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Antifungal Combinations against *Candida albicans* Biofilms In Vitro

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***Candida* biofilms display increased resistance to most antifungal agents. We have evaluated the efficacy of combinations of fluconazole (FLC), amphotericin B, and caspofungin (CSP) against *Candida albicans* biofilms in vitro. Indifference was observed for all the combinations of paired antifungal agents when a checkerboard titration method was used. Time-kill experiments revealed an antagonistic effect of high FLC doses with CSP.**

Candida albicans readily forms biofilms, consortia of cells that coexist as an organized community, attached to a solid substratum that is enveloped within an exopolysaccharide matrix (4, 12). Biofilms are a well-described phenomenon which have gained notoriety from their ability to resist antimicrobials and immune cell challenge (4, 12).

In this study, we initially assessed the effects of antifungal combinations on biofilms by a checkerboard microdilution method with biofilms formed on the wells of microtiter plates and an XTT-based colorimetric assay (10). From these experiments we calculated the $SMIC_{80}$ for each drug alone and in combination, the fractionary inhibitory concentration (FIC), and the FIC indices of the paired combinations of antifungal agents. By use of the interpretation of FICs recommended by Hindler (6), indifference (FIC index of >0.5 to ≤ 4) was observed for all combinations of paired antifungal agents. Fluconazole (FLC) did not alter amphotericin B (AMB) activity, resulting in an FIC index of 1.00. The combination of AMB and caspofungin (CSP) showed an FIC index of 0.56, indicating indifference with a trend towards additivism. A calculated FIC index of 2.00 for the FLC-CSP combination also indicated indifference but with a trend towards antagonism which was evident at high FLC concentrations.

The interactions observed in the checkerboard microtiter plate testing combining the different antifungal agents were confirmed in time-kill curve experiments according to the methodology described before by our group (11). Log plots of decreased biofilm viability versus time for the different combinations used are presented in Fig. 1. When FLC at a concentration of 16 μg/ml was combined with 2 μg of AMB/ml, we found a nearly identical killing curve compared to that of AMB alone at 2 μg/ml. FLC at a dose of 64 μg/ml slightly inhibited AMB at 2 μg/ml. FLC at either concentration slightly decreased the effect of AMB at 0.5 μg/ml (Fig. 1A). There was no difference when AMB was used at a concentration of 2 μg/ml alone or in combination with CSP at concentrations of 0.125 or 0.5 μg/ml, and only a slight, nonsignificant improvement of AMB at 0.5 μg/ml in combination with either CSP concentration was observed (Fig. 1B). The time-kill kinetics of the combination of FLC and CSP showed a clear inhibitory effect compared to that for CSP alone with nearly identical killing curves for all tested concentration combinations (Fig. 1C).

Previous studies by our group and others have demonstrated lack of activity of FLC against *C. albicans* biofilms, increased resistance to AMB, and efficacy of CSP against *C. albicans* biofilms (1–5, 7, 9–12). The expanding armamentarium of antifungal drugs including agents with different molecular targets should also open new possibilities for exploring the usefulness of combination therapy. In the present study we have examined the effects and interactions of AMB, FLC, and CSP used in combination for the treatment of *C. albicans* biofilms in vitro. A checkerboard broth microdilution method was used to examine the effects of antifungal combinations against *C. albicans* biofilms. In general, these experiments pointed towards indifference for all antifungal combinations tested. Results of the effects of antifungal combinations were confirmed using time-kill methods. Because the FLC concentrations used in these experiments were high, the antagonistic effects of high FLC concentrations, particularly in combination with CSP, were evident. It was also observed that, even though combinations of AMB and CSP showed in general an indifferent effect, the use of these two agents in combination against *C. albicans* biofilms may still benefit from the rapid killing by high concentrations of AMB and the more sustained effect of physiological concentrations of CSP. This approach to therapy could be appealing in a clinical setting, particularly if biofilm resistance is due to the presence of a few "persister" cells able to withstand antimicro-

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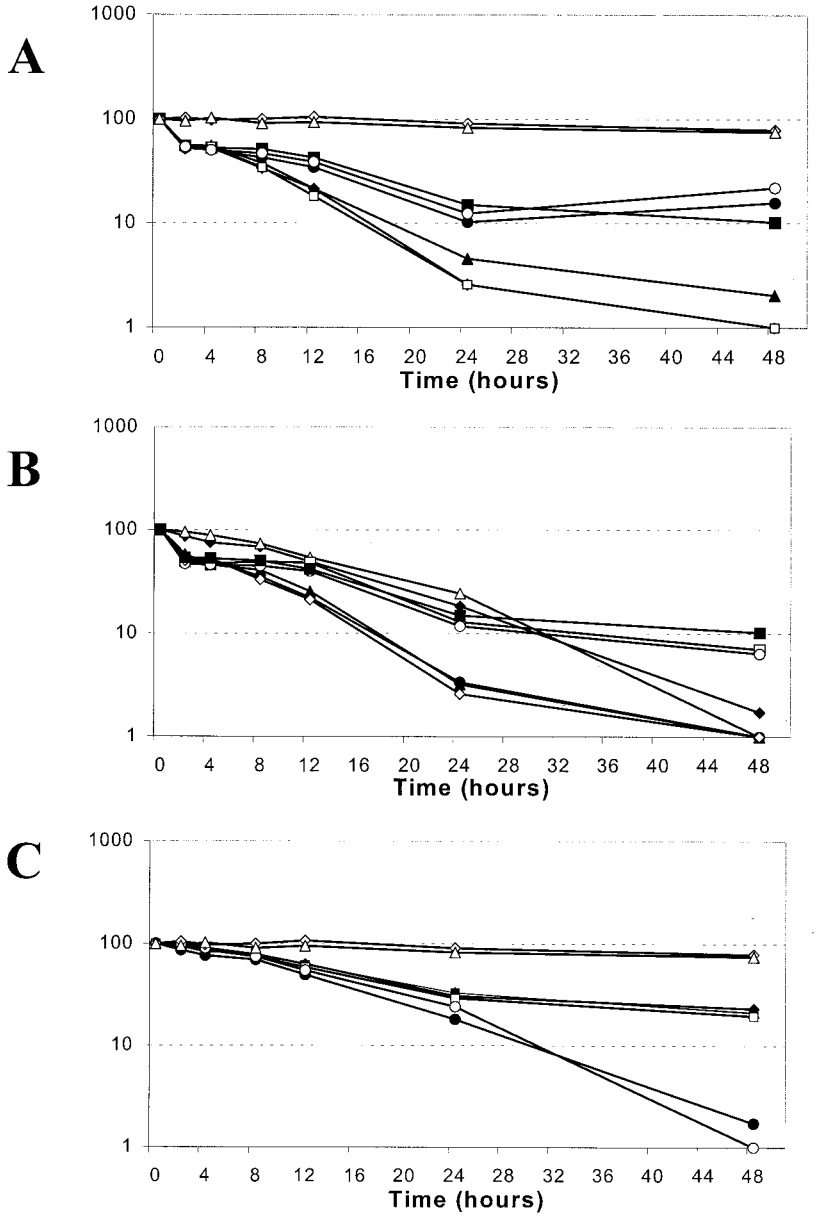

FIG. 1. (A) Log plots of killing kinetics of FLC-AMB combinations against preformed biofilms of *C. albicans* 3153A. Symbols: △, FLC (16 μg/ml); ◇, FLC (64 μg/ml); ○, FLC (16 μg/ml)-AMB (0.5 μg/ml); ●, FLC (64 μg/ml)-AMB (0.5 μg/ml); ◆, FLC (16 μg/ml)-AMB (2 μg/ml); ▲, FLC (64 μg/ml)-AMB (2 μg/ml); ■, AMB (0.5 μg/ml); □, AMB (2 μg/ml). (B) Log plots of killing kinetics of AMB-CSP combinations against preformed biofilms of *C. albicans* 3153A. Symbols: ■, AMB (0.5 μg/ml); ◇, AMB (2 μg/ml); □, AMB (0.5 μg/ml)-CSP (0.125 μg/ml); ○, AMB (0.5 μg/ml)-CSP (0.5 μg/ml); ▲, AMB (2 μg/ml)-CSP (0.125 μg/ml); ●, AMB (2 μg/ml)-CSP (0.5 μg/ml); ◆, CSP (0.125 μg/ml); △, CSP (0.5 μg/ml). (C) Log plots of killing kinetics of FLC-CSP combinations against preformed biofilms of *C. albicans* 3153A. Symbols: △, FLC (16 μg/ml); ◇, FLC (64 μg/ml); ▲, FLC (16 μg/ml)-CSP (0.125 μg/ml); ◆, FLC (64 μg/ml)-CSP (0.125 μg/ml); □, FLC (16 μg/ml)-CSP (0.5 μg/ml); ■, FLC (64 μg/ml)-CSP (0.5 μg/ml); ●, CSP (0.125 μg/ml); ○, CSP (0.5 μg/ml). Results are mean values for each condition tested and are expressed as percentages of reduction in absorbance by the XTT assay. Standard deviations were within 5% of the mean values and are not depicted in the graphs.

bial treatment, as suggested by other authors for bacterial biofilms (8).

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REFERENCES

1. **Bachmann, S. P., K. VandeWalle, G. Ramage, T. F. Patterson, B. L. Wickes, J. R. Graybill, and J. L. López-Ribot.** 2002. In vitro activity of caspofungin

against *Candida albicans* biofilms. Antimicrob. Agents Chemother. **46:**3591–3596.
2. **Baillie, G. S., and L. J. Douglas.** 1999. *Candida* biofilms and their susceptibility to antifungal agents. Methods Enzymol. **310:**644–656.
3. **Chandra, J., P. K. Mukherjee, S. D. Leidich, F. F. Faddoul, L. L. Hoyer, L. J. Douglas, and M. A. Ghannoum.** 2001. Antifungal resistance of candidal biofilms formed on denture acrylic in vitro. J. Dent. Res. **80:**903–908.
4. **Douglas, L. J.** 2003. *Candida* biofilms and their role in infection. Trends Microbiol. **11:**30–36.
5. **Hawser, S. P., and L. J. Douglas.** 1995. Resistance of *Candida albicans* biofilms to antifungal agents in vitro. Antimicrob. Agents Chemother. **39:** 2128–2131.
6. **Hindler, J.** 1995. Antimicrobial susceptibility testing, p. 5.18.11–15.18.20. *In* H. D. Isenberg (ed.), Clinical microbiology procedures handbook. American Society for Microbiology, Washington, D.C.
7. **Kuhn, D. M., T. George, J. Chandra, P. K. Mukherjee, and M. A. Ghannoum.** 2002. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. Antimicrob. Agents Chemother. **46:**1773–1780.
8. **Lewis, K.** 2001. Riddle of biofilm resistance. Antimicrob. Agents Chemother. **45:**999–1007.
9. **Ramage, G., S. Bachmann, T. F. Patterson, B. L. Wickes, and J. L. López-Ribot.** 2002. Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. J. Antimicrob. Chemother. **49:**973–980.
10. **Ramage, G., K. Vande Walle, B. L. Wickes, and J. L. López-Ribot.** 2001. Standardized method for in vitro antifungal susceptibility testing of *Candida albicans* biofilms. Antimicrob. Agents Chemother. **45:**2475–2479.
11. **Ramage, G., K. VandeWalle, S. P. Bachmann, B. L. Wickes, and J. L. López-Ribot.** 2002. In vitro pharmacodynamic properties of three antifungal agents against preformed *Candida albicans* biofilms determined by time-kill studies. Antimicrob. Agents Chemother. **46:**3634–3636.
12. **Ramage, G., B. L. Wickes, and J. L. López-Ribot.** 2001. Biofilms of *Candida albicans* and their associated resistance to antifungal agents. Am. Clin. Lab. **20:**42–44.