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Valinomycin Affects the Morphology of *Candida albicans*

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Abstract Microbial metabolites were screened for inhibitors of hyphal growth in *Candida albicans*. Inhibitory activity was found among metabolites of a culture of an actinomycete, which had been isolated from soil. The active substance inhibited hyphal growth and induced growth as a chain of yeast cells under hyphal growth induction conditions. The active substance was purified and analyzed with ^{1}H-NMR, ^{13}C-NMR and mass spectra. The substance was identified as valinomycin, and commercial valinomycin inhibited hyphal growth as effectively as the purified metabolite. The effective concentration was from 0.49 to 62.5 μg/ml. Valinomycin also inhibited hyphal growth in other dimorphic fungi, *Candida tropicalis* and *Aureobasidium pullulans*. These results suggest that valinomycin may be a useful tool for understanding the morphological transition of dimorphic fungi.

Keywords *Candida albicans*, dimorphism, hyphal growth, morphology, valinomycin

Introduction

Infections by opportunistic fungal pathogens have increased dramatically and have been a major medical problem in recent years; however, there are only five antifungal drug classes: polyenes, flucytosine, azoles, squalene epoxidase inhibitors, and echinocandins [1～3]. New potential targets are required for antifungal development. *Candida albicans* is the major opportunistic fungal pathogen in humans, and it can reversibly switch between yeast and hyphal forms [4]. Morphological mutants with hyphal growth defects have low virulence compared to the wild type [5], suggesting that the hyphal growth inhibition will reduce the pathogenicity. Understanding the mechanism of this dimorphism should provide valuable information to develop antifungal therapies.

Genetic approaches have identified several genes involved in the regulation of hyphal growth, suggesting that the morphological transition is controlled by two signal cascades, the mitogen-activated protein kinase (MAPK) and cAMP-PKA transduction pathways [6]. On the other hand, several environmental factors, such as temperature, pH and nourishment, are involved in this dimorphism. For example, high temperature (≥37°C), neutral pH (≥6.5), and nutrient starvation induce hyphal growth [7], and low temperature, acidic pH (4 to 6) and enriched media induce yeast growth [8]. Additives such as *N*-acetyl-D-glucosamine, proline and serum induce hyphal growth [4, 9, 10] and conversely, farnesoic acid and phenylethanol induce yeast growth [8, 10]. Although the effective concentration is high, cysteine also induces yeast growth [10]. Inhibitors of hyphal growth (inducers of yeast growth) are not only useful tools to analyze the dimorphism, but are also interesting as the lead compound to develop antifungal agents.

We focused on inhibitors of hyphal growth to understand the dimorphism of *C. albicans* and screened substances that inhibit hyphal growth and induce yeast-like growth under hyphal growth induction conditions. Here we demonstrate that hyphal growth was inhibited by valinomycin (potassium ionophore), and discuss the effect of valinomycin on the growth and morphology of *C. albicans*.

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Materials and Methods

Microorganisms, Media and Culture Conditions

Candida albicans IFO1061 was used in this study. The organism was maintained on YPD agar consisting of 1.0% yeast extract (Difco), 2.0% polypeptone, 2.0% glucose and 2.0% agar. The cells were subcultured in a 15-ml test tube containing 4 ml YPD medium at 30°C for 24 hours with shaking for several experiments.

To examine the effect of valinomycin on the growth and morphology of various microorganisms, yeasts and fungi were cultured as follows: 96-well plates containing SPG medium [0.17% yeast nitrogen base without $(NH_4)_2SO_4$ and without amino acids (Difco), 0.1% L-proline, and 2.0% D-galactose, pH 5] were inoculated with *C. albicans* IFO1061, *C. albicans* TUA6 (provided by Dr. Umeyama, National Institute of Infectious Disease) and *C. tropicalis* No. 559-9 [11], and subcultured in 4 ml YPD medium at 30°C for 24 hours. Plates containing YPD medium were also inoculated with *Aureobasidium pullulans* NBRC4466, *Saccharomyces cerevisiae* OHNY2 [12], *Schizosaccharomyces pombe* JY741 (provided by Dr. Shimoda, Osaka City Univ.) and *C. boidinii* AKU4618, which were subcultured in 4 ml YPD medium at 30°C for 24 hours. These plates were incubated at 30°C for 24 hours to observe the growth and morphology of the above microorganisms. Spores of *Aspergilus niger* ATCC6275 and *Penicillium chrysogenum* IFO4626, which were cultured on YPD agar at 30°C for 7 days, were suspended in 4 ml YPD medium to an optical density of 0.01 to 0.015 at 600 nm. The spore suspensions were incubated at 30°C for 3 days to observe the growth and morphology of the fungi.

Induction of Hyphal Growth

To induce hyphal growth, yeast cells incubated in YPD medium were inoculated in SPG medium, modified Lee's medium [13] [M-Lee; 0.5% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.25% K_2HPO_4, 0.5% NaCl, 1.25% D-galactose, 0.05% L-alanine, 0.13% L-leucine, 0.1% L-lysine, 0.01% L-methionine, 0.007% L-ornithine, 0.05% L-proline, 0.05% L-threonine, and 0.0001% biotin, pH 5] and RPMI-1640 medium (Sigma) with 10% fetal bovine serum (FBS; ICN biomedicals) and incubated at 30°C for 24 or 48 hours. The morphology of more than 300 cells was observed microscopically and the ratio of hyphal cells was calculated.

Screening of a Hyphal Growth Inhibitor

Microbial products were screened for hyphal growth inhibitors. The strains isolated from soil samples were cultured in a test tube containing 3 ml glucose - bouillon medium (GB; 1.0% Polypepton, 1.0% beef extract, 1.0% D-glucose and 0.3% NaCl, pH 7) at 30°C for 7 days with shaking. After cultivation, 3 ml acetone was added, and the cells were crushed ultrasonically. The cell disruptant was centrifuged at 2,300 *g* for 10 minutes and 20 μl of the supernatant was added to 160 μl of SPG medium in a 96-well plate for screening. *C. albicans* cells subcultured in YPD medium were harvested by centrifugation, washed twice with PBS (0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4 and 0.29% $Na_2HPO_4 \cdot 12H_2O$, pH 5), and then suspended in PBS (optical density of 0.3 to 0.35 at 600 nm). The cell suspensions were additionally diluted 10 times with SPG medium. Twenty μl of SPG medium containing yeast cells was added to 180 μl of SPG medium containing each microbial metabolite in a 96-well plate. After incubation at 30°C for 24 hours, the cell morphology was observed by microscopy.

Measurement of the Inhibitory Activity of Hyphal Growth

The dilution assay method was used to measure inhibitory activity. Two-fold serial dilution of the active substance or reagents was performed with SPG medium in a 96-well plate. SPG solutions (100 μl) diluted by 11 steps were prepared in the plate. *C. albicans* cells subcultured in YPD medium were harvested, washed twice with PBS, and then suspended in PBS to an optical density of 0.3 to 0.35 at 600 nm. The cell suspension was additionally diluted 50 times with SPG medium. 100 μl of the suspension was added to 100 μl of the SPG solution containing the active substance or reagents in a 96-well plate. After incubation at 30°C for 24 hours, the cell morphology was observed by microscopy. Farnesoic acid was provided from Dr. Tanaka, Osaka City University.

Growth-rate Determination

To examine the effect of valinomycin on the cell growth of *C. albicans*, yeast cells grown in YPD medium were inoculated into 20 μl of SPG and RPMI-1640 media with or without 1.0 μg/ml valinomycin to an optical density of 0.025 to 0.03 at 600 nm, and incubated at 30°C for 48 hours. 1.0 ml was sampled at each time point and the cells were harvested, washed twice with PBS, and then freeze-dried for 2 days. The dry weight of cells was determined. Cell morphology at each time point was also observed by microscopy.

Results and Discussion

Screening for Inhibitors

We screened substances that inhibit hyphal growth and induce yeast growth under hyphal growth induction conditions of *C. albicans*. Previously we examined the relationship between the culture medium and the morphology [14]. Cells grown in YPD liquid medium had a yeast form (>95%), whereas many cells in M-Lee, SPG, and RPMI-1640 liquid media had a hyphal form after 24 hours of culture. The ratios of cells with a hyphal form in M-Lee and SPG (>90%) were higher than the ratio in RPMI-1640 (>60%). There was no difference in the appearance of hyphal cells grown in M-Lee and SPG. SPG medium was used here for screening.

Approximately 500 strains of microorganisms (almost all of them, actinomycetes) from soil samples were collected at various sites in Japan, and their cultures were examined for inhibitory activity. Only one strain (No. 3-2), isolated from soil in a bamboo grove in Osaka prefecture, was found to give reproducible activity. Cells in SPG medium containing metabolites grow as chains of yeast cells. After 24 hours of culture, the average number of cells in the chain was eight.

Identification of the Active Substance as Valinomycin

No. 3-2 strain was cultured in GB medium at 30°C with shaking. The active substance was isolated from the cultured broth and then identified as valinomycin (Fig. 1) through mass and NMR analysis (data not shown).

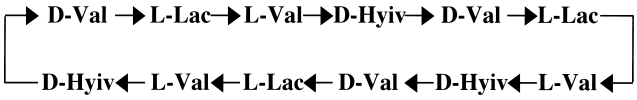

Fig. 1 Structure of valinomycin.

Inhibitory Activity of Valinomycin against Hyphal Growth

We then examined the effect of valinomycin on cell morphology (Table 1). Valinomycin inhibited hyphal growth under hyphal growth induction conditions and induced growth as chains of yeast cells. The morphology induced by valinomycin is shown in Fig. 2Bb. The yeast growth in YPD medium was not inhibited by valinomycin (100 μg/ml), suggesting that valinomycin specially acts on hyphal cells. Valinomycin effectively inhibited hyphal growth and the induction of yeast-like growth over a wide range of 0.49 to 62.5 μg/ml (Table 1). The cells in SPG medium did not grow at 125 μg/ml of valinomycin. On the other hand, farnesoic acid, cysteine and phenylethanol, which are known as inducers of yeast growth, were not effective over a wide range (Table 1): in particular, no activity was detected with farnesoic acid. This difference from the reported result might be due to differences in the assay method or the strain of *C. albicans* used.

Morphological Change of Hyphal Cells by the Addition of Valinomycin

We observed morphological changes of hyphal cells by the addition of valinomycin. Cells cultured in SPG medium had a hyphal form (>90%) after 12 hours of culture. The ratio of hyphal cells decreased to 14% and below by the addition of valinomycin at the beginning of culture (Fig. 2A). Also, with the addition of valinomycin at 9 hours of culture, the ratio of hyphal cells increased with time until 12 hours of culture and chains of yeast cells developed from hyphae after 12 hours of culture. The morphology of the chains is shown in Fig. 2Bc. The ratio of hyphal cells without chains subsequently decreased with time (Fig. 2A). These results also suggest that valinomycin induces the morphological transition from hypae to chains of yeast cells.

Table 1 Inhibitory activity of valinomycin against hyphal growth

Substance	Initial conc. (μg/ml)	Dilution										
		1	2	4	8	16	32	64	128	256	512	1024
Valinomycin	125	N	Y	Y	Y	Y	Y	Y	Y	Y	H	H
Farnesoic acid	94400	H	H	H	H	H	H	H	H	H	H	H
Cysteine	12100	Y	Y	Y	H	H	H	H	H	H	H	H
Phenylethanol	40	N	N	Y	Y	H	H	H	H	H	H	H

Control (no addition of substances) showed hyphal growth. The method is described in Materials and Methods. Y: chain of yeast cells, H: hyphae, N: no growth.

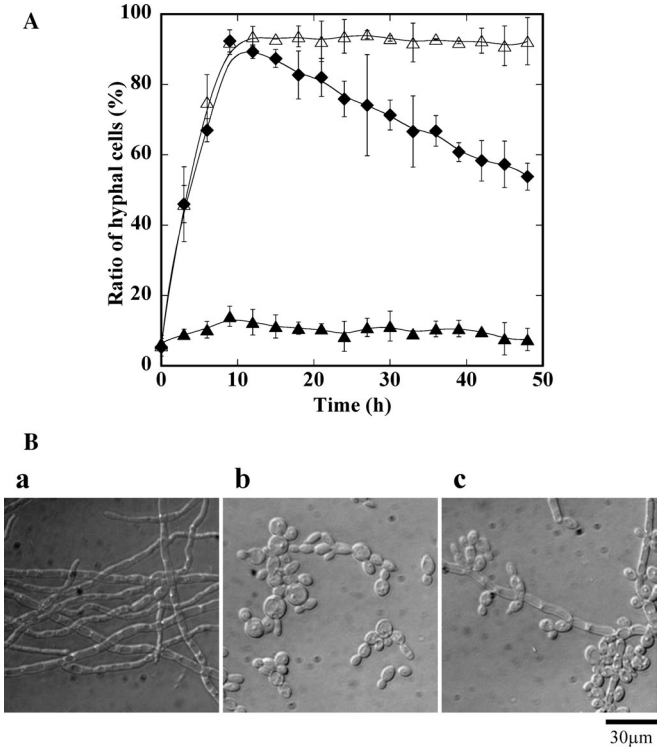

Fig. 2 (A) Morphological transition from the hyphal form with the addition of valinomycin. (B) Morphology of cells incubated for 24 hours in SPG with (b) or without (a) 1.0 μg/ml of valinomycin or in SPG, to which 1.0 μg/ml of valinomycin was added after 9 hours of incubation (c).

(A) Time courses of hyphal cell ratios are shown in cells incubated in SPG with (closed triangle) or without (open triangle) 1.0 μg/ml of valinomycin or in SPG, to which 1.0 μg/ml of valinomycin was added after 9 hours of incubation (closed diamond). The hyphal cell ratio was judged as follows: each value represents the average of three independent assays±S.D. Here, chains of yeast cells were defined as a chain of 3 or more cells and a chain was counted as one cell. Even if hyphae involved branching, it was considered as one cell. If hypahe caused chains of yeast cells (>2 cells), the hyphae were counted as one cell except in the case of the hyphal form.

Effect of Valinomycin on the Cell Growth of *C. albicans*

Cells grew as chains of yeast cells in SPG medium with valinomycin (1.0 μg/ml), however, the growth was not better than without valinomycin (Fig. 3A). To confirm whether valinomycin induces growth as chains of yeast cells without delaying growth, we examined the growth and morphology in RPMI-1640 medium with or without valinomycin. The cells grew as chains of yeast cells in RPMI-1640 medium with valinomycin (Fig. 3B) and there was no large difference between the dry weights of cells cultured with and without valinomycin after 24 hours of culture (Fig. 3A). As previously described, the ratio of hyphal cells in RPMI-1640 medium was lower than that in SPG medium, although the ratio of hyphal cells exceeded 60%. If there was a significant distinction between the growth speeds of hyphal cells and chains of yeast cells, differences should occur in those dry cell weights. These results therefore suggest that valinomycin induces growth as chains of yeast cells without delaying growth in RPMI-1640 medium.

Effect of Valinomycin on the Growth and Morphology of Other Microorganisms

Valinomycin, a potassium ionophore, is known as an antibiotic against tubercle bacillus; however the action of valinomycin on the morphology of fungi and yeast is not well known. The antimicrobial activities of valinomycin on several yeasts and fungi were determined by two-fold serial dilution. The resulting minimum inhibitory concentrations (MICs) are shown in Table 2. In all yeasts and fungi, the MICs were 25 μg/ml and above. Subsequently, we examined the effect on the morphology of these strains. We expected morphological changes of *Aspergillus niger* and *Penicillium chrysogenum* by the addition of valinomycin because the strains grow as hyphae; however, no change was observed between 0.1 and 100 μg/ml of valinomycin. On the other hand, in dimorphic fungi (*C. albicans* TUA6, *C. tropicalis* No. 559-9 and *Aureobasidium pullulans*

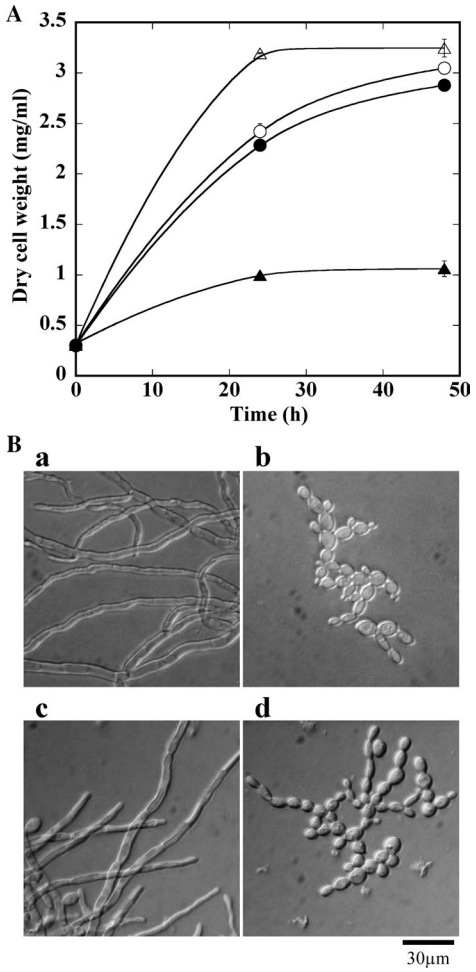

Fig. 3 (A) Effect of valinomycin on cell growth. Growth in each medium is shown. (B) Effect of valinomycin on cell morphology.

(A) Open triangle: SPG; closed triangle: SPG+1.0 μg/ml of valinomycin; open circle: RPMI-1640; closed circle: RPMI-1640+1.0 μg/ml of valinomycin. (B) Cells subcultured in YPD medium were incubated at 30°C for 24 hours in SPG medium with (b) or without (a) 1.0 μg/ml of valinomycin or in RPMI-1640 medium with (d) or without (c) 1.0 μg/ml of valinomycin.

NRBC4466), valinomycin induced growth as yeast or chains of yeast cells, suggesting that valinomycion effects morphological changes in dimorphic fungi.

Conclusions

We screened for hyphal growth inhibitors in *C. albicans* from microbial metabolites. Inhibitory activity was found in metabolites of an actinomycete isolated from soil and the active substance was purified. As a result of structural analysis, we found that valinomycin inhibited hyphal growth and induced growth as chains of yeast cells under hyphal growth induction conditions. Valinomycin was also highly effective in the induction of growth as chains of yeast cells compared with farnesoic acid, cysteine and phenylethanol. Valinomycin is toxic to humans and has not previously been used as a medicine. It is therefore difficult to use as an antifungal agent, although it is a useful tool for understanding the dimorphism of *C. albicans*. Valinomycin is a potassium ionophore, and we have found few reports of intracellular potassium concentration involved in dimorphism. To our knowledge, the relationship between germ tube formation in *C. albicans* and the intracellular sodium/potassium ratio has been described in only one paper [15]. Future detailed analysis using valinomycin will lead to understanding of the relationship between dimorphism and intracellular potassium concentration.

Table 2 Effect of valinomycin on the growth and morphology of various microorganisms

Strains	MIC*1 (μg/ml)	MICY*2 (μg/ml)
Dimorphic fungi		
C. albicans IFO1061(yeast)*3	>100	—
C. albicans IFO1061	100	0.4
C. albicans TUA6	100	0.4
C. tropicalis No. 559-9	100	0.4
Aureobasidium pullulans NBRC4466	50	6.25
Yeasts		
Saccharomyces cerevisiae OHNY2	>100	—
Schizosaccharomyces pombe JY741	25	—
C. boidinii AKU4618	>100	—
Filamentous fungi		
Aspergilus niger ATCC6275	>100	n*4
Penicillium chrysogenum IFO4626	100	n

*1 Minimum inhibitory concentration.
*2 Minimum concentration inducing yeast-like growth.
*3 Cells were cultured in YPD medium to induce yeast growth.
*4 Valinomycin did not induce yeast-like growth between 0.1 and 100 μg/ml.

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