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Fungicidal Mechanism of Action of D0870 against *Cryptococcus neoformans* under Acidic Conditions

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The fungicidal mechanism of the triazole D0870 against *Cryptococcus neoformans* under acidic conditions was investigated. D0870 reduced the intracellular K^+ content of *C. neoformans* at pH 4 to about half the value at pH 7 after 12 h of incubation. The 50% inhibitory concentrations of D0870 for ergosterol biosynthesis were almost the same at both pH 4 (0.017 μg/ml) and 7 (0.014 μg/ml); however, D0870 caused a marked accumulation of an unknown lipid and methylated sterols in *C. neoformans* cultured at pH 4. Extracted fractions containing the unknown lipid or methylated sterols showed strong fungicidal activities against *C. neoformans* both at pH 4 and 7 in phosphate-citrate buffer not containing D0870. Gas chromatographic-mass spectrometric analysis showed that the unknown lipid was obtusifolione. These results suggest that D0870 kills *C. neoformans* by disturbing the permeability of the cell membrane through the accumulation of obtusifolione and methylated sterols in the cell membrane under acidic conditions.

D0870 is a novel triazole antifungal agent which has a broad spectrum of activity and more potent in vitro and in vivo activities than fluconazole against yeasts and filamentous fungi (16). We have also reported that D0870 has fungicidal activity against *Cryptococcus neoformans* in vitro and in vivo (9) and that the anticryptococcal activity of D0870 was greatly enhanced under acidic conditions (9). Although acidic conditions are restricted in vivo except in macrophage phagolysosomes, the fungicidal action of D0870 could partly explain the excellent therapeutic activity of this drug against experimental pulmonary cryptococosis in mice (9). In this study, aimed at the elucidation of the fungicidal mechanism of D0870 against *C. neoformans*, we investigated the effect of culture medium pH on the uptake of D0870 by *C. neoformans*, intracellular K^+ content, and the sterol biosynthesis of *C. neoformans* treated with D0870.

MATERIALS AND METHODS

Antifungals. D0870 was synthesized at Fuji Central Research Laboratory, Mochida Pharmaceutical Co., Ltd., Shizuoka, Japan. In all experiments, D0870 was dissolved in dimethyl sulfoxide (DMSO) and added to the culture. The final concentration of DMSO was 1%.

Fungi. *C. neoformans* TIMM 0362 was kindly provided by Hideyo Yamaguchi, Research Center for Medical Mycology, Teikyo University School of Medicine, Tokyo, Japan. The isolate was stored at −196°C in Sabouraud dextrose broth containing 10% DMSO. Prior to each experiment, the isolate was cultured for 24 h at 30°C in Sabouraud dextrose broth.

Effect of D0870 and culture medium pH on intracellular K^+ content and cell viability of *C. neoformans*. *C. neoformans* was added to 40 ml of fungal synthetic amino acid medium (SAAMF) (pH 7 or 4) (6, 16) containing D0870 at a concentration of 6.25 μg/ml to give a final inoculum of 2×10^6 cells/ml. Each flask was incubated at 30°C with shaking at 120 rpm. After 0, 6, and 12 h of incubation, cells were harvested by centrifugation (700 × *g*, 10 min, 4°C), washed twice with 0.25 M sucrose, and resuspended in the same solution at a cell suspension of 4×10^6 cells/ml. A portion (1.5 ml) of this cell suspension was recentrifuged, and cells were suspended in 1.5 ml of 5% trichloroacetic acid aqueous solution and boiled at 90°C for 10 min. The boiled samples were centrifuged, and the K^+ concentration of the supernatant was measured with an Atomic Absorption Spectrophotometer (model z-8100; Hitachi Ltd., Tokyo, Japan). For the determination of numbers of CFU, 0.1-ml portions were removed from the flasks at the end of each incubation time, serially diluted with sterilized saline, and plated on Sabouraud dextrose agar. Plates were incubated at 37°C for 48 h.

Effect of culture medium pH on uptake of D0870 by *C. neoformans*. *C. neoformans* was added to 8 ml of SAAMF (pH 7 or 4) containing 2.80 KBq of [^{14}C]D0870 (specific activity, 1.82 GBq/mmol; ZENECA Pharmaceuticals, Macclesfield, England) to give a final inoculum of 10^9 cells/ml. Each tube was incubated at 30°C with shaking. Portions (1 ml) were removed from the tube after 0, 30, 60, and 120 min of incubation, added to 4 ml of saline containing unlabeled D0870 (10 μg/ml), and centrifuged for 5 min at 1,500 × *g*. Cells were washed twice with 5 ml of the same solution and transferred to the vial containing 10 ml of Atomlight (NEN-Dupont, Markham, Ontario, Canada) for the measurement of radioactivity.

Effect of culture medium pH on ergosterol biosynthesis in *C. neoformans*. *C. neoformans* was added to 10 ml of SAAMF (pH 4 or 7 containing D0870 at concentrations of 0.003 to 1 μg/ml and 74 kBq of sodium [U-^{14}C]acetate (specific activity, 2.07 GBq/mmol; Amersham International, Buckinghamshire, England) to give a cell suspension of 2×10^6 cells/ml. After incubating at 30°C for 12 h with shaking, the cells were harvested by centrifugation and washed once with distilled water. Lipids were extracted from whole cells with chloroform-methanol (1:2, vol/vol), streaked onto thin-layer chromatography plates (20 by 20 cm; catalog no. 5583; Merck, Darmstadt, Germany), and developed in a solvent system consisting of *n*-heptane–diisopropylether–acetic acid (60:40:4, vol/vol/vol) (13). Radioactivity in each lipid band was analyzed with a Bio-imaging Analyzer (BAS 2000; Fuji Photo Film Co., Ltd., Tokyo, Japan) (10).

Fungicidal activity of extracted fractions containing an unknown lipid or methylated sterols against *C. neoformans*. Each lipid fraction was prepared as follows: *C. neoformans* was added to 600 ml of SAAMF (pH 4) containing D0870 at a concentration of 12.5 μg/ml to give a cell suspension of 2×10^7 cells/ml. After incubating at 30°C for 12 h with shaking, lipids were extracted and fractionated basically according to the method described above, except that benzene-ethylacetate (9:1, vol/vol) was used as the solvent system. Bands were visualized with I_2 vapor, and those of the unknown lipid and methylated sterols were scraped from the plate and eluted with chloroform-methanol (2:1, vol/vol). The extract was evaporated to dryness, dissolved in DMSO and used for the killing assay described below. One unit of the unknown lipid or methylated sterols was defined as the amount extracted from 10^{10} cells of *C. neoformans* TIMM 0362 cultured for 12 h in SAAMF (pH 4) containing 12.5 μg of D0870 per ml. *C. neoformans* was added to 100 μl of phosphate-citrate buffer (pH 4 or 7) containing extracted lipids at various concentrations to give a cell suspension of 10^5 cells/ml. After incubating at 30°C for 24 h with shaking, the number of CFU in each culture was determined as described above.

Analysis of the unknown lipid accumulated in *C. neoformans*. The unknown lipid fraction was dissolved in chloromethane and identified by gas chromatographic-mass spectrometric analysis (11).

Statistical analysis. Student's *t* test, with significance at P values of <0.05 and <0.01, was used to compare data.

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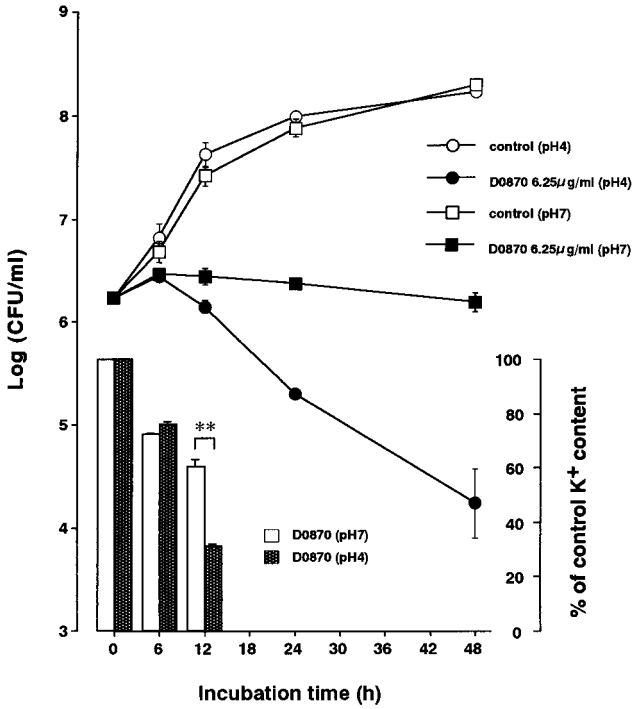

FIG. 1. Effects of D0870 and culture medium pH on intracellular K^+ content and cell viability of *C. neoformans* TIMM 0362. Results are the means ± standard errors of the means (error bars) of three experiments. **, $P < 0.01$ compared with the K^+ content of *C. neoformans* incubated with D0870 at pH 7.

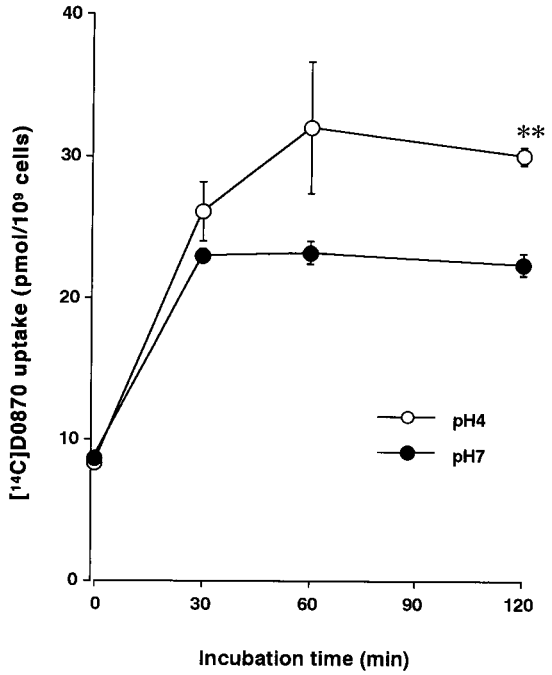

FIG. 2. Effect of culture medium pH on the uptake of [^{14}C]D0870 by *C. neoformans* TIMM 0362. Results are the means ± standard errors of the means (error bars) of three experiments. **, $P < 0.01$ compared with the drug uptake at pH 7.

RESULTS AND DISCUSSION

D0870 showed fungicidal activity against *C. neoformans* in SAAMF at pH 4 (9). Generally, azole antifungals have at least two distinct modes of action against fungi–(i) inhibition of ergosterol biosynthesis in fungi (13, 14) and (ii) direct membrane damage in the fungal cells (1, 2, 4, 7, 12, 14)–and fungistatic and fungicidal activities of azole compounds are thought to be attributable to the former and the latter, respectively (1, 12). Therefore, since cell membrane damage could be one of the fungicidal mechanisms of D0870 against *C. neoformans*, we examined the effect of the culture medium pH on intracellular K^+ content, a marker of membrane permeability disorder, of this yeast together with the uptake of D0870 into *C. neoformans*. Figure 1 shows the effects of D0870 and culture medium pH on the intracellular K^+ content and the cell viability of *C. neoformans*. After 12 h of incubation, just before D0870 killed the yeast cells, D0870 at a concentration of 6.25 μg/ml reduced the intracellular K^+ content of *C. neoformans* cultured in SAAMF at pH 4 to 31.4% of the control value, which was significantly ($P < 0.01$) less than the value at pH 7, 60.3%. At pH 4, the uptake of D0870 by 10^9 cells of *C. neoformans* during 120 min of incubation was 30 pmol, which was significantly greater than the uptake at pH 7, 22 pmol (Fig. 2). These results suggest that D0870, readily taken up by *C. neoformans* under acidic conditions, kills *C. neoformans* through disturbing the permeability of the cell membrane. However, the membrane permeability disorder induced by D0870, unlike those induced by imidazole antifungal agents (1, 2, 4, 7), needed a longer incubation. Therefore, our finding suggests that D0870 disturbs the membrane permeability not by a direct interaction with the cell membrane molecules (17) but by a time-dependent alteration in the cell membrane structure. Aiming to elucidate the fungicidal mechanism of D0870, we further investigated the effect of culture medium pH on sterol biosynthesis in *C. neoformans*.

The 50% inhibitory concentrations of D0870 for ergosterol biosynthesis from [^{14}C]acetate in *C. neoformans* cultured in

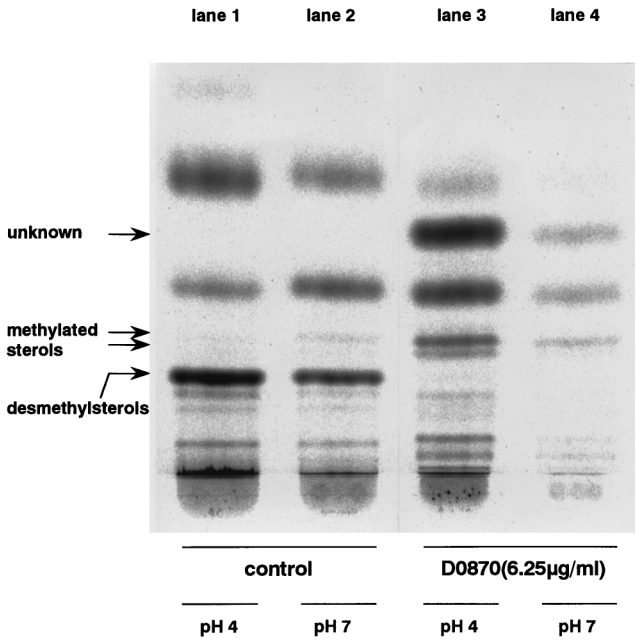

FIG. 3. Effects of D0870 and culture medium pH on [^{14}C]acetate incorporation into lipids in *C. neoformans* TIMM 0362. The radioluminogram presented is representative of three independent experiments.

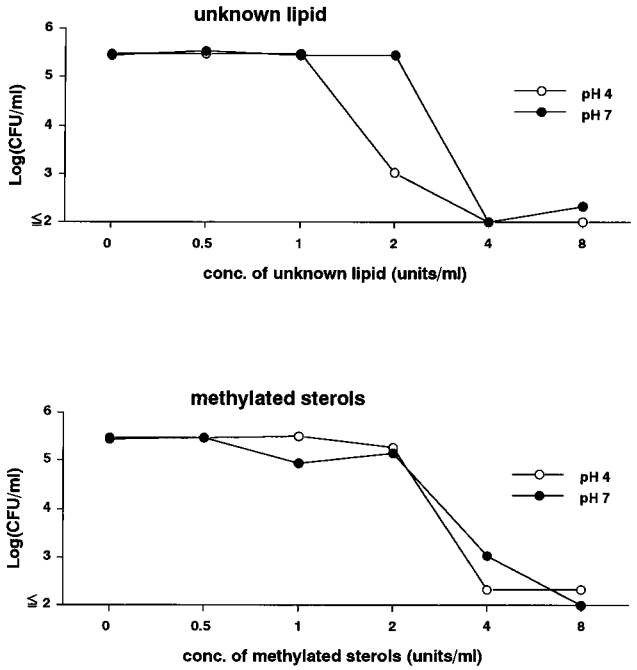

FIG. 4. Effects of exogenous unknown lipid and/or methylated sterols on the cell viability of *C. neoformans* TIMM 0362 in phosphate-citrate buffer (pH 4 or 7). Yeasts were incubated in each buffer containing extracted lipids at 30°C for 24 h. One unit of unknown lipid or methylated sterols was defined as the amount of each lipid extracted from 10^{10} cells of *C. neoformans* TIMM 0362 cultured for 12 h at 30°C in SAAMF (pH 4) containing 12.5 μg of D0870 per ml. Each result presented is representative of three independent experiments.

SAAMF at pH 4 was 0.017 μg/ml and almost the same as the 50% inhibitory concentration at pH 7, 0.014 μg/ml. However, the autoradioluminogram at pH 4 was very different from that at pH 7, as shown in Fig. 3. The level of total radioactivity in the lipid fraction derived from cells incubated with D0870 at pH 4 was 19-fold higher than that at pH 7 (Fig. 3, lanes 3 and 4, respectively), which shows that lipid biosynthesis in *C. neoformans* was enhanced by D0870 at pH 4. Furthermore, remarkable accumulation of methylated sterols and an unknown lipid were observed in *C. neoformans* cultured in SAAMF at pH 4 containing 6.25 μg of D0870 per ml (the numbers of cells used for lipid extraction were almost the same between lanes 1 and 2 or lanes 3 and 4, respectively, although the data are not shown).

Since these lipids, especially the unknown lipid, may be fungicidal for *C. neoformans* under acidic conditions, we investigated the effect of exogenous unknown lipid or methylated sterols on cell viability in phosphate-citrate buffer at pH 4 and 7. We used fractions containing the unknown lipid and methylated sterols extracted from the thin-layer chromatography plates and defined the amount of these lipids as described in Materials and Methods. Figure 4 shows the effects of extracted fractions containing the unknown lipid or methylated sterols on the cell viability of *C. neoformans* in phosphate-citrate buffer at pH 4 and 7. Phosphate-citrate buffer was used to mimic the fungistatic state of yeasts treated with D0870. In this buffer, yeasts stopped growing but maintained their viability during incubation. Exogenous unknown lipid, at concentrations higher than 2 U/ml, showed strong fungicidal activity against *C. neoformans* at pH 4, which was stronger than that at pH 7. Methylated sterols at concentrations higher than 4 U/ml also showed strong fungicidal activity in the same buffer at both pH 4 and 7, and the fungicidal activity was less affected by the pH of the buffer. Iwatani et al. (8) suggested that the fungi would become unstable and more susceptible to physical damage when the cellular sterol composition changed. Thus, *C. neoformans* cultured with D0870, especially if it accumulates obtusifolione in its membrane, might become susceptible to the low pH of the medium and be damaged synergistically.

The unknown lipid was expected to be obtusifolione, since Vanden Bossche et al. have found that itraconazole shows an excellent anticryptococcal activity through the accumulation of major intermediates like ebricol and obtusifolione (15); obtusifolione has been reported to increase the permeability and fragility of the membrane by its strong bilayer-disturbing effect (3, 5, 15). However, since obtusifolione is not commercially available and the R_f value for obtusifolione has also not been reported, we tried to determine the structure of the unknown lipid by comparing the mass spectrum of its main peak with that reported for obtusifolione (11); that were identical (data not shown).

These results strongly suggest that D0870, readily taken up by *C. neoformans* under acidic conditions, kills *C. neoformans* by disturbing the permeability of the cell membrane through the accumulation of obtusifolione and methylated sterols in the cell membrane under acidic conditions.

ZENECA Pharmaceuticals terminated the development of D0870 this March. In the clinical trial (phase II), they used fairly high doses of D0870 for the therapy of fluconazole-resistant oral candidiasis. However, in Japan, Mochida Pharmaceutical Co., Ltd., is still continuing to develop D0870, using lower doses than those used by ZENECA.

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