Activated Lactoferrin and Fluconazole Synergism Against Candida albicans and Candida glabrata Vaginal Isolates

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OBJECTIVE: To evaluate the fungistatic activity of activated lactoferrin (ALF), fluconazole (FCN) individually and in combination against Candida vaginal isolates as well as to measure the time to recovery from the fungistatic effects after exposure in vitro to threshold minimal inhibitory concentrations (MIC).

STUDY DESIGN: Fungistasis patterns for ALF (2.5 mg/mL) and FCN (0.25 mg/mL) were tested at threshold MIC against vaginal isolates of C albicans (n = 5) and C glabrata (n = 5) grown in Sabouraud’s dextrose broth against 10^5 yeast inoculum at 37°C for 48 hours by microscale optical density (OD) assay according to the following criteria: “Total stasis” indicates that an agent elicited no change or a change in turbidity < 0.1 OD unit for > 48 hours (complete growth inhibition), “stasis recovery” (SR) is the time point at which turbidity of a previous stasis system shows an upward growth trend for > 0.1 OD unit (recovery from growth inhibition), and “partial stasis” (PS) is proliferation after stasis recovery, measured as a percentage relative to growth control at any time (incomplete growth inhibition).

RESULTS: For ALF (2.5 mg/mL), the mean SR time was 15.6 ± 2 hours for C albicans (n = 5) and 27.5 ± 2 hours for C glabrata (n = 5). The SR patterns for FCN were strain dependent and showed a wide range of deviation for both Candida species; accordingly, the values were 15.8 ± 9 hours for C albicans and 25.5 ± 12 hours for C glabrata. After 48 hours exposure to C albicans, ALF and FCN elicited a mean PS of 27.5 ± 2% and 24.8 ± 7%, respectively. The PS values at 48 hours showed a marked variation between C glabrata isolates, 29.1 ± 24% for ALF and 21.5 ± 38% for FCN. However, a combination of ALF and FCN at their threshold MIC showed significant drug synergism, causing total stasis of both species of Candida isolates. Thus, no SR for any Candida isolate was detected at or beyond 48 hours. Conversely, native lactoferrin failed to demonstrate such

The ALF/FCN combination is a novel antifungal potentiation system that could be effective for clinical management of VVC.

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potent synergism with FCN against either Candida species.

CONCLUSION: The combination of ALF and FCN at the threshold MIC elicited potent synergism, leading to total fungistasis of C albicans and C glabrata vaginal pathogens. ALF is a new class of fungistatic agent with a mode of action distinct from that of azoles. (J Reprod Med 2004;49:800–807)

Keywords: candidiasis, vulvovaginal; lactoferrin; fluconazole.

Vulvovaginal candidiasis (VVC) is a common fungal infection of the female genital tract. Conventional treatment consists primarily of repeated, often monthly, short courses of topical vaginal therapy prescribed with the first appearance of symptoms. Fluconazole (FCN) is a frequently used therapeutic in the clinical management of VVC due to its high water solubility and wide tissue distribution after oral administration. The cellular target for FCN activity is identified as cytochrome P450-dependent C14α-demethylase, an active component of the ergosterol biosynthetic pathway. Accordingly, FCN causes ergosterol depletion and accumulation of 14α-methyl-sterols in the plasma membrane of Candida species. However, long-term use of FCN often leads to drug resistance in Candida strains. FCN resistance has been linked to (1) up-regulation of the ERG11 gene, which encodes the drug target enzyme C14α-demethylase; (2) reduced affinity of azoles to cellular targets; (3) accumulation of a less toxic ergosterol intermediate, C14α-methyllecosteol; and (4) active efflux of FCN away from Candida spp, at least 2 types of efflux pumps, the ATP binding cassette transporter superfamily and the major facilitators, are known to contribute to drug resistance.

Lactoferrin (LF) is a major innate defense factor in exocrine secretions and neutrophil granules. The ability of LF to bind 2 Fe^{3+} ions with high affinity in cooperation with 2 bicarbonate ions is an essential characteristic that contributes to its major biologic properties, including antimicrobial activity. Accordingly, induction of iron-deprivation stasis is the mechanism by which LF protects mucosal surfaces, including vaginal epithelia from pathogens. LF also binds with high affinity to specific pore-forming channel proteins on the microbial cell surface and alters the membrane diffusion of antimicrobial agents. Fungistasis and membrane drug diffusion effects of native LF against Candida spp with limited efficacy have been reported.

Recently, a molecular activation process to enhance the antimicrobial activity of native LF has been developed. This resulting, activated LF (ALF), is a microbial blocking agent several fold more potent than native LF, with specific activity against pathogens without affecting commensal flora, such as vaginal lactobacilli.

In a previous study we reported potent fungistasis activity for ALF against vaginal isolates of C albicans and C glabrata for an extended period, 96 hours. Furthermore, ALF has effectively blocked the attachment of Candida hyphae and blastospores to vaginal epithelia. This study was undertaken to further elucidate the potential of ALF to elicit antifungal synergism with FCN against C albicans and C glabrata vaginal isolates.

Materials and Methods

LF

LF (lot 10099751) purified from cow’s milk was provided by DMV International Nutritional, Veghel, the Netherlands. A 2% (wt/vol) native solution was prepared by dissolving LF (2.0 g) in 100 mL sterile PBS (pH 7.2). A 2% (wt/vol) ALF solution was prepared in accordance with Naidu using deionized water. Native LF (2.0 g) was dissolved in 100 mL sterile buffer solution containing 1 mM EDTA (Versene NA™, Dow Chemicals, Freeport, Texas), 10 mM NaHCO3 (Fisher, Fairlawn, New Jersey) and 100 mM NaCl (Sigma Chemicals, St. Louis, Missouri). After adjusting the pH to 8.2 (with NaHCO3), food grade pectin (0.02 g; CU 201, Herbstreith & Fox, Nürenburg, Germany) was added to this solution with gentle stirring for partial immobilization of the dissolved LF. Formation of immobilized LF was confirmed by gel filtration chromatography using a Sephacryl S-200 high-resolution column (Amersham Biosciences, Piscataway, New Jersey).
FCN

FCN (Diflucan® intravenous infusion glass bottles, Pfizer Roerig, New York, New York) in 0.9% saline was obtained as 2 mg/mL stock solution and stored at 26°C until used. FCN was diluted in glass vials to required working concentrations in 0.9% saline prior to testing.

Vaginal Candida Isolates

Clinical strains of Candida spp were isolated from VVC patients at the Microbiology Laboratory, Mayo Clinic, Scottsdale, Arizona. Isolates were identified and speciated to C albicans and C glabrata according to MicroScan (Dade Behring, Dearfield, Illinois, and Vitek Microbial ID system. BioMerieux, Durham, North Carolina). C albicans (n = 5) and C glabrata (n = 5) were received as slant cultures on Sabouraud's dextrose (SD) agar. After revival, pure yeast cultures were stored on Microbank™ beads (Pro-Lab Diagnostics, Austin, Texas) at -80°C. Yeast were grown in SD broth overnight at 35°C for all experimental purposes.

Microscale Optical Density (OD) Assay (MODA)

Antifungal activity of ALF, native LF and FCN against C albicans and C glabrata was evaluated in vitro. A 100-μL volume of sterile SD broth (2× concentration) was added to all 96 wells of a sterile microtiter plate (Costar® 3596, Corning, New York). ALF, native LF, FCN or sterile EDTA/bicarbonate buffer (control), each 50 μL in volume, was added to wells and inoculated with 50 μL yeast suspension containing ~10^5 yeast/mL (diluted from a precalibrated [OD 1.0 at 600 nm] solution of 10^8 yeast/mL). Yeast growth was monitored at 35°C as turbidity change in broth at different time points by measuring OD at 600 nm using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, California). Prior to OD measurement, the contents of each well were mixed with a sterile pipette to obtain a uniform yeast suspension. Wells containing SD broth without yeast inoculum served as the sterility control. Wells containing SD broth inoculated with yeast but without antifungal (i.e., ALF, LF or FCN) served as the positive growth control. Based on the above working controls of sterility as well as growth, when a test strain proliferates typically under defined conditions of inoculation and incubation, the turbidity (OD) changes in the microbial growth media, as measured at 600 nm with the following criteria for MODA: “Total stasis” indicates that an agent elicited no change or a change in turbidity ≤ 0.1 OD unit for ≥ 48 hours (complete growth inhibition), “stasis recovery” is the time point when turbidity of a previous stasis system showed an upward growth trend for ≥ 0.1 OD unit; and “Partial stasis” is proliferation after stasis recovery, measured as a percentage relative to growth control at any time (incomplete growth inhibition).

Measurement of Yeast Generation Time

From the MODA experiment, the total number of viable yeast cells was enumerated at 0, 24 and 48 hours and compared with the viable counts of inocula to calculate generation time for each Candida species under a given test condition. A 200-μL Candida cell suspension from the microtiter plate was centrifuged, and the treatment solutions were aspirated to remove antifungal exposure. Yeast cells were resuspended in 0.5 mL normal saline and serially diluted 10-fold in normal saline. Selected dilutions were plated in duplicate on SD agar for total yeast counts using an Autoplate® 4000 device (Spiral Biotech, Norwood, Massachusetts). Plating was performed in an exponential log dilution setting on the spiral autolater. Agar plates were incubated at 35°C for 24–48 hours, and the total colony counts were estimated using an automated infrared Q-count device (Spiral Biotech). Data were expressed as colony-forming units per milliliter of growth medium.

Statistical Analysis

Interaction between ALF and FCN was assessed at each time by using a 2-way analysis-of-variance model. Differences < 0.05 were considered significant. All p values and confidence intervals are 2 sided.

Results

Native LF and ALF Effects with FCN

Native LF, ALF at 2.5 mg/mL concentration and FCN at 0.25 mg/mL dosage were tested for fungistatic activity either alone or in combination against vaginal Candida isolates (Figure 1). Stasis recovery time points for C albicans strain NTRL809A were estimated at 13.8 and 15.4 hours for ALF and FCN, respectively. Native LF (2.5 mg/mL) under similar test conditions (inoculum 10^5 yeast) failed to elicit fungistasis. Partial stasis values were 86% and 69% at 24 hours and 28% and 34% at 48 hours for ALF and FCN, respectively. A combination of ALF and FCN, however, resulted in total stasis of C albicans growth for up to 48 hours, significantly higher
(p<0.0001) than ALF or FCN, individually. Native LF in combination with FCN showed an additive effect at 24 hours that progressively diminished within 48 hours.

*C. glabrata* strain NTRL131G in the presence of ALF and FCN showed stasis recovery at 29.6 and 9.3 hours, respectively. Native LF was ineffective against *C. glabrata* under similar test conditions. ALF elicited total stasis at >24 hours, and 55% partial stasis was observed after 48 hours of exposure. Partial stasis values for FCN were 21% at 24 hours and 4% at 48 hours. Finally, a combination of these 2 agents resulted in fungistasis of *C. glabrata*, significantly more potent (p<0.0001) than ALF and FCN alone measured at their individual dosages at 48 hours. The native LF and FCN mixture demonstrated a drug potentiation effect at 24 hours that progressively diminished within 48 hours.

**ALF and FCN Effects on Yeast Generation Time**

The generation (doubling) time for *C. albicans* was estimated as 110 minutes in SD broth with 10^5 yeast inoculum (Table I). A total of 13 generations were required for *C. albicans* strain NTRL809A to reach stationary growth phase in the controls. Treatment with ALF or FCN reduced yeast multiplication to 4 replicates by prolonging the generation time to 360 minutes. The ALF/FCN combination treatment, however, significantly eliminated (p<0.0001) the doubling of *C. albicans*; therefore, no generation time was detected.

*C. glabrata* strain NTRL131G at 10^5 inoculum in SD broth replicated after 110 minutes and reached the stationary growth phase after 16 generations under control conditions. The presence of ALF and FCN in growth medium retarded the yeast replication to 2 and 10 generations, respectively. Accordingly, treatments with ALF and FCN extended the doubling time of *C. glabrata* to 720 minutes (2 doublings) and 144 minutes (10 doublings), respectively. Accordingly, FCN was less effective against *C. glabrata*. Finally, ALF/FCN treatment, in combination, resulted in significant antifungal synergism (p<0.0001), therefore, no generation time was observed.

These data suggested that ALF (2.5 mg/mL) and FCN (0.25 mg/mL) as individual treatments were partially effective against VVC pathogens. Howev-
Table 1  Effects of ALF, Native LF, FCN Alone and Combinations on Yeast Cell Replication Rates of C. albicans (NTRL 809A) and C. glabrata (NTRL 131G) Vaginal Isolates

<table>
<thead>
<tr>
<th>Test</th>
<th>C. albicans inoculum (3.0 x 10^3)</th>
<th>C. glabrata inoculum (2.7 x 10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yeast count</td>
<td>Generations</td>
</tr>
<tr>
<td>Control</td>
<td>1.0 x 10^9</td>
<td>13</td>
</tr>
<tr>
<td>LF</td>
<td>2.2 x 10^9</td>
<td>13</td>
</tr>
<tr>
<td>ALF</td>
<td>4.0 x 10^6</td>
<td>4</td>
</tr>
<tr>
<td>FCN</td>
<td>2.8 x 10^6</td>
<td>4</td>
</tr>
<tr>
<td>LF+FCN</td>
<td>1.5 x 10^6</td>
<td>3</td>
</tr>
<tr>
<td>ALF+FCN</td>
<td>1.2 x 10^5</td>
<td>0</td>
</tr>
</tbody>
</table>

ALF and native LF were tested at 2.5 mg/mL and FCN at 0.25 mg/mL against yeast grown at 35°C in SD broth and measured by MODA. Viable yeast counts were performed on SD agar with growth solutions from MODA at 0 (inoculum) and 24 hours, using an automated spiral plating system.

er, a combined dose of their threshold minimal inhibitory concentrations (MIC) resulted into a potent synergism and elicited stasis against both vaginal isolates of C. albicans and C. glabrata strains.

ALF/FCN Synergism Against Candida Isolates (n = 10)

Based on the above data, the spectrum of fungistatic synergism between ALF and FCN was further evaluated in different vaginal isolates of C. albicans (n = 5) and C. glabrata (n = 5) strains. Stasis recovery and partial stasis values for individual Candida isolates following treatment with ALF (2.5 mg/mL) and FCN (0.25 mg/mL) either alone or in combination are shown in Table II. The data were further elaborated as percentage growth pattern for each strain, with average values for each group of Candida species at defined time points following treatment with both antifungal agents (Figure 2).

The average stasis recovery for C. albicans isolates was estimated as 15.6 ± 2 hours and 15.8 ± 9 hours for ALF and FCN, respectively. The average partial stasis values after 48 hours drug exposure were 27.5 ± 2% for ALF and 24.8 ± 7% for FCN. Unlike their individual doses, the combination of ALF and FCN resulted in significant synergism (p < 0.0001), causing total fungistasis with all 5 C. albicans test strains.

ALF and FCN susceptibility patterns showed a high degree of deviation between the 5 test isolates of C. glabrata. The average value for stasis recovery was 27.5 ± 2 hours, and partial stasis after 48 hours exposure was 29.1 ± 24% with ALF treatment. After FCN exposure, an average stasis recovery was observed at 25.5 ± 12 hours, and partial stasis after 48 hours was estimated at 21.5 ± 38%. Despite the marked drug susceptibility deviations among individual strains, ALF and FCN, when combined,

Table II  Stasis Recovery (SR) and Partial Stasis Values for Vaginal Candida Isolates Following Treatment with ALF and FCN Either Alone or in Combination

<table>
<thead>
<tr>
<th>Test strain</th>
<th>ALF (2.5 mg/mL)</th>
<th>FCN (0.25 mg/mL)</th>
<th>ALF + FCN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SR time (h)</td>
<td>PS% (48 h)</td>
<td>SR time (h)</td>
</tr>
<tr>
<td>C. albicans</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTRL809A</td>
<td>13.8</td>
<td>27.9</td>
<td>15.4</td>
</tr>
<tr>
<td>NTRL358A</td>
<td>15.6</td>
<td>6.9</td>
<td>13.0</td>
</tr>
<tr>
<td>NTRL931A</td>
<td>17.7</td>
<td>19.7</td>
<td>9.4</td>
</tr>
<tr>
<td>NTRL128A</td>
<td>16.2</td>
<td>57.6</td>
<td>8.4</td>
</tr>
<tr>
<td>NTRL224A</td>
<td>14.8</td>
<td>7.0</td>
<td>16.1</td>
</tr>
<tr>
<td>C. glabrata</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTRL131G</td>
<td>29.6</td>
<td>55.1</td>
<td>9.3</td>
</tr>
<tr>
<td>NTRL807G</td>
<td>25.3</td>
<td>5.6</td>
<td>43.1</td>
</tr>
<tr>
<td>NTRL814G</td>
<td>25.2</td>
<td>5.6</td>
<td>25.6</td>
</tr>
<tr>
<td>NTRL127G</td>
<td>30.3</td>
<td>50.9</td>
<td>24.9</td>
</tr>
<tr>
<td>NTRL224G</td>
<td>27.3</td>
<td>28.2</td>
<td>24.6</td>
</tr>
</tbody>
</table>

TS = total stasis, ND = nondetectable.
showed significant synergism (p < 0.0001) causing total fungistasis of all 5 test strains of C. glabrata.

**Discussion**

Resistance to FCN is highly prevalent among Candida pathogens; therefore, any development of a new antifungal agent and/or synergist to overcome such drug resistance is urgent. Since VVC is a recurrent infection in the immunocompromised host, wherein a Candida pathogen proliferates with slow-steady kinetics, such factors as the infectious dose of blastospores and antifungal activity for an extended period need to be balanced in the development of any therapeutic regimen. In a previous study we estimated that ~10^5 blastospores of Candida are required to infect a 1-cm² surface of the vaginal epithelium and that the yeast cells replicated at a rate of ~110/min under optimal conditions. Furthermore, we have shown that ALF (5 mg/mL) elicited fungistasis of C. albicans and C. glabrata at 10^5 inoculum for 96 hours and significantly blocked both hyphal and blastospore interactions with vaginal epithelia. In the present study we demonstrated the synergistic potential of ALF with FCN at threshold MIC to inhibit vaginal Candida pathogens.

In 1994, our laboratory first reported the ability of LF to bind specific receptors on pathogens and potentiate diffusion of antibiotics across microbial membranes. Furthermore, LF interactions with the pathogen surface seem to enhance an array of antimicrobial effects, including stasis, adhesion blockade, altered membrane diffusion rates, opson-
ic activity, intracellular damage and plasmid removal.\textsuperscript{13-16,25} The development of ALF technology is essentially to capture and enhance the multiple antimicrobial spectrum of native LF.\textsuperscript{23} Thus, apart from stasis and adhesion blockade effects, ALF seems to enhance membrane diffusion of FCN by binding to Candida surfaces; to limit the iron-dependent cytochrome P450, the FCN cellular target, as well as other components of the electron transport system; and thus to cause synergistic potentiation of FCN. In this study, ALF/FCN synergism demonstrated potent fungistatic efficacy against both \textit{C. albicans} and FCN-resistant \textit{C. glabrata}.

Synergism between ≥2 drugs results from the combination of different antimicrobial mechanisms. FCN synergism with terbinafine, ibuprofen, sodium salicylate and propyl paraben against \textit{C. albicans} has been reported.\textsuperscript{26,27} Synergism of LF and LF-derived peptides with FCN, itraconazole, amphotericin B and 5-fluorocytosine against \textit{Candida} spp has also been reported.\textsuperscript{18-22} A cationic peptide derived from the N-terminus region of LF (LFcin) has been reported to disrupt the fungal cell membrane and elicit fungicidal activity against \textit{C. albicans}.\textsuperscript{18,19} That group further reported cidal synergism between FCN and LFcin B (0.1 mg/mL) against an azole-resistant \textit{C. albicans} strain when tested at a low yeast inoculum of $5 \times 10^2$ yeast in low-ionic RPMI growth medium, for 15 hours.\textsuperscript{19} Cationic peptides cause cytolysis by inducing membrane perturbations of microbial and eucaryotic cells in low ionic environments.\textsuperscript{28} Thus, most cationic peptides, including LFcins, fail to function in physiologic saline.\textsuperscript{29,30} Unlike LFcin B, ALF demonstrated anti-\textit{Candida} efficacy and FCN synergism in high-ionic, iron-rich, regular SD broth against $10^5$ blastospore inocula over 48 hours of stasis. Moreover, ALF/FCN synergism resulted in stasis, independent of the species, strain or FCN-resistance characteristics of vaginal \textit{Candida}. The cidal lytic activity of cationic peptides could leave intracellular yeast debris, including mycotoxins, on a diseased, compromised vaginal mucosal surface; that is highly undesirable. Based on viable yeast recovery data, we clearly showed that ALF/FCN synergism is a stasis phenomenon. Furthermore, the microbial blocking activity of ALF reported in our earlier study could detach and exclude \textit{Candida} from the vaginal mucosa to complement fungistasis.

Lupetti et al also reported potentiation of FCN with noncandidal concentrations of an N-terminal peptide (1-11) of human LF.\textsuperscript{22} In their study, a higher inoculum, 6 logs (10\textsuperscript{6} yeast), of FCN-resistant \textit{C. albicans}, when treated with hLF peptide (8 μM) and FCN (0.2 mg/mL), showed 0.64- and 0.76-log viable yeast reduction, respectively, which aggregated to 1.4-log inhibition. A combination of both treatments caused a 1.86-log reduction, indicating that the reported phenomenon was an additive, not synergistic. Furthermore, survival of $>10^4$ cells of \textit{C. albicans} from hLF peptide/FCN treatment suggested limited antifungal activity of the combination. In our study the yeast generation time data suggested that ALF/FCN mixtures eliminated any budding or replication of \textit{C. albicans} and \textit{C. glabrata} at 5-log inocula with no detectable stasis recovery over 48 hours. Also, the ALF/FCN mixtures demonstrated true synergism to the extent of about 70% and 560% significantly greater efficacy (p < 0.0001) than ALF and FCN individual and combined treatment for \textit{C. albicans} and \textit{C. glabrata}, respectively.

In conclusion the ALF/FCN combination is a novel antifungal potentiation system that could be effective for clinical management of VVC. The cumulative effect of fungistasis and adhesion blockade activity, besides clearing the infection load, makes ALF a promising antimicrobial agent for protection of the vaginal mucosa.

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**References**