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# Heterogeneity of Action Mechanisms Among Antimycotic Imidazoles

INDER JIT SUD AND DAVID S. FEINGOLD*

*Department of Dermatology, Tufts-New England Medical Center, and Department of Medicine (Dermatology), Boston Veterans Administration Medical Center, Boston, Massachusetts 02130*

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The three imidazole antimycotics clotrimazole, miconazole, and ketoconazole all inhibit the demethylation of lanosterol to ergosterol, resulting in inhibition of growth of *Saccharomyces cerevisiae*; this is a fungistatic action. At higher concentrations clotrimazole and miconazole are fungicidal, whereas ketoconazole is not. The fungicidal action reflects direct membrane damage by the imidazoles. Evidence for this is that ketoconazole is markedly less active than the other imidazoles in its ability to allow methylene blue entry into cells and to disrupt liposome model membranes. The possible clinical significance of these findings is discussed.

The antimycotic imidazoles clotrimazole and miconazole inhibit susceptible fungi by two mechanisms (I. J. Sud and D. S. Feingold, J. Invest. Dermatol., in press). At low concentrations they block demethylation of lanosterol to the major fungal sterol ergosterol (6, 9, 12, 14). Fungistatic concentrations of clotrimazole and miconazole increase lanosterol/ergosterol ratios in *Saccharomyces cerevisiae* (Sud and Feingold, in press). Accumulation of the bulky lanosterol molecule is likely responsible for the growth inhibition (10). Under strictly anaerobic conditions, *S. cerevisiae* cannot synthesize sterols and will not grow unless a sterol and an unsaturated free fatty acid are added to the medium (1). Miconazole and clotrimazole have a sharply elevated minimal inhibitory concentration (MIC) for the yeast growing anaerobically under these conditions. At a concentration of the imidazoles 30 to 60 times the usual aerobic MIC, however, these imidazoles are fungicidal both aerobically and anaerobically (Sud and Feingold, in press). The fungicidal action is distinct from the fungistatic one described above. At higher concentrations the imidazoles are rapidly fungicidal as evidenced by viable counts and the entry of methylene blue into the cells within minutes of drug addition (Sud and Feingold, in press). It is likely that the locus of the immediate lethal effect of these imidazoles is on fungal membranes, and this is further reflected in the liposome disruptive ability of the imidazoles (13, 17), especially prominent when the liposome model membranes are rich in free unsaturated fatty acids.

We refer to the low-concentration, fungistatic effect on sterol synthesis as the sterol synthesis inhibition and to the rapid fungicidal action as direct membrane damage (DMD). When we examined the action of the new imidazole ketoconazole in the systems described above a remarkable difference was seen. Against *S. cerevisiae* ketoconazole has a potent sterol synthesis inhibition action, but showed little or no DMD effect. This fact may have important clinical ramifications as will be discussed.

## MATERIALS AND METHODS

**Organism and growth conditions.** *S. cerevisiae* ATCC 287 was grown aerobically and anaerobically as we have described in detail (Sud and Feingold, in press). The broth (pH 7.0) cultures were incubated at 34°C. For anaerobic cultures, the broth was supplemented with ergosterol (20 μg/ml) and Tween 80 (10 μg/ml).

**Imidazole susceptibility.** Serial twofold dilutions of the imidazoles were employed to determine the MICs. The drugs examined were ketoconazole (Janssen R&D Inc.), miconazole (Johnson and Johnson Inc.) and clotrimazole (Delbay Pharmaceuticals, Inc.). All the imidazoles were used as the free base. The inoculum size was about $10^3$ organisms per ml. The MIC was the lowest concentration of the antimicrobial agent causing inhibition as judged by the absence of turbidity. Stock solutions of the imidazoles were made in dimethyl sulfoxide; the concentration of this solvent when added to broth was always 1% or less.

**Lanosterol/ergosterol ratios.** For studies of the sterol composition, flasks containing broth with various concentrations of drugs were inoculated at $10^4$ cells per ml and then incubated aerobically for 24 h. Cultures showing turbidity were processed further for isolation and determination of sterol composition as described elsewhere (Sud and Feingold, in press).

Briefly, the cells were treated with 5% trichloroacetic acid, washed, and then saponified with methanolic KOH. The petroleum ether extracts were then subjected to gas-liquid chromatography to determine ergosterol and lanosterol content of cells.

**Effect of imidazoles on viability.** Broths containing various concentrations of the imidazoles were inoculated with a log-phase culture to give a starting population of approximately $10^3$ organisms per ml. The cultures were shaken aerobically at 34°C, and samples taken over a 9-h time span were plated on agar plates after appropriate dilutions for determination of viable counts.

**Methylene blue uptake.** Details of the method used to determine methylene blue uptake have been described in detail (Sud and Feingold, in press). Briefly, the drug-treated cells were exposed to aqueous methylene blue, and the percentage of uniformly stained cells was determined microscopically. Control experiments showed that cells killed either by boiling for 5 min or by exposure to formaldehyde were 100% stained, whereas fewer than 3% of cells from growing cultures were stained.

**Liposome preparation and testing.** Liposomes were prepared and their imidazole susceptibility was tested as previously described by us (7). The liposomes contained phosphatidylcholine, dicetyl phosphate, and oleic acid in molar ratios of 60:10:30. Glucose was used as a marker molecule. Liposomes were exposed to various drug concentrations for 1 h at room temperature. Glucose released was measured enzymatically by a modification (13) of the method described by Kinsky et al. (8).

## RESULTS

Table 1 shows the MICs for *S. cerevisiae* for the three tested imidazoles aerobically and anaerobically. Ketoconazole had an MIC similar to that of clotrimazole under aerobic conditions; miconazole was more potent in this system. However, the anaerobic MIC value was very different for ketoconazole than for the others (Table 1). There was growth of *S. cerevisiae* at the highest ketoconazole concentration employed, 200 μg/ml ($3.8 \times 10^{-4}$ M). This finding suggests that ketoconazole has a very weak or no DMD action on *S. cerevisiae* since under these anaerobic conditions DMD is the only operative antifungal mechanism (Sud and Feingold, in press).

As with the other imidazoles, ketoconazole at subinhibitory levels also caused an increase in the lanosterol/ergosterol ratio. In Table 2 results with ketoconazole are compared with those we have already reported for clotrimazole and miconazole (Sud and Feingold, in press). At $3 \times 10^{-7}$ M ketoconazole the ratio was 0.92, whereas control cells had a ratio of 0.05. Increase of the ratio was first detected at $3 \times 10^{-8}$ M ketoconazole. It seems clear that ketoconazole like clotrimazole and miconazole blocks demethylation of lanosterol to ergosterol at low concentrations; very likely this is an important mechanism in vivo as well as in vitro (14).

Table 3 shows the comparative effects of ketoconazole and miconazole on methylene blue uptake by *S. cerevisiae* as a function of imidazole concentration. There was very little methylene blue staining caused by ketoconazole even at a $10^{-3}$ M concentration. Methylene blue staining induced by miconazole began at $10^{-5}$ M and approached 100% by $10^{-4}$ M. This correlates quite well with the lethal effect of miconazole. Results with clotrimazole were similar to those with miconazole (Sud and Feingold, in press).

Figure 1 shows the striking differences in the effect of miconazole, clotrimazole, and ketoconazole on viable counts of *S. cerevisiae* as a function of drug concentration and time. Miconazole and clotrimazole showed prompt fungicidal action at $10^{-4}$ M and above under these conditions. As shown in the figure ketoconazole caused no killing even at $10^{-3}$ M. Growth inhibition with increasing ketoconazole concentrations began at $3.3 \times 10^{-7}$ M and was prompt and complete at

TABLE 2. *Lanosterol/ergosterol ratios with growth of S. cerevisiae aerobically at the indicated concentrations of the imidazole*

| Imidazole concentration (M) | Lanosterol/ergosterol ratio in cells grown in the presence of[a]: | | |
|---|---|---|---|
| | Miconazole | Clotrimazole | Ketoconazole |
| 0 | 0.09 | 0.10 | 0.05 |
| $1 \times 10^{-10}$ | 0.09 | 0.08 | ND |
| $1 \times 10^{-9}$ | 0.23 | 0.08 | 0.05 |
| $3 \times 10^{-9}$ | ND | 0.10 | ND |
| $1 \times 10^{-8}$ | 0.52 | 0.13 | 0.04 |
| $3 \times 10^{-8}$ | 1.15 | 0.76 | 0.12 |
| $1 \times 10^{-7}$ | NG | 1.56 | 0.52 |
| $3 \times 10^{-7}$ | ND | NG | 0.92 |
| $1 \times 10^{-6}$ | ND | ND | NG |

[a] Abbreviations: ND, not done; NG, no growth.

TABLE 1. *MICs of miconazole, clotrimazole, and ketoconazole for S. cerevisiae*

| Growth condition | MIC (μg/ml, M) of: | | |
|---|---|---|---|
| | Miconazole | Clotrimazole | Ketoconazole |
| Aerobic | 0.097, $2.0 \times 10^{-7}$ | 0.39, $1.1 \times 10^{-6}$ | 0.39, $7.4 \times 10^{-7}$ |
| Anaerobic | 12.5, $2.6 \times 10^{-5}$ | 12.5, $3.6 \times 10^{-5}$ | >200[a], $>3.8 \times 10^{-4}$ |

[a] Highest ketoconazole concentration tested.

TABLE 3. *Methylene blue staining of imidazole-treated S. cerevisiae*

| Imidazole concn (M) | % of cells stained[a] | |
|---|---|---|
| | Miconazole | Ketoconazole |
| 0 | 1 | 2 |
| $10^{-6}$ | 4 | 1 |
| $10^{-5}$ | 32 | 3 |
| $10^{-4}$ | 97 | 8 |
| $10^{-3}$ | NT | 11 |

[a] Cells were exposed to imidazole for 1 h. Subsequently they were exposed to methylene blue and examined microscopically in a Petroff-Hausser chamber; 200 cells were counted. NT, Not tested.

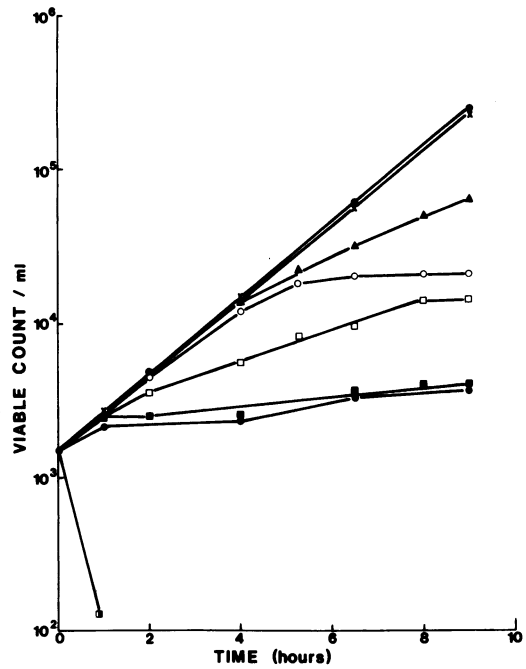


FIG. 1. *Effect of ketoconazole, miconazole, and clotrimazole on growth of S. cerevisiae. Imidazoles were added to growing cultures at time 0. Growth was measured by plating dilutions of cultures. Symbols: control (●); ketoconazole at 1 × $10^{-7}$ M (×); 3.3 × $10^{-7}$ M (▲); 1 × $10^{-6}$ M (○); 1 × $10^{-5}$ M (□); 1 × $10^{-4}$ M (■); 1 × $10^{-3}$ M (◎); clotrimazole and miconazole at 1 × $10^{-4}$ M (◨).*

$10^{-4}$ M. However, under no conditions we tested was it fungicidal.

Figure 2 compares the three imidazoles in their effect on liposomes. The liposomes used consisted of phosphatidyl choline-dicetyl phosphate-oleic acid in molar ratios of 60:10:30. Such liposomes were shown previously to be very susceptible to miconazole and clotrimazole (13). In the experiments shown in the figure, similar results for marker release as a function of the imidazole concentration were seen with miconazole and clotrimazole. Ketoconazole caused much less marker release in the system at all concentrations tested up to 100 μg/ml.

## DISCUSSION

Miconazole and clotrimazole have at least two distinct antifungal mechanisms, sterol synthesis inhibition and DMD (Sud and Feingold, in press). Ketoconazole differs fundamentally from clotrimazole and miconazole in that it has very weak or no DMD action when compared to the others. This is reflected in a lack of fungicidal action of ketoconazole (Fig. 1), an absence of an effect of ketoconazole anaerobically (Table 1), and inability of ketoconazole to foster methylene blue entry into *S. cerevisiae* (Table 3).

Ketoconazole is being hailed as "a major innovation for treatment of fungal disease" (3). Unlike other imidazoles it gives substantial blood levels after oral administration, and toxicity observed so far is minimal (2, 3, 5, 11). It may be that the DMD effect of the imidazoles in mammalian cells is responsible for imidazole toxicity in humans and, hence, the apparent minimal toxicity of ketoconazole versus miconazole in the early clinical studies.

The dual mechanism of action of clotrimazole and miconazole might explain the extremely rare development of clinically important fungal resistance to clotrimazole and miconazole. Our experience also is that it is extremely difficult to isolate imidazole resistant strains (unpublished observation). Since ketoconazole lacks DMD action the emergence of resistant strains in clinical settings may present a greater problem with this imidazole than with clotrimazole or miconazole.

The absence of a rapid fungicidal action of ketoconazole may also present a problem, especially during long-term treatment of fungal in-

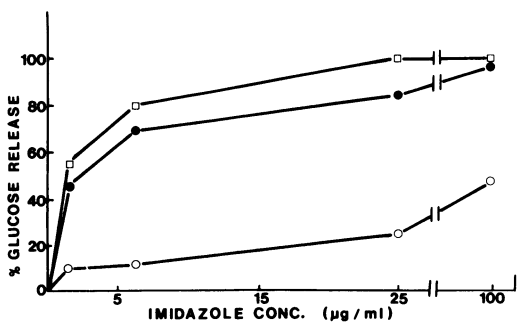


FIG. 2. *Effect of ketoconazole* (○), *miconazole* (●), *and clotrimazole* (□) *on glucose marker release from liposomes prepared from egg phosphatidylcholine, dicetyl phosphate, and oleic acid (molar ratio, 60:10:30).*

fections in compromised hosts in whom a lethal antifungal effect is important theoretically. It is true that the terms "cidal" and "static" are relative ones. For example, drugs that in themselves do not cause a lethal action might set in motion biochemical events that result in cell death. The penicillins are an example. The action of certain penicillins on the cell wall results in activation of destructive lytic enzymes in most susceptible organisms, but not in all. Williams et al. (16) and Graybill et al. (4) reported that ketoconazole has a lethal effect on *Cryptococcus neoformans* after prolonged incubation (18 to 72 h). Thus, it may be that ketoconazole ultimately causes cell death in some fungi, but this is not the case with *S. cerevisiae* under all conditions in which we tested the drug.

Demonstration of the heterogeneity in activities among the imidazoles should make possible study of structure-function relationships among them. Design of optimal imidazoles for various clinical situations may be possible. Ketoconazole appears to be an important step forward in antifungal therapy, but there is likely room for improvement.

## ACKNOWLEDGMENTS

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