Combination Therapy of Murine Mucormycosis or Aspergillosis with Iron Chelation, Polyenes, and Echinocandins

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Liposomal amphotericin B (LAmB) combined with either micafungin or deferasirox was synergistic in previous murine studies with mucormycosis or aspergillosis. We hypothesized that triple therapy using LAmB, micafungin, and deferasirox could further improve outcomes of mucormycosis or aspergillosis. Triple therapy improved survival and reduced tissue fungal burden of mice with mucormycosis and to a lesser extent with aspergillosis. Continued investigation into the use of triple therapy against mucormycosis and aspergillosis is warranted.

Invasive pulmonary aspergillosis (IPA) and mucormycosis are among the most common life-threatening mold infections and are occurring with increasing frequency, in immunocompromised patients (2, 14, 25). Despite antifungal therapy, infections with these two molds result in more than 50% mortality rates (3, 4, 21). Clearly, new modalities are needed to prevent these infections and/or improve the clinical outcome of infected patients.

Recent data demonstrated that iron acquisition from the host represents a critical virulence factor for fungi causing mucormycosis and aspergillosis (5, 11), and patients suffering from elevated available serum iron (12) or increased bone marrow iron stores (15) are at increased risk of developing mucormycosis and aspergillosis, respectively. Previous studies showed that lipid formulations of amphotericin B combined with either echinocandins or the iron chelator deferasirox (Def) were synergistic against mucormycosis (8, 10) or experimental IPA (9). Therefore, we hypothesized that triple therapy using liposomal amphotericin B (LAmB), micafungin (MICA), and Def could further improve outcomes of mice with mucormycosis or IPA.

For mucormycosis, the streptozotocin-induced diabetic ketoacidotic (DKA) mouse model was used as we previously described (6). To detect potential synergy with triple therapy, mice were infected intravenously with Rhizopus oryzae 99-880 at a high inoculum of $2 \times 10^9$ spores to mitigate the protective effect of dual therapy previously seen with LAmB plus Def (8) or LAmB plus MICA (10). For the IPA model, BALB/c mice were immunosuppressed by two doses of cyclophosphamide and cortisone acetate on days $-2$ and $+3$ relative to infection, as we have previously described (19). Mice were infected with Aspergillus fumigatus strain AF293 by aerosolizing $1.2 \times 10^{10}$ conidia in our inhalational chamber (19). For both models, LAmB (Astellas Pharma US) in 5% dextrose water (5DW) (15 mg/kg of body weight/day for mucormycosis or 3 mg/kg/day for IPA) or MICA (Astellas Pharma US) in phosphate-buffered saline (PBS; 1 mg/kg/day) treatment started on day +1 relative to infection and continued daily for 5 days. Both drugs were given intravenously. LAmB was given at 3 mg/kg/day for IPA because earlier studies showed that treating mice with LAmB at 5 mg/kg/day resulted in >70% survival of infected mice (data not shown), which would make it difficult to evaluate for synergy. Def (Novartis Pharmaceuticals) (10 mg/kg twice a day [b.i.d.], given by oral gavage in 0.5% Klucel [8]) was started on day +1 and continued every day for 7 doses for mucormycosis or every other day for 4 doses for IPA. These treatment regimens were chosen based on our previous studies (9, 10). Placebo mice in both animal models were given 5DW and 0.5% Klucel. Survival (Kaplan-Meier curves) and fungal burden in brain and kidneys (for mucormycosis) or lungs (for IPA) were used as primary and secondary endpoints, respectively. Fungal burden was measured by quantitative PCR (qPCR) assay with a conidial standard curve (1, 7). All qPCR results are expressed as log$_{10}$ spore equivalents per gram of tissue.

**Mucormycosis model.** For mucormycosis, triple therapy was superior to all other treatments (i.e., placebo and monotherapy or dual therapy) in increasing 28-day survival of infected mice (40% survival for triple therapy versus 0 to 11% for all other treatments, $P < 0.05$) (Fig. 1A). To define the effect of triple therapy on the tissue fungal burden, DKA mice were infected with R. oryzae as described above. Because placebo-treated mice die within 72 to 96 h postinfection, we initiated treatment 8 h postinfection and continued until the morning of day 2 (3 doses total). Mice were euthanized on day 3, and tissue fungal burden was determined by qPCR as described before for brains and kidneys since they represent the primary and sec-
secondary target organs. Triple therapy resulted in significant reductions in brain and kidney fungal burdens compared to those for all other treatments \( (P < 0.0001) \) (Fig. 1B and C). We also detected potential antagonism between MICA and Def since kidneys harvested from mice treated with Def plus MICA had higher fungal burdens than did those from MICA- or Def-treated mice \( (P < 0.001) \) (Fig. 1B).

**Aspergillosis model.** For IPA, only triple therapy improved 28-day survival of infected mice over that for placebo (70% survival for triple therapy versus 31% survival for placebo, \( P = 0.042 \) by log rank test). MICA treatment strongly trended to improve survival of mice over that with placebo \( (P = 0.085) \). Additionally, triple therapy improved survival over that with all dual combination treatments or for mice treated with LAmB \( (P < 0.05) \). Triple therapy did not significantly improve survival over that with Def \( (P = 0.09) \) or MICA monotherapy (Fig. 2A). Surprisingly, survival of mice treated with the combination of Def and MICA was worse than the survival of those treated with MICA alone \( (P = 0.006) \).

To define the impact of antifungal therapy on lung fungal burden, the authors investigated the effect of triple therapy in the murine model of IPA. The results showed that triple therapy improved lung fungal burden over that with placebo \( (P < 0.05) \) and dual therapy by the nonparametric Mann-Whitney test. This finding highlights the potential synergy of combining MICA with Def and LAmB for treating mold infections.
burden, mice were infected and treated as described above starting 24 h after infection and continuing until the morning of day 3 postinfection (3 doses total). At 96 h postinfection, mice were euthanized and tissue fungal burden was determined by qPCR as described earlier (1). Triple therapy resulted in a significant reduction in lung fungal burden compared to that with placebo (log_{10} conidial equivalents/g of tissue for placebo = 3.56, versus 1.63 for triple therapy, P = 0.0028 by Wilcoxon rank sum test). Additionally, triple therapy reduced fungal burden below that with other treatments except for that with LAmB or MICA (Fig. 2B). Finally, although dual combination therapy with LAmB and MICA demonstrated an antagonistic effect in reducing lung fungal burden compared to monotherapy with LAmB or MICA (P < 0.05), these results were not corroborated with the survival studies.

Several animal studies demonstrated the benefit of using a dual combination of LAmB and echinocandins or LAmB and Def over monotherapy in treating IPA (9, 17, 24) or mucormycosis (8, 10, 22), while others showed an indifferent effect (23). We show in this study that triple combination therapy consistently enhanced survival and reduced the tissue fungal burden of mice infected with mucormycosis compared to placebo, monotherapy, or dual drug therapy. A lesser effect was found with mice infected with IPA since triple therapy improved survival and reduced tissue fungal burden compared to those for placebo-treated mice but not compared to monotherapy. Indeed, case reports of patients with mucormycosis (18, 20) or aspergillosis (13, 16) treated with dual or triple combinations have described various outcomes. Interestingly, MICA therapy strongly trended to be equally as effective as triple therapy in protecting mice from IPA. One surprising finding was the possible detrimental effect that we detected when MICA and Def were used in combination without the presence of LAmB. This finding requires additional confirmation and, if confirmed, requires mechanistic exploration.

Given the poor outcomes of mold infections with current treatments, continued investigation of the potential for triple therapy using polyenes, echinocandins, and iron chelation to improve outcomes of IPA and mucormycosis infections is warranted.

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