

MYCOLOGY

Effect of fluvastatin and pravastatin, HMG-CoA reductase inhibitors, on fluconazole activity against *Candida albicans*

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Synergy between fluvastatin, at clinically unachievable concentrations, and fluconazole against *Candida albicans* has been reported. The purpose of the present study was to evaluate the in-vitro activity of fluconazole alone and in combination with clinically achievable concentrations of pravastatin and fluvastatin against *C. albicans*. In-vitro susceptibility and synergy testing were performed against clinical isolates of *C. albicans* with fluconazole, pravastatin and fluvastatin. Both checkerboard method and time-kill studies were performed. MICs for fluconazole ranged from 0.5 (susceptible) to >256 mg/L (resistant) at 24 h. All isolates had MICs >2 mg/L for both statins. No synergy or antagonism was observed with fluconazole in combination with either agent against any isolate of *C. albicans* by the checkerboard assay or time-kill studies. Clinically achievable concentrations of pravastatin and fluvastatin did not affect the in-vitro activity of fluconazole against *C. albicans*.

Introduction

The emergence of resistance to commercially available antifungal compounds is increasing and there is much interest in the development of new antifungal drugs or combination therapy of antifungal agents with other agents to improve efficacy or decrease toxicity, or both [1]. In 1997, Chin *et al.* reported synergy between fluvastatin, a cholesterol-lowering agent, and fluconazole and itraconazole by both checkerboard and time-kill studies against *Candida albicans* and *Cryptococcus neoformans* [2]. However, the concentrations of fluvastatin used (4–≥32 mg/L) were not clinically achievable. The reported C_{max} for fluvastatin after doses of 20–40 mg/day ranged from 100 to 300 ng/ml [3]. The potential utility of combination therapy of fluconazole and statins is unclear, as the concentrations of fluvastatin used by Chin *et al.* were clinically unachievable.

These investigators also found that pravastatin, lova-

statin and simvastatin did not have activity when tested against these strains of fungi. Lovastatin and simvastatin must be converted from the lactone form, which is inactive, to the active acid form by contact with digestive fluids, the gut wall, or the liver [4]. Most of the agents used in their report were in the lactone form which would not be expected to show any clinical effect *in vivo*. Hence, the purpose of this study was to evaluate the in-vitro activity of fluconazole alone and in combination with clinically achievable concentrations of pravastatin and fluvastatin against sensitive and resistant strains of *C. albicans*.

Fluvastatin sodium and pravastatin sodium are both water-soluble cholesterol-lowering agents which act through inhibiting 3-hydroxy-3-methylglutarylco-enzyme A (HMG-CoA) reductase, which is the rate-limiting step in cholesterol synthesis. Fluconazole is the first of the subclass of synthetic triazole antifungal agents. It is a highly selective inhibitor of fungal cytochrome P-450 sterol C-14 α -demethylation. It is hypothesised that synergic fungicidal effects could occur between these two agents. HMG-CoA reductase inhibition would decrease levels of cytosolic cholesterol or cholesterol precursors which are needed in fungal cell membrane synthesis, therefore decreasing the propagation of fungi.

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Materials and methods

Pharmacological agents

The following drugs were obtained from the manufacturers as laboratory powders: fluconazole (Pfizer Inc., New York, NY, USA), fluvastatin sodium (Novartis Pharmaceuticals Corporation East, Hanover, NJ, USA) and pravastatin sodium (Bristol-Myers Squibb Company, Princeton, NJ, USA). Stock solutions were prepared with the weight adjusted according to the potency of each drug and then stored at -20°C until used.

Isolates

Eight isolates of *C. albicans* were used in this experiment. All isolates were obtained from the Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio, TX, USA.

In-vitro susceptibility testing

MICs were performed in duplicate for each isolate of *C. albicans* against fluconazole, pravastatin and fluvastatin following the National Committee for Clinical Laboratory Standards (NCCLS) guidelines for antifungal susceptibility testing (M27-A) [5]. RPMI 1640 (American Biorganics, Niagara Falls, NY, USA) buffered to pH 7.0 with 0.125 morpholinepropanesulphonic (MOPS) buffer was used as the test medium. The plates were inoculated with $c. 10^3$ cfu of *C. albicans*/ml and incubated at 35°C for 48 h. MICs were defined as an 80% reduction in turbidity compared with the growth control at 24 and 48 h and each isolate was classified as susceptible, intermediate or resistant to fluconazole based on the NCCLS breakpoints [5].

In-vitro synergy testing

Checkerboard technique. The checkerboard technique with broth microdilution was used to evaluate the effects of pravastatin and fluvastatin in combination with fluconazole against each isolate of *C. albicans*. Each combination was tested in duplicate. The concentration range for fluconazole was 0.06–256 mg/L and for fluvastatin and pravastatin was 0.016–2 mg/L and 0.008–1 mg/L, respectively. Dilutions of fluconazole and HMG-CoA reductase inhibitors were made with a two-fold diluter (Dynatech Autodiluter III). Briefly, the columns in each well of a microtitration tray contain the same amount of fluconazole being diluted along the x axis and the rows in each well contain the same amount of either fluvastatin or pravastatin being diluted on the y axis. Each plate was inoculated with 10^5 cfu of *C. albicans*/ml, incubated at 35°C for 48 h and read as described for in-vitro susceptibility testing.

Time-kill studies. Three *C. albicans* isolates with variable susceptibility patterns to fluconazole were

selected for evaluation by time-kill studies. Two susceptible isolates (*C. albicans* 99-508, MIC 0.5 mg/L and *C. albicans* 99-594, MIC 4 mg/L) and a resistant isolate (*C. albicans* 99-506, MIC >256 mg/L) were used according to the NCCLS proposed breakpoint for fluconazole against *C. albicans*. Before testing, isolates were subcultured twice on Sabouraud dextrose agar. Three to five colonies from 24-h plates were suspended in sterile water and matched to a 0.5 McFarland turbidity standard. Appropriate volumes of the fungal suspensions were added to 25 ml of RPMI 1640 medium buffered to pH 7.0 with 0.165 M MOPS buffer to yield an initial inoculum $c. (1-5) \times 10^5$ cfu/ml. The following concentrations of fluconazole, pravastatin and fluvastatin were tested alone and in combination against each isolate: fluconazole 10 and 30 mg/L, pravastatin 0.25 mg/L and fluvastatin 1 mg/L. All tubes were incubated at 35°C with continuous agitation in an orbital shaker. Samples were drawn at 0, 4, 8, 12, 24, 36 and 48 h, diluted if necessary, then plated on Sabouraud dextrose agar plates by means of a spiral plater (Spiral Biotech, Bethesda, MD, USA). The plates were incubated at 35°C for 24 h and colony counts were determined with a laser colony counter (CASBA 4, Spiral Biotech). The limit of quantification was 1×10^2 cfu/ml. Antifungal carry-over was controlled by use of the spiral plater. This device dispenses a 50- μl sample in a logarithmically decreasing fashion from the centre to the edge of the agar plates. Colonies were counted from the outer edge, which has been shown to be unaffected by residual antibiotic in bacterial studies [6]. All time-kill experiments were run in duplicate.

Data analysis

To evaluate the effect of the combinations in the checkerboard studies, the fractional inhibitory concentration (FIC) index was calculated for each isolate according to the following formula: FIC index = MIC of drug A in combination/MIC of drug A alone + MIC of drug B in combination/MIC of drug B alone. Synergy was defined as an FIC index <0.5 [7].

For the time-kill studies, plots of mean colony counts (\log_{10} cfu/ml) versus time were constructed and fungicidal ($\geq 99.9\%$ reduction in cfu/ml compared with the starting inoculum) or fungistatic ($<99.9\%$ reduction in cfu/ml compared with the starting inoculum) activity was determined. Synergy was defined as $\geq 2 \log_{10}$ decrease in cfu/ml at 24 h with the combination when compared with the most active single agent and the number of surviving organisms for the antimicrobial combination was $\geq 2 \log_{10}$ less than the initial inoculum. Indifference was defined as a $<1 \log_{10}$ change in cfu/ml at 24 h between the combination and the most active single agent. Antagonism was defined as $\geq 2 \log_{10}$ increase in cfu/ml at 24 h with the combination when compared with the most active single agent. Finally, the percentage reduction in col-

ony counts at 24 h in comparison to the growth controls and the respective fluconazole concentrations was calculated for each isolate.

Results

Susceptibility testing

Six of the *C. albicans* isolates were susceptible to fluconazole with an MIC \leq 8 mg/L. *C. albicans* 99-494 had an MIC of 16 mg/L (susceptible dose-dependent) and *C. albicans* 99-506 had an MIC \geq 256 mg/L (resistant) (Table 1). All the isolates had MICs $>$ 2 mg/L against pravastatin and fluvastatin.

Synergy testing

Checkerboard studies. None of the eight isolates of *C. albicans* showed enhanced in-vitro inhibitory activity of fluconazole in combination with pravastatin or fluvastatin. MICs of the combinations were similar to

the MICs of the agents alone and none of the isolates showed a greater than four-fold decrease in the MICs or an FIC index \leq 0.5. Hence, all the isolates were considered to be indifferent to the combination of the two agents and no combination was antagonistic.

Time-kill. Two susceptible isolates of *C. albicans* (99-508 and 99-594) and one highly resistant isolate (99-506) were used to evaluate the effect of fluconazole in combination with pravastatin and fluvastatin by time-kill curves. No statistically significant reduction in colony count was observed between fluconazole 10 and 30 mg/L for each isolate. Neither pravastatin nor fluvastatin showed a significant reduction in colony count compared to the growth control. For all three isolates of *C. albicans*, the time-kill curves for pravastatin and fluvastatin essentially superimposed the growth control. The azoles alone and in combination displayed a *c.* 80% reduction in colony count that was observed among all three isolates. None of the isolates showed a significant reduction in colony count at 24 and 48 h with the combination of agents (fluconazole 10 mg/ml and fluconazole 30 mg/L in combination with pravastatin 0.25 mg/L and fluvastatin 1 mg/L) compared to the two concentrations of fluconazole alone. As no differences were observed between the three isolates, only *C. albicans* 99-506 kill curves are presented in Fig. 1.

There was a statistically significant difference between fluconazole-resistant and fluconazole-susceptible isolates and their percentage reduction in colony counts with fluconazole compared with the growth control curve ($p = 0.0026$, 69 SD 6% versus 83 SD 9%).

Table 1. In-vitro activity of fluconazole against *C. albicans*

<i>C. albicans</i> strain no.	MIC (mg/L)	Interpretation
99-508	0.5	S
99-595	2	S
99-536	2	S
99-513	4	S
99-519	4	S
99-594	4	S
99-494	16	S-DD
99-506	$>$ 256	R

S, susceptible; S-DD, susceptible dose-dependent; R, resistant.

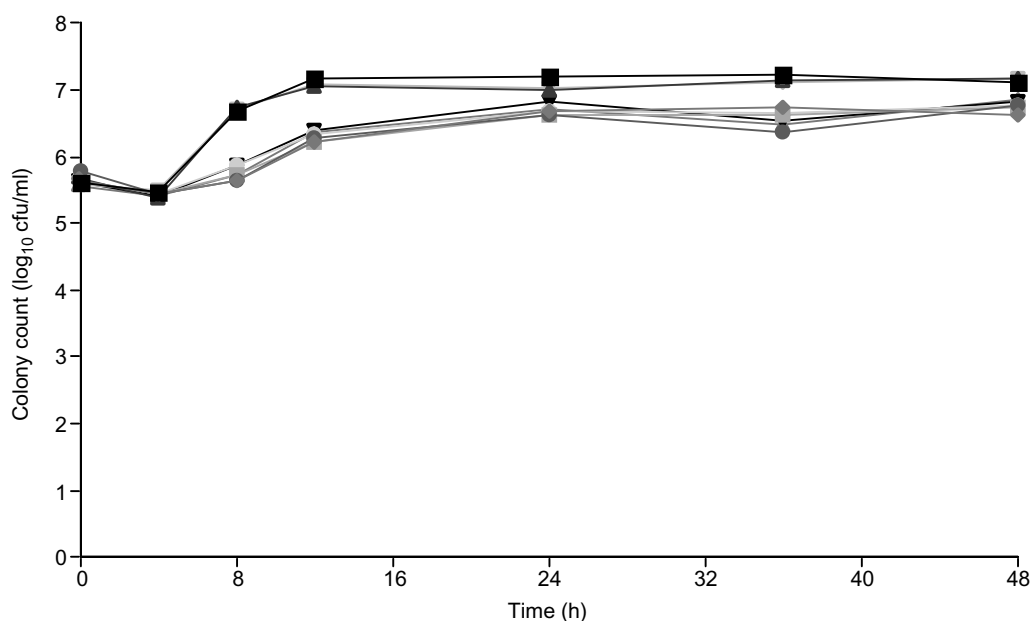


Fig. 1. Time-kill studies of fluconazole alone and in combination with fluvastatin or pravastatin against *C. albicans* 99-506. ■, growth control; ◆, fluconazole 10 mg/L; ●, fluconazole 30 mg/L; ▲, pravastatin 0.25 mg/L; ▼, fluvastatin 1 mg/L; ■, fluconazole 10 mg/L + pravastatin; ●, fluconazole 30 mg/L + pravastatin; ▼, fluconazole 10 mg/L + fluvastatin; ▲, fluconazole 30 mg/L + fluvastatin.

Discussion

Nosocomial bloodstream infections are an important cause of morbidity and mortality in the USA. Between 1984 and 1989, the National Nosocomial Infections Surveillance of the Centers for Disease Control showed a 10-fold increase in the incidence of candidaemia among hospitalised patients [8]. Today, many hospitals report that *C. albicans* isolates account for 8–15% of all hospital-acquired bloodstream infections [9, 10]. *Candida* spp. are now the fourth leading cause of nosocomial bloodstream infections and have the highest mortality (40%) of all nosocomial bloodstream infections [9]. The explanation of this dramatic increase in fungal infections is multifactorial and includes the use of broad-spectrum antibacterial agents, central vascular catheters and surgical procedures. Other important factors related to the increased incidence of candidaemia are improvements in medical therapies and technologies that allow for the increased survival of chronically immunosuppressed patients and the increased use of immunosuppressive agents [9, 10].

In the 1950s, the sole systemic drug for the treatment of fungal diseases was amphotericin B. Gradually, other therapies were developed such as 5-fluorocytosine in the 1970s and the azoles in the 1980s and 1990s. Since the introduction of these agents, some species of *Candida* now exhibit intrinsic antimicrobial resistance, leading to the need to develop new therapies for treating these infections [1]. The possibility that HMG-CoA reductase inhibitors could exhibit synergy in combination with azoles would help provide an alternative regimen for treatment.

The study by Chin *et al.* found that higher concentrations of fluvastatin (16–>128 mg/L) caused 80% inhibition of *C. albicans* growth and concentrations of 64–128 mg/L were fungicidal (>99.9% killing). When used in combination with fluconazole, the activity of fluvastatin against *C. albicans* was 4–8-fold greater. Synergy testing between fluvastatin and fluconazole or itraconazole demonstrated fungicidal activity with both combinations [2]. In the present study, when fluvastatin and pravastatin were used in clinically relevant concentrations (1 mg/L and 0.25 mg/L, respectively) synergy between fluconazole and the statins was not seen.

In time-kill studies, Chin *et al.* showed fluconazole 16 mg/L to be fungistatic against *C. albicans*, but the addition of fluvastatin 4 mg/L to fluconazole 4 mg/L displayed fungicidal activity. Killing curves show undetectable cfu/ml with the combination of agents, but the azole alone displayed growth after a few hours of fungistatic activity. Time-kill studies in the present study found no change in the fungistatic activity of fluconazole at either concentration (10 and 30 mg/L) in combination with fluvastatin or pravastatin. Combina-

tion testing did not result in fungicidal activity at any concentration or in any combination.

If the findings of Chin *et al.* could have been confirmed with clinically relevant in-vivo concentrations of pravastatin and fluvastatin, combination azole and statin therapy may have provided a unique treatment option for resistant fungi. Demonstration of synergy with this combination would require clinical trials to confirm laboratory observations. Unfortunately, there appears to be little reason to pursue clinical testing at this time.

Klepser *et al.* looked at the concentration-response characteristics of fluconazole against *C. albicans* and observed a small range of concentrations in which improvement in antifungal activity was seen (0.5–2 × MIC) [11]. MICs used in their study were either ≤0.125 or 0.25 mg/L whereas those in the present study were much higher. Previous studies from this laboratory looked at time-kill studies for three isolates of *C. albicans* [12]. Isolates were classified as susceptible, susceptible-dose dependent and resistant to fluconazole, which are similar to the isolates used in this study. Concentrations ≤0.5 × MIC did not show a response in any of the studies mentioned above, including the present study, and fungistasis was noted in concentrations >2 × MIC.

In summary, the results of this study suggest that statins do not affect the activity of fluconazole against *C. albicans*, as evaluated by the checkerboard assay and time-kill studies. The lack of a positive interaction between fluconazole and the statins is probably explained by lower concentrations of statins used in this study.

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