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Effect of miconazole on diO-C_6-(3) accumulation in mitochondria of *Candida albicans*

Hyeong-Cheol Yang, Hideaki Taguchi, Kazuko Nishimura and Makoto Miyaji

Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1-8-1, Inohana, Chuo-ku, Chiba 260, Japan

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A flow cytometric method was used to investigate the effect of miconazole (MCZ) on yeast-form cells of *Candida albicans*. Relative changes in electric potential of mitochondrial and cytoplasmic membranes were assessed by 3,3′-dihexyloxacarbocyanine iodide (diO-C_6-(3)) and bis-(1,3-dibutyl-barbituric acid) trimethine oxonol (diBA-C_4-(3)) stainings, respectively. When *C. albicans* was exposed to MCZ at 10 μg/ml (a fungistatic concentration) for 2 h, no change appeared in cytoplasmic membrane potential, which was revealed by constant fluorescence intensity of diBA-C_4-(3)-stained cells. On the other hand, the cells lost the ability to accumulate diO-C_6-(3) in mitochondria by MCZ treatment. Time- and dose-responses in fluorescence intensity reflected that MCZ affected the mitochondrial activity of *C. albicans*.

Key Words——*Candida albicans*; diO-C_6-(3); membrane potential; miconazole; mitochondria.

During the past decade, flow cytometry (FCM) has become widely used in biological and medical sciences. Several fluorochromes including cell viability probes developed for fluorescence microscopy have been used successfully for FCM. Antifungal activity was also assessed rapidly by FCM with such cell viability probes as ethidium bromide (O'Gorman and Hopfer, 1991; Pore, 1991). As indicators of cell viability, membrane potential-sensitive dyes, of which fluorescence intensity varies with the magnitude of trans-membrane electric potential, have recently come into use. Viability assessment of *Candida* spp. treated with amphotericin B was carried out with bis-(1,3-dibutyl-barbituric acid) trimethine oxonol (diBA-C_4-(3)) and 3,3′-dipentyloxacarbocyanine iodide (diO-C_5-(3)) (Carter et al., 1993; Ordónez and Wehman, 1995).

The plasma membrane potential of bacteria has been evaluated with fluorescent carbocyanine, which accumulated in cytoplasm of inside-negative potential (Mason et al., 1993). On the other hand, eucaryotes such as mammalian cells accumulated the dye exclusively in their mitochondria, which have relatively high electric potential (inside-negative), and the intensity of fluorescence reflected the trans-membrane potential of that organelle (Johnson et al., 1981). Mitochondrial activity of mammalian cells was assessed with rhodamine 123, which has identical properties to carbocyanine (Al-Rubeai et al., 1991a, b). However, it should be considered that the fluorescence of carbocyanine-stained cells was partially affected by the electric potential of the plasma membrane, which is the first barrier against entry into the cell. Thus, the plasma membrane should be observed concurrently to know whether the change in fluorescence intensity of carbocyanine comes from the mitochondrial membrane or the plasma membrane. For this purpose, oxonol dyes such as diBA-C_4-(3) should be useful, since dyes which have a negative potential in each molecule have frequently been employed to measure the electric potential of the plasma membrane (Seamer et al., 1992).

In the present study we used a carbocyanine dye, 3,3′-dihexyloxacarbocyanine iodide (diO-C_6-(3)), with FCM to estimate mitochondrial activity of *Candida albicans* exposed to miconazole (MCZ). Several action mechanisms of MCZ were reported previously (Swamy et al., 1974; Van den Bossche et al., 1978; Sud and Feingold, 1981; Bastide et al., 1982). Among the antifungal activities of MCZ, an effect on mitochondria was also reported, in which the activity of mitochondrial ATPase was inhibited by MCZ (Portillo and Gancedo, 1984). Until now, however, it was not certain that MCZ affects mitochondria in cytoplasm. In this paper the feasibility of using diO-C_6-(3) to detect the mitochondrial response to MCZ in intact *C. albicans* cells was demonstrated. The effect of MCZ on plasma membrane was also investigated with an oxonol dye, diBA-C_4-(3).

Materials and Methods

Organism and chemicals *Candida albicans* ATCC 10231 was maintained on potato-dextrose agar (Difco). MCZ was provided by Mochida Pharmaceutical (Japan) and dissolved in dimethyl sulfoxide to make a stock solution of 2 mg/ml. Antimycin A was purchased from Sigma Chemical. 3,3′-Dihexyloxacarbocyanine (diO-C_6-(3)) and bis-(1,3-dibutylbarbituric acid) trimethine oxonol (diBA-C_4-(3)) were purchased from Molecular Probes (U.S.A.) and stocked in 50% ethanol and stored at 5°C.

Culture condition and treatment with antimycin A and

MCZ *Candida albicans* was grown in yeast nitrogen base (Difco) supplemented with 0.5% glucose (YNBG) at 30°C with agitation (120 rpm). Cells were harvested at mid-exponential phase, resuspended at a cell concentration of 5×10^5 per ml in YNBG medium containing an inhibitor (antimycin A or MCZ), and incubated at 30°C. The optical density (OD) of the cell suspensions was measured at 540 nm with a spectrophotometer (UV-160, Shimadzu, Japan).

Cell staining An aliquot (400 μl) of the cell suspension which had been treated with the inhibitor was added to 1.6 ml of prewarmed YNBG medium containing the membrane potential-sensitive dye. The staining solutions were gently shaken for 15 min at 30°C and applied to microscopic observation and flow cytometric analysis. Final concentrations of diO-C_6-(3) and diBA-C_4-(3) were 5 ng/ml and 1 μg/ml, respectively.

Fluorescence microscopy and flow cytometric analysis One drop of the staining solution was placed on a glass slide and covered with a cover glass for microscopic observation. Fluorescence was examined under a fluorescence microscope (BH-2, Olympus, Japan) equipped with filters (dichroic mirror, 510 nm; excitation filter, 455-490 nm; barrier filter, 515 nm long pass). Flow cytometric measurement was performed with a cell sorter (CS-20, Showa Denko, Japan) equipped with an argon laser (GLG 3300, NEC, Japan). Excitation was at 488 nm and 400 mW. The aperture of the nozzle was 100 μm in diam. Emission filters were 520 nm long pass interference and 530 nm dichroic. About 5,000 cells were flowed per test.

Results

Fluorescence microscopic image of diO-C_6-(3)-stained *C. albicans* cells Examination of non-treated cells of *C. albicans* stained with the cationic membrane potential-sensitive dye, diO-C_6-(3), revealed that the dye accumulated in mitochondria exclusively (Figs. 1A, B). The morphology of mitochondria which formed branching aggregates was almost identical to that of *Saccharomyces cerevisiae* observed by Koning et al. (1993). The length of mitochondria was variable, and the diameter was 0.2-0.4 μm. To confirm that diO-C_6-(3) actually stained mitochondria, the cells were treated with 10 μg/ml of antimycin A, which is known to inhibit electron transport of mitochondria and dissipate the membrane potential. These cells showed weak or no fluorescence within the plasma after staining with diO-C_6-(3), which indicates that the carbocyanine dye accumulated exclusively in mitochondria of normal membrane potential.

Figs. 1C and 1D show the effect of MCZ on diO-C_6-(3) stainability of mitochondria. Treatment at 10 μg/ml, the minimum inhibitory concentration, for 2 h completely inhibited the accumulation of diO-C_6-(3) in mitochondria. Little or no mitochondrial fluorescence was observable.

Quantitation of fluorescence intensity of diO-C_6-(3) by FCM *Candida albicans* cells were stained with diO-C_6-(3) for 15 min, which was enough to saturate mitochondria with the dye, and their fluorescence was analyzed by FCM. The contour of cell populations is shown in Fig. 2. The fluorescence intensity (perpendicular axis) of each cell was drawn as a function of cell size indicated by forward light scatter (horizontal axis). Mitochondrial activi-

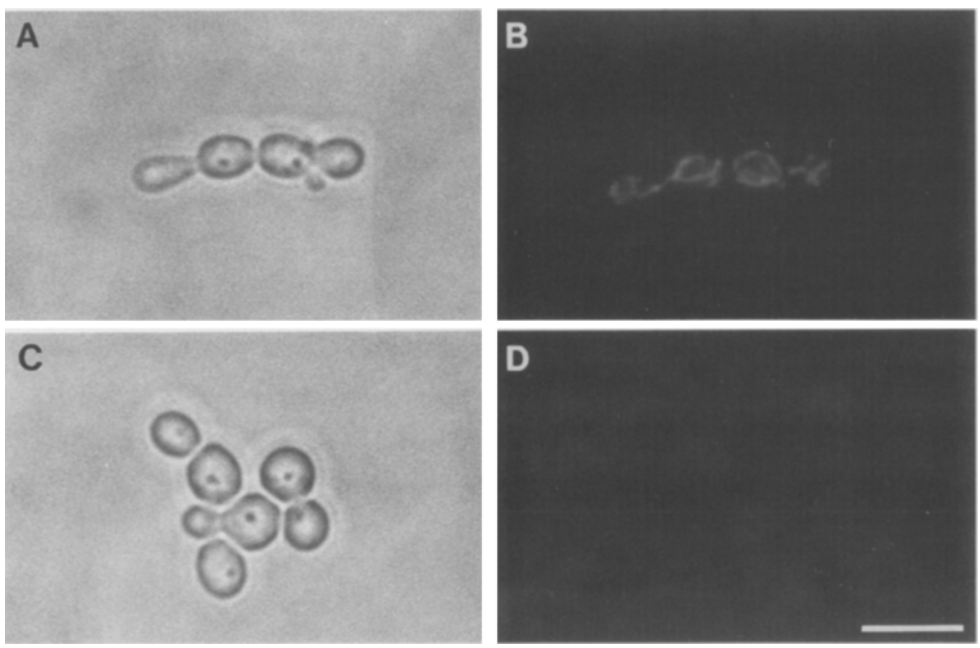

Fig. 1. Photomicrographs of diO-C_6-(3)-stained cells of *C. albicans*.
A. Bright field image of non-treated cells stained with 5 ng/ml diO-C_6-(3) for 15 min. B. Fluorescence image of the cells in Fig. 1A. C. Bright field image of MCZ-treated cells (at 10 μg/ml for 2 h) stained with diO-C_6-(3). D. Fluorescence image of the cells in C. Scale bar is 10 μm.

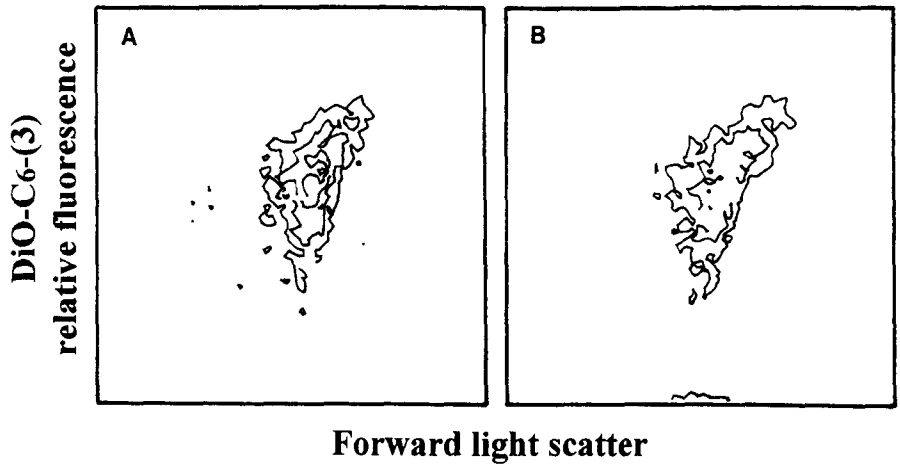

Fig. 2. Contour plots of non-treated cells of *C. albicans* stained with diO-C_6-(3).
A. Cells in mid-exponential growth phase in YNBG medium were stained with 5 ng/ml diO-C_6-(3) for 15 min. B. Cells in the same growth phase were resuspended at a cell concentration of 5×10^5 per ml in prewarmed YNBG medium and incubated at 30°C for 2 h. Staining was performed under the same conditions as in A.

ty of cells of mid-exponential phase (Fig. 2A) and early exponential phase (Fig. 2B) monitored by their fluorescence showed no considerable difference between them. Since a decrease in the fluorescence appeared at the beginning of the stationary phase, all the tests were performed during mid-exponential phase to acquire constant light-intensity of fluorescence in control cells.

Effect of MCZ on the cell growth and the ability to accumulate diO-C_6-(3) in cells Figure 3 illustrates the change in growth curve of *C. albicans* induced by MCZ treatment at various concentrations. There was no effect on the growth when cells were exposed to MCZ at 0.1 μg/ml, and complete suppression appeared at 10 μg/ml.

To estimate the effects of MCZ treatment on the accumulation of diO-C_6-(3), the cells exposed to MCZ (10 μg/ml) for various periods were stained and flowed to the flow cytometer. Figure 4 shows the decline of ability to accumulate the carbocyanine dye in cells that was induced by MCZ treatment. Severe decrease in fluorescence intensity appeared at 30 min (Fig. 4B) and continued with time of incubation. After exposure to MCZ for 2 h, the cells scarcely accumulated diO-C_6-(3), which could be confirmed by microscopic observation (Fig. 1D).

Dose-dependence of the fluorescence intensity on the concentration of MCZ is shown in Fig. 5. Cells were treated with MCZ at several concentrations arranged from 0 to 10 μg/ml for 2 h. Distributions of fluorescence intensity were altered in the groups treated with different concentrations of MCZ. At 0.1 μg/ml, MCZ did no effect

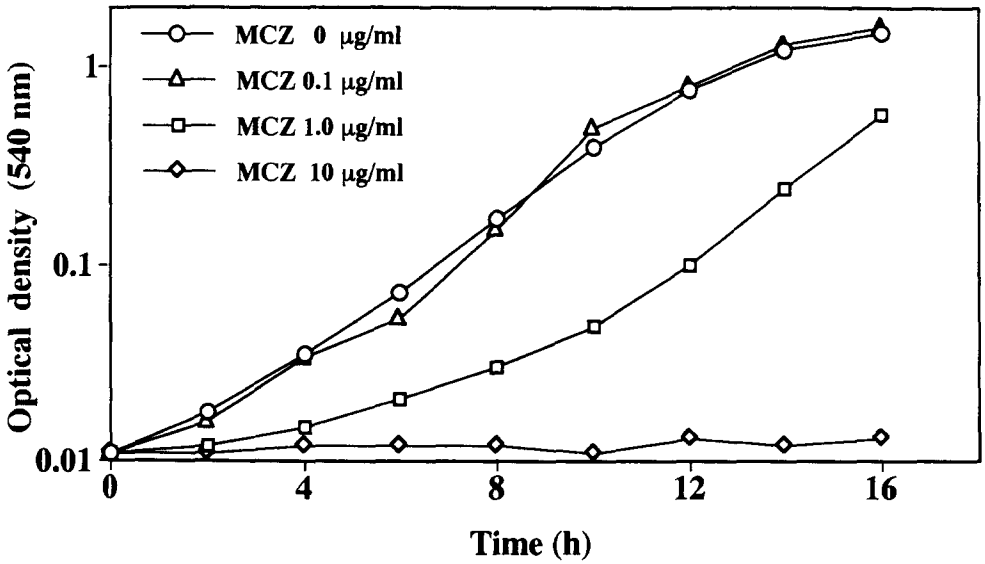

Fig. 3. Effects of increasing concentrations of MCZ on the growth of *C. albicans*.
Cells (5×10^5 per ml) were incubated in YNBG medium containing MCZ at 30°C.

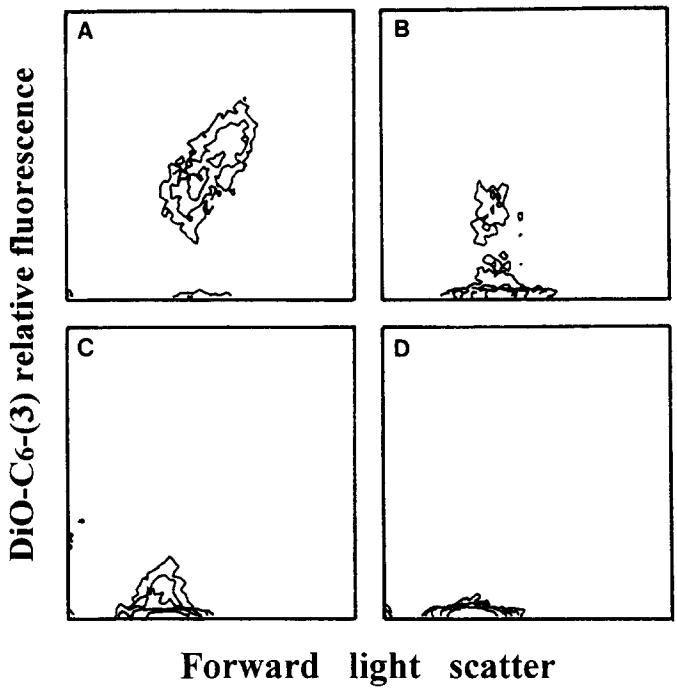

Fig. 4. Contour plots of MCZ-treated cells of *C. albicans* stained with diO-C_6-(3).
Cells (5×10^5 per ml) were incubated in YNBG medium containing 10 μg/ml MCZ at 30°C. The cells were exposed to MCZ for 0 (A), 30 (B), 60 (C) and 120 (D) min and stained with diO-C_6-(3) for 15 min.

on the fluorescence, which showed the same distribution as that of the control cells. With higher concentrations of MCZ the histogram shifted to the left. Fluorescence intensity was reduced significantly by MCZ treatment at the concentration of complete growth suppression (10 μg/ml) as revealed in Fig. 3, and the distribution did not overlap with that of the control group.

Effect of MCZ treatment on staining with diBA-C_4-(3)
The possibility that MCZ alters the electric potential of the plasma membrane was investigated. Control cells did not incorporate fluorescent oxonol dye, diBA-C_4-(3), in the cytoplasm because of their inside-negative membrane potential. Thus no or weak fluorescence was observable inside cells under the fluorescence microscope. The fluorescence intensity of control cells was quantitated by FCM (Fig. 6A). Although treated with MCZ at the concentration of 10 μg/ml for 2 h, at which severe reduction of fluorescence of diO-C_6-(3) was observed (Fig. 3, 4), *C. albicans* showed similar fluorescence intensity to control cells, as shown in Fig. 6. Therefore, MCZ at 10 μg/ml did not affect the membrane potential of cytoplasm during exposure for 2 h.

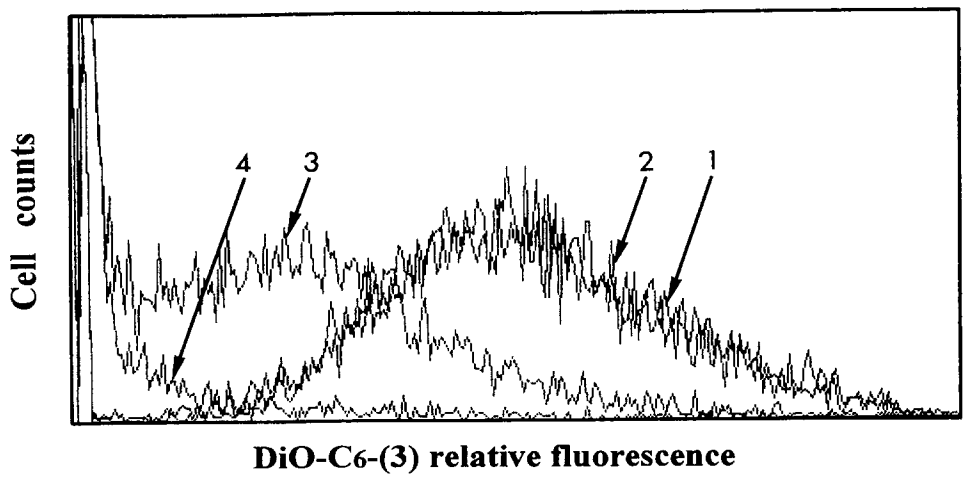

Fig. 5. A single parameter histogram of MCZ-treated cells of *C. albicans* stained with diO-C_6-(3).
Incubation was carried out at 30°C with MCZ at concentrations of 0 (1), 0.1 (2), 1.0 (3) and 10 (4) μg/ml. After treatment for 2 h, cells were stained with diO-C_6-(3).

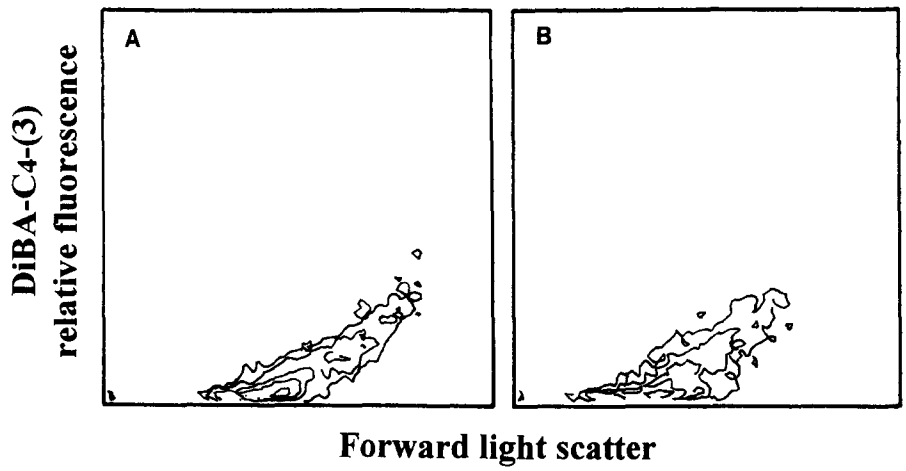

Fig. 6. Contour plots of *C. albicans* cells stained with diBA-C_4-(3).
A. Non-treated cells were stained with diBA-C_4-(3) at a concentration of 1 μg/ml for 15 min. B. MCZ-treated (at a concentration of 10 μg/ml for 2 h) cells were stained with diBA-C_4-(3).

Discussion

At present, the most plausible mechanism of MCZ fungistatic action is that the antimycotic inhibits demethylation of lanosterol to ergosterol, resulting in inhibition of fungal growth (Van den Bossche et al., 1978). Many other reports also deal with the physiological effects of MCZ on fungi, including suppression of ATP (Odds et al., 1985), change of cell permeability and exogenous respiration (Swamy et al., 1974). Inhibition of mitochondrial ATPase by MCZ has also been demonstrated (Portillo and Gancedo, 1984). However, no direct evidence to link MCZ action to mitochondria in situ has been presented.

This research shows that the ability of *C. albicans* to accumulate diO-C_6-(3) in mitochondria was altered by MCZ treatment. Generally, cationic membrane potential dyes, such as rhodamine and carbocyanine dyes, are known to concentrate on mitochondria of eucaryotic cells, and they have been employed to assess the electric potential of mitochondrial membranes, an indicator of mitochondrial activity (Al-Rubeai et al., 1991a). The higher the fluorescence intensity is, the more active are the mitochondria. From the decline of ability to accumulate diO-C_6-(3) in cells (Figs. 4, 5), it is supposed that MCZ affected the activity of mitochondria. Moreover, the conformity between the growth inhibition (Fig. 3) and reduction of fluorescence (Fig. 5) suggests that mitochondria are a target of MCZ fungistatic action. Considering the properties of carbocyanine dye, however, we should be careful in drawing such a conclusion. Another potential barrier against penetration of diO-C_6-(3) into mitochondria is the plasma membrane, which should sustain an inside-negative electric potential for the dye to enter the cytoplasm from the medium. The reduction of fluorescence intensity caused by MCZ thus derives from dissipation of potential in either or both of the cytoplasmic and mitochondrial membranes. However, the use of diBA-C_4-(3) revealed that plasma membrane was not involved in the variation in fluorescence intensity of MCZ-treated cells, which indicates that mitochondrial membrane was the principal barrier to the dye.

In summary, the results described here indicate that MCZ might affect the mitochondria of *C. albicans*. It was hard to confirm from our experiment that MCZ inhibited mitochondrial ATPase, which was reported previously (Portillo and Gancedo, 1984). Although the mechanism of reaction between mitochondria and MCZ could not be elucidated, this report should be of value in further investigations of MCZ action mechanism and antifungal action of other azole drugs.

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