Original Article

Synergistic Antifungal Effect of Lactoferrin with Azole Antifungals against Candida albicans and a Proposal for a New Treatment Method for Invasive Candidiasis

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SUMMARY: The combination of lactoferrin with fluconazole has been reported to synergistically enhance the antifungal activity of fluconazole against Candida spp. and inhibit the hyphal formation in fluconazole-resistant strains of Candida albicans. In this study, we investigated the association between the therapeutic effects of this combination and the pharmacological characteristics of fluconazole and itraconazole and the variation in these effects with differences among the strains in terms of the susceptibility and resistance mechanisms. Lactoferrin enhanced the growth-inhibitory activity of fluconazole against two different ergosterol mutants but not against pump mutants or an azole-susceptible strain; but increased the activity of itraconazole against all the strains tested in this study. Exogenous iron cancelled the synergistic effect, which suggests that the iron-chelating function of lactoferrin may contribute to the synergism. Besides, radiolabeled fluconazole assays revealed that lactoferrin did not affect the intracellular concentrations of fluconazole, thereby indicating that these synergistic effects were not due to the alteration of the intracellular uptake of the drug. The development of new clinical treatments and therapeutic method against resistant Candida will depend on our understanding of the resistance mechanisms and methods to overcome them by the application of suitable drug combinations with synergistic effects. The results of this study might contribute to the improvement of our understand of the mechanisms underlying the resistance of Candida strains.

INTRODUCTION

Candidiasis is the commonest invasive mycosis encountered in clinical settings, even in the current “echinocandin era,” and it remains refractory to treatment and has a high mortality of 30% or higher (1). Although fluconazole (FLCZ) and echinocandins are the primary choices for therapy against invasive candidiasis, more effective treatments or prophylactic methods need to be developed.

Lactoferrin (LF) is a broad-spectrum antimicrobial peptide against bacteria, fungi, viruses, and protozoa and shows potent synergism with FLCZ in azole-susceptible isolates of Candida albicans obtained from neonates with sepsis (2); this synergism might also be applicable for refractory candidiasis. Wakabayashi et al. also showed that a combination of FLCZ with LF-related compounds exerted synergistic effects on cell growth, even in the case of azole-resistant C. albicans (3), but it has not yet been elucidated exactly how combination with LF will influence effectiveness of different drugs against various resistance mechanisms. Candida spp. are known to acquire azole resistance by at least three different mechanisms: altered sterol synthetic pathway from native ergosterol due to ERG3 mutation, resulting in the production of non-toxic alternative sterol in the presence of azoles (4); decreased substrate affinity due to mutations in the target molecule, 14-alpha-demetlylase, which is encoded by ERG11 (5); and decreased intracellular concentrations of drugs due to overexpression of genes encoding efflux pumps, such as CDR1 and CaMDR (6). Here, we investigated the effects of LF combinations with FLCZ and itraconazole (ITCZ) on strains that exhibit one or more of the abovementioned resistance mechanisms.

MATERIALS AND METHODS

Fungal strains: The following C. albicans strains were tested: SC5314, an azole-susceptible strain; CAE3DU3, the erg3 disrupted mutant (7); Darlington strain, a clinical strain carrying mutations in both erg3 and erg11 (4,8,9) (kindly provided by Dr. John E. Bennett, NIAID, NIH, Md., USA); C26, a CDR1 overexpressing mutant (10) (provided by Dr. S. Maesaki, Saitama Medical School, Saitama, Japan); and C40, a CaMDR overexpressing mutant (10) (see Table 1).
Antifungal susceptibility test: Antifungal susceptibilities were determined using a slightly modified version of the CLSI M27A method (11). FLCZ (Pfizer Japan, Tokyo, Japan) and ITCZ (Janssen Pharmaceuticals, Cork, Ireland) were dissolved in dimethylsulfoxide (DMSO; Wako, Osaka, Japan) and diluted with RPMI 1640 medium (with l-glutamine, without NaHCO₃, and supplemented with 2% glucose; pH 7.0 from Gibco BRL, Paisley, Scotland) with 0.165 mol/l 3-morpholinopropanesulfonic acid (MOPS); the latter was prepared according to the manufacturer’s instructions. All solutions were sterilized by passing them through filters (pore size, 0.22 μm; Millipore, Bedford, Mass., US, Millex®-GV Syringe Filtering Unit) and added to the microtiter plates. Tested ranges of antifungal susceptibilities were 0.0625–64 μg/ml and 0.0156–16 μg/ml for FLCZ and ITCZ, respectively. The 50% inhibitory concentrations (IC₅₀s) were defined as the minimal concentrations required to inhibit 50% of the growth compared to control (without treatment).

Preparation of LF and iron: Bovine LF (Wako) was dissolved in RPMI1640 media, and the tested LF concentration in combination with azoles was 200 μg/ml, which represents the LF concentration in peripheral blood (12), while 2-fold serially diluted concentrations of LF, from 0 to 6,400 μg/ml, were tested to evaluate the antifungal effect of LF alone. Iron sulfate II and iron chloride III were added at 10, 50, or 100 μM.

Cell preparation: Each C. albicans strain was grown on a Sabouraud dextrose agar (SDA) plate for 24 h at 35°C. Five colonies of each strain were scraped out from the plate and suspended in 5 ml of phosphate buffered saline (PBS). Cell densities were counted by plating, and the radioactivity per cell was counted by plating, and the radioactivity per cell.

Growth-inhibition assay: One hundred microliter of the final cell suspension was applied to each well of a sterile 96-well plastic flat-bottom plate with a cover (MicrotestTM Tissue Culture Plate, 96 well, flat bottom with low evaporation lid; Becton Dickson, Sparks, Md., USA), and 100 μl of the prepared antifungal solutions were added to each well at series of concentrations. The plates were incubated for 48 h at 35°C without agitation, and the turbidity was measured by absorption at 620 nm, using an automated microplate reader (Multiskan Ascent; Labsystems, Helsinki, Finland). All experiments were performed in triplicate.

Checkerboard analysis: The synergistic growth-inhibitory effects of antifungal-LF combinations were verified by the checkerboard method reported by Eliopoulos and Moellering with slight modification (13). Briefly, the fractional inhibitory concentration (FIC) indexes were calculated as a summation of the IC₅₀ for drug A in the combination/IC₅₀ for drug A alone and the IC₅₀ for drug B in the combination/IC₅₀ for drug B alone. When the IC₅₀s were greater than the highest concentrations tested, the highest concentrations were substituted for the IC₅₀s. The effects of the drugs were interpreted to be indicative of synergy, indifference, or antagonism when the FIC indexes were <1, 1 to 4, or 4<, respectively (14).

Evaluation of intracellular FLCZ concentrations: Intracellular concentrations of FLCZ were measured by a protocol as previously reported (15). Briefly, each tested C. albicans strain was inoculated in yeast nitrogen base broth (Difco Labs., Detroit, Mich., USA) with 2% glucose (pH 6.0) and incubated with or without LF. ³H-thymidine-labeled FLCZ (³H-FLCZ), which was kindly provided by Pfizer Japan Inc., was added to the overnight culture to achieve a final specific radioactivity of 8.35 kBq/ml (0.225 μCi/ml). Immediately or 1 h after the addition, excessive ³H-FLCZ was removed by filtration and a brief wash, and the radioactivity of the incorporated ³H-FLCZ was measured by LSC-5001 liquid scintillation counter (Aloka, Tokyo, Japan). The radioactivity was corrected for the control background level using heat-killed cells. Viable cell numbers were also counted by plating, and the radioactivity per cell was calculated. Results were expressed as counts per minute (cpm) per 10⁶ cells. All experiments were performed in triplicate.

Statistical analysis: The data were analyzed using analysis of variance. Unless otherwise indicated, the data are presented as the mean ± standard error (SE) of triplicates. The error bars represent the SE, and the data are representative of 2 or more individual experiments.

RESULTS

LF synergistically enhances the growth-inhibitory ef-

<table>
<thead>
<tr>
<th>C. albicans strain</th>
<th>Characteristic</th>
<th>Origin or reference</th>
<th>IC₅₀ of FLCZ alone (μg/ml)</th>
<th>IC₅₀ of FLCZ/LF combination (μg/ml)</th>
<th>FIC index of FLCZ/LF combination</th>
<th>IC₅₀ of ITCZ alone (μg/ml)</th>
<th>IC₅₀ of ITCZ/LF combination (μg/ml)</th>
<th>FIC index of ITCZ/LF combination</th>
<th>IC₅₀ of LF alone (μg/ml)</th>
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<td>2 (I)</td>
<td>0.125</td>
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<td>0.28(S)</td>
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<td>(9)</td>
<td>0.25/16/25</td>
<td></td>
<td>&gt;16</td>
<td>2/50</td>
<td>&lt;0.13(S)</td>
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<tr>
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<td>0.016/200</td>
<td>&lt;0.03 (S)</td>
<td>&gt;4,000</td>
<td>&gt;0.5/100</td>
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¹: The FIC indexes are calculated as described in the text, and the interpretation is indicated in the parenthesis; S, synergy; I, indifference.
Fig. 1. Inhibition of *C. albicans* growth by fluconazole (FLCZ) or itraconazole (ITCZ) in combination with lactoferrin (LF). The growth of each *C. albicans* strain measured by optical density at 620 nm (OD$_{620}$) is shown. Data are presented as means of triplicates. Strains are SC5314 for A and E, CAE3DU3 for B and F, Darlington for C and G, and C26 for D and H. Closed squares (•) are data for FLCZ or ITCZ alone, open squares (○) are for FLCZ or ITCZ with LF. LF significantly enhanced the growth inhibitory effects of FLCZ only against CAE3DU3 and Darlington strain while LF significantly enhanced the growth inhibitory activity of ITCZ against all strains tested.
effects of FLCZ and ITCZ: The IC_{50}s of FLCZ and ITCZ for the strains used are summarized in Table 1. Strains other than SC5314 were extremely resistant to FLCZ and ITCZ alone. LF alone exhibited no antifungal activity, even at a concentration of 6,400 \mu g/ml, although it did increase yeast growth in the media to some extent, contrary to our expectations. LF significantly enhanced the growth-inhibitory effects of FLCZ in CAE3DU3 or in the Darlington strain (Fig. 1B and C), leading to a significant reduction of the IC_{50}s (Table 1); however, the effect of the LF-FLCZ combination remained unclear in the case of SC5314 and the pump mutants (Fig. 1A and D).

The effect of the LF-FLCZ combination was limited only to ergosterol mutants; however, LF significantly enhanced the growth-inhibitory activity of ITCZ against all strains tested, irrespective of theirazole susceptibilities and resistance mechanisms (Fig. 1E–H), which led to a significant reduction in the IC_{50}s (Table 1). Thus, LF seemed to exert different effects depending on whether it was combined with FLCZ or ITCZ.

To verify the effects of the combinations, the FIC indexes were calculated (Table 1). The FIC indexes of the LF-FLCZ and LF-ITCZ combinations were lower than 1, except in the case of that of the LF-FLCZ combination against SC5314 and the pump mutants. Therefore, LF was verified to exert a synergistic action with FLCZ and ITCZ, thereby enhancing their growth-inhibitory activity.

Exogenous iron cancels the synergism: We sought to examine whether exogenous iron canceled the effect of LF, since LF is known to be an iron chelator. Both iron sulfate II and iron chloride III reversed the resistance of the Darlington strain against FLCZ, at concentrations of 50 \mu mol or higher (Fig. 2). Thus, exogenous iron cancelled the synergistic effect of LF with FLCZ, suggesting that the chelating function of LF may be related to the synergism. It is plausible that additional iron is essential for the operation of the defense mechanism of Candida against azoles and that it may have play some role in the promotion or suppression of ergosterol synthesis in the Candida cell membrane.

LF does not increase the intracellular concentrations of FLCZ: LF did not promote the uptake of \textsuperscript{3}H-FLCZ for any of the C. albicans strains tested (Fig. 3). The statistically significant decrease observed for the SC5314 strain might be attributable to experimental errors, and since the decrease was not marked, it is unlikely to contribute to the effect. These findings suggest that the synergistic effect of LF is not due to a change in FLCZ uptake.

DISCUSSION

Innate immunity plays an important role in protection against systemic candidiasis, and LF is an anti-infective peptide secreted from human cells that acts directly on Candida cells at the site of inflammation. LF is believed to exert its effects via the inhibition of Fe^{3+} uptake by microorganisms, which is essential to them, but recent studies have revealed other biochemical actions of LF (16,17). For instance, it has been reported that binding to bacterial polysaccharide contributes to the reduction in the inflammation of the oral mucosa (16) and that lactoferricin, the N-terminus of LF, has fungicidal activity similar to that of defensin (17). Another study also revealed that LF synergistically enhanced the antifungal effects of the azole class of antifungal agents (18), and we focused on this synergistic effect in this study.

Although studies indicate that the synergistic effect of LF is evident in C. albicans strains that have low susceptibility to azoles (18), it remains to be clarified whether this particular synergism also depends on the resistance mechanisms adopted by the strain. Our data suggest that the antifungal synergism of the LF-FLCZ combina-
tion depends on the azole susceptibility of the strain and the resistance mechanisms adopted by it, while that of the LF-ITCZ combination is independent both these factors. This reason behind the differences in the effects of the LF-FLCZ and LF-ITCZ combinations could not be fully elucidated, but can be partially explained by previously reported findings. According to Goldberg et al., a positively charged molecule such as lysozyme intensifies the hydrophobicity of the cell surface of microorganisms (19). LF is one such positively charged molecule and can be speculated to have a similar effect although it has not yet been proven. ITCZ is also hydrophobic, and therefore, its antifungal activity might be enhanced by hydrophobic interaction, although the detailed mechanisms are not understood (20).

Clearly, the difference in the synergism between SC5314 and CAE3DU3 is due to the latter’s ergosterol mutation, although the other strains have such different genetic backgrounds that it is still impossible to ascertain what characteristic or characteristics actually control their response to synergism. Assays using radiolabeled FLCZ also revealed that LF did not alter the intracellular level of FLCZ, suggesting that the synergistic effect of LF is not due to the modification of FLCZ uptake. In addition, exogenous iron canceled the synergism, and thus the chelating function of LF might contribute to its synergistic antifungal activity.

With regard to the temperatures, 37°C is the internal temperature of healthy mammals, and 35°C is the optimum temperature for the growth of *Candida*; which may explain both the effectiveness of fevers in fighting candidiasis and why undernourished or otherwise compromised patients and individuals exposed to cold tend to succumb to the infection.

Our previous study revealed that FLCZ had antifungal activity in vivo against CAE3DU3, which is extremely resistant to FLCZ in vitro; this finding suggested the existence of some intrinsic factors that might potentiate the antifungal activity of FLCZ (7). Therefore, our results suggest that both FLCZ and ITCZ can act in tandem with endogenous LF, thereby increasing their clinical efficacy against *C. albicans* to an extent greater than that expected by the traditional in vitro susceptibility of the organisms. In addition, reports indicate that LF concentrations decreased under certain conditions, such as those found in AIDS patients (21), and thus, exogenous LF can be considered a promising therapy to potentiate the antifungal activity of FLCZ and ITCZ when endogenous LF is insufficient.

In conclusion, the development of new clinical treatments and therapeutic approaches against *Candida* resistance will depend on our understanding of the mechanisms underlying this resistance, and the application of various combinations exhibiting synergistic action. The findings of this study shed some light in this regard.

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Conflict of interest None to declare.

**REFERENCES**


