Effect of Chemosensitizers on Minimum Inhibitory Concentrations of Fluconazole in *Candida albicans*

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Key Words

Candida albicans • Fluconazole • Chemosensitizers

Abstract

Objective: To evaluate the effect of chemosensitizers on the in vitro activity of fluconazole against *Candida albicans* strains. **Materials and Methods:** Using Clinical Laboratory Standard Institute method, antifungal activity of fluconazole was determined alone and in combination with 16 chemosensitizers that included verapamil, reserpine, quinine, quinidine, gemfibrozil, lansoprazole, tamoxifen, diltiazem, desipramine, nicardipine, cyclosporine, chlorpromazine, prochlorperazine, promethazine, thioridazine, and trifluoperazine. Further studies were done using double combinations of selected chemosensitizers with fluconazole (28 combinations). For testing combinations, half of the minimum inhibitory concentration (MIC) of each agent was selected in order to avoid the effect of the drug alone. One reference strain (ATCC90028) and one clinical isolate of *C. albicans* were used for testing the in vitro activity. Broth dilution method was used to determine the MICs of fluconazole and chemosensitizers. **Results:** Of the 16 chemosensitizers tested, 3 exhibited in vitro activity by increasing fluconazole susceptibility to 7-fold. The MICs of the reference strain and clinical isolate for fluconazole were 5.5 and 0.55 µg/ml, respectively, and these were reduced to 0.76 µg/ml by gemfi-

brozil, 0.83 µg/ml by quinine, and 0.76 µg/ml by chlorpromazine in the reference strain, with MIC reduction to 0.08 µg/ml by all three chemosensitizers in the clinical isolate. Some double combinations reduced the MIC of fluconazole to 10- to 100-fold, even when the chemosensitizers were not effective alone. **Conclusion:** The most effective double combinations were those of chlorpromazine with either reserpine or nicardipine.

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Introduction

The wide use of fluconazole for the treatment of *Candida albicans* infections and other mycoses is currently hindered by the appearance of resistant strains [1–3]. The most prevalent resistance pattern appears to be decreased drug accumulation due to increased expression of efflux pumps on the plasma membrane of fungal cells, either the major facilitators such as multidrug resistance (MDR) [4, 5] and *FLU* [6, 7] or the ABC transporters *CDR1* [5, 8] and *CDR2* [9, 10].

It has been demonstrated that drug-extruding efflux pumps in *C. albicans* possess sequences with a high degree of homology to the product of human MDR-1 gene that confers MDR to anticancer drugs [11]. Examples of MDR-reversing agents (chemosensitizers) include calci-

um channel blockers (verapamil, nifedipine), calmodulin inhibitors (trifluoperazine, prochlorperazine), reserpine, quinine, progesterone, tamoxifen, cephalosporins, and cyclosporine A [12].

A wide range of compounds were previously studied for synergistic effects with fluconazole in *C. albicans*: cyclosporine, FK506 [13], chlorpromazine, fluphenazine, amitriptyline, clomipramine, antiarrhythmic drugs, proton pump inhibitors, platelet aggregation inhibitors [14, 15], terbinafine, propafenones [16], quinolone antibiotics [17] and milbemycins [18]. Potent synergistic effects were also shown between ibuprofen, verapamil, β -estradiol, progesterone, and azole antifungals: fluconazole, itraconazole, and voriconazole on *Candida* species [19] as was reviewed extensively by Afeltra et al. [20]. Most of the studies were conducted in *Candida* strains that hyperexpress active efflux pumps and it was demonstrated that the inhibition of these pumps may lead to reversal of the resistance to antifungals [13, 21].

The present study sought to investigate the effectiveness of a combination of fluconazole with a wide range of compounds commonly used in clinical practice for a variety of indications, including cardiovascular (reserpine, quinidine, quinine, verapamil, diltiazem, nicardipine, gemfibrozil), gastrointestinal (lansoprazole), psychotropic (chlorpromazine, prochlorperazine, promethazine, thioridazine, trifluoperazine, desipramine), and an estrogen antagonist (tamoxifen).

Materials and Methods

Chemicals

Chemosensitizers (verapamil, reserpine, quinine, quinidine, gemfibrozil, lansoprazole, tamoxifen, diltiazem, desipramine, nicardipine, cyclosporine, chlorpromazine, prochlorperazine, promethazine, thioridazine, trifluoperazine) were purchased from Sigma Chemicals Co. (St. Louis, Mo., USA). Fluconazole was supplied by Pfizer (Groton, Conn., USA). Fluconazole and modulators were dissolved in methanol and prepared as saturated solutions.

Strains and Media

A strain of *C. albicans* was isolated from a hospitalized patient from the Jordan University Hospital and was confirmed by biochemical tests. A reference strain of *C. albicans* ATCC90028 was used as a control. Until testing, the yeasts were kept frozen in brain-heart broth (Difco Laboratories, Detroit, Mich., USA) with 5% glycerol. For each experiment, the strains were subcultured twice on Sabouraud agar (Difco) for 24 h at 35°C to ensure viability and purity. The inoculum suspension was prepared by picking five colonies of at least 1 mm in diameter and suspending them in 5 ml of sterile distilled water. Suspensions with a cell density of $1-5 \times 10^6$ cells/ml were prepared in sterile distilled water.

Table 1. MICs of fluconazole and chemosensitizers against reference and clinical isolate strains of *C. albicans*

Compound	Reference strain	Clinical isolate
Fluconazole	5.5	0.55
Reserpine	55	55
Verapamil	5.5	55
Diltiazem	55	55
Nicardipine	5.5	55
Gemfibrozil	0.05	55
Quinine	5.5	55
Quinidine	55	5.5
Lansoprazole	55	55
Desipramine	0.55	5.5
Tamoxifen	55	55
Cyclosporine	55	5.5
Chlorpromazine	0.55	5.5
Prochlorperazine	5.5	0.55
Promethazine	55	5.5
Thioridazine	0.55	0.05
Trifluoperazine	55	55

Determination of Minimum Inhibitory Concentrations of Fluconazole and Chemosensitizers

Minimum inhibitory concentrations (MICs) of fluconazole alone and in combination with chemosensitizers were determined according to the CLSI M27-A2 protocol for broth dilution antifungal susceptibility testing of yeasts established by the National Committee for Clinical Laboratory Standards [22]. A dilution range of 0.01–100 μ g/ml of the chemosensitizers was used. The experiments were done in triplicate for each compound or combination. For testing combinations half the MIC was selected for each agent so as to avoid the effect of the drug alone. The mean concentration in the tube with no turbidity and that in the previous tube were calculated and considered as MIC.

The fungal suspension was added to give an inoculum size of 10^4 cells in the final mixture of the chemosensitizer and fluconazole. The medium was mixed thoroughly and incubated at 37°C for 24 h. Control tubes containing the chemosensitizers or fluconazole in the nutrient media with the microorganisms were also included. The blank tubes were prepared by mixing methanol (solvent) with nutrient media and microorganisms. Preliminary experiments showed that the solvent did not exhibit antimicrobial activity at the concentration used. The experiments were carried out in duplicate 3 times.

Results

Antifungal Activities of Chemosensitizers

The MICs of fluconazole and chemosensitizers tested on both the reference strain and clinical isolate of *C. albicans* are summarized in table 1. Both strains were sus-

Table 2. MICs ($\mu\text{g/ml}$) of fluconazole alone and in combination with the chemosensitizers against reference and clinical isolate strains of *C. albicans*

Drug	Reference strain	Clinical isolate
Fluconazole	5.5	0.55
Fluconazole + reserpine	0.83	0.83
Fluconazole + verapamil	0.83	0.83
Fluconazole + diltiazem	0.83	8.32
Fluconazole + nicardipine	0.08	8.32
Fluconazole + gemfibrozil	0.76	0.08
Fluconazole + quinine	0.83	0.08
Fluconazole + quinidine	8.32	1.51
Fluconazole + lansoprazole	8.32	8.32
Fluconazole + desipramine	8.32	1.51
Fluconazole + tamoxifen	0.01	0.83
Fluconazole + cyclosporine	8.32	8.32
Fluconazole + chlorpromazine	0.76	0.08
Fluconazole + prochlorperazine	1.51	1.51
Fluconazole + promethazine	8.32	1.51
Fluconazole + thioridazine	0.83	0.83
Fluconazole + trifluoperazine	8.32	8.32

Concentrations of chemosensitizers were 1/2 of MIC stated in table 1.

Table 3. MICs of selected double chemosensitizer combinations with fluconazole against the reference strain of *C. albicans*

	RES	NIC	GEM	QUI	TAM	CYC	CHL
FC	55	55	5.5	0.06	5.5	55	0.55
FC + RES	0.83	–					
FC + NIC	0.08	0.11	–				
FC + GEM	0.76	<0.01	0.06	–			
FC + QUI	0.83	0.11	0.15	0.56	–		
FC + TAM	0.01	0.01	0.01	0.01	1.06	–	
FC + CYC	8.32	0.01	0.11	0.05	<0.01	0.01	–
FC + CHL	0.76	0.01	0.01	0.11	0.61	0.06	0.61

FC = Fluconazole; RES = reserpine; NIC = nicardipine; GEM = gemfibrozil; QUI = quinine; TAM = tamoxifen; CYC = cyclosporine; CHL = chlorpromazine.

ceptible to fluconazole (MIC 5.5 $\mu\text{g/ml}$ in reference strain and 0.55 $\mu\text{g/ml}$ in the clinical isolate).

Most of the MIC values of chemosensitizers were 10- to 100-fold higher than those for fluconazole for the clinical isolate and the MIC for prochlorperazine was similar to that of fluconazole. The MICs were up to 10-fold higher than those for fluconazole in the reference strain, sug-

Table 4. MICs of selected double chemosensitizer combinations with fluconazole against a clinical isolate of *C. albicans*

	RES	NIC	GEM	QUI	TAM	CYC	CHL
FC	0.55	55	55	55	55	5.5	5.5
FC + RES	0.83	–					
FC + NIC	8.32	0.11	–				
FC + GEM	0.08	1.06	0.11	–			
FC + QUI	0.08	0.11	1.06	1.06	–		
FC + TAM	0.83	1.06	0.01	0.01	0.11	–	
FC + CYC	8.32	1.06	1.06	0.11	0.11	1.06	–
FC + CHL	0.08	0.01	0.01	0.11	1.06	0.01	0.11

FC = Fluconazole; RES = reserpine; NIC = nicardipine; GEM = gemfibrozil; QUI = quinine; TAM = tamoxifen; CYC = cyclosporine; CHL = chlorpromazine.

gesting only weak antifungal activity. Only the MICs of verapamil, nicardipine, quinine and prochlorperazine were similar to that of fluconazole in the reference strain. Interestingly, gemfibrozil, desipramine and chlorpromazine appeared to be more active than fluconazole in the reference strain (MICs 0.06 $\mu\text{g/ml}$ for gemfibrozil, 0.55 $\mu\text{g/ml}$ for desipramine and 0.55 $\mu\text{g/ml}$ for chlorpromazine), while thioridazine was more active than fluconazole in both the reference strain (MIC 0.55 $\mu\text{g/ml}$) and the clinical isolate (MIC 0.05 $\mu\text{g/ml}$).

Effect of Chemosensitizers on the Susceptibility of C. albicans to Fluconazole

The MICs of fluconazole used with a variety of chemosensitizers are shown in table 2. The sensitivity of the reference *C. albicans* strain was increased dramatically when fluconazole was combined with reserpine, verapamil, diltiazem (MICs 0.83 $\mu\text{g/ml}$), nicardipine (MIC 0.08 $\mu\text{g/ml}$), tamoxifen (MIC 0.01 $\mu\text{g/ml}$), prochlorperazine (MIC 1.51 $\mu\text{g/ml}$) and thioridazine (MIC 0.83 $\mu\text{g/ml}$), while gemfibrozil, quinine and chlorpromazine increased the susceptibility to fluconazole of both the reference strain (MIC 0.76 $\mu\text{g/ml}$ for gemfibrozil, 0.83 $\mu\text{g/ml}$ for quinine and 0.76 $\mu\text{g/ml}$ for chlorpromazine) and clinical isolate (MICs 0.08 $\mu\text{g/ml}$ for all three compounds).

Effect of Simultaneous Combinations of Chemosensitizers on the Susceptibility of C. albicans to Fluconazole

When double chemosensitizer combinations with fluconazole were used against the reference strain (table 3), a 10- to 100-fold shift in fluconazole sensitivity when

compared to a single combination was observed with quinine + cyclosporine, chlorpromazine + reserpine, chlorpromazine + nicardipine, reserpine + gemfibrozil (MICs <0.01 µg/ml), reserpine + cyclosporine (MIC 0.01 µg/ml), gemfibrozil + cyclosporine (MIC 0.06 µg/ml), quinine + reserpine, and chlorpromazine + gemfibrozil (MIC 0.11 µg/ml).

Regarding the clinical isolate which was more susceptible to fluconazole than the reference strain, the following double chemosensitizer combinations were found to cause an 8-fold increase in fluconazole susceptibility compared to a single combination (table 4): chlorpromazine + reserpine, chlorpromazine + tamoxifen, chlorpromazine + nicardipine, gemfibrozil + tamoxifen, tamoxifen + nicardipine (MICs 0.01 µg/ml), reserpine + nicardipine (MIC 0.11 µg/ml), and nicardipine + cyclosporine (MIC 1.06 µg/ml).

Combinations of chlorpromazine with either reserpine or nicardipine produced a shift in fluconazole susceptibility in both the reference strain and the clinical isolate of *C. albicans*.

Discussion

Our data show that most combinations of fluconazole with chemosensitizers increase the susceptibility of fluconazole in either of the *C. albicans* strains. Few compounds (gemfibrozil, quinine and chlorpromazine) were able to change the MIC of fluconazole in both the clinical isolate and the reference strain.

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It has previously been reported that double combinations of chemosensitizers at nontoxic levels can be effectively used to reverse resistance in cancer cells [23, 24] and in a malarial parasite [25]. Because we have demonstrated in this study that double combinations increased the antifungal effect of fluconazole, even when the chemosensitizers were not effective alone, this study may facilitate clinical investigations of ‘cocktails’ of different chemosensitizers with fluconazole that could improve the efficacy and safety of such combinations in patients infected with *Candida*.

Further studies are needed that will investigate mechanisms of this synergistic effect using *Candida* species that express or lack active efflux pumps.

Conclusion

Our results show that the most promising combinations of chemosensitizers are chlorpromazine with either reserpine or nicardipine, since these combinations caused a shift in fluconazole sensitivity in both the reference strain and the clinical isolate of *C. albicans*, although these compounds did not produce an antifungal effect on their own.

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