

Pneumocandin L-743,872 Enhances the Activities of Amphotericin B and Fluconazole against *Cryptococcus neoformans* In Vitro

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Cryptococcus neoformans infections in patients with AIDS are often incurable, despite aggressive antifungal therapy. Combination regimens with additive or synergistic drugs could provide additional options for treating cryptococcal meningitis. We evaluated the efficacy of combination therapies using L-743,872, a pneumocandin antifungal drug, and amphotericin B or fluconazole against 18 strains of *C. neoformans*, including 11 *C. neoformans* var. *neoformans*, 3 *C. neoformans* var. *gattii*, and 4 fluconazole-resistant isolates. The combination of subinhibitory concentrations of L-743,872 with amphotericin B significantly enhanced amphotericin B activity against *C. neoformans* as measured by turbidity (antifungal susceptibility studies using the National Committee of Clinical and Laboratory Standards method), quantitative CFU, and tetrazolium salt reduction assays. Similarly, the addition of subinhibitory concentrations of L-743,872 to fluconazole enhanced fluconazole activity, but the effect was less dramatic than for the pneumocandin-amphotericin B combination. A marked synergism was observed in all combinations of amphotericin B and L-743,872 (fractional inhibitory concentration index [FIC] of ≤ 0.5). Fluconazole-resistant strains showed a susceptibility to amphotericin B and L-743,872 which was comparable to that of susceptible isolates. Combinations of pneumocandin with fluconazole revealed different activities for the various strains, including synergism (FIC < 1.0), additivity (FIC = 1.0), and autonomy (FIC between 1.0 and 2.0). Combination studies with fluconazole and L-743,872 showed additive and autonomous activities against fluconazole-resistant isolates. No antagonistic interactions (FIC > 2.0) were observed for any combination of L-743,872 with either amphotericin B or fluconazole. The results of this study suggest that L-743,872 can enhance the efficacy of fluconazole or amphotericin B in vitro and indicate a potential role for L-743,872 in combination therapy against *C. neoformans*.

Cryptococcus neoformans is an opportunistic fungal pathogen which causes life-threatening meningoencephalitis in approximately 5 to 10% of patients with AIDS (4, 18). Cryptococcal meningoencephalitis in the setting of AIDS is usually incurable and has a high mortality despite aggressive therapy (15). Standard therapy for cryptococcosis remains amphotericin B with or without 5-flucytosine (5, 6). AIDS patients with *C. neoformans* infections who survive the initial presentation are treated with lifelong suppressive therapy to reduce the likelihood of recurrent infection. For suppression therapy fluconazole is presently the agent of choice (6, 16). DNA typing analysis of initial and relapse isolates from patients with recurrent *C. neoformans* meningoencephalitis consistently reveals persistence of the same strain despite antifungal therapy (3, 15). This implies that *C. neoformans* meningoencephalitis is incurable in patients with AIDS because antifungal therapy does not eradicate the infection. The difficulties involved in the therapy of cryptococcosis indicate an urgent need for new therapeutic strategies.

Recently, a new class of powerful antifungal agents has become available: the pneumocandins. These compounds are cyclic hexapeptides which inhibit cell wall 1,3- β -D-glucan synthesis (2). Some pneumocandin derivatives (L-705589, L-731373, and L-733560) are fungicidal in vitro and have a potent in vivo activity against *Candida* spp., *Pneumocystis carinii*, and *Aspergillus fumigatus*. However, these pneumocandin

derivatives have weak activity against *C. neoformans*, both in vitro and in vivo (1, 2).

Since pneumocandins have a different mechanism of action than either amphotericin B or fluconazole, we hypothesized that pneumocandin-amphotericin B and pneumocandin-fluconazole combinations may be more active against *C. neoformans* than amphotericin B or fluconazole alone. In this study pneumocandin L-743,872 was shown to enhance the activities of both amphotericin B and fluconazole against *C. neoformans* in vitro.

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MATERIALS AND METHODS

Strains. Eighteen clinical and environmental *C. neoformans* strains were included in this study. Six clinical isolates were obtained from cerebrospinal fluid of AIDS patients from the Bronx, New York (J10, J11, and J15), and from Belo Horizonte, Minas Gerais, Brazil (C1, C2, and C3). Four environmental isolates were obtained from pigeon excreta collected in the Bronx, New York (B5 and B10), and Belo Horizonte, Minas Gerais, Brazil (E1 and E2). These 10 isolates were assigned to *C. neoformans* var. *neoformans* on the basis of no color change on canavanine-glycine-bromothymol blue agar (10). *C. neoformans* ATCC 24067 serotype D was obtained from the American Type Culture Collection (ATCC; Rockville, Md.). In addition, three strains of *C. neoformans* var. *gattii* (NIH 34, ATCC 24065, and ATCC 32608) were also studied. Four fluconazole-resistant isolates (96-734, 96-806, 96-824, and 96-1034) were generously provided by Deanna Sutton, Fungus Testing Laboratory, The University of Texas Health Science Center at San Antonio. All the strains were maintained on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) slants at 4°C and were grown in Sabouraud dextrose broth (Difco) at 30°C overnight before each experiment.

Antifungal drugs. Pneumocandin L-743,872 was synthesized by the Department of Medicinal Chemistry at Merck Research Laboratories, Rahway, N.J. Fluconazole was provided by Roerig-Pfizer (New York, N.Y.). Amphotericin B was obtained from Boehringer GmbH (Mannheim, Germany). Stock solutions of L-743,872 and fluconazole were prepared in sterile distilled water (5 mg/ml). Fresh solutions were used for all assays.

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Antifungal susceptibility assays. The standard method for antifungal susceptibility testing proposed by the National Committee for Clinical Laboratory Standards (NCCLS; document M27-P) was used (12). Briefly, in vitro susceptibility testing was performed by a broth macrodilution technique in RPMI 1640 medium (Sigma Chemical Co., St. Louis, Mo.) containing L-glutamine, without bicarbonate, and buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS). Serial twofold dilutions of each drug stock solution were prepared at 10 times the strength of the final drug concentration, and 0.1-ml aliquots were dispensed in polystyrene plastic tubes (12 by 75 mm; Falcon 2054, Becton Dickinson, Lincoln Park, N.J.). Inocula of 500 to 2,500 cells/ml were prepared and incubated at 35°C for 72 h. Final drug concentrations ranged from 128 to 0.03 µg/ml for fluconazole and L-743,872 and from 2 to 0.0075 µg/ml for amphotericin B. Amphotericin B and L-743,872 minimal inhibitory concentrations (MICs) were defined as the lowest drug concentration at which there was an absence of growth. Fluconazole MIC was defined as the lowest drug concentration which resulted in a visual turbidity less than or equal to 80% inhibition compared with that produced by the control without antifungal agent.

Combination testing was performed by the checkerboard method recommended by the NCCLS (12). Instead of using 0.1-ml aliquots of each drug at 10× (as was done with the single-drug experiments), we used 50-µl aliquots of each drug at 20 times the targeted final concentration. The rest of the protocol was unaltered. The efficacies of combinations of fluconazole or amphotericin B with pneumocandin L-743,872 were evaluated. Drug interaction was classified as either synergistic, additive, autonomous, or antagonistic based on the fractional inhibitory concentration (FIC) index. The FIC index is the sum of the FICs for each drug, and the FIC is calculated as follows: for drug A the FIC equals the MIC of drug A in combination divided by the MIC of drug A alone. Drug-drug interactions are considered synergistic if the FIC index is less than 1.0, additive if the FIC is equal to 1.0, autonomous (indifferent) if the FIC index is between 1 and 2, and antagonistic if the FIC index is greater than 2 (7, 13). Synergism was further subclassified as strong (FIC ≤0.50) and weak (FIC between 0.50 and 1.0) (13).

After the MICs were recorded, aliquots of 100 µl of each tube were spread on Sabouraud dextrose agar plates (Difco) to determine the number of colonies per milliliter (1 colony = 1 CFU). Plates were counted after incubation for 72 h at 35°C. CFU counts were performed only for the strain ATCC 24067.

XTT reduction assay. XTT assays were performed as described (11). A fresh solution of XTT [(2,3)-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide; Sigma] was prepared by dissolving 0.5 mg/ml in phosphate-buffered saline (PBS), with heating at 60°C for 30 min. Coenzyme Q was added to a final concentration of 40 µg/ml. Twofold dilutions of each drug, alone or in combination, were prepared in sterile PBS and added to a suspension of 3.0×10^6 *C. neoformans* cells per ml for 1 h at 37°C. Only strain ATCC 24067 was tested by this assay. Following incubation, the cells were collected by centrifugation at $3,000 \times g$ for 7 min at 4°C, and the cell pellets were washed with 1 ml of distilled water. Aliquots of freshly prepared XTT (0.4 ml) were then added to each tube. The tubes were incubated for 1 h at 37°C, and then 100 µl from each supernatant was placed in a well of a 96-well enzyme-linked immunosorbent assay plate (Corning, New York). A_{450} s were measured with a microplate reader (Ceres 900Hdi; Bio-Tek Instruments, Inc., Winooski, Vt.). The percentage of fungal cell damage was calculated by the equation $[1 - (A_{450} \text{ C. neoformans incubated with drugs}/A_{450} \text{ C. neoformans incubated without drugs})] \times 100$. Control tubes contained *C. neoformans* cells in PBS. For drug interaction studies, amphotericin B (at concentrations ranging from 0.03 to 1.0 µg/ml) and fluconazole (ranging from 0.25 to 4 µg/ml) were studied in combination with a single subinhibitory concentration of pneumocandin L-743,872: 8 µg/ml in the case of amphotericin B and 16 µg/ml in the case of fluconazole.

Statistical analysis. The results were obtained as means and standard deviations of at least three repetitions carried out for each compound, alone and in combination. Statistical significance was evaluated by analysis of variance (ANOVA) followed by Bonferroni *t* test. Significance values calculated with parametric methods were confirmed with nonparametric methods (Kruskal Wallis Test). A *P* value of less than 0.05 was considered to be significant. Statistical analysis was done using the Primer for biostatistics: the program (McGraw Hill Inc., New York, N.Y.).

RESULTS

Antifungal susceptibility data. The MIC ranges and the MICs required to inhibit 50 and 90% of the isolates (MIC₅₀s and MIC₉₀s, respectively) of amphotericin B, fluconazole, and L-743,872 are shown in Table 1. The combination of subinhibitory concentrations of amphotericin B with subinhibitory concentrations of L-743,872 resulted in a median eightfold reduction in both the amphotericin and L-743,872 MICs (range of 4- to 16-fold) (Table 2). All the interactions between amphotericin B and L-743,872 were strongly synergistic (FIC index ≤0.5) for both variety *gattii* and variety *neoformans* isolates as well as for fluconazole-resistant strains. When fluconazole

TABLE 1. In vitro activities of amphotericin B, fluconazole, and pneumocandin L-743,872 against 18 *C. neoformans* isolates

Antifungal agent	MIC (µg/ml) ^a		
	Range	50%	90%
Amphotericin B	0.0625–0.50	0.25	0.50
Fluconazole	1–128	4	32
Pneumocandin L-743,872	16–32	16	32

^a 50% and 90%, MICs at which 50 and 90% of the strains, respectively, are inhibited.

and L-743,872 were tested in combination, there was a median twofold reduction in the MICs of both drugs (range of one- to fourfold for both drugs) (Table 3). Twenty-two percent of the interactions were synergistic (FIC index = 0.75), 50% were additive (FIC index = 1.0), and the remaining 28% were autonomous (FIC index = 1.5 or 2.0). Combinations of fluconazole and L-743,872 against fluconazole-resistant isolates revealed additivity (two strains) or autonomy (two strains).

The effects of the combinations of subinhibitory concentrations of amphotericin B and L-743,872 on the CFU of strain ATCC 24067 are shown in Fig. 1. Marked reductions in CFU were observed when amphotericin B (at 0.0075, 0.015, and 0.03 µg/ml) was combined with L-743,872 (at 4, 8 and 16 µg/ml). For all combinations, the reduction in CFU was significantly greater (*P* < 0.05) than that observed with single drugs alone. Likewise, Fig. 2 shows the effects of combinations of subinhibitory concentrations of fluconazole (at 0.25, 0.50, and 1.0 µg/ml) and L-743,872 (at 4, 8, and 16 µg/ml) on the growth of *C. neoformans* ATCC 24067. For most combinations of L-743,872 and fluconazole, the addition of L-743,872 significantly enhanced the activity of fluconazole by reducing CFU (*P* < 0.05). At a fluconazole concentration of 1 µg/ml the addition of either 4 or 8 µg of L-743,872 per ml had no effect

TABLE 2. Mode of interaction of amphotericin B with pneumocandin L-743,872 in 18 *C. neoformans* isolates

Strain	MIC (µg/ml)				FIC		
	AMB ^a		L-743,872		Index ^b	AMB	L-743,872
	Alone	Com-bined	Alone	Com-bined			
24067	0.0625	0.015	32	4	0.38	0.25	0.125
J10	0.25	0.03	16	2	0.25	0.125	0.125
J11	0.25	0.03	32	4	0.25	0.125	0.125
J15	0.25	0.06	32	8	0.50	0.25	0.25
B5	0.25	0.03	32	2	0.19	0.125	0.06
B10	0.50	0.06	16	2	0.25	0.125	0.125
C1	0.25	0.03	16	2	0.25	0.125	0.125
C2	0.25	0.015	16	1	0.12	0.06	0.06
C3	0.25	0.03	32	4	0.25	0.125	0.125
E1	0.50	0.03	32	4	0.19	0.06	0.125
E2	0.50	0.03	32	4	0.19	0.06	0.125
NIH34 ^c	0.50	0.06	32	4	0.25	0.125	0.125
24065 ^c	0.25	0.06	16	4	0.50	0.25	0.25
32608 ^c	0.50	0.06	32	4	0.25	0.125	0.125
96-734 ^d	0.50	0.125	32	8	0.50	0.25	0.25
96-806 ^d	0.50	0.125	32	4	0.38	0.25	0.125
96-824 ^d	0.50	0.06	32	4	0.25	0.125	0.125
96-1034 ^d	0.50	0.06	16	2	0.25	0.125	0.125

^a AMB, amphotericin B.

^b Note that all the combinations were markedly synergistic (FIC index ≤0.5).

^c Variety *gattii* isolates.

^d Fluconazole-resistant isolates.

TABLE 3. Mode of interaction of fluconazole with pneumocandin L-743,872 in 18 *C. neoformans* isolates

Strain	MIC ($\mu\text{g/ml}$)				FIC		
	FLU ^a		L-743,872		Index ^b	FLU	L-743,872
	Alone	Com- bined	Alone	Com- bined			
24067	2	0.5	32	16	0.75	0.25	0.5
J10	1	0.5	16	8	1.0	0.5	0.5
J11	4	2	32	16	1.0	0.5	0.5
J15	2	2	32	16	1.5	1.0	0.5
B5	4	2	32	8	0.75	0.5	0.25
B10	2	0.5	16	8	0.75	0.25	0.5
C1	4	2	16	8	1.0	0.5	0.5
C2	1	0.5	16	8	1.0	0.5	0.5
C3	1	1	32	16	1.5	1.0	0.5
E1	4	1	32	16	0.75	0.25	0.5
E2	1	1	32	16	1.5	1.0	0.5
NIH34 ^c	1	0.5	32	16	1.0	0.5	0.5
24065 ^c	8	4	32	16	1.0	0.5	0.5
32608 ^c	8	4	16	8	1.0	0.5	0.5
96-734 ^d	32	16	32	16	1.0	0.5	0.5
96-806 ^d	64	32	16	16	1.5	0.5	1.0
96-824 ^d	32	16	32	16	1.0	0.5	0.5
96-1034 ^d	128	128	16	16	2.0	1.0	1.0

^a FLU, fluconazole.

^b Note that for 4 isolates the combination was synergistic (FIC index <1.0), for 9 isolates it was additive (FIC index = 1.0), and for 5 isolates it was autonomous or indifferent ($1.0 < \text{FIC index} \leq 2.0$).

^c Variety *gattii* isolates.

^d Fluconazole-resistant isolates.

on the number of CFU. However, the addition of 16 μg of L-743,872 per ml to 1 μg of fluconazole per ml significantly reduced CFU.

XTT reduction assay. Results obtained with XTT assays performed using strain ATCC 24067 are shown in Fig. 3 and 4.

The XTT reduction assay measures fungal cell damage. Pneumocandin alone at 8 $\mu\text{g/ml}$ produced no cell damage as measured by XTT reduction. At 16 $\mu\text{g/ml}$ the XTT assay indicated an average damage of 35%. The combination of increasing concentrations of amphotericin B (from 0.03 to 1 $\mu\text{g/ml}$) with 8 μg of L-743,872 per ml resulted in significant increases in the percentage of fungal cell damage compared to the effect of amphotericin B alone (Fig. 3). In contrast, incubation of cells treated with fluconazole in the presence of L-743,872 at 16 $\mu\text{g/ml}$ resulted in similar values of fungal cell damage. It is remarkable that, for all combinations, we observed that addition of pneumocandin (at 16 $\mu\text{g/ml}$) clearly enhanced the ability of fluconazole to damage *C. neoformans* cells (Fig. 4). Although the effect was independent of fluconazole concentration, the combination of fluconazole and pneumocandin augmented the damage level to 56 to 65% in comparison to the 35% damage level due to pneumocandin alone.

DISCUSSION

The addition of subinhibitory concentrations of the pneumocandin L-743,872 to amphotericin B significantly enhanced amphotericin B activity against *C. neoformans* as measured by turbidity, quantitative CFU, and XTT reduction assays. A marked synergistic interaction between amphotericin B and L-743,872 was observed for the 18 strains tested, regardless of whether the isolates were variety *gattii* or variety *neoformans* or resistant to fluconazole. Resistance to fluconazole did not alter susceptibility to amphotericin B or L-743,872 or the benefits obtained from the combination of these two drugs. Similarly, the addition of subinhibitory concentrations of L-743,872 to fluconazole enhanced fluconazole activity against *C. neoformans*, but the effect was less dramatic than for the pneumocandin-amphotericin B combination. A synergistic interaction was demonstrated only in 22% of the strains tested. However, combination of fluconazole and L-743,872 still enhanced fluconazole activity, as demonstrated by reduced CFU and in-

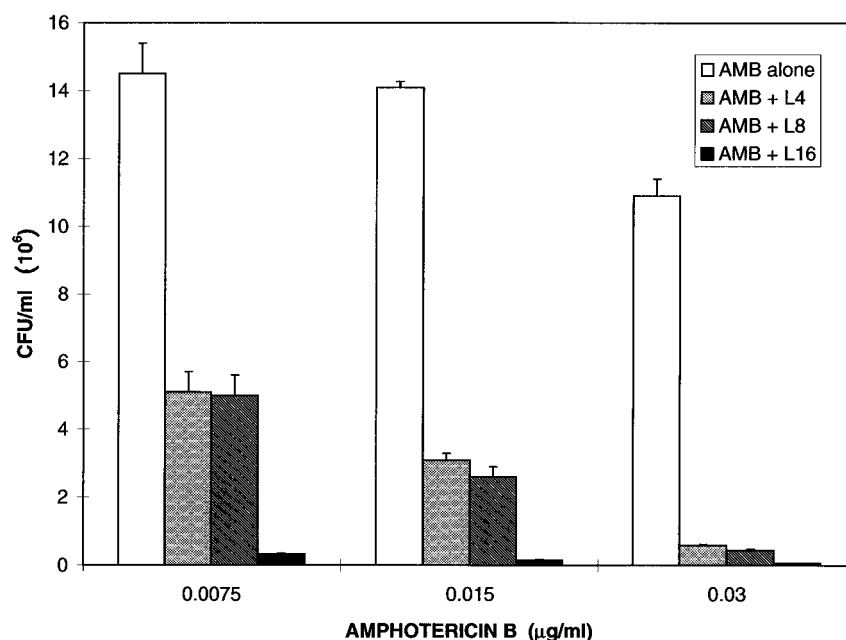


FIG. 1. Effects of amphotericin B (AMB) and pneumocandin L-743,872 on CFU of *C. neoformans* ATCC 24067. Pneumocandin L-743,872 concentrations were 4 $\mu\text{g/ml}$ (L4), 8 $\mu\text{g/ml}$ (L8), and 16 $\mu\text{g/ml}$ (L16). CFU were determined after 72 h of incubation under NCCLS conditions. *P* values were calculated by *t* test after significance by ANOVA was established. Data are the averages of three repetitions, and error bars denote standard deviations from the mean.

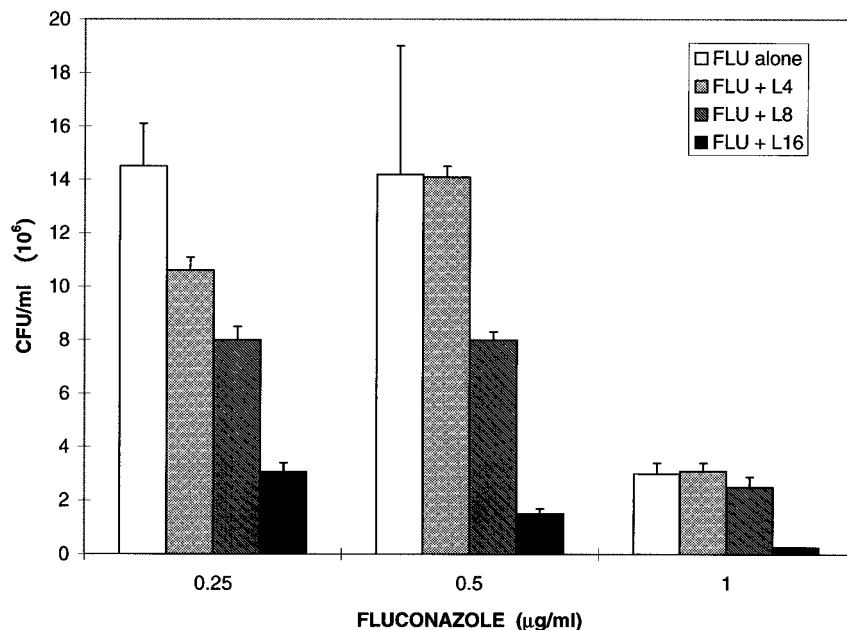


FIG. 2. Effects of fluconazole (FLU) and pneumocandin L-743,872 on CFU of *C. neoformans* ATCC 24067. Pneumocandin L-743,872 concentrations were 4 µg/ml (L4), 8 µg/ml (L8), and 16 µg/ml (L16). CFU were determined after 72 h of incubation under NCCLS conditions. *P* values were calculated by *t* test after significance by ANOVA was established. Data are the averages of three repetitions, and error bars denote standard deviations from the mean.

creased percentage of damage observed when fluconazole was combined with subinhibitory concentrations of L-743,872 against *C. neoformans* ATCC 24067 (Fig. 2 and 4). Combination of fluconazole and L-743,872 demonstrated an additive or autonomous mode of interaction for fluconazole-resistant strains. However, these effects were also shown by the combination of fluconazole and L-743,872 in fluconazole-susceptible isolates. No differences were observed between varieties *gattii* and *neo-*

formans regarding the effects of combinations of amphotericin B or fluconazole with L-743,872. Antagonistic interactions were not observed for any of the strains tested. Overall these results indicate that addition of L-743,872 can potentiate the activities of both amphotericin B and fluconazole against *C. neoformans* in vitro.

The results obtained with the XTT reduction assays were in good agreement with those obtained by the conventional NC-

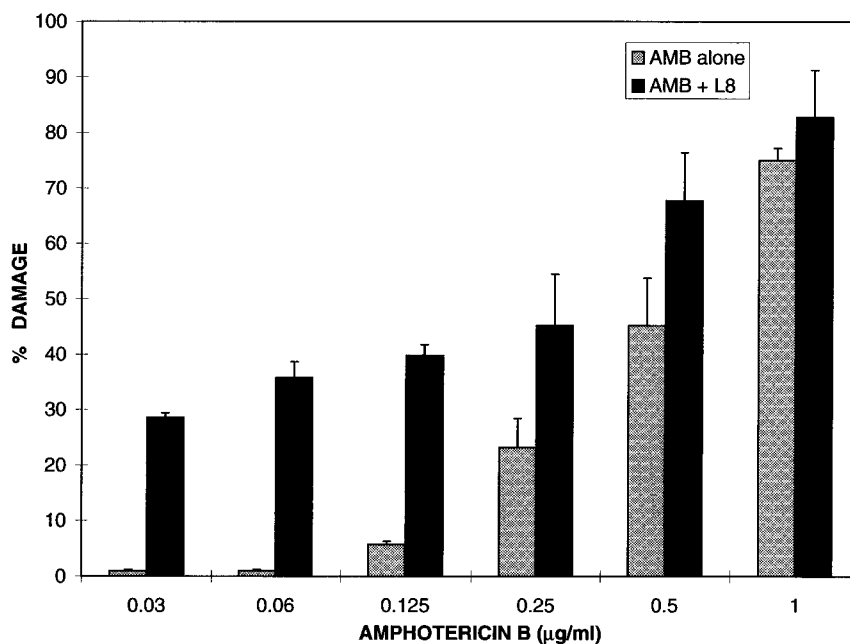


FIG. 3. Effects of amphotericin B (AMB) and pneumocandin L-743,872 on *C. neoformans* ATCC 24067 cell viability as measured by XTT colorimetric assay. The AMB concentration was variable and the pneumocandin concentration was constant at 8 µg/ml (L8). Pneumocandin alone produced no damage at a concentration of 8 µg/ml. This experiment was done three times with similar results. Error bars denote standard deviations from the mean.

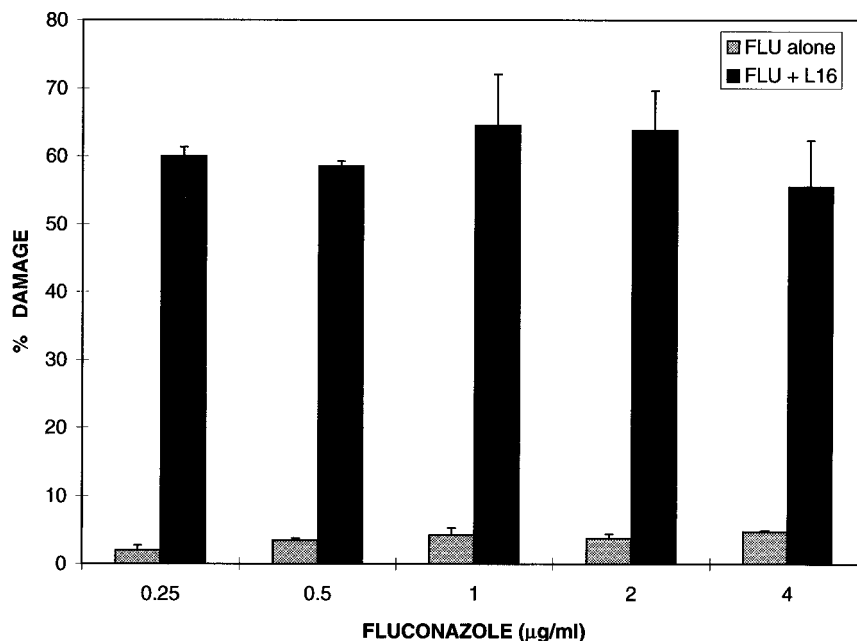


FIG. 4. Effects of fluconazole (FLU) and pneumocandin L-743,872 on *C. neoformans* ATCC 24067 cell viability as measured by XTT colorimetric assay. The FLU concentration was variable and the pneumocandin concentration was constant at 16 µg/ml (L16). Pneumocandin alone at a concentration of 16 µg/ml produced 35% of the damage. This experiment was done three times with similar results. Error bars denote standard deviations from the mean.

CLS method. The XTT assay is based on the fact that mitochondrial respiratory activity can be measured by a simple colorimetric test in which tetrazolium salts are reduced by active mitochondria (9, 17). In the XTT assay live cells reduce XTT, whereas dead cells do not. Incubation of *C. neoformans* with amphotericin B produced a large decrease in XTT reduction consistent with fungicidal activity. In contrast, incubation of *C. neoformans* with fluconazole produced only a small decrease in XTT reduction by fungal cells consistent with fungistatic activity. Several studies have documented the usefulness of tetrazolium dye reduction in assaying for susceptibility to antifungal agents (9, 17). The XTT reduction assay has the advantage of same-day results and could potentially be useful in antifungal susceptibility testing if standardized.

Although the mechanism(s) by which pneumocandin L-743,872 enhances amphotericin B and fluconazole activities was not investigated, one can speculate as to potential mechanisms based on the known effects of these drugs on fungal cells. Pneumocandins inhibit 1,3-β-glucan synthesis and are fungicidal drugs (2). Amphotericin B is believed to act primarily by damaging the fungal cell membrane after binding to fungal sterols and is usually a fungicidal drug (8). Fluconazole is believed to interfere with fungal sterol synthesis and is primarily a fungistatic drug (14). Both amphotericin B and fluconazole must traverse the fungal cell wall to reach their respective targets. Therefore we speculate that L-743,872 enhances amphotericin B and fluconazole activity by inhibiting cell wall synthesis and increasing the access of these drugs to the cell membrane.

Previous studies have evaluated the efficacy of other pneumocandin derivatives against *C. neoformans*, alone or in combination with amphotericin B. Abruzzo et al. (1) evaluated the activities of three pneumocandin analogs (L-733,560, L-705,589, and L-731,373) in a disseminated cryptococcosis mouse model and found that these drugs were ineffective in reducing CFU in the studied organs. Additionally, Bartizal et

al. (2) showed that drug combination studies with pneumocandin L-733,560 (a hybrid of L-705,589 and L-731,373) and amphotericin B revealed additive-to-synergistic effects against *C. neoformans* in vitro (FICs, 0.32 to 0.98). When L-733,560 was combined with fluconazole, indifferent or additive effects were found (FICs, 0.57 to 1.07). Our studies with L-743,872 confirm the results of Bartizal et al. (2) with L-733,560, but L-743,872 appears to be more effective in combination with amphotericin B as measured by lower FICs (FICs, 0.12 to 0.50). Bartizal et al. (2) also demonstrated that pneumocandin L-733,560 alone exhibited weak anti-*C. neoformans* activity in vitro and suggested that the relative resistance of *C. neoformans* to pneumocandins resulted from absence of 1,3-β-D-glucans in the cell wall of this yeast, differential penetration or access of the compound to the target, and/or undefined resistance mechanisms.

In summary, our results indicate that pneumocandin L-743,872 enhances the activities of amphotericin B and fluconazole against *C. neoformans* in vitro. These findings suggest that the addition of pneumocandin to amphotericin B or fluconazole regimens could be useful in the treatment of *C. neoformans* infection. However, susceptibility testing for *C. neoformans* is poorly standardized, and we caution against extrapolating these results to clinical situations without additional testing. Nevertheless our results are hopeful for the possibility that L-743,872 (or other pneumocandins) can be useful against *C. neoformans* in combination with amphotericin B or fluconazole and suggest testing combination therapy in animal models.

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