Antifungal activity of clotrimazole against *Candida albicans* depends on carbon sources, growth phase and morphology

Lydia Kasper,1 Pedro Miramón,1† Nadja Jablonowski,1 Stephanie Wisgott,1 Duncan Wilson,1‡ Sascha Brunke1,2 and Bernhard Hube1,2,3

1Department of Microbial Pathogenicity Mechanisms, Leibniz Institute for Natural Product Research and Infection Biology – Hans Knoell Institute, Jena, Germany
2Integrated Research and Treatment Center, Sepsis und Sepsisfolgen, Center for Sepsis Control and Care (CSCC), University Hospital, Jena, Germany
3Friedrich Schiller University, Jena, Germany

Vulvovaginal candidiasis, a superficial infection caused predominantly by the pathogenic fungus *Candida albicans*, is frequently treated with clotrimazole. Some drug formulations contain lactate for improved solubility. Lactate may modify *C. albicans* physiology and drug sensitivity by serving as a carbon source for the fungus and/or affecting local pH. Here, we explored the effects of lactate, in combination with pH changes, on *C. albicans* proliferation, morphology and clotrimazole sensitivity. Moreover, we determined the influence of growth phase and morphology per se on drug sensitivity. We showed that utilization of lactate as a carbon source did not promote fast fungal proliferation or filamentation. Lactate had no influence on clotrimazole-mediated killing of *C. albicans* in standard fungal cultivation medium but had an additive effect on the fungicidal clotrimazole action under in vitro vagina-simulative conditions. Moreover, clotrimazole-mediated killing was growth-phase and morphology dependent. Post-exponential cells were resistant to the fungicidal action of clotrimazole, whilst logarithmic cells were sensitive, and hyphae showed the highest susceptibility. Finally, we showed that treatment of pre-formed *C. albicans* hyphae with sublethal concentrations of clotrimazole induced a reversion to yeast-phase growth. As *C. albicans* hyphae are considered the pathogenic morphology during mucosal infections, these data suggest that elevated fungicidal activity of clotrimazole against hyphae plus clotrimazole-induced hyphae-to-yeast reversion may help to dampen acute vaginal infections by reducing the relative proportion of hyphae and thus shifting to a non-invasive commensal-like population. In addition, lactate as an ingredient of clotrimazole formulations may potentiate clotrimazole killing of *C. albicans* in the vaginal microenvironment.

INTRODUCTION

The fungus *Candida albicans* is both a harmless commensal on human mucosal surfaces of the oral, gastrointestinal and vaginal tracts, and an important human pathogen. Under certain predisposing conditions, *C. albicans* can cause superficial but also life-threatening systemic infections. Vulvovaginal candidiasis (VVC) is arguably the most common superficial infection in otherwise healthy individuals, and *C. albicans* is the *Candida* sp. that is most frequently associated with VVC (Ashkar & Fries, 2010; Mendling & Brasch, 2012). Overall, 75% of women experience at least one episode of VVC in their lifetime, and 5–10% suffer from recurrent VVC (Sobel, 2007).

Acute VVC can be treated topically with polyenes, ciclopirox olamine or azoles (Mendling & Brasch, 2012; Sobel, 2013). Azoles, which inhibit fungal ergosterol biosynthesis, have proven to be effective in short-term treatment of vaginal candidiasis (Edelman & Grant, 1999), and clotrimazole is one of theazole antifungals used most commonly in this context (Plempel, 1982; Sobel, 2013). We have
shown recently that low levels of clotrimazole reduce but do not block fungal growth, and thus can effectively inhibit *C. albicans*-induced damage of vaginal epithelial cells *in vitro* (Wächter *et al.*, 2011). Furthermore, we found that these low clotrimazole levels are sufficient to dampen the vaginal inflammation response to *C. albicans* infection (Wilson *et al.*, 2013).

A 500 mg vaginal ovule of clotrimazole for 1-day treatment (Canesten GYN Once Kombi; Bayer) is supplied in an acidic formulation containing lactate, the ion salt of lactic acid, to improve drug solubility and bioavailability (Mendling & Plempel, 1982). Lactic acid is a natural component of vaginal secretions (Owen & Katz, 1999) and is believed to be generated mainly by vaginal lactobacilli (Boskey *et al.*, 2001). Clotrimazole ovule application may lead to a rise in vaginal lactate levels and may therefore affect *C. albicans* growth and clotrimazole sensitivity: altered levels of lactic acid/lactate may change the local pH in the vaginal cavity, which may have an impact on growth and membrane integrity of the infecting *C. albicans* cells. For example, the environmental pH affects the morphogenetic transition of *C. albicans* between the yeast and hyphal growth form (Biswas *et al.*, 2007), which is known to be critical for pathogenesis of candidal vaginitis (Sobel *et al.*, 1984). Recent studies have shown that growth on lactate as a carbon source affects *C. albicans* cell-wall properties, interaction with immune cells and virulence (Ene *et al.*, 2012a, b, 2013).

In this study, we analysed the possible effects of lactate in clotrimazole formulations on growth and drug sensitivity of *C. albicans*. We investigated the effect of pH changes as well as the impact of fungal growth phase and morphology on the clotrimazole antifungal activity. Finally, we determined the clotrimazole action under conditions mimicking the physiological environment in the vaginal cavity, by using a vagina-simulative medium (VSM) (Owen & Katz, 1999; Sosinska *et al.*, 2008) at CO₂ and O₂ levels typical for the vagina.

**METHODS**

**Strains and growth conditions.** Experiments were performed with the *C. albicans* clinical isolate SC5314 (Gillum *et al.*, 1984). Yeasts were cultured overnight at 30 °C and 180 r.p.m. in liquid YPD medium (1% yeast extract, 1% peptone, 2% glucose,) to post-exponential growth phase. To obtain yeasts in exponential growth phase, overnight pre-cultures were diluted to an OD₆₀₀ of 0.2 in fresh YPD and incubated for 4 h at 30 °C and 180 r.p.m. Liquid media with different carbon sources were prepared based on defined minimal medium [1 × yeast nitrogen base (YNB) with ammonium sulfate; BD Biosciences], and 2% glucose (111 mM; Roth) or 2% D/L-lactate (178 mM; Sigma) was added as carbon source. The media were buffered with 75 mM sodium tartrate (pH 3.0, 3.5, 4.0, 4.5, and 5.0), 75 mM potassium phosphate (pH 6.0) or 75 mM HEPE (pH 7.0 or 7.5). None of these buffers can be utilized as a carbon source by *C. albicans* (data not shown).

Microbial growth under vagina-simulative conditions was monitored in modified VSM (Owen & Katz, 1999; Sosinska *et al.*, 2008) consisting of 58 mM NaCl, 18 mM KOH, 2 mM Ca(OH)₂, 1.75 mM glycerol, 6.7 mM urea, 33 mM glucose and 6.7 g YNB L⁻¹, as well as lactic acid (22 mM) and acetic acid (17 mM), at a pH of 4.2. Cells in VSM were cultured in vagina-typical gas conditions of 6% CO₂ and 7% O₂ (Sosinska *et al.*, 2008). Clotrimazole (Bayer Vital GmbH) was added from a 25 mM stock in DMSO.

**Yeast growth analyses.** Growth curve experiments were performed in 1 × YNB medium (see above) with yeasts inoculated from a logarithmic pre-culture at a starting OD₆₀₀ of 0.05. Growth was measured by monitoring OD₆₀₀ per well every 30 min in a 200 µl volume in a 96-well plate (TPP) using a Tecan Infinite M200 microplate reader at 30 °C with intermittent shaking. Generation times were calculated after subtracting the OD₆₀₀ of medium controls.

**C. albicans survival assays.** Survival in the presence of clotrimazole was assayed for yeasts inoculated from logarithmic or post-exponential YPD pre-cultures (see above) to a cell number of 5 × 10⁵ ml⁻¹ in 1 × YNB medium or VSM (see above). Cells were incubated in glass vials with rotary shaking for 5 h. For evaluation of survival, cells were plated directly after inoculation (0 h) as well as after 5 h incubation on YPD plates, and c.f.u. were counted to calculate viable cell numbers ml⁻¹. The survival rate was then determined as the percentage of the 0 h value: % = (viable cell count at 5 h/viable cell count at 0 h) × 100.

**Hyphal growth analysis.** Hyphal growth was analysed by inoculating an overnight YPD pre-culture to a cell density of 4 × 10⁴ cells ml⁻¹ into 1 × YNB medium buffered to pH 7.5 (see above) or RPMI with fetal bovine serum (RPMI + FBS). Filamentation was induced by a temperature change to 37 °C at 5% CO₂ with contact induction on plastic (24-well plates; TPP). After fixation with 4% paraformaldehyde (Histofix; Roth), the morphology was evaluated by reverse microscopy (Axio Vert.A1; Zeiss).

**Statistical analysis.** The graphs show means ± SD of at least three experimental replicates. A statistical analysis was performed with a two-way ANOVA followed by an unpaired Student’s t-test with Bonferroni multiple comparison correction. Significant differences were determined at P < 0.05, P < 0.01 or P < 0.001.

**RESULTS**

**Influence of lactate and pH on yeast and hyphal growth**

Lactate in clotrimazole drug formulations may serve as a carbon source for *C. albicans* at the site of treatment or lead to pH changes, which in turn could influence fungal growth behaviour. To determine the impact of lactate/lactic acid and pH changes on *C. albicans in vitro*, we monitored proliferation and filamentation in YNB-based minimal medium over a pH range of 3.0–7.5 with lactate as the sole carbon source. At pH values above 4.0, lactate is present as an ion salt, whilst at lower pH values, lactic acid (pKₐ = 3.86) is present in the undissociated form. Glucose as a preferential carbon source for *C. albicans* was used for comparison. The results are summarized in Table 1.

As expected, yeast growth in medium containing lactate as a carbon source was generally reduced compared with growth in glucose-containing medium, as shown by prolonged generation times. In addition, in contrast to glucose-containing cultures, growth in lactate-containing cultures was more pH...
Table 1. Summarized phenotypes of C. albicans yeast and filamentous growth depending on pH and carbon source

Results are shown as means ± SD of three experimental replicates. Significant differences: *P<0.05; ** P<0.01; *** P<0.001 between glucose and lactate samples at the same pH; #P<0.05; ##P<0.01; ###P<0.001 compared with pH 4.5 as a vagina-typical pH.

<table>
<thead>
<tr>
<th>pH</th>
<th>Yeast generation time (h)†</th>
<th>Filamentation (%)††</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Lactate</td>
</tr>
<tr>
<td></td>
<td>3.0 1.49 ± 0.15***</td>
<td>11.28 ± 3.41###</td>
</tr>
<tr>
<td></td>
<td>3.5 1.29 ± 0.08**</td>
<td>6.27 ± 1.83</td>
</tr>
<tr>
<td></td>
<td>4.0 1.38 ± 0.30</td>
<td>4.83 ± 0.43</td>
</tr>
<tr>
<td></td>
<td>4.5 1.42 ± 0.13</td>
<td>4.13 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>5.0 1.52 ± 0.06</td>
<td>5.27 ± 1.19</td>
</tr>
<tr>
<td></td>
<td>6.0 1.42 ± 0.05</td>
<td>4.09 ± 0.76</td>
</tr>
<tr>
<td></td>
<td>7.0 1.46 ± 0.06**</td>
<td>6.80 ± 1.07</td>
</tr>
<tr>
<td></td>
<td>7.5 1.59 ± 0.15***</td>
<td>8.32 ± 4.46##</td>
</tr>
</tbody>
</table>

†Cells were grown in 1 × YNB minimal medium at 30 °C (yeast growth) or 37 °C and 5% CO2 (filamentous growth) with 2% glucose or 2% lactate as the carbon source.
††Filamentation rate (percentage of hyphal and pseudohyphal cells on total cell count) was determined after 6 h.

-dependent and was thus significantly reduced at strongly acidic pH (pH 3) and neutral/alkaline pH (pH 7.5) (Table 1). Of note, a combination of lactate and glucose led to a significant reduction in yeast growth compared with glucose alone (Fig. 1).

Induction of filamentous growth was analysed under hyphae-inducing conditions (37 °C and 5% CO2 on plastic). As observed previously (Biswas et al., 2007), pH values below 6.0 did not support filamentation of C. albicans, whilst filaments (hyphae and pseudohyphae) were present at pH 6.0, 7.0 and 7.5 after 6 h (Table 1). This was also true after 3 and 8 h (data not shown). The filamentation rate (percentage of cells forming filaments) in lactate-containing media was much lower than in glucose-containing media (Table 1), and filaments were generally much shorter (16.0 ± 6.9 μm for lactate; 46.5 ± 9.9 μm for glucose after 6 h, pH 7.0). A combination of glucose and lactate, however, allowed similar filamentation as with glucose alone (data not shown).

Taken together, lactate as the sole carbon source did not promote fast yeast growth or induce significant filamentation. In combination with glucose, lactate had a slight inhibitory effect on yeast growth. Changes in pH were well tolerated by glucose-grown yeasts but led to decreased growth with lactate at very low or high pH values.

**Influence of lactate and pH on growth inhibition and killing of C. albicans by clotrimazole**

As shown in our previous studies, clotrimazole levels in the low micromolar range reduce yeast as well as filament growth in a fungistatic fashion, by inhibition of ergosterol biosynthesis (Wächtler et al., 2011). Clotrimazole concentrations above 100 μM are known to have fungicidal effects on yeasts, probably by a distinct mechanism independent of ergosterol biosynthesis via direct physicochemical membrane damage (Iwata et al., 1973; Mendling et al., 1982; Sud & Feingold, 1981). By affecting C. albicans growth and morphology, lactate may interfere with these fungistatic and fungicidal drug actions of clotrimazole.

To determine the effect of pH and carbon source on the fungistatic activity of clotrimazole, we recorded growth curves in the presence of 1, 10 or 100 μM clotrimazole (Fig. 2a). Cell densities reached in the absence of the drug were lower in lactate-grown cultures than in glucose-grown cultures, consistent with the slow growth rate with lactate as a carbon source (Table 1, Fig. 1). In the presence of glucose, a significant growth reduction compared
with untreated control samples was evident at all three tested clotrimazole concentrations. Lactate-containing cultures showed a growth reduction for clotrimazole concentrations of 10 and 100 \( \mu \text{M} \) but not for 1 \( \mu \text{M} \). Similar trends were seen for the different pH conditions.

To determine whether the fungicidal action by clotrimazole on yeast cells was influenced by lactate or pH, we monitored \( C. \text{ albicans} \) survival by recording the number of c.f.u. by plating cells recovered after 5 h incubation with clotrimazole. As relevant concentrations with clearly discernible effects on fungal growth, we chose 100 \( \mu \text{M} \) clotrimazole, expected to be in the lower limit of fungicidal drug action, and 400 \( \mu \text{M} \) clotrimazole, expected to have clear fungicidal effects (Sud & Feingold, 1981). C.f.u. numbers after treatment were compared with the initial inoculum to detect possible fungicidal drug effects (c.f.u. counts lower than that of the inoculum; % survival <100%). Yeast proliferation (% survival >100%) was observed in control samples containing no drug in the presence of glucose as carbon source (glucose or glucose + lactate) but not in the presence of lactate alone or without any carbon source (DMSO-treated samples; Fig. 2b). Treatment with

---

**Fig. 2.** Effect of carbon source and pH on growth inhibition of \( C. \text{ albicans} \) by clotrimazole. (a) Logarithmic \( C. \text{ albicans} \) pre-cultures were inoculated in 1 \( \times \) YNB medium with 2 % glucose or 2 % lactate to an OD\(_{600}\) of 0.05. Growth was monitored in the presence of 1, 10 or 100 \( \mu \text{M} \) clotrimazole or DMSO (vehicle control) at 30 °C to promote growth in the yeast form. Shown is the difference in the maximum and minimum OD\(_{600}\) reached during 24 h incubation. (b) Logarithmic \( C. \text{ albicans} \) pre-cultures were inoculated in 1 \( \times \) YNB medium with 2 % glucose, 2 % lactate, 2 % glucose + 2 % lactate or without a carbon source, buffered to pH 4.5 or 7.5, to a starting cell density of \( 5 \times 10^5 \text{ ml}^{-1} \). Cells were incubated for 5 h at 30 °C with 100 or 400 \( \mu \text{M} \) clotrimazole or DMSO. Shown are survival rates on a logarithmic scale, calculated from c.f.u. counts of yeasts plated before and after clotrimazole treatment. Significant differences: **\( P < 0.01 \), ***\( P < 0.001 \), compared with DMSO controls at the respective pH; *\( P < 0.05 \), ***\( P < 0.001 \), compared with pH 4.5 as a typical pH of the vagina.
100 µM clotrimazole was not sufficient to cause fungal killing at pH 4.5 and even allowed yeast proliferation in glucose-containing samples. However, a low fungicidal effect was observed in samples buffered to pH 7.5. In contrast, 400 µM clotrimazole caused clear fungicidal effects independent of pH and carbon source.

These data showed that growth with either glucose or lactate as a carbon source can be inhibited by clotrimazole. Furthermore, the sensitivity of *C. albicans* to the fungicidal activity of clotrimazole was not overly influenced by lactate in standard cultivation medium.

**Lactate affects clotrimazole sensitivity of *C. albicans* under vagina-simulative conditions**

*In vitro* experiments often do not reflect key physiological aspects of the *in vivo* setting. In the vaginal cavity, for example, other nutrients and carbon sources besides glucose and lactate are likely to affect *C. albicans* growth, morphology and drug sensitivity. To improve analysis of clotrimazole sensitivity in an *in vitro* system, we made use of a VSM, mimicking the physiological conditions during growth in the vaginal cavity. The medium composition is based on known concentrations and constituents of the vaginal fluid of healthy pre-menopausal women (Owen & Katz, 1999; Sosinska et al., 2008). Besides lactate and glucose, the medium contains glycerol and acetic acid as potential carbon sources for *C. albicans*, and is buffered to a pH of 4.2. This pH reflects the expected situation during vaginal infection, as *Candida* vaginitis is associated with a physiological pH of 4.0–4.5 (Linhares *et al.*, 2011). To determine the influence of physiological and altered lactate concentrations in this system, we modified the VSM-typical lactate concentration of 22 mM to 0, 22, 50 or 100 mM. Experiments were performed at 37 °C under vagina-typical gas conditions (6% CO₂ and 7% O₂) (Sosinska *et al.*, 2008) and were compared with atmospheric gas conditions (~0.04% CO₂ and ~21% O₂). As described previously, under these conditions, the cells grew predominantly in non-hyphal forms (Sosinska *et al.*, 2008).

We monitored fungal survival (i.e. fungicidal drug activity) by recording the number of c.f.u. from cells recovered after 5 h treatment with clotrimazole and comparing with the number of c.f.u. of the initial inoculum. Similar to our experiments in 1 × YNB medium with comparable pH (Fig. 2b, pH 4.5), we observed a low inhibitory effect of 100 µM clotrimazole when incubated under atmospheric gas conditions. Importantly, under vagina-simulative gas conditions, the sensitivity of *C. albicans* to the drug was increased (Fig. 3). Survival was lowest with 22 mM lactate. In addition, vagina-typical lactate levels promoted growth in DMSO-treated control samples, as cells in medium with 22 mM lactate reached the highest viable cell count under both gas conditions. Treatment with 400 µM clotrimazole led to complete killing of the yeast independent of lactate or gas conditions.

In conclusion, under *in vitro* vagina-simulative conditions, lactate had a positive effect on the fungicidal action of clotrimazole.

![Fig. 3. Clotrimazole sensitivity of *C. albicans* under vagina-simulative conditions. Logarithmic *C. albicans* pre-cultures were inoculated in VSM with 0, 22, 50 or 100 mM lactate at a starting cell number of 5 × 10⁶ ml⁻¹. Clotrimazole was added at a concentration of 100 or 400 µM. Control samples contained DMSO (vehicle control). Cells were incubated for 5 h at 37 °C under atmospheric (~0.04% CO₂ and ~21% O₂) or vagina-typical (6% CO₂, 7% O₂) gas conditions. Shown are survival rates on a logarithmic scale, calculated from c.f.u. counts of yeasts plated before and after clotrimazole treatment. Significant differences: *P*<0.05, **P*<0.01, ***P*<0.001 compared with untreated controls at the respective lactate concentration; #P<0.05, ###P<0.001 compared with corresponding sample without lactate.](image-url)
Clotrimazole sensitivity depends on growth phase and morphology

At the onset of treatment of VVC, the vaginal \textit{C. albicans} population is likely to consist of a mixture of cells in different growth phases (actively growing or stationary phase cells) and morphologies (yeast or hyphal growth). We therefore investigated the effect of lactate on clotrimazole antifungal activity against different growth phases and morphologies of \textit{C. albicans}. As \textit{C. albicans} grows predominantly in non-hyphal forms in VSM medium (Sosinska et al., 2008), we used standard YNB-based minimal medium for these experiments. \textit{C. albicans} cells were incubated in the respective media in the presence or absence of clotrimazole, and survival was monitored by recording c.f.u. from cells recovered after 5 h incubation. The fungicidal effects of 400 \( \mu \)M clotrimazole on yeasts at 30 \( ^\circ \)C presented in Fig. 2(b) were with logarithmic pre-cultures. In contrast, with yeasts pre-grown to post-exponential phase, clotrimazole was not fungicidal under any of the tested carbon sources and pH conditions at 30 \( ^\circ \)C (Fig. 4a).

To test the influence of fungal morphology, cells were cultured under conditions that promote hypha formation (pH 7.5, 37 \( ^\circ \)C) in the absence or presence of clotrimazole. Strikingly, under these conditions, clotrimazole exhibited exceptionally high fungicidal activity (Fig. 4b, right panel). Parallel samples at pH 4.5 (Fig. 4b, left panel) showed no such fungicidal effect at 100 \( \mu \)M clotrimazole but, compared with incubation at 30 \( ^\circ \)C (Fig. 2b, left panel), a higher sensitivity to 400 \( \mu \)M clotrimazole was observed. The differences in drug sensitivity were independent of the presence of lactate.

Fig. 4. \textit{C. albicans} growth phase affects clotrimazole sensitivity. Post-exponential (a) or logarithmic (b) \textit{C. albicans} pre-cultures were inoculated in 1 \( \times \) YNB medium with 2\% glucose, 2\% lactate, 2\% glucose + 2\% lactate or without a carbon source, buffered to pH 4.5 or 7.5, at a starting cell density of 5 \( \times 10^5 \) ml\(^{-1}\). Cells were incubated for 5 h with 100 or 400 \( \mu \)M clotrimazole or DMSO at 30 \( ^\circ \)C (a) or 37 \( ^\circ \)C (b). Shown are survival rates on a logarithmic scale, calculated from c.f.u counts of yeasts plated before and after clotrimazole treatment. Significant differences: *\( P < 0.05 \), ***\( P < 0.001 \), compared with DMSO controls at the respective pH; *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \), comparing samples at pH 4.5 and 7.5.
Taken together, drug sensitivity was strongly affected by growth phase and temperature, with increased sensitivity of logarithmic-phase cells compared with post-exponential-phase cells and stronger fungicidal effects at 37 °C. Maximum sensitivity to clotrimazole was achieved under conditions promoting hyphal growth.

Inhibition of hyphal elongation and maintenance by clotrimazole

As hyphal morphology plays a critical role in the pathogenesis of candidal vaginitis (Sobel et al., 1984), we aimed to directly study the effect of clotrimazole on hyphal growth. Previous experiments focused on the induction of filamentation in the presence of the drug (Odds et al., 1985; Wächter et al., 2011). However, during treatment of VVC, a significant portion of the C. albicans population has probably already formed hyphae. We were thus interested in the impact of clotrimazole on pre-formed hyphae. We incubated C. albicans under hyphae-inducing conditions (on plastic, 37 °C, neutral pH) in 1 × YNB medium with 2% glucose or in strong hyphae-inducing RPMI with 10% fetal bovine serum (RPMI + FBS). After an initial 3 h incubation in the absence of drug, the formed hyphal cells were exposed to clotrimazole or DMSO (control) for 3 h. Fresh medium without clotrimazole was then added to follow the resumption of hyphal growth after treatment for a further 3 h.

Experiments in 1 × YNB medium showed that even low clotrimazole concentrations (1 μM) were sufficient to inhibit hyphal elongation. Hyphal length was significantly reduced in clotrimazole-treated samples as compared with DMSO controls (Fig. 5a, c). Interestingly, following the removal of clotrimazole (post-incubation 3 h), growth inhibition was maintained. Low azole concentrations (1 and 10 μM) permitted very low levels of hyphal elongation (24 μm at the end of the pre-incubation to 39 μm after 3 h in the presence of 1 μM clotrimazole), whilst 100 μM clotrimazole completely blocked hyphal elongation. This, in addition to the results obtained for clotrimazole-mediated killing under similar conditions (Fig. 4b), suggested that 100 μM clotrimazole may be fungicidal to C. albicans hyphae.

Experiments in RPMI + FBS gave similar results. Again, a growth-inhibitory effect of low clotrimazole concentrations was observed, whilst 100 μM clotrimazole completely blocked hyphal growth (Fig. 5b, d). Hyphae formed in RPMI + FBS were longer than in 1 × YNB medium, and hyphal branching was observed regularly (Fig. 5d). Interestingly, treatment with 1 or 10 μM clotrimazole reversed the hyphae growth programme and induced growth of secondary yeast cells (yeast cells that originate from hyphal cells; Fig. 5d, lower panels).

Taken together, these data showed that low clotrimazole concentrations are sufficient to inhibit filament elongation when added to hyphal cells, and can even induce reversion to yeast morphology. Higher clotrimazole concentrations (100 μM) completely block hyphal elongation, possibly due to direct fungicidal action on hyphal cells.

DISCUSSION

Influence of lactate on C. albicans growth and clotrimazole sensitivity

The presence of lactate, either as a natural compound of the vaginal fluid or as an ingredient in drug formulations, can potentially alter the clotrimazole sensitivity of C. albicans. These effects could be due to lactate-induced pH changes or utilization of lactate as a carbon source.

The data presented here showed that, compared with glucose, lactate is a poor carbon source for C. albicans, supporting only slow growth and not promoting a significant shift to hyphal growth at neutral pH. Slow growth, especially at a pH below 4.0, as well as slight inhibition of growth in glucose cultures with added lactate can possibly be explained by weak acid stress in presence of undissociated lactic acid (pKₐ=3.86) (Davis, 2009). Besides the effect of lactate on fungal growth described here, Ene et al. (2012a) showed lactate-dependent changes to C. albicans cell-surface properties, stress resistance and drug sensitivity.

We did not detect any influence of lactate on clotrimazole sensitivity, either in the form of growth inhibition or as fungal killing, in standard culture medium (1 × YNB). Additionally, under yeast growth-favouring conditions (30 °C), the medium pH had little influence. This is in agreement with previous studies that showed C. albicans susceptibility to azoles at neutral but also low pH (Danby et al., 2012).

In contrast, cultivation in VSM, especially under physiological gas conditions of 6% CO₂ and 7% O₂, increased the sensitivity of C. albicans to clotrimazole in the presence of lactate. These data showed that clotrimazole is more effective in an environment that mimics the physiological conditions during vaginal colonization than in standard laboratory growth medium. Similar tendencies were found for fluconazole in a previous study (Moosa et al., 2004). One possible reason may be a synergistic action of lactate with the acetic acid present in VSM, by causing weak acid stress (Costa et al., 2013; Moosa et al., 2004), which may potentiate the drug-generated stress. In addition, different fungal replication rates in vagina-simulative compared with standard atmospheric gas conditions, as described by Sosinska et al. (2008), may influence drug sensitivity.

Consequently, we showed that lactate as a supplement of clotrimazole drug formulations can be beneficial not only for drug solubility but also by providing an additive positive effect by the inhibition of C. albicans growth. These in vitro data can be seen as a basis for future in vivo analyses, for example in a mouse model of vaginal candidiasis (Stevens et al., 2002), to analyse whether the additional beneficial effect of lactate holds true in an in vivo setting and to obtain more information on the general suitability of lactate addition to vaginal clotrimazole preparations for the treatment of patients.
Determinants of *C. albicans* clotrimazole sensitivity

(a) YNB glucose pH 7.5

(b) RPMI + FBS

(c) YNB glucose pH 7.5

(d) RPMI + FBS
Fig. 5. Clotrimazole inhibits hyphal elongation and maintenance. Overnight C. albicans pre-cultures were inoculated in 1 × YNB medium with 2 % glucose at pH 7.5 (a, c) or in RPMI with 10 % fetal bovine serum (RPMI + FBS) (b, d), at a starting cell density of 4 × 10^6 ml^-1 in 24-well-plates. After a pre-incubation for 3 h at 37 °C and 5 % CO₂, clotrimazole (1, 10 or 100 μM) or DMSO (vehicle control) was added and cells were incubated for additional 3 h. After a washing step to remove exogenous clotrimazole, cells were incubated for a further 3 h in fresh medium without the drug. Cells were fixed with 4 % paraformaldehyde and analysed by inverse microscopy. (a, b) Quantification of hyphal length. Quantification was not possible in RPMI + FBS post-incubation samples due to extensive branching (ν, not determined). Significant differences: *P<0.05, **P<0.01, ***P<0.001, compared with DMSO-treated controls at the respective time points; ****P<0.001, compared with 3 h sample. (c, d) Representative microscopic images. Examples of secondary yeast cells are indicated with white arrowheads.

Our observations are also relevant for understanding the interplay of C. albicans with bacteria during vaginal colonization and disease. Lactobacilli have been shown to directly inhibit C. albicans growth in vitro by producing lactic acid (Boskey et al., 2001; Köhler et al., 2012). Several studies have associated VVC with a low number of lactobacilli in the vagina (Mendling & Brasch, 2012). In this setting, one would expect lower levels of vaginal lactate, which could in turn be reversed by administration of the lactate-containing clotrimazole drug.

Influence of growth phase and morphology on C. albicans clotrimazole sensitivity

At the onset of VVC treatment, the vaginal C. albicans population is likely to consist of a mixture of cells in different growth phases and in different morphological states. These subpopulations may be differentially susceptible to the fungistatic (inhibition of ergosterol biosynthesis) and/or fungicidal action (direct physicochemical membrane damage) of clotrimazole (Iwata et al., 1973; Sud & Feingold, 1981). Two earlier studies addressed this question, focusing on distinct growth states. Whilst Niimi et al. (1985) found an increased sensitivity of C. albicans hyphae compared with stationary yeast cells to the fungicidal action of clotrimazole, Beggs et al. (1987) described how stationary cells are resistant to clotrimazole killing whilst logarithmic cells are killed effectively. In this study, we directly compared the clotrimazole sensitivity of logarithmic- and post-exponential-phase yeast cells, as well as hyphae. Our data confirmed that fungal cells in the exponential growth phase are much more sensitive to clotrimazole than post-exponential-phase cells. Importantly, we demonstrated that sensitivity of C. albicans to 100 μM clotrimazole was strongly increased under hyphae-inducing conditions (37 °C, pH 7.5). These data were supported by the complete lack of elongation of hyphal cells treated with 100 μM clotrimazole, again suggesting a direct fungicidal action of clotrimazole against this C. albicans morphotype. Thus, our data imply that clotrimazole-mediated killing depends on both the growth phase and the morphological state of C. albicans, with hyphal cells being the most sensitive.

Clotrimazole concentrations in the vaginal fluid during and after treatment with the vaginal ovule are expected to be high in the fungicidal range (Mendling & Plempel, 1982). However, drug levels inside vaginal epithelia encountered by invading fungal cells may be much lower. Interestingly, we observed that even these low, non-lethal clotrimazole concentrations strongly inhibited the elongation of pre-formed hyphae and even induced a reversion to the yeast growth form. One likely explanation for this phenomenon is an elevated production of the quorum-sensing molecule farnesol due to drug treatment (Hornby & Nickerson, 2004). Inhibition of hyphal formation from yeast cells has been shown previously by addition of clotrimazole preceding the hyphae-inducing conditions (Odds et al., 1985; Wächterl et al., 2011). We showed here that inhibition of hyphal elongation even occurred in C. albicans cells that had already undergone the yeast-to-hypha transition. This is significant because, by the time clotrimazole is administered therapeutically (i.e. following the onset of symptoms), C. albicans cells will probably have already formed hyphae. Invasive hyphal growth probably occurs concomitant with epithelial damage and induction of a pro-inflammatory immune response during acute vaginal C. albicans infection (Moyes et al., 2010). As hyphae are more sensitive to the fungicidal action of clotrimazole, and sublethal clotrimazole levels promote a morphological transition from hyphae to yeast, we thus propose the following mechanisms for clotrimazole resolution of acute vaginal infections: initial drug treatment, associated with high levels of clotrimazole, reduces the relative proportion of hyphae in the infecting C. albicans population by selectively killing this cell type. In the vaginal microenvironment, this strong fungicidal effect may be even potentiated by the presence of lactate. Subsequent lower levels of clotrimazole induce yeast over hyphal growth, thus promoting the formation and maintenance of a non-invasive commensal C. albicans population. This is associated with reduced vaginal cell damage and reduced inflammation (Wächterl et al., 2011; Wilson et al., 2013). This is associated with reduced vaginal cell damage and reduced inflammation. In summary, the therapeutic activity of antifungal drugs, such as clotrimazole, appears to be the net result of a complex interplay of multiple factors, including fungal killing, growth inhibition, morphological manipulation and dampened inflammation.

ACKNOWLEDGEMENTS

This work was funded by Bayer Vital and supported by the resources and facilities at the Hans Knoell Institute Jena, Germany. We thank Melanie Kahl for excellent assistance with the laboratory work.
REFERENCES


Mending, W. & Plempel, M. (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), 43–47.


