The iron chelator deferasirox enhances liposomal amphotericin B efficacy in treating murine invasive pulmonary aspergillosis

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Objectives: Increased bone marrow iron levels in patients with haematological malignancies is an independent risk factor for developing invasive pulmonary aspergillosis (IPA), suggesting an important role for iron uptake in the pathogenesis of IPA. We sought to determine the potential for combination therapy with the iron chelator deferasirox + liposomal amphotericin B (LAmB) to improve the outcome of murine IPA compared with LAmB monotherapy.

Methods: In vitro MIC and minimum fungicidal concentration (MFC) values of the iron chelator, deferasirox, for Aspergillus fumigatus were determined by microdilution assay. In addition, we studied the efficacy of deferasirox alone or combined with LAmB in treating immunocompromised mice infected with A. fumigatus via inhalation.

Results: Deferasirox was cidal in vitro against A. fumigatus, with an MIC and MFC of 25 and 50 mg/L, respectively. Deferasirox monotherapy modestly prolonged survival of mice with IPA. Combination deferasirox + LAmB therapy synergistically improved survival and reduced lung fungal burden compared with either monotherapy alone.

Conclusions: Iron chelation therapy with deferasirox alone or in combination with LAmB is effective in treating experimental IPA. Further study of deferasirox is warranted as adjunctive therapy for IPA infections.

Keywords: Aspergillus fumigatus, LAmB, IPA

Introduction

A rise in the incidence of invasive pulmonary aspergillosis (IPA) in immunocompromised patients has occurred during the last two decades.1 Despite the development of new therapeutic agents, mortality with IPA remains >50%.2 Clearly new modalities are needed to prevent IPA and/or improve the clinical outcome of infected patients.

Recent laboratory studies demonstrate that iron acquisition is essential for the growth and virulence of Aspergillus.3 Evidence that invasive aspergillosis is associated with iron overload in patients with haematological malignancies has been found in case studies.4 Most recently, Kontoyiannis et al.5 reported that an increased bone marrow iron level was an independent risk factor for developing IPA in high-risk patients. Therefore, chelating host iron with an appropriate agent might improve the outcome of IPA.

Deferasirox is the first orally available iron chelator approved by the FDA, with an indication for the treatment of transfusion-related iron overload. We have found that deferasirox is highly active against Mucorales, and has significant efficacy both as monotherapy and in combination therapy with lipid polyenes for the treatment of murine mucormycosis.6 We sought to determine the potential efficacy of deferasirox against IPA.

Materials and methods

Culture conditions

A. fumigatus clinical strain AF293 was used for all experiments. To prepare the inoculum for in vitro or in vivo studies, A. fumigatus was grown on Sabouraud dextrose agar (SDA) plates for 2 weeks at 37°C. For susceptibility experiments, A. fumigatus was starved of iron by growing on SDA in the presence of 1 mM ascorbic acid and 1 mM
Susceptibility testing and animal model

The MIC was determined for deferasirox following the CLSI M38-A2 method on iron-starved conidia. The MFC was determined by spotting samples from all of the 96-well plate on SDA plates and incubating at 37°C for 5 days. The MFC was defined as the least concentration of the drug at which the organism failed to grow on the SDA plate.

BALB/c male mice were immunosuppressed by cyclophosphamide and cortisone acetate given at 250 mg/kg on day –2 relative to infection and repeated on day +3 at 200 mg/kg for cyclophosphamide and 250 mg/kg for cortisone acetate. This regimen resulted in a duration of leucopenia up to +7 to +8 days relative to infection. Mice were infected with A. fumigatus by aerosolizing 1.2 x 10^12 conidia in our inhalational chamber. Immediately after exposure to aerosolized conidia, three mice were sacrificed, and the lungs were homogenized and quantitatively cultured to determine the infectious inoculum. Deferasirox (Novartis Pharmaceuticals) was administered at 10 mg/kg by oral gavage in 0.5% hydroxypropylcellulose (Klucel) twice daily every other day for a total of four doses. This treatment regimen was chosen based on previous efficacy against Rhizopus oryzae in neutropenic mice. Deferasirox was initiated either 24 h post-infection (delayed therapy) or 2 days prior to infection (prophylactic therapy). Liposomal amphotericin B (LAmB; Gilead Sciences) was diluted in 5% dextrose water (5DW) and administered via tail-vein injection at a dose of 3 mg/kg/day starting 24 h post-infection and continued daily for five doses. Placebo mice were given 5DW and 0.5% Klucel. All mice were randomly assigned for treatment regimens. The primary endpoint was time of leucopenia up to +7 to +8 days relative to infection. Mice were infected with A. fumigatus by aerosolizing 1.2 x 10^12 conidia in our inhalational chamber. Immediately after exposure to aerosolized conidia, three mice were sacrificed, and the lungs were homogenized and quantitatively cultured to determine the infectious inoculum. Deferasirox (Novartis Pharmaceuticals) was administered at 10 mg/kg by oral gavage in 0.5% hydroxypropylcellulose (Klucel) twice daily every other day for a total of four doses. This treatment regimen was chosen based on previous efficacy against Rhizopus oryzae in neutropenic mice. Deferasirox was initiated either 24 h post-infection (delayed therapy) or 2 days prior to infection (prophylactic therapy). Liposomal amphotericin B (LAmB; Gilead Sciences) was diluted in 5% dextrose water (5DW) and administered via tail-vein injection at a dose of 3 mg/kg/day starting 24 h post-infection and continued daily for five doses. Placebo mice were given 5DW and 0.5% Klucel. All mice were randomly assigned for treatment regimens. The primary endpoint was time to death of moribund mice. As a secondary endpoint lung fungal burden was determined 96 h post-infection by homogenization using gentle rolling in Whirl-Pak bags containing 1 mL of saline, and plated on SDA for quantification of tissue fungal burden.

For histopathological examination, lungs were collected 6 days post-infection, fixed in 10% zinc-buffered formalin, paraffin embedded, sectioned and stained with Gomori Methenamine Silver stain for microscopic examination. All procedures involving mice were approved by the institutional animal use and care committee, according to the National Institutes of Health guidelines for animal housing and care.

Statistical analysis

The non-parametric log-rank test was used to determine differences in survival times, whereas differences in lung fungal burden were compared by the non-parametric Mann–Whitney test.

Results

Deferasirox has a modest but significant effect against A. fumigatus in vitro and in vivo

Initially, we determined the in vitro activity of deferasirox against A. fumigatus. After a 48 h incubation period, deferasirox had an MIC of 25 mg/L and an MFC of 50 mg/L. Next, mice were infected via inhalation and treated with 10 mg/kg of deferasirox alone given twice daily starting 24 h post-infection. Mice treated with deferasirox had modestly improved time to death compared with placebo-treated mice (P = 0.007 by log rank test) (Figure 1). A qualitative histopathological examination of lungs harvested from mice treated with deferasirox showed fewer fungal abscesses, which contained conidia-shaped fungal elements, compared with lungs harvested from placebo-treated mice, which had abscesses containing hyphal elements (data not shown).

Deferasirox enhanced activity of LAmB in treating murine IPA

Subsequently, mice were infected with A. fumigatus as above and treated with 3 mg/kg/day LAmB, deferasirox at 10 mg/kg twice a day, a combination of both, or placebo, with treatment starting 24 h post-infection. LAmB + deferasirox significantly improved time to death compared with placebo (P = 0.006; Figure 2a). The LAmB + deferasirox combination was superior in efficacy to either drug alone (P < 0.04). LAmB monotherapy also prolonged survival of mice compared with placebo (P < 0.04). Finally, there was a strong trend for deferasirox monotherapy to improve survival compared with placebo in this set of experiments despite the average infectious inoculum being 4-fold higher than the average inoculum used in Figure 1 (a comparison between uninfected control mice (n = 11) and mice with IPA treated with placebo or deferasirox at 10 mg/kg twice a day (n = 24 in each arm from three separate experiments). Deferasirox was given every other day for a total of four doses starting 24 h post-infection. A. fumigatus AF293 average inhaled inoculum was 7 x 10^2 conidia. *P ≤ 0.007 versus placebo by log rank test.

To define the impact of antifungal therapy on lung fungal burden, mice were infected and treated as above, starting 24 h after infection and continued until the morning of day 3 post-infection (three doses total). Only combination treatment of deferasirox with LAmB reduced tissue fungal burden compared with placebo or either drug alone (P < 0.03). There was no significant difference between organ fungal burden in mice treated with either monotherapy versus placebo (Figure 2b).

We next tested the efficacy of deferasirox administered prior to infection at day –2 and continued every other day after infection for a total of four doses (i.e. prophylactic treatment). LAmB was administered 24 h post-infection and continued every day for a total of five doses as above. Deferasirox did not improve time to death compared with placebo (P > 0.05), whereas LAmB monotherapy did improve time to death compared with placebo (P = 0.043). Combination therapy of LAmB with deferasirox mediated a significant improvement in time to death versus placebo (P < 0.05). Additionally, combination therapy
with LAmB was superior to deferasirox monotherapy (P<0.05), and there was a trend to improve time to death compared with LAmB monotherapy (P=0.078) (Figure 2c).

When the experiment was repeated to determine the effect of prophylactic therapy on fungal burden (treatment continued until 3 days after infection, when mice were euthanized), combination LAmB+deferasirox therapy reduced tissue fungal burden compared with placebo or either monotherapy (P<0.05 for all comparisons). Neither of the monotherapy arms demonstrated efficacy in reducing lung fungal burden when compared with placebo (Figure 2d).

**Discussion**

Iron is required by virtually all microbial pathogens for growth and virulence, and the efficacy of iron chelators in treating fungal infections (other than IPA) has been shown in recent animal studies.6,9

In this study, we demonstrate that deferasirox has an *in vitro* activity against *A. fumigatus* with an MIC and MFC of 25 and 50 mg/L, respectively. We previously found deferasirox to be more active against fungi belonging to the order Mucorales in which the MIC90 and MFC90 were found to be 6.25 mg/L.6 It is unclear why higher concentrations of deferasirox are required for inhibiting the growth of *A. fumigatus*. It is possible that Mucorales are more sensitive to iron deprivation than *A. fumigatus*. Alternatively, *A. fumigatus* might have a higher capability of trapping iron (enhanced capability to either acquire external or maintain internal stores of iron), which would result in a requirement for higher concentrations of deferasirox to deplete its iron stores. The decreased susceptibility of *A. fumigatus* to deferasirox *in vitro* was also seen *in vivo* since...
deferasirox only modestly enhanced survival time of mice infected with *A. fumigatus* when compared with placebo-treated mice. The enhanced survival time of mice treated with deferasirox is probably due to chelation of iron since we already demonstrated that administration of free iron reverses the protective effects of deferasirox and another iron chelator (deferiprone) in mice infected with *R. oryzae*. There are no published data on deferasirox pharmacology in rodents. In previous experience with deferasirox in our neutropenic mouse model we have found evidence that higher doses of deferasirox (>10 mg/kg twice per day given every other day) are toxic, resulting in inferior efficacy in mice infected with *R. oryzae*. Therefore, we selected the dose based on this prior experience.

A recent study demonstrated synergy between iron chelators including lactoferrin, ciclopirox and deferiprone in inhibiting *A. fumigatus* conidial growth in vitro when combined with amphotericin B, ketoconazole or fluconazole. Therefore, we sought to evaluate the benefit of combination deferasirox + LAmB therapy for IPA. We found both drugs to act synergistically against IPA. In contrast to lactoferrin, ciclopirox and deferiprone, deferasirox is already approved for use in humans by the FDA, as well as by the European Medicines Agency. Given the poor outcomes of IPA with current treatments, continued investigation of the potential for combination deferasirox–polyene therapy to improve survival in IPA is warranted.

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### Transparency declarations

None to declare.

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