Phytosphingosine-1-phosphate is a signaling molecule involved in miconazole resistance in sessile Candida albicans cells.

Running title: Sphingolipids play a role in miconazole resistance

Davy Vandenbosch¹, Anna Bink², Gilmer Govaert², Bruno P. A. Cammue², Hans J. Nels¹, Karin Thevissen² and Tom Coenye¹*

¹Laboratory of Pharmaceutical Microbiology, Ghent University,
Harelbekestraat 72, B-9000, Ghent, Belgium
²Centre of Microbial and Plant Genetics, K.U.Leuven, Kasteelpark Arenberg 20, B-3001, Heverlee, Belgium

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* Corresponding author:

Tom Coenye
Laboratory of Pharmaceutical Microbiology
Ghent University
Tel.: +32 9 2648141
Fax: +32 9 2648195
Email address: Tom.Coenye@UGent.be
Abstract

Previous research has shown that 1% - 10% of sessile Candida albicans cells survive treatment with high doses of miconazole (a fungicidal imidazole). In the present study, we investigated the involvement of sphingolipid biosynthetic intermediates in this survival. We observed that the gene LCB4, coding for the enzyme that catalyzes the phosphorylation of dihydrosphingosine and phytosphingosine, is important in governing miconazole resistance of sessile Saccharomyces cerevisiae and C. albicans cells. Addition of 10 nM phytosphingosine-1-phosphate (PHS-1-P) drastically reduced the intracellular miconazole concentration and significantly increased the miconazole resistance of a hypersusceptible C. albicans heterozygous LCB4/lcb4 mutant, indicating a protective effect of PHS-1-P against miconazole-induced cell death in sessile cells. At this concentration of PHS-1-P we did not observe any effect on the fluidity of the cytoplasmatic membrane. The protective effect of PHS-1-P was not observed when the efflux pumps were inhibited or when tested in a mutant without functional efflux systems. Also, the addition of PHS-1-P during miconazole treatment increased the expression of genes coding for efflux pumps, leading to the hypothesis that PHS-1-P acts as a signaling molecule and enhances the efflux of miconazole in sessile C. albicans cells.
Introduction

*Candida albicans* is a fungal pathogen frequently causing nosocomial infections in immunocompromised hosts. Adhesion of the cells to biotic or abiotic surfaces results in the formation of a complex three-dimensional biofilm consisting of yeast cells and filaments embedded in a self-produced exopolymeric matrix. These sessile cells show an increased antifungal resistance compared to planktonic cells, resulting in recurrent infections which are very difficult to treat. Therefore, there is an urgent need to unravel the molecular mechanisms of resistance of biofilms to find new and effective therapies (3, 14, 19, 24).

Azoles are widely used to treat *Candida* infections. These antifungal compounds decrease the production of ergosterol by interacting with cytochrome P450 and inhibiting the 14α-demethylation of lanosterol. As ergosterol is an important constituent of the cytoplasmatic membrane, treatment withazole antifungals leads to growth inhibition (31). Besides this fungistatic mechanism of action, recent data indicate a fungicidal effect for miconazole (an imidazole) against *Candida* spp. cells in suspension and in young and mature biofilms (17, 28). Accumulation of reactive oxygen species appears to be involved in this process, although it is likely that other mechanisms are also involved in the fungicidal activity (10, 16, 27).

Despite the observed fungicidal activity of miconazole also against biofilms, 1% - 10% of the sessile *C. albicans* cells survive exposure to high levels of this antifungal agent (28).

Previous research has shown that membrane rafts, which are tightly packed domains of sphingolipids and sterols, and both the sphingolipid and ergosterol content of the membrane are critical factors in the mode of action of miconazole against yeast cells (11). However, possible mechanisms of miconazole resistance related to membrane rafts and more specifically sphingolipids have not been investigated in *C. albicans* biofilm cells. Sphingolipids are characterized by their typical long chain amino-alcohol backbone which can be phosphorylated or form more complex structures after acylation with fatty acids.
(ceramides) and further addition of sugar residues (12). Sphingolipids are incorporated in different cellular membranes including the cytoplasmic membrane. Because of their physico-chemical properties, sphingolipids are involved in a broad range of biological functions, including intracellular transport, cell-cell interaction and molecular sorting (23). Membrane rafts are involved in cell signaling, sorting of membrane-bound proteins and maintenance of polarity during mating (2, 9, 29).

The aim of the present study was to investigate the role of sphingolipids in the resistance of \textit{C. albicans} sessile cells to miconazole.

Materials and methods

Strains

The strains used in this study are listed in Supplementary Table 1. A stock culture of all these strains was kept at -80°C. Cells were transferred to Sabouraud dextrose agar (Oxoid, Basingstoke, UK) plates and incubated at 37°C for 48 h.

Determination of miconazole susceptibility of \textit{S. cerevisiae} deletion mutants

Suspensions of \textit{S. cerevisiae} BY4741 and BY4741-derived deletion mutants, containing approximately 10^7 cells/ml were prepared in Yeast-Pepton-Dextrose (YPD) medium (BD, Franklin Lakes, NJ). Biofilms were grown in round-bottomed 96-well microtitre plates (SPL Lifesciences, Pocheon, Korea) containing 100 µl cell suspension per well (12 replicates per strain). After 1 h incubation at 37°C, the supernatant was removed and the wells were rinsed with 100 µl of physiological saline (0.9% NaCl) to remove unattached cells. The microtitre plates were further incubated for 24 h at 37°C after addition of 100 µl YPD medium to each well. Subsequently, the mature biofilms were rinsed with 100 µl physiological saline before treatment with miconazole (1000 µg/ml) (Certa, Braine-l’Alleud, Belgium). To this end, 100
µl of a miconazole suspension in phosphate buffered saline (PBS) containing 2% DMSO (Sigma-Aldrich, St Louis, MO) was added to 6 biofilms of each strain and 100 µl of PBS containing 2% DMSO to the other 6 biofilms (control). After 24 h incubation at 37°C the supernatant was removed and 120 µl of a diluted resazurin solution (CellTiter-Blue 1:6 in physiological saline) (Promega, Leiden, The Netherlands) was added to each well. Fluorescence was measured (λ_{ex} 535 nm; λ_{em} 590 nm) after 2 h incubation at 37°C using an Envision microtitre plate reader (Perkin Elmer, Wellesley, MA). For each deletion mutant, a relative value for the susceptibility to miconazole was calculated. To this end, the ratio of the average fluorescence of miconazole-treated biofilms to untreated biofilms (both corrected for the blank) was compared between the wild type (WT) and each mutant.

### Analysis of gene expression in C. albicans sessile cells

*C. albicans* biofilms were grown on silicone disks in 24-well microtitre plates and treated with miconazole and PHS-1-P or miconazole alone as described previously (28). Biofilm cells were collected and washed with physiological saline. Untreated *C. albicans* sessile cells were used as control. Cell disruption, RNA purification and DNase treatment were performed according to the manufacturers’ instructions (RiboPure-Yeast kit, Applied Biosystems, Carlsbad, CA). The isolated RNA was concentrated with an Amicon Ultra filter (Millipore, Billerica, MA) and subsequently diluted with DEPC-treated water until a final volume of 15 µl. The iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) was used for the reverse transcriptase (RT) reaction. To this end, 1 µl reverse transcriptase and 4 µl reaction mix were added to each tube (5 min at 25°C, 30 min at 42°C and 5 min at 85°C). After development of a forward and a reverse primer for the genes *LCB1, LCB2, LCB3, LCB4, KSR1, SUR2, LAG1, YDC1, HSX11, DPL1, SCS7, AUR1, IPT1, MIT1, CDR1, CDR2, MDR1, RIP* and *LSC2* (Supplementary Table 2) and testing their specificity, real-time PCR (CFX96 Real Time...
System, Bio-Rad) was performed using the iQ SYBR Green Supermix (Bio-Rad). The expression levels of the genes of interest were normalized using two reference genes (RIP and LSC2). Experiments were performed as five independent biological repeats each consisting of six technical repeats and analyzed with the Bio-Rad CFX Manager software (Bio-Rad).

Determination of the susceptibility of \textit{C. albicans} sessile cells to miconazole

Biofilms of \textit{C. albicans} SC5314 WT, the \textit{LCB4/lcb4} mutant and DSY1050 (in which \textit{CDR1}, \textit{CDR2} and \textit{MDR1} are inactivated) (22) were grown in 96-well microtitre plates and treated with miconazole as described above. Biofilm susceptibility to miconazole was also determined in the presence of the sphingolipid biosynthetic intermediates dihydrosphingosine-1-phosphate (DHS-1-P, 10 nM and 100 nM) (Sigma-Aldrich) or phytosphingosine-1-phosphate (PHS-1-P, 10 nM and 100 nM) (Avanti Polar Lipids, Alabaster, AL), the oxidative phosphorylation inhibitor carbonyl cyanide m-chlorophenylhydrazone (CCCP, 5 µM) (Sigma-Aldrich) and/or the serine palmitoyltransferase (\textit{LCB1}) inhibitor myriocin (10 µM) (Sigma-Aldrich). The susceptibility to miconazole was evaluated with the resazurin based cell viability assay as described above.

Pyrene lateral diffusion assay

The lateral diffusion was measured by the excimerization of pyrene. This small molecule can bind to the long chain fatty acids of the cytoplasmatic membrane. Excited pyrene monomers are able to form a complex with unexcited pyrene molecules leading to the formation of pyrene dimers, which emit fluorescence at a higher wavelength (1, 4). Biofilms of \textit{C. albicans} SC5314 WT and the \textit{LCB4/lcb4} mutant were grown in a 96-well microtitre plate and treated as described above, with and without the addition of sphingolipid biosynthetic intermediates (DHS-1-P or PHS-1-P). For each experimental condition, the cells of six biofilms were
collected and were resuspended in 10 ml PBS with 0.25% formaldehyde. The cell suspensions were centrifuged (4 min at 5000 rpm), washed twice with PBS containing 0.25% formaldehyde and further diluted until an absorption of 0.25 at 450 nm was reached. One ml of these suspensions was incubated with pyrene (Sigma-Aldrich, final concentration 10 µM) for 20 min at 37°C. The cells were washed to remove excess pyrene and resuspended in 1 ml PBS. Aliquots of 200 µl were added to the wells of a black 96-well microtitre plate (Perkin Elmer) and fluorescence was measured ($\lambda_{\text{ex}}$ 340 nm; $\lambda_{\text{em}}$ 380 nm and 480 nm). The ratio of pyrene dimers to pyrene monomers (fluorescence at 480 nm divided by fluorescence at 380 nm) was calculated for each condition and is directly proportional to the membrane fluidity.

**Determination of intracellular miconazole concentration**

Biofilms of *C. albicans* SC5314 WT and the *LCB4/lcb4* mutant were grown in a 96-well microtitre plate and treated as described above, with and without the addition of PHS-1-P. For each experimental condition, the cells of six biofilms were collected, rinsed three times with physiological saline to remove extracellular miconazole and finally resuspended in a mixture of 70% acetonitrile / 30% PBS. Glass beads were added to the cell suspensions and the cells were subsequently lysed by shaking with the Precellys24 (Bertin Technologies, Montigny-le-Bretonneux, France) for 20 sec at 6000 rpm. The cell lysate was transferred to a new tube to adjust the concentration of acetonitrile to 30% and add trifluoroacetic acid to 0.1%. The tubes were centrifuged for 30 min at 13000 rpm after which the intracellular miconazole concentration was determined using HPLC, as described previously (11).

**Statistical analysis**

Statistical analysis was performed using the non-parametric Mann-Whitney U Test (SPSS Statistics 17.0 software).
Results

Determination of miconazole susceptibility of *S. cerevisiae* deletion mutants

The susceptibility to miconazole of biofilms of *S. cerevisiae* mutants affected in genes involved in the sphingolipid biosynthesis was investigated and was compared to that of the WT (Figure 1). Seven mutants (Δlcb5, Δysr3, Δlag1, Δlac1, Δydc1, Δype1 and Δscs7) did not show an altered susceptibility. Sessile Δlcb4 cells, deficient in the phosphorylation of dihydrosphingosine and phytosphingosine, were hypersusceptible to miconazole, while sessile Δlcb3 cells, deficient in the dephosphorylation of DHS-1-P and PHS-1-P, and sessile Δdpl1 cells, deficient in the breakdown of DHS-1-P and PHS-1-P, were more resistant to miconazole than the WT. The deletion of *SUR1*, *CSG2* or *CSH1*, involved in the mannosylation of inositol-phosphorylceramide, the deletion of *IPT1*, encoding inositolphosphotransferase, and the deletion of *SUR2*, encoding dihydrosphingosine hydroxylase, resulted in an increased resistance of sessile cells of the corresponding mutants to miconazole.

Expression of genes involved in sphingolipid biosynthesis in *C. albicans* sessile cells

The expression levels of all genes involved in the sphingolipid biosynthesis were determined in sessile *C. albicans* cells after miconazole treatment and compared with those in untreated sessile *C. albicans* cells (Figure 2 and Table 1). Four genes, *KSR1*, *YDC1*, *LCB4* and *DPL1*, were significantly (p < 0.05) upregulated after miconazole treatment. The highest upregulation was observed for *LCB4* (3.1 fold). *DPL1* (2.2 fold upregulated) is involved in the breakdown of DHS-1-P and PHS-1-P. *KSR1* (2.3 fold upregulated) and *YDC1* (2.1 fold upregulated) are involved in the formation of dihydrosphingosine and phytosphingosine, respectively.
Determination of the susceptibility of *C. albicans* sessile cells to miconazole

In line with the observed miconazole hypersusceptibility of sessile *S. cerevisiae Δlcb4* cells, also sessile cells of the heterozygous *C. albicans LCB4/lcb4* mutant were hypersusceptible to miconazole (Figure 3). Addition of DHS-1-P (final concentration: 10 nM and 100 nM) during biofilm growth and miconazole treatment did not significantly alter the susceptibility of the WT and the *LCB4/lcb4* mutant. In contrast, the *LCB4/lcb4* mutant showed significantly increased resistance (p < 0.05) to miconazole after addition of PHS-1-P (final concentration: 10 nM and 100 nM) during biofilm growth and miconazole treatment. This effect was not dose-dependent and an optimal effect was obtained at 10 nM. A similar, but less explicit change in susceptibility to miconazole was obtained for the WT after supplementation of the medium with PHS-1-P. As control, we tested the effect of PHS-1-P in combination with the *LCBI* inhibitor myriocin, which blocks the first step of the sphingolipid biosynthesis (18). The significantly increased susceptibility to miconazole of the WT SC5314 after the addition of 10 µM myriocin decreased again in combination with PHS-1-P (10 nM).

When CCCP (5 µM) was added simultaneously with PHS-1-P (10 nM), no changes in the susceptibility to miconazole were observed for the WT and the *LCB4/lcb4* mutant, compared to the condition without PHS-1-P. Application of only CCCP did not influence the susceptibility to miconazole of the WT and the *LCB4/lcb4* mutant. Addition of PHS-1-P (10 nM) during biofilm growth and miconazole treatment of the triple mutant DSY 1050 did also not alter miconazole resistance.

Pyrene lateral diffusion assay

The relative fluidity of the cytoplasmatic membrane was similar in untreated *C. albicans* sessile cells of the WT and the *LCB4/lcb4* mutant (1.39 ± 0.11 and 1.58 ± 0.19, respectively). Treatment with miconazole resulted in a significant decrease (p < 0.05) in membrane fluidity
for both strains (1.04 ± 0.06 for the WT and 0.88 ± 0.13 for the LCB4/lcb4 mutant). This decrease was significantly more pronounced (p < 0.05) for the LCB4/lcb4 mutant than for the WT. Addition of PHS-1-P during biofilm growth and miconazole treatment significantly (p < 0.05) increased the fluidity of the cytoplasmatic membrane of the WT (1.22 ± 0.05), but did not affect the membrane fluidity of the LCB4/lcb4 mutant (0.86 ± 0.11).

Intracellular miconazole concentration

The intracellular miconazole concentration after 24 h treatment was significantly higher (p < 0.05) in sessile LCB4/lcb4 mutant cells than in sessile WT cells (2.1 µg/1000 cells and 1.5 µg/1000 cells, respectively) (Table 2). Addition of PHS-1-P (to a final concentration of 10 nM) significantly decreased the intracellular miconazole concentration to 0.6 µg/1000 cells in sessile LCB4/lcb4 mutant cells, while only a slight decrease was observed (1.3 µg/1000 cells) for the WT sessile cells. The addition of CCCP during miconazole treatment (in the presence of 10 nM PHS-1-P) significantly increased the intracellular miconazole concentration in sessile cells of the WT and the LCB4/lcb4 mutant (1.8 µg/1000 cells for both strains).

Expression of genes coding for efflux pumps in C. albicans sessile cells

The expression levels of CDR1, CDR2 and MDR1 were determined in sessile C. albicans cells after treatment with miconazole and PHS-1-P and compared with those in sessile C. albicans cells treated with miconazole alone. All three genes were highly upregulated (132 fold for CDR1, 29 fold for CDR2 and 22 fold for MDR1) due to the addition of PHS-1-P during miconazole treatment. The expression levels of CDR1, CDR2 and MDR1 in untreated sessile LCB4/lcb4 mutant cells were not significantly different (p > 0.05) compared with those in untreated sessile WT cells (2.3 fold, 1.2 fold and 2.9 fold, respectively).
Discussion

We previously observed that 1% - 10% of cells in a C. albicans biofilm survived treatment with high doses of miconazole (28). Other studies have suggested a role for sphingolipids in governing miconazole resistance in planktonic yeast cultures (11, 21), and in the present study we focused on the role of sphingolipid biosynthesis in the molecular mechanisms underlying the resistance of C. albicans biofilms to miconazole.

The screening of S. cerevisiae deletion mutants affected in sphingolipid biosynthesis revealed the involvement of LCB4 in governing resistance to miconazole, as biofilms of the corresponding deletion mutant showed an increased susceptibility to miconazole. LCB4 encodes a sphingosine kinase which is involved in the phosphorylation of dihydrosphingosine and phytosphingosine (20). The increased transcription of LCB4 in miconazole treated sessile C. albicans cells confirmed its involvement in miconazole resistance. Furthermore, a significant upregulation was observed for the genes KSR1 and YDC1, which encode enzymes involved in the formation of dihydrosphingosine and phytosphingosine, respectively.

Increased levels of DHS-1-P and PHS-1-P are known to play a role in mediating resistance to heat stress (26), probably due to induction of TPS2 transcription and trehalose accumulation (8). In contrast, a rapid intracellular accumulation of DHS-1-P and PHS-1-P results in a reduced growth rate and may be even lethal for the cells (15, 32). The observed overexpression of DPL1, involved in the breakdown of DHS-1-P and PHS-1-P to a long chain aldehyde and ethanolamine phosphate, is probably necessary to maintain balanced levels of DHS-1-P and PHS-1-P.

DHS-1-P and PHS-1-P seem to be important in resistance to miconazole. To elucidate their role, experiments were performed using a heterozygous C. albicans LCB4/lcb4 mutant. Sessile cells of this LCB4/lcb4 mutant were hypersusceptible to miconazole as compared to the WT. We also observed a higher intracellular miconazole concentration in treated sessile...
cells of the *LCB4/lcb4* mutant than in sessile WT cells. Addition of PHS-1-P (10 nM) during biofilm growth and miconazole treatment significantly increased the resistance to miconazole of the *LCB4/lcb4* mutant (similar to that of the WT without PHS-1-P addition) and drastically reduced the intracellular miconazole levels, clearly pointing to a protective effect of PHS-1-P against the action of miconazole. The intracellular miconazole concentration in sessile WT cells was not altered by the addition of PHS-1-P. However, a slightly increased resistance to miconazole was observed in the presence of PHS-1-P. When we inhibited *LCB1* by adding myriocin, a similar increase in resistance was noted upon addition of PHS-1-P, confirming the protective effect of the latter against miconazole. In contrast, DHS-1-P has no protective effect against miconazole treatment in sessile *C. albicans* cells as the susceptibility to miconazole was unchanged when this sphingolipid intermediate was added to the WT and the *LCB4/lcb4* mutant.

To investigate whether PHS-1-P has a direct or indirect effect on the structure of the cytoplasmatic membrane of sessile *C. albicans* cells resulting in altered miconazole uptake, the fluidity of the cytoplasmatic membrane was measured using a pyrene lateral diffusion assay. The membrane fluidity of untreated sessile cells of the WT and the *LCB4/lcb4* mutant did not differ. A more rigid structure of the cytoplasmatic membrane of both strains after treatment with miconazole was noticed. Changes in the composition of the cytoplasmatic membrane due to stress situations have been described previously (25). Addition of PHS-1-P to sessile cells of the *LCB4/lcb4* mutant had neither a direct nor an indirect effect on the fluidity of the cytoplasmatic membrane, indicating that changes in membrane fluidity are not responsible for this protective effect. Previous research has shown that several cellular processes are regulated by sphingolipid long chain bases. In *S. cerevisiae*, sphingolipid long chain bases are thought to activate the protein kinase Phk1 and Phk2, leading to an activation of downstream pathways playing a role in actin cytoskeleton dynamics, regulation of
transcription and translation, stress resistance and cell growth (7). Still, more research is needed to understand the exact regulatory mechanisms and the connections to other metabolic pathways (6). Knowledge about the role of sphingolipids in fungal pathogens like C. albicans is even more limited. Pasrija et al. (21) found that the disruption of certain sphingolipid biosynthetic genes in C. albicans, with exception of Δlcbl, lead to a mislocalization of efflux pumps and increased susceptibility to drugs. The role of PHS-1-P on efflux pumps was investigated by chemically inhibiting these pumps (using CCCP) and by studying a triple mutant in which all these efflux pumps were inactivated. The addition of the oxidative phosphorylation inhibitor CCCP (5,13) significantly increased the intracellular miconazole levels, both for the WT and the LCB4/lcb4 mutant, and counteracted the protective effect of PHS-1-P. Similarly, addition of PHS-1-P did not increase resistance to miconazole in the triple efflux pump mutant. Furthermore, the expression of the genes coding for efflux pumps in C. albicans SC 5314 was highly upregulated when PHS-1-P was added during miconazole treatment. These data indicate that PHS-1-P directly or indirectly acts on efflux pumps in miconazole treated sessile C. albicans cells.

In conclusion, a protective effect of PHS-1-P against miconazole treated C. albicans biofilms was observed. As the structure of the cytoplasmatic membrane remained unaffected, it is likely that PHS-1-P acts as a signaling molecule and that it enhances the export of miconazole. The regulatory mechanism involved in this process remains unclear so far.

Acknowledgements

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respectively. We would like to thank Dominique Sanglard (University Hospital of Lausanne, Switzerland) for providing strain DSY1050.
References


Figure legends

FIGURE 1: Relative susceptibility to miconazole of S. cerevisiae BY4741 deletion mutants compared to the susceptibility of the WT. The values are the mean of six replicates. Error bars represent SEM. Significant differences (p < 0.05) are marked with an asterisk.

FIGURE 2: Sphingolipid biosynthesis pathway in C. albicans. Genes with significantly increased expression levels (p < 0.05) after miconazole treatment of sessile cells compared to untreated sessile cells are underlined.

FIGURE 3: Relative miconazole susceptibility of SC5314 WT, LCB4/lcb4 mutant and DSY1050 after addition of dihydrosphingosine-1-phosphate (DHS-1-P), phytosphingosine-1-phosphate (PHS-1-P), carbonyl cyanide m-chlorophenylhydrazone (CCCP) and myriocin (or combinations). Values higher than 1 indicate a higher resistance, while values lower than 1 indicate a higher susceptibility compared to the WT. Values marked with an asterisk are significantly different from the values of the corresponding strain without addition of the sphingolipid intermediate. Data presented are the mean and SEM of three independent experiments on six biofilms.
TABLE 1: Fold change in expression levels of genes involved in the sphingolipid biosynthesis in sessile *C. albicans* cells after miconazole treatment, compared to untreated cells. Data presented are the mean of five independent experiments. Genes with significantly different expression levels (p < 0.05) are marked with an asterisk.

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<th>Gene</th>
<th>Average fold change</th>
<th>SEM</th>
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<tr>
<td>YDC1</td>
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TABLE 2: Intracellular miconazole concentrations in C. albicans SC5314 WT and the LCB4/lcb4 mutant with addition of 10 nM PHS-1-P and/or 5 µM CCCP. The results are the mean and standard deviation of at least four replicates. (1)Significantly different compared to WT. (2)Significantly different compared to LCB4/Δlcb4 without PHS-1-P. (3)Significantly different compared to LCB4/Δlcb4 + PHS-1-P without CCCP.

<table>
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<th>Intracellular miconazole concentration (µg/1000 cells)</th>
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<td></td>
<td>Without CCCP</td>
<td>With CCCP</td>
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<tr>
<td>SC5314 WT</td>
<td>1.5 ± 0.2</td>
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<tr>
<td>SC5314 WT + PHS-1-P</td>
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<tr>
<td>LCB4/Δlcb4</td>
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<td>1.8 ± 0.1(3)</td>
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