mg/ml), intraperitoneal MCFG (1 mg/kg/day), and control. CFU counts, as a parameter of *A. fumigatus* burden in the lungs of IPA mice at 4 hours after day

3-treatment, are shown. * $P < 0.05$ (Student's-*t* test)

Figure 3

Histopathology of lung tissues. Both lungs were obtained from IPA mice 4 hours after three days treatment with a combination of intraperitoneal MCFG and aerosolized L-AMB, aerosolized L-AMB, intraperitoneal MCFG, and saline alone as control. The lungs obtained from aerosolized L-AMB- and combination-treated mice showed obviously smaller number of hyphae and less foci of inflammation compared to intraperitoneal MCFG and control mice. HE; hematoxylin-eosin, GMS; Grocott's methenamine silver nitrate (GMS) stain.

Figure1

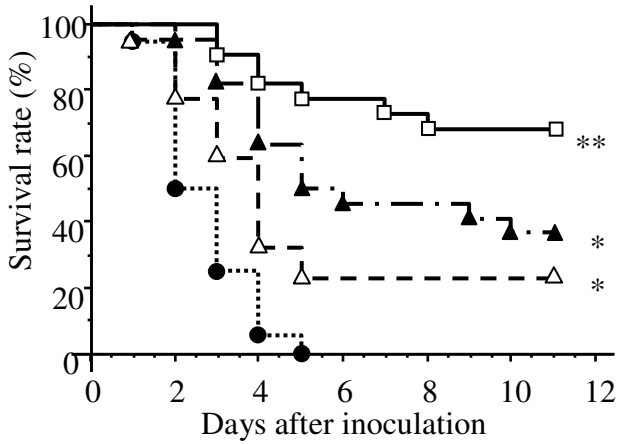
Survival rate (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
Days after inoculation
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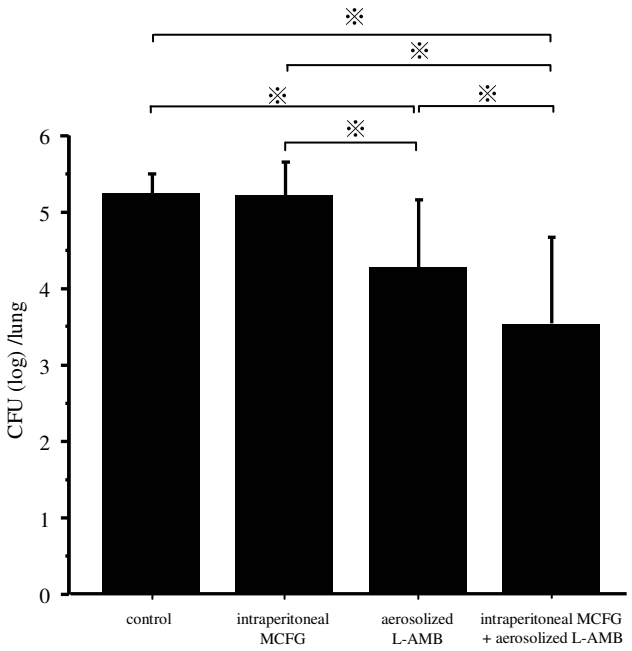
Figure2


※
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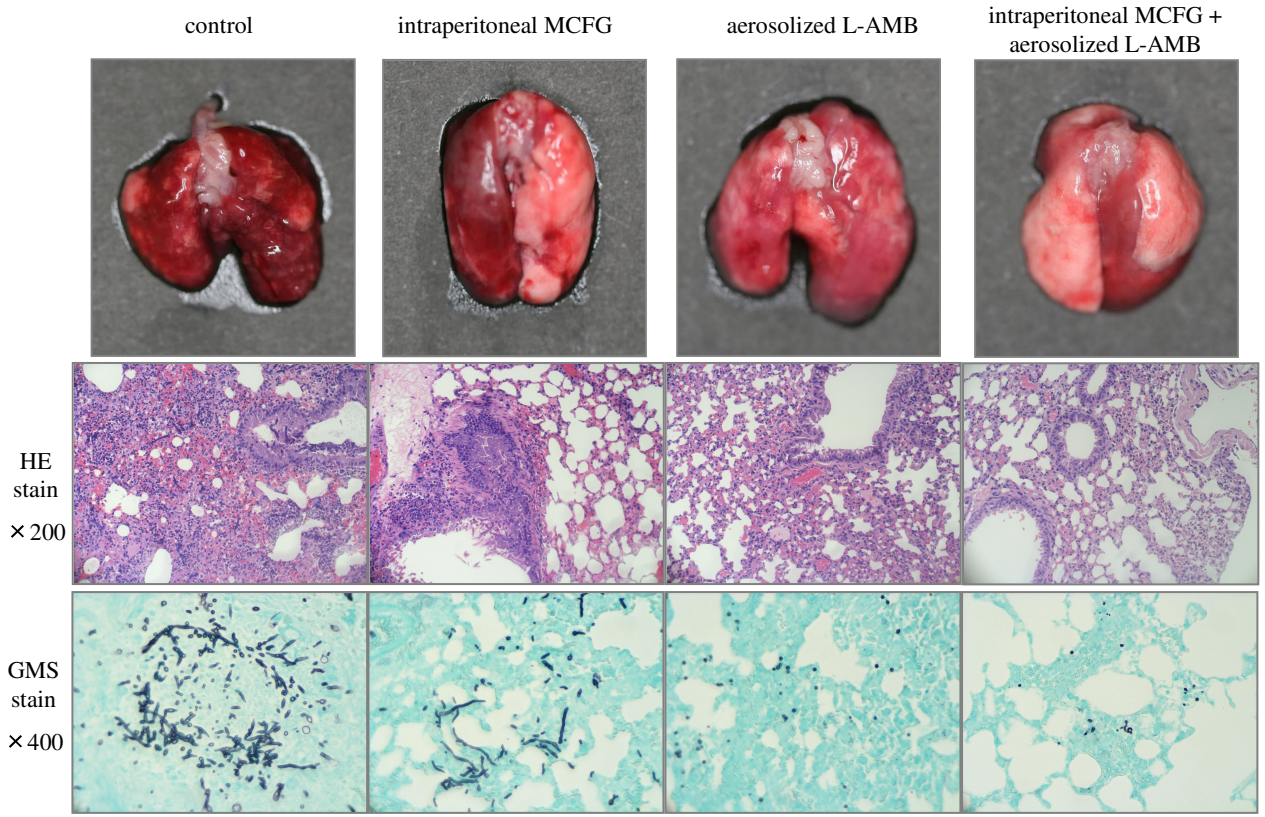
6
5
4
3
2
1
0
CFU (log) /lung
control
intraperitoneal MCFG
aerosolized L-AMB
intraperitoneal MCFG + aerosolized L-AMB

Figure3


control
intraperitoneal MCFG
aerosolized L-AMB
intraperitoneal MCFG +
aerosolized L-AMB
HE
stain
×200
GMS
stain
×400