ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1977, p. 16-25
Vol. 12, No. 1

Printed in U.S.A.

Antagonistic Action of Lipid Components of Membranes from *Candida albicans* and Various Other Lipids on Two Imidazole Antimycotics, Clotrimazole and Miconazole

HIDEYO YAMAGUCHI

Department of Microbiology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

Received for publication 9 February 1977

The growth-inhibitory activity of two imidazole antimycotics, clotrimazole and miconazole, against *Candida albicans* was significantly reversed when lipid extracts from protoplast membranes of the same organism were added to the assay medium together with the drugs. Of four major classes of lipids further separated from them, viz., phospholipids, triglycerides, sterol esters, and free sterols, the former two were capable of counteracting both drugs, whereas the latter two were not. However, even with phospholipids or triglycerides, no antagonism was noted when they were saturated by catalytic hydrogenation before use. The antagonistic effect of varying classes of commercial lipids, including phospholipids, acylglycerides, sterols, and fatty acids, was also studied by means of the agar diffusion technique. Significant antagonism to both drugs was observed with: (i) phospholipids with an unsaturated acyl group; (ii) acylglycerides, the ester portion of which consists of unsaturated fatty acid; (iii) ultraviolet-activated sterols; and (iv) unsaturated fatty acids of *cis*-configuration. By contrast, none of the saturated phospholipids and acylglycerides nor sterols was effective as an antagonist. With the exception only of lauric acid, all of a series of saturated fatty acids and unsaturated *trans*-fatty acids ranging from C_8 to C_{18} in chain length were either minimally effective or completely ineffective. Essentially, there was no qualitative difference between clotrimazole and miconazole in the response to these various lipids.

Both clotrimazole (Bay b 5097; bis-phenyl-[2-chloro-phenyl]-1-imidazolyl methane) and miconazole (R14889; 1-[2,4-dichloro-β-[(2,4-dichlorobenzyl)oxy]phenethyl]imidazole nitrate) are promising imidazole antimycotics active against a wide range of pathogenic fungi. Our previous studies showed that when *Candida albicans* cells were exposed to relatively high concentrations of clotrimazole, leakage of various types of small molecules was induced, such as K^+, amino acids, inorganic phosphate, and nucleotides (19, 20, 21, 34). As with clotrimazole, miconazole also has proved to cause loss of those cellular components from susceptible fungal cells (31). Our current interest is directed to understanding the details of the mechanism of action on membranes of these imidazole derivatives. There is a large number of antimicrobial agents which have been demonstrated to have a membrane-active property. However, in no case has the specific site or component in the susceptible membrane that can be involved in the binding and/or the action of a drug been clarified. The only exception is the case of polyene antibiotics which appear to interact preferentially with sterols existing in the membrane of susceptible organisms (24).

Early evidence for the interaction between polyenes and sterols was provided by Gottlieb et al. (13-15) and Lampen et al. (25), who showed that growth-inhibitory and other actions of polyene antibiotics could be offset by sterols in the medium. Results have also been published which showed that certain types of phospholipids are preferentially effective in antagonism to the antibiotic action of several other membrane-active agents such as tyrothricin (1), polymyxins (11), pyrrolnitrin (30), and mycobacillin (16, 17). These studies encouraged us to test some membrane fractions, especially those of lipid nature. These were prepared from *C. albicans* for their ability to counteract the antifungal activity of imidazole drugs, with the hope that information about the actual site of drug action on the fungal cell membrane would be obtained. To extend this study and to learn the lipid specificity of these drugs, further investigations were carried out with various

classes of synthetic and naturally occurring lipid compounds.

MATERIALS AND METHODS

Organisms. *C. albicans*, strain MTU 12021, sufficiently susceptible to both clotrimazole and miconazole, was used as the test organism for antifungal activity of the imidazole drugs in the presence or absence of various samples and also as the source of membrane preparations.

Chemicals. Clotrimazole was furnished by Bayer Yakuhin Co., Ltd. (Osaka) and miconazole was obtained from Mochida Pharmaceutical Co. (Tokyo). Stock solutions of each drug (20 mg/ml) were made with dimethyl sulfoxide and stored at −20°C. The lytic enzyme "Zymolyase" (23) was a generous gift from Y. Yamamoto, Research Institute, Kirin Brewing Co. (Takasaki). All of the acylglycerides, including mono-, di-, and triglycerides, and all of the phospholipids were purchased from P-L Biochemicals Inc. Sterols and their related compounds, and fatty acids, including their sodium salts, methyl esters, and cholesterol esters, were all obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo), except for cholesterol and ergosterol which were the products of Applied Science Laboratories, Inc. (State College, Pa.).

Preparation of protoplast membranes. One loopful of *C. albicans* cells from a freshly grown slope culture was inoculated into a 500-ml flask containing 200 ml of GPY broth (2% glucose, 1% peptone [Difco], and 0.5% yeast extract [Difco], wt/vol), and the culture was incubated at 37°C for 24 h with vigorous shaking. The culture then contained 8 to 10 mg (dry weight equivalent) of cells per ml. The cells were harvested by centrifugation and washed three times with saline and once with 0.05 M potassium phosphate buffer, pH 7.2. The final pellet was suspended in the same buffer containing mannitol (1 M) and β-mercaptoethanol (1 mM) to a concentration of about 7.5 mg (dry weight) of cells/ml. To a 100-ml portion of this cell suspension 100 U of Zymolyase was added, and the mixture was shaken at 37°C for 2 h, when almost all cells had been converted into protoplasts, as determined by examination under a phase-contrast microscope. The protoplasts were separated by centrifugation of 0°C for 5 min at 800 × *g* in a refrigerated centrifuge, washed twice at 0°C with 100 ml of phosphate buffer containing mannitol (0.8 M), and finally suspended in 100 ml of buffered mannitol (0.8 M) containing $MgCl_2$ (10 mM). Protoplast membranes were prepared from this suspension essentially according to the method of Longley et al. (26). Finally, the protoplast membranes were lyophilized and stored in a vacuum desiccator over silica gel at −20°C.

Trypsin digestion of membranes. Procedures were essentially the same as those reported by Tsukagoshi et al. (33). A 24-h digest of lyophilized membranes in phosphate buffer was subjected to centrifugation at 20,000 × *g* for 60 min. After separating the supernatant fluid, the sediment was washed once with phosphate buffer, solubilized in hot KOH (0.3 N), and then dialyzed against 1,000 volumes of the same buffer at 4°C for 24 h. The dialyzed preparation was stored at 4°C (trypsin-digested membranes).

Extraction of lipids from membranes. Lipids were extracted from lyophilized membranes successively with ethanol (90%, vol/vol) and with chloroform-methanol (2:1, vol/vol) by the method of Hunter and Rose (18). All extracts were combined, filtered through a sintered glass filter (no. 4), washed as outlined by Folch et al. (12), and then evaporated to constant dry weight under nitrogen. They were dissolved in an appropriate amount of chloroform and stored in a sealed ampoule (lipid extract).

The membrane residue was further treated with hot HCl (0.7 N) in methanol and subsequently with light petroleum according to the procedures of Kates (22) for extraction of bound lipids to minimize the lipid content therein (defatted membranes).

Solubilization of membranes. Before assaying for antagonizing effect, lyophilized membrane preparations for testing were solubilized with hot KOH (0.3 N) and then dialyzed against 0.05 M phosphate buffer at 4°C for 24 h.

Fractionation of lipids. Phospholipids and neutral lipids in lipid extracts from protoplast membranes were separated by column chromatography. A solution of lipid extract in chloroform was passed through silicic acid as described by Borgeström (4, 5), with only phospholipids retained by the absorbent, and subsequently recovered by desorption with chloroform-methanol (2:1, vol/vol). Neutral lipids were further fractionated by the column chromatographic method of Carrol (8), using activated magnesium silicate (Florisil) as stationary-phase and hexane-ether mixtures, with stepwise increase in polarity, for elution. Sterol esters, triglycerides, and free sterols were separated as the major fractions in this order. Each lipid fraction was evaporated to dryness under a stream of nitrogen and weighed. A solution of the phospholipid fraction was made with chloroform-methanol (2:1) and for the other lipid fractions in ethanol.

Hydrogenation of lipids. To obtain hydrogenated phospholipids containing sufficiently low amounts of partial hydrogenolysis products, hydrogenation was done by shaking under hydrogen of about 10 mg of the phospholipid fraction separated as described above and 1 mg of palladium-on-carbon (10%) catalyst in 4 ml of ethanol. The reaction was practically complete in 15 to 20 min at room temperature and atmospheric pressure. Hydrogenated triglycerides were also prepared by the same procedure. To determine the extent of unsaturation, the hydrogenated samples of phospholipids in 4 ml of chloroform or those of triglycerides in 5 ml of ethanol, in an ice bath, were treated with 1 ml of a 0.1 N solution of dibromopyridine sulfate. Blanks were run concurrently. The resulting solution was stirred at 4°C for 10 min after which time 4 ml of 10% KI was added. After the addition of 20 ml of water, remaining bromide (as I_2) was determined by titration with 0.1 N sodium thiosulfate and starch. The absence of I_2 uptake indicated completion of the hydrogenation of both lipid samples.

Test for antagonism in liquid medium. Cultures

were grown on yeast nitrogen base (Difco) supplemented with 0.15% L-asparagine and 1% glucose (YNB-AG medium) at 37°C for 24 h on a shaker, and then diluted with the same fresh medium so as to give a cell suspension containing about 2×10^5 viable cells per ml. Twofold dilutions of clotrimazole and miconazole, extending from 0.18×10^{-6} to 22.4×10^{-6} M and from 0.53×10^{-6} to 134×10^{-6} M, respectively, were prepared in a 1-ml volume of sterile YNB-AG medium (final concentration of dimethyl sulfoxide, 1%) in 10- by 100-mm tubes. When membrane materials dissolved in phosphate buffer were tested, a 0.5-ml volume was added to each series of tubes containing graded concentrations of drug and then mixed together. Solutions of lipid extracts and fractions were all made in ethanol, except for phospholipids, which were dissolved in chloroform-methanol (2:1). Upon testing, each drug-containing tube successively received 0.45 ml of phosphate buffer and 0.05 ml of a suspension of *C. albicans* cells. After 48 h of incubation at 37°C, samples were taken from each tube for the determination of the number of viable cells. The assay was made on GPY agar plates by a conventional plating technique (20). The minimum inhibitory concentration (MIC) was defined as the lowest concentration of drug at which the viable number did not exceed 10 times the initial number of cells in the inoculum.

Test of antagonism on agar plates. The antagonizing ability of various classes of authentic lipid compounds against anti-*Candida* activity of clotrimazole and miconazole was tested by applying the procedures for the conventional agar diffusion method as described by Bennett et al. (3) because of simplicity and higher susceptibility. Yeast morphology agar (Difco) was used as the assay agar, into which an appropriate amount of a 24-h culture of *C. albicans* was seeded so that the agar contained about 10^4 viable cells per ml. Solutions of the drug in dimethylsulfoxide, in a volume of 0.1 ml, were diluted with 1.9 ml of sterile water and then mixed together with 0.1 ml of the lipid solution to be tested to give the indicated concentrations of drug and lipid. Solutions of all classes of lipids were made in ethanol, except phospholipids and fatty acid sodium salts, which were dissolved in chloroform-methanol (2:1) and water, respectively. The mixture of drug and lipid was left standing at room temperature for 1 h and then applied to the agar plates to fill the punched wells. The diameter of the well was 8 mm. After 24 h of incubation at 37°C, the plates were inverted and the clear zones were read with a caliper. The potency of the antagonistic effect of the lipid tested on each antimycotic was expressed as a decrease in the diameter (in milliliters) of the inhibitory zone for a definite concentration of the drug.

RESULTS

Effect of different membrane preparations from *C. albicans*. Experiments were conducted to learn whether each of the two imidazole antimycotics, clotrimazole and miconazole, would more preferentially interact with either proteins or lipids of the membrane of susceptible organisms. For this purpose, *Candida* protoplast membranes and three fractions of proteinous or lipid nature derived from them were examined for their effect on the anti-*Candida* activity of the two drugs.

As shown in Table 1, with the addition of solubilized protoplast membranes to a final concentration of 2 mg (dry weight) per ml, the MIC of both clotrimazole and miconazole was increased fourfold as compared with the value for each drug alone. Almost the same extent of the reversal of the anti-*Candida* action of the two drugs was attained when they were combined with 2 mg (dry weight) of trypsin-digested membranes per ml or 0.2 mg (dry weight) of lipid extracts per ml. In all cases, antagonistic effect increased in relation to the increasing amounts of these membrane materials present in the medium. By contrast, defatted membranes, which were practically free of lipids, at concentrations up to 2 mg (dry weight)/ml, had no effect on the MIC value of the two drugs against *C. albicans*.

Effect of several major lipid fractions from *C. albicans* protoplast membranes. Further experiments were attempted to examine what class(es) of membrane lipids is(are) principally responsible for the antagonism. By means of column chromatography, the following four major lipid fractions were separated from lipid extracts of protoplast membranes: phospholipids, sterol esters, triglycerides, and free sterols. Judged from the recovery expressed on a weight basis, the former two were present in greater amounts than the latter two.

Table 2 shows that among these four classes of lipids, only phospholipids and triglycerides were capable of counteracting the two drugs: the MIC of clotrimazole and miconazole was

TABLE 1. *Effect of protoplast membranes and several fractions prepared therefrom on anti-Candida activity of imidazole antimycotics*

Membrane fraction added	Concn (mg [dry wt]/ml)	MIC ($\times 10^{-6}$ M)	
		Clotrimazole	Miconazole
None		1.4	4.2
Whole membranes	1	1.4	8.4
	2	5.6	16.8
Trypsin-digested membranes	1	2.8	16.8
	2	5.6	33.6
Defatted membranes	1	1.4	4.2
	2	1.4	4.2
Lipidic extracts	0.1	2.8	16.8
	0.2	5.6	33.6
	0.4	11.2	134.0

TABLE 2. *Effect of several major lipid fractions from protoplast membranes on anti-Candida activity of imidazole antimycotics*

Expt	Lipid fraction added	Concn (mg [dry wt]/ml)	MIC ($\times 10^{-6}$ M)	
			Clotrimazole	Miconazole
1	None		1.4	4.2
	Phospholipids	0.2	5.6	67.2
		0.4	11.2	134.0
	Triglycerides	0.2	1.4	8.4
		0.4	5.6	33.6
	Sterol esters	0.2	1.4	4.2
		0.4	1.4	4.2
	Free sterols	0.2	1.4	4.2
		0.4	1.4	4.2
2	None		1.4	4.2
	Phospholipids	0.4	22.4	134.0
	Phospholipids, hydrogenated	0.4	0.7	2.1
	Triglycerides	0.4	5.6	33.6
	Triglycerides, hydrogenated	0.4	1.4	4.2

increased 8- to 32-fold in the presence of 0.4 mg (dry weight) of phospholipids per ml and 4- to 8-fold in the presence of the comparable concentration (on a dry weight basis) of triglycerides, as compared with the value for lipid-free controls. However, after being almost fully saturated by catalytic hydrogenation as confirmed by the absence of I_2 uptake, both of the resulting fractions of phospholipids and triglycerides completely lost their antagonizing ability toward the two drugs.

Relationship between zone size and drug concentration as the basis for estimating the antagonistic ability of various lipids. As illustrated in Fig. 1, the dose response curve obtained for clotrimazole by the conventional agar diffusion method was linear and free from curvilinear deviation throughout its entire range. A similar curve was also obtained for miconazole, although it leveled off at concentrations greater than 2 mM. Based on these dose response curves, 0.75 mM for both clotrimazole and miconazole was chosen as the drug concentration suitable for testing the antagonistic effect of various lipids; this concentration for each drug, used alone, coincidentally showed a diameter of about 25 mm for the inhibitory zone.

Effect of phospholipids. Of five lecithins tested, only egg lecithin and L-α-dioleoyl-lecithin showed the antagonistic effect on the anti-*Candida* action of the two imidazole drugs; either one of these lecithins at concentrations as high as 0.13 mM and 0.06 mM caused an appreciable decrease in zone diameter for 0.75 mM clotrimazole and miconazole, respectively (Table 3). By contrast, none of the other three lecithins with saturated acyl groups, namely, L-α-dipalmitoyl-lecithin, L-α-distearoyl-lecithin and reduced-egg lecithin, was shown to have antagonistic effect. Not only egg lecithin but two other naturally occurring phospholipids, i.e., egg phosphatidyl ethanolamine and bovine phosphatidyl serine, were capable of antagonizing the two drugs.

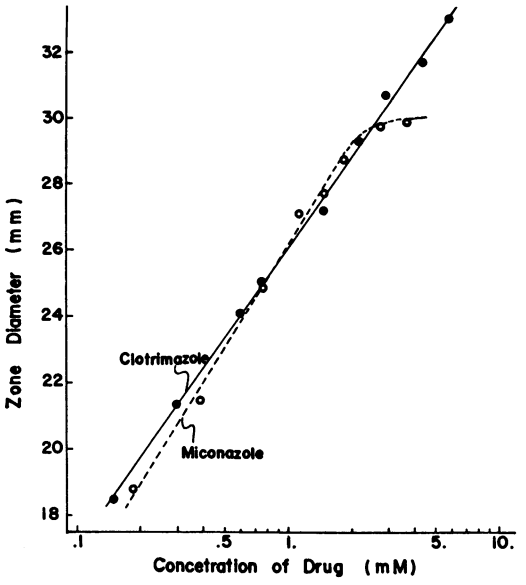

FIG. 1. *Assay for the anti-Candida activity of clotrimazole and miconazole.*

Effect of acylglycerides. Several mono-, di-, and triacylglycerides that possess acyl groups consisting of a single species of fatty acid (either palmitic acid, stearic acid, or oleic acid) were tested for their antagonistic effect. Substantial antagonism was produced by those acylglycerides that possessed one or more oleic acid residues as their ester moiety (Table 4),

TABLE 3. *Reversal of anti-Candida activity of imidazole antimycotics by naturally occurring and synthetic phospholipids*[a]

Expt	Phospholipid tested (mM)	Decrease in diameter of inhibitory zone (mm)[a]	
		Clotrimazole (0.75 mM)	Miconazole (0.75 mM)
A	Lecithin (egg)		
	0.5	4.0	5.5
	0.25	3.0	4.5
	0.13	1.5	3.0
	Lecithin (reduced egg)		
	0.5	0[b]	0[b]
	0.25	0[b]	0
	0.13	0[b]	0
	Phosphatidyl ethanolamine (egg)		
	0.5	3.5	5.5
	0.25	1.0	2.0
	0.13	0[b]	2.0
	Phosphatidyl serine (bovine)		
	0.5	5.0	5.0
	0.25	3.0	2.5
	0.13	0.5	0[b]
	Dipalmitoyl-L-α-lecithin		
	0.5	0	0
	0.25	0[b]	0
	0.13	0[b]	0[b]
	Distearoyl-L-α-lecithin		
	0.5	0[b]	0[b]
	0.25	0	0[b]
	0.13	0	0
	Dioleoyl-L-α-lecithin		
	0.5	5.0	5.0
	0.25	3.5	4.0
	0.13	2.0	2.5
B	Lecithin (egg)		
	0.13	1.5	3.5
	0.06	0[b]	2.0
	0.03	0	0
	Dioleoyl-L-α-lecithin		
	0.13	2.0	3.5
	0.06	1.5	2.5
	0.03	0[b]	1.0

[a] The diameter of the inhibitory zone for 0.75 mM clotrimazole and miconazole was 25.5 and 24.5 mm in experiment A, and 26.0 and 25.0 mm in experiment B, respectively.

[b] There was an increase in diameter of inhibitory zone.

but not by others. It was also noted from the results that the extent of drug-inactivating ability of oleyl glycerides increased with the number of oleyl groups present in the molecule; triolein, diolein, and mono-olein were effective in this order. The reversing effect of triolein appears to be comparable in degree with that of egg lecithin when expressed on a molar basis.

Effect of sterols and related compounds. Of seven sterols with an intact steroid nucleus (ergosterol, lanosterol, dehydrocholesterol, stigmasterol, cholesterol, β-sitosterol, and β-cholestanol), all were practically ineffective as antagonists to both drugs (Table 5). In contrast to the above sterols, ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3), the activated forms of ergosterol and dehydrocholesterol, respectively, showed a potent ability to inactivate the two drugs. To a lesser extent, an antagonistic effect was exerted by squalene, a polyenoic hydrocarbon known as an essential intermediate of sterol biosynthesis.

Effect of fatty acids, esters, and alcohols. A series of saturated and unsaturated fatty acids were examined for their antagonistic effect. The results are summarized in Tables 6 and 7. All of the saturated fatty acids ranging in chain length between C_6 and C_{18} were unable to coun-

TABLE 4. *Reversal of anti-Candida activity of imidazole antimycotics by mono-, di-, and triacylglycerides*

Expt	Acylglyceride tested (mM)	Decrease in diameter of inhibitory zone (mm)[a]	
		Clotrimazole (0.75 mM)	Miconazole (0.75 mM)
A	Monoacylglycerides		
	α-Monopalmitin (0.5)	0	0
	α-Monostearin (0.5)	0	0[b]
	α-Monoolein (0.5)	2.5	3.5
	Diacylglycerides		
	1,2-Dipalmitin (0.5)	0[b]	0[b]
	1,2-Distearin (0.5)	0	0
	1,2-Diolein (0.5)	4.0	5.0
	Triacylglycerides		
	Tripalmitin (0.5)	0.5	0
	Tristearin (0.5)	0.5	0.5
	Triolein (0.5)	5.5	5.0
B	α-Monoolein		
	0.25	0.5	2.0
	0.13	0[b]	0
	0.06	0	0[b]
	1,2-Diolein		
	0.25	2.0	3.5
	0.13	0	1.5
	0.06	0[b]	0[b]
	Triolein		
	0.25	3.0	4.5
	0.13	1.5	3.0
	0.06	0	1.0

[a] The diameter of the inhibitory zone for 0.75 mM clotrimazole and miconazole used alone was 26.5 and 25.5 mm in experiment A, and 26.0 and 25.0 mm in experiment B, respectively.

[b] There was an increase in diameter of inhibitory zone.

teract both clotrimazole and miconazole, with the only exception being lauric acid (C_{12}). On the other hand, among unsaturated fatty acids, several long-chain members, i.e., palmitoleic acid ($C_{16:1}$), oleic acid ($C_{18:1}$), α-linoleic acid ($C_{18:2}$), γ-linolenic ($C_{18:3}$), and arachidonic acid ($C_{20:4}$), were demonstrated to be potently antagonistic against both drugs. They are all *cis*-fatty acids, and among them there was no significant difference in potency of antagonistic action. When compared on a molar basis, these unsaturated fatty acids were more than 10 times as effective as egg lecithin or triolein. In contrast to oleic acid, its *trans*-isomer, olaidic acid, was much less active as an antagonist. Similarly, *trans*-2-hexadecenoic acid ($C_{16:1}$) or any other *trans*-monoenoic acid ranging in chain length between C_4 and C_8 was scarcely active.

In comparison with free oleic acid, the sodium salt of oleic acid showed almost the same extent of inactivating ability, whereas that of the methyl or cholesterol ester of oleic acid was far less. Oleyl alcohol was only slightly effective in antagonism (Table 8).

DISCUSSION

Several investigators working on protoplast membranes of *Saccharomyces cerevisiae* (6, 26) and *Candida utilis* (28) coincidentally demonstrated that, like membranes from many other organisms, yeast cell membranes usually contain proteins and lipids as the two major components which account for 80 to 90% of the dry weight of the membrane. The results of the present study using *C. albicans* protoplast membranes showed that those membrane components which can be involved in the antagonistic interaction with the two membrane-active imidazole antimycotics, clotrimazole and miconazole, are lipid in nature and that, of several different lipid fractions from the fungal membranes, phospholipids and triglycerides are preferentially effective in this antagonistic action, whereas the hydrogenated products from them are completely ineffective. There are several reports of lipids in yeasts showing that phospholipid and neutral lipid fractions derived from whole cells and/or protoplast membranes of *C. albicans* (10, 29) and some other yeasts,

TABLE 5. *Reversal of anti-Candida activity of imidazole antimycotics by sterols and related compounds*

Expt	Compound tested (mM)	Decrease in diameter of inhibitory zone (mm)[a]	
		Clotrimazole (0.75 mM)	Miconazole (0.75 mM)
A	Ergosterol		
	0.5	0	1.5
	0.25	0	1.0
	Lanosterol		
	0.5	1.0	2.0
	0.25	0	1.0
	Cholesterol		
	0.5	0	0.5
	0.25	0	0
	Dehydrocholesterol		
	0.5	0	1.5
	0.25	0	0.5
	β-Cholestanol		
	0.5	0.5	0.5
	0.25	0	0.5
	Stigmasterol		
	0.5	0	1.5
	0.25	0	0
	β-Sitosterol		
	0.5	0	0.5
	0.25	0	0
	Ergocalciferol		
	0.5	6.0	7.5
	0.25	5.5	6.0
	Cholecalciferol		
	0.5	6.0	8.0
	0.25	4.5	6.0
	Squalene		
	0.5	1.5	2.5
	0.25	1.0	2.0
B	Ergocalciferol		
	0.25	4.5	6.5
	0.13	4.0	5.0
	0.06	1.5	2.5
	Cholecalciferol		
	0.25	4.5	6.5
	0.13	3.5	5.0
	0.06	1.5	3.0
	Squalene		
	0.25	1.0	1.5
	0.13	0	1.0
	0.06	0	0

[a] The diameter of the inhibitory zone for 0.75 mM clotrimazole and miconazole, used alone, was 25.5 and 24.5 mm in experiment A, and 26.0 and 25.0 mm in experiment B, respectively.

such as *C. utilis* and *S. cerevisiae* (2, 7, 9, 18, 26, 32), are characterized by the fatty acid composition in which several unsaturated long-chain members predominate, e.g., palmitoleic acid, oleic acid, linoleic acid, and linolenic acid.

On the basis of these data and ours, we are led to the conclusion that of various components of cellular membranes of susceptible *Candida* cells, phospholipids and triglycerides with unsaturated acyl groups, are substantially effective in antagonism against the anti-*Candida* action of imidazole drugs. It, in turn, suggests that such types of membrane lipids have a special avidity for the drugs and that they may play a role in drug susceptibility of the membrane. Consistent results were obtained from the experiments with a wide range of classes of synthetic and naturally occurring authentic lipids. Moreover, they provided more detailed information on the lipid preference of the imidazoles.

In this study, a significant antagonistic ability was demonstrated for naturally occurring phospholipids (e.g., egg lecithin) and synthetic unsaturated lecithins (e.g., L-α-dioleoyl-lecithin) but not for reduced egg lecithin nor any synthetic saturated lecithins (e.g., L-α-dipalmitoyl-lecithin). Considering this and the fact that acyl groups of naturally occurring phospholipids generally contain two species of unsaturated fatty acids, palmitoleic acid and oleic acid, in relative abundance, it is probable that the unsaturated acyl group in the molecule of lecithin and other types of phospholipids may play a critical role in exerting antagonistic interaction with the imidazole drugs. It remains to be answered whether or not the antagonizing ability of unsaturated phospholipids is influenced by the type of polar group in their molecule. With acylglycerides, such unsaturated acyl group-dependent antagonism was also shown.

Of various sterols and related compounds, activated forms of certain sterols such as ergocalciferol and cholecalciferol were shown to be effective in antagonism. However, none of those sterols that possess an intact steroid nucleus containing a 3β-OH group and a saturated or unsaturated hydrocarbon side chain was effective. Among them were ergosterol and cholesterol, which are known as the major sterol of fungal cells and animal cells, respectively. It looks likely, therefore, that imidazole antimycotics are different from the polyene antibiotics, e.g., amphotericin B and nystatin, in their lipid preference. Growth inhibition by the latter group of antifungals has been reported to be protected preferentially by several sterols including ergosterol and cholesterol (13). More recently, Halder and Bose (16, 17) have studied the mechanism of antagonism of the antifungal action by certain sterols and phospholipids of a membrane-active polypeptide antibiotic, mycobacillin. Working with several derivatives of cholesterol and with components of lecithin molecules (egg), they reached the conclusion that the 3 β-OH group of the former compounds and the oleic acid compo-

TABLE 6. *Reversal of anti-Candida activity of imidazole antimycotics by various saturated and unsaturated fatty acids*

Fatty acid tested (0.5 mM)	Decrease in diameter of inhibitory zone (mm)	
	Clotrimazole (0.75 mM)	Miconazole (0.75 mM)
Saturated		
Hexanoic acid (caproic acid, $C_{6:0}$)	0	0
Octanoic acid (caprylic acid, $C_{8:0}$)	0	0.5
Decanoic acid (capric acid, $C_{10:0}$)	0.5	1.0
Dodecanoic acid (lauric acid, $C_{12:0}$)	5.0	6.0
Tetradecanoic acid (myristic acid, $C_{14:0}$)	0.5	1.0
Hexadecanoic acid (palmitic acid, $C_{16:0}$)	0	0
Octadecanoic acid (stearic acid, $C_{18:0}$)	0.5	0.5
Unsaturated:		
trans-2-Butenoic acid (crotonic acid, $C_{4:1}$)	0	0
3-Hexenoic acid ($C_{6:1}$)	0	0.5
2-Octenoic acid ($C_{8:1}$)	0	0.5
2-Hexadecenoic acid ($C_{16:1}$)	1.0	2.0
cis-9-Hexadecenoic acid (palmitoleic acid, $C_{16:1}$)	11.5	12.0
cis-9-Octadecenoic acid (oleic acid, $C_{18:1}$)	12.0	12.5
trans-9-Octadecenoic acid (elaidic acid, $C_{18:1}$)	3.0	4.5
cis-9,*cis*-12-Octadecadienoic acid (α-linoleic acid, $C_{18:2}$)	120	130
cis-6,*cis*-9,*cis*-12-Octadecatrienoic acid (γ-linolenic acid, $C_{18:3}$)	9.5	10.5
cis-5,*cis*-8,*cis*-11,*cis*-14-Eicosatetraenoic acid (arachidonic acid, $C_{20:4}$)	12.5	12.0

nent of the latter are responsible for the antagonistic interaction with the antibiotic (17). Although the imidazole drugs are different from mycobacillin in their response to sterols, they appear to share a common property in that their antifungal action is considerably antagonized by unsaturated lecithin but not by saturated lecithin. There are some other membrane-active agents, e.g., tyrothricin (1), polymyxins (11), pyrrolnitrin (30), and surfactin (33), in which growth inhibition has been reported to be reversed by certain phospholipids including naturally occurring lecithin and phosphatidyl ethanolamine. In any case, however, no evidence is available for the involvement of acyl groups of the phospholipid molecule in the antagonistic interaction with an antimicrobial agent.

Tests with a series of saturated and unsaturated fatty acids revealed that long chain, unsaturated fatty acids of *cis*-configuration, e.g., palmitoleic acid and oleic acid, were effective in antagonism against the imidazole drugs to an extent much greater than that of the phospholipids or acylglycerides containing the corresponding fatty acid residues. It was also demonstrated that not only saturated fatty acid (with the only exception of lauric acid) but also unsaturated fatty acids of *trans*-configuration were all practically ineffective as antagonists. These results lead us to the possibility that the presence of an aliphatic hydrocarbon chain with one or more double-bond(s) arranged in *cis*-configuration in the fatty acid molecule may enable it to more potently antagonize the imidazole drugs. This is in accord with the above-mentioned result suggesting that, in the case of the antagonistic effect exerted by phospholipids and acylglycerides, an unsaturated acyl group is an essential component.

For the moment no data are available as to the mechanism of antagonistic action of unsaturated *cis*-fatty acids, free or esterified to glycerol, toward the imidazole drugs. Nevertheless, besides the strong hydrophobic and lipophilic nature of both clotrimazole and miconazole, reports from our laboratory and others, indicating potent membrane activity of the two drugs against susceptible organisms as well as lecithin (egg) liposomes (9, 12, 14), suggest the possibility that the antagonism may result from interaction between the antagonist of lipid nature and the drug to form inactive complexes. However, it cannot be ruled out that the imidazole drugs may inhibit biosynthesis of some essential unsaturated fatty acids and/or unsaturated lipids in fungal cells. As compared with free oleic acid on a molar basis, the antagonistic ability of its sodium salt appeared to be similar, but its methyl or cholesterol ester or

TABLE 7. *Reversal of anti-Candida activity of imidazole antimycotics by graded concentrations of certain fatty acids*

Fatty acid added	Concn (mM)	Decrease in diameter of inhibitory zone (mm)	
		Clotrimazole (0.75 mM)	Miconazole (0.75 mM)
Lauric acid ($C_{12:0}$)	0.25	4.0	4.5
	0.13	1.5	2.5
	0.06	0	1.0
2-Hexadecenoic acid ($C_{16:1}$)	0.25	0	2.0
	0.13	0	0
	0.06	0	0
Palmitoleic acid ($C_{16:1}$)	0.25	11.0	10.5
	0.13	8.0	9.0
	0.06	6.0	7.5
Oleic acid ($C_{18:1}$)	0.25	10.5	11.0
	0.13	8.5	9.5
	0.06	6.0	7.5
Elaidic acid ($C_{18:1}$)	0.25	1.0	3.5
	0.13	0	2.5
	0.06	0	1.5
α-Linoleic acid ($C_{18:2}$)	0.25	11.0	12.0
	0.13	9.5	9.5
	0.06	7.0	7.0
γ-Linolenic acid ($C_{18:3}$)	0.25	7.5	8.0
	0.13	5.5	6.5
	0.06	3.0	4.0
Arachidonic acid ($C_{20:4}$)	0.25	11.5	11.5
	0.13	9.5	9.0
	0.06	7.0	8.0

TABLE 8. *Reversal of anti-Candida activity of imidazole antimycotics by oleic acid and its derivatives*

Compound added	Concn (mM)	Decrease in diameter of inhibitory zone (mm)	
		Clotrimazole[a]	Miconazole[a]
Oleic acid	0.5	12.0	12.5
	0.25	10.0	11.0
	0.13	8.0	9.5
Oleic acid, sodium salt	0.5	10.5	12.0
	0.25	8.5	10.0
	0.13	6.5	8.0
Oleic acid, methyl ester	0.5	2.5	2.5
	0.25	1.5	1.5
	0.13	0.5	1.0
Oleic acid, cholesterol ester	0.5	2.0	2.5
	0.25	1.5	1.5
	0.13	0	1.0
Oleyl alcohol	0.5	2.5	1.5
	0.25	1.0	1.5
	0.13	0	0.5

[a] The final concentration of clotrimazole or miconazole applied to the well was 0.75 mM.

oleyl alcohol was much less active; the activity of L-α-dioleoyl-lecithin and triolein was intermediate. It is possible, therefore, that such a potent activity of oleic acid and probably of other unsaturated *cis*-fatty acids, occurring in a free form, may be due to their negative charge which reacts to the positively charged imidazole ring in the drug molecule.

ACKNOWLEDGMENTS

I am indebted to Bayer Yakuhin Co., Ltd. and Mochida Pharmaceutical Co. for providing clotrimazole and miconazole, respectively.

I also thank Akiko Ohi for skillful technical assistance and K. Iwata for encouragement and helpful discussions.

LITERATURE CITED

1. **Baker, Z., R. W. Harrison, and B. F. Miller.** 1941. Inhibition by phospholipids of the action of synthetic detergents on bacteria. J. Exp. Med. 74:621.
2. **Baraud, J., M. A. Maurice, and C. Napias.** 1970. Composition et répartition des lipides au sein des cellules de *Saccharomyces cerevisiae*. Bull. Soc. Chem. Biol. 52:421–432.
3. **Bennett, J. V., J. L. Brodie, E. J. Benner, and W. M. M. Kirby.** 1966. Simplified, accurate method for antibiotic assay of clinical specimens. Appl. Microbiol. 14:170–177.
4. **Borgeström, B.** 1952. Investigation on lipid separation methods. Separation of phospholipids from neutral fat and fatty acids. Acta Physiol. Scand. 25:101–110.
5. **Borgeström, B.** 1952. Investigation on lipid separation methods. Separation of cholesterol esters, glycerides and free fatty acids. Acta Physiol. Scand. 25:111–119.
6. **Boulton, A. A.** 1965. Some observations on the chemistry and morphology of the membranes released from yeast protoplasts by osmotic shock. Exp. Cell Res. 37:343–359.
7. **Brown, C. M., and A. H. Rose.** 1969. Fatty acid composition of *Candida utilis* as affected by growth temperature and dissolved-oxygen tension. J. Bacteriol. 99:371–378.
8. **Carrol, K. K.** 1961. Separation of lipid classes by chromatography on florisil. J. Lipid Res. 2:135–141.
9. **Castelli, A., G. P. Littarru, and G. Barbaresi.** 1969. Effect of pH and CO_2 concentration changes on lipids and fatty acids of *Saccharomyces cerevisiae*. Arch. Mikrobiol. 66:34–39.
10. **Combs, T. J., J. J. Guarneri, and M. A. Pisano.** 1968. The effect of sodium chloride on the lipid content and fatty acid composition of *Candida albicans*. Mycologia 60:1232–1239.
11. **Few, A. V.** 1955. The interaction of polymyxin E with bacterial and other lipids. Biochim. Biophys. Acta 16:137–145.
12. **Folch, J., M. Lees, and G. H. Sloane-Stanley.** 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497–509.
13. **Gottlieb, D., H. E. Carter, J. H. Sloneker, and A. Ammann.** 1958. Protection of fungi against polyene antibiotics by sterols. Science 128:361.
14. **Gottlieb, D., H. E. Carter, J. H. Sloneker, L. C. Wu, and E. Gaudy.** 1961. Mechanism of inhibition of fungi by filipin. Phytopathology 51:321–330.
15. **Gottlieb, D., H. E. Carter, L. C. Wu, and J. H. Sloneker.** 1960. Inhibition of fungi by filipin and its antagonism by sterols. Phytopathology 50:549–603.
16. **Halder, A., and S. K. Bose.** 1971. Reversal of mycobacillin inhibition reaction by sterols and phospholipids.

J. Antibiot. 24:779–784.
17. **Halder, A., and S. K. Bose.** 1973. Mechanism of sterol and lipid antagonism of a polypeptide antibiotic, mycobacillin. J. Antibiot. 26:358–361.
18. **Hunter, K., and A. H. Rose.** 1972. Lipid composition of *Saccharomyces cerevisiae* as influenced by growth temperature. Biochim. Biophys. Acta 260:639–653.
19. **Iwata, K.; and H. Yamaguchi.** 1973. Mechanism of action of clotrimazole. 1. Survey of the principal action on *Candida albicans* cells. Jpn. J. Bacteriol. 28:513–521.
20. **Iwata, K., H. Yamaguchi, and T. Hiratani.** 1973. Mode of action of clotrimazole. Sabouraudia 11:158–166.
21. **Iwata, K., H. Yamaguchi, Y. Kanda, and M. Osumi.** 1976. Action mechanism of clotrimazole, p. 315–322. *In* K. Iwata (ed.), Yeasts and yeast-like microorganisms in medical science. Proceedings of the Second International Specialized Symposium on Yeasts, Tokyo 1972, University of Tokyo Press, Tokyo.
22. **Kates, M.** 1964. Simplified procedures for hydrolysis or methanolysis of lipids. J. Lipid Res. 5:132–135.
23. **Kitamura, K., T. Kaneko, and Y. Yamamoto.** 1971. Lysis of viable yeast cells by enzymes of *Arthrobacter luteus*. Arch. Biochem. Biophys. 145:402–404.
24. **Lampen, J. O.** 1966. Interference by polyenic antifungal antibiotics (especially nystatin and filipin) with specific membrane functions, p. 111–130. *In* Biochemical studies of antifungal drugs. Sixteenth Symposium of Soc. Gen. Microbiology London 1966, Cambridge University Press, London.
25. **Lampen, J. O., P. M. Arnow, and R. S. Saferman.** 1960. Mechanism of protection by sterols against polyene antibiotics. J. Bacteriol. 80:200–206.
26. **Longley, R. P., A. H. Rose, and B. A. Knights.** 1968. Composition of the protoplast membrane from *Saccharomyces cerevisiae*. Biochem. J. 108:401–412.
27. **McMurrough, I., and A. H. Rose.** 1971. Effect of temperature variation on the fatty acid composition of *Candida utilis*. J. Bacteriol. 107:753–758.
28. **Mendosa, C. G., and J. R. Villaneuva.** 1967. Preparation and composition of the protoplast membrane of *Candida utilis*. Biochim. Biophys. Acta 135:189–195.
29. **Nishi, K., H. Ichikawa, K. Tomochika, A. Okabe, and Y. Kanemasa.** 1973. Lipid composition of *Candida albicans* and the effect of growth temperature on it. Acta Med. Okayama 27:73–81.
30. **Nose, M., and K. Arima.** 1969. On the mode of action of a new antifungal antibiotic, pyrrolnitrin. J. Antibiot. 22:135–143.
31. **Sreedhara Swamy, K. H., M. Sirsi, and G. Ramananda Rao.** 1974. Studies on the mechanism of action of miconazole: effect of miconazole on respiration and cell permeability of *Candida albicans*. Antimicrob. Agents Chemother. 5:420–425.
32. **Suomalainen, H., and T. Nurminen.** 1970. The lipid composition of cell wall and plasma membrane of baker's yeast. Chem. Phys. Lipids 4:247–256.
33. **Tsukagoshi, N., G. Tamura, and K. Arima.** 1970. A novel protoplast-bursting factor (Surfactin) obtained from *Bacillus subtilis* IAM 1213. II. The interaction of surfactin with bacterial membranes and lipids. Biochim. Biophys. Acta 196:211–214.
34. **Yamaguchi, H., and K. Iwata.** 1974. Mechanism of action of clotrimazole. 2. Effects on the cell membrane of *Candida albicans*. Jpn. J. Bacteriol. 29:379–385.