Candida albicans adhesion, invasion and damage of vaginal epithelial cells: stage-specific inhibition by clotrimazole and bifonazole

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running title: stage specific inhibition by azoles
Clotrimazole and bifonazole are highly effective antifungal agents against mucosal *Candida albicans* infections. Here we examined the effects of low levels of clotrimazole and bifonazole on the ability of *C. albicans* to adhere, invade and damage vaginal epithelial cells. Although adhesion and invasion were not affected, damage was greatly reduced upon azole treatment. This clearly indicates that low levels of azoles influence specific activities of *C. albicans* during distinct stages of vaginal epithelia infections.
The human pathogenic fungus *Candida albicans* is commonly found on mucosal surfaces, such as the vagina. Here, the fungus may persist as a harmless colonizer but can also cause disease. Around 75% of all women experience at least one episode of vulvo-vaginal candidiasis (VVC) in their lifetime (23). Some predisposing factors for VVC, such as diabetes or pregnancy, have been identified; however, VVC is also common among otherwise healthy women (8). Fungal adhesion to epithelium is a prerequisite for colonization and infections are characterized by invasion of vaginal epithelial cells (7, 22, 25). Additionally, hyphal formation contributes to symptomatic vaginal infections by damaging epithelial tissue (9, 24, 30). Therefore, a number of factors may be responsible for VVC but the exact pathogenicity mechanisms of vaginal *Candida* infections remain poorly understood.

Imidazoles, which block fungal ergosterol biosynthesis, have been proven to be effective against vaginal candidiasis (5). However, due to the pharmacokinetic properties of azoles, the major fraction of an applied dose remains on the surface or in the stratum corneum, leading to reduced therapeutic concentrations. Clotrimazole is one of the most commonly used imidazoles for the treatment of VVC and can persist at inhibitory levels in vaginal secretions for up to 3 days following a single treatment. (1, 15, 17). Bifonazole also inhibits the ergosterol biosynthetic pathway, but has higher fungicidal activity than clotrimazole due to additional inhibition of terpenoid biosynthesis (2). Furthermore, clotrimazole and bifonazole have been shown to affect *C. albicans* farnesol production, and to reduce virulence *in vivo* (2, 7, 14, 21). In this study we sought to dissect the effects of clotrimazole and bifonazole on *C. albicans* interactions with vaginal epithelial cells by analyzing adhesion and invasion, as well as epithelial damage in the presence of these two drugs.

*C. albicans* strain SC5314 (10, 13), was used for all experiments. Clotrimazole and bifonazole (Bayer AG) stocks were prepared in DMSO and used at final
concentrations of 0, 0.01, 0.1, 1, 10, 100 µM in indicated media. *C. albicans* growth
rates in liquid YPD were determined at 30 °C by measuring optical density at 600 nm
every 30 min using an Infinite M200 ELISA reader (16). Filamentation of *C. albicans*
was induced with RPMI-1640 (PAA) at 37°C on plastic surfaces. Interactions of *C.*
*albicans* with the vaginal epithelial cell line A-431 (Deutsche Sammlung von
Mikroorganismen und Zellkulturen GmbH, Germany - ACC 91) (12) were performed
as described previously (26). Briefly, *C. albicans* were co-incubated with A-431 cells
for 1, 3 or 24 h in DMEM medium supplemented with 1 or 10 µM clotrimazole or
bifonazole at 37°C and 5% CO₂. Following co-incubation, the number of adherent (1 h)
and invading (3 h) cells was determined by fluorescence microscopy as described
(26) and morphology was monitored after 24 h. Epithelial cell damage was measured
at 24 h by monitoring the release of LDH using the Cytotoxicity Detection Kit (LDH,
Roche Applied Science) according to the manufacturer’s instructions. All experiments
were performed in duplicates at least three times. The data were analyzed using
either 2way ANOVA to compare the relative growth or ANOVA (Dunnett’s multiple
comparison test) to compare treated versus untreated controls and *p*-values of *<
0.05 and **< 0.01 considered significant.

Bifonazole concentrations of 0.01 and 0.1 µM and clotrimazole concentrations of 0.01
µM had no significant influence on growth or filamentation of *C. albicans* under the
conditions tested. Treatment of *C. albicans* with 100 µM of either drug almost
completely blocked yeast growth (data not shown). Concentrations of 10 µM (both
azoles) and 1 µM (clotrimazole only) inhibited yeast growth, resulting in generation
times of over 4.3 h, compared to 2.5 h for untreated control (Tab. 1). Both azoles had
an even stronger effect on filamentous growth: bifonazole- (10 – 100 µM) and
clotrimazole- (0.1 – 100 µM) treatment strongly reduced the formation of long hyphae
in RPMI-1640 from 47 µm in the untreated control to around 18 µm in the presence of
azoles after 3 h of hyphal induction (Tab. 1). Bifonazole at 1 µM moderately inhibited filamentation (Tab. 1). Even after longer incubation periods of 24 hours, concentrations of 10 and 100 µM for both azoles and 1 µM for Clotrimazole had blocked the ability of C. albicans to form long filaments (data not shown). These data are in agreement with previous findings that clotrimazole and bifonazole inhibit both yeast growth and filamentation of C. albicans (4, 19, 27) and that hyphae are more effectively inhibited by these azoles than yeast cells (18). Based on the observed inhibitory effects on yeast growth and filamentation, we used clotrimazole and bifonazole at concentrations of 1 or 10 µM to analyze the effect on C. albicans-vaginal epithelial cell interactions.

Irrespective ofazole treatment, more than 98% of yeast cells formed germ tubes following 3 h incubation in contact with epithelial cells; however, treatment with clotrimazole or bifonazole strongly reduced the average filament length similar to RPMI induced filamentation (Fig. 1, Tab. 1). Approximately 5 - 15% (ranging from 2.92x10^3 to 1.49x10^4) of inoculated yeast cells adhered tightly to vaginal epithelium cells after 1 h. Adhesion was not affected by azole treatment, with or without azole pretreatment (Tab. 1 and data not shown), resulting in adherence of 5.81x10^3 to 7.46x10^3 cells. Similarly, azole treatment did not influence the invasion of epithelial cells by C. albicans (Fig. 1, Tab. 1). However, the invading (intracellular) portions of azole treated fungal cells were considerably shorter than without treatment and did not undergo inter-epithelial invasion (penetration from one epithelial cell to another adjacent cell) (Fig. 1). Therefore, although clotrimazole and bifonazole strongly reduced the formation of long filament of C. albicans, these azoles did not influence adhesion to, or initial invasion into vaginal epithelial cells. In contrast, both azoles had a striking effect on the ability of C. albicans to elicit cellular damage. Co-incubation of untreated C. albicans with vaginal epithelial cells resulted in the release of 157 ng/ml
LDH after 24 h (Fig. 2). The addition of 10 µM bifonazole, 1 µM clotrimazole or 10 µM clotrimazole prevented epithelial damage (Fig. 2, Tab. 1). Bifonazole at a lower concentration (1 µM) reduced epithelial damage by 76% compared to the untreated control (Fig. 2, Tab. 1).

Together these data demonstrate that low levels of clotrimazole and bifonazole, which had only moderate effects on yeast growth, did not affect adhesion or primary invasion, but prevented vaginal epithelial cell damage by *C. albicans*.

For the establishment of *C. albicans* colonization and infection of epithelial surfaces, adhesion to host cells is a prerequisite. During interaction with epithelial cells, adhesion and hyphal formation are linked: contact and adhesion stimulates hyphal formation and hyphal formation enhances adhesion (9, 29, 30). Interestingly, although treatment with clotrimazole or bifonazole resulted in shorter germ tubes, the actual percentage of yeast cells, which formed germ tubes was not affected (data not shown). Moreover, these short germ tubes retained full adhesive potential (Fig. 1, Tab. 1). This is in accordance with results from Odds *et al.*, (1988) (20), which showed that sub-MICs of ketoconazole and clotrimazole have no significant influence on *C. albicans* adhesion to vaginal epithelial cells. However, in other studies, sub-lethal concentrations of ketoconazole and/or fluconazole were shown to reduce adhesion of *C. albicans* to buccal epithelial cells (6) or endothelial cells (11), suggesting an azole- and/or host cell type-specific effect.

Moreover, the short hyphae produced by azole-treated *C. albicans* also invaded vaginal epithelial cells to the same degree as untreated cells (Tab. 1), indicating that azole treatment does not prevent initial epithelial invasion. Significantly, these azole-treated invasive hyphae were unable to damage vaginal epithelial cells, clearly demonstrating that initial fungal invasion is not sufficient to cause epithelial damage.
Importantly, this data implies that clotrimazole and bifonazole are capable of interfering with cellular damage caused by *C. albicans*, after invasion of vaginal epithelia. Consistent with these findings, fluconazole and voriconazole have been shown to reduce tissue destruction in reconstituted oesophageal epithelium when administered early after *C. albicans* infection (3). Although primary invasion was not affected by azole treatment, we did observe differences in the invasion patterns following treatment: secondary invasion of adjacent epithelial cells was not observed in the presence of clotrimazole or bifonazole (Fig. 1). After initial invasion into an epithelial cell, *C. albicans* hyphae do not persist within this primary epithelial cell (like some facultative intracellular bacteria), but further penetrate through the first epithelial cell and into adjacent epithelial cells (Wächtl and Hube, unpublished data). Such inter-epithelial invasion properties have been shown to depend on hyphal extension and to be essential for damage. We therefore speculate that inhibition of hyphal extension is responsible for the observed reduction in epithelial damage upon clotrimazole and bifonazole treatment.

Taken together, low levels of the two azoles, clotrimazole and bifonazole, have strong inhibitory effects on a distinct stage of *C. albicans*-vaginal epithelial cell infection: the ability to cause damage. Therefore, we conclude that treatment with clotrimazole or bifonazole, at levels which have only a moderate effect on fungal growth, may alleviate disease symptoms *in vivo* by preventing epithelial damage and possibly by reducing attraction of further tissue damaging neutrophils (28). Our data also suggest that targeting a distinct fungal virulence attribute or stage of infection is a reasonable and realistic antifungal strategy.
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Figure 1. Representative fluorescent pictures of *C. albicans* invasion into epithelial cells - untreated (a), treated with 1 µM bifonazole (b), treated with 1 µM clotrimazole (c). *C. albicans* hyphae are stained differentially: panel 1 – red - extracellular part of *C. albicans* hyphae; panel 2 – blue - extracellular and internalized *C. albicans* hyphae; panel 3 – overlay from panel 1 and 2, yellow - epithelial cell membranes; panel 4 – DIC. Arrows mark internalized hyphae. Numbers indicate epithelial cells invaded by *C. albicans*. Note that all hyphae have the capacity to invade epithelial cells, but that only untreated *C. albicans* hyphae penetrate through the first epithelial cell. Bar = 10 µm.

Figure 2. Damage of vaginal epithelial cells is inhibited by bifonazole and clotrimazole. Vaginal epithelial damage was determined by measuring LDH release. Bifonazole at 1 µM inhibited damage compared to untreated (Ctr) infections or infections with DMSO only (Ctr + DMSO); 10 µM bifonazole and 1 µM / 10 µM clotrimazole blocked damage entirely. *, significant difference compared to the untreated control ($p < 0.01$)

References


17. Mendling, W., and M. Plempel. 1982. Vaginal secretion levels after 6 days, 3 days and 1 day of treatment with 100, 200 and 500 mg vaginal tablets of clotrimazole and their therapeutic efficacy. Chemotherapy 28 Suppl 1:43-7.


Table 1. Summarized phenotypes of *C. albicans* wild type cells treated with 1 µM or 10 µM bifonazole and clotrimazole, respectively, compared to the untreated wild type control. Yeast growth in liquid YPD presented as generation time [h] during exponential growth phase. Filament length (in µm) was determined either following induction in RPMI in liquid cultures after 3 h or on vaginal epithelial cells in DMEM after 3 h of co-incubation. Adhesion to and invasion of vaginal epithelial cells compared to 100 % adhesion and invasion of the untreated control. Damage was calculated as LDH release in ng/ml by vaginal epithelial cells after 24 h. *p < 0.05, **p <0.01 significant difference compared to untreated control.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Time point</th>
<th>Untreated control</th>
<th>Bifonazole 1 µM</th>
<th>Bifonazole 10 µM</th>
<th>Clotrimazole 1 µM</th>
<th>Clotrimazole 10 µM</th>
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</thead>
<tbody>
<tr>
<td>Yeast growth (log phase)</td>
<td>2.53 ± 0.12</td>
<td>2.69 ± 0.07</td>
<td>5.20 ± 1.69*</td>
<td>4.26 ± 1.08*</td>
<td>6.23 ± 3.41**</td>
<td></td>
</tr>
<tr>
<td>Filament length (RPMI) [µm]</td>
<td>3 h</td>
<td>46.9 ± 17</td>
<td>33 ± 9.2*</td>
<td>17.5 ± 3.5**</td>
<td>19.1 ± 6.1**</td>
<td>18.5 ± 7.2**</td>
</tr>
<tr>
<td>Filament length (vaginal cells) [µm]</td>
<td>3 h</td>
<td>46.1 ± 3.8</td>
<td>27.3 ± 14.2**</td>
<td>23.3 ± 11.0**</td>
<td>19.2 ± 7.8**</td>
<td>16.9 ± 5.3**</td>
</tr>
<tr>
<td>Adhesion [%]</td>
<td>1 h</td>
<td>100</td>
<td>109.6 ± 34</td>
<td>99.6 ± 18.7</td>
<td>94 ± 26.9</td>
<td>109 ± 37.4</td>
</tr>
<tr>
<td>Invasion [%]</td>
<td>3 h</td>
<td>100</td>
<td>92.2 ± 6.7</td>
<td>101.5 ± 35</td>
<td>91.3 ± 30.5</td>
<td>91.8 ± 29.3</td>
</tr>
<tr>
<td>Damage [ng/ml]</td>
<td>24 h</td>
<td>157.0 ± 23.8</td>
<td>37.3 ± 47.5**</td>
<td>4.6 ± 22.6**</td>
<td>-0.7 ± 12.8**</td>
<td>-3.7 ± 9.0**</td>
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