Iron Restriction to Clinical Isolates of *Candida albicans* by the Novel Chelator DIBI Inhibits Growth and Increases Sensitivity to Azoles *In Vitro* and *In Vivo* in a Murine Model of Experimental Vaginitis

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ABSTRACT *Candida albicans* is an important opportunistic pathogen causing various human infections that are often treated with azole antifungals. The U.S. CDC now regards developing candidal antifungal resistance as a threat, creating a need for new and more effective antifungal treatments. Iron is an essential nutrient for all living cells, and there is growing evidence that interference with iron homeostasis of *C. albicans* can improve its response to antifungals. This study was aimed at establishing whether withholding iron by currently used medical iron chelators and the novel chelator DIBI could restrict growth and also enhance the activity of azoles against clinical isolates of *C. albicans*. DIBI, but not deferoxamine or deferiprone, inhibited the growth of *C. albicans* at relatively low concentrations *in vitro*, and this inhibition was reversed by iron addition. DIBI in combination with various azoles demonstrated stronger growth inhibition than the azoles alone and greatly prolonged the inhibition of cell multiplication. In addition, the administration of DIBI along with fluconazole (FLC) to mice inoculated with an FLC-sensitive isolate in a model of experimental *C. albicans* vaginitis showed a markedly improved clearance of infection. These results suggest that iron chelation by DIBI has the potential to enhance azole efficacy for the treatment of candidiasis.

KEYWORDS *Candida albicans*, DIBI, antifungal agents, azoles, iron, synergy

*C*andida spp. are commonly associated with health care-associated infections in the United States as well as worldwide (1). An estimated 46,000 *Candida* infections occur among hospitalized patients in the United States each year, and of the more than 20 species of *Candida* yeasts that can cause infection in humans, the most common is *C. albicans* (2). As *Candida* typically inhabits the skin and mucosa, candidiasis is often superficial in nature (3); these infections are incredibly common affecting millions of people worldwide (4). While this is rarely life threatening, it can significantly impact a patient's quality of life (4, 5) and may spread to other individuals or become invasive (4). Vulvovaginal candidiasis (VVC) is the most common gynecological infection in healthy women (4), as 70% to 75% of women will experience this mucosal fungal infection at least once during their lives (6), and VVC can become recurrent due in part to azole resistance (7). With the limited range of available antifungal drug classes (5, 8) and growing incidence of azole resistance (7, 9–11), agents that might enhance or broaden the efficacies of the azole antifungals would be attractive.

While *C. albicans* colonizes the skin and mucosal surfaces of healthy individuals,
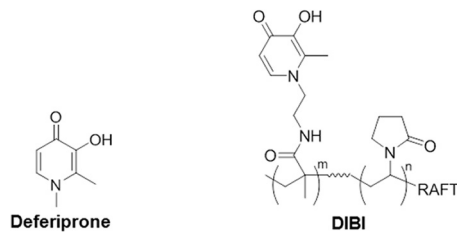
FIG 1 Structures of deferiprone and DIBI. Deferiprone is a hydroxypyridinone iron chelator (molecular mass, 139 Da) with similar chelating functionality to DIBI, an iron-chelating polymer (molecular mass, 9 kDa) containing 9 hydroxypyridinone groups per molecule.

predisposing host factors are implicated in infection (12). It is becoming increasingly clear that the innate immune response is an important host defense against *Candida* infection (13), with iron availability and sequestration being important components of susceptibility and immunity (14–16). Interestingly, estrogen administration has been shown to increase iron availability in the host (17–19), with a correspondingly increased susceptibility to infection (17, 19). Estrogen administration is also a requirement to predispose mice to experimental candidal vaginitis (7, 20).

Iron acquisition mechanisms of *C. albicans* have also been related to its virulence (21, 22). Iron is an irreplaceable essential nutrient for all pathogenic bacteria and fungi, as it is integral for the activity of various key enzyme systems of metabolism, repair, and defense (23). Therefore, targeting iron acquisition provides a potential basis for a new approach to antimicrobials (24). Interference with microbial iron nutrition *in vivo* might also provide an enhancement of the activities of antifungal agents. In this regard, daughter cells from *C. albicans* iron-limited biofilms have been shown to be more sensitive to amphotericin B (25). Additionally, lactoferrin, a key iron-withholding defense protein of vertebrate animals and essential for restricting iron availability to pathogens *in vivo* (26), has been shown to improve the *in vitro* efficacies of various antifungal agents, including fluconazole (FLC) (27, 28).

The elucidation of the roles of iron supply and iron restriction in enhancing FLC and other azole activities might be aided by the use of iron-specific chelators that can withdraw and deny nutritional iron from *C. albicans*. DIBI, recently patented by Chelation Partners Incorporated (29), is a high-affinity iron chelator which has been found to provide strong iron-specific growth inhibition of *C. albicans* (30). DIBI is the developmental code name for a new hydroxypyridinone functional iron-chelating polymer shown in Fig. 1. DIBI has chemical similarities to clinically used deferiprone as shown, but DIBI provides a platform on which adjacent pyridinone groups can together bind iron with a higher avidity than that provided by deferiprone (M. T. C. Ang, R. Gumbau-Brisa, D. S. Allan, R. McDonald, M. J. Ferguson, B. E. Holbein, and M. Bierenstiel, submitted for publication). In this study, we investigated the influence of iron withdrawal by DIBI on the activity of azole antifungals with *C. albicans* clinical isolates *in vitro* and *in vivo*.

RESULTS

Effect of DIBI on *C. albicans* growth. DIBI inhibited *C. albicans* growth and was superior to the other chelators tested. The MIC$_{80}$ (80% reduction) values for 3 iron chelators were determined for 3 strains of *C. albicans* (Table 1). MICs were read after 24 h

TABLE 1 MICs of iron chelators deferoxamine, deferiprone, and DIBI against 3 strains of *C. albicans*

C. albicans strain	MIC (μg/ml)[a]		
	Deferoxamine	Deferiprone	DIBI
SC5314	>1,280	160	2
Ca3969	>1,280	80	2
Ca5031	>1,280	80	2

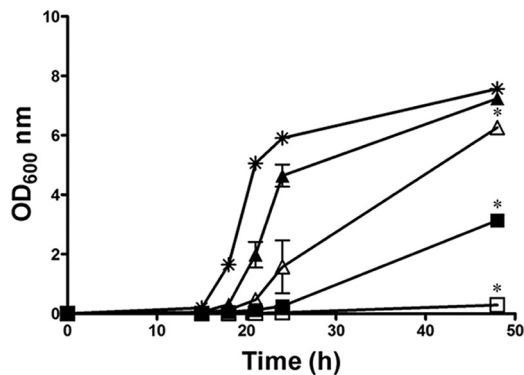[a]Values reported are 48-h results from 4 replicates within 2 independent experiments.

FIG 2 DIBI growth inhibition of *C. albicans* is iron reversible. RPMI (✳), RPMI with DIBI at 1 (△) or 10 μg/ml (□), and RPMI supplemented with 1 μM iron with DIBI at 1 (▲) or 10 μg/ml (■) were inoculated with *C. albicans* strain Ca3969. Growth was measured as OD at 600 nm over 48 h. Data points are means ± SEMs from 2 independent experiments. *, $P < 0.05$ for DIBI 1 or 10 μg/ml or RPMI plus iron plus DIBI 10 μg/ml versus RPMI, for DIBI 1 μg/ml versus DIBI 10 μg/ml, for DIBI 1 μg/ml versus RPMI plus iron plus DIBI 1 μg/ml, and for DIBI 10 μg/ml versus RPMI plus iron plus DIBI 10 μg/ml.

(data not shown) and 48 h to examine prolonged inhibition from iron sequestration. While deferoxamine was noninhibitory at the concentrations tested, deferiprone and DIBI both inhibited the growth of these strains. However, deferiprone required 80 to 160 μg/ml for growth inhibition at 48 h, while DIBI was inhibitory at 2 μg/ml. These MICs corresponded to 0.57 to 1.15 mM for deferiprone and only 0.02 μM for DIBI.

Iron dependence of DIBI inhibition. DIBI provided dose-dependent and iron-reversible growth inhibition when tested against both a reference strain (SC5314, data not shown) and a clinical strain (Ca3969) (Fig. 2). The growth of *C. albicans* over 48 h was suppressed by DIBI addition compared to that in the control medium. This repression was dose dependent, as the inhibitory effect of DIBI at 10 μg/ml was greater than that observed by the addition of 1 μg/ml and persisted until 48 h. DIBI inhibition of growth was also reversed directly by iron addition, and the extent of reversal was iron concentration dependent (Fig. 2).

Improvement of azole activity with DIBI. Azole MICs, including MIC_{80} and MIC_{50} values, for several *C. albicans* strains were determined in RPMI medium (Table 2). The majority of the strains shared similar sensitivity profiles: FLC sensitive (MIC_{50} = 0.12 to 0.25 μg/ml, MIC_{80} = 0.5 to 1 μg/ml) and susceptible or susceptible-dose dependent for itraconazole ([ITC] MIC_{50} = 0.03 to 0.06 μg/ml, MIC_{80} = 0.25 to 0.5 μg/ml). In contrast, strain LP1158-07 was resistant to both azoles (FLC, MIC_{80} and MIC_{50} > 8 μg/ml; ITC, MIC_{80} and MIC_{50} ≥ 1 μg/ml). In regard to clotrimazole (CLT), although there are no definitive interpretive breakpoints (31), most strains shared similar profiles (MIC_{50} < 0.005 μg/ml, MIC_{80} = 0.005 to 0.03 μg/ml), with MICs for strain LP1158-07 at 0.03 μg/ml (MIC_{50}) and 0.5 to 1 μg/ml (MIC_{80}).

Checkerboard assays were performed to assess DIBI synergy with FLC. For this investigation of two independent fungistatic agents, the more stringent MIC_{80} endpoint was used to more clearly interpret the interaction between the chelator and the azole. Furthermore, MIC_{80} was used for studies with DIBI alone. The results showed that

TABLE 2 Azole MICs against 5 strains of *C. albicans*

| *C. albicans* strain | MIC_{80} (MIC_{50}) (μg/ml)[a] | | |
	FLC	ITC	CLT
96113	0.5 (0.25)	0.5 (0.06)	0.01 (<0.005)
SC5314	0.5 (0.12)	0.5 (0.03)	0.03 (<0.005)
Ca3969	1 (0.25)	0.5 (0.06)	0.03 (<0.005)
Ca5031	0.5 (0.25)	0.25 (0.03)	0.005 (<0.005)
LP1158-07	>64 (16)	8 (1)	0.5–1 (0.03)

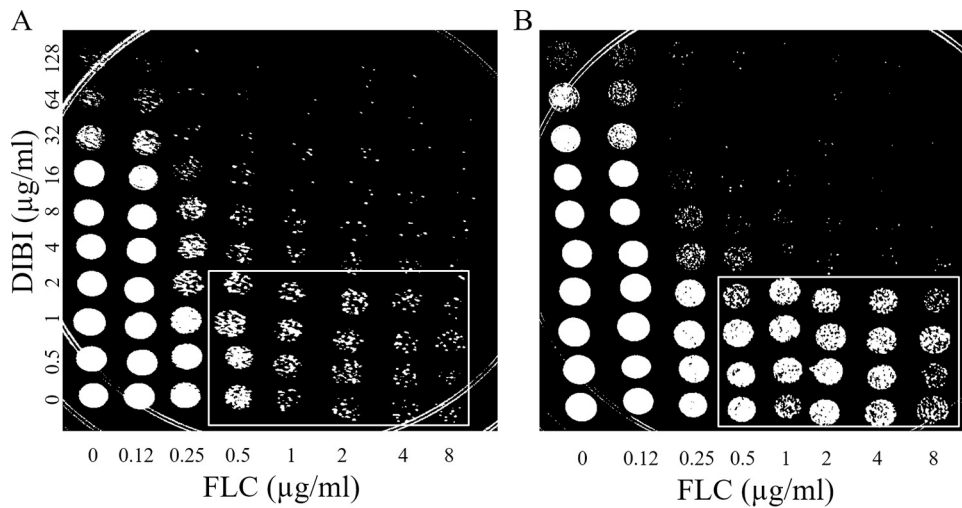[a]Values reported are from at least 2 independent experiments.

FIG 3 Recovery growth of *C. albicans* is inhibited after exposure to a combination of fluconazole (FLC) and DIBI. A 5-μl volume from each well of a checkerboard assay that had been inoculated with Ca5031 was spotted onto a YPD agar plate following 24-h (A) or 48-h (B) exposure to the agent. Recovery plates were incubated for 24 h before being visually inspected and photographed. Images are representative of results observed in 3 independent experiments.

DIBI was synergistic with FLC when tested against strain 96113, with fractional inhibitory concentration indices (FICIs) of 0.38 at 24 h and 0.3 to 0.38 at 48 h. In addition, spot plates were prepared from the wells on the basis of a method similar to that outlined by Fiori and Van Dijck (32). Recovery spots prepared from typical 24-h checkerboards (Fig. 3A) showed killing (reduced recovery of viable cells) for FLC alone at ≥0.5 μg/ml and with DIBI alone at ≥32 μg/ml. Increasing concentrations of DIBI together with FLC at 0.25 μg/ml resulted in decreasing recovery of viable CFU in a synergistic manner. Recovery spots prepared from 48-h checkerboard plates (Fig. 3B) indicated tolerance to FLC at 0.5 to 8 μg/ml with DIBI at 0 to 2 μg/ml, recoveries being greater from these wells than was observed for 24 h when comparing the outlined section in Fig. 3B to that in 3A. However, the combination of FLC at ≥0.25 μg/ml with DIBI at ≥4 μg/ml resulted in little to no recovery growth. Similar results were observed for spot plating of checkerboards testing Ca3969 with FLC, for both Ca5031 and Ca3969 with ITC, and via disk diffusion assays as a complementary visual assessment (data not shown).

The improvement of azole activity by DIBI was also assessed using a growth inhibition/kill type assay system as shown for four stains with FLC, ITC, or CLT (Fig. 4). Azole addition alone resulted in a partial growth inhibition of all strains, including the azole-resistant isolate LP1158-07. The DIBI concentrations tested provided either no (96113) or partial inhibition (all other strains) over 72 h. While the combination of DIBI with each of the azoles provided total growth inhibition for most strains tested (Fig. 4B to F), it resulted in a moderate fungicidal effect over 72 h for strain 96113 with FLC (Fig. 4A).

Improvement of FLC by DIBI *in vivo*. Experimental vaginitis in estrogenized mice was examined with FLC-sensitive strain 96113 (Fig. 5). The treatments included DIBI (40 mg/kg) alone administered topically on days 3 to 6 postinoculation, FLC (25 mg/kg) alone administered by oral gavage on days 3 and 5 postinoculation, and DIBI plus FLC administered similarly. Vaginal fungal burden was evaluated on day 7 postinoculation. DIBI alone had no effect on vaginal fungal burden. The organism burden was highly variable in the FLC-treated animals but was significantly reduced compared to that of the vehicle control ($P < 0.0001$). The addition of DIBI with FLC did not reduce the fungal burden further when statistically compared to that of FLC-treated mice but did enhance the rate of clearance (39% of treated mice) compared to that with FLC alone (10% of treated mice) ($P < 0.007$). Higher concentrations of DIBI (80 mg/kg) had no additional effects (data not shown). The overall results were supported by the qualitative scoring
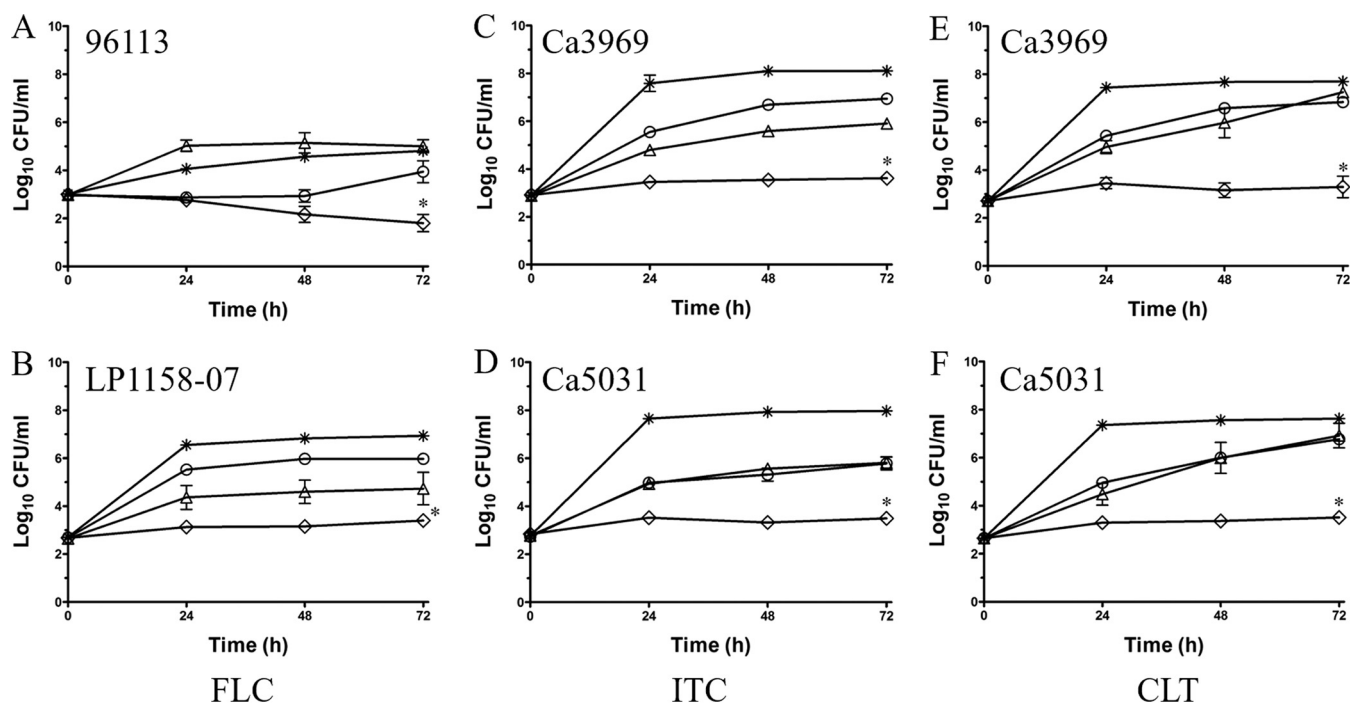
FIG 4 Long-term growth inhibition of *C. albicans* by combinations of DIBI with fluconazole (FLC), itraconazole (ITC), and clotrimazole (CLT). RPMI (✳), RPMI with DIBI alone (△), RPMI with azole alone (○), and RPMI with DIBI and azole combined (◇) were inoculated with azole-susceptible strains (96113, Ca3969, Ca5031) or the FLC-resistant isolate (LP1158-07), as indicated. (A) 1 μg/ml DIBI, 1 μg/ml FLC; (B) 10 μg/ml DIBI, 128 μg/ml FLC; (C) 10 μg/ml DIBI, 0.5 μg/ml ITC; (D) 10 μg/ml DIBI, 0.25 μg/ml ITC; (E and F) 10 μg/ml DIBI, 0.03 μg/ml CLT. Viable cell counts were determined by plate counting over 72 h. Data points are means ± SEMs from 3 independent experiments. *, $P < 0.01$ for DIBI plus azole versus RPMI, DIBI alone, or azole alone.

of lavage fluid for yeast/hyphae of individual animals; cleared animals had no visible organisms present (score, 0), FLC or DIBI-plus-FLC-treated animals had scores ranging from 1 to 4, and vehicle- and DIBI-treated animals had scores ranging from 3 to 4. In other experiments, the FLC-resistant strain LP1158-07 was similarly tested. The results showed no effect of FLC alone at 25 or 50 mg/kg or in combination with DIBI at 40 mg/kg (data not shown).

DISCUSSION

This study investigated the roles that iron supply and its *in situ* sequestration by iron chelation have in relation to growth inhibition and azole sensitivity of *C. albicans in vitro* and *in vivo*. Deferoxamine and deferiprone are iron chelators used clinically in humans for the treatment of iron overload disorders. Deferoxamine was completely noninhibitory and deferiprone was only weakly inhibitory for clinical strains of *C. albicans*, a finding consistent with our previous results for the reference strain ATCC 10231 and also for *Candida vini* (30). DIBI is a relatively low-molecular-weight (9 kDa) hydroxypyridinone-containing polymeric chelator with strong selective iron-binding characteristics (Fig. 1), and it was found to strongly inhibit *C. albicans* in an iron-reversible manner. DIBI inhibition occurred at very low concentrations in comparison to the chemically related hydroxypyridinone, deferiprone. On a comparative molar concentration basis, DIBI was >10,000-fold more inhibitory than deferiprone. Thompson et al. (33) also demonstrated that deferoxamine was not inhibitory for a number of bacterial pathogens and reported that deferiprone (also known as Apo-L1) and the related Apo-6619, while exhibiting better activity than deferoxamine, were nevertheless only modest growth inhibitors. The deferiprone MICs observed for *C. albicans* were in the mM range, which is similar to those found for various bacterial pathogens. Given the >10,000-fold increased activity found for DIBI, it would appear to be a good candidate for a stand-alone growth inhibitor and also as an enhancing agent for antibiotics. Zarember et al. (34) demonstrated the synergy between deferiprone and
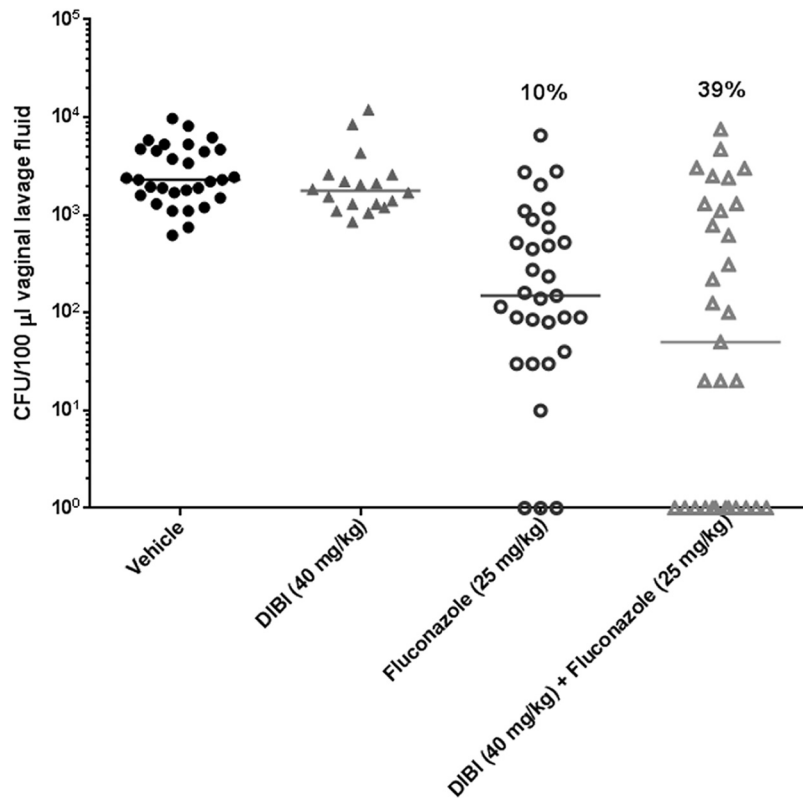
FIG 5 Improvement of fluconazole (FLC) by DIBI *in vivo*. Experimental murine vaginitis was established using FLC-sensitive strain 96113. Animals were given DIBI alone (40 mg/kg), FLC alone (25 mg/kg), or a combination of DIBI plus FLC. Vaginal fungal burden was evaluated at day 7 postinoculation. Results are expressed as CFU/100 μl vaginal lavage fluid. Percentages indicate rates of fungal clearance. Results are cumulative of 5 independent experiments.

azoles for *Aspergillus fumigatus* conidia using a checkerboard assay. Other agents that impact microbial essential iron and other metals have also been shown to affect azole sensitivity. Members of the tetracycline family have been shown to enhance FLC activity in *C. albicans* as well as chelate metal ions. Shi et al. (35) proposed that minocycline enhances FLC penetration due to Ca efflux, while Fiori and Van Dijck (32) concluded that doxycycline enhancement of FLC was related to its iron-binding activity. The tetracycline family of compounds, including minocycline and doxycycline, are known to bind iron, and this iron interaction can neutralize these agents, interfering with antibiotic uptake (36). However, the tetracyclines also bind Co and Ni (37) as well as Ca and Mg (38). Thus, it is possible the iron addition that negated the doxycycline enhancement of FLC activity, as observed by Fiori and Van Dijck (32), may have been in part related to iron neutralization of doxycycline, or the iron might have displaced other doxycycline-sequestered essential metals such as Co, Ni, Mg, or Ca, making these available to *C. albicans*. We have shown that DIBI is strongly iron selective and does not bind Ca, Mg, Co, or Ni (30); therefore, it is unlikely that the sequestration of these other essential metals was related to its observed activity. Prasad et al. (39) reported that iron limitation resulted in membrane leakiness of *C. albicans* in association with an increased sensitivity to various antifungals, including FLC. However, their studies employed the addition of bathophenanthroline sulfonate (BPS), a chelator that is known not to be iron specific and can also bind Ni, Cu, Co, and Zn (40). Therefore, BPS used at relatively high concentrations may have had other metal-sequestering activities in addition to that for iron.

We have demonstrated a strong ability of DIBI at low concentrations to improve the activities of various azoles, including fluconazole, itraconazole, and clotrimazole. DIBI at concentrations as low as 1 μg/ml in combination with an azole provided strong and

prolonged inhibition of the growth of various clinical isolates of *C. albicans in vitro*, including one which is azole resistant. The *in vivo* results showing that DIBI administered intravaginally to infected mice along with oral treatment with FLC promoted an enhanced clearance of the *C. albicans* vaginitis provide further evidence of an improvement of the azoles by DIBI. The 96113 strain used was also more sensitive to FLC when combined with DIBI *in vitro*; fungistasis was observed *in vitro*. Two additional aspects of the infection results warrant further discussion. First, infection fungal burdens in FLC-treated mice (with both FLC alone and with FLC and DIBI) were quite variable compared to those found for either sham-treated controls or DIBI-treated mice. A likely explanation is that the FLC concentration used was suboptimal in an attempt to promote potentiation by DIBI. Additionally, FLC administered orally is highly bioavailable, rapidly entering the systemic circulation for delivery to tissues, including into vaginal secretions, but with >60% of the FLC load being eliminated nonmetabolized and intact via urinary excretion (41). It seems possible that an additional local delivery of FLC via urine at the opening of the vagina might be a source of variations in effective vaginal FLC concentrations in the infected mice. In this regard, it would be interesting to investigate intravaginal administration of FLC (or an alternate azole, especially CLT, which is typically used topically in humans), as this approach would possibly provide a less variable response to azole therapy. Second, DIBI alone *in vivo* had minimal effects on vaginal fungal burden in contrast to the *in vitro* results. The exception was the lack of *in vitro* activity against strain 96113 that was also used *in vivo*. However, it is notable that control growth for this strain *in vitro* was less robust than for other strains, which could account for the differences observed. Nevertheless, the *in vivo* results were reflective of the *in vitro* results. Further studies will be needed to determine if DIBI alone *in vivo* has any appreciable antimicrobial effects. However, when considering the synergistic activity of DIBI with the azoles, it is interesting to consider how the concentration of DIBI might impact the outcome. DIBI displays fungistatic activity on its own at higher concentrations *in vitro*, but at lower concentrations, it provides iron-restricted yet continued slowed growth as measured by CFU yield. This condition of iron-restricted growth resulting from DIBI exposure improved the response to FLC (and to the other azoles) *in vitro*. Our results suggest that DIBI is providing an iron stress by restricting the supply to *C. albicans in vivo*, resulting in an enhanced susceptibility to FLC, a result fully consistent with that observed *in vitro*. The finding that estrogen treatment increases available host iron for candidal and other urogenital infections (17–19) is noteworthy as well. Thus, DIBI may help to sequester estrogen-mobilized excess iron available in the vaginal sections of the mice during infection and create an iron-limiting condition.

Unfortunately, DIBI had no enhancing or potentiating effect on an FLC-resistant isolate *in vivo*, in contrast to the *in vitro* results. A possible explanation is that DIBI requires some effect of the azole in order to provide any potentiation. While FLC had a modest effect alone on the FLC-resistant isolate *in vitro*, there was no effect of FLC *in vivo*, even at high concentrations. Additional studies will be necessary to investigate this mechanism further.

Also noteworthy is that our studies showed no adverse toxic effects with intravaginal administration of DIBI alone in mice. More comprehensive testing of DIBI toxicity has been completed separately. Both acute and chronic systemic toxicity testing in male and female rats receiving daily intravenous (i.v.) administration of DIBI at up to 200 mg/kg (highest dosage tested) for 14 consecutive days showed no significant adverse effects. This included the rate of food consumption, modification in weight gain, and parameters of hematological, clinical chemistry, organ weight, and tissue histopathological changes (B. E. Holbein, unpublished results). Thus, DIBI appears safe for use in conjunction with azoles for the treatment of fungal infections.

Our results with DIBI provide stronger evidence for a direct role of iron in azole sensitivity. On this basis, we hypothesize a rational and direct physiological link between azole sensitivity and iron availability as centered on the cytochrome P450 14alpha-demethylase, which is the primary azole target and essential for ergosterol

synthesis (39, 42). This is a heme (iron)-dependent enzyme system and therefore an iron-dependent target. The binding of the azoles and the subsequent inactivation of this enzyme as their known mode of action prevents demethylation of lanosterol (43–45) and correspondingly depletes the ergosterol content of membranes (46). This in turn leads to an accumulation of methylated sterols and induces changes in membrane physiology, such as leakiness and altered membrane-bound enzymes, resulting in the cessation of growth of *C. albicans* (44, 46).

We propose that iron chelation by DIBI results in reduced iron availability and uptake from the fungal growth environment, leading to the suppressed production of functional iron-dependent enzymes, including the cytochrome P450-dependent ergosterol demethylase. Reduced quantities of this azole target could then improve relative inactivation efficiency by azoles (reduced target content), while a loss of membrane integrity from impaired ergosterol synthesis could favor improved influx of additional azole. These complementary responses could act together to improve the overall azole inhibition of growth. Consistent with this proposed mode of action is the finding that exposure to ketoconazole induced an upregulation of various genes related to both P450 demethylase activity and iron uptake acquisition systems in *C. albicans* (47), indicating an increased need for iron in response to azole exposure.

The data presented here support the use of specific iron chelators that withhold iron necessary for fungal growth as a promising avenue for enhancing azole effectiveness in the treatment of fungal infections. Given its apparent lack of toxicity and activity at low concentrations, DIBI appears to have strong potential in this regard and warrants further investigation.

MATERIALS AND METHODS

Yeast strains. The *C. albicans* strains used in this study are listed in Tables 1 and 2. The reference strain ATCC MYA-2876 (SC5314, clinical origin) was received from Malcom Whiteway (National Research Council, Montreal, Quebec, Canada); the reference strain ATCC 96113 and the fluconazole-resistant clinical strain LP1158-07 were from the Fidel lab culture collection. All other strains were clinical isolates obtained from the Capital District Health Authority, Halifax, Nova Scotia (Ca3969 blood specimen, Ca5031 oral specimen). The isolates were subcultured on a regular basis from frozen stock at $-80°C$ and maintained at 4°C on Trypticase soy agar plates with 5% sheep blood (Becton Dickinson) or yeast extract-peptone-dextrose (YPD) agar plates (Difco BD).

Iron chelators and antifungals for *in vitro* susceptibility tests. DIBI as previously reported (Chelation Partners Inc.) (30), deferoxamine (Novartis), and deferiprone (Sigma-Aldrich) were tested for their antifungal activity against *C. albicans*. All three chelators were dissolved in water. FLC (Sigma-Aldrich) was dissolved in water. ITC and CLT (Sigma-Aldrich) were both dissolved in dimethyl sulfoxide (DMSO). Stocks were filter-sterilized (0.2-μm filter) prior to dilution in water or medium.

Cultivation and assay media. RPMI 1640 medium (with L-glutamine, without NaHCO$_3$, and supplemented with 2% glucose; Sigma-Aldrich), pH 7.0, buffered with 0.165 M morpholinepropanesulfonic acid ([MOPS] Sigma-Aldrich) was routinely used. RPMI agar plates were prepared using 1.5% (wt/vol) agar (Bioshop Canada) addition. YPD agar plates used for checkerboard spot recovery plating contained 1% (wt/vol) yeast extract (Bacto; Becton Dickinson), 2% (wt/vol) peptone (Bacto; Becton Dickinson), 2% (wt/wt) glucose (Sigma-Aldrich), and 2% (wt/wt) agar (Acumedia; Neogen).

Susceptibility testing in broth microdilution assays. MICs for the iron chelators were determined in RPMI medium. Serial dilutions of each chelator were tested using the following ranges: deferoxamine and deferiprone, 0.63 to 1280 μg/ml; DIBI, 0.06 to 128 μg/ml. Microtiter plates were inoculated with 0.5×10^3 to 2.5×10^3 CFU/ml *C. albicans* prepared from overnight cultures in RPMI medium and incubated at 35°C for 48 h. MIC endpoints were defined as the lowest concentration of chelator that inhibited growth by 80% (MIC$_{80}$, visual read) compared to that of a positive control. All experiments were replicated two times.

Azole MICs were determined according to the CLSI standard reference method for broth dilution antifungal susceptibility testing of yeasts (48, 49), with the following modifications. Serial dilutions of azoles were assayed in the following ranges: FLC, 0.12 to 64 μg/ml; ITC, 0.01 to 8 μg/ml; and CLT, 0.005 to 4 μg/ml. Microtiter plates were inoculated with 0.5×10^3 to 2.5×10^3 CFU/ml *C. albicans* prepared from overnight cultures and incubated at 35°C for 24 h. The MIC$_{80}$ and MIC$_{50}$ endpoints were determined by visual observation as relative to the growth of an azole-free control. All experiments were repeated a minimum of two times.

Effect of RPMI iron concentration and DIBI on growth. The effects of DIBI addition and RPMI iron concentration were determined for *C. albicans* strain Ca3969 in RPMI alone, RPMI plus DIBI 1 μg/ml, RPMI plus DIBI 10 μg/ml, RPMI plus DIBI 1 μg/ml plus 1 μM iron, and RPMI plus DIBI 10 μg/ml plus 1 μM iron. Aqueous ferric di-citrate (Sigma-Aldrich) was used as an exogenous iron source. Ten milliliters of each test medium was inoculated with Ca3969 at a final dilution of 1:2,000 from an overnight culture in RPMI medium first adjusted to an optical density (OD) of 0.12. Cultures were

incubated at 35°C with shaking, and OD readings were taken over a 48-h period. The experiments were replicated two times.

Synergy testing by checkerboard assay. DIBI and FLC were tested in a checkerboard assay in 96-well plates in a similar manner to that described by Fiori and Van Dijck (32), with the following modifications. The DIBI concentrations tested ranged from 0.5 to 128 μg/ml and the range of FLC was from 0.12 to 8 μg/ml. Microtiter plates were inoculated with strains 96113 and Ca5031 as described above for azole MIC tests and incubated at 35°C for 48 h. Synergy was defined as an FICI of $\leq$0.5 (50) using MIC_{80} values. For additional visualization of checkerboard results, a multichannel pipette was used to spot 5 μl from each well of the checkerboard assay plate onto a recovery YPD agar plate (32) at 24 and 48 h postexposure. Recovery plates were incubated at 35°C and were inspected after 24 h of incubation. The experiments were repeated a minimum of two times.

Azole improvement by measuring replicative growth. The beneficial effects of DIBI with FLC, ITC, and CLT were examined using a growth curve procedure. Test media were inoculated with *C. albicans* prepared as described above to obtain an initial viable count of approximately 10^3 CFU/ml in the following medium combinations: RPMI alone, RPMI plus DIBI alone (1 μg/ml for 96113 and 10 μg/ml for LP1158-07, Ca3969, and Ca5031), RPMI plus azole alone (FLC at 1.0 μg/ml for 96113 and 128 μg/ml for LP1158-07; ITC at 0.25 μg/ml for Ca5031 and 0.5 μg/ml for Ca3969; CLT at 0.03 μg/ml for both Ca5031 and Ca3969), and RPMI plus DIBI plus azole (concentrations as above). The cultures were incubated for 72 h at 35°C with shaking. Viable counts were measured every 24 h. Each experiment was replicated 3 times.

Murine vaginitis model. Female C3H/HeNCrl mice, 8 to 10 weeks old and weighing ~20 g, were obtained from Charles River Laboratories (Wilmington, MA). All animals were housed and handled according to institutionally recommended guidelines. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Louisiana State University Health Sciences Center (LSUHSC). The estrogen-dependent model has been described previously (51). Briefly, animals ($n = 6$/group) were injected subcutaneously with estradiol valerate (0.1 mg dissolved in 100 μl sesame oil) 3 days prior to and 4 days after vaginal inoculation. One day prior to inoculation, a blastospore culture of the *C. albicans* isolate(s) to be used in each study was prepared. On the day of inoculation, blastospores were collected and washed once with phosphate-buffered saline (PBS) and resuspended at 2.5×10^6/ml in PBS for an inoculum of 5×10^4 cells/20 μl PBS. For inoculation, animals were anesthetized "to effect" by isoflurane inhalation. To anesthetized animals, 5×10^4 blastospores in 20 μl PBS were pipetted directly into the vagina, using a Pipetman. Topical treatments of 5% poly-vinylpyrrolidone (PVP) vehicle only or 4% or 8% DIBI (40 or 80 mg/kg, respectively) dissolved in vehicle (20 μl) were given once daily starting on day 3 and continued through day 6. Fluconazole (25 or 50 mg/kg) or PBS vehicle was given via oral gavage on days 3 and 5. Animals given both DIBI and fluconazole followed the same treatment regimens. On day 7, animals were sacrificed and the vaginal cavity was lavaged with 100 μl of PBS. The lavage fluid was examined microscopically for yeast/hyphae, qualitatively scored (0 to 4 based on volume of organisms present), and serially diluted and cultured for quantitative enumeration of organisms (expressed as CFU/100 μl lavage fluid).

Statistical analysis. The data are reported as means $\pm$ standard errors. For *in vitro* studies, the data were analyzed using a two-way analysis of variance (ANOVA) with Bonferroni's posttest (GraphPad Prism software). For *in vivo* studies, the data were analyzed using the Mann-Whitney U test (GraphPad Prism software). Additional one-way ANOVA, Tukey, and chi-square analyses were performed for clearance data using SAS 9.4 software. Significant differences were defined as a P value of $<$0.05.

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