Mode of Action of Miconazole on *Candida albicans*: Effect on Growth, Viability and K⁺ Release

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Miconazole at 10 µg ml⁻¹ inhibited the growth of exponential phase cultures of *Candida albicans* and released intracellular K⁺. Higher concentrations of miconazole were, however, required to cause cell death: at neutral pH, complete killing occurred at 30 µg ml⁻¹, while at pH 3.0 or 4.5, there was only partial killing with miconazole up to 80 µg ml⁻¹. Efficient killing of *C. albicans* by miconazole occurred both at low temperature and when cells were incubated in buffer alone. It is proposed that both the fungistatic and fungicidal actions of miconazole are due to its direct interaction with the cellular membranes of *C. albicans* rather than to an inhibition of biochemical reactions. Divalent cations protected *C. albicans* from both the fungistatic and fungicidal effects of miconazole and this was probably due to a competition between the ions and miconazole in its positively charged form for negatively charged binding sites. *Candida albicans* increased in resistance to miconazole-induced K⁺ release during the stationary phase of a batch culture. Development of this resistance required efficient aeration.

INTRODUCTION

Miconazole is a synthetic imidazole derivative with broad-spectrum antimicrobial action against fungi and Gram-positive bacteria (Godefroi *et al.*, 1969). It has been used successfully in the treatment of topical fungal infections including vaginal candidiasis (Godts *et al.*, 1971), tinea pedis (Brugmans *et al.*, 1970), onychomycosis (Achten *et al.*, 1977) and pityriasis versicolor (Van Cutsem & Reyntjens, 1978). It has also been partially successful in the treatment of systemic mycoses (Stevens, 1977). Miconazole affects membrane function in a number of systems. In *Candida albicans*, alterations in the transport properties of the plasma membrane were found at concentrations of miconazole lower than that required to inhibit growth (Van den Bossche, 1974). Leakage of K⁺ and ultraviolet-absorbing materials has also been reported (Swamy *et al.*, 1974). Ultrastructural studies have shown that miconazole causes widespread disruption of the cellular membranes of *C. albicans* (De Nollin & Borgers, 1974, 1976). In addition, miconazole affects the function *in vitro* of erythrocytes (Swamy *et al.*, 1976*b*), lysosomes (Swamy *et al.*, 1976*a*) and mitochondria (Dickinson, 1977). Yamaguchi (1977, 1978) suggested that miconazole associated with free unsaturated fatty acids and those within phospholipids and thus could disrupt membrane structure. However, Van den Bossche *et al.* (1978) have suggested that the effect of miconazole on cell membranes in *C. albicans* is due to inhibition of sterol demethylation thus preventing the synthesis of ergosterol, the major sterol in fungal membranes. De Nollin *et al.* (1977) observed a drop in the amount of peroxidase enzymes in *C. albicans* and *Saccharomyces cerevisiae* following incubation with miconazole and suggested that cell death was due to a build-up of H₂O₂.

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The present study was carried out to extend our knowledge of the effect of miconazole on the growth and viability of *C. albicans* and the loss of K^+ from this organism.

METHODS

Organisms and growth conditions. *Candida albicans* strains 6406, 723 and 6979 were obtained from the Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine. Unless otherwise stated, all experiments were carried out using strain 6406. Cultures were maintained on slopes of Yeast Morphology Agar (Difco) (YMA) and stored at 4 °C. Unless otherwise stated, liquid cultures were grown in the synthetic yeast medium of Davies (1953) containing 2 % (w/v) glucose (SYG medium). Yeast Nitrogen Base (Difco) containing 2 % (w/v) glucose (YNBG medium) was also used. Weekly inoculum cultures were prepared by inoculating 100 ml SYG medium from the slopes, and these were stored at 4 °C after overnight incubation at 37 °C in an orbital incubator.

Measurement of inhibition of growth by miconazole. To 100 ml SYG medium in 250 ml conical flasks was added a sufficiently large inoculum to give an initial A_{600} of about 0.1. After 4 to 5 h in an orbital incubator at 37 °C, the growth rate was exponential. Miconazole (kindly given by Janssen Pharmaceutica, Beerse, Belgium), dissolved in methanol at 5 mg ml⁻¹, was then added to the cultures to give the required concentration. Further growth was followed turbidimetrically at 600 nm.

Measurement of release of K^+ by miconazole. Release of K^+ was determined using a K^+ -sensitive electrode (type BH 115, Electronic Instruments Ltd, Richmond, Surrey) connected to a measuring unit C33B-2 and Vibron electrometer 33B-2 (Electronic Instruments) with output to a potentiometric recorder, as described by Gale (1974). Cultures of *C. albicans* (either 1 l or 100 ml) were harvested, washed twice with 0.05 M-Tris/HCl buffer pH 7.5 (made with deionized water) and resuspended in this buffer to give 10 mg dry wt ml⁻¹ ($A_{600} = 33$). Two ml of this suspension was diluted into 18 ml of the same Tris/HCl buffer pH 7.5 in the electrode vessel, except for the experiments in which the effect of cell density on K^+ release was studied, when 1 ml and 4 ml of the stock suspension were diluted into 19 ml and 16 ml buffer, respectively. Miconazole was added to the cells from the stock solution in methanol and average K^+ release rates in nmol min⁻¹ (mg cells)⁻¹ were calculated for each 1 min period after adding miconazole, using a previously constructed calibration curve. Release of K^+ from each sample was followed for up to 30 min. The intracellular K^+ content of cells was assayed by measuring the amount of K^+ released on incubation of 2 ml of the washed cell suspension in a boiling water bath for 20 min prior to diluting into 18 ml buffer in the electrode vessel.

Measurement of loss of viability induced by miconazole. To produce populations of single cells suitable for viability counting, cultures were incubated at 37 °C without shaking. Initially, the effect of miconazole was tested directly in the culture medium, while, for studying the effect of pH and cell density, samples of the cells were harvested, washed and resuspended in Tris/HCl buffer pH 7.5 and Na₂HPO₄/citric acid buffers pH 4.5 and 3.0, at A_{600} values of 2.0 and 6.0. Miconazole, dissolved in methanol as described above, was added to 10 ml of cell suspension in glass tubes (diam. approx. 15 mm) and incubated at 37 °C. At appropriate times, 0.5 ml was removed from each tube, diluted into 4.5 ml sterile 0.85 % (w/v) NaCl, and treated for 2 min in a Sonicleaner type 6441 A (Dawe Instruments Ltd, Western Avenue, Acton, London W3). Cell suspensions were diluted a further 10⁴ or 10⁵ times in sterile saline before spreading 0.5 ml on to each of three YMA plates. After 24 h at 37 °C, colonies were counted and the number of viable units per ml of original suspension was calculated. The total number of cells in the first dilution was counted in an Improved Neubauer haemocytometer after sonication for 2 min. The counting was done using phase contrast microscopy and approximately 600 cells were counted in each sample. Percentage viability was calculated from the ratio of viable to total count.

RESULTS

Effect of miconazole on the growth of C. albicans

When added to a culture of *C. albicans* in mid-exponential phase, miconazole at 10 µg ml⁻¹ caused complete cessation of growth, while at 1 µg ml⁻¹ growth continued unchecked. At intermediate concentrations, there was partial inhibition of growth (Fig. 1a). The degree of inhibition by miconazole at 10 µg ml⁻¹ was greater the earlier in the exponential phase that it was added, but if it was added before exponential growth was established, there was less inhibition (Fig. 1b). The decreased effect of miconazole when added in the later stages of growth may have been due, at least in part, to an effect of the increased cell density, since when miconazole was added to a series of cultures inoculated at different cell densities, the inhibition was greater the lower the cell density (Fig. 1c).

Fig. 1. Effect of miconazole on the growth of *C. albicans* in 2% (w/v) glucose synthetic yeast (SYG) medium. (a) After 4 h incubation, miconazole was added to give concentrations of 10 (Δ), 5 ($\blacksquare$), 3 ($\square$), 1 ($\bullet$) or 0 ($\circ$) $\mu\text{g ml}^{-1}$. (b) Miconazole was added to give 10 $\mu\text{g ml}^{-1}$ after incubation for 0 h (Δ), 4 h ($\blacksquare$), 6 h ($\square$) or 8 h ($\bullet$); control without miconazole ($\circ$). (c) After 4 h incubation, miconazole was added to give 10 $\mu\text{g ml}^{-1}$ to cultures having approximate A_{600} values of 0.7 (∇), 2.5 ($\blacksquare$), 3.5 ($\blacktriangle$) or 5.5 ($\bullet$); open symbols are for the respective controls without miconazole. (d) After 5 h incubation, miconazole was added to give 10 $\mu\text{g ml}^{-1}$ to cultures containing 45 mM- MgCl_2 and/or 85 mM-KCl: miconazole plus KCl ($\blacktriangle$), miconazole plus MgCl_2 ($\bullet$), miconazole plus MgCl_2 plus KCl ($\blacksquare$), miconazole alone (Δ), MgCl_2 plus KCl ($\square$), control with no additions ($\circ$).

Addition of either Mg^{2+} or Ca^{2+} to SYG medium prevented miconazole from inhibiting the growth of *C. albicans*. The concentration of divalent cations required to exhibit a protective effect depended upon the concentration of miconazole to which *C. albicans* was exposed. Divalent cations were only effective when added to a culture of *C. albicans* before or immediately after adding miconazole. An example of the protective effect of MgCl_2 , and the failure of KCl to have a similar effect, is given in Fig. 1d. Miconazole at 10 $\mu\text{g ml}^{-1}$ inhibited exponentially growing static cultures of *C. albicans* as completely as shaken cultures.

Effect of miconazole on the viability of C. albicans

When added to samples of an early-exponential phase static culture of *C. albicans*, miconazole was fungicidal at 30 to 40 $\mu\text{g ml}^{-1}$ but not at 20 $\mu\text{g ml}^{-1}$. At 37 °C killing was almost complete within 2 h (Table 1a); at 1 °C the rate of killing was slower, although after 24 h there was no difference in the percentage of cells surviving. Cultures at the end of the growth phase were much more resistant to killing by miconazole and an appreciable percentage survived 72 h contact with 80 $\mu\text{g ml}^{-1}$ (Table 1b). This was apparently due to the drop in pH which occurred during growth rather than to the increase in cell density, since a threefold difference in cell density did not affect the fungicidal activity of miconazole, whereas there was a marked pH dependency (Table 2). The pH of cultures of *C. albicans* grown in SYG medium dropped from an initial value of 6.5 to about 3.0 during growth. As with the effect on growth inhibition, Mg^{2+} but not K^+ protected *C. albicans* from the fungicidal action of miconazole.

Effect of miconazole on K⁺ release from C. albicans

Kerridge *et al.* (1976b) reported that *C. albicans* 6406 developed a resistance to miconazole-induced K^+ release during the stationary phase of a shaken batch culture. This was confirmed for cells harvested at 24 h (Fig. 2a), 48 h (Fig. 2b) and 72 h after inoculation. The release of K^+ from cells harvested after 72 h incubation was negligible in the presence of miconazole up to 40 $\mu\text{g ml}^{-1}$. Resistant cells regained their sensitivity to miconazole on re-inoculation into fresh medium.

Table 1. *Survival of C. albicans grown for 15 h and 48 h at 37 °C in static culture when exposed at 37 °C to miconazole*(a) 15 h (exponential phase) culture ($A_{600} = 2.6$)

Miconazole ($\mu\text{g ml}^{-1}$)	Percentage of cells surviving after exposure for:				
	0 h	2 h	4 h	8 h	24 h
0	100	61	50	60	37
20	90	26	24	25	31
30	69	6	1	1	< 1
40	72	2	2	< 1	< 1

(b) 48 h (early-stationary phase) culture ($A_{600} = 6.0$)

Miconazole ($\mu\text{g ml}^{-1}$)	Percentage of cells surviving after exposure for:		
	24 h	48 h	72 h
0	96	103	85
10	106	58	53
20	105	66	45
40	114	67	39
80	76	27	13

Table 2. *Effect of cell density and pH on the ability of miconazole to kill C. albicans grown for 15 h in static culture (exponential phase) when exposed at 37 °C to miconazole for 24 h after washing and resuspension in buffer*

Miconazole ($\mu\text{g ml}^{-1}$)	Percentage of cells surviving					
	Initial $A_{600} = 6.0$			Initial $A_{600} = 2.0$		
	pH 3.0	pH 4.5	pH 7.5	pH 3.0	pH 4.5	pH 7.5
0	63	40	60	78	50	78
10	64	42	29	77	44	16
20	62	43	15	60	46	1
40	52	39	< 1	52	34	< 1
80	59	26	< 1	54	8	< 1

Gale (1974), in using K^+ release measurements to study the effect of amphotericin B methyl ester (AME) on *C. albicans*, defined a standard release concentration (SRC) of AME as the amount required to raise the K^+ release rate by $1 \text{ nmol min}^{-1} (\text{mg cells})^{-1}$ above the background leakage rate in 8 min. This is not a suitable measure of miconazole action since this antifungal agent produces an increase followed by a decrease in K^+ release rate in contrast to AME which causes the K^+ release rate to increase until the cells become severely depleted of K^+ . An SRC can be defined more conveniently in terms of the concentration of miconazole required to give a maximum K^+ release rate of $1 \text{ nmol min}^{-1} (\text{mg cells})^{-1}$ above the background leakage rate. Although SRCs are arbitrarily defined, sensitivities can be quoted in terms analogous to minimum growth inhibitory concentrations measured by traditional methods. The SRC of miconazole increased in a linear fashion from 1 to $10 \mu\text{g ml}^{-1}$ for *C. albicans* in exponential phase to about $40 \mu\text{g ml}^{-1}$ for cells grown for 72 h in shaken batch culture. Thereafter, there was a steeper rise in the SRC which was found to be due to cell death and loss of intracellular K^+ . However, the increase in resistance to miconazole-induced K^+ release which occurred between the end of the growth phase and the point of cell death in culture could not be accounted for in terms of altered intracellular K^+ content.

In the same way as the growth inhibitory activity of miconazole was less, the greater

Fig. 2. Pattern of miconazole-induced K^+ release from *C. albicans* harvested from shaken batch culture in (a) early-stationary phase (24 h growth) and (b) mid-stationary phase (48 h growth). Miconazole was added to give 40 ($\circ$), 20 ($\bullet$) or 10 ($\square$) $\mu\text{g ml}^{-1}$. Note the different ordinate scales in (a) and (b).

Table 3. Effect of the density of cell suspensions on miconazole-induced K^+ release from *C. albicans*

Phase of growth	Cell density (mg dry wt ml^{-1})	Miconazole ($\mu\text{g ml}^{-1}$)	MRR* [$\text{nmol min}^{-1} (\text{mg dry wt})^{-1}$]
Early-exponential (6 h)	2.0	10	0.49
	1.0	10	1.90
	0.5	10	6.40
Mid-stationary (48 h)	2.0	20	0.65
	1.0	20	1.28
	0.5	20	2.72

* MRR, Maximum rate of K^+ release, determined as $\text{nmol min}^{-1} (\text{ml buffer})^{-1}$ and converted to $\text{nmol min}^{-1} (\text{mg dry wt cells})^{-1}$.

the density of growth (Fig. 1c), so its ability to release K^+ from *C. albicans* was less, the greater the density of the cell suspension (Table 3).

Candida albicans strains 723 and 6979 also developed resistance to miconazole-induced K^+ release during the stationary phase of a shaken batch culture and suffered cell death and loss of intracellular K^+ after 3 d in culture. Similar behaviour was observed when *C. albicans* 6406 was grown in YNBG medium instead of SYG medium. These phenomena are therefore not due entirely to either the strain of organism or the growth medium used.

Increase in resistance to miconazole-induced K^+ release did not occur during the stationary phase of static cultures of *C. albicans*. The SRC for miconazole increased from about 1 $\mu\text{g ml}^{-1}$ for cells from exponential phase static cultures to about 10 $\mu\text{g ml}^{-1}$ at the end of the growth phase (2 to 3 d incubation). There was then no change in SRC and no cell death in cultures for up to 18 d (longer periods were not studied), both in contrast to shaken batch cultures.

Sensitivity of *C. albicans* to miconazole-induced K^+ release during the exponential phase of shaken batch cultures depended on the culture volume. All cultures were inoculated with cells from an early-stationary phase culture with an SRC of about 10 $\mu\text{g ml}^{-1}$. In 100 ml cultures, there was a decrease in the SRC to about 1 $\mu\text{g ml}^{-1}$ during exponential growth, followed by an increase to about 10 $\mu\text{g ml}^{-1}$ again at the end of the growth phase. However, in 1 l cultures, the SRC did not change significantly during growth, remaining at about 10 $\mu\text{g ml}^{-1}$ throughout. The increase in SRC which occurred during the stationary phase was independent of culture volume.

DISCUSSION

The action of miconazole on growth and K^+ release from *C. albicans* occurred without a delay, suggesting first that membrane damage was more likely to have been due to a direct interaction of miconazole with the plasma membrane than to an effect on ergosterol synthesis (particularly since K^+ release is measured in buffer which does not support growth), and second that growth inhibition was likely to have been a consequence of the leakage of intracellular contents induced by miconazole. The inverse relationship between cell density and miconazole-induced K^+ release was not due to metabolism to inactive product(s), since Van den Bossche *et al.* (1975) recovered [3H]miconazole entirely unchanged from *C. albicans*. It was probably a result of miconazole binding to the cell wall, thus depleting the buffer of miconazole, while not reaching the plasma membrane (Cope, 1980). The greater the effect of miconazole on growth of exponential phase cultures the earlier it was added was likely to have been due partly to a similar cell density effect, although other factors, e.g. pH and release of secondary metabolites, may also have affected growth inhibition in the constantly changing environment of the batch culture.

The action of Mg^{2+} in protecting *C. albicans* from miconazole-induced growth inhibition and viability loss might have been due either to an equilibration with intracellular Mg^{2+} , thus preventing its depletion from the cell, or to competition between Mg^{2+} and miconazole in its positively charged form (pK_a for the imidazole group is 6.65) for negatively charged sites within the cell. Although the former adequately explains a similar protective action of a mixture of Mg^{2+} and K^+ against polyene action on yeasts (Liras & Lampen, 1974; Kerridge *et al.*, 1976a), it is unlikely in the present context, since miconazole causes more indiscriminate membrane damage than the polyenes, resulting in the release of small molecules and ultraviolet-absorbing materials as well as ions (Swamy *et al.*, 1974). The effect of divalent cations may play a role in the variation in sensitivity of *C. albicans* to miconazole when incubated in different growth media (Swamy *et al.*, 1974; Van den Bossche *et al.*, 1975; Hoepflich & Huston, 1976).

Higher concentrations of miconazole were required to kill *C. albicans* than to inhibit growth, suggesting that additional action is involved. Support for this is provided by the ultrastructural studies of De Nollin & Borgers (1974, 1976) which showed more widespread damage to the intracellular structure of *C. albicans* at fungicidal than at fungistatic concentrations. Miconazole killed *C. albicans* under conditions where there was no growth, i.e. after washing and resuspension in buffer or when incubated at 1 °C. Although killing was slower at 1 °C, it was still too efficient to be accounted for in terms of an inhibition of sterol demethylation (Van den Bossche *et al.*, 1978) or an inhibition of synthesis of peroxidase enzymes (De Nollin *et al.*, 1977). The sterol demethylation theory has other disadvantages since (1) it does not account for the action of miconazole against Gram-positive bacteria which do not synthesize sterols, (2) external addition of demethylated sterols does not affect the minimum growth inhibitory concentration of miconazole for *C. albicans* (Yamaguchi, 1977) and (3) sterol demethylation appears not to be a vital function since mutants of *C. albicans* lacking demethylated sterols are viable, although their mean generation times are about twice that of the wild-type under the same conditions (Pierce *et al.*, 1978). The results are more consistent with a cumulative effect of miconazole due to direct interaction first with the plasma membrane, causing growth inhibition, then with intracellular membranes, leading to widespread cellular disorganization and death. The action would be limited by diffusion rather than by the rates of enzyme reactions, and inhibition of biochemical reactions would occur as a consequence of membrane disruption.

The earlier observation that *C. albicans* develops a resistance to miconazole-induced K^+ release during the stationary phase of a batch culture (Kerridge *et al.*, 1976b) has been confirmed, although it was not possible to test whether there was a similar effect on miconazole-induced killing since the static cultures used for viability determination did

not develop resistance to miconazole-induced K^+ release during the stationary phase. This phenotypic miconazole resistance may account for the lesser effect of miconazole on growth inhibition when added at the start of a batch culture (cells still in stationary phase) than when added during exponential growth (Fig. 1*b*).

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