Pseudohyphal growth induced by exposure of yeast cells to subinhibitory levels of antifungal azoles in *Candida tropicalis*.

* Takahito Suzuki¹, Shin-Ichi Iwaguchi¹ and Teijiro Kamihara².

¹Department of Biological Science, Faculty of Science, Nara Women's University, Kitauoya, Nara-shi, 630-8506, Japan

²Osaka University of Health and Sport Sciences, Kumatori, Osaka 590-0496, Japan

Received December 28, 2001

Summary: The azole class of compounds kills fungal cells through an inhibition of ergosterol biosynthesis resulting in malfunction and breakdown of cell membranes. Subinhibitory levels of these compounds were found bringing about a transition from yeast form to pseudohyphal one in *Candida tropicalis*, as well as ethanol reported previously. The other classes of inhibitors for ergosterol biosynthesis, terbinafine and amorolfine, also caused the transition. These findings implicated the function of ergosterol for filamentous growth. Although depolarized cell growth was observed to precede filamentation during the process of ethanol-induced pseudohyphal formation in this organism, no distinct depolarization was found to occur during the process induced by these antifungal compounds.

Key Words: antifungal agents, *Candida tropicalis*, pseudohyphal growth

Introduction

Fungal dimorphism, the interconversion between two different growth forms according to environmental conditions, is a simple model of cellular morphogenesis as well as considered an important virulence trait of many pathogenic fungi (Cutler, 1991; Calderone 1994; Odds, 1994; Lo *et al.*, 1997). *Candida albicans* and its related petroleum-assimilating species *C. tropicalis* are opportunistic pathogens of warm-blooded animals and humans, growing in either yeast or filamentous form depending on environmental conditions. The clinical importance of candidiasis has increased recently due to the onset of AIDS and medical procedures including immunosuppressive therapy and organ transplants. The developmental transition from yeast form to the filamentous form of these *Candida* species is considered to contribute to early steps in invasion of epithelial tissues (Fidel & Sobel, 1994).

Recent investigations on fungal dimorphism have been progressively done in the baker's yeast *Saccharomyces cerevisiae* as well as in *C. albicans*. It has turned out that diploid cells of *S. cerevisiae*, with a bipolar mode of bud site selection, can undergo a switch to a pseudohyphal form in response to nitrogen starvation (Gimeno *et al.*, 1992). This filamentous growth form represents a dramatic change in the cellular program in which the cells elongate, adopt a unipolar budding pattern, remain physically connected in chains, and invade the agar (Kron *et al.*, 1994). The haploid cells of this organism with an axial mode of bud site selection, also respond to nutrients deprivation by penetrating the agar, a process termed the invasive growth,

in which growth behavior is switched to a bipolar mode of bud site selection (Roberts & Fink, 1995). In *C. albicans*, filament formation contributes to the pathogenicity of this organism, since the defects in the formation are associated with reduced virulence in the mouse model (Lo et al., 1997).

Azole antifungals work as the inhibitor of both ergosterol biosynthesis and filament formation (Hawser *et al.*, 1996; Hawser and Islam, 1999). Ergosterol is the fungal sterol similar to animal cholesterol, but with significant structural differences. Sterols are known to play a structural role in membrane integrity. Most of the antifungal compounds in medical and agricultural use affect sterol synthesis or function. However, little is known of the precise physiological roles that ergosterol plays in the yeast cell (Parks & Casey 1995). Significant implications of the function of ergosterol have been reported as follows. Ergosterol depletion by action of azole leads to a drastic change in membrane fluidity accompanied by drastic reduction of amino acid transport in *C. albicans* (Ansari & Prasad 1993). Ergosterol levels were shown to be paralleled with viability data, using cells of a sterol auxotrophic mutant of *S. cerevisiae* which contained higher levels of the sterol exhibiting greater tolerances to heat and ethanol (Swan & Watson 1998). An increase in the proportion of ergosterol, unsaturated fatty acid levels and the maintenance of phospholipid biosynthesis seems to be responsible for ethanol tolerance in *S. cerevisiae* and *Kloeckera apiculata* (Alexandre *et al.*, 1994). Aberrant budding and perturbation of mating efficiency were reported in ergosterol auxotrophs of *S. cerevisiae* grown on media supplemented with various sterols other than ergosterol, which indicated sterol alterations gave effects on conjugation (Tomeo et al., 1992).

Here, we report another case of implications of the function of ergosterol, in which exposure of yeast cells to subinhibitory levels of antifungal azoles induced pseudohyphal growth in *C. tropicalis*.

Materials and Methods

Yeast cultures. The strain used in this study was *C. tropicalis* Pk233. Yeast cells were precultured as colonies on YPD solid medium as described previously (Suzuki *et al.*, 1998). Sabouraud medium contained (g/L): Bacto Neopeptone (Difco), 10 and glucose, 20. Inocula were prepared by seeding a loopfull of cells into 100 ml of semi-defined medium (SDM, pH 6.0) containing (g/L): KH_2PO_4 , 3.4; NaH_2PO_4 , 3.9; glucose, 20; vitamin-free Casamino acids (Difco), 5; $(\text{NH}_4)_2\text{SO}_4$, 5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.125; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.025; KCl, 0.425; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.125; and biotin, 20×10^{-6} . Inoculated cultures were incubated at 30°C with shaking (90 rpm) for approximately 30 h. They were then diluted two hundred fold into a fresh SDM (control medium) and incubated at the same temperature (control culture). The medium supplemented with an antifungal agent was prepared by adding the agent to the control medium. At various times during incubation, 5 to 10 ml portions of each culture were taken for either fixation by adding an equal volume of 6% (w/v) formaldehyde solution in 0.2 M K-phosphate buffer (pH 6.5) or fixation in 55% (w/v) ethanol. The former fixed sample was used for monitoring growth by measuring turbidity at 600 nm and for the observation of cell shape by microscopy. The latter one for observation of DAPI-stained DNA by fluorescence microscopy, according to the method described

previously (Suzuki *et al.*, 1998).

Thin layer chromatography (TLC). Yeast cells grown in Sabouraud media for 48 h in the presence or absence of antifungal agents were used for analyzing sterol composition of membranes. Contents of ergosterol and its precursors were examined by TLC according to the method described by Cannon and Kerridge (1988).

Photomicroscopy. Photomicrographs were taken with an Olympus BH-2-RFL microscope equipped with a phase-contrast apparatus or an Olympus BX51-34-FLD microscope attached with a Nomarski apparatus. Photographs were taken on Kodak Tri-X Pan film (ISO 400).

Determination of the minimal inhibitory concentration (MIC). MIC was defined here as the concentration of drug that showed 50% inhibition of the growth of *C. tropicalis* in Sabouraud media grown at 30°C for 48 h. In the case of 5-fluorouracil (5FC), SDM was used instead of the Sabouraud one. The methods were according to the protocol described by McGinnis (1980). The concentrations of drugs that induced filamentous growth in this organism were measured in SDM as described above.

Antifungal agents and chemicals. Aculeacin A was kindly given to us by Dr M. Miyata at Gifu Pharmaceutical University, Japan. Amphotericin B, brefeldin A, 5FC and ketoconazol were purchased from Sigma Chemical Co. (St Louis, MO, USA). Miconazole was from Janssen Pharmaceutica, Beerse, Belgium, and clotrimazole from Bayer, Leverkusen, Germany. Terbinafine and amorolfine were from Sandoz, Vienna, Austria. Antifungal agents were dissolved in dimethyl sulfoxide (DMSO). Final concentrations of DMSO were less than 0.1% v/v, which did not affect growth rates or morphogenetic transition.

Results and Discussion

Induction of yeast to pseudohyphal growth by antifungal agents that inhibit ergosterol biosynthesis in *C. tropicalis*

Hawser *et al.*, (1996) described that some antifungal agents including amphotericin and azole compounds inhibited filamentation at sub-MIC values in *C. albicans*. We carried out similar experiments in the related yeast *C. tropicalis* and, the contrary to our expectations, we found azole antifungals including ketoconazol, miconazole and clotrimazole induced filamentation in this organism when they were supplemented to the growth media at subinhibitory concentrations (Table 1). On the other hand, amphotericin B, brefeldin A and 5-fluorocytosin did not induce filamentatous growth at all in this organism. Nystatin, another type of inhibitor of cell membrane biosynthesis, did not induce filamentatous growth. All azole antifungal agents have been known to work principally by inhibition of cytochrome P450 14 α -demethylase (Lyman and Walsh, 1992). This enzyme is in the sterol biosynthesis pathway that leads from lanosterol to ergosterol. By TLC, the content of ergosterol of membranes was shown to decrease with an increase in the content of lanosterol after incubating *C. tropicalis* cells in the presence of these azole compounds at the MIC, which seemed reasonable because ergosterol levels were parallel with viability data (Swan & Watson 1998). Since the filamentous growth induced by these azole compounds occurred at subinhibitory concentrations, at the order of ten to the minus one to minus two of the MIC (Table 1), their inhibitory action on sterol biosynthesis would not be the direct cause for the filamentation. We also examined the content of

ergosterol at sub-MIC values by TLC, however, no parallel relationships were demonstrated between the content of ergosterol and the frequency of occurrence of filamentation. Therefore, filamentation of this organism by the supplement of azole compounds did not simply be due to the decrease in ergosterol contents in membranes of this organism. We also examined another allylamine antifungal agent, terbinafine, which exerts its action by interfering with fungal ergosterol biosynthesis, through inhibition of the enzyme squalene epoxidase (Lyman and Walsh, 1992). Amorolfine, a morpholine derivative that blocks delta 14 reduction and delta 7-8 isomerisation, resulting in depletion of ergosterol and accumulation of ignosterol in fungal cytoplasmic membrane (Lyman and Walsh, 1992). These two agents were also found induce filamentous growth at sub-MIC values in this organism (Table 1). Therefore, some causal relationships should be present between the induction of filamentous growth and the supplement of antifungal agents that affect ergosterol biosynthesis.

Table 1. Comparisons of MIC and the transition of yeast to filamentous growth in the presence of antifungal agents in *C. tropicalis*.

Antifungal agent	MIC	Filamentous growth at subinhibitory concentrations	
Clotrimazole	50	Yes	(>0.50)
Ketoconazole	2.5	Yes	(>0.025)
Miconazole	12.5	Yes	(>0.10)
Terbinafine	50	Yes	(>0.50)
Amorolfine	1.0	Yes	(>0.025)
Aculeacin A	0.03	No	
Amphotericin B	2.5	No	
Brefeldin A	25	No	
Nystatin	12.5	No	
5-fluorocytosin	12.5	No	

MIC; Minimal inhibitory concentration (mg/L)

Values in parentheses represent concentrations causing filamentous growth.

In the case of ethanol-induced pseudohyphal formation in this organism, a decrease in the molar ratio of the saturated acid content of phospholipids, especially of palmitic acid in phosphatidylinositol was associated with filamentous growth (Uejima et al., 1987). The occurrence of filamentous growth may be due to an increase in plasma membrane fluidity through a decrease in the sterol:phospholipid and sterol:protein ratios and an increase in unsaturation index (Alexandre *et al.*, 1998). Even a slight change in sterol content could affect the phospholipid composition by affecting the transfer rate of phospholipids by phospholipid-transfer proteins, especially by a phosphatidylinositol-transfer protein (Szolderits *et al.*, 1989), which might also be a cause for the filamentous growth in this organism. Numbers of separate signaling pathways leading to hyphal formation have been shown in *C.*

albicans, which are stimulated with various environmental cues (Ernst, 2000). One promising clue to elucidate the phenomenon described here is that changes in ergosterol biosynthesis in membranes, which could not be detected enough by TLC, or the presumed increase in plasma membrane fluidity by the supplement of these antifungal agents may trigger some signaling pathway leading to filamentous growth in *C. tropicalis*. Examinations of occurrence of such signaling pathways, as well as further analysis on the constituents of plasma membranes of azole-induced filamentous cells in this organism will clarify these problems.

Morphological characterization of filamentous growth induced by antifungal agents. Further examinations of morphology of the filamentous growth were performed on the culture of SDM supplemented with ketoconazole (40 μ g/L). In order to clarify

Figure 1. Growth curves of *C. tropicalis* Pk233 cultured in the absence (○) or presence (■) of ketoconazole (40 μ g/L).

the events leading up to the formation of pseudohyphae, cell morphology was examined at various times on the ketoconazole-supplemented culture (ketoconazole culture) and control culture. At 0 time when ketoconazole was added and the total count of inoculated cells was 2×10^6 /ml, unbudded cells accounted for 90% of the cell population, since the inoculum was from a stationary phase culture. After 2 h of incubation, the control culture entered into log-phase (Fig.1). While the control culture showed apparently monophasic growth, the ketoconazole culture showed biphasic growth, changing when the turbidity of the culture reached around 1.0 (Fig.1). Doubling time of the mid-log phase was 1.8 h in the control culture.

In the ketoconazole culture, doubling time was 2.5 h during the first phase and 8 h during the second phase.

The second phase started at 12 to 14 h after inoculation, when the turbidity of culture reached more than 1.0. The control culture did not show any significant presence of filamentous cells throughout its growth cycle. Filamentous cells appeared in the ketoconazole culture at 14 to 16 h when the culture entered into the second phase. The filamentous form induced with this agent was characteristic for pseudohyphae by the following criteria

Figure 2. A and C. Appearance of DAPI-stained filamentous cells grown in SDM supplemented with ketoconazole (40 $\mu\text{g/L}$) for 16 h when examined with a fluorescence microscope with Nomarsky optics. B and D. The same cells as in (A) and (C), respectively, by fluorescence microscopy. Arrow heads indicate constrictions at septal junctions. Bar represents 20 μm .

which had been described by McGinnis (1980); (1) filamentous growth resulted from a blowing-out process and subsequent-appearing basal constrictions of each new filament, without the separation of each filament from its parental cell (Fig. 2A and C), (2) the terminal cell of a pseudohypha was typically shorter or equal to the preceding cell just behind the first septum (Fig. 2A and C), (3) the walls contained marked constrictions at septa, and (4) side branches were constricted at their point of origin and there was a septum at the origin of the branch (Fig. 2C). An initial stage of pseudohyphal formation in the ketoconazole culture was different from that in the ethanol-supplemented culture (Suzuki *et al.*, 1998). In the latter culture, depolarized cell growth precedes filamentation: individual yeast cells appeared to alter their shape from an ellipsoidal one to a spherical one and new-born daughter buds from spherical mother cells swelled up isodiametrically. Until late stage of the first phase of growth in the ethanol culture, several spherical yeast cells formed a chain and each cell of the chain

increased its cell volume until the end of the first growth-phase (Suzuki *et al.*, 1998). On the other hand, in the ketoconazole culture of this work, new-born filamentous daughter cells appeared from the mother yeast cells apparently without such depolarized growth. Some of the mother cell also produced the second pseudohyphal filament without swelling during the second phase (Fig. 2a to c). Until 24 h of incubation in the ketoconazole culture, some of the pseudohyphal filaments spread their branches out through the budding process (Fig. 2c). DAPI-staining revealed the location of nuclear DNA during the process of pseudohyphal development. In the developing pseudohypha, a nucleus was not located on the terminal cell but on the mother one just behind the septum, suggesting the nucleus mainly remained in the mother cell (Fig. 2 C and D). In the case of *C. albicans* true hyphal formation, the nucleus is located on the terminal cell and mitosis occurs at the center of the terminal one (Sudbery, 2001). On the other hand, mitosis took place across the septum (Fig 2 A and B), and then one of the daughter nuclei segregated into the terminal cell in this organism. These nuclear behaviors coincided with the description on the mitosis of *C. albicans* pseudohyphal development (Sudbery, 2001).

In order to elucidate these morphological events during pseudohyphal development induced by the supplementation of antifungal azole agents, we are now in progress to identify genes expressed preferentially during the development by subtractive gene cloning as described previously (Imanishi *et al.*, 2001).

Acknowledgements

We wish to express our thanks to Dr. Machiko Miyata at Gifu Pharmaceutical University for her kindly giving us aculeacin A. This work was supported in part by a grant in aid (no. 13640664) for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

- Alexandre, H., Rousseaux, I., Charpentier, C. (1994). Relationship between ethanol tolerance, lipid composition and plasma membrane fluidity in *Saccharomyces cerevisiae* and *Kloeckera apiculata*. FEMS Microbiol Lett

- 142:17-22
- Ansari, S., Prasad, R. (1993). Effect of miconazole on the structure and function of plasma membrane of *Candida albicans*. FEMS Microbiol Lett 15:93-8
- Calderone, A.R. (1994). Molecular pathogenesis of fungal infections. Trends Microbiol 2:461-463
- Cannon, R.D, Kerridge, D. (1988). Correlation between the sterol composition of membranes and morphology in *Candida albicans*. J Med Vet Mycol 26:57-65
- Cutler, J.E. (1991). Putative virulence factors of *Candida albicans*. Ann Rev Microbiol 45:187-218
- Ernst, J.F. (2000). Transcription factors in *Candida albicans*: environmental control of morphogenesis. Microbiol 146:1763-1774
- Fidel, P.Jr., Sobel, J.D. (1994). The role of cell-mediated immunity in candidiasis. Trends Microbiol 2: 202-206
- Gimeno, C.J., Ljungdahl, P.O., Styles, C.A., Fink, G.R. (1992). Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS. Cell 68:1077-1090
- Hawser, S., Francolini, M., Islam, K. (1996). The effects of antifungal agents on the morphogenic transformation by *Candida albicans in vitro*. J Antimicrob Chemother 38:579-587
- Hawser, S., Islam, K. (1999). Comparisons of the effects of fungicidal and fungistatic antifungal agents on the morphogenetic transformation of *Candida albicans*. J Antimicrob Chemother 43: 411-413
- Imanishi, Y., Kawai, T., Iwaguchi, S.-I., Suzuki, T., Kamihara, T., Yokoyama, K., Nishimura, K. (2001). Subtractive gene cloning using Dynabeads Oligo(dT)₂₅ for elucidation of pseudohyphal formation in *Candida tropicalis*. Jpn J Med Mycol 42:243-251
- Kron,, S.J., Styles, C.A., Fink, G.R. (1994). Symmetric cell division in pseudohyphae of the yeast *Saccharomyces cerevisiae*. Mol Biol Cell 5: 1003-1022
- Lo, H.J., Kohler, J.R., Didomenico, B., Loebenberg, D., Cacciapuoti, A., Fink, G.R. (1997). Nonfilamentous *C. albicans* mutants are avirulent. Cell 90:939-949
- Lyman, C.A., Walsh, T.J. (1992). Systemically administered antifungal agents. A review of their clinical pharmacology and therapeutic applications. Drugs 44:9-35
- McGinnis, W.R. (1980). Laboratory handbook of medical mycology. Academic Press, New York
- Odds, F.C. (1994). *Candida* species and virulence. ASM news 60:313-318
- Parks, L.W., Casey, W.M. (1995). Physiological implications of sterol biosynthesis in yeast. Annu Rev Microbiol 49:95-116
- Roberts, R.L., Fink, G.R. (1995). Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth. Genes Dev 8:2974-2985
- Sudbery, P.E. (2001). The germ tubes of *Candida albicans* hyphae and

- pseudohyphae show different patterns of septin ring location. *Mol Microbiol* 41:19-31
- Suzuki, T., Imanishi, Y., Iwaguchi, S.-I., Kamihara, T. (1998). Depolarized cell growth precedes filamentation during the process of ethanol-induced pseudohyphal formation in the yeast *Candida tropicalis*. *Microbiol* 144:403-410
- Swan, T.M., Watson, K. (1998). Stress tolerance in a yeast sterol auxotroph: role of ergosterol, heat shock proteins and trehalose. *FEMS Microbiol Lett* 169:191-197
- Szolderits, G., Hermstetter, A., Paltauf, F., Daum, G. (1989). Membrane properties modulate the activity of a phosphatidylinositol transfer protein from the yeast, *Saccharomyces cerevisiae*. *Biochim Biophys Acta* 986:301-309
- Tomeo, M.E., Fenner, G., Tove, S.R., Parks, L.W. (1992). Effect of sterol alterations on conjugation in *Saccharomyces cerevisiae*. *Yeast* 8:1015-24
- Uejima, Y., Koga, T., Kamihara, T. (1987). Enhanced metabolism of phosphatidylinositol in *Candida tropicalis* in association with filamentous growth caused by ethanol. *FEBS Lett* 214:127-129