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Antibacterial Activity of Antimycotic Miconazole against Methicillin-Resistant *Staphylococcus aureus*

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Abstract Miconazole was estimated to have a minimum inhibitory concentration of 0.78 μg/ml against the methicillin-resistant strains of *Staphylococcus aureus* (MRSA). The antibacterial activity of miconazole against MRSA was bacteriostatic in cation-adjusted Mueller-Hinton broth, but bacteriocidal in saline solution. The anti-MRSA activity of miconazole did not show a significant change under the susceptibility-testing conditions such as various inoculum sizes, pHs, or cations (Ca^{++} or Mg^{++}) which was added to the medium. However, the anti-MRSA activity of miconazole was completely suppressed by lipophilic α-tocopherol (50 μg/ml), but definitely not by water-soluble ascorbic acid.

Key words: Miconazole, antibacterial activity, MRSA, α-tocopherol

Strains of methicillin-resistant *Staphylococcus aureus* (MRSA) were first reported in Europe in the early 1960s, shortly after the introduction of methicillin into clinical use [3, 8]. They have subsequently spread throughout the world [5], and now pose a serious problem to hospitalized patients as well as health care personnel [4, 13]. Since strains of MRSA are usually resistant to many antibiotics [1, 11], they are extremely difficult to eradicate. Although many anti-staphylococcal agents have been developed, very few agents are available to treat MRSA infections [4, 9, 10]. Therefore, it is necessary to search for new drugs with anti-MRSA activity.

Miconazole, an imidazole derivative, has been used as an antifungal drug since it has been developed in the late 1960s [6]. It was also reported to have antibacterial activity against gram-positive bacteria including *Erysipelothrix insidiosa, Streptococcus pyogens, Staphylococcus aureus, Bacillus anthracis, Staphylococcus hemolyticus,* and *Staphylococcus faecalis*. However, even at a high concentration level of 1,000 μg/ml, it did not appear to show any activity against gram-negative bacteria such as *Salmonella pullorum gallinarum, Escherichia coli, Pseudomonas aeruginosa, Pasteurella pseudotuberculosis,* and *Bordetella bronchiseptica* [18]. In contrast to the above, a gram-negative *Helicobacter pylori*, which is a causal agent of type B gastritis, was found to represent antibacterial susceptibility to miconazole in a range of 2–32 μg/ml [20]. In addition, according to the study by Sud *et al.* [17] which was performed with normal strains of *Staphylococcus aureus*, miconazole exhibited bacteriocidal activity, and α-tocopherol inhibited the antibacterial activity of miconazole completely.

In this study, we explored a new antibacterial activity of miconazole against MRSA, and in order to characterize the action, the anti-MRSA and the antifungal activities of miconazole were compared under various conditions such as inoculum sizes, medium pHs, and cations or antioxidants added to the medium.

Materials and Methods

Strains, Media, and Cultures

The gram-positive bacteria of *Staphylococcus aureus*; 12598 (normal), 25923 (normal), 11632 (penicillin-resistant), and 29247 (penicillin-resistant), 33591 (methicillin-resistant), 33592 (methicillin-resistant), and the yeast *Saccharomyces cerevisiae* 7754 were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). For measuring the antimicrobial activity, bacterial inoculum was cultured on a Nutrient Yeast Glucose (NYG) agar (0.8% nutrient broth, 0.5% yeast extract, 0.1% glucose) plate at 37°C for 16 h, whereas yeast inoculum was cultured on a Sabouraud Dextrose agar (1% bactopeptone, 4% dextrose, 1.8% agar) plate at 35°C for 24 h.

Antioxidative and Antimicrobial Compounds

Antioxdants, ascorbic acid and α-tocopherol, and antimicrobials, miconazole, methicillin, penicillin G, and

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tetracycline, were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). All antimicrobial compounds with an exception of methicillin were first dissolved at 100-fold in dimethyl sulfoxide and further diluted 2-fold with the same solvent. They were also diluted 10-fold with the broth medium needed for the antimicrobial test. Because of its solubility, methicillin was dissolved in distilled water and sterilized by membrane filtration.

Determination of MICs

Antibacterial and antifungal activities of compounds were tested according to M7-A3 [14] and M27-T [15] methods approved by The National Committee for Clinical Laboratory Standards (NCCLS), respectively. Briefly, a diluent (0.3 ml) of the compound was added to the broth medium (3 ml) containing fresh inoculum, and it was then cultured without shaking. For the antibacterial test, a cation-adjusted Mueller-Hinton broth (CAMHB) (BBL, Cockeysville, MD, U.S.A.), with an inoculum size of 10^5 CFU/ml and 37°C incubation temperature was used whereas an RPMI 1640 (Sigma) broth, with an inoculum size of 10^3 CFU/ml and 35°C incubation temperature, was employed for the antifungal test. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that showed no visible microbial growth after 48 h of incubation. Assays were performed in triplicate for each experiment.

Time Killing Curve

Time killing studies of miconazole against the methicillin-resistant *S. aureus* were performed in CAMHB medium and saline solution. The culture tubes with an appropriate concentration of miconazole and an inoculum (5.8×10^7 CFU/ml) were carefully prepared as described above. Samples were taken at the indicated time intervals, and 0.1 ml of the samples was plated onto the NYG agar plates after serial dilutions were made in a sterile saline. These plates were incubated at 37°C for 2 days and the number of CFU was determined.

Results and Discussion

Antibacterial Activity of Miconazole against the Antibiotic-Resistant Strains of *S. aureus*

The antibacterial activity of miconazole was estimated against the various kinds of *S. aureus* strains with the M7-A3 method [14] of NCCLS (Table 1). Against the normal (antibiotic-unresistant) strains, miconazole exhibited nearly identical antibacterial activity to methicillin, although it was somewhat inferior compared to penicillin G and tetracycline. In addition, miconazole revealed an MIC of 3.13 μg/ml against the penicillin-resistants. As reported previously [1, 11], clinical antibiotics including methicillin, penicillin G, and tetracycline exhibited no effect against the methicillin-resistant *S. aureus* (MRSA), even at a high concentration of 100 μg/ml. However, miconazole inhibited the growth of MRSA completely at a low concentration of 0.78 μg/ml. Thus, it is likely that miconazole exhibited strong antibacterial activity against the normal strains of *S. aureus* as shown previously [18, 17]. In addition, antimycotic miconazole is thought to have strong antibacterial activity against not only the penicillin-, but also the methicillin-resistant (MRSA) strains of *S. aureus*.

Effect of Test Conditions on the Anti-MRSA Activity of Miconazole

The antimicrobial activities of miconazole against methicillin-resistant *S. aureus* (MRSA) and *S. cerevisiae* have been investigated under various conditions such as inoculum sizes, medium pHs, and cations (Table 2). Initially, the MICs of miconazole were estimated after adjusting the inoculum size to 10^3 CFU/ml, 10^5 CFU/ml, 10^7 CFU/ml. The MIC values against MRSA did not change more than 4-fold, but the MICs against *S. cerevisiae* were greatly enhanced with increasing of the inoculum size, as expected [7]. Secondly, the MICs of miconazole were estimated after adjusting the pH of the medium to 3, 5, and 7 with 0.2 M MOPS buffer. The anti-MRSA activity was similar

Table 1. Antibacterial activity of miconazole against antibiotic-resistant strains of *S. aureus*.

S. aureus strains	MIC (μg/ml)*			
	Miconazole	Methicillin	Penicillin G	Tetracycline
Normal (unresistant)				
ATCC 12598	3.13	3.13	0.10	0.39
ATCC 25923	3.13	1.56	0.10	0.78
Penicillin-resistant				
ATCC 11632	3.13	3.13	100	0.78
ATCC 29247	3.13	3.13	100	25
Methicillin-resistant				
ATCC 33591	0.78	>100	>100	>100
ATCC 33592	0.78	>100	>100	>100

*The minimum inhibitory concentrations (MIC) of compounds were determined using the macrodilution broth method (M7-A3) of NCCLS.

Table 2. Effect of several conditions on the antimicrobial activities of miconazole against methicillin-resistant *S. aureus* (MRSA) and *S. cerevisiae*.

Conditions		MIC (μg/ml)	
		MRSA	*S. cerevisiae*
Inoculum (CFU/ml)	10^3	0.78	0.03
	10^5	0.78	6.25
	10^7	1.56	50
pH	3	ND	1.56
	5	1.56	0.10
	7	0.78	0.03
Cation (10 mM)	None	0.78	0.03
	Ca^{++}	0.78	25
	Mg^{++}	0.78	25

*The anti-MRSA and the antifungal activities of miconazole were tested against *S. aureus* ATCC 33591 and *S. cerevisiae* ATCC 7754. ND, not determined.

Table 3. Effect of antioxidants on the antimicrobial activities of miconazole against methicillin-resistant *S. aureus* (MRSA) and *S. cerevisiae*.

Antioxidants (μg/ml)		MIC (μg/ml)	
		MRSA	*S. cerevisiae*
α-Tocopherol	0	0.78	0.03
	25	12.5	0.03
	50	>100	0.03
	100	>100	0.39
Ascorbic acid	0	0.78	0.03
	25	0.78	0.03
	50	0.78	0.03
	100	0.78	0.03

*The anti-MRSA and the antifungal activities of miconazole were tested against *S. aureus* ATCC 33591 and *S. cerevisiae* ATCC 7754.

at both pHs, where the test organism can grow. In addition, the antifungal activity was greatly lowered by decreasing of medium pH as reported by other investigators [21]. Finally, after adding Mg^{++} or Ca^{++} ions at 10 mM (final concentration) to the medium, the antimicrobial activities of miconazole were estimated. The MICs against MRSA were determined at the concentration of 0.78 μg/ml, regardless of adding cations. However, as shown previously [2], the MICs against *S. cerevisiae* were highly enhanced by the addition of divalent cations to the medium. Thus, it is thought that the anti-MRSA activity of miconazole, unlike its antifungal activity, is mostly unaffected by inoculum sizes (10^3-10^7 CFU/ml), medium pHs (3-7), or cations (Ca^{++} or Mg^{++}). Based on these results, it is surmised that the antibacterial action of miconazole is remarkedly different from its antifungal action.

Effect of Antioxidants on the Antimicrobial Activity of Miconazole

In order to find out what kinds of effects the antioxidants have on the antimicrobial activities of miconazole against MRSA and *S. cerevisiae*, the MICs of miconazole were estimated in the media with different concentrations of ascorbic acid or α-tocopherol (Table 3). Ascorbic acid was found to have no effect on the anti-MRSA and antifungal activities of miconazole. However, when α-tocopherol of ≥ 50 μg/ml was added to the medium, the anti-MRSA activity of miconazole did not appear even at a high concentration of 100 μg/ml. Its antifungal activity was also greatly (16-fold) decreased by α-tocopherol of 100 μg/ml, although it did not completely disappear as seen with antifungal activity. Thus, it is thought that the antimicrobial activity of miconazole is adversely affected by the lipophilic α-tocopherol, but definitely not by the water-soluble ascorbic acid. In addition, this effect is considered to be more serious on the anti-MRSA compared to the antifungal action. On the other hand, it has been conceived that the antibacterial action of miconazole is caused by its membrane damage [6]. Miconazole was also reported to activate the peroxide-generating system in a fungal cell [19]. Considering all these results, it is inferred that the bacterial membrane damage of miconazole may be associated with the membrane oxidation and the action is suppressed by lipophilic antioxidant α-tocopherol. In addition, these results seem to indicate that the antifungal activity of miconazole is related to the lipophilic oxidation of the cell in a certain extent, although it is primarily correlated with an inhibition of ergosterol biosynthesis.

Killing Kinetics of Miconazole against MRSA

The killing kinetics of miconazole against MRSA were investigated in saline solution (Fig. 1) and CAMHB medium (Fig. 2), respectively. When MRSA was exposed to different concentrations of miconazole in saline solution, miconazole of 6.25 μg/ml, 3.13 μg/ml, or 1.56 μg/ml was enough to kill all viable cells of MRSA within 6 h, 12 h, and 24 h, respectively. These results were in accordance with the study [17] of Sud *et al.*, in which the experiments were performed with the normal strains of *S.aureus* in saline solution. However, miconazole in CAMHB medium did not show a complete killing against MRSA, even at a high concentration of 100 μg/ml. The similar results were obtained with the normal strains of *S. aureus* (data not shown). Thus, it is likely that the bactericidal activity of miconazole functions most efficiently in saline solution. Moreover, it is supposed that the bactericidal activity of miconazole is antagonized by certain components in the medium or it is limited to the inactivity of test organism.

At present, it is known that vancomycin is the last reliable antibiotic against MRSA infection [12]. However, this is jeopardized by the already increasing resistance to this agent [16]. Thus, it is considered that the strong antibacterial activity of miconazole against MRSA has a

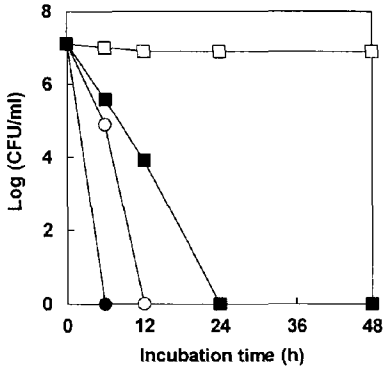

Fig. 1. Time killing curves of miconazole against methicillin-resistant *S. aureus* (MRSA) ATCC 33591 in saline solution.
Symbols indicate the treated concentration (μg/ml) of miconazole; 0 (□), 1.56 (■), 3.13 (○), 6.25 (●).

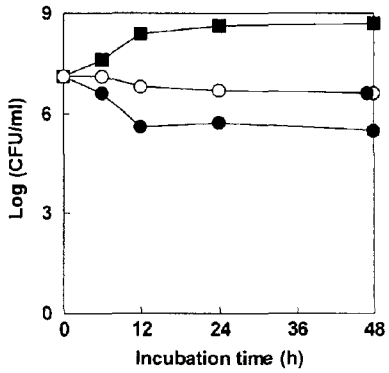

Fig. 2. Time killing curves of miconazole against methicillin-resistant *S. aureus* (MRSA) ATCC 33591 in cation-adjusted Mueller-Hinton broth.
Symbols indicate the treated concentration (μg/ml) of miconazole; 0 (■), 50 (○), 100 (●).

great significance. In addition, it is expected that further study on the antibacterial mode of action of miconazole may represent a new antibacterial target, and the chemical structure of miconazole may be used as a model for the chemical synthesis of new anti-MRSA agents.

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