Drug Development and Industrial Pharmacy, 22(3), 237-242 (1996)

Development of Anticandidal Delivery Systems: (I) Anticandidal Activities of Antifungal Agents and Synergistic Combination with Other Drugs

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ABSTRACT

Susceptibility testing with antifungal agents, e.g., minimum inhibitory concentration (MIC) determination, is performed to obtain reliable data that permit selection of the most suitable agents for treatment of an infective condition. To determine the drugs that provide maximum effectiveness against oral candidiasis, the MICs of various antifungal agents were determined. Also, synergism between two chosen antifungal agents was evaluated, and the effect of benzocaine, an anesthetic, and hydrocortisone, an antiinflammatory agent, on their MICs was examined. It was observed that among all the drugs tested, clotrimazole was the most promising candidate for use as an oral local antifungal. The combination of clotrimazole and chlorhexidine resulted in a decrease in the MIC. While the addition of hydrocortisone to this combination resulted in a slight increase in the MIC, the inclusion of benzocaine resulted in a substantial decrease in the MIC of the antifungal agent combination.

INTRODUCTION

Candida albicans is a dimorphic fungus that is a normal resident of the oral cavity, gastrointestinal tract, and female genital tract of humans. However, under conditions like trauma and immunological compromise, this organism may invade various sites, like the oral cavity, gastrointestinal tract, respiratory tract, urinary tract, genitals, central nervous system, cardiovascular system, and other organs/tissues (1,2).

The choice of antifungal agent used in the treatment of candidiasis is dependent on the severity and nature of the infection. Typically, polyenes (amphotericin B and nystatin), azoles (miconazole and clotrimazole), and flucytosine are preferred. In the treatment of oral candidiasis, antimicrobial agents like chlorhexidine

(which in addition to being an anticandidal drug is also effective against other oral infective conditions) may be prescribed (3,4). Other antimicrobial agents that may be prescribed for oral diseases include quaternery ammonium compounds like cetylpyridinium chloride and hexadecyltrimethyl ammonium bromide (5).

Susceptibility tests are performed for antifungal agents to provide reliable data that may permit selection of the most suitable agents for the treatment of an infective condition. Determination of minimum inhibitory concentration (MIC) is a quantitative method for such measurement.

Treatment of oral disease states often involves the prescription of mouthwashes or lozenges containing one or more antifungal agents (6). To determine the antifungal agents that may provide the maximum effectiveness against oral candidiasis, various antifungals were evaluated on the basis of their MICs. Also, the effect of combining two chosen antifungal agents on their MIC was determined. In addition, the effect of adding benzocaine, an anesthetic, and hydrocortisone, an antiinflammatory agent, which may serve to minimize the pain and the extent of inflammation secondary to a candidal infection, was examined.

MATERIALS

Two strains of *Candida albicans*, ATCC #10231 and ATCC #44505, were obtained from American type culture collection (ATCC, Rockville, MD). ATCC #10231 is the strain recommended for antifungal activity studies (USP) and ATCC #44505 is a reference strain from the oral mucosa. RPMI 1640 (R 5382) was used as the medium for susceptibility testing, and morpholinepropanesulfonic acid (MOPS) was used as the buffer system for dissolving the medium. Sabouraud's dextrose agar was used as the growth medium for the cultures.

METHODS

Assay Medium Preparation

The buffer solution was prepared by dissolving 34.53 g of MOPS (0.165 M) in 1000 ml of deionized water. A preweighed amount (10.4 g) of RPMI 1640 was then dissolved in 1000 ml of the buffered solution and the pH adjusted to 7 with sodium hydroxide solution (10 N). The resulting medium was sterilized by filtration through a microbiologic filter (0.22 nm) and stored at 4°C until used. Sterility checks on the medium were performed regularly (7,8).

To prepare the Sabouraud's dextrose agar medium, 65 g of the powder was weighed and dissolved in 1000 ml of deionized water and the solution was sterilized by autoclaving at 121°C and 15 psi for 20 min.

Inoculum Preparation

Inocula were prepared by using the spectrophotometric method. The organism pellet, which was obtained from ATCC, was suspended in 5 ml of RPMI 1640 medium, and a loopful of this suspension was inoculated onto the Sabouraud's dextrose agar and incubated at 35°C for 48 hr. Five colonies, at least 1 mm in diameter, were picked from the culture plate using a disposable plastic loop (10 μl), and suspended in 5 ml of sterile saline solution (0.85%). The resulting yeast suspension was vortexed for 15 sec and its turbidity adjusted, using sterile saline, to 85% transmission at 530 nm. This procedure yields a cell suspension containing $1–5 \times 10^6$ organisms/ml. The solution was then diluted with the RPMI 1640 medium to provide a working inoculum of $1–5 \times 10^4$ organisms/ml. Colony counts were performed to confirm the number of organisms in each ml of the inoculum (3,7,8).

Microtiter Plate Preparation

Sterile, individually wrapped, plastic microtiter plates, each containing 96 round-bottom wells, were used. Stock solutions of each antifungal agent (1280 μg/ml) were prepared in dimethylformamide. The stock solutions were then diluted 1:10 with RPMI 1640 medium. Ten twofold serial dilutions were carried out for each antifungal solution to obtain a range of concentrations (0.125–64 μg/ml). These solutions (100 μl each) were dispensed in the microtiter plates, from Wells 1 through 10 of each row (high to low concentration). Well 11 contained the organism in the medium devoid of any antimicrobial agent, as the growth control, and Well 12 contained only the medium, as the sterility check (7).

Minimum Inhibitory Concentration Determination

Each well of the microtiter plate was inoculated, using a repeat pipettor, with 10 μl of the inoculum preparation. The inoculum sequence proceeded from Well 11 to 1. The plate was incubated at 35°C and the tur-

bidity in the wells was checked after 24 and 48 hr incubation. Before checking for turbidity, the plates were agitated for 5 min using a reciprocating-action shaker. The wells were then checked for growth inhibition and the MIC of each drug was determined (7).

To determine if the drug is fungistatic or fungicidal at its respective MIC, a loopful of the above solution was inoculated onto the Sabouraud's dextrose agar and further incubated at 35°C for 48 hr. Absence of growth demonstrated the fungicidal nature of the antifungal agent.

Synergism Between Antifungal Agents

To minimize the formation of resistant strains, two antifungal agents can be used in conjunction for the therapy of disease states. To evaluate whether or not synergism exists between two antifungal agents, they were combined, in a 1:1 w/w ratio, and twofold serial dilutions were performed so as to obtain a range of drug concentrations. In addition, the two antifungal agents were also combined in a 1:1 MIC ratio and dilutions of this solution were performed as well. The MICs of the drug combinations were determined by the broth microdilution method described above. The results obtained could be expressed as the fractional inhibitory concentration (FIC), by dividing the MIC for the combination by the MIC for each agent alone. Synergism exists if the FIC is less than 1 or if a fourfold decrease in the MIC of the agents in combination is observed (9).

Antifungal, Anesthetic, and Antiinflammatory Agent Interaction

The effect of the antiinflammatory agent and the anesthetic, alone and in combination, on the MIC of the two combined antifungal agents was determined. The two antifungal agents were combined in a 1:1 (w/w) ratio and serial dilutions were performed as described above, to obtain a series of concentrations. The anesthetic, benzocaine, and the antiinflammatory agent, hydrocortisone, were added to the above solutions, alone and in combination. The dilutions were performed such that the final concentration of the two antifungal agents together was in the desired range, while that of benzocaine and hydrocortisone was 1250 μg/ml each in all the solutions. The microtiter plates were incubated at 35°C for 48 hr and any change in the MIC of the antifungal agents was determined.

To determine if variation in the concentration of anesthetic and antiinflammatory agent affects the MIC of the antifungal agents, the concentration of the antifungal agents was maintained at the MIC while that of benzocaine and hydrocortisone was varied in turn, and the presence or absence of growth was monitored.

All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Minimum Inhibitory Concentration Determination

The MICs obtained for the various antifungal agents tested are compared in Table 1. The most effective antifungal agents, in terms of their ability to inhibit the growth of *Candida albicans* (strains 10231 and 44505), are flucytosine and clotrimazole. Of all the antifungal agents tested, clotrimazole, miconazole, chlorhexidine, nystatin, and the cetylpyridinium chloride have been approved for topical use. All of the above have been incorporated into some form of oral preparation, such as lozenge, mouthwash, or buccal tablet.

Effect of Strain Type

The results in Table 1 indicate that the MICs for the various antifungal agents did not vary greatly from one strain type to another. Some minor variation was observed in the case of amphotericin B, clotrimazole, miconazole, and chlorhexidine. The rest of the antifungals tested yielded identical MIC values for both the *Candida albicans* strains, at both the end time points tested.

Effect of Time of MIC Determination

The time at which the reading is taken does have some effect on the MIC values; higher MIC values were often obtained at 48 hr than at 24 hr. In the case of chlorhexidine and hexadecyltrimethyl ammonium bromide, no effect of the time of end-point determination was seen.

It has been recommended that the end-point values for MIC determination be taken after 24 hr for the imidazoles and 5-flucytosine, and after 48 hr for the polyenes (3). Since the time for end-point determination for chlorhexidine and quaternary ammonium compounds has not been specified, the MICs were determined at both times, i.e., 24 and 48 hr for all the antifungal agents.

In order to evaluate the antifungals based on their MIC values, the effectiveness of a particular antifungal

Table 1

MICs forAntifungal Agents

	Antifungal Activity (MIC, μg/ml)							
	ATCC #10231				ATCC #44505			
Anticandidal Agent	24 hr	c/s	48 hr	c/s	24 hr	c/s	48 hr	c/s
Polyene								
Amphotericin B	1.0	c	2.0	c	2.0	c	4.0	c
Nystatin	4.0	c	8.0	c	4.0	c	8.0	c
Azole								
Clotrimazole	1.0	s	2.0	c	0.125	c	4.0	c
Miconazole	1.0	s	8.0	c	0.25	s	8.0	c
Quater. Ammonium Salt								
Cetylpyridinium chloride	4.0	c	8.0	c	4.0	c	8.0	c
Cetrimide	8.0	c	8.0	c	8.0	c	8.0	c
Bisguanide								
Chlorhexidine	16.0	c	16.0	c	8.0	c	8.0	c
Pyrimidine Derivative								
5-Fluorocytosine	0.125	c	0.25	c	0.125	c	0.25	c

c/s = fungicidal/fungistatic against *Candida albicans* (n = 3).

was determined based on the specific recommendation for that antifungal. For those antifungals with no specified time for end-point determination in MIC studies, the 48 hr value was considered.

Choice of Antifungal Agents

Of the various antifungal agents tested, amphotericin B and 5-flucytosine have been recommended for systemic candidiasis only. The rest of the antifungals can be used for topical and local application. Of these antifungals, clotrimazole exhibited the lowest MIC value against both the strains of *Candida albicans* (24 hr end point). Hence clotrimazole was chosen as one of the anticandidal agents for further studies.

Chlorhexidine has been approved for use against a variety of oral disease conditions like aphthous stomatitis, denture stomatitis, oral cancer, gingivitis, and plaque, in addition to its use as an anticandidal drug. In addition, chlorhexidine had a reasonably low MIC against the oral strain of *Candida albicans* (#44505) and was one of the antifungal agents that did not show time-dependency in its efficacy. Therefore, chlorhexidine was chosen as the second anticandidal agent.

Synergism Between Antifungal Agents

Combination Based on Weight of the Antifungal Agents

Chlorhexidine and clotrimazole were combined in equal amounts, and since their respective 24 hr MICs against ATCC #44505 were 8 μg/ml and 0.125 μg/ml, their combined concentration varied from 8 μg/ml to 0.0156 μg/ml using serial dilution. The study was performed only on the oral strain. The results obtained are tabulated in Table 2.

The MIC obtained after 24-hr incubation (0.25 μg/ml) does not demonstrate any synergistic effect of the two drugs. However, at the end of 48 hr, the increase in the MIC value (1 μg/ml) was not as significant as that obtained when the combination was not used; rather a considerable decrease in the MIC as compared to that of both clotrimazole and chlorhexidine alone was observed.

Combination Based on MIC of the Antifungal Agents

To get a clearer picture of the synergism, the two drugs were also combined at their respective MICs, i.e., 8 μg/ml of chlorhexidine was combined with 0.125 μg/ml of clotrimazole, and serial dilutions of this solution were made to obtain a range of concentrations, with the lowest drug concentration solution containing 0.06 μg/ml of chlorhexidine and 9.8×10^{-4} μg/ml of clotrimazole.

The results obtained are also tabulated in Table 2 and demonstrate that at both 24 and 48 hr, the MIC of the combination of drugs is 2 μg/ml for chlorhexidine and 0.03 μg/ml for clotrimazole. This significant (greater than fourfold) decrease in the individual MICs demonstrates the presence of synergism between the two drugs.

Table 2

Synergism on Combination of Antifungals and Other Drugs

Anticandidal Combination[a] and Adjuvants[b]	Antifungal Activity (MIC, μg/ml)			
	24 hr	c/s	48 hr	c/s
Chlorohexidine	8.0	c	8.0	c
Clotrimazole	0.125	c	4.0	c
A	0.25	c	1.0	c
B	2.0 & 0.03	c	2.0 & 0.03	c
A + Hydroxortisone	1.0	s	2.0	c
A + Benzocaine	0.015	c	0.015	c
A+ Hydrocortisone and Benzocaine	0.015	c	0.015	c

[a]Anticandidal combination: Chlorhexidine and Clotrimazole combinated at (A) 1:1 μg/ml ratio and (B) 1:1 MIC ratio.

[b]Adjuvant = hydrocortisone and/or benzocaine (1250 μg/ml each).

c/s = fungicidal/fungistatic against *Candida albicans* (ATCC #44505) (n = 3).

Antifungal, Antiinflammatory Agent, and Anesthetic Interaction

The effect of hydrocortisone and benzocaine on the MIC of chlorhexidine and clotrimazole in combination was determined on the combination with the same weight proportions of the two antifungals. This was done since the final formulation to be proposed would contain the same amount of chlorhexidine and clotrimazole in combination.

Effect of Hydrocortisone

The effect of hydrocortisone on the MIC of the chlorhexidine-clotrimazole combination is presented in Table 2. Hydrocortisone increased the MIC of the antifungal drug combination at the 24 hr time point. The value obtained after 24 hr was 1 μg/ml, and the value after 48 hr was 2 μg/ml.

The increase resulting from the inclusion of hydrocortisone may be due to the mechanism of action of the antifungals. Both chlorhexidine and clotrimazole exert their inhibitory action by interacting with the sterols in the cell walls of the organism, which results in the formation of leaky cell walls and thus loss of important nutrients from the cell (10). Hydrocortisone, a progestational steroid added externally to the solution, may react with the antifungals and consequently decrease their effective concentration.

Effect of Benzocaine

Addition of benzocaine resulted in a decrease in the MIC value of the antifungal combination to 0.015 μg/ml, and remained at this value even after 48 hr (Table 2). The reason for this synergistic action of benzocaine on the MIC of chlorhexidine and clotrimazole in combination is unclear. Benzocaine is a local anesthetic that reacts with the nerve endings. It has been reported that on local application, benzocaine is inserted into the lipid bilayer of cell membranes (11). This orientation of benzocaine in the lipid bilayer may result in the perturbation of the barrier properties of the membrane, thus resulting in increased partitioning of the antifungal agents into the cell membrane. Alternately, benzocaine may act as a carrier for the antifungal agents during its passage into the cell membrane. More studies need to be conducted to provide a better insight into this synergistic effect.

Effect of Hydrocortisone and Benzocaine in Combination

On addition of 1250 μg/ml of both hydrocortisone and benzocaine to the series of antifungal solutions containing chlorhexidine and clotrimazole in combination, the MIC was found to be the same as that obtained when benzocaine alone was used (0.015 μg/ml) (Table 2). Thus, the synergistic action between benzocaine and the antifungal drugs was not affected by the opposing antagonistic action of hydrocortisone.

Effect of Varying Hydrocortisone and Benzocaine Concentration

The results obtained on varying the concentration of benzocaine and hydrocortisone, in turn, in the antifungal solution are shown in Table 3.

The microtiter wells showed no presence of growth when the benzocaine concentration was decreased up to

Table 3

Effect of Varying Hydrocortisone and Benzocaine Concentrations

Hydrocortisone (μg/ml)	Benzocaine (μg/ml)	Antifungal Activity[a] 24 hr	c/s	48 hr	c/s
1250	100	–	c	–	c
1250	750	–	c	–	c
1250	500	–	c	–	c
1250	250	–	c	+	
1250	125	+		+	
1750	500	–	c	–	c
1500	500	–	c	–	c
1000	500	–	c	–	c
750	500	–	c	–	c
500	500	–	c	–	c

[a]Chlorhexidine and clotrimazole combined at 1:1 μg/ml ratio.
c/s = fungicidal/fungistatic against *Candida albicans* (ATTC #44505).
+ indicated presence of growth; – indicated absence of growth (n = 3).

500 μg/ml from the 1250 μg/ml used in the initial studies. However, when the concentration was decreased to 250 μg/ml presence of growth was observed after 48 hr.

Therefore, the concentration of benzocaine was maintained at 500 μg/ml and that of hydrocortisone varied. No effect of change of hydrocortisone concentration was observed (Table 3).

CONCLUSION

A combination therapy with antifungal, anti-inflammatory, and anesthetic properties could serve as a very efficacious treatment against oral candidiasis. It would, in addition to treatment of the candidal infection, prevent the formation of resistant strains, and also combat the secondary manifestations of oral diseases.

ACKNOWLEDGMENTS

The authors would like to acknowledge research support to Dr. M. Nair (currently at Warner-Lambert Company, 170 Tabor Road, Morris Plains, NJ 07950) from Parke-Davis Chair Endowment and Sintong Corporation.

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