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Benzyl Alcohol Increases Diffusion Limit of Nuclear Membrane in *Saccharomyces cerevisiae* Cells

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ABSTRACT

Objective: Fungi are invasive species responsible for infections in many people around the world and which severely affect the immune system. The opportunistic pathogenic species, such as Candida species and *Aspergillus fumigatus*, can cause death in people with weakened immune systems. Natural medicines derived from plants are often used to treat fungal diseases. In connection with our efforts to unearth possible cellular targets of antimicrobial agents, in this study, we aimed to determine the functional consequences of benzyl alcohol treatment on the nuclear membrane.

Materials and Methods: We analysed the nuclear membrane distortions caused by benzyl alcohol in *Saccharomyces cerevisiae* cells using Nup49-GFP reporter strain. We also studied cellular distributions of various fluorescently tagged nuclear-cytoplasmic shuttling proteins to determine any functional disturbances in nuclear pore complexes upon benzyl alcohol treatment. Localization of 51.5 kDa protein LexA-NES-GFP and 61.8 kDa protein Pho4(Δ157-164)-GFP to the nucleus in yeast cells was key for evaluating the effect upon diffusion limit of pores.

Results: By analyzing the distribution of fluorescently tagged nuclear localization signal or nuclear export signals bearing reporter proteins between the nucleus and cytoplasm, we have shown that the nuclear membrane becomes leaky upon benzyl alcohol treatment.

Conclusion: The diffusion limit across the nuclear membrane in yeast cells is increased upon benzyl alcohol treatment. We believe that these findings not only increase our understanding of the mode of action of benzyl alcohol bearing antifungal agents, but also help widening their use.

Keywords: Benzyl alcohol, yeast, antifungal activity, nuclear membrane, nuclear pore complex

INTRODUCTION

Fungal infections are among the common causes of death, especially in individuals with a weakened immune system. These infections may be neglected, but they can be dangerous, contrary to common belief. There are few and limited treatments available to treat invasive fungal infections. A number of natural medicines derived from plants are used to treat the fungal diseases. Discovery of antifungal agents is much slower than antibacterial agents. Because fungi are eukaryotes, fungal-damaging substances can also often damage the host, so the antifungal agent developments are usually slow.

As a hallmark of eukaryotic cells, the genetic material is separated in a compartment surrounded by the nuclear membrane. The nuclear membrane separates DNA and translation, allowing transcription to be regulated in various ways. It also allows for differential processing and selective export of the synthesized mRNA.

The nuclear envelope consists of double lipid bilayers, the outer and inner nuclear membranes (1). The outer layer is continuous with the endoplasmic reticulum, which hosts ribosomes that carry out translation. Nuclear pore complexes (NPCs), which are large multiprotein structures that pass through the double bilayer structure, allow and regulate the passage of substances into

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and out of the nucleus. They are the gatekeepers mediating the exchange of essential compounds between the nucleoplasm and the cytoplasm (2-6). Ions and small metabolites can freely diffuse through NPCs. However, for the transport of large molecules with MW >40 kDa, active transport is necessary (7-12). This active transport into and out of the nucleus is governed by a large family of transport receptors called karyopherins in yeast (7, 13, 14). Although NPC structures differ between species such as size and variation in individual proteins, their overall structures are conserved from yeasts to mammals (3, 5, 10).

Benzyl alcohol is a known membrane fluidizer, and recently we demonstrated that it is active against yeast cells (15). In this study, we set out to determine its cellular target in *Saccharomyces cerevisiae* cells. For this purpose, we studied possible defects in the yeast nuclear membrane structure and function upon treatment with benzyl alcohol. We believe that these studies not only increase our understanding of the mechanism of action of alcoholic antifungal agents, but also open up new possibilities for the therapy of fungal infections.

MATERIALS AND METHODS

Strains and Growth Conditions

YPH499 (*MATa ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1*) (16) was used as the *Saccharomyces cerevisiae* strain in this study. pRS315-GFP-*NUP49,* pADH1-*NLS-2*GFP-TRP1, pADH1-*NES*-YFP-TRP1, pRS315-*npl3*[S411A]-GFP, pRS314-*LexA-NES*-GFP and pRS314-*Pho4*(Δ157-164)-GFP reporters were a generous gift from Prof. Karsten Weis, and used according to the previously described protocols (20). Yeast cells were grown until the early exponential phase in yeast extract peptone glucose medium (YPD) at 25°C while shaking. Then cells were treated with 1% benzyl alcohol for 15 minutes and analysed using fluorescence microscopy as explained below. 1% benzyl alcohol corresponds to 10xMIC value.

Minimum Inhibitory Concentration (MIC) Measurement

The minimum inhibitory concentration (MIC) measurement was performed in a similar procedure in our previous study (15). *S. cerevisiae* cells were cultured overnight at 25°C in YPD broth and were suspended in YPD to give a final density of 1×10^6 CFU/mL. Benzyl alcohol dissolved in dimethyl sulfoxide (DMSO) was prepared with the serial dilution method and put in a 24-well microtiter plate. After that, *S. cerevisiae* cells were added to each well. Suspension of yeast cells in the medium without benzyl alcohol or any other additives as well as yeast cells in the medium with only DMSO were tested as controls. 24-well microtiter plates were incubated at 25 °C. The MIC was determined after 48 h. Viability of yeasts was deduced based on turbidity.

Extracellular pH Measurement

S. cerevisiae cells were incubated at 25°C in 20 mL of YPD until the exponential growth phase, harvested and washed twice with sterilized dH_2O. The pellet was then resuspended in sterilized dH_2O. About 50 mg wet weight of yeast cells were used for each experiment. 2% glucose was added and incubated for 20 min while shaking. At this point, 5 and 10 mM benzyl alcohol was added, and extracellular pH was recorded using an HI 98127 water proof pH meter (HANNA, USA).

Fluorescence Microscopy

Still images of non-fixed cells were captured at room temperature with a wide-field epifluorescence microscope (Axio Imager.A1; Carl Zeiss MicroImaging) equipped with 100x NA 1.45 oil immersion objective (Plan-Fluar; Carl Zeiss MicroImaging), a Cascade:1K camera (Photometrics) and Metamorph software (Universal Imaging). At least 1000 cells were analysed for each data set. Representative figures for each analysis were combined together after adjusting contrast and brightness.

MTT Mitochondrial Functionality Assay

Yeast cells were incubated at 25°C in 20 mL of YPD until the exponential growth phase, harvested and then resuspended in sterilized dH_2O, 1:1 (w/v). 0.05 M glucose, 0.4 mg/ml MTT, 1% benzyl alcohol was added to yeast cells and incubated at 30 °C for 15 min. The MTT reduction was determined using a spectrophotometer at 570 nm.

RESULTS

Nuclear Membrane is Distorted in Benzyl Alcohol Treated Yeast Cells

We have previously reported the antifungal activities of various terpenoids as well as benzyl alcohol (with MIC value of 5-10 mM) against yeast *Saccharomyces cerevisiae* (15). Recently, we extended our studies on the mode of action of benzyl alcohol. We have shown that the fungal cell wall is impaired by the action of benzyl alcohol and the cell membrane integrity is disrupted (17). The damage on the cell membrane triggers H^+ ion intake, revealed by an increase in extracellular pH (Figure 1).

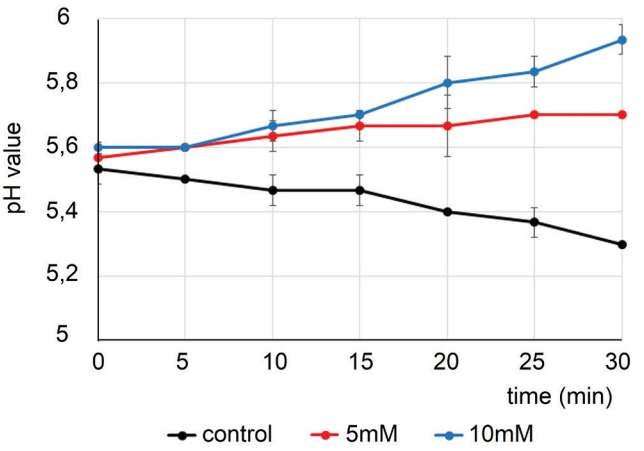

Figure 1. Effect of benzyl alcohol on the extracellular pH of yeast cells. Control corresponds to the measurements without any benzyl alcohol addition. The data represent the average of at least three independent experiments.

Furthermore, visualization of the nuclear membrane by means of labelling the nuclear pore complex protein Nup49 with GFP, revealed that yeast cells had highly deformed nuclear membranes (Figure 2) (17, 18). The effect of benzyl alcohol on the nuclear envelope can be seen as early as 15 min after its application.

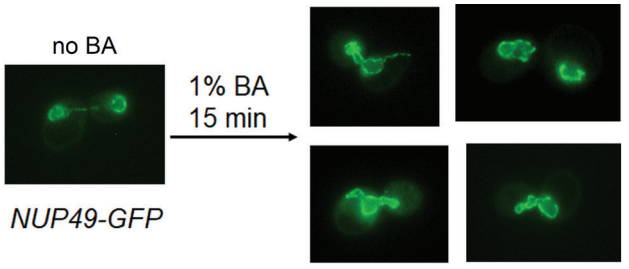

Figure 2. Nuclear envelope phenotype of benzyl alcohol treated cells. a) Yeast cells were transformed with pRS315-GFP-*NUP49* and analyzed using fluorescence microscopy. b) Yeast cells, transformed with pRS315-GFP-*NUP49,* were treated with 1% benzyl alcohol for 15 min and analyzed with fluorescence microscopy. Bars, 5 µm.

Transport through the Nuclear Membrane is Compromised in Benzyl Alcohol Treated Yeast Cells

Later, we tested whether the benzyl alcohol treated cells mislocalize the NLS-GFP (SV40 nuclear localization signal reporter protein fused with GFP) and NES-YFP (SV40 nuclear export signal reporter protein fused with YFP) reporters (Figure 3). In wild type cells with functional NPC, we expect the NLS reporter protein to be found entirely in the nucleus and the NES reporter protein to be excluded from the nucleus and found in the cytoplasm (20). As shown in Figure 2, both reporters were mislocalized upon benzyl alcohol treatment in some yeast cells, pointing to a defect in NPC function (21, 22). Note that mislocalization of these reporters takes somewhat longer than (ca. 1h) the morphological changes observed at the nuclear envelope (15 min).

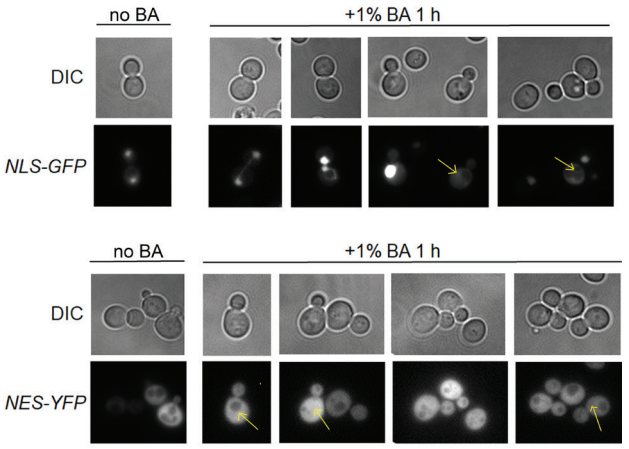

Figure 3. NLS-GFP and NES-YFP reporters were mislocalized in benzyl alcohol treated cells. Wild type cells were transformed with pADH1-NLS-2GFP-TRP1 and pADH1-NES-YFP-TRP1 reporters, and analyzed before and after benzyl alcohol treatment, with fluorescence microscopy. Arrows show the nucleus. Bars, 5 µm.

In order to demonstrate defects in NPC function in benzyl alcohol treated cells in a more quantitative manner, we used a reporter based on the yeast shuttling protein Npl3 described by Madrid et al. (20, 23). Npl3 is mainly nuclear and Npl3-GFP localizes correctly to the nucleus both in the absence and presence of benzyl alcohol (data not shown). However, while Npl3[S411A]-GFP reporter localizes mainly to the nucleus before treatment, benzyl alcohol treated cells show a marked increase in cells with cytoplasmic Npl3[S411A]-GFP reporter signal (Figure 4).

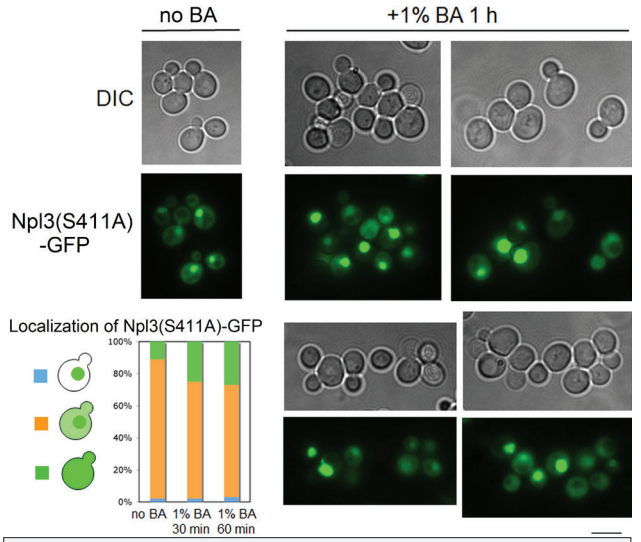

Figure 4. Npl3[S411A]-GFP reporter was mislocalized in benzyl alcohol treated cells. Wild type cells were transformed with pRS315-*npl3*[S411A]-GFP reporter, and analyzed before and after benzyl alcohol treatment, with fluorescence microscopy. Bars, 5 µm.

Diffusion Limit through the Nuclear Membrane is Increased in Benzyl Alcohol Treated Yeast Cells

In order to test the diffusion limit through NPCs, we used reporter proteins LexA-NES-GFP and Pho4(Δ157-164)-GFP. Both 51.5 kDa protein LexA-NES-GFP and 61.8 kDa protein Pho4(Δ157-

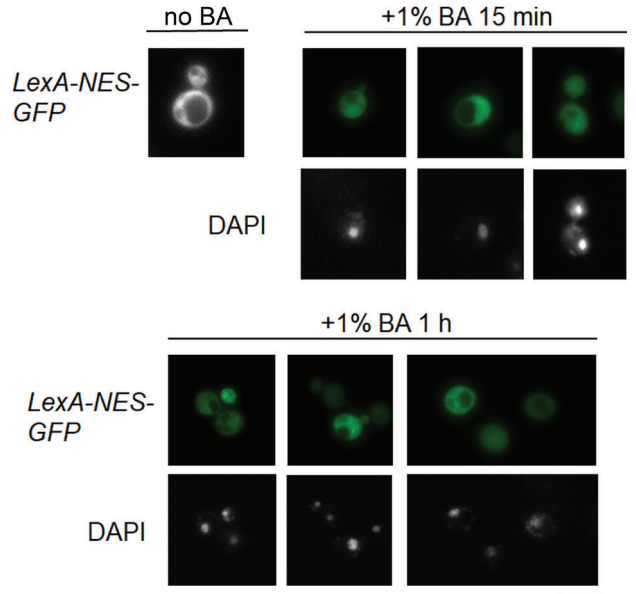

Figure 5. Diffusion limit is increased in wild type cells upon benzyl alcohol treatment. Wild type cells were transformed with pRS314-*LexA-NES*-GFP reporter, and analyzed before and after benzyl alcohol treatment, with fluorescence microscopy. Bars, 5 µm.

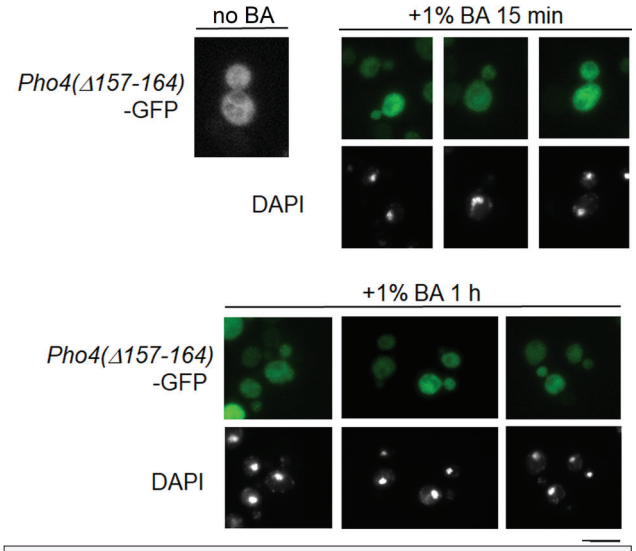

Figure 6. Diffusion limit is increased in wild type cells upon benzyl alcohol treatment. Wild type cells were transformed with pRS314-*Pho4*(Δ157-164)-GFP reporter, and analyzed before and after benzyl alcohol treatment, with fluorescence microscopy. Bars, 5 µm.

164)-GFP were excluded from the nucleus in yeast cells, but mislocalized inside the nucleus upon benzyl alcohol treatment (Figures 5 and 6). Thus, the diffusion limit increased upon benzyl alcohol application.

Mitochondria is Intact in Benzyl Alcohol Treated Yeast Cells

Disruption of the cell membrane as well as defects in nuclear pore function raises the question whether other organelles are also affected. In order to test this question, we performed an MTT mitochondrial functionality assay (24). This analysis revealed that benzyl alcohol treated cells have functional mitochondria, with no detectable loss in MTT activity (Figure 7).

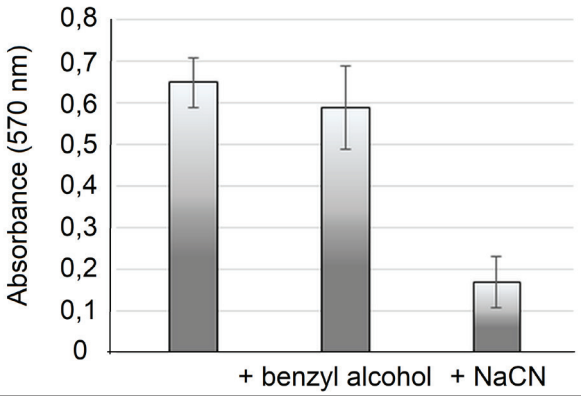

Figure 7. Mitochondria remains intact upon benzyl alcohol treatment. Optical density was measured at 570 nm using a spectrophotometer in order to determine the MTT reduction by yeast cells.

DISCUSSION

We have previously shown that benzyl alcohol acts as an antifungal agent against *S. cerevisiae* cells (15), and causes defects in the cell wall and cell membrane structures (17). In this study, we wanted to unearth any effects of benzyl alcohol on the organelle membranes of yeast cells. Visualization of the nuclear membrane by means of labelling the Nup49 protein a protein in the structure of nuclear pore complex that is embedded to the nuclear membrane with GFP, revealed that benzyl alcohol treated yeast cells had highly deformed nuclear membranes (Figure 2).

We have recently reported that certain stress conditions, including benzyl alcohol treatment, cause a reduction in Pom34 protein levels in up to 20% of untreated cells after incubation with the compound (19). Benzyl alcohol treatment mislocalized *POM34* mRNA towards the cytoplasmic fraction, reducing the translation of Pom34 protein. Since Pom34 is a protein in the structure of nuclear pore complexes (NPC), the gateways between the nucleus and cytoplasm, we asked whether benzyl alcohol treatment might affect NPC function. We first tested whether the benzyl alcohol treated cells mislocalized the NLS-GFP (SV40 nuclear localization signal reporter protein fused with GFP) and NES-YFP (SV40 nuclear export signal reporter protein fused with YFP) reporters (Figure 3). In wild type cells with functional NPC, we expect NLS reporter protein to be found entirely in the nucleus and the NES reporter protein to be excluded from the nucleus and found in the cytoplasm (20). However, both reporters were mislocalized upon benzyl alcohol treatment in some yeast cells, pointing to a defect in NPC function (21, 22).

In order to demonstrate defects in NPC function in benzyl alcohol treated cells in a more quantitative manner, we used a reporter based on the yeast shuttling protein Npl3 described by Madrid et al. (20, 23). Npl3 is a nuclear protein, and accordingly Npl3-GFP is exclusively nuclear in yeast cells, spectroscopically no detectable cytoplasmic signal in wild-type cells is observed. The Npl3[S411A]-GFP mutant, which cannot undergo Ser411 phosphorylation, in contrast, partially mislocalizes to the cytoplasm (20, 23). Although Npl3[S411A]-GFP reporter localized mainly to the nucleus in wild type cells before treatment, benzyl alcohol treatment caused an increase in cells with cytoplasmic Npl3[S411A]-GFP (Figure 4). This result is similar to the mislocalization of the same reporter in NPC defective cells (20).

Ions and small metabolites can freely diffuse through NPCs; however, molecules possessing a mass >40 kDa cannot diffuse and have to be actively transported (25). In order to test the diffusion limit through NPCs, we used reporter proteins LexA-NES-GFP and Pho4(Δ157-164)-GFP, having an MW greater than the diffusion limit in yeast cells (26, 27). Both 51.5 kDa protein LexA-NES-GFP and 61.8 kDa protein Pho4(Δ157-164)-GFP were excluded from the nucleus in wild type cells, but mislocalized inside the nucleus upon benzyl alcohol treatment (Figure 5 and 6). This result is also similar to the mislocalization of the same

reporters in NPC defective cells (20). Thus, we conclude from these observations that wild type cells treated with benzyl alcohol show various defects in nuclear pore function and diffusion limit is increased in these cells.

CONCLUSION

Considering our previous results showing that benzyl alcohol inhibits translation of Pom34 protein, a transmembrane protein in the structure of NPC, we propose that this antifungal agent targets NPC structures. Benzyl alcohol causes structural and functional defects in the NPC, which perhaps contributes to the antifungal activity of this molecule (28). These observations extend our understanding of the mechanism of action of membrane fluidizer alcohols against yeast cells.

Acknowledgement: pRS315-GFP-*NUP49,* pADH1-*NLS-2*GFP-TRP1, pADH1-*NES*-YFP-TRP1, pRS315-*npl3*[S411A]-GFP, pRS314-*LexA-NES*-GFP and pRS314-*Pho4*(Δ157-164)-GFP reporters are generous gift of Prof. Karsten Weis (20).

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