Biochemical Pharmacology, Vol. 31, No. 16, pp. 2609–2617, 1982.
Printed in Great Britain.

0006-2952/82/162609-09 $03.00/0

THE INTERACTION OF MICONAZOLE AND KETOCONAZOLE WITH LIPIDS

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(*Received* 19 *January* 1982; *accepted* 3 *March* 1982)

Abstract—*Staphylococcus aureus* can be protected by unsaturated unesterified fatty acids against the growth inhibitory effects of miconazole and ketoconazole observed at concentrations $>10^{-6}$ M and $>10^{-5}$ M, respectively. Miconazole's fungicidal activity is partly antagonized by oleic acid. However, the effect of ketoconazole on the viability of *Candida albicans* was not affected by this fatty acid. Cytochrome oxidase and ATPase activities are more sensitive to miconazole (10^{-5} M) than to ketoconazole ($>10^{-4}$ M) and also liposomes are more susceptible to lysis induced by miconazole. Using differential scanning calorimetry it is shown that high concentrations of miconazole shift the lipid transition temperature of multilamellar vesicles to lower values without affecting the enthalpy of melting. Ketoconazole induces a broadening of the main transition peak only. It is suggested that miconazole changes the lipid organization without binding to the lipids, whereas ketoconazole is localized in the multilayer without having an important direct effect on the lipid organization. The results indicate that miconazole, and to a lesser extent ketoconazole, at doses that can be reached by topical application only, interfere with a third target (the two others are ergosterol synthesis and fatty acid elongation plus desaturation). It is hypothesized that the induced change in lipid organization may play some role in miconazole's topical antibacterial and fungicidal activity, whereas it does not seem to play a significant role in ketoconazole's activities.

Miconazole‡ is an imidazole derivative with broad-spectrum activity against most pathogenic yeasts, fungi and Gram-positive bacteria of medical interest. It is used topically or intravenously and to a limited extent orally [1, 2].

Ketoconazole§, a more recently developed imidazole derivative, has a spectrum of antifungal activity which qualitatively resembles that of miconazole. Most noteworthy is ketoconazole's absorption from the gastrointestinal tract and distribution profile making it suitable for the oral treatment of superficial and deep mycosis [3].

Both miconazole [4] and ketoconazole [5] affect ergosterol synthesis in yeast cells resulting in an accumulation of 14α-methylsterols known to disturb membrane and cell properties. At concentrations higher than those needed to affect sterol metabolism or after longer incubation periods, miconazole also affects the nature of free and esterified fatty acids. A shift from unsaturated (18:1) to saturated and shorter (16:0) fatty acids occurs [6]. An accumulation of saturated fatty acids was also observed in yeast incubated in the presence of ketoconazole (unpublished results). This effect enhances the membrane disturbances, decreases growth and may lead to decreased activity of membrane-bound enzymes.

Saccharomyces cerevisiae can grown anaerobically if ergosterol and an unsaturated fatty acid are present in the medium. Sud and Feingold [7, 8] determined under these conditions the minimum inhibitory concentration (MIC) for miconazole and ketoconazole. They found that the MIC for miconazole was increased 64 times by anaerobiosis. The MIC found for ketoconazole was 7.4×10^{-7} M under aerobic and $>3.8 \times 10^{-4}$ M under anaerobic conditions. This may indicate that, next to the ergosterol synthesis and fatty acid desaturation and elongation (both aerobic processes) miconazole affects another target too, whereas the molecular bases for ketoconazole's antimycotic properties only seem to be interference with both aerobic processes.

The present study was designed to examine the interaction of miconazole and ketoconazole with free and esterified fatty acids. Therefore the antagonistic activity of fatty acids and phospholipids on miconazole and ketoconazole's antibacterial and antimycotic activity was investigated. The effects of both antimycotics on cytochrome oxidase and adenosine triphosphatase activities in the presence and absence of fatty acid supplements were studied. Differential scanning calorimetry has been employed to study the interaction of miconazole and ketoconazole with phospholipids.

The impact that a direct effect of miconazole and ketoconazole on lipids may have on their antimycotic and antibacterial activity is discussed.

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‡ (±) - 1 - [2 - (2,4 - dichlorophenyl) - 2 - [(2,4 - dichlorophenyl)methoxy]ethyl] - 1H - imidazole mononitrate.

§ (±)-*cis*-1-acetyl - 4 - {4 - [[2 - (2,4 - dichlorophenyl) - 2 - (1H - imidazol - 1 - yl - methyl) - 1,3 - dioxolan - 4 - yl]methoxy]phenyl}piperazine.

MATERIALS AND METHODS

Strains, inocula and media

Inocula of *Candida albicans* (strain ATCC 28516) were prepared and grown in casein hydrolysate–yeast extract–glucose medium (CYG-medium) as previously described [4, 5].

Staphylococcus aureus B 180 was maintained on tryptose-agar slants and subcultured twice in tryptose broth (Tb) for 24 hr at 37°. A 0.2-ml aliquot was inoculated in 100 ml of the same medium and grown aerobically at 37° for 64 hr with shaking. A 1-ml aliquot of a dilution of this culture, containing 3×10^8 cells/ml, was used to inoculate 100 ml of Tb and grown for 24 hr as before. The experimental media were then inoculated with 1-ml aliquots of the diluted culture (containing 3×10^8 cells/ml).

Casein hydrolysate–yeast extract–glucose medium was prepared as previously described [9], tryptose broth contained 20 g of tryptose (Difco), 5 g of sodium chloride and 1 g of glucose per litre of distilled water, tryptose-agar is similar with the exception that 15 g agar is added per litre.

Growth and antagonism studies

C. albicans was grown aerobically in a reciprocating shaker at 37° in 100 ml casein hydrolysate–yeast extract–glucose medium (CYG-medium). The inoculum added contained 15×10^6 cells.

Miconazole nitrate was dissolved in dimethylsulfoxide (DMSO), ketoconazole was dissolved in water acidified with 0.1 N HCl (final pH 2.4) and passed through a 0.22 μm Millipore filter. Dilution of ketoconazole was made in sterile water. Both drugs were added immediately before inoculation.

Staphylococcus was grown aerobically at 37° for 24 hr by shaking in a reciprocating shaker. Miconazole nitrate and ketoconazole solutions were prepared and added to the medium as described above.

The fatty acids and phosphatidylcholines were dissolved in ethanol. In all experiments, the freshly prepared solutions were added to the media immediately before inoculation.

Controls were similarly set up with equivalent quantities of DMSO, acidified water and ethanol (final concentrations 0.10%, 0.05% and 0.25%, respectively).

Total count method

The number of cells per unit of Tb medium were determined with a Coulter Counter [4].

Viable count method

Samples were withdrawn from 24 hr cultures and decimally diluted in sterile 0.85% saline. Aliquots of 0.1 ml from the undiluted and diluted *Staphylococcus* or yeast suspensions were plated on each of 4 replicate tryptose-agar plates or on each of 6 replicate Sabouraud agar plates, respectively. These were incubated for 72 hr at 37° and the colonies counted.

Uptake of [^{3}H]*miconazole or* [^{3}H]*ketoconazole by* S. aureus

[^{3}H]Miconazole (sp. act. 189 mCi/mmole) was dissolved in DMSO and added to Tb immediately before inoculation with *S. aureus*. [^{3}H]Ketoconazole (sp. act. 170.5 mCi/mmole) was dissolved in water acidified with 0.1 N HCl (pH 2.4). After 24 hr of growth, cells were harvested by centrifugation and 3 times washed with physiological NaCl solution. The cells were resuspended in 1 ml of twice distilled water, transferred into glass counting vials and digested with 0.2 ml $HClO_4$ and 0.4 ml H_2O_2 for 3 hr at 80°. After addition of 10 ml of scintillator solution, the radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer.

Preparation of liposomes

Release studies. Liposomes were prepared by a modified Yeagle *et al.* [10] method. Phosphatidylcholine (PC) from egg yolk (Sigma) with the following fatty acid composition: 16:0, 37.4%, 18:0, 12.9%; 18:1, 34.2%; 18:2, 15.5% was used. Cholesterol (Aldrich) was recrystallized from ethanol and dried at 56° at 200 mg Hg before use. Five μM PC and 5 μM cholesterol solubilized in chloroform were mixed and evaporated to dryness under nitrogen. The residue was further dried in a dessicator for 30 min. The dried film was gently dispersed in 4 ml of 1 mM [U-^{14}C]glucose (sp. act. 2 mCi/mmole) in 10 mM NaCl. The solution was sonicated in ice under nitrogen for 30 min in a Braun Sonic®-300 Sonicator with a microprobe and then centrifuged at 100,000 *g* for 30 min at 4°. The supernatant thus obtained was passed through a Sephadex G-50 (medium) column pre-equilibrated with 1 mM glucose in 10 mM NaCl. Vesicles containing trapped [^{14}C]glucose were eluted in the void volume (4 ml). The radioactivity (Go) of 1 ml aliquots was determined immediately after collection using a liquid scintillation counter. One ml aliquots were incubated in the presence of 10^{-4} M of miconazole or ketoconazole and/or solvent (DMSO or acidified water, pH 2.4 respectively) at 37° for 1, 2, 3 or 4 hr. Then the samples were applied to a Sephadex G-50 column and the radioactivity determined in the void volume (Gx).

$$\% \text{ glucose release} = 100\left(1 - \frac{Go}{Gx}\right).$$

Differential scanning calorimetry studies. Multilamellar vesicles of dipalmitylphosphatidylcholine (DPPC, Sigma) were prepared at a lipid concentration of 55 μmole/ml in Tris–HCl buffer (10^{-2} M, pH 7.3) as described previously [11]. Miconazole and ketoconazole were incorporated in variable amounts in the lipid film prior to the liposome formation.

Differential scanning calorimetry

Aliquots (0.1 ml) of the liposome suspension were placed in sealed inoxydable sample pans. A reference sample was similarly prepared using 0.1 ml of the Tris–HCl buffer. Measurements were carried out on a Setaram DSC III differential scanning calorimeter (Lyon, France) operating at a heating rate of 2°/min [12].

Preparation of yeast subcellular fractions

Commercially available baker's yeast ('Gist en Spiritus Fabrieken', Brugman, Ghent, Belgium) was used to prepare cell-free supernatants (S 1500 *g*) or 'microsomal' fractions (P 100,000 *g*). The cell-free

supernatant is the supernatant collected after a 5 min centrifugation at 1500 *g* of, in 0.1 M sodium phosphate buffer (pH 7.0), broken yeast cells [13]. To prepare a 'microsomal' fraction the cell-free supernatant was centrifuged at 10,000 *g* for 20 min, the supernatant thus obtained was centrifuged for 60 min at 100,000 *g*. The pellet was resuspended in 0.1 M Tris–HCl buffer (pH 8.4) and called 'microsomal' fraction. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Cat. No. 500.0006).

Cytochrome oxidase

The effects of miconazole and ketoconazole alone or together with free fatty acids or phospholipids on the cytochrome *c* oxidase activity were determined with the method of Wharton and Tzagoloff [14] using the S 1500 *g* fraction (0.68 mg protein per ml).

Adenosine triphosphatase

The effects of miconazole and ketoconazole on Mg, Na, K-ATPase activity and the antagonistic effects of free fatty acids and phospholipids were studied using a 'microsomal' fraction (0.5 mg protein per ml) of baker's yeast. The method used was an adaptation of a previously published one [15]. The reaction mixture contained 0.1 M Tris–HCl buffer pH 8.4, 10 mM KCl, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 107 mM sucrose, 162 mM mannitol, 5 μM bovine serum albumin, 2.5 mM EDTA and 0.125 mg protein in a volume of 2 ml. The incubation (30 min at 30°) and determination of P_i was as described previously [15].

RESULTS

Effects of miconazole, ketoconazole and fatty acids on growth of S. aureus

As seen in Fig. 1 miconazole at concentrations $>3 \times 10^{-6}$ M completely blocked growth of *S. aureus*. At 3×10^{-6} M only a small number of cells was found. The addition of 10^{-5} M or 1.5×10^{-5} M oleic acid (18:1) to cultures containing 3×10^{-6} M miconazole partly antagonized miconazole's growth inhibitory effect. Growth inhibition was antagonized by more than 90% when 3×10^{-5} M of oleic acid was added simultaneously to 3×10^{-6} M miconazole (Fig. 1, Table 1); about 70% antagonism was seen when 5×10^{-5} M oleic acid was added to cultures containing 5×10^{-6} or 10^{-5} M miconazole (Fig. 1). Antagonism was not further enhanced by increasing the oleic acid concentration $\geq 10^{-4}$ M. This may be related to oleic acid's inhibitory effects observed when such concentrations were added to *S. aureus* cultures (Figs. 1 and 2). Vaccenic acid resembles oleic acid in its growth inhibitory (Fig. 2) and antagonistic (Fig. 3) properties. Palmitoleic acid (16:1) and linoleic acid (18:2) are much more inhibitory, reaching almost 100% inhibition at 10^{-4} M. Stearic acid (18:0) is without toxic effect (Fig. 2), it also is devoid of antagonistic properties (Fig. 3) just as palmitic acid (16:0). Linoleic acid can antagonize miconazole's antibacterial activity. However at concentrations higher than 3×10^{-5} M it is too toxic itself (Figs. 2 and 3) to be of further use. It is of

Table 1. Effects of miconazole on the viability of *S. aureus**

Concentration (M) Miconazole	Concentration (M) Oleic acid	Viable cells % of control
0	1.5×10^{-5}	104.2 (101.4, 107.0)
0	3.0×10^{-5}	106.1 ± 33.4 (5)
3×10^{-6}	0	9.3 ± 2.7 (4)
3×10^{-6}	1.5×10^{-5}	56.3 (55.7, 56.9)
3×10^{-6}	3.0×10^{-5}	90.6 (86.6, 94.5)

* Cells were grown for 24 hr at 37° in tryptose broth supplemented with miconazole and/or oleic acid. The number of viable cells in the control cultures was $4.8 \pm 0.5 \times 10^9$ cells per ml (mean of 4 experiments). No significant effect of the solvents, DMSO and ethanol, was observed.

The figures given are mean values ± S.D. followed by the number of experiments in parentheses.

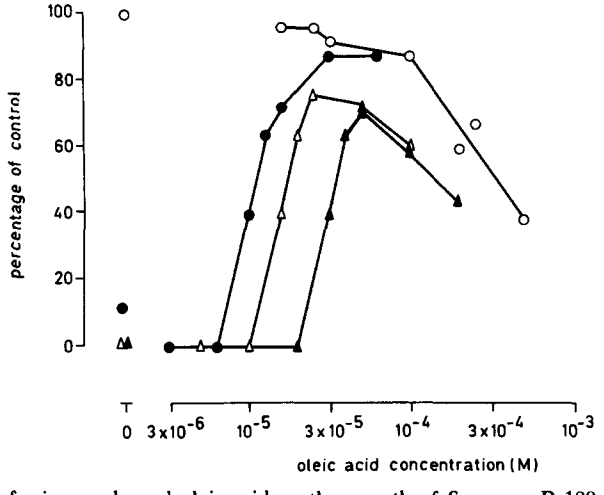

Fig. 1. The effect of miconazole and oleic acid on the growth of *S. aureus* B 180. Cells were grown aerobically at 37° in the presence of solvents and various concentrations of oleic acid and 0 (○), 3×10^{-6} M (●), 5×10^{-6} M (△) or 10^{-5} M (▲) miconazole. After 24 hr the total number of cells was determined.

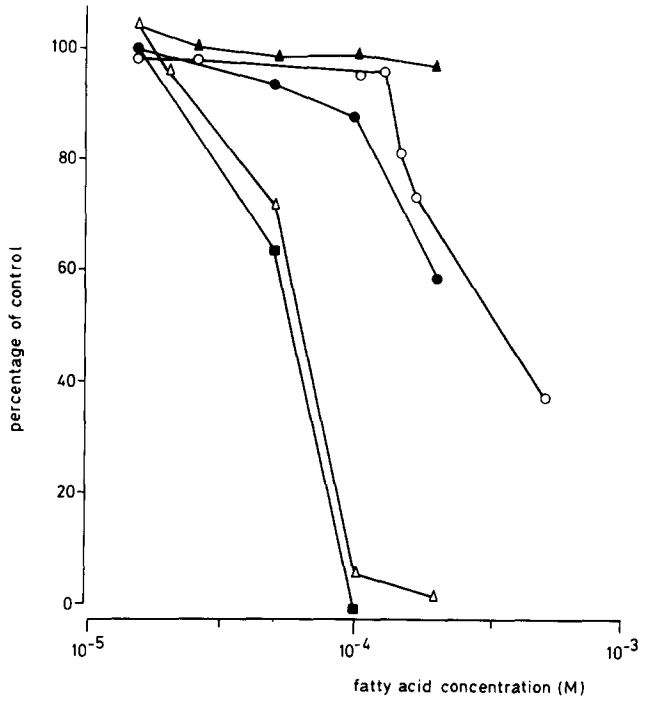

Fig. 2. Effects of oleic acid (○), vaccenic acid (●), linoleic acid (△), stearic acid (▲) and palmitoleic acid (■) on the growth of *S. aureus*. Further details are given in the legend to Fig. 1.

interest to note that palmitoleic acid is devoid of antagonistic properties.

Antagonism by oleic acid is not affected by the sequence of addition. As shown in Table 2 similar results were obtained when oleic acid was added before or after miconazole.

The effect of dioleoyl phosphatidylcholine (DOPC) on the growth inhibitory effects of miconazole is much less pronounced than that of free oleic acid. In fact, 50% antagonism was seen only when 10^{-4} M of DOPC was added to *S. aureus* cultures containing 5×10^{-6} M of miconazole (Table 3).

Ketoconazole is much less active against *S. aureus*; about 90% inhibition of growth was observed at 5×10^{-5} M (Fig. 4). Although the growth inhibitory effects of 3 and 4×10^{-5} M are antagonized by

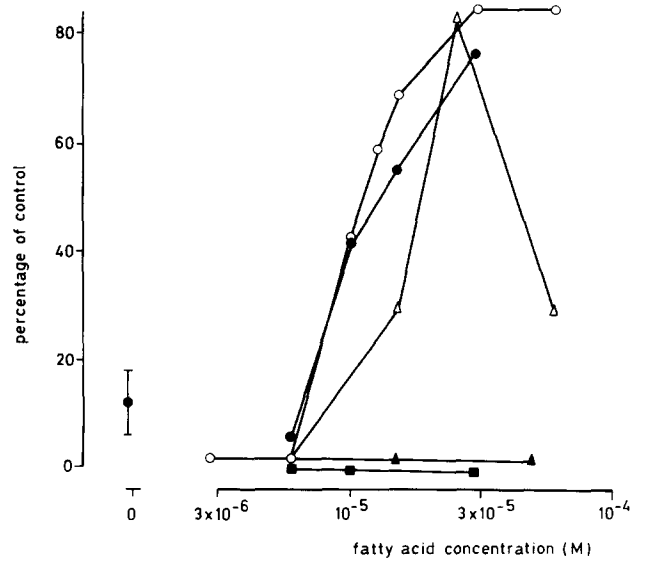

Fig. 3. Effects of oleic acid (○), vaccenic acid (●), linoleic acid (△), stearic acid (▲) and palmitoleic acid (■) on the growth inhibitory effect of 3×10^{-6} M miconazole on *S. aureus*. The effect of miconazole on growth in the absence of fatty acids is the mean value of 12 experiments ± S.D. Further details are given in the legend to Fig. 1.

Table 2. Effects of 3×10^{-6} M miconazole and oleic acid on growth of *S. aureus**

Concentration (M)		Per cent of control			
		A		B	
Miconazole	Oleic acid	1 hr	2 hr	1 hr	2 hr
0	0	91.2	78.7	87.6	80.4
0	3×10^{-5}	88.2		87.7	
3×10^{-6}	0	16.9	5.7	19.4	15.0
3×10^{-6}	10^{-5}	49.5	54.0	31.1	52.9
3×10^{-6}	1.5×10^{-5}	62.2	66.4	54.6	67.9
3×10^{-6}	3×10^{-5}	71.3	82.9	72.2	72.4

* *S. aureus* was grown for 24 hr at 37° in tryptose broth supplemented with miconazole and/or oleic acid and/or the solvents DMSO and ethanol.

† Growth in the absence of solvents, miconazole and oleic acid was taken as 100%.

A, micronazole added at 0 hr, oleic acid 1 or 2 hr later; B, oleic acid added at 0 hr, miconazole 1 or 2 hr later.

Table 3. Effects of miconazole and dioleoyl phosphatidyl-choline (DOPC) on growth of *S. aureus**

Concentration (M)		
Miconazole	DOPC	Percentage of control
5×10^{-6}	0	0.4
	10^{-5}	1.2
	2.5×10^{-5}	10.2
	5.0×10^{-5}	15.1
	10^{-4}	50.1

* Cells were grown for 24 hr at 37° in tryptose broth supplemented with miconazole and/or DOPC and/or solvents.

10^{-4} and 1.5×10^{-4} M of oleic acid, the concentration of this fatty acid needed to antagonize the inhibition induced by 5×10^{-5} M of ketoconazole is toxic itself.

Effects of oleic acid on the uptake of [^{3}H]*miconazole and* [^{3}H]*ketoconazole by* S. aureus

At the concentrations used oleic acid did not affect the uptake (P = 0.83) of miconazole or ketoconazole when added simultaneously to *S. aureus* cultures (Table 4) indicating that the antagonism observed is not due to a captation of miconazole or ketoconazole outside the cell.

Effects of miconazole, ketoconazole and oleic acid on the viability of C. albicans

A complete fungicidal effect was seen after a 24 hr cultivation period of *C. albicans* in the presence of 3×10^{-4} M miconazole (Fig. 5). At 10^{-4} M 44,668 viable cells per ml of medium were found, i.e. much lower than the 150,000 cells per ml present at the start. At 10^{-5} M a small number of multiplications took place. These effects of miconazole on the viability were partly antagonized by the addition of oleic acid. The growth inhibition seen in the presence of 5×10^{-8} and 10^{-7} M was not antagonized by oleic acid as was the growth inhibition induced by ketoconazole concentrations up to 3×10^{-4} M. It should be noted that under the growth conditions used the fungicidal activity of ketoconazole (10^{-4} and 3×10^{-4} M) after 24 hr of contact is comparable to that of miconazole (Fig. 5).

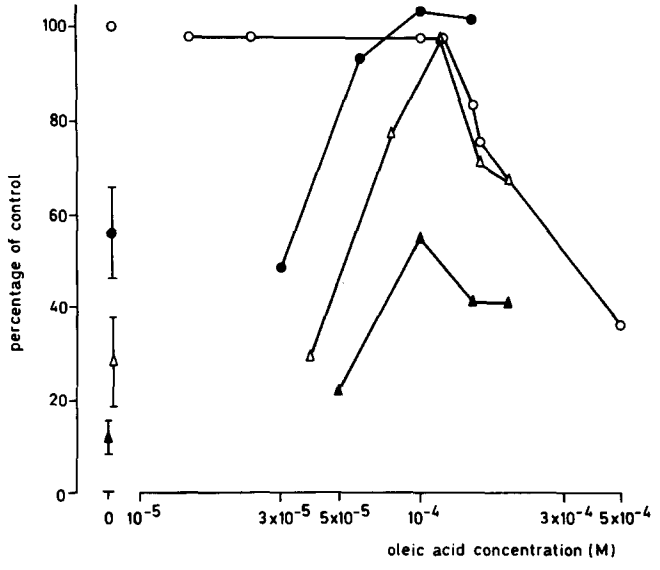

Fig. 4. Effects of ketoconazole and oleic acid on the growth of *S. aureus*. Ketoconazole concentrations: 0 (○), 3×10^{-5} M (●), 4×10^{-5} M (△), 5×10^{-5} M (▲). Further details are given in the legend to Fig. 1.

Table 4. Effect of oleic acid on the uptake of [^{3}H]miconazole and [^{3}H]ketoconazole by *S. aureus**

Drug	Concentration (M)	Oleic acid (M)	Cellular content of drug (pmoles/10^9 cells)
Miconazole	2×10^{-6}	0	371 ± 35 (3)
		2×10^{-5}	535 (512–558)
	3×10^{-6}	0	890 ± 299 (8)
		3×10^{-5}	771 ± 236 (10)
Ketoconazole	3×10^{-5}	0	2927 ± 529 (3)
		6×10^{-5}	2435 ± 380 (4)
		10^{-4}	2689 ± 420 (4)

* *S. aureus* was grown for 24 hr at 37° in tryptose broth supplemented with [^{3}H]miconazole, [^{3}H]ketoconazole, solvent and different concentrations of oleic acid. Figures in parentheses are the number of experiments or cellular contents of the drug when only 2 experiments were done.

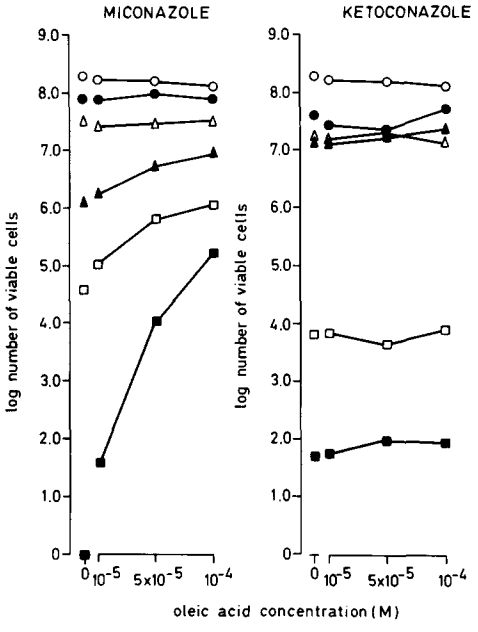

Fig. 5. Effects of miconazole and ketoconazole on the viability of *C. albicans*. Cells were grown aerobically at 37° in the presence of solvents and various concentrations of oleic acid and 0 (○), 5×10^{-8} M (●), 10^{-7} M (△), 10^{-5} M (▲), 10^{-4} M (□), 3×10^{-4} M (■) miconazole or ketoconazole. After 24 hr the number of viable cells was determined.

Effects of miconazole and ketoconazole on liposomes

Liposomes composed of egg phosphatidylcholine:cholesterol (1:1) were found to be very susceptible to miconazole. Miconazole caused a rapid release of the marker [^{14}C]glucose; the effect of ketoconazole was much slower (Fig. 6). In fact, the ketoconazole-induced release of the marker is only 15% more than that of liposomes incubated in the presence of solvent.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) provides valuable information on the thermotropic properties of lipid vesicles. Figure 7 shows the pattern characteristic of multilamellar vesicles: a pretransition at 32° and a main transition at 42°. In the presence

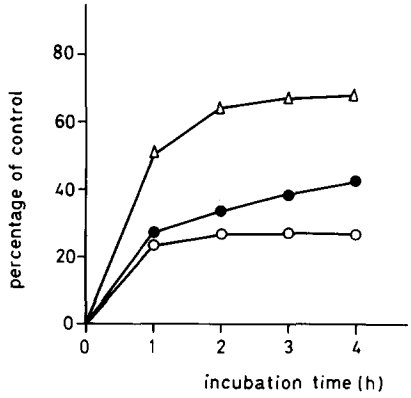

Fig. 6. Effects of solvent (○) and 10^{-4} M of micronazole (△) and ketoconazole (●) on the release of the marker [^{14}C]glucose from liposomes consisting of egg phosphatidylcholine:cholesterol (1:1).

Table 5. Enthalpy of melting*

Liposomes	Molar ratio	ΔH (main transition) (kcal/mole)
DPPC/drug	100/0	8.0
DPPC/ketoconazole	100/5	8.2
	100/10	8.1
	100/20	7.8
	100/25	8.0
	100/30	8.2
DPPC/miconazole	100/5	7.8
	100/10	8.0
	100/20	8.2
	100/25	8.4
	100/30	7.8

* Lipid concentration 55 μmoles/ml. Liposomes were formed in a Tris–HCl buffer (10^{-2} M, pH 7.3).

of ketoconazole (Fig. 7A) the pretransition vanishes but no significant shift of the main DPPC transition is observed even if the main transition peak becomes broader. This may indicate that ketoconazole is localized in the multilayer without having great effect on lipid organization. The fact that both miconazole and ketoconazole do not affect significantly the enthalpy of melting suggests that neither drug binds to the lipids (Table 5). However, the peak displace-

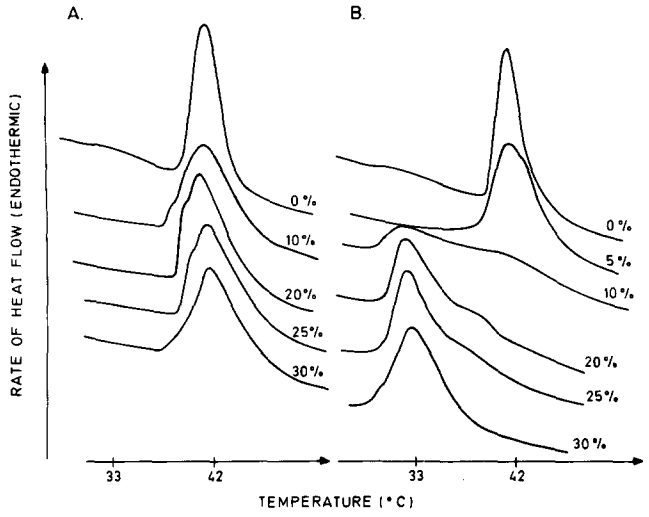

Fig. 7. Differential scanning calorimetry of DPPC multilamellar vesicles containing increasing molar amounts of ketoconazole (A) or miconazole (B). Lipid concentration: 55 μmoles/ml. Liposomes were formed in Tris–HCl buffer (10^{-2} M, pH 7.3). Drug:lipid molar ratios are indicated in the figure.

ment obtained in the presence of miconazole is highly suggestive of a change in lipid organization (fig. 7B).

Effects of miconazole and ketoconazole on the cytochrome oxidase and ATPase activity in subcellular fractions of baker's yeast

Cytochrome oxidase activity of a cell-free supernatant, containing mitochondria, microsomes and soluble fractions (S 1500 *g*) of baker's yeast is much more sensitive to miconazole than to ketoconazole. Fifty per cent inhibition was reached with 6.6×10^{-5} M miconazole whereas 2.8×10^{-4} M ketoconazole was needed (Fig. 8). The addition of 10^{-4} M oleic acid almost completely antagonized the miconazole effect. The effect of ketoconazole was not antagonized by 10^{-4} M oleic acid, the highest concentration that could be used.

Oleic acid (18:1) can be replaced by linoleic acid (18:2) but not by palmitic (16:0) and stearic acid

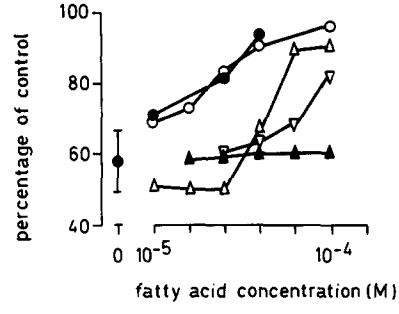

Fig. 9. Effect of oleic acid (○), linoleic acid (●), dioleoyl phosphatidylcholine (△), palmitic acid (▽) and stearic acid (▲) on the miconazole affected cytochrome oxidase activity. Miconazole concentration: 4×10^{-5} M. The result obtained in the absence of fatty acids or DOPC is the mean value of 20 experiments ± S.D.

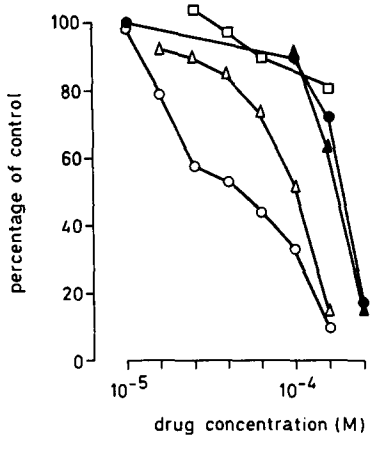

Fig. 8. Effects of miconazole (○) and ketoconazole (●) on the cytochrome oxidase activity in the absence and presence of oleic acid. Oleic acid concentrations added in the presence of miconazole: 6×10^{-5} M (△), 10^{-5} M (□) and ketoconazole: 10^{-4} M (▲).

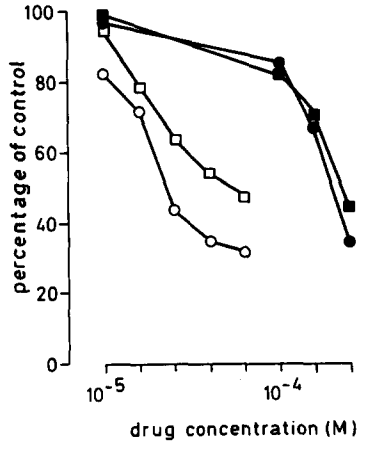

Fig. 10. Effect of miconazole (○) and ketoconazole (●) on the microsomal ATPase activity in the absence or presence of 10^{-4} M oleic acid (miconazole + oleic acid: □, ketoconazole + oleic acid: ■).

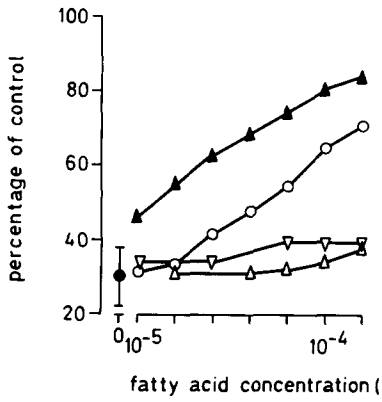

Fig. 11. Effect of oleic acid (○), stearic acid (△), DOPC (▲) and DPPC (▽) on the miconazole affected ATPase activity. Miconazole concentration: 4×10^{-5} M. The result obtained in the absence of fatty acids or phospholipids is the mean value of 15 experiments ± S.D.

(18:0) (Fig. 9). Higher concentrations of DOPC are needed to antagonize miconazole's inhibitory effects on the cytochrome oxidase.

Miconazole also is a better inhibitor of the microsomal ATPase (P 100,000 *g*) than ketoconazole (Fig. 10). Fifty per cent was achieved with 3.6×10^{-5} M miconazole whereas 3×10^{-4} M ketoconazole was needed. The ketoconazole-induced inhibition could not be antagonized by oleic acid; that of miconazole could partly be antagonized (Fig. 10). Again, oleic acid could not be replaced by stearic acid (Fig. 11). However, in contrast with the miconazole-induced inhibition of the cytochrome oxidase (Fig. 9) that of the ATPase was much better antagonized by DOPC (Fig. 11). Dipalmitoylphosphatidylcholine (DPPC) had no effect.

DISCUSSION

Staphylococcus aureus and *Candida albicans* can be protected by unsaturated, unesterified fatty acids against the antibacterial and fungicidal properties of miconazole. This is not simply due to a change of the absorption profile of miconazole since the uptake of miconazole by *S. aureus* in the presence of oleic acid appears to be comparable to that observed in the absence of this unsaturated fatty acid. Furthermore when compared on a molar basis, more than 10 times more dioleoyl phosphatidylcholine (DOPC) than oleic acid is needed to antagonize miconazole's growth inhibitory effects on *S. aureus*. If this antagonism would have been due to a captation of miconazole outside the cell a more pronounced antagonism with DOPC could be expected. DOPC is also much less active in antagonizing miconazole's inhibitory effects on the mitochondrial cytochrome oxidase. However, it is a better antagonizing agent of the miconazole-induced inhibition of the microsomal ATPase where no membrane barrier for DOPC is present. The inefficacy of dipalmitylphosphatidylcholine (DPPC), palmitic and stearic acid to antagonize miconazole's activity suggests that the unsaturated acyl group plays an important role.

Ketoconazole also affects the growth of *S. aureus*. However, as compared with miconazole almost 16 times more ketoconazole (5×10^{-5} M) is needed. Another fundamental difference between miconazole and ketoconazole was found by comparing their fungicidal properties and the antagonizing effects of oleic acid. Miconazole's killing property is antagonized, at least partly, by the addition of oleic acid whereas the effect of ketoconazole on the viability of *C. albicans* was not affected at all.

Miconazole is also a more effective inhibitor of the mitochondrial cytochrome oxidase and microsomal ATPase activity, which are both enzymes known to require a fluid lipid environment for optimal activity [16, 17].

The results presented here also confirm the previously described [18, 19] rapid destructive action of miconazole on liposome membrane systems and the much less direct membrane damage observed with ketoconazole [8].

An even more important difference between both antimycotics was observed using differential scanning calorimetry. The shift of the main lipid transition temperature observed in the presence of miconazole and the inability of ketoconazole to induce such a shift indicates that miconazole adopts in the lipid layer an orientation completely different from that of ketoconazole. The miconazole-induced shift of the lipid transition temperature to lower values is highly suggestive of a change in lipid organization. The different mode of organization of miconazole and ketoconazole in the lipid matrix was further proven by using a recently described method [20] of lipid conformational analysis. This theoretical procedure demonstrates for ketoconazole a drug orientation with the piperazine moiety inserted into the hydrophobic region of the bilayer and the dichlorophenyl group into the hydrophilic phase. Miconazole, however, maintains its dichlorophenyl groups in the hydrophobic phase. The results of this computational approach will be published elsewhere.

It should be noted that all the effects described here are observed at relatively high concentrations of the drugs as compared to the concentrations at which they induce an inhibition of sterol synthesis, fatty acid elongation and desaturation and growth of fungal cells. Doses of miconazole $\geq 3 \times 10^{-6}$ M (≥ 1.5 μg/ml) and, in some experiments, ketoconazole concentrations as high as 10^{-4} M (53.1 μg/ml) were needed. Such high concentrations might be reached by topical application only. In fact, in healthy subjects, a single oral dose of 200 mg or 400 mg ketoconazole gave peak plasma concentrations of about 3 or 6 μg/ml respectively [21]. Oral bioavailability of miconazole is even much lower. Following an oral dose of 1000 mg in healthy volunteers, peak plasma concentrations of about 1.2 μg/ml occurred after 2–4 hr [2]. Fifteen minutes after an intravenous infusion of 1 g miconazole a plasma level of 7.5 μg/ml was found, i.e. 1.5×10^{-5} M, whereas 50% inhibition of, for example, the cytochrome oxidase, was seen at 6.6×10^{-5} M only.

The studies discussed here indicate that an alteration of the fluidity characteristics by a miconazole-induced change in lipid organization may be part of miconazole's topical, antibacterial and fungicidal properties. However, under the present

experimental conditions miconazole and ketoconazole have at 10^{-4} M comparable fungicidal properties. This suggests that interference with the third target (the two others are the ergosterol synthesis and fatty acid desaturation and elongation) in the *Candida* strain used in this study is not needed by ketoconazole for its fungicidal activity.

The interaction of miconazole and ketoconazole with lipid constituents of, for example bacteria, yeast and mammalian cell membranes, are under investigation. Although there may be a preferential interaction profile with the different acyl moieties, the fatty acids and phospholipids used in this study are not only present in, for example fungal cells, but also in mammalian cells. Therefore, the high doses needed to interact with the third target are in favour of a selective toxicity.

Acknowledgements—We are grateful to Dr. Paul A. Janssen for his interest in our work. Appreciation is expressed to H. Vanhove for help in preparing the manuscript and to B. Wouters for typing it. This work was supported by a grant from I.W.O.N.L.

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