The iron chelator deferasirox protects mice from mucormycosis through iron starvation

Ashraf S. Ibrahim,1,2 Teclegiorgis Gebermariam,1 Yue Fu,1,2 Lin Lin,1 Mohamed I. Huseinny,1 Samuel W. French,1,3 Julie Schwartz,4 Christopher D. Skory,5 John E. Edwards Jr.,1,2 and Brad J. Spellberg1,2

1Division of Infectious Diseases, Los Angeles Biomedical Research Institute at Harbor–UCLA Medical Center, Torrance, California, USA.
2Department of Medicine and 3Department of Pathology, UCLA David Geffen School of Medicine, Los Angeles, California, USA.
4Charles River Laboratories, Davis, California, USA. 5National Center for Agricultural Utilization Research, USDA, Peoria, Illinois, USA.

Mucormycosis causes mortality in at least 50% of cases despite current first-line therapies. Clinical and animal data indicate that the presence of elevated available serum iron predisposes the host to mucormycosis. Here we demonstrate that deferasirox, an iron chelator recently approved for use in humans by the US FDA, is a highly effective treatment for mucormycosis. Deferasirox effectively chelated iron from Rhizopus oryzae and demonstrated cidal activity in vitro against 28 of 29 clinical isolates of Mucorales at concentrations well below clinically achievable serum levels. When administered to diabetic ketoacidotic or neutropenic mice with mucormycosis, deferasirox significantly improved survival and decreased tissue fungal burden, with an efficacy similar to that of liposomal amphotericin B. Deferasirox treatment also enhanced the host inflammatory response to mucormycosis. Most importantly, deferasirox synergistically improved survival and reduced tissue fungal burden when combined with liposomal amphotericin B. These data support clinical investigation of adjunctive deferasirox therapy to improve the poor outcomes of mucormycosis with current therapy. As iron availability is integral to the pathogenesis of other infections (e.g., tuberculosis, malaria), broader investigation of deferasirox as an antiinfective treatment is warranted.

Introduction

Mucormycosis is a highly lethal infection caused by fungi belonging to the order Mucorales of the class Zygomycetes (1, 2). Recent data have demonstrated a striking increase in the frequency of mucormycosis cases (3–5). Unfortunately, despite aggressive surgical debridement and antifungal therapy, mortality from mucormycosis remains at least 50% (2, 6). In patients with central nervous system involvement, prolonged neutropenia, or disseminated disease, mortality is 80–100% despite therapy (2, 4, 5, 7). Because of its unacceptable high mortality rate, and the morbidity of disseminated disease, recent interest has been generated in the possibility of utilizing iron chelation therapy for mucormycosis. Based on this role of iron sequestration in innate immunity, recent interest has been generated in the possibility of utilizing iron chelation therapy as a treatment for infectious diseases. Mucormycosis is a particularly promising target for iron chelation therapy given the exceptional iron requirement for growth and pathogenicity of Mucorales (12–15). For example, it is likely that diabetic ketoacidosis and other systemic acidoses predispose patients to developing mucormycosis by inducing dissociation of iron from sequestering proteins, resulting in elevated available serum iron (2, 16, 17).

The potential therapeutic role of iron chelation therapy for mucormycosis was initially obscured by the paradoxically increased risk of developing mucormycosis during treatment with deferoxamine (18). However, while deferoxamine is an iron chelator from the perspective of the human host, it serves as a xenosiderophore to Mucorales, which are able to specifically bind to deferoxamine-iron complexes, strip the iron from the chelator through an energy-dependent reductive process, and facilitate iron uptake (12, 18). Furthermore, administration of deferoxamine worsens survival of animals with mucormycosis (12–15). In contrast, other iron chelators do not act as iron siderophores for Mucorales. Specifically, treatment of Rhizopus-infected mice (16) or guinea pigs (13) with the iron chelator deferiprone markedly improved survival. Unfortunately, while available in India and Europe, deferiprone is not approved by the US FDA for use in humans.

In 2005, deferasirox became the first orally bioavailable iron chelator approved for use by the FDA, with an indication for treatment of iron overload in transfusion-dependent anemias. Given the important role of iron in the pathogenesis of mucormycosis, as well as the current availability of deferasirox for use in humans, we sought to define the potential efficacy of deferasirox iron chelation therapy against experimental mucormycosis in vitro and in vivo.

Results

Deferasirox kills Mucorales by iron starvation. We have previously found that iron starvation causes the rapid expression of the high-affinity iron permease gene (rFTR1) in Rhizopus oryzae (19), the most common cause of mucormycosis (2, 20). Therefore, to determine whether deferasirox effectively chelates iron from R. oryzae, we extracted RNA and performed RT-PCR on R. oryzae 99-880 grown for 1 hour at 37°C in potato dextrose broth (PDB) supplemented with 350 μM ferric chloride (iron replete), 350 μM ferric chloride plus 2 mM...
Deferasirox (iron depleted), or 2 mM deferasirox plus supersaturating 6 mM ferric chloride. The rFTR1 gene was expressed in the presence of deferasirox, but not in media supplemented with ferric chloride alone or in the presence of deferasirox plus supersaturating ferric chloride (Figure 1A). The possibility of contaminating genomic DNA was excluded by lack of a detectable band when PCR (rather than RT-PCR) was performed on RNA samples.

To confirm protein expression under chelating conditions, the gene encoding GFP was cloned behind the rFTR1 promoter (rFTR1p) (Figure 1B). R. oryzae M16 spores transformed with the rFTR1p::GFP or actin promoter::GFP (ACT1p::GFP) (constitutively expressed positive control) constructs were incubated overnight with deferasirox, deferasirox plus supersaturating ferric chloride, or iron-replete media. M16 spores transformed with the constitutive ACT1p::GFP construct were fluorescent regardless of growth conditions (Figure 1C). In contrast, M16 spores transformed with rFTR1p::GFP were only fluorescent in the presence of deferasirox (Figure 1C). Similarly, by flow cytometry, less than 1% of untransformed or rFTR1p::GFP-transformed spores grown in iron-replete conditions were fluorescent (Figure 1C). In contrast, 43% of spores transformed with rFTR1p::GFP and grown in the presence of deferasirox were fluorescent. Collectively, these data confirmed that deferasirox induced an iron-starvation response in R. oryzae.

We next determined the deferasirox susceptibilities of multiple clinical isolates of Mucorales (Table 1). The MIC$_{90}$ (minimum concentrations at which 90% of isolates were inhibited) of deferasirox against Mucor spp, non-oryzae Rhizopus spp, and R. oryzae were 3.12–6.25 μg/ml. Minimum fungicidal concentrations (MFCs) were similar to MICs, and deferasirox was cidal for 28 of 29 (97%) isolates. Of note, slight growth was seen within the first 12 hours even at extremely high concentrations of deferasirox. However, the fungi were dead by 24 hours even at low concentrations of deferasirox, suggesting that deferasirox cidality was time dependent rather than concentration dependent.

To determine if the cidal effect of deferasirox was related to chelation of iron, we repeated susceptibility testing for 4 strains of R. oryzae. Deferasirox was added at a concentration equivalent to the previously established MFC. In half the wells, iron was added at a molar ratio to saturate the deferasirox. For all 4 isolates, addition of iron completely reversed the fungicidal effects of the deferasirox, resulting in significant visible fungal growth despite the presence of the drug.
Deferasirox is effective against *R. oryzae* in vivo in diabetic ketoacidotic mice. To determine deferasirox’s in vivo efficacy, diabetic ketoacidotic mice were infected via the tail vein with 2.2 × 10⁴ spores of *R. oryzae* 99-892. The mice were treated by oral gavage with 1, 3, or 10 mg/kg deferasirox twice daily or placebo for 7 days. Deferasirox significantly improved survival compared with controls (Figure 2A). While there was no statistical difference between the time to death of mice treated with the different doses of deferasirox, a dose-response trend was observed, with more surviving mice at higher doses.

To determine the impact of deferasirox on tissue fungal burden, diabetic ketoacidotic mice were infected via the tail vein with 4.2 × 10⁴ spores of *R. oryzae* 99-892 as above. Mice were treated with deferasirox (10 mg/kg twice daily), deferasirox plus saturating ferric chloride, or placebo. Treatment was begun 16 hours after infection and administered daily for 3 days. Deferasirox resulted in a greater than 10-fold decrease in day 4 brain and kidney (primary target organs) fungal burdens compared with placebo or deferasirox plus saturating ferric chloride (Figure 2B). By histopathology, kidneys of deferasirox-treated mice had no visible hyphae, whereas kidneys of mice treated with placebo or deferasirox plus saturating ferric chloride had extensively filamented fungi (Figure 2C). Furthermore, mice treated with saturating iron had a striking absence of neutrophil influx to the sites of infection, while neutrophil influx was prominent in the kidneys of mice treated with deferasirox.

Deferasirox was considered fungicidal when the ratio of MFC to MIC was less than or equal to 4 (40).

### Table 1

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<th>Fungicidal?²</th>
<th>MIC (µg/ml) 48 hours</th>
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²Deferasirox was considered fungicidal when the ratio of MFC to MIC was less than or equal to 4 (40).
Deferasirox protects diabetic ketoacidotic mice from hematogenously disseminated *R. oryzae* infection. Mice infected via the tail vein were treated with placebo (hydroxypropylcellulose carrier), deferasirox, or deferasirox plus iron (FeCl₃, 10 mg/kg) to reverse the effect of iron chelation. (A) Survival of mice (*n* ≥ 7 per group) infected with *R. oryzae* 99-892 (2.2 × 10⁴ spores) and treated with various doses of deferasirox. (B) Brain and kidney fungal burden of mice (*n* = 11 per group) infected with *R. oryzae* 99-892 (4.2 × 10⁴ spores) and treated with placebo, deferasirox (10 mg/kg twice daily), or deferasirox plus iron. Organs were harvested on day 4 after mice received 3 daily treatments. Data are displayed as median ± interquartile ranges. The y axis reflects the lower limit of detection of the assay. (C) H&E-stained kidney sections of mice infected with *R. oryzae* 99-892 and treated with deferasirox, deferasirox plus ferric chloride, or placebo as described in B. Arrows indicate *R. oryzae* hyphae in tissue. Sections are representative of findings throughout organs in all mice in the respective groups. Original magnification, ×400. (D) Survival of diabetic ketoacidotic mice (*n* = 24 from 3 separate experiments with similar results) infected via the tail vein with *R. oryzae* 99-880 (average inoculum, 1.3 × 10⁸ spores) and 24 hours later treated with 10 mg/kg deferasirox twice daily for 7 days. *P* < 0.05 for survival and *P* < 0.002 for tissue fungal burden compared with placebo or deferasirox plus ferric chloride.

CFU/mouse; range, 3.8 × 10⁴ to 9.7 × 10⁴). Mice were treated for 7 days, starting 24 hours after infection, with deferasirox (10 mg/kg, twice daily) or placebo. As controls, infected or uninfected mice were treated with deferoxamine (50 mg/kg, i.p.), which is known to supply iron to, rather than chelate iron from, *R. oryzae*. Deferasirox was protective compared with placebo or deferoxamine (*P* = 0.009). As expected, deferoxamine worsened survival of infected mice compared with placebo (*P* = 0.047) and had no effect on uninfected mice (Figure 3).

Effects of iron chelation versus excess iron on host immune response. The diminished polymorphonuclear leukocyte influx seen in kidneys of mice treated with saturating ferric chloride versus iron chelation suggested that excess iron might directly suppress inflammation. Therefore, we determined the impact of deferasirox iron chelation therapy on Th1/Th2 and inflammatory cytokine responses at sites of infection. Diabetic ketoacidotic mice were infected with 3.1 × 10⁴ spores of *R. oryzae* 99-892 as above and treated with deferasirox, deferasirox plus ferric chloride, or placebo. On day 4 of infection, spleens and kidneys (which are heavily infected in the disseminated model; refs. 21, 22) were processed for intracellular and whole-organ cytokine determination. Deferasirox resulted in a significant increase in both Th1 and Th2 splenocyte frequencies compared with saturating ferric chloride or placebo (Figure 4A). Deferasirox-treated mice had significantly higher splenic levels of the canonical proinflammatory cytokines TNF and IFN-γ than mice treated with saturating iron or placebo (Figure 4B). Mice treated with deferasirox also had significantly higher kidney levels of IFN-γ (Figure 4B). In contrast, splenic and renal concentrations of Th2 cytokines, including IL-4, IL-5, and IL-10, did not significantly differ between the groups (data not shown).

Because differences in Th2 cytokine levels were not observed, and therefore lower Th2 cytokine levels could not explain increased Th1 cytokine levels in mice treated with deferasirox, we sought to determine whether deferasirox treatment affected Treg or lymphocyte apoptosis frequencies. The frequencies of CD4⁺IL-10⁺ or CD4⁺CD25⁺Foxp3⁺ lymphocytes were not significantly different between the groups (data not shown). There was also no significant difference in splenocyte apoptosis between the groups (data not shown).

**Efficacy of deferasirox therapy in combination with liposomal amphotericin B.** To determine whether deferasirox was synergistic with liposomal amphotericin B (LAmB), diabetic ketoacidotic mice were infected via the tail vein with the more virulent *R. oryzae* 99-880 isolate and treated with placebo, LAmB alone at 15 mg/kg/d i.v. for 4 days, deferasirox alone at 10 mg/kg orally twice daily for 7 days, or a combination of LAmB and deferasirox. Both deferasirox and LAmB monotherapy significantly improved survival compared with placebo (Figure 5A). However, the combination of deferasirox plus LAmB significantly improved survival versus placebo and both monotherapies (Figure 5A).

We also determined the impact of combination therapy on tissue fungal burden. Treatment began 24 hours after infection and was administered daily for 2 days because a substantial number of deaths was anticipated in the placebo group by day 3 (Figure 2D and Figure 5A). On day 3, kidneys and brains were removed, homogenized, and quantitatively cultured. The kidney fungal burden of mice treated with deferasirox alone was not significantly different from that of mice treated with placebo (Figure 5B). LAmB monotherapy and combination therapy decreased kidney fungal burden versus placebo (Figure 5B). In contrast, in the brain, only combination deferasirox plus LAmB significantly
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reduced fungal burden versus placebo, and combination therapy also significantly reduced brain fungal burden versus both monotherapies (Figure 5B).

**Efficacy of deferasirox in neutropenic, infected mice.** To determine whether deferasirox was also effective in the setting of neutropenia, mice were myeloablated with cyclophosphamide. Two days later, mice were infected via the tail vein with $2.7 \times 10^3$ spores of *R. oryzae* 99-892. Initial dose response studies suggested that, in contrast to the diabetic ketoacidotic model, optimal outcomes were achieved with dosing of deferasirox every other day rather than every day (data not shown), as we have previously described with the iron chelator deferoxamine (16). Treatment with deferasirox (10 mg/kg twice every other day for 5 doses starting 24 hours after infection) significantly improved time to death compared with placebo treatment (Figure 6). In contrast, deferasirox administered daily did not significantly improve time to death versus placebo.

**No specific toxicity of deferasirox in neutropenic mice could be identified.** Because deferasirox dosing every other day was optimal in the neutropenic model, we sought to identify any potential toxicity caused by daily dosing of deferasirox in neutropenic mice. Mice were made neutropenic with cyclophosphamide as above but were not infected. The mice were treated with deferasirox 10 mg/kg twice daily for 7 days, 10 mg/kg twice every other day for 4 doses, or placebo. Terminal bleeds were performed on day 3 or day 8 to measure complete blood count, serum chemistries, and liver function tests. Histopathology was performed on a number of organs, including brain, heart, lungs, liver, gallbladder, spleen, kidneys, gastrointestinal tract (including stomach, small intestine, and large intestine), and bone marrow (both smears and core). No differences in white cell counts, absolute neutrophil counts, platelet counts, hemoglobin levels, serum chemistries (including creatinine, blood urea nitrogen, or electrolytes), or liver function tests (including AST, ALT, or bilirubin) were identified between the 3 groups at either time point (data not shown). By histopathology, no organ-specific toxicity attributable to deferasirox was identified, including no evidence of alterations in hematopoiesis.

**Discussion**

The role of iron sequestration in innate host defense against infection has gained increasing attention of late (8–11, 23, 24). Nevertheless, iron chelation therapy has not been rigorously studied against any type of infection, and regulatory agencies have not approved iron chelators for the treatment of any infectious disease. Here we show that deferasirox induced an iron-starvation
response in *R. oryzae* and was cidal for multiple clinical isolates from 2 different genera of the family Mucorales. The slight fungal growth that occurred within 12 hours even at high concentrations of deferasirox, and the cidal seen at 24 hours even at low concentrations of deferasirox, suggested that deferasirox is a time-dependent killer of Mucorales. Furthermore, the MICs and MFCs of deferasirox against these isolates were well below clinically achievable peak (~38 μg/ml) and trough serum levels (~17 μg/ml) of the drug at steady state when administered at the FDA-approved starting dose (20 mg/kg/d) (25). These in vitro susceptibility data indicate that treatment of infected patients should be feasible based on pharmacokinetic/pharmacodynamic principles.

The efficacy of deferasirox in treatment of mice with mucormycosis was comparable to the efficacy of LAmB. Furthermore, deferasirox was effective in both diabetic ketoacidotic and neutropenic murine models. Importantly, the combination of deferasirox and LAmB was synergistic. In particular, combination deferasirox plus LAmB dramatically reduced brain fungal burden compared with either drug alone. These data are concordant with our recent, successful treatment of a patient with advanced, refractory cerebral mucormycosis by adding salvage deferasirox therapy to LAmB (26).

Reversal of the protective effect of deferasirox by administration of free iron indicates that the drug’s mechanism of protection was likely iron chelation. Furthermore, it is known that deferasirox chelation is specific for iron and that other trace metals and cations are not affected by the drug (27, 28). However, deferasirox was associated with an enhancement of the suppressed inflammatory response in diabetic ketoacidotic mice. The mechanisms by which iron levels modulated inflammation are not clear, as we found no evidence of an impact on Th2 cytokines, Tregs, or plasmocyte apoptosis. Our findings are consistent with previous studies demonstrating that iron overload suppresses cell-mediated immune responses and inflammation, including production of canonical Th1 cytokines such as IFN-γ or IL-12 (29–31). However, reduction of iron levels with chelators other than deferasirox has also been shown to suppress Th1 polarization and inflammatory responses and to favor Th2 responses (32, 33), so the effects of intracellular iron levels and iron chelation on host immune responses appear to be protein.

Deferasirox appeared to be somewhat more effective in diabetic ketoacidotic mice than in neutropenic mice. Although we hypothesized that the diminished activity of daily dosing in neutropenic mice might be due to a toxic effect of deferasirox, an extensive evaluation failed to identify any toxicity. Specifically, there was no evidence of exacerbation of or delayed recovery from bone marrow ablation by chemotherapy. Nor was there any evidence of renal or hepatic dysfunction by laboratory testing or any specific organ toxicity by histopathological evaluation. It is known that leukocytes form an extensive storage pool for iron in mammals, and increased erythropoiesis results in depletion of iron stores (8, 23, 34). Furthermore, injury to rapidly dividing intestinal epithelial cells by cyclophosphamide may inhibit uptake of iron from dietary sources. Hence, the alteration in maximally effective deferasirox dosing in neutropenic mice may relate to altered iron homeostasis in hosts myeloablated with cyclophosphamide. Alternatively, the enhanced efficacy of deferasirox in the diabetic ketoacidotic mouse model might reflect the ability of this iron chelator to efficiently chelate serum iron previously freed by proton-mediated dissociation from iron-sequestering proteins (2, 16, 17).

Little data has been published regarding the safety of deferasirox in myeloablated hosts. In preclinical investigations of deferasirox, no toxicities were seen in non–iron-overloaded marmosets after 39 weeks of treatment with up to 40 mg/kg/d, and during subacute treatment (4 weeks), doses up to 65 mg/kg/d were well tolerated, with no adverse effects (27). In clinical investigations (25, 28, 35), deferasirox was very well tolerated at doses up to 40 mg/kg/d for up to 12 days, and over months to years, at up to 30 mg/kg/d. However, there have been rare postmarketing reports of cytopenias...
in patients taking deferasirox, particularly in chronically transfused patients with underlying marrow dyscrasias (i.e., myelodysplastic syndrome) (36). Similarly, cases of acute renal failure in patients taking deferasirox have been reported (36). The relationship between drug and toxicity in such cases remains unclear.

Given the demographics of aging in the US population, the incidence of cancer and neutropenia will likely continue to rise in the coming decade. Furthermore, an epidemic of obesity in the US presages a worsening of the current epidemic of diabetes, and the frequency of organ transplantation, with its associated immunosuppression, is on the rise (37). Because of the increasing frequency of each of these major risk factors, it is likely that mucormycosis will continue to increase in incidence. Given the poor outcomes with maximal therapy, there is a critical need to develop novel therapies for this highly lethal infection. We have found that the FDA-approved iron chelator deferasirox has cidal activity against multiple strains of Mucorales at clinically achievable concentrations of the drug. Deferasirox was effective as monotherapy and was synergistic with LAmB, resulting in enhanced clearance of the fungus from the brain and improved survival. These results support the need for a clinical trial to determine the efficacy of adjunctive deferasirox in addition to polynye therapy in patients with mucormycosis. These data also support the need for continued investigation of deferasirox as an antifungal therapy for other diseases in which iron availability plays a crucial role in pathogenesis, including tuberculosis, malaria, and others.

Methods

*R. oryzae* and culture conditions. *R. oryzae* 99-880 is a clinical isolate from a brain abscess of a diabetic patient. *R. oryzae* 99-892 is a clinical, pulmonary isolate. *R. oryzae* 99-880 and 99-892 were provided by the Fungus Testing Laboratory at the University of Texas Health Science Center, San Antonio, Texas, USA. *R. oryzae* M16 is a pyrF-null mutant (pyrF encodes orotidine 5′-phosphate decarboxylase, an enzyme required for uracil synthesis) generated by A.S. Ibrahim from *R. oryzae* 99-880 by chemical mutagenesis. This mutant exhibited a stable phenotype for uracil auxotrophy even when plated more than 1 × 10^6 spores. Organisms were grown on potato dextrose agar (PDA) for 4 days at 7°C. For M16, PDA was supplemented with 100 μg/ml uracil. In some experiments, *R. oryzae* was starved for iron by growth on yeast nitrate base (YNB) (Difco; BD) supplemented with complete supplemental medium without uracil (YNB+CSM-URA; formulation/100 ml, 1.7 g YNB without amino acids, 20 g glucose, 0.77 g CSM-URA) in the presence of 1 mM of ascorbic acid and ferrozine. The sporangiospores were collected in endotoxin-free PBS containing 0.01% Tween-80, washed with PBS, and counted with a hemacytometer to prepare the final inocula.

Effect of deferasirox on the expression of rFTR1. *R. oryzae* 99-880 plugs taken from a confluent PDA plate were grown in PDB overnight at 37°C with shaking. Mycelia were collected aseptically and transferred to fresh PDB flasks containing 350 μM ferric chloride (to suppress the expression of rFTR1; ref. 19), 350 μM ferric chloride plus 2 mM deferasirox to test iron chelation, or 2 mM deferasirox plus 6 mM ferric chloride to supersaturate the added deferasirox. The flasks were incubated for 1 hour at 37°C with shaking. Mycelia were collected by filtration and ground in liquid nitrogen using mortar and pestle. Total RNA was extracted using the RNeasy Plant Mini kit (QIAGEN) with RLC buffer. The RNA was reverse transcribed with oligo-dT primer using the SuperScript First-Strand Synthesis System (Invitrogen) to generate first-strand cDNA. The products were diluted and used in PCR to detect the expression of rFTR1 and 18S rDNA gene. The sequences of the PCR primers were as follows: sense primer 5′-TCAGAGAAGGACTTGAAGC-3′ and anti-sense primer 5′-TAAGTAGCC-GTATTGTCC-3′ for rFTR1 to amplify of *R. oryzae* (19); and sense primer 5′-CATGTTGAGATTGTAAGATAG-3′ and anti-sense primer 5′-AGTC-CAAATTGAAGTGAACTC-3′ for the 18S rDNA gene. The PCR products were separated on a 2% agarose gel containing 0.1 μg/ml ethidium bromide. Both primer sets were designed to amplify a 400-bp band.

To study the effect of deferasirox on the expression of rFTR1, we used GFP as a reporter system. A 1.5-kb fragment upstream of the rFTR1 ORF was PCR amplified from genomic DNA of strain 99-880 using the following primer pair: sense primer 5′-GCTCTAGATCATCCTCAACCATCAATT-3′ and anti-sense primer 5′-TGCGGCGGTGCTTTTGTATTCTCTTGGA-3′. A 2.1-kb fragment containing the constitutively expressed actin promoter was also PCR amplified from genomic DNA of strain 99-880 to use as a control using the following primer pair: sense, 5′-GCTCTAGATGGTTATATCGATTAGA-3′; and anti-sense, 5′-GTACGGCCGCATAACGGAACCGTTATCG-3′. Amplified fragments were ligated into the XbaI and EagI sites of plasmid pyr225b (38). GFP was amplified from pGFPB21-43.31 (39) and cloned downstream of either promoter into EagI-SalI sites of the plasmid. Finally, a 2.1-kb fragment representing the ORF of pyrF and its native promoter was cloned into SpeI-Clal sites of the plasmid to serve as a selection marker. Plasmids containing GFP driven by either rFTR1p or ACT1p were transformed into *R. oryzae* M16 with microprojectile particle bombardment (Bio-Rad) (38). Transformants were selected on YNB+CSM-URA plates grown at 37°C for 5–7 days following bombardment. Isolates were then tested for uracil auxotrophy by passaging transformants on plates containing minimal medium without uracil and incubating the plates at 37°C. Purified transformants selected on uracil-negative plates were analyzed by Southern blotting.

Expression of rFTR1p and ACT1p was studied in transformants grown in iron-replete medium (i.e., YNB+CSM-URA) or iron-depleted conditions (i.e., YNB+CSM-URA supplemented with 2 mM deferasirox). Additionally, to reverse the effect of deferasirox, transformants were grown in YNB+CSM-URA supplemented with 2 mM deferasirox and supersaturating 6 mM ferric chloride. Finally, M16 transformed with empty plasmid (pyr225b-pyrF without GFP) was used as a negative control. Spores (1 × 10^4/ml) were inoculated in the above-mentioned media and incubated overnight at 37°C. A drop of the culture was mounted on a slide, and GFP expression was visualized in *R. oryzae* with a Leica DMRXE confocal microscope using an excitation wavelength of 488 nm. Additionally, a 1-ml sample from each culture was also used to quantify the fluorescence emission using a FACSCaliber (BD) flow cytometer. Spores were gated by forward and side scatter and fluorescence measured in FL1.

Susceptibility testing. MIC and MFC were determined for deferasirox by the method of Epstein-Ingoff using *R. oryzae* spores starved for iron (40). Cidality was defined as difference of less than or equal to 4-fold between MIC and MFC (40).

Murine models. For in vivo infection, BALB/c male mice (≥20 g) were rendered diabetic with a single i.p. injection of 210 mg/kg streptozotocin in 0.2 ml citrate buffer 10 days prior to fungal challenge (41). Glycosuria and ketonuria were confirmed in all mice 7 days after streptozotocin treatment. For the neutropenic mouse model, mice were injected with a single i.p. dose of 200 mg/kg cyclophosphamide (Bristol-Myers Squibb) 2 days prior to infection with *R. oryzae*. This treatment regimen resulted in pancytopenia from day −2 to day 5 relative to infection, with recovery of cell counts beginning on day 6 after infection (42). To confirm the inocula for i.v. infection, dilutions were streaked on PDA plates containing 0.1% Triton, and colonies were counted following a 24-hour incubation period at 37°C. For the intranasal infection, 10^7 spores in 20 μl of 0.01% Tween-80 in PBS were placed on the nostrils of ketamine-sedated (100 mg/kg) mice (43). To confirm the inoculum, mice were sacrificed immediately after inhaling *R. oryzae* spores; lungs were homogenized and plated on PDA containing 0.1% Triton; and colonies...
were counted following incubation at 37°C. For both models, the primary efficacy endpoint was time to death. In some experiments, as a secondary endpoint, brain and kidney fungal burden (the primary target organs) (16, 21) was determined by homogenization by rolling a pipette on organs placed in Whirl-Pak bags (Nasco) containing 1 ml saline. The homogenate was serially diluted in 0.85% saline and then quantitatively cultured on PDA plates containing 0.1% Triton. Values were expressed as log_10 CFU/g tissue. Finally, for histopathological analysis, infected organs were collected from mice and fixed in 10% zinc formalin. Fixed tissues were embedded in paraffin, and 5-μm sections were stained with H&E for light microscopy examination.

**Treatment regimens.** LamB diluted in 5% dextrose water was obtained from Gilead and was administered at a dose of 15 mg/kg daily via the tail vein for 4 days. Deferasirox (Novartis) was suspended in 0.5% hydroxypropyl methylcellulose (Klucz; Hercules Inc.) and administered by oral gavage at 1, 3, or 10 mg/kg twice daily or every other day. Treatment was begun 24 hours after infection and continued for a total of 5 or 7 doses. Control groups were treated with the diluent, 5% dextrose water, and 0.5% Klucz.

In some experiments, a saturating dose of free iron was administered with deferferox. Deferasirox is known to form molecular complexes with ferric iron (Fe^{3+}) in a 1:2 ratio of iron to deferferox (13). Based on the known molecular weights of ferric chloride (FeCl₃; molecular weight 162.22 g/mol) and deferasirox (molecular weight 373.4 g/mol), a 2.8-mg/kg dose of FeCl₃ was calculated to result in a significant excess of Fe^{3+} versus a 10-mg/kg dose of deferasirox given to an 18-g mouse: Fe^{3+} moles = (0.0028 g/kg × 0.018 kg [mouse wt] / 162.22 g/mol) × 3 = 3 × 10^{-7} mol; versus deferferox moles = (0.01 g/kg × 0.018 kg [mouse wt] / 373.4 g/mol) = 5 × 10^{-7} mol; ratio = 1:1.7 moles of Fe^{3+} to deferasirox, which is more than that required to achieve a saturating 1:2 ratio.

**Spleenic lymphocyte frequencies and whole-organ cytokines.** Splenic Th1 (CD4^+IFN-γ+IL-4+) and Th2 (CD4^+IFN-γ-IL-4-) lymphocyte frequencies were determined as we have previously described (44). In brief, splenic homogenates were passed through 70-μm filters followed by rbc lysis with 0.15 M ammonium chloride. The cells were fixed with Cytofix buffer (BD Biosciences—Pharmingen), and stained with 10 μg/ml of FITC-conjugated anti-mouse CD4 (clone RM4-5), PE-conjugated anti-mouse IFN-γ (clone XMG1.2), or isotype control (clone R3-14); allophycocyanin-conjugated (APC-conjugated) anti-mouse IL-4 (clone 11B11) or isotype control (clone R3-34); or APC-conjugated anti-mouse IL-10 (clone JESS-166/3) or isotype control (clone A95-1) (all from BD Biosciences—Pharmingen). In separate experiments, the frequency of CD4^+CD25^hiFoxp3^+ Tregs was determined using the Mouse Regulatory T Cell Staining Kit (eBioscience) per the manufacturer's recommendation. The frequency of apoptosis was determined using the Annexin FITC Apoptosis Kit (BD Biosciences—Pharmingen).

Cells were washed, and 3-color flow cytometry was performed on a BD FACScan instrument calibrated with CalibRITE beads (BD Biosciences—Pharmingen) using FACSComp software as per the manufacturer's recommendations. During data acquisition, CD4^+ lymphocytes were gated by concatenation of forward and side scatter and FITC–anti-CD4 antibody fluorescence properties. Data for each sample were acquired until 10,000 CD4^+ lymphocytes were analyzed.

**Whole-organ cytokines.** Spleens and kidneys were homogenized in 1 ml of PBS. The homogenates were pelleted at maximum speed on a tabletop centrifuge at 4°C. The supernatants were assayed for cytokines using the Cytometric Bead Array Murine Inflammatory Cytokine kit (BD Biosciences—Pharmingen) per the manufacturer's instructions.

**Deferasirox toxicity studies.** Toxicity of deferasirox in neutropenic mice was evaluated. Mice were rendered neutropenic as described above and treated with deferasirox at 10 mg/kg twice every day or every other day for 7 days. Mice from 3 different groups (i.e., placebo, deferasirox daily treatment, and deferasirox every other day treatment) were sacrificed on day 3 or 8, and blood was collected and sent to Charles River Laboratories for evaluation. Additionally, bone marrow smears were prepared from femurs, and tissues were collected, preserved in zinc-buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with H&E. The resulting slides were examined by a board-certified veterinary pathologist at Charles River Laboratories.

All procedures involving mice were approved by the institutional animal use and care committee of the Los Angeles Biomedical Research Institute, following the NIH guidelines for animal housing and care.

**Statistics.** The nonparametric log-rank test was used to determine differences in survival times of the mice. Differences in tissue fungal burdens, splenic lymphocyte frequencies, and whole-organ cytokines in the infected organs were compared by the Mann Whitney U test. P values of less than 0.05 were considered significant.

**Acknowledgments**

This work was supported by Public Health Service grants R01 AI63503-01A2 and R21 AI064716-01A2 and research and educational grants from Gilead Sciences Inc. and Novartis Pharmaceuticals to A.S. Ibrahim. B.J. Spellberg is supported by Public Health Service grant K08 AI060641 and American Heart Beginning Grant-in-Aid 0665154Y and is a Liu Young Investigator in Biomedical Research. Y. Fu is supported by American Heart Beginning Grant-in-Aid 0665041Y. J.E. Edwards is supported by Public Health Service grants R01 AI19990 and AI63382 and an unrestricted Freedom to Discover Grant for Infectious Disease from Bristol-Myers Squibb. Research described in this manuscript was conducted in part at the research facilities of the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center. We also thank June Kwon-Chung for her collegial interactions regarding our development of systems to study the molecular genetics of the Mucorales. A.S. Ibrahim, Y. Fu, J.E. Edwards, and B.J. Spellberg have equity in NovaDigm Therapeutics Inc.

Received for publication April 6, 2007, and accepted in revised form June 13, 2007.

Address correspondence to: Ashraf S. Ibrahim, Division of Infectious Diseases, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, 1124 W. Carson St., Torrance, California 90502, USA. Phone: (310) 222-6424; Fax (310) 782-2016; E-mail: ibraham@labiomed.org.

This work was presented in part at Focus on Fungal Infections 17 in San Diego, California, USA, on March 7–9, 2007. Abstract P-0024.