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Effect of nagilactone E on cell morphology and glucan biosynthesis in budding yeast *Saccharomyces cerevisiae*

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ABSTRACT

Nagilactones are norditerpene dilactones isolated from the root bark of *Podocarpus nagi*. Although nagilactone E has been reported to show antifungal activities, its activity is weaker than that of antifungals on the market. Nagilactone E enhances the antifungal activity of phenylpropanoids such as anethole and isosafrole against nonpathogenic *Saccharomyces cerevisiae* and pathogenic *Candida albicans*. However, the detailed mechanisms underlying the antifungal activity of nagilactone E itself have not yet been elucidated. Therefore, we investigated the antifungal mechanisms of nagilactone E using *S. cerevisiae*. Although nagilactone E induced lethality in vegetatively growing cells, it did not affect cell viability in non-growing cells. Nagilactone E-induced morphological changes in the cells, such as inhomogeneous thickness of the glucan layer and leakage of cytoplasm. Furthermore, a dose-dependent decrease in the amount of newly synthesized (1, 3)-β-glucan was detected in the membrane fractions of the yeast incubated with nagilactone E. These results suggest that nagilactone E exhibits an antifungal activity against *S. cerevisiae* by depending on cell wall fragility *via* the inhibition of (1, 3)-β-glucan biosynthesis. Additionally, we confirmed nagilactone E-induced morphological changes of a human pathogenic fungus *Aspergillus fumigatus*. Therefore, nagilactone E is a potential antifungal drug candidate with fewer adverse effects.

1. Introduction

Novel antifungal drugs with new modes of action are urgently required owing to an increase in opportunistic fungal infections in immunocompromised patients and the elderly [1]. Current targets of antifungals are limited to the functions and structures unique to fungi; for example, ergosterol, the cell wall and cytosine deaminase. Polyene macrolide antifungals typified by amphotericin B (AmB) directly bind ergosterol in the plasma membrane to form pores in the membrane [2]. These drugs have excellent antifungal potencies but can cause nephropathy. Azole antifungals such as miconazole, fluconazole, and itraconazole inhibit ergosterol biosynthesis [3]. However, clinical isolates have been reported to show resistance to azoles [4]. The most recently developed echinocandins, including micafungin, anidulafungin, and caspofungin, inhibit (1, 3)-β-glucan synthase, thereby weakening the fungal cell wall [5]. As the structure and biosynthesis of fungal cell wall are unique to fungi, it is an excellent target for the development of antifungal drugs [6,7].

Nagilactones are norditerpene dilactones isolated from the root bark of *Podocarpus nagi*, an evergreen tree belonging to Podocarpaceae that grows mainly in western Japan. They exhibit biological activities such as plant growth inhibitory and antiherbivory effects [8,9]. Among them, nagilactones C, D, and F show insect-feeding-deterrent activities [10]. Nagilactone C also showed insecticidal [11] and potent antitumor activities [12]. In contrast, nagilactone E (Fig. 1) has been reported to show antifungal activities against a nonpathogenic budding yeast *Saccharomyces cerevisiae* and two human opportunistic fungal pathogens, *Candida albicans* and *Pityrosporum ovale* [13] in addition to potent cytotoxic activities against P388 murine leukemia cells [14]. The antifungal activity of nagilactone E is weaker than that of antifungal drugs on the market, but nagilactone E enhances the antifungal activity of phenylpropanoids such as anethole and isosafrole against *S. cerevisiae*

Abbreviations: AmB, amphotericin B; Triton X-100, polyethylene glycol *tert*-octylphenyl ether; MCFG, micafungin; MIC, minimum growth inhibitory concentration; ME, malt extract; CFU, colony forming unit

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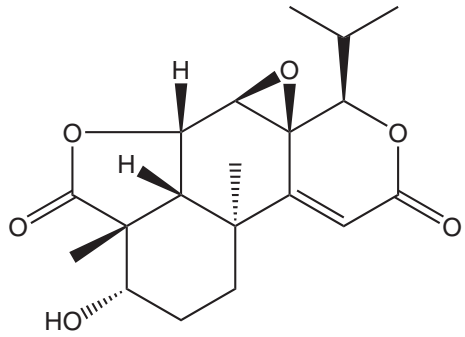

Fig. 1. The structure of nagilactone E.

and *C. albicans* [13,15]. Thus, nagilactone E combined with other drugs has potential for clinical application with less severe adverse effects.

The antifungal mechanisms of nagilactone E itself have not yet been reported. Concerning synergistic effects in combination of drugs, the action mode of one drug is prominently enhanced by another drug, which restricts drug resistance [16,17]. In the case of nagilactone E, such a phenomenon possibly occurs. Therefore, to gain insight into the pharmacological application of nagilactone E with less severe adverse effects in combination with other drugs, analyzing the action mechanisms of nagilactone E alone is expected to provide beneficial cues. Herein, we investigated the antifungal mechanism of nagilactone E against *S. cerevisiae,* which is a model fungus, based on cell viability, changes in cell morphology, visualization of (1, 3)-β-glucan, and (1, 3)-β-glucan synthase activity. In addition, we examined the effect of nagilactone E on a human pathogenic fungus *Aspergillus fumigatus* and its morphology.

2. Materials and methods

2.1. Chemicals

Nagilactone E was from laboratory stock [13]. AmB was purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyethylene glycol *tert*-octylphenyl ether (Triton X-100) was obtained from Alfa Aesar (Lancashire, UK). Micafungin (MCFG) was a gift from Astellas Pharma Inc. (Tokyo, Japan). The drugs were dissolved in dimethyl sulfoxide (DMSO) prior to the following experiments. All other chemicals used were of analytical grade.

2.2. Measurement of cell growth and viability

The parent strain of *S. cerevisiae* BY4741 was obtained from the Yeast Knock Out Strain Collection (Thermo Scientific Open Biosystems, Waltham, MA, USA). The yeast cells were grown overnight at 30 °C with vigorous shaking in YPD medium consisting of 1% Bacto-yeast extract (Difco Laboratories, Detroit, MI, USA), 2% Bacto-peptone (Difco Laboratories), and 2% D-glucose prior to experiments unless stated otherwise.

A wild-type strain *Aspergillus fumigatus* NBRC 5840 was obtained from the Biological Resource Center, NITE (Tokyo, Japan). The fungus was maintained on 2.5% malt extract (ME; Oriental Yeast Co., Ltd., Tokyo, Japan) agar plates.

The *in vitro* minimum growth inhibitory concentration (MIC) of nagilactone E against *S. cerevisiae* was determined by the two-fold broth dilution method described previously [18]. Cells were harvested, washed with 2.5% ME.broth medium, diluted to 1×10^6 cells/mL in the same medium, and then incubated with various concentrations of nagilactone E at 30 °C for 24 h in a 96-well plate (the final volume of cell suspension at 100 μL). The MIC of nagilactone E was determined as the lowest concentration in which no visible growth was observed. All assays were repeated at least three times.

The *in vitro* MIC of nagilactone E against *A. fumigatus* was determined as described above except that the fungus was directly suspended at 1×10^6 germinated spores/mL in ME medium prior to the MIC assay.

For time-kill assay, the yeast cells were diluted to 1×10^7 cells/mL in ME medium containing 0, 100, or 200 μg/mL nagilactone E. Cell suspensions were incubated with vigorous shaking at 30 °C. Aliquots were withdrawn from the suspensions and then spread on YPD plates. The cell viability was determined as the number of colony forming units (CFUs). Data are means ± standard deviations of triplicate experiments.

2.3. Leakage of potassium ions and 260 nm-absorbing materials

The *S. cerevisiae* cells cultured overnight in YPD medium were harvested by centrifugation and washed with 50 mM sodium succinate buffer (S-buffer, pH 6.0). The yeast cells were then suspended at a cell density of 1×10^8 cells/mL in S-buffer containing 0, 100, or 200 μg/mL nagilactone E. Cell suspensions were incubated with vigorous shaking at 30 °C. The supernatants obtained after removal of the cells by centrifugation were used to quantify K^+ ions released from the cells. The quantification was performed with a K^+ ion assay kit based on the tetraphenylborate method [19,20]. The supernatants were also used to measure leakage of 260-nm-absorbing materials as an index of non-specific plasma membrane disruption, which represents the leakage of intracellular nucleosides, nucleotides, and other related compounds with absorption optima at around 260 nm [20,21]. AmB and Triton X-100 were used as positive controls of agents causing leakage of intracellular potassium ions and 260 nm-absorbing materials, respectively. Finally, the cell viability in S-buffer was also determined as CFUs after a 72-h incubation. Data are means ± standard deviations of triplicate experiments.

2.4. Cell wall chitin staining with calcofluor white

Cell wall chitin staining with calcofluor white was performed according to the methods of Yutani et al. [22] with slight modifications. The cells of *S. cerevisiae* BY4741 (10^7 cells/mL) were incubated with 0 and 200 μg/mL nagilactone E at 30 °C with vigorous shaking for 72 h. The cells were washed with deionized water and then incubated in deionized water at 25 °C for 1 h. Calcofluor white (Sigma-Aldrich) at 1 mg/mL was added to the cell suspension. After incubation at 25 °C for 5 min in the dark, chitin was visualized using a fluorescence microscope (excitation wavelength, 330 nm; emission wavelength, 385 nm).

2.5. Cell wall glucan staining with aniline blue dye

The cells of *S. cerevisiae* BY4741 (10^7 cells/mL) were incubated with vigorous shaking in ME medium containing 0 and 200 μg/mL nagilactone E at 30 °C for 72 h. The cells were washed with deionized water and then incubated in deionized water at 25 °C for 1 h. Aniline blue dye (Wako Pure Chemicals Industries, Osaka, Japan) at 5 mg/mL was added to the cell suspension. After incubation at room temperature for 5 min in the dark, (1, 3)-β-glucan of cell wall was visualized using a fluorescence microscope (excitation wavelength, 330 nm; emission wavelength, 385 nm).

2.6. Antifungal assay under hyperosmotic conditions

An antifungal assay under hyperosmotic conditions using sorbitol was performed according to the methods of Frost et al. [23] with slight modifications. Briefly, cells were suspended to 5×10^5 cells/mL in yeast nitrogen base (Difco Laboratories) containing 0.67% glucose, 3 mM leucine, 12 mM adenine, and 0.67 mM uracil. Sorbitol at 0.8 M was further added to the medium as an osmotic support. The culture was then incubated in the presence of various concentrations of