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In Vitro Kill Curves of a New Semisynthetic Echinocandin, LY-303366, against Fluconazole-Sensitive and -Resistant *Candida* Species

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In vitro killing by a new semisynthetic echinocandin, LY-303366, was characterized using clinical isolates of fluconazole-sensitive (Y58) and -resistant (Y180) *Candida albicans* as well as *Candida glabrata* (Y7) and *Candida krusei* (Y171). The 24-h kill curves for Y58 and Y180 demonstrated dose-independent killing of between 1 and 2 $\log_{10}$ with LY-303366 at concentrations of 0.1, 1, 10, 50, 100, and 1,000 times the MIC. Regrowth did not occur at 24 h with either *C. albicans* isolate at the aforementioned LY-303366 concentrations. At their MICs, LY-303366 and amphotericin B produced similar killing kinetics in cultures of Y58, Y180, Y7, and Y171, while all cultures exposed to fluconazole at its MIC demonstrated stasis or growth over 24 h.

LY-303366 is a novel semisynthetic echinocandin antifungal agent that acts as a potent noncompetitive inhibitor of (1,3)-β-D-glucan synthase, an enzyme essential to the structural integrity of the fungal cell wall (3, 5). LY-303366 has demonstrated excellent in vitro (7, 9, 10, 15) and in vivo (2, 12–15) activity against *Aspergillus* species, *Pneumocystis carinii*, and fluconazole-sensitive and -resistant *Candida* species. Given the dramatic rise in *Candida* infections (1), the emergence of previously uncommon fungal pathogens (8), and the therapeutic and spectral limitations of the few presently available antifungal agents (11), the development and characterization of new antifungal agents such as LY-303366 are imperative. The purpose of this study was to characterize in vitro killing by LY-303366 by constructing 24-h kill curves for both fluconazole-sensitive and -resistant *Candida albicans* as well as *Candida glabrata* and *Candida krusei*. In addition, the killing kinetics of LY-303366, fluconazole, and amphotericin B were compared.

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Stock solutions of LY-303366 (Lilly Research Laboratories, Indianapolis, Ind.), amphotericin B (Bristol-Myers Squibb Canada, Saint-Laurent, Quebec, Canada), and fluconazole (Pfizer Canada, Kirkland, Quebec, Canada) were prepared from standard powders with dimethyl sulfoxide as the diluent. RPMI 1640 medium (Sigma Chemical Company, St. Louis, Mo.) was used for antifungal agent susceptibility testing (6) and kill curve experiments. Sabouraud dextrose agar (Becton Dickinson, Cockeysville, Md.) was used to subculture *Candida* isolates and perform colony counts.

Two clinical isolates (Y58 [fluconazole sensitive] and Y180 [fluconazole resistant]) of *C. albicans*, one isolate of *C. glabrata* (Y7), and one isolate of *C. krusei* (Y171) were obtained from patient stocks collected by the Department of Clinical Microbiology at the Health Sciences Centre in Winnipeg, Manitoba, Canada. Isolates were stored at −80°C in skim milk and subcultured twice on Sabouraud dextrose agar before use. Each strain of *Candida* was tested for susceptibility to LY-303366, amphotericin B, and fluconazole by the National Committee for Clinical Laboratory Standards M27-T macrodilution reference method (6). The LY-303366 MIC end point was defined as the lowest concentration of LY-303366 that resulted in 100% inhibition of visible growth. Antifungal agent susceptibility tests were repeated in triplicate on separate occasions.

Kill curve construction began with the inoculation of 20 ml of RPMI 1640 medium with two or three *Candida* colonies taken from a Sabouraud dextrose agar plate. The culture was incubated with shaking for approximately 16 h at 37°C. One-milliliter aliquots of the culture (approximately 10^7 CFU/ml) were then added to a growth control flask containing 9 ml of RPMI 1640 medium and to test flasks containing 8.9 ml of RPMI 1640 medium plus 0.1 ml of antifungal agent concentrate. LY-303366 was tested at concentrations of 0.001, 0.01, 0.1, 1, 10, 50, 100, and 1,000 times the MIC for both the fluconazole-sensitive (Y58) and -resistant (Y180) *C. albicans* isolates. The comparators, amphotericin B and fluconazole, were tested against all four *Candida* species isolates at their MICs. Initial colony counts were performed on all cultures, the antifungal agent was added, and the flasks were incubated at 37°C with shaking. Aliquots of 100 μl were removed from cultures at 1, 2, 3, 4, 5, 6, 12, and 24 h, serially diluted in cold (4°C) sterile 0.85% NaCl, and plated onto Sabouraud dextrose agar. Plates were incubated at 35°C for 18 h, and numbers of viable colonies were recorded. Experiments were performed in triplicate on separate occasions. Kill curves were constructed by plotting mean $\log_{10}$ CFU per milliliter versus time. Log_{10} kill values for LY-303366, amphotericin B, and fluconazole, at their MICs, were calculated after 24 h of incubation with the antimicrobial agent, with a positive value indicating killing and a negative value representing growth relative to the original inoculum.

The antifungal agent MICs for isolates Y58, Y180, Y7, and Y171 are presented in Table 1. All four isolates possessed similar susceptibilities to LY-303366 and amphotericin B, but

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TABLE 1. MICs of LY-303366, amphotericin B, and fluconazole for four clinical isolates of *Candida* species

Candida isolate	MIC (μg/ml) of:		
	LY-303366	Amphotericin B	Fluconazole
C. albicans Y58	0.04	0.5	0.5
C. albicans Y180	0.04	0.5	128
C. glabrata Y7	0.08	1	32
C. krusei Y171	0.16	1	32

the fluconazole susceptibilities of isolates Y180, Y7, and Y171 were significantly lower than that of isolate Y58 (Table 1).

Kill curves for the two isolates of *C. albicans*, Y58 and Y180, grown in cultures supplemented with antimicrobial agent concentrations ranging from 0.001 to 1,000 times the MIC are presented in Fig. 1. LY-303366 displayed concentration-independent killing of 1 to 2 $\log_{10}$ CFU/ml for all concentrations tested between 0.1 and 1,000 times the MIC for both the fluconazole-sensitive (Y58) and -resistant (Y180) strains of *C. albicans*. LY-303366 at a concentration of 0.001 times the MIC did not appear to significantly affect the growth of either Y58 or Y180 (Fig. 1). Regrowth was not seen at 24 h in cultures of either Y58 or Y180 at LY-303366 concentrations greater than or equal to 0.1 times the MIC (Fig. 1).

Figure 2 depicts fungal killing with LY-303366, amphotericin B, and fluconazole at their MICs. LY-303366 and amphotericin B killing followed similar kinetics over 24 h for both fluconazole-sensitive (Y58) and -resistant (Y180) *C. albicans* (Fig. 2) as well as *C. glabrata* (Y7) and *C. krusei* (Y171) (data not shown). For all four *Candida* isolates, LY-303366 and amphotericin B, at their MICs, each resulted in an approximately 1.5 $\log_{10}$ kill which reached a maximum after 5 to 6 h of exposure, and there was no regrowth at 24 h (Fig. 2 and data not shown). LY-303366 and amphotericin B demonstrated significantly greater killing than fluconazole against all four *Candida* isolates (Fig. 2 and data not shown). At 24 h, LY-303366 and amphotericin B demonstrated similar residual inocula, while fluconazole cultures showed stasis or growth for the entire 24 h (Table 2).

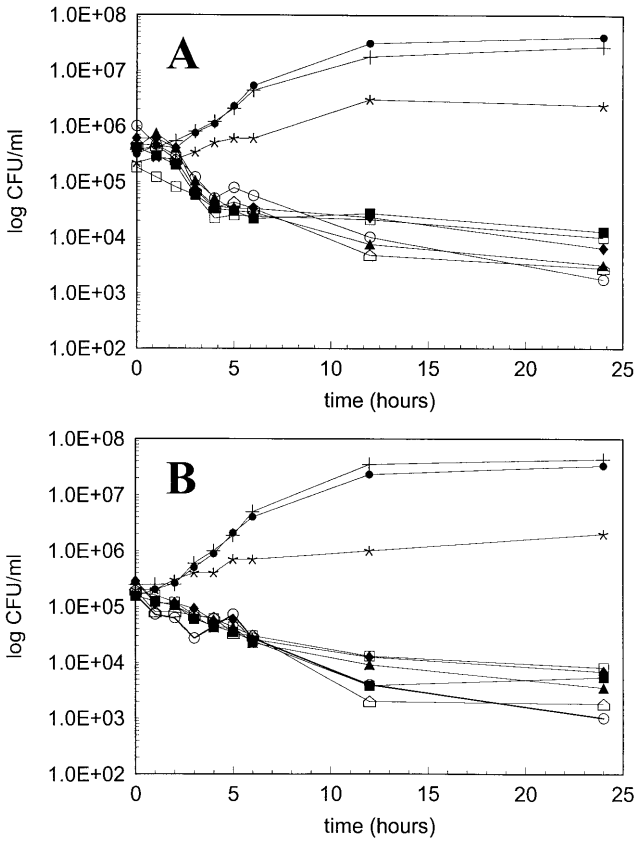

FIG. 1. Twenty-four-hour fungal kill curves for LY-303366 at concentrations equivalent to 0.001, 0.01, 0.1, 1, 10, 50, 100, and 1,000 times the MIC against fluconazole-sensitive *C. albicans* Y58 (A) and fluconazole-resistant *C. albicans* Y180 (B). Symbols: ●, growth control; +, MIC × 0.001; ★, MIC × 0.01; □, MIC × 0.1; ■, MIC × 1; ◆, MIC × 10; ▲, MIC × 50; ⬠, MIC × 100; ○, MIC × 1,000.

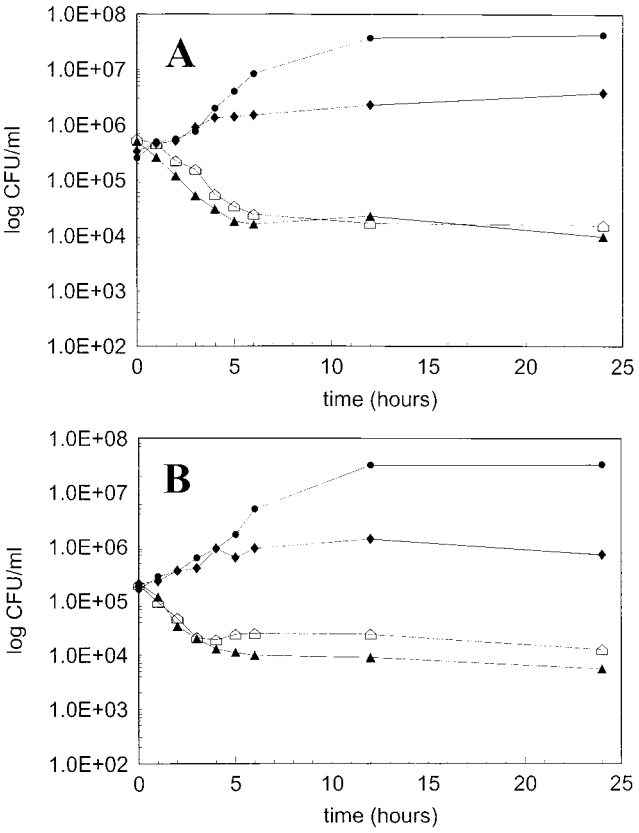

FIG. 2. Twenty-four-hour fungal kill curves for LY-303366, amphotericin B, and fluconazole at concentrations equivalent to their MICs against fluconazole-sensitive *C. albicans* Y58 (A) and fluconazole-resistant *C. albicans* Y180 (B). Symbols: ●, growth control; ◆, fluconazole; ⬠, LY-303366; ▲, amphotericin B.

Discussion. LY-303366 is a new semisynthetic echinocandin antifungal agent which has demonstrated significant in vitro and in vivo activity against fluconazole-sensitive and -resistant *Candida* species, *Aspergillus* species, and *Pneumocystis carinii* (2, 7, 9, 10, 12–14). LY-303366 demonstrated dose-independent killing of 1 to 2 $\log_{10}$ against *C. albicans*. LY-303366 was equally active against fluconazole-susceptible and -resistant *C. albicans* strains. In addition, against fluconazole-sensitive and -resistant *Candida* species, LY-303366 demonstrated killing activity similar to that of amphotericin B and significantly greater than that of fluconazole (Fig. 2). However, it has been previously demonstrated that amphotericin B, unlike LY-303366, results in concentration-dependent killing at concentrations greater than the MIC (4).

TABLE 2. Killing of *Candida* species cultures determined after 24 h of incubation with LY-303366, amphotericin B, or fluconazole at the MIC

Candida isolate	Log_{10} CFU killed/ml with[a]:		
	LY-303366	Amphotericin B	Fluconazole
C. albicans Y58	1.31 ± 0.25	1.67 ± 0.41	−1.41 ± 0.16
C. albicans Y180	1.22 ± 0.06	1.68 ± 0.16	−1.03 ± 0.44
C. glabrata Y7	1.53 ± 0.18	1.51 ± 0.24	−1.21 ± 0.24
C. krusei Y171	1.56 ± 0.19	1.31 ± 0.30	−1.32 ± 0.12

[a] Log_{10} CFU killed per milliliter following 24 h of incubation with the antifungal agent was calculated as the difference between the 24-h value and that of the initial inoculum. A positive log_{10} value indicates killing, while a negative log_{10} value represents growth.

The observation that killing by LY-303366 at 0.1 times the MIC is similar to that at the MIC and multiples thereof suggests that the definition of the LY-303366 MIC end point as no visible growth may underestimate the antifungal activity of LY-303366. Ernst and coworkers have recently published a similar report demonstrating that LY-303366 at one-eighth its MIC resulted in killing activity similar to that at the MIC in isolates of *Candida* species, using identical MIC end point criteria (4). Clearly, an alternative LY-303366 MIC end point which accurately reflects the antifungal activity of this compound must be chosen. Ernst and coworkers have suggested that a clearer relationship between MIC and antifungal activity may be obtained by using antibiotic medium 3 instead of RPMI 1640 (4).

Our work suggests, as does the work of other investigators (4), that LY-303366 has potential as a fungicidal alternative to amphotericin B in the treatment of infections with fluconazole-sensitive and -resistant *Candida* species. Further in vitro and in vivo characterization of LY-303366 is warranted.

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