

Action of Antifungal Imidazoles on *Staphylococcus aureus*

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In *Staphylococcus aureus*, using the imidazoles miconazole and ketoconazole, detailed studies of minimal inhibitory concentrations, kinetics of growth, viability, and release of intracellular K^+ confirm that the two imidazoles work differently in this bacterium. Miconazole is bactericidal at low concentrations and causes release of cellular K^+ . Ketoconazole has no bactericidal effect at any tested concentration and has little effect on K^+ permeability of *S. aureus*; it slows growth at high concentration. This is reflected in a low minimal inhibitory concentration for miconazole and a high one for ketoconazole. The probable mechanisms of the bacteriostatic and bactericidal effects of the imidazoles are discussed in light of these results and the previously described antifungal mechanisms of the drugs. α -Tocopherol blocks the action of both imidazoles.

The imidazole antifungal agents are active against gram-positive bacteria (6, 12). It is generally believed that the major antifungal mechanism of the imidazoles is interference with ergosterol synthesis (7, 14). Since sterols are not an important class of compounds in bacteria, the antibacterial action of the imidazoles is unexplained. We have examined in detail the action of the imidazoles miconazole and ketoconazole on gram-positive bacteria.

In *Saccharomyces cerevisiae*, we have previously shown that miconazole and ketoconazole differ in their actions (8). At low concentrations, both antimicrobial agents interfere with one or more steps in the oxidative demethylation of lanosterol to ergosterol; this action is fungistatic. At higher concentrations, miconazole causes membrane damage which is fungicidal; ketoconazole does not share this activity (8, 9). Our studies of the action of the two imidazoles on *Staphylococcus aureus* show that miconazole and ketoconazole also differ in their effects on this organism. Miconazole is bactericidal at low concentrations. Ketoconazole has no bactericidal action and is only bacteriostatic at high concentrations.

MATERIALS AND METHODS

Organism and growth conditions. *S. aureus*, a clinical isolate, was grown in tryptic soy broth (Difco Laboratories) at 37°C, shaking on a rotary water bath shaker. α -Tocopherol was added as D- α -tocopherol acid succinate (Sigma Chemical Co.).

Imidazole susceptibility. Minimum inhibitory concentrations (MICs) of the imidazoles toward *S. aureus* were determined by employing serial twofold dilutions of drugs in broth. The imidazoles examined were

ketoconazole (Janssen R&D, Inc.) and miconazole (Ortho Pharmaceutical Corp.). Stock solutions of drugs were made in dimethyl sulfoxide; the final concentration of this solvent when added to liquid media was 1% or less. The tubes were inoculated at 2×10^3 cells ml^{-1} and incubated for 24 h. The MIC was the lowest concentration of the drug causing inhibition of turbid growth. These conditions were strictly followed.

MICs toward various gram-positive cocci were done by using serial 10-fold imidazole dilutions from 0.1 to 100 μg ml^{-1} . The following gram-positive clinical isolates were examined: *S. aureus*, *S. epidermidis*, *Streptococcus pyogenes*, *Diplococcus pneumoniae*, *Bacillus cereus*, and group D streptococci.

Growth kinetic experiments. The effect of imidazoles on growth was determined by exposing logarithmically growing cultures with about 10^4 cells ml^{-1} to various concentrations of drugs. Viable counts were determined in duplicate at intervals by plating appropriate dilutions of cultures on agar plates; the plates were incubated for 24 h before enumeration of colonies.

Viability studies. Actively growing cells were diluted into potassium phosphate buffer (0.1 M; pH 7.0) to give 2×10^3 cells ml^{-1} and then exposed to various concentrations of imidazoles. After 3 h, 0.1-ml samples were plated on agar plates; the number of viable cells was determined by counting colonies after 24 h of incubation at 37°C.

Measurement of release of K^+ . K^+ release was determined by using a K^+ -specific electrode as previously described (1). An *S. aureus* culture in early stationary phase growth was washed twice with Tris-hydrochloride buffer (0.05 M; pH 7.4), and the cells were suspended in the same buffer at a density of 5×10^9 colony-forming units ml^{-1} . After addition of the imidazole to 3 ml of cell suspension, K^+ release from the bacteria (extracellular K^+) was determined at intervals during a 2-h incubation at room temperature. Total cellular K^+ was measured after a cell suspension was incubated for 30 min in a boiling water bath. The

K^+ released was calculated in terms of percentage of total cellular K^+ released by using the formula [(extracellular K^+ in sample at indicated time) - (extracellular K^+ in control at time 0)/(total cellular K^+)] \times 100.

Fatty acid composition. A growing culture of *S. aureus* at a cell concentration of about 10^6 ml $^{-1}$ was exposed to ketoconazole ($100 \mu\text{g ml}^{-1}$) for 6 h. The cells were harvested by centrifugation, washed twice with distilled water, and saponified for isolation of free fatty acids, followed by gas-liquid chromatography of methyl esters as described previously (11). Hydrogenation of methyl esters was performed in methanol by bubbling hydrogen in the presence of platinum oxide. The branched-chain fatty acid esters were identified by comparison with a standard mixture from Supelco, Inc. (GLC-110). Quantitative measurements were done by triangulation of peaks.

RESULTS

MICs. Group D streptococci were resistant to both imidazoles at a concentration of $100 \mu\text{g ml}^{-1}$ (Table 1). All other gram-positive bacteria were susceptible to $10 \mu\text{g}$ of miconazole or less per ml. In all cases, the MICs were at least 10-fold higher for ketoconazole.

The MIC of miconazole for the *S. aureus* isolate was $1.56 \mu\text{g ml}^{-1}$, with a sharp endpoint. Under the same conditions, the MIC for ketoconazole was $25 \mu\text{g ml}^{-1}$. Growth in the presence of $100 \mu\text{g}$ of α -tocopherol per ml raised the MIC for both imidazoles to over $100 \mu\text{g ml}^{-1}$.

Growth studies. Figures 1 and 2 show the growth of *S. aureus* in the presence of various concentrations of miconazole and ketoconazole. Miconazole (Fig. 1) had no effect on the growth rate of the organism at 0.01 and $0.1 \mu\text{g ml}^{-1}$. The doubling time was 30 min. In $1.0 \mu\text{g}$ of miconazole per ml, growth inhibition was obvious after a few hours of incubation; however, by 24 h, the culture achieved stationary phase. At concentrations of 10 and $100 \mu\text{g ml}^{-1}$, rapid killing of the inoculum was seen.

With ketoconazole, the results of the same experiment were strikingly different (Fig. 2). At 0.01 , 0.1 , 1.0 , and $10 \mu\text{g ml}^{-1}$, the doubling time of 30 min was the same as that of the control. By

TABLE 1. Imidazole susceptibility of some gram-positive isolates

Organism	MIC ($\mu\text{g ml}^{-1}$) ^a	
	Miconazole	Ketoconazole
<i>Staphylococcus aureus</i>	10	100
<i>Staphylococcus epidermidis</i>	0.1	10
<i>Streptococcus pyogenes</i>	10	100
<i>Streptococcus pneumoniae</i>	10	100
Group D streptococci	100	100
<i>Bacillus cereus</i>	10	100

^a Serial 10-fold dilutions were done for these MICs.

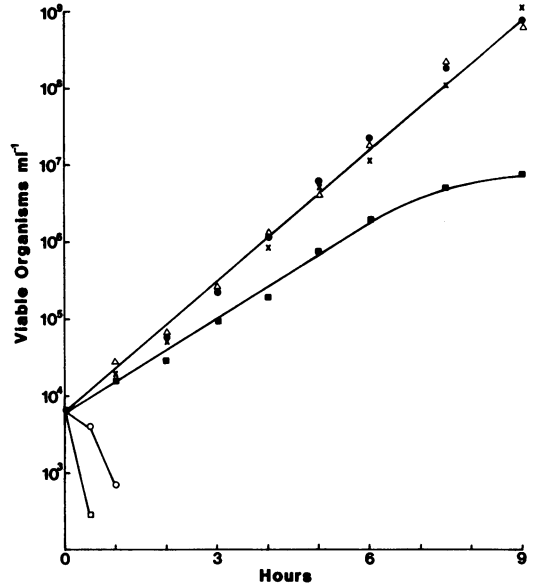


FIG. 1. The indicated concentrations of miconazole were added to growing *S. aureus* cultures. At intervals, duplicate samples were taken for viable counts. Symbols: control (●); $0.01 \mu\text{g ml}^{-1}$ (×); $0.1 \mu\text{g ml}^{-1}$ (Δ); $1.0 \mu\text{g ml}^{-1}$ (■); $10 \mu\text{g ml}^{-1}$ (○); $100 \mu\text{g ml}^{-1}$ (□).

4 h of incubation, it was clear that ketoconazole at $100 \mu\text{g ml}^{-1}$ caused inhibition of the growth of *S. aureus*; however, at 24 h, growth was heavy even in this flask. The inoculum size in these experiments was 10 times that for the MIC determination, which explains growth of *S. au-*

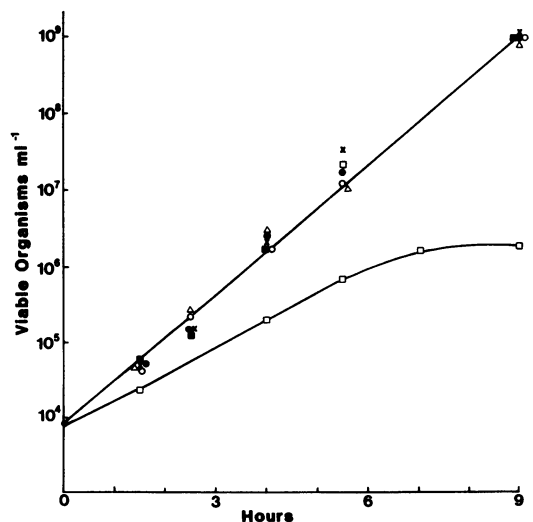


FIG. 2. This experiment is identical to that depicted in Fig. 1, except that ketoconazole was used in lieu of miconazole.

res at $100 \mu\text{g ml}^{-1}$ and a reported MIC of $25 \mu\text{g ml}^{-1}$.

Viability studies. To look more carefully at the lethal effect of the imidazoles on *S. aureus*, the experiments tabulated in Table 2 were performed. After 3 h of exposure to miconazole, extensive killing was seen at $6.25 \mu\text{g}$ of miconazole per ml, some killing was observed at 3.12 and $1.56 \mu\text{g ml}^{-1}$, and no killing was observed below a miconazole concentration of $1.56 \mu\text{g ml}^{-1}$. In the presence of lethal miconazole concentrations, killing began within minutes. Even at $100 \mu\text{g}$ of ketoconazole per ml, no drop in viability of the inoculum was seen after 3 h of incubation in buffer.

Imidazole-induced K^+ release. Figure 3 shows the leakage of cellular K^+ induced by various concentrations of miconazole and ketoconazole from *S. aureus*; the data shown are averages of two experiments. Miconazole, at 100 and $10 \mu\text{g ml}^{-1}$, caused a steady release of K^+ ; the rate of release was linear with time for about 1 h, by which time well over 50% of the intracellular K^+ had leaked out. At $1 \mu\text{g}$ of miconazole per ml, the rate of release was slow; about 30% of the cellular K^+ leaked out in 2 h, compared with 10% in the control cells. Lower concentrations of miconazole had no effect. Ketoconazole was much less effective in causing K^+ leakage; about 20% of the cellular K^+ was released at a concentration of $100 \mu\text{g ml}^{-1}$. Lower concentrations of the drug were without any effect on K^+ leakage (data not shown).

Effect of Ketoconazole on cellular fatty acids. As reported in the literature (4), the fatty acids in *S. aureus* consisted mainly of branched-chain saturated acids, with $\text{C}_{15:0}$ anteiso as the major acid (41%); smaller amounts of straight-chain saturated fatty acids ($\text{C}_{14:0}$, $\text{C}_{16:0}$, and $\text{C}_{18:0}$) were also present (Table 3). Unsaturated fatty acids were not detected. The fatty acid profile

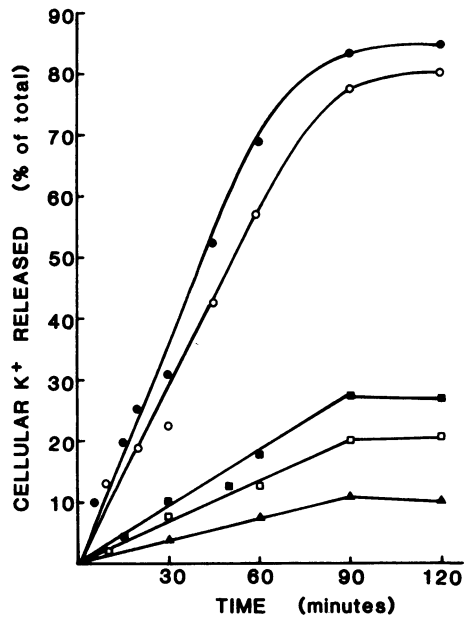


FIG. 3. Imidazole-induced K^+ release from *S. aureus*. Symbols: miconazole at 100 (●), 10 (○), and 1.0 (■) $\mu\text{g ml}^{-1}$; ketoconazole at $100 \mu\text{g ml}^{-1}$ (□); and no imidazole (△).

remained unchanged after hydrogenation. There were two unidentified fatty acid peaks, with the last peak in the profile comprising 14% of the total fatty acids. Qualitatively, the ketoconazole-treated cells contained similar fatty acids. However, there was a twofold increase in the amount of the slow-migrating unknown fatty acid paralleled by an almost similar decrease in the amount of $\text{C}_{15:0}$ anteiso fatty acid. Only minor changes in the amounts of other fatty acids were noted.

DISCUSSION

It has been demonstrated previously that the imidazole antimycotics inhibit fungal growth by interfering with the demethylation of lanosterol to ergosterol, the major fungal sterol (8, 9, 14). This action on *S. cerevisiae* or *Candida albicans* or both has been observed with all imidazoles tested: clotrimazole (9), miconazole (8), miconazole nitrate (14), and ketoconazole (8, 13). In addition to this fungistatic action, at higher concentrations clotrimazole and the miconazoles are rapidly fungicidal toward these organisms (8). Our studies suggest that the fungicidal action is separate from the sterol synthesis inhibition and represents direct membrane damage caused by the imidazoles. Ketoconazole differs from the other three imidazoles in that although it shares with them the action on sterol synthesis, it does not cause membrane damage and is

TABLE 2. Effect of 3-h exposure to ketoconazole and miconazole on viability of *S. aureus* in buffer

Imidazole concn ($\mu\text{g ml}^{-1}$)	No. of colonies ^a	
	Miconazole	Ketoconazole
0.19	199	183
0.39	187	194
0.78	206	198
1.56	69	212
3.12	51	206
6.25	1	194
12.5	3	203
25	0	187
50	0	219
100	ND ^b	214

^a Average of duplicate viable counts.

^b ND, Not done.

TABLE 3. Effect of ketoconazole on fatty acid composition of *S. aureus*

<i>S. aureus</i> cells	% of total fatty acid							
	C _{14:0} iso	C _{14:0}	C _{15:0} anteiso	3.6 ^a	C _{16:0}	C _{17:0} anteiso	C _{18:0}	15.3 ^a
Untreated	3.4	7.7	40.9	Tr ^b	12.2	4.8	16.8	14.0
Ketoconazole treated	5.4	11.7	24.5	1.3	12.5	2.1	13.9	28.5

^a Unknown acid; retention time given in minutes.

^b Tr, Trace.

not fungicidal for *S. cerevisiae* or *C. albicans*, even at 100 µg ml⁻¹ (2×10^{-4} M) (8).

Sterols are not important molecules biochemically or structurally in bacteria. Thus, one would not expect the imidazoles to have bacteriostatic action against *S. aureus*. One would expect bactericidal action if the bacterial plasma membrane were susceptible to direct membrane damage by the imidazole, as is the case with miconazole in fungi (8, 9). That miconazole causes membrane damage in *S. aureus* is strongly suggested by the K⁺ release experiments (Fig. 3); extensive loss of cellular K⁺ occurred after treatment with 10 µg of miconazole per ml. Miconazole-induced K⁺ release has been reported also for *C. albicans* (2). Ketoconazole, on the other hand, had very little effect on the membrane permeability of K⁺; at the highest concentration tested (100 µg ml⁻¹), only an additional 10% of the total K⁺ was lost in 2 h compared with the control cells. This correlates with the finding that ketoconazole has no lethal action on *S. aureus*.

Ketoconazole, however, does have a bacteriostatic action on *S. aureus*, as evidenced by slowing of growth with 100 µg ml⁻¹ (Fig. 2) and an MIC of 25 µg ml⁻¹. The Janssen group has shown that at higher concentrations of imidazole than those effecting sterol synthesis in *C. albicans*, the composition of fatty acids is altered; C_{18:2} content increases at the expense of C_{18:1}, and saturated fatty acid levels increase (14). *S. aureus* lacks unsaturated fatty acids. The major changes caused by bacteriostatic levels of ketoconazole were a decreased level of C_{15:0} anteiso and an increased level of one of the two unknown fatty acids. No significant changes in straight-chain saturated fatty acids were seen. Qualitatively, these changes were similar to those seen when *S. aureus* was grown anaerobically (unpublished data). This indirectly suggests that the effect of ketoconazole on fatty acid composition is secondary in nature.

It has previously been suggested by us (10) and others (5, 15) that unsaturated fatty acids are an important cellular target for imidazole action. In model membrane systems, the presence of increased unsaturated fatty acid levels markedly

increases the membrane damage caused by clotrimazole or miconazole (10). Our observation that there are no unsaturated fatty acids in *S. aureus* under the conditions used in the studies described herein suggests that there are other targets for imidazole membrane damage.

It is intriguing that α-tocopherol antagonizes the action of miconazole on *S. aureus*. We have previously observed that α-tocopherol also antagonizes the imidazole-induced membrane damage to liposome model membranes (unpublished data). De Nollin et al. have suggested that activation of oxidative enzymes is responsible for some of the imidazole damage in *C. albicans* (3). This would not explain the α-tocopherol effect in model membranes. Other explanations are that the membrane damage involves free radicals that are scavenged by α-tocopherol, that α-tocopherol directly inactivates the imidazoles, or that the α-tocopherol blocks the imidazole binding sites.

ACKNOWLEDGMENT

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