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# Deferoxamine augments growth and pathogenicity of *Rhizopus*, while hydroxypyridinone chelators have no effect

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**Deferoxamine augments growth and pathogenicity of *Rhizopus*, while hydroxypyridinone chelators have no effect.** Deferoxamine (DFO), when used in dialysis patients, is a well recognized risk factor for the development of mucormycosis caused by *Rhizopus*. This study compares, both *in vivo* and *in vitro*, the effects produced on *Rhizopus* by DFO and by two chelators of the hydroxypyridinone class, L1 and CP94. Experimental systemic mucormycosis was induced in the guinea pig by an i.v. injection of two different strains of *Rhizopus*: *R. microsporus* and *R. arrhizus*. Concomitant i.p. administration of DFO for four days shortened animal survival ($P < 0.05$), whereas concomitant administration of either L1 or CP94 did not. *In vitro* radioiron uptake by *R. microsporus* was 100-fold higher from the $^{55}$ferric complex of DFO than of L1 or CP94. *In vitro* fungal growth was stimulated sevenfold by the ferric complex of DFO ($P < 0.0001$) but not significantly by the ferric complex of either L1 or CP94. These results indicate that the ferric complex of DFO but not that of L1 or CP94 specifically stimulates both the iron uptake and the growth of *Rhizopus*. They suggest that the risk of developing mucormycosis should be minimal with L1 or CP94, as opposed to DFO.

The most severe side effect of deferoxamine (DFO) therapy in dialysis patients is the development of mucormycosis, caused by *Rhizopus*. Recently, an international registry reported 46 dialysis patients who developed this DFO-associated fungal infection, which was fatal in 41 of 46 (89%) of the patients [1]. Apart from clinical data, other evidence linking DFO and mucormycosis has accumulated during the past few years. Experimentally induced mucormycosis is aggravated by the administration of DFO or its iron complex feroxamine (FO) [2–4]. Moreover, *Rhizopus* has been shown to take up radioiron from radiolabeled FO and to enhance its growth in the presence of FO [5].

New classes of iron or aluminum chelators, chemically unrelated to DFO, are in the process of intense investigation. Among these, the class of the 3-hydroxypyridin-4-ones has gained the greatest interest, the two most studied compounds being the 1, 2-dimethyl derivative (CP 20, better known as "L1" and recently named deferiprone) and the 1,2-diethyl derivative (CP 94) [6, 7].

We compared the effects of these new chelators with those of DFO upon *Rhizopus in vitro* as well as *in vivo*. This study shows that, in contrast to DFO, neither L1 nor CP94 stimulate *Rhizopus'* growth *in vitro* or aggravate experimental mucormycosis.

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## Methods

### *Fungal strains*

Two clinical strains of *Zygomycetes*, order *Mucorales*, family *Mucoraceae*, genus *Rhizopus* were studied. The first was *Rhizopus microsporus var. rhizopodiformis*, referred here in as *R. microsporus* (B51321, ATCC 66276), the second was *Rhizopus arrhizus* (B51322, ATCC 66275). Details on both strains have previously been reported [8]. The strains were lyophilized and maintained on Sabouraud dextrose agar (Difco, Detroit, Michigan, USA). Sporangiospores (spores) were prepared by growing the fungi on the same medium for 10 days. The mycelium was scraped in sterile deionized water (Milli Q, Millipore, Bedford, Massachusetts, USA); the sporulated mycelial suspension was vigorously agitated for 10 minutes in the presence of glass beads (mean diameter of ca. 2 mm) and then filtered through gauze. The eluate containing spores was washed twice with water and concentrated by centrifugation to obtain a suspension free of hyphal fragments which contained about $10^7$ spores/ml, as estimated with a Bürker hemocytometer. Spores were stored at 4°C in the presence of penicillin and streptomycin at 30 and 70 μg/ml, respectively [9].

### *Chelators*

DFO was the commercially available Desferal$^R$ (Ciba Geigy, Basel, Switzerland). FO or $^{55}$FO were prepared as previously described [10] by mixing stoichiometrically DFO with $FeCl_3$ or $^{55}FeCl_3$, respectively (Amersham Intern., Buckinghamshire, UK). L1 (1,2-dimethyl-3-hydroxypiridin-4-one) and CP94 (1,2-diethyl-3-hydroxypyridin-4-one) were provided by Dr. M. Stockham (British Technology Group, London, UK) and by Dr. J. Yi (Ciba Geigy, Basel, Switzerland). Ferric complexes were prepared by mixing L1 and CP94, respectively, with $^{55}FeCl_3$, with a stoichiometry of 3:1 (ligand:iron).

### *In vivo experiments*

*Animals.* Normal, non-predisposed male Albino guinea pigs (Pirbright strain) weighing 500 ± 50 g were infected i.v. with 293 colony forming units (CFU) of *R. microsporus*/g body wt and

with 3,550 CFU of *R. arrhizus*/g body wt. Details on these fungal inocula have previously been reported [2].

*Experimental protocol.* For each of the two fungal strains, five experimental groups were studied, each consisting of six animals. All 60 animals were infected. Those of group 1 remained untreated, while those of groups 2 to 5 received four i.p. injections (1 ml) of either saline (group 2), DFO (group 3), L1 (group 4) or CP94 (group 5). Injections were given on four consecutive days (day −1 to day +2, with reference to the infection). All three chelators were dissolved in saline and dosed at 50 mg/kg body wt. In three more experimental groups, each of the three studied chelators was administered at the same dosage to non-infected animals.

*Assessment.* Animals were observed daily; survival was recorded up to day 28. At autopsy, cultures from eight organs per animal (lung, liver, spleen, left kidney, brain, heart, left eye and skin of the back) were performed on Sabouraud agar at 37°C.

### *In vitro experiments*

*Fungal growth and fungal iron uptake.* Experiments were carried out as recently reported [5]. The growth of *R. microsporus* was studied by a turbidimetric determination at 400 nm [11], performed after cultivation in BDM (basal defined medium), a synthetic medium designed for mammalian cell cultivation, having an iron content of 1.36 μM [12]. $10^6$ spores were incubated in 2.1 ml of medium containing 40% human serum, in the presence or absence of ferric complexes of the three chelators at 1 μM. After 24 hours at 37°C, fungal elements were centrifuged and washed. Pellets were harvested and analyzed.

*Iron transfer.* $^{55}$Fe-radiolabeled $Fe(L1)_3$ or FO at 30 μM were added to serum from healthy volunteers and separated by agarose gel electrophoresis (Ciba Corning, Palo Alto, California, USA), run in 0.05 M veronal buffer, pH 8.6. The serum had following characteristics: iron content 16.7 μM; transferrin 2.40 mg/ml, (30 μM); and iron saturation of transferrin 24%. After electrophoresis, the gel was dried; autoradiography was carried out with Hyperfilm™-β max (Amersham Int., Buckinghamshire, UK).

### *Statistical analysis*

Mean values are given together with SEM. Statistical significance between study groups was determined by the two-tailed probability Mann-Whitney U test (comparison of survival) or by the one-factor ANOVA test.

## Results

### *In vivo*

Table 1 compares the effects of L1 and CP94 versus DFO upon the course of experimental mucormycosis caused by *R. microsporus* and *R. arrhizus*, respectively. The results obtained with these two strains are similar. DFO (group 3) significantly shortened mean survival, from 5.0 ± 0.5 to 3.2 ± 0.2 days and from 7.8 ± 1.1 to 4.8 ± 0.3 days, respectively ($P < 0.05$). In contrast, neither L1 nor CP94 (groups 4 and 5) exerted a significant effect when compared to saline. The effect of DFO upon survival of the infected animals was significantly different from that of either L1 or CP94. The animals died from disseminated mucormycosis, as indicated by positive fungal cultures obtained in 91 to 100% of the organs cultured within each experimental group.

**Table 1.** Effect of different chelators on the outcome of experimental mucormycosis

| Treatment | Survival (days, mean ± SEM) | $P^a$ |
|---|---|---|
| *R. microsporus* | | |
| none | 4.0 ± 0.2 | |
| saline | 5.0 ± 0.5 | * |
| DFO[b] | 3.2 ± 0.2 | |
| L1[b] | 4.0 ± 0.2 | * ** |
| CP94[b] | 4.7 ± 0.3 | |
| *R. arrhizus* | | |
| none | 9.5 ± 0.7 | |
| saline | 7.8 ± 1.1 | * |
| DFO[b] | 4.8 ± 0.3 | |
| L1[b] | 16.2 ± 4.0 | * ** |
| CP94[b] | 8.7 ± 1.0 | |

[a] Mann-Whitney U test (two-tailed probability). * $P \leq 0.05$; ** $P \leq 0.01$

[b] 50 mg · $kg^{-1}$ of deferoxamine (DFO), the 1,2-dimethyl- and the 1,2-diethyl derivative of 3-hydroxypyridin-4-one ("L1" and "CP94", respectively), administered i.p. in 1 ml saline for four consecutive days (days −1 to +2 of infection)

Non-infected animals, given either DFO, L1 or CP94 at 50 mg/kg for four consecutive days, all remained alive until the end of the study period (28 days).

### *In vitro*

Figure 1A shows that 1 μM FO significantly stimulated the fungal growth of *R. microsporus* in BDM containing 40% human serum (7.3-fold increase, $P < 0.0001$). In contrast, equimolar ferric complexes of L1 or CP94 did not significantly modify the fungal growth rate. Phase contrast microscopy confirmed the effect of the three ferri-chelators on *Rhizopus* growth. When added to 40% human serum, which inhibits fungal growth, FO strongly stimulated the growth of *Rhizopus*, whereas $Fe(L1)_3$ or $Fe(CP94)_3$ only minimally affected its growth (not shown).

Fungal radioiron uptake from the $^{55}$Fe complex of the three chelators was compared in Figure 1B. *R. microsporus* accumulated about 100-fold more radioiron when bound to DFO (1,131 ± 63 pmol/$10^6$ fungal particles) than when bound to L1 or CP94 (13 ± 1 and 11 ± 3 pmol/$10^6$ fungal particles, respectively). The *in vitro* effects of DFO, both upon fungal growth and upon fungal iron accumulation, were significantly different from the effects observed for L1 and CP94.

Autoradiography of an agarose gel showed that, in the presence of human serum, nearly 50% of the radioiron was released from $Fe(L1)_3$, but not from FO to apotransferrin (Fig. 2).

## Discussion

The present results confirm that DFO significantly aggravates mucormycosis, induced in non-predisposed guinea pigs by the i.v. injection of spores from two different species of *Rhizopus*. The degree of shortening of animal survival induced by DFO is similar to that previously reported, indicating consistency in the model of experimental infection used as well as in the drug's

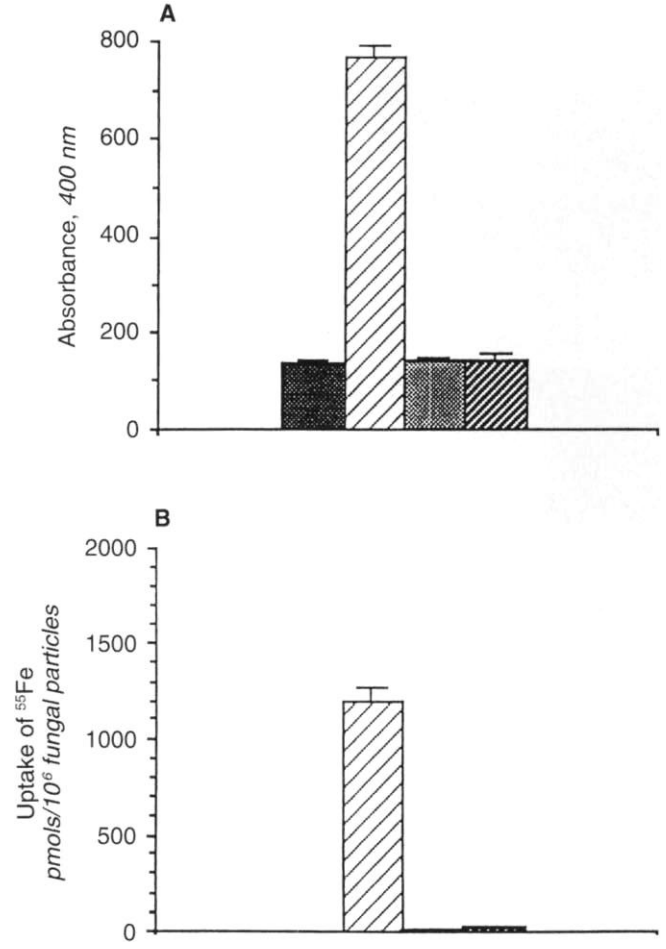


**Fig. 1.** *Growth (A), measured by turbidimetry, and radioiron uptake (B) of* Rhizopus, *cultured for 24 hours in BDM with 40% human serum alone (control) or supplemented with the iron complex of deferoxamine and the iron complex of the 1,2-dimethyl- and of the 1,2-diethyl derivative of 3-hydroxypyridin-4-one ("L1" and "CP94"), respectively.* Symbols are: (in A) (▩) BDM + 40% serum (control); (▨) + FO; (▩) + $Fe(L1)_3$; (▨) + $Fe(CP94)_3$; and in (B) ( ) $^{55}FO$; (▩) $^{55}Fe(L1)_3$; (▨) $^{55}Fe(CP94)_3$.

effect [2]. Abe et al reported similar results in mice; all mice pretreated with DFO or FO died within five days after i.v. inoculation of *R. arrhizus*, whereas the mortality in the infected but untreated animals was only 20% through the three-week experimental period [3]. More recently, DFO was successfully used to produce rhinocerebral mucormycosis in mice which were challenged intraethmoidally with *R. arrhizus* [4].

During the last few years, the mechanism of this effect of DFO on *Rhizopus* has been investigated. When challenged to iron limitation, *Rhizopus* does not synthesize DFO but an unrelated siderophore, called rhizoferrin, consisting of two citric acid and one diaminobutane residues [13]. Nevertheless, *Rhizopus* can mobilize iron presented by the exogenous hydroxamate siderophore FO. Figure 1B confirms the intense fungal accumulation of $^{55}Fe$ from 1 μM $^{55}FO$. Even at a $^{55}FO$ concentration as low as 0.01 μM, $^{55}Fe$ uptake occurred, leading to disruption of serum fungistasis and to stimulation of the *in vitro* growth of *Rhizopus* in a serum-containing medium [5, 14].

Figure 1A shows the significant enhancement of the *in vitro* growth of *Rhizopus* induced by the addition of 1 μM FO. This siderophore-mediated iron uptake and growth stimulation is considered to play a key role in the aggravation of experimental mucormycosis by DFO as well as in the pathogenesis of DFO-mediated human mucormycosis [5].

How specific is this interaction between *Rhizopus* and FO? In a previous study, we compared the effect of FO upon three different fungal genera and found that FO had a greater effect on *Rhizopus* than on the two other studied species, that is, radioiron uptake from $^{55}FO$ by *R. microsporus* was eightfold and 40-fold greater than that by *Aspergillus fumigatus* and by *Candida albicans*, respectively [5].

In the present study, the question on the specificity of the interaction between *Rhizopus* and FO was addressed at the chelator level: how specific to FO is the siderophore effect on *Rhizopus*? For comparison, two compounds of the 3-hydroxypyridin-4-one class were chosen for several reasons. First, this class of chelators is chemically unrelated to hydroxamate siderophores, such as DFO. Second, L1 has been investigated as a chelator of iron both in cell culture and in animal studies, and it has been administered orally to several hundreds of patients with iron overload with encouraging results [7, 15, 16]. Third, some preliminary evidence in rats indicates that L1 is also an effective chelator of aluminum, as L1 was comparable to DFO in its effect on aluminum removal from the bone in uremic rats with aluminum intoxication [17]. Furthermore, a pilot study suggests that the aluminum complex of L1 and its glucoronide conjugate are readily removed by both hemo- and peritoneal dialysis in patients [18]. CP94, being possibly a more efficacious iron chelator than L1 [19], was also included in the present study.

*In vitro* radioiron uptake by *Rhizopus* from the $^{55}$ferric complex of L1 or CP94 is ± 100-fold lower than from equimolar $^{55}FO$ (Fig. 1B). Correspondingly, iron-bound L1 or CP94 at 1 μM does not significantly influence the *in vitro* growth rate of *Rhizopus* in a serum-containing culture medium. This is in sharp contrast with equimolar FO, which increases fungal growth 7.3-fold (Fig. 1A). The low rate of fungal iron uptake from ferric L1 or CP94 could possibly be explained by the release of iron from these chelators to apotransferrin, as observed by autoradiography (Fig. 2) and as reported by others [20].

This different *in vitro* handling by *Rhizopus* of hydroxypyridinones compared to DFO results in different chelator effects on experimental mucormycosis. Neither L1 nor CP94 influenced the course of this infection, whether induced by *R. microsporus* or by *R. arrhizus*. The effect of both chelators is therefore significantly different from the observed DFO effect. The lack of effect of L1 or CP94 cannot be attributed to underdosing, as the dosage used yielded a 1.5- and 1.3-fold higher molar concentration, respectively, than for DFO when compared on an iron-binding equivalence base (1:1 for the DFO-iron complex and 3:1 ligand-iron complex for L1 and CP94). Although the hydroxypyridinone chelators are primarily designed for oral use [6, 15, 16], it is highly unlikely that the absence of effect on mycormycosis should be due to the i.p. route used, as the effectiveness (degree of iron mobilization) of L1 and CP94 by the i.p. and by the oral route are reported to be identical [21].

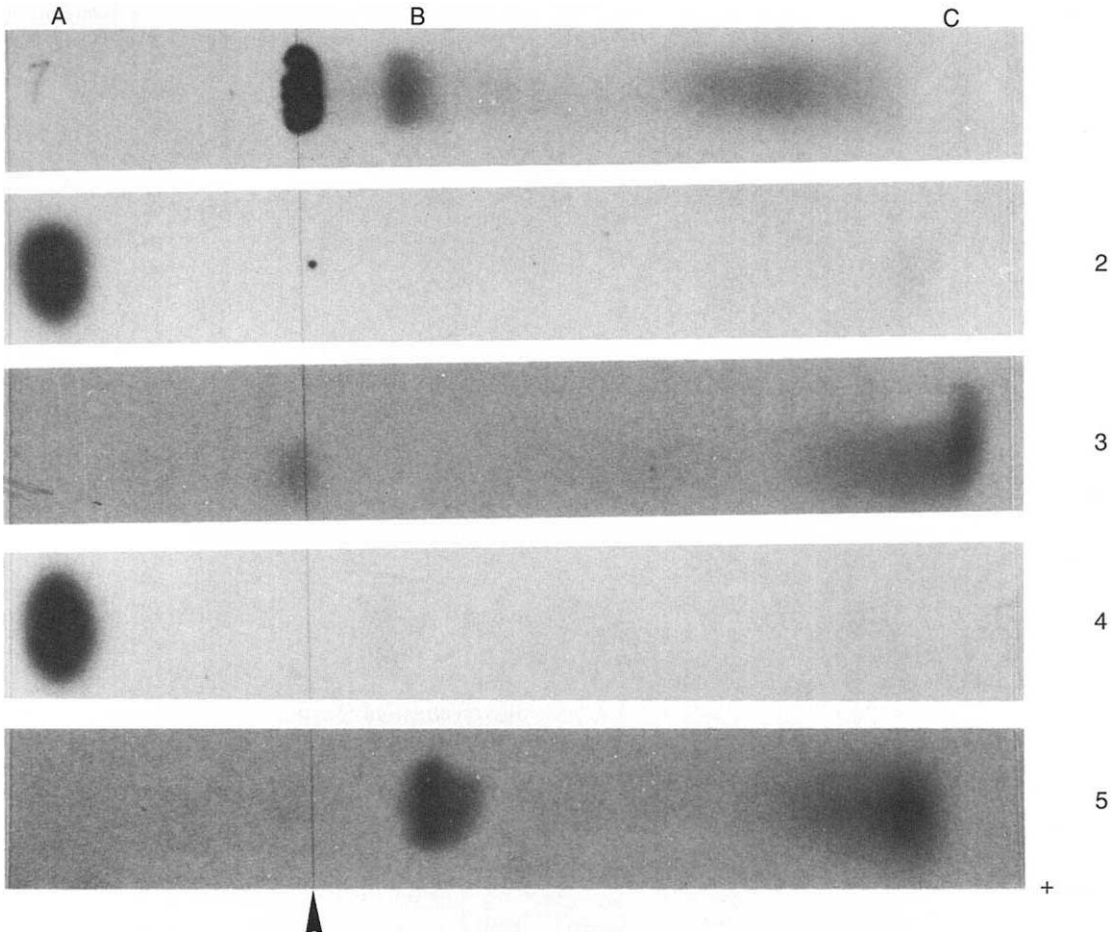


**Fig. 2.** *Autoradiography of agarose gel with $^{55}$Fe-radiolabeled human transferrin alone [1], FO alone [2], Fe(L1)$_3$ alone [3] and with either 30 μM FO with human serum [4] or 30 μM Fe(L1)$_3$ with human serum [5].* Specific migration bands are shown: A for FO, B for transferrin, C for Fe(L1)$_3$. The starting line is indicated by an arrow.

The present study was not designed to assess the potential usefulness of L1 or CP94 in replacement of DFO in the management of the aluminum- or iron overloaded dialysis patient. The effectiveness of L1, given cumulatively to more than 450 non-uremic patients with iron overload, is well documented. The main concern is the occurrence of transient agranulocytosis in 5 of the 450 patients [22]. The long-term use of L1 as chelator of aluminum in uremic patients has not been reported yet. However, if the drug proves to be effective and safe in this patient population, our study indicates that *Rhizopus* handles L1 and CP94 differently from DFO and it suggests that the risk of developing mucormycosis should be minimal with L1 or CP94, in contrast with DFO. It also shows that *in vitro* studies on the relationship between chelators and *Rhizopus* can help predict *in vivo* consequences. The same has been found for other microorganisms [23]. As DFO remains at present the standard drug in clinical chelation therapy, guidelines restricting the use of DFO and lowering its dosage in dialysis patients should be applied in the hope of lessening the risk of mucormycosis [24]. However, it remains to be proved that using the lower DFO dosage proposed at a recent consensus conference [24] will be effective in reducing the risk of mucormycosis.

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