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Iron Acquisition: A Novel Prospective on Mucormycosis Pathogenesis and Treatment

Ashraf Ibrahim[1,2,*], **Brad Spellberg**[1,2], and **John Edwards Jr.**[1,2]

[1]Department of Medicine, Los Angeles Biomedical Institute at Harbor-UCLA Medical Center, Torrance, California

[2]Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles California

Abstract

Purpose of review—Mucormycosis is an increasingly common fungal infection with an unacceptably high mortality despite first-line antifungal therapy. Iron acquisition is a critical step in the causative organsisms' pathogenetic mechanism. Therefore, abrogation of fungal iron acquisition is a promising therapeutic strategy to impact clinical outcomes for this deadly disease.

Recent findings—The increased risk of mucormycosis in patients in renal failure receiving deferoxamine iron chelation therapy is explained by the fact that deferoxamine actually acts as a siderophore for the agents of mucormycosis, supplying previously unavailable iron to the fungi. The iron liberated from deferoxamine is likely transported into the fungus by the high affinity iron permease. In contrast, two other iron chelators, deferiprone and deferasirox, do not supply iron to the fungus and were shown to be cidal against *Zygomycetes* in vitro. Further, both iron chelators were shown to effectively treat mucormycosis in animal models, and one has been successfully used as salvage therapy for a patient with rhinocerebral mucormycosis.

Summary—Further investigation and development of iron chelators is warranted as adjunctive therapy for mucormycosis.

Keywords

Mucormycosis; Rhizopus; Iron chelation; Deferasirox; deferiprone

I. Introduction

Mucormycosis is a life-threatening infection caused by fungi of the class *Zygomycetes,* order Mucorales. Fungi belonging to the family Mucoraceae, and specifically the species *Rhizopus oryzae* (*Rhizopus arrhizus*), are by far the most common cause of infection [1]. Strong clinical evidence has implicated iron availability as a major regulator of *Zygomycetes* virulence. This review will focus on the mechanism of iron uptake in *R. oryzae* and describe recent data suggesting the potential for iron chelators to be potential novel agents in the treatment of mucormycosis.

II. Overview of Mucormycosis

The agents of mucormycosis are opportunistic pathogens that almost uniformly affect immunocompromised hosts [2]. Patients with diabetic ketoacidosis are particularly

*Corresponding author: Ashraf Ibrahim, Ph.D. Division of Infectious Diseases, Harbor-UCLA Medical Center, 1124 West Carson St. RB2, Torrance, CA 90502, 310-222-6424 (phone), 310-782-2016 (fax), Ibrahim@labiomed.org.

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susceptible to mucormycosis. Patients immunocompromised by cytotoxic chemotherapy or corticosteroid therapy are also susceptible to mucormycosis. A marked increase in the incidence of mucormycosis has occurred over the past two decades (Fig. 1). Similar increases have been reported by major stem cell transplant centers [3,4]. Given the increasing prevalence of diabetes, cancer, and organ transplantation in the aging United States population, the rise in incidence of mucormycosis is anticipated to continue unabated for the foreseeable future.

III. Available Antifungal Therapies for Mucormycosis

The standard therapy for invasive mucormycosis includes reversal of the underlying predisposing factors (if possible), emergent, wide-spread surgical debridement of the infected area (Fig. 2), and adjunctive antifungal therapy [5-8]. Amphotericin B (AmB) remains the only antifungal agent approved for the treatment of invasive mucormycosis [5-8]. Because the fungus is relatively resistant to AmB, high doses are required, which frequently results in nephrotoxicity [8]. Also, in the absence of surgical removal of the infected focus (such as excision of the eye in patients with rhinocerebral mucormycosis), antifungal therapy alone is rarely curative [5,6]. Even when surgical debridement is combined with high-dose AmB, the mortality associated with mucormycosis exceeds 50% [8], and in disseminated disease approaches 100% [9]. Because of this unacceptably high mortality rate, and the extreme morbidity of highly disfiguring surgical therapy (Fig. 2), it has been imperative to develop new strategies to treat and prevent invasive mucormycosis.

The nephrotoxicity of AmB has prompted clinicians in practice to adopt the use of lipid formulations of AmB, which are less nephrotoxic than AmB and can be administered at higher doses for a longer period of time [6,10]. Most recently, a retrospective review of outcomes in patients with rhino-orbital-cerebral mucormycosis suggested that combination therapy with lipid polyene plus caspofungin was superior to monotherapy with lipid polyenes [11]. Nevertheless, there is a great need for additional therapeutic strategies to improve outcomes in patients with these deadly infections.

IV. The Role of Iron in Mucormycosis Pathogenesis

A. Increased available serum iron is a risk factor for mucormycosis

Iron is required by virtually all microbial pathogens for growth and virulence [12]. In mammalian hosts, very little serum iron is available to microorganisms because it is highly bound to carrier proteins such as transferrin [13]. Sequestration of iron by serum is a major host defense mechanism against *R. oryzae* in particular [13]. The organism grows poorly in serum and this growth inhibition is reversed when exogenous iron is added [13,14].

Importantly, patients with elevated levels of available serum iron are uniquely susceptible to infection by *R. oryzae* and other *Zygomycetes*, but not to other pathogenic fungi, such as *Candida* or *Aspergillus* [6,8]. For example, patients treated with the iron chelator, deferoxamine, have a markedly increased incidence of invasive mucormycosis, which is associated with a mortality of >80% in these patients [15]. While deferoxamine acts as an iron chelator with respect to the human host, its effect on *R. oryzae* is just the opposite. Deferoxamine predisposes patients to *Rhizopus* infection by acting as a siderophore, which supplies previously unavailable iron to the fungus [14]. *Rhizopus* obtains iron from the iron-deferoxamine complex by intracellular transport of the reduced iron without deferoxamine internalization [16]. This transport is likely mediated by high-affinity iron permeases (Fig. 3).

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Patients with diabetic ketoacidosis have elevated levels of available serum iron, likely due to release of iron from binding proteins in the presence of acidosis [13]. Artis *et al.* showed that sera collected from patients with diabetic ketoacidosis supported growth of *R. oryzae* in the presence of acidic pH (7.3-6.88) but not in the presence of alkaline pH (7.78-8.38) [13]. Furthermore, adding exogenous iron to sera allowed *R. oryzae* to grow profusely at acidic conditions but not at pH 7.4. Finally, simulated acidotic conditions decreased the iron-binding capacity of sera collected from normal volunteers, suggesting that acidosis temporarily disrupts the capacity of transferrin to bind iron. Therefore, the increased susceptibility to mucormycosis of patients with diabetic ketoacidosis is likely due at least in part to an elevation in available serum iron during diabetic ketoacidosis, due to proton-mediated dissociation of iron from transferrin (Fig. 3).

B. Mechanisms and chemistry of iron assimilation in fungi

General concepts—Iron is present in two readily available ionization states, Fe^{2+} (ferrous) and Fe^{3+} (ferric). Because of its ability to exist in either of these two states, iron has the ability to donate and accept electrons, and therefore can participate in a wide variety of cellular oxidation-reduction reactions. However, the chemical properties of iron place two limitations on its cellular accumulation and utilization by microorganisms. First, the metal is mainly found in nature in an insoluble state, typically comprised of Fe^{3+} hydroxides [12]. The insolubility of Fe^{3+} hydroxides limits the ability of microorganisms to transport the iron intracellularly. Therefore, fungi have devised a variety of strategies to overcome this problem, as discussed below.

The second problem limiting iron utilization by fungi is that iron is potentially toxic because of its ability to catalyze the production of oxygen free radicals via the Fenton reaction [17] or the Haber-Weiss reaction [17]. Iron catalyzed production of oxygen free radicals leads to cellular injury by causing oxidative damage to a wide variety of cellular substrates [12]. Therefore, proper storage of excess iron is essential to prevent toxicity. For instance, soon after uptake, iron can be found in the ferrous form bound to polyphosphates in vacuoles of *S. cerevisiae* [18]. Alternatively, iron can be stored as part of iron-rich proteins (ferritins). To date, the only fungi identified that store iron in ferritins are members of the class *Zygomycetes* [19]. Three types of iron-rich proteins have been identified in *Zygomycetes*: 1) mycoferritin, which is closely related to the mammalians ferritins [12]; 2) bacterioferritin [20]; and 3) zygoferritin, which is unique to *Zygomycetes* [12]. Also, fungi can store iron as part of small proteins called siderophores, which specialize in obtaining iron from the environment [19]. This mechanism of storage is common among fungi belonging to the ascomycetes and basidiomycetes classes.

Iron uptake mechanisms—Three general mechanisms of iron uptake have been identified in fungi. These include: 1) a reductive iron uptake that involves reduction of the ferric form into the ferrous and subsequent transport by a permease [21-24]; 2) a siderophore permease that facilitates the uptake of siderophore-sequestered iron [25-27]; and 3) an uptake system for acquiring iron from haemin [28]. In the reductive system, fungi can use any of the following three methods to reduce ferric iron into the more soluble ferrous form: i) a low affinity iron reductase (*Km*, 40 μM) functions in iron-rich environments to reduce Fe^{3+} to Fe^{2+}. Subsequently, Fe^{2+} is likely transported into the cell by the action of the low affinity iron permease. This iron permease also transports other bivalents elements, such as calcium and magnesium [12]; ii) an iron regulated high-affinity ferric reductase (*Km,* 0.15 μM) that reduces Fe^{3+} into Fe^{2+} and operates in iron-depleted environments, such as those present in the host. Even in hosts predicted to have elevated available serum iron, such as patients with diabetic ketoacidosis, most iron remains bound to carrier molecules, and free serum iron would still be present in submicromolar concentrations that induce the high-

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affinity rather than the low-affinity uptake system. The produced Fe^{2+} is further oxidized into Fe^{3+} by the action of a membrane copper oxidase before being transported across the cell membrane by a high-affinity iron permease [12,29]. The oxidation of ferrous iron back into ferric form is considered necessary to introduce specificity to transporting only iron into the cell. The copper oxidase and high-affinity iron permease exists as a complex enzyme [29] and their expression, as well as the expression of the high-affinity reductases, is controlled by the transcriptional regulator *AFT1,* which functions in low concentrations of iron [30]; and iii) non-enzymatic reduction of Fe^{3+} and transport of Fe^{2+}. Phenolic compounds such as anthranilate and 3-hydroxyanthranilate are known to maintain a reduced environment to release and prolong the existence of Fe^{2+} at the fungal membrane until transport occurs [18]. However, the role of these compounds in solubilizing iron is considered to be limited compared to the enzymatic reduction processes.

The role of reductase/permease systems in *Zygomycetes* iron metabolism— We have cloned the high-affinity iron permease of *R. oryzae* and found the putative rFtr1p to have significant homology to known fungal high affinity iron permeases from *C. albicans* (46% identity) and *S. cerevisiae* (44% identity) [31]. As well, multiple regions of the predicted rFtr1p showed significant homology with putative transmembrane domains from *S. cerevisiae* [29] and *C. albicans FTR1* [24]. Importantly, the putative REGLE motif, in which the glutamic acid residue is believed to interact directly with iron [29], was conserved in the predicted protein sequences of *FTR1* from the three organisms and was embedded in a hydrophobic region. The *rFTR1* was expressed in iron-depleted and not in iron-rich media. This iron-regulated expression of *rFTR1* was also accompanied by an iron-regulated activity of ferric reductase that was induced or suppressed in low or high concentrations of iron, respectively (unpublished data). These data indicate that *rFTR1* is likely to act in concert with ferric reductase to supply the cell with iron under iron-depleted conditions. Finally, *rFTR1* restored the ability of an *ftr1* null mutant of *S. cerevisiae* to grow on iron-limited medium and to take up radiolabeled iron, whereas *S. cerevisiae* transformed with the empty vector did not [31]. Recently, we have established the in vivo expression of rFtr1p in *R. oryzae* hyphae (unpublished data) and inhibition of rFtr1p expression by RNA-interference reduced the virulence of *R. oryzae* in diabetic ketoacidosis mouse model [32].

The role of siderophores in *Zygomycetes* iron metabolism—Fungi can produce siderophores, which provide the cell with much needed iron by chelating ferric iron [27,33]. To acquire iron, fungi can utilize their own secreted siderophores, siderophores secreted by other organisms (xenosiderophores), or both [12,14,16]. Siderophores supply iron to the host cell by one of the following four mechanisms: 1) Direct transfer of iron across the plasma membrane without entrance of the siderophore into the cell. In this case the transfer of iron is not an enzymatic membrane-reductive event, but rather an exchange between the gathering siderophore and an internal storage compound [34]; 2) Direct transfer of iron without entrance of the siderophore into the cell after reducing the chelated Fe^{3+} into Fe^{2+} [16]. This method is common among fungi utilizing iron from xenosiderophores [16]; 3) A shuttle mechanism encompassing the uptake of the entire siderophore-iron complex into the cell. Once internalized, iron is released by a reductase or by direct ligand exchange in which the recipient siderophore becomes the storage compound and the gathering siderophore is released into the environment to capture more iron [19]; and 4) An esterase-reductase mechanism by which Fe^{3+} is released from ferric triacetylfusarinine C (a siderophore belonging to the hydroxamate family, the most common fungal siderophores) by breaking the ester bond following internalization of the iron-siderophore complex [35]. The released Fe^{3+} is reduced and stored while the siderophore excreted to capture another iron molecule.

Zygomycetes are known for secreting rhizoferrin, a siderophore that belongs to the polycarboxylate family [36]. This siderophore supplies *Rhizopus* with iron through a

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receptor-mediated, energy dependant process [16,36]. However, it is not currently known by which mechanism of uptake this siderophore supplies the organism with iron. What is known is that rhizoferrin is inefficient in obtaining iron from the serum [14,16], and therefore the contribution of the organism's endogenous siderophores to its virulence is likely minimal. Additionally, because of their antigenic properties, siderophores may not be effective iron scavengers in the host since they elicit an immune response [37].

Rhizopus can also utilize siderophores secreted by other organisms as xenosiderophores in their quest for iron. A prime example of the use of xenosiderophores by *Rhizopus* is provided by the clinical experience with the bacterial siderophore, deferoxamine [14]. In contrast to rhizoferrin, *in vitro* studies utilizing radiolabeled deferoxamine in serum demonstrated that *R. oryzae* efficiently liberates ferric iron from deferoxamine extracellularly before taking up the iron. This step is an energy-dependent, and requires the reduction of Fe^{3+} to Fe^{2+} prior to transporting the iron intracellularly, suggesting the involvement of the reductase/permease system [16].

The role of haemin utilization in *Zygomycetes* iron metabolism—*C. albicans* [28] and *Histoplasma capsulatum* [38] can utilize haemin as a source of iron. Haemin uptake kinetics in *C. albicans* have demonstrated two phases: a rapid phase of haemin binding followed by a slower uptake phase, both of which were induced in iron-depleted conditions [28]. The putative *C. albicans* haem oxygenase gene (*CaHMX1*) was required for iron assimilation from haemin and its expression was induced in iron deprived conditions, by haemin, and by a shift from 30 to 37°C. However, *CaHMX1* was not involved in the uptake of haemin since a *Cahmx1* null mutant was able to take up haemin similar to the wild-type. Finally, the three different iron uptake systems in *C. albicans* (reductive, siderophore and haemin) are regulated independently from each other, emphasizing the independence of the haemin uptake system. The *Rhizopus* genome project revealed two homologues (RO3G_07326 and RO3G_13316) of the *CaHMX1*. These two *R. oryzae* homologues may provide a means for invasive *R. oryzae* to obtain iron from host hemoglobin. Since iron is usually present in abundance in the human blood, the presence of these homologues might explain the angioinvasive nature of *R. oryzae*.

A potentially novel mode of iron acquisition by *Zygomycetes*—Another method of iron acquisition in fungi includes the acidification of the environment when the fungi are grown under anaerobic conditions. For example, under acidic conditions, *S. cerevisiae* [18] and *Neurospora crassa* [12] can accumulate iron at the cell surface and mobilize the iron by excreted hydroxy acids, such as citric acid, to transport iron intracellularly. This method might have *in vivo* relevance for *Zygomycetes,* especially in diabetic ketoacidotic patients, however, to date, no exploration of the role of such a mechanism in *Zygomycetes* iron acquisition has been undertaken.

V. Preliminary evidence of the benefit of iron chelation therapy for mucormycosis

The central role of iron metabolism in the pathogenesis of mucormycosis suggests the possibility of utilizing effective iron chelators as adjunctive antifungal therapy. In fact, in addition to deferoxamine, other experimental iron chelators have been studied *in vitro* against *R. oryzae* [15]. In contrast to deferoxamine, these other iron chelators did not allow the organism to take up iron, and did not support its growth *in vitro* in the presence of iron (Table 1) [15].

Furthermore, while deferoxamine significantly worsened disseminated *R. oryzae* infection in guinea pigs, one of the other chelators had no impact on *in vivo* infection and the other

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chelator more than doubled the mean survival time of infected guinea pigs [15]. This latter agent, deferiprone, is approved for clinical use as an iron chelator in Europe and India, and is available on a compassionate use basis for iron overload in the United States. We have confirmed the ability of deferiprone to inhibit growth of *Zygomycetes* in vitro (Table 1) and confirmed its efficacy in our diabetic-ketoacidotic murine model of *R. oryzae* infection [39]. Further, in 2005, deferasirox became the first orally bioavailable iron chelator approved for use by the United States (US) Food and Drug Administration (FDA), with an indication for treatment of transfusion-dependent iron overload. We found deferasirox to be effective at chelating iron from *R. oryzae* and demonstrated cidal activity in vitro against *Zygomycetes* at concentrations well below clinically achievable serum levels (Table 1). Additionally, deferasirox significantly improved survival of diabetic ketoacidotic or neutropenic mice with mucormycosis, with efficacy comparable to that of liposomal amphotericin B. Most importantly, deferasirox synergistically improved survival and reduced tissue fungal burden when combined with liposomal amphotericin B [40]. A recent study using *Drosophila melanogaster* as a model host also demonstrated that deferasirox significantly protected wild-type flies infected with *R. oryzae* when compared with placebo-treated flies [41]. Finally, deferasirox was recently successfully used as a salvage therapy to treat a patient with rhinocerebral mucormycosis who was failing months of polyene treatment [42]. A phase II clinical trail to determine the safety and efficacy of using deferasirox in combination with liposomal amphotericin B is currently underway.

VI. Conclusion

Mucormycosis is an increasingly common infection in immunocompromised patients, and the morality with standard therapy remains unacceptably high. The agents of mucormycosis are uniquely susceptible to variations in environmental iron concentrations. Therefore, abrogation of iron acquisition by the agents of mucormycosis is a promising therapeutic strategy to impact clinical outcomes. Iron chelators that cannot be utilized to supply *Zygomycetes* with iron have efficacy in treating experimental mucormycosis and at least one of them (i.e. deferasirox) has been successfully used in a salvage therapy for a case of rhinocerebral mucormycosis. Given the fact that deferasirox is FDA approved and deferiprone is approved for clinical use in Europe and India, further development of these agents, as well as other novel iron chelators, is warranted as adjunctive therapy for mucormycosis.

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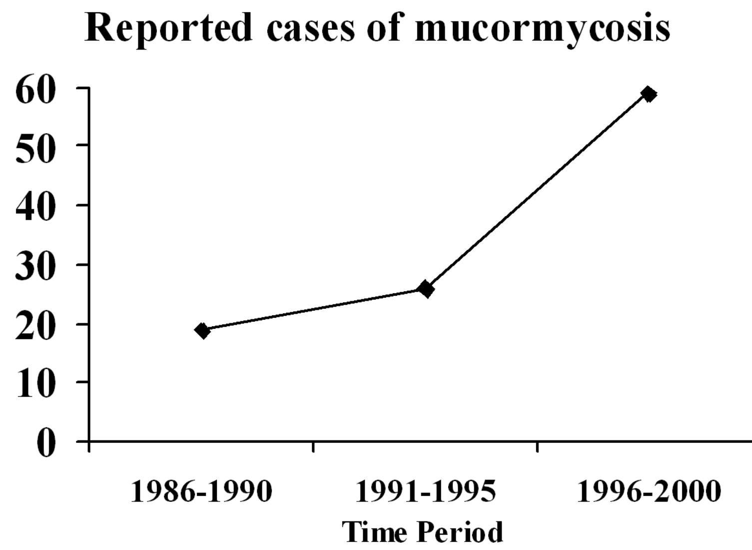

Figure 1. Increasing frequency of mucormycosis

Data adapted from [10].

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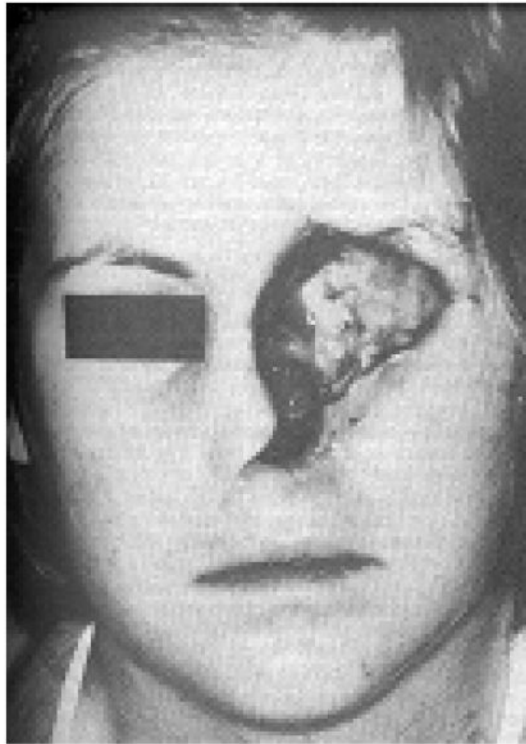

Figure 2.
Photograph of a teen patient who recovered from rhinocerebral mucormycosis but was left with a facial defect.

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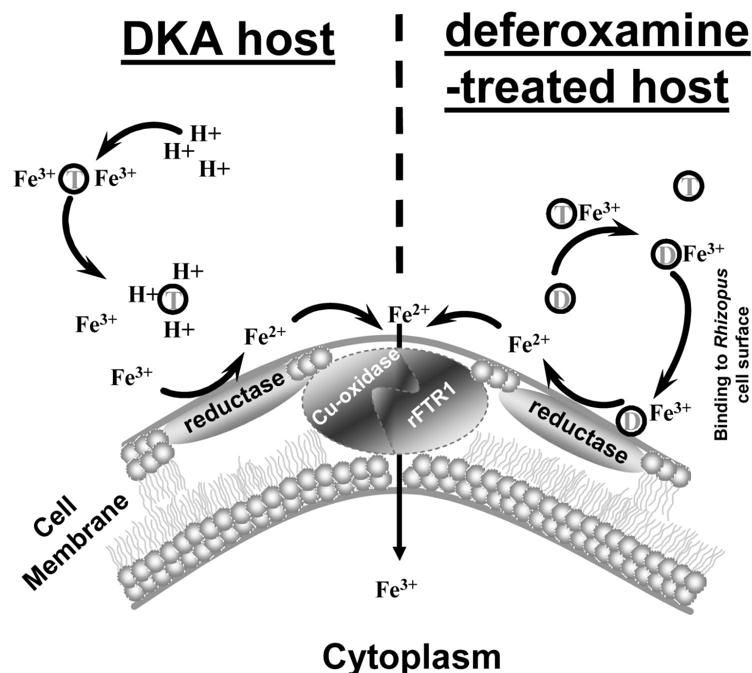

Figure 3. Proposed mechanisms of iron assimilation by *Zygomycetes* in conditions of elevated available serum iron

In patients in diabetic ketoacidosis (DKA), low pH conditions cause proton-mediated displacement of ferric iron (Fe^{3+}) from serum carrier molecules, including transferrin (T). Ferric iron is then reduced at the cell surface to ferrous iron (Fe^{2+}). In contrast, deferoxamine (D) directly chelates iron from transferrin, resulting in ferroxamine (iron-deferoxamine complex). Ferroxamine then binds to unidentified receptor(s) on the surface of *Zygomycetes*. The fungus then liberates the iron from ferroxamine by reduction at the cell surface, solubilizing ferrous iron from ferroxamine. In both cases, ferrous iron is then reoxidized back to ferric iron by copper oxidase (Cu-oxidase). High affinity iron permease (*rFTR1*), which physically complexes with copper oxidase in yeast, transports ferric iron nearly simultaneously to the oxidation step. Note that the oxidation to ferric iron prior to transport introduces specificity to iron transport by electrochemically separating the trivalent ferric iron from other divalent cations.

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Table 1

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of iron chelators against zygomycetes (adapted from [39,40]).

Iron Chelator	24 hours		48 hours	
	MIC (μg/ml)	MFC (μg/ml)	MIC (μg/ml)	MFC (μg/ml)
Deferiprone	3.12	100	6.25	6.25
Deferasirox	3.12-12.5	3.12-12.5	3.12-6.25	3.12-6.25
Deferoxamine	>100	>100	>100	>100