Fluconazole treatment hyperpolarizes the plasma membrane of Candida cells

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Five pathogenic Candida species were compared in terms of their osmotolerance, tolerance to toxic sodium and lithium cations, and resistance to fluconazole. The species not only differed, in general, in their tolerance to high osmotic pressure (C. albicans and C. parapsilosis being the most osmotolerant) but exhibited distinct sensitivities to toxic sodium and lithium cations, with C. parapsilosis and C. tropicalis being very tolerant but C. krusei and C. dubliniensis sensitive to LiCl. The treatment of both fluconazole-susceptible (C. albicans and C. parapsilosis) and fluconazole-resistant (C. dubliniensis, C. krusei and C. tropicalis) growing cells with subinhibitory concentrations of fluconazole resulted in substantially elevated intracellular Na⁺ levels. Using a diS-C₃(3) assay, for the first time, to monitor the relative membrane potential (ΔΨ) of Candida cells, we show that the fluconazole treatment of growing cells of all five species results in a substantial hyperpolarization of their plasma membranes, which is responsible for an increased non-specific transport of toxic alkali metal cations and other cationic drugs (e.g., hygromycin B). Thus, the combination of relatively low doses of fluconazole and drugs, whose import into the tested Candida strains is driven by the cell membrane potential, might be especially potent in terms of its ability to inhibit the growth of or even kill various Candida species.

Keywords fluconazole resistance, sodium tolerance, membrane potential, combinatorial stress, Candida

Introduction

Yeasts belonging to the genus Candida are associated with infections of immunocompromised patients and cause life-threatening invasions of the bloodstream and organs [1,2]. The virulence of various Candida species depends on their ability to survive under a broad range of stress conditions caused by nutrition availability in host tissues, immune cells or antimycotics. Osmotic stress, among others, is an obstacle to overcome, as it may play an important role in the progress of the infection [3] and the cell salt tolerance is a key physiological parameter. Candida species are, in general, osmotolerant yeasts [4], and similarly to many other yeast species, including the model Saccharomyces cerevisiae, Candida cells maintain a low intracellular concentration of toxic sodium or lithium cations (in the μM range) but a relatively high concentration (200–300 mM) of potassium cations, which plays an important role in many biological processes such as the regulation of cell volume, pH and membrane potential [5]. To maintain an optimally high intracellular ratio between potassium and sodium, yeast cells use a broad range of various transport mechanisms and substrate specificities to mediate the uptake and efflux of alkali metal cations. For an efficient efflux of toxic sodium and lithium cations or surplus potassium, yeasts employ two active transporters, a Na⁺(K⁺)/H⁺ antiporter and a Na⁺(K⁺)-ATPase [5,6].

Candida species differ in their salt tolerance. A direct comparison of four Candida species [4] showed that C. albicans is relatively osmotolerant, but C. parapsilosis...
can grow in the presence of even higher concentrations of external salts and tolerates the lowest intracellular K\(^+\)/Na\(^+\) ratio. \emph{Candida dubliniensis} is in contrast relatively osmo-sensitive. These findings correlate with the characterization of Na\(^+\)(K\(^+\))/H\(^+\) antiporters of the three \emph{Candida} species upon expression in \emph{S. cerevisiae}. The Na\(^+\)(K\(^+\))/H\(^+\) antiporter of the most halotolerant \emph{C. parapsilosis} had a much higher transport activity than the antiporter of the most osmosensitive \emph{C. dubliniensis} [7]. Besides the low activity of its Na\(^+\)(K\(^+\))/H\(^+\) antiporter, \emph{C. dubliniensis}'s Na\(^+\)-ATPase is also much less expressed and active than its homologue in the closely related but much more osmotolerant \emph{C. albicans} [8]. As some of the yeast alkali-metal-cation transporters do not exist in mammalian cells (e.g., Trk potassium-uptake systems or Ena Na\(^+\)-ATPases), they may serve as possible antifungal drug targets.

An important factor in the development of candidemia is the species-specific susceptibility to antifungal agents. The introduction of treatment with azoles in the 1980s resulted on the one hand in a decrease in infections caused by relatively fluconazole-susceptible \emph{C. albicans} strains, and on the other hand in a rapid increase of non-\emph{C. albicans} infections caused by species resistant to azoles. Azoles block the synthesis of ergosterol via the inhibition of 4\(\alpha\)-demethylase, which in turn results in a change in the composition of the plasma membrane (higher lanosterol content) and cell wall (changed chitin/glucans ratio) in \emph{C. albicans}, \emph{C. krusei}, \emph{C. parapsilosis} and \emph{C. tropicalis} [9]. Fluconazole (FLC) is one of the most frequently used azoles in both the therapy and prophylaxis of candidemia thanks to its suitable and reliable pharmacokinetic parameters [10–12] and many studies were devoted to characterizing its activity in detail [13]. Using the model yeast \emph{S. cerevisiae}, FLC was shown to modulate plasma-membrane parameters such as rigidity, heterogeneity, and water penetration [14] that influence numerous cell activities. For example, ergosterol depletion leads to the inactivation of vacuolar ATPase accompanied by an impaired vacuolar acidification that causes alterations in the secretome and wall proteome of \emph{C. albicans} cells [15]. The presence of FLC also affects the tolerance of \emph{C. albicans} to salts. Subinhibitory concentrations of FLC increased the intracellular content of toxic sodium cations in both FLC-susceptible and FLC-resistant \emph{C. albicans} strains [16].

In this study, we aimed to answer the question of whether the observed combination effect of subinhibitory concentrations of FLC and NaCl is species-specific and what mechanism is responsible for the increased sodium content in \emph{Candida} cells in the presence of FLC. We focused on five clinically important species differing in many biological aspects and used the most frequently isolated species \emph{C. albicans}, its closest relative \emph{C. dubliniensis}, the naturally FLC-resistant \emph{C. krusei}, the halotolerant \emph{C. parapsilosis} and the species associated with neutropenia and malignancy, \emph{C. tropicalis} [4,17–21]. First, we characterized the salt tolerance of \emph{C. krusei} and \emph{C. tropicalis} and compared it with the other three species. We then studied the combinatory effect of FLC and salts on the five species and measured the cation content in cells. In order to estimate the relative membrane potential of cells in various growth conditions, we optimized a fluorescence diS-C\(_3\)(3) assay for \emph{Candida} species. We show for the first time that FLC-treatment results in a plasma-membrane hyperpolarization and that the combination effect of low FLC and salt concentrations is caused by an increased uptake of alkali metal cations that is driven by the higher plasma-membrane potential.

### Materials and methods

#### Strains, media and growth conditions

The commonly used laboratory strains \emph{C. albicans} SC5314, \emph{C. dubliniensis} CD36, \emph{C. krusei} ATCC6258, \emph{C. parapsilosis} CBS604 and \emph{C. tropicalis} ATCC750 were employed. Cells were grown in YPD medium (1% yeast extract, 2% Bacto peptone, and 2% glucose) supplemented as indicated at 30°C. Agar (2%) was added to allow for solidification of the plates. Salts or sorbitol were added before autoclaving, drugs (fluconazole; Ardez Pharma, Kosor, Czech Republic; 2 mg/ml aqueous stock solution and hygromycin B (MP Biomedicals, Santa Ana, CA, USA); 50 mg/ml aqueous stock solution) were sterilized by filtration and added to the media after autoclaving.

The phenotypes of the species in the presence of salts, FLC and hygromycin B were studied both on solid and in liquid media. Classical drop tests were performed to compare the growth capacity of \emph{Candida} species. Yeast cells (grown overnight on a fresh YPD plate without supplements) were resuspended in sterile water to the same initial OD\(_{600}\) (approx. 1). Ten-fold serial dilutions were prepared (grown overnight on a fresh YPD plate without supplements) were resuspended in sterile water to the same initial OD\(_{600}\) (approx. 1). Ten-fold serial dilutions were prepared and 3 \(\mu\)l aliquots spotted on a series of plates containing the indicated concentrations of supplements. Plates were incubated at 30°C for 3 or 7 d, and digital greyscale images of growing colonies were taken using a Nikon Coolpix 7000 camera. Each drop test was repeated at least three times and representative results are shown. To compare the growth in liquid media, an E1x808 BioTek 96-well plate reader was used [22]. Cells growing overnight in YPD were diluted to OD\(_{600}\) = 0.002 in YPD with supplements at the indicated concentrations. Aliquots (100 \(\mu\)l) were distributed into the plate wells and their growth at 30°C was monitored over 48 h. Gen5 software (BioTek, Prague, Czech Republic) was used to determine the V\(_{\text{max}}\) value for each growth curve by performing linear regression, calculating the slope for each curve and reporting the steepest slope as the
\( V_{\text{max}} \) (mOD \(_{600}\)/min). Growth rate was determined in quadruplicate in three separate experiments. The plotted values of \( V_{\text{max}} \) are the mean ± standard error of three separate experiments.

**Sodium content assay**

To estimate the intracellular sodium content, cells growing overnight in YPD were diluted in 20 ml of fresh YPD with or without fluconazole to OD\(_{600}\) = 0.15 and incubated at 30°C to OD\(_{600}\) = 0.6. As yeast cells grown under standard conditions maintain a very low intracellular concentration of toxic Na\(^+\) (on the order of \(\mu\)M), the cells need to be pre-loaded with NaCl before [Na\(^+\)]\(_{\text{in}}\) measurement. For the sodium preloading, fresh YPD supplemented with NaCl (and fluconazole when indicated) was added to the cell culture and cultivation followed at 30°C for 60 or 120 min. The cell culture was then harvested, washed with H\(_2\)O, and the pellets resuspended in a buffer containing 20 mM MES (2-(N-morpholino)-ethanesulfonic acid), 0.1 mM MgCl\(_2\) and adjusted with Ca(OH)\(_2\) to pH 5.5. Three aliquots of cells were withdrawn immediately, filtered, acid extracted, and the concentration of Na\(^+\) in the extracts was estimated by atomic absorption spectroscopy [23]. The plotted values are the mean ± standard error (SE) of three separate experiments.

**Relative membrane potential assay**

The fluorescence assay for monitoring relative membrane-potential changes was adapted from [24–26] as follows: cells grown overnight in YPD were diluted in 20 ml of fresh YPD with or without fluconazole to OD\(_{600}\) = 0.15 and incubated at 30°C to OD\(_{600}\) = 0.6. Cells were harvested, washed twice with a MES-TEA buffer (10 mM MES adjusted with triethanolamine to pH 6.0), and resuspended in the same buffer to OD\(_{600}\) = 0.2. A diS-C\(_3\)(3) (3,3-dipropylthiacarbocyanine iodide) fluorescence probe (Sigma-Aldrich, St Louis, MO, USA; 0.1 mM stock solution in ethanol) was added to 3 ml of cell suspension to a final probe concentration of 0.2 \(\mu\)M. When indicated, fluconazole was added to the cell suspension in MES-TEA buffer immediately after the fluorescence probe just before the fluorescence measurement. CCCP (carbonyl cyanide p-chlorophenylhydrazone, Sigma-Aldrich; 50 mM stock solution in dimethyl sulfoxide) or amiodarone (Sigma-Aldrich; 20 mM stock solution in dimethyl sulfoxide) were added to the measured samples to the final indicated concentration when the fluorescence signal reached its equilibrium. Fluorescence emission spectra were measured with an ISS PC1 spectrofluorometer. The excitation wavelength was 531 nm, and emission intensities were measured at 560 and 580 nm. The staining curves (i.e., the dependence of the emission intensity ratio I\(_{580}/I_{560}\) on the duration of staining \(t\)) were fitted as described in [27], and the value of the intensity ratio at equilibrium was estimated. The shown values are the mean ± SE of three separate experiments.

**Results**

First, we compared the salt tolerance of \(C.\) krusei and \(C.\) tropicalis to the salt tolerance of the other three species whose salt tolerance we had compared previously [4], i.e., with highly salt-tolerant \(C.\) parapsilosis, salt-tolerant \(C.\) albicans and relatively salt-sensitive \(C.\) dubliniensis. The growth of each of the five species was tested on a series of plates containing a broad range of salt concentrations (0.5–2.5 M NaCl, 0.25–1 M LiCl and 0.5–2.7 M KCl). The three salts were chosen as a non-toxic solute that mainly increased the osmotic pressure (KCl), a moderately toxic solute that increased the osmotic pressure (NaCl) and a highly toxic solute (LiCl) whose toxicity prevents its use at concentrations causing a high osmotic stress. Figure 1A shows the growth of the five species at salt concentrations where crucial differences among the species were observed. \(Candida\) krusei is more osmotolerant than \(C.\) dubliniensis, but it is extremely sensitive to the presence of toxic lithium cations. On the other hand, \(C.\) tropicalis appears to be a rather osmosensitive species but tolerates toxic lithium cations, as well as \(C.\) parapsilosis. Similarly, we also compared the ability of the five species to grow in the presence of FLC ranging from 0.5–200 \(\mu\)g/ml. Figure 1B demonstrates that FLC was very toxic for \(C.\) albicans cells, less toxic for \(C.\) dubliniensis, \(C.\) parapsilosis and \(C.\) tropicalis and not toxic for \(C.\) krusei. \(Candida\) krusei growth was inhibited by FLC concentrations higher than 100 \(\mu\)g/ml, i.e., the concentration at which the other four species did not grow at all (data not shown). The use of a series of plates with increasing concentrations of salts and fluconazole helped us to choose the subinhibitory concentrations of salts and FLC that did not significantly affect growth. The subinhibitory concentrations of FLC used were 0.5 (for \(C.\) albicans and \(C.\) parapsilosis) or 10 \(\mu\)g/ml (for the other three species). Figure 5B shows that the growth at these FLC concentrations was not significantly inhibited.

**Fluconazole affects salt tolerance**

When we tested the growth of \(Candida\) cells in the presence of FLC together with a salt (both at subinhibitory concentrations), we observed a combinatorial inhibitory effect for all five species. The tests of FLC and salt combinations were performed first on plates and then confirmed in liquid media. Figures 2A and 2B show in detail the results obtained for the FLC-resistant and simultaneously relatively salt-sensitive species \(C.\) dubliniensis. \(Candida\)
Fig. 1  Tolerance of Candida species to salts and fluconazole (FLC). Ten-fold serial dilutions of overnight-grown cells were prepared and 3 μl aliquots spotted on a series of YPD plates containing the indicated concentrations of salts (A) or FLC (B).

Fig. 2  Growth of Candida species in presence of subinhibitory concentrations of salts, fluconazole (FLC) and their combination. Ten-fold serial dilutions of overnight-grown cells of Candida species were prepared and 3 μl aliquots spotted on a series of plates supplemented as indicated (A and C). (B) Growth of C. dubliniensis cells in liquid YPD supplemented as indicated. The growth rate $V_{max}$ values are the mean ± standard error of three separate experiments.

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**Dubliniensis** grew well in the presence of 10 μg/ml FLC or moderate concentrations of salts (e.g., 1 M NaCl or 1 M KCl) but when the two compounds were mixed, its growth was severely inhibited. The level of inhibition was higher when NaCl was used, probably due to the toxicity of sodium cations. Nevertheless, a combinatorial effect observed for non-toxic KCl and FLC suggested that the presence of fluconazole affected the general osmotolerance of *C. dubliniensis* cells. To confirm this, we tested the combination of FLC and a non-ionic solute (1 M sorbitol). As with the salts, cells were able to grow well in the presence of 1 M sorbitol, but as soon as FLC was added together with sorbitol, the growth of cells was significantly inhibited (Fig. 2A and 2B). This result confirmed that the presence of fluconazole influenced the general osmotolerance of *C. dubliniensis* cells.

Similar tests with appropriate specific subinhibitory concentrations of salts and FLC were performed for the other four species and the obtained results are summarized in Figure 2C. For all four species, the combination of subinhibitory concentrations of FLC and salt inhibited their growth, and this inhibition was, as with *C. dubliniensis*, more pronounced with NaCl than with KCl. Table 1 summarizes the used subinhibitory FLC concentration and the lowest concentrations of salts at which the combinatorial effect of both compounds was clearly visible for each species. In summary, we found for both FLC-tolerant/sensitive and/or salt-tolerant/sensitive species a combination

**Table 1** Subinhibitory concentrations of fluconazole (FLC) and the lowest concentrations of salts that when combined caused a significant inhibition of growth of species.

<table>
<thead>
<tr>
<th>Species</th>
<th>FLC (μg/ml)</th>
<th>NaCl (M)</th>
<th>KCl (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida albicans</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Candida dubliniensis</td>
<td>10</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Candida krusei</td>
<td>10</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>10</td>
<td>0.5</td>
<td>1</td>
</tr>
</tbody>
</table>

![Fig. 3](image-url) Influence of fluconazole (FLC) on intracellular sodium content in Candida cells. (A) Overnight-grown cells were preloaded with NaCl (0.5 M NaCl in YPD for *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*; 0.25 M for *C. dubliniensis*) and subinhibitory concentrations of FLC for 60 min, then the intracellular sodium content was estimated. (B) Overnight-grown cells were cultivated in fresh YPD medium with or without subinhibitory concentrations of FLC for two cell cycles. The cells were then incubated in YPD supplemented with NaCl (0.5 M for *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*; 0.25 M for *C. dubliniensis*) and the intracellular sodium content was estimated after 60 and 120 min. The plotted values are the mean ± standard error of three separate experiments.

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of subinhibitory concentrations that significantly affected their growth.

**Fluconazole treatment increases intracellular sodium content**

Our previous results suggested that FLC increases the content of toxic sodium cations in *C. albicans* cells [16]. To determine whether the presence of FLC increases the sodium content in all five species, we first measured the intracellular sodium content in *Candida* cells preloaded with NaCl and simultaneously treated with FLC (at the concentrations given in Table 1) for 60 min (Fig. 3A). All species were incubated with 0.5 M NaCl except *C. dubliniensis*, which was preloaded with 0.25 M NaCl. Figure 3A shows that the internal sodium content in *Candida* species differed and reflected their salt tolerance. Salt-sensitive *C. dubliniensis* cells had the highest intracellular Na\(^+\) concentration, although they were preloaded with a lower amount of NaCl (Fig. 3A). Surprisingly, we did not observe the presence of FLC during cell preloading to have any effect. In all five species, the intracellular content of sodium was the same whether fluconazole was added or not. The results obtained suggested that FLC had no immediate effect on sodium content and that it needed to be present during cell growth to influence cation homeostasis.

To verify the effect of FLC treatment during cell growth and division, a second series of sodium content measurements was performed with cells that were grown in the presence of subinhibitory concentrations of FLC for two cell cycles prior to Na\(^+\) preloading. Preloading (with the same NaCl concentrations as mentioned above) took 60 or 120 min. The FLC-treatment resulted in a subsequent increase in the intracellular sodium content in the cells of all five species (Fig. 3B). After 60 min of preloading with NaCl, FLC-treated cells contained about 40% (*C. albicans*) to 80% (*C. parapsilosis*) more sodium than control cells grown without FLC. In the growth tests, (Fig. 2) all *Candida* species tolerated 1 M NaCl however, the FLC-treatment resulted in a sharp increase in [Na\(^+\)]\(_{\text{in}}\) though the...
cells were preloaded with much lower concentrations of NaCl (0.5 M for C. albicans, C. krusei, C. parapsilosis and 0.25 M for C. dubliniensis) and for a relatively short time (60–120 min). These results suggested that the changes in the plasma-membrane composition resulting from FLC treatment either altered the rate of uptake or efflux of sodium cations across the plasma membrane. As both FLC-treated and control cells contained more sodium after 60 min of preloading than after 120 min (Fig. 3B), we concluded that the induction/activation of sodium exporting systems (mainly Ena ATPases) is functional in both types of cells. No specific transporters for the uptake of toxic sodium cations have been identified in yeast cells. The only reported exception is the phosphate-Na + cotransporter Pho89 in S. cerevisiae [28] which is only active in cells starved of phosphate and incubated in an alkaline external pH. Under conditions of NaCl stress, sodium transport into the cells is non-specific. It follows the sodium concentration gradient and is driven by the plasma-membrane potential [5]. Thus, the observed increased sodium content could result from a plasma-membrane hyperpolarization caused by a change in the membrane composition resulting from growth in the presence of FLC. To verify this hypothesis, we first optimized the technique used for estimating the relative plasma-membrane potential (ΔΨ) in S. cerevisiae cells for the five Candida species, confirmed its applicability in a series of experiments with hyperpolarizing and depolarizing compounds, and finally estimated the relative membrane potential in FLC-treated cells.

**Measurement of relative membrane potential**

The relative plasma-membrane potential and its changes in the five Candida species was measured using a diS-C₃(3) assay. This assay is based on the potential-dependent distribution of a diS-C₃(3) fluorescence probe across the cell membrane and it was successfully used to monitor the changes in the plasma-membrane potential in S. cerevisiae [24,25]. The wavelength of maximum emission changes, when the probe is bound to intracellular components, and the position of the emission spectrum thus reflects the actual intracellular probe concentration. To confirm that the diS-C₃(3) assay is a suitable technique for monitoring membrane potential changes in Candida cells, we first measured the diS-C₃(3) probe staining curves in YPD grown cells (Fig. 4A) and then monitored the influence of a depolarizing (CCCP) and a hyperpolarizing (amiodarone) drug on the relative ΔΨ of all five Candida species. The protonophore CCCP causes either a small reduction or an almost total loss of membrane potential depending on the dose, and amiodarone was shown to both increase and decrease the ΔΨ of S. cerevisiae depending on dose [29] and to have a synergistic inhibitory effect with FLC on C. albicans cells [30]. Reproducible staining curves were obtained for all five species; however, there were differences among them suggesting that the Candida species significantly differ either in their plasma-membrane composition or in their ΔΨ or both (Fig. 4A). The relative plasma membrane potential is usually represented as the emission intensity I₅₈₀/I₅₆₀ ratio at the staining equilibrium [25]. This ratio differed from 2.78 ± 0.16 for C. tropicalis to 3.97 ± 0.18 for C. dubliniensis and was much higher for all five Candida species than the ratio measured for similarly grown and treated S. cerevisiae (approx. 1.8). Nevertheless, the addition of either hyperpolarizing amiodarone or depolarizing CCCP resulted in a corresponding increase or decrease in the staining curve (shown for C. albicans in Fig. 4B) and confirmed the reliability of this technique for estimating the relative membrane potential of Candida cells.

**Fluconazole treatment increases relative membrane potential**

To determine if FLC influences the ΔΨ of Candida cells, we compared the relative membrane potential in three samples of cells for each species. Overnight-grown cells were divided into three aliquots and incubated in fresh YPD in the absence (aliquots 1 and 2) or presence (aliquot 3) of appropriate subinhibitory FLC concentrations (Table 1) for two cell cycles (approx. 240 min). The cells were then transferred to the MES-TEA buffer, the probe was added and fluorescence measured. The first aliquot served as a control, the second aliquot was used to monitor the immediate effect of FLC, which was added to cells together with the diS-C₃(3) probe just before the fluorescence measurement, and the third aliquot of cells served to monitor the changes in ΔΨ resulting from the presence of fluconazole during cell growth. We observed no changes in the ΔΨ of any Candida species when FLC was added together with the fluorescence probe (Fig. 5A) and this result confirmed our conclusion that FLC had no immediate effect on the sodium content in Candida cells (Fig. 3A). On the other hand, the cells of all five species that grew for two cell cycles in the presence of subinhibitory concentrations of FLC exhibited a significant increase in I₅₈₀/I₅₆₀ ratio that implied a hyperpolarization of their plasma membranes (Fig. 5A). The relative hyperpolarization was species-specific; nevertheless, the treatment of growing cells with subinhibitory concentrations of FLC increased their relative ΔΨ in all Candida species. To confirm that FLC treatment hyperpolarizes the plasma membrane, we performed a series of drop tests on YPD plates with a toxic cationic compound (hygromycin B). Hygromycin B enters into the yeast cells in proportion to their plasma-membrane...
potential, i.e., hyperpolarized cells are more sensitive to this drug [31–33]. Candida species tolerated quite high concentrations of hygromycin B (Fig. 5B), however, some of them (C. albicans, C. dubliniensis and C. parapsilosis) were more resistant than the others (C. krusei and C. tropicalis). Thus, we used two subinhibitory concentrations of hygromycin B (100 μg/ml for C. krusei and C. tropicalis, and 200 μg/ml for C. albicans, C. dubliniensis and C. parapsilosis), which did not cause a significant inhibition of growth (Fig. 5B). When FLC and hygromycin B were combined, the growth of all five Candida species was strongly inhibited (Fig. 5B). The subinhibitory concentrations of FLC increased the sensitivity of all five Candida species to the hygromycin B whose toxicity depends on the level of cell membrane potential.

**Discussion**

The virulence of Candida species depends on many environmental conditions, including extracellular pH and the concentration of alkali metal cations. For example, intracellular potassium concentration was shown to be involved in the yeast-to-hyphae morphological switch of C. albicans [34], and high extracellular concentrations of alkali metal cations were shown to affect C. albicans virulence traits such as germ tube formation, adhesion, and hydrophobicity [35]. Our long-term aim is to understand the regulation of the maintenance of alkali-metal-cation homeostasis in pathogenic yeast species and to contribute towards identifying new targets for antifungal therapy.

Testing the tolerance of five Candida species to alkali-metal-cation salts revealed significant differences among these species despite the fact that they possess homologous genes encoding alkali-metal-cation transporters in their genomes. Though all five species could be classified as relatively osmotolerant (compared to the model yeasts S. cerevisiae and Schizosaccharomyces pombe), they exhibited different levels of tolerance to different salts. Whereas C. krusei was rather osmotolerant but highly sensitive to relatively low concentrations of toxic lithium cations, C. tropicalis was the most osmosensitive among the five tested species but it tolerated, similarly to C. parapsilosis, very high concentrations of LiCl (Fig. 1A). One of the reasons for the observed variations in salt tolerance might be the difference in the level of expression and/or activity of cation exporting systems, Ena ATPases and Cnh1 cation/proton antiporters in these species [7,8].
As with salt tolerance, we observed significant differences in species sensitivity to fluconazole, with C. krusei being by far the most FLC-resistant (Fig. 1B).

Our results showed that though the various Candida species had different levels of salt and fluconazole tolerance, all of them were sensitive to the combination of species-specific subinhibitory concentrations of these compounds (Fig. 2) and in all of them, fluconazole treatment resulted in an increase in the intracellular content of toxic sodium cations (Fig. 3B). Thus, the previously observed phenomenon of a combinatorial inhibitory effect of FLC and salts in C. albicans [16] seems to be rather general in all Candida species. Nevertheless, the level of inhibition was species-specific, as the most resistant to the combinatorial effect of salt and FLC was the most halotolerant (and relatively FLC-susceptible) C. parapsilosis and the most FLC-resistant C. krusei (Figs. 1 and 2). As the combination of subinhibitory concentrations affected the growth of both FLC/salt sensitive and FLC/salt tolerant species, we suggest that the observed synergistic interaction was a result of a general change in cytosolic cation homeostasis and most probably also in the osmotic balance.

Having established the requisite experimental platform for the estimation of membrane potential changes, we were able to show that the observed higher content of sodium in FLC-treated cells most likely resulted from a higher non-specific uptake of sodium driven by the increased membrane potential (negative inside). We did not observe an immediate effect of FLC addition when measuring the intracellular sodium content, nor when estimating the relative membrane potential (Figs. 3A and 5A). The cell had to grow and divide in the presence of FLC before the changes in cation content and membrane potential could be observed. These results suggested that it was the different composition of the plasma membrane in FLC-treated cells [9] that led to the hyperpolarization of the plasma membrane, and that this hyperpolarization was the basis of the increased cell sensitivity to toxic cationic compounds, e.g., hygromycin B (Fig. 5B). This conclusion of ours is supported by two earlier observations. First, some azoles (miconazole, ketoconazole) influence K+ homeostasis immediately after their addition to cells, but the short-term presence of FLC does not affect the potassium content in S. cerevisiae [36]. Second, a synergistic effect of amiodarone and azoles in C. albicans has been described [30] and we showed that both compounds had a hyperpolarizing effect on the plasma membrane, amiodarone an immediate one (Fig. 4B) and FLC during cell growth and division (Fig. 5A).

In summary, we show for the first time that FLC treatment results in a hyperpolarization of the plasma membranes of five strains of both FLC-susceptible and FLC-tolerant Candida species and that the synergistic effect of subinhibitory concentrations of FLC and many other cationic drugs is most probably caused by an increased uptake of these compounds driven by the higher plasma-membrane potential.

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