ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1995, p. 1070–1076 Vol. 39, No. 5
0066-4804/95/$04.00+0

In Vitro Evaluation of the Pneumocandin Antifungal Agent L-733560, a New Water-Soluble Hybrid of L-705589 and L-731373

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Received 6 December 1994/Returned for modification 17 January 1995/Accepted 24 February 1995

Lipopeptide L-733560 is a hybrid analog of L-731373 and L-705589. All are water-soluble semisynthetic pneumocandin Bo derivatives. In vitro susceptibility testing of L-705589, L-731373, and L-733560 against more than 200 clinical isolates consisting of eight *Candida* species, *Cryptococcus neoformans*, and three *Aspergillus* species was performed by the broth microdilution methods. All three pneumocandins exhibited potent anti-*Candida* activity and moderate anti-*C. neoformans* activity. However, anti-*Aspergillus* activity was demonstrated only by an agar disk diffusion method. Antifungal agent-resistant *Candida* species and *C. neoformans* showed susceptibility comparable to that of susceptible isolates. Growth inhibition kinetic studies against *Candida albicans* revealed fungicidal activity within 3 to 5 h. Drug combination studies with pneumocandins and amphotericin B revealed indifferent activity against *C. albicans* and additive effects against *C. neoformans* and *Aspergillus fumigatus*. The activities of the compounds were not dramatically affected by the presence of serum. Resistance induction studies showed that the susceptibility of *C. albicans* MY1055 was not significantly altered by repeated exposure to subinhibitory concentrations of L-733560. Erythrocyte hemolysis studies indicated minimal hemolytic potential with pneumocandins. Results from preclinical evaluations and development studies performed thus far indicate that the pneumocandins should be safe, broad-spectrum fungicidal agents and potent parenteral antifungal agents.

The significance of the increasing incidence of fungal infections (19, 34, 37) and the identification of new opportunistic mycotic diseases especially in immunodeficient patients (4, 5, 13, 14) have been thoroughly documented. The severities of these infections (26, 38), the lack of sensitive, rapid diagnostic tests for the identification of fungal infections (17, 25, 30), and the emergence of increasing numbers of isolates resistant to antifungal agents (11, 22, 35) reinforce the critical necessity for new and effective, fungicidal chemotherapeutic agents.

A new generation of semisynthetic amine derivatives of the natural product pneumocandin Bo (L-688786) is being developed. These compounds show enhanced potencies and expanded spectra of antifungal activity, notably, against *Aspergillus fumigatus*. The fungicidal mode of action of this class of compounds, via inhibition of cell wall 1,3-β-D-glucan synthesis, is especially attractive. The polysaccharide 1,3-β-D-glucan is not present in mammalian cells. However, it is a fundamental cell wall component that provides structural integrity and osmotic stability for fungi and *Pneumocystis carinii* cysts.

L-733560 is a semisynthetic hybrid analog of L-731373 and L-705589, which are derivatives of the natural product pneumocandin Bo (12). All three are water-soluble cyclic hexapeptides containing a fatty acyl side chain and are 10- to greater than 100-fold more potent 1,3-β-D-glucan synthesis inhibitors than narrow-spectrum pneumocandin Bo (6, 7, 29, 32). The pneumocandins have exhibited potent anti-*Candida* (6–8) and anti-*P. carinii* activities in vivo (6, 8, 36), with excellent pharmacokinetics in rodents and rhesus monkeys (20). Additionally, both L-705589 and L-733560 displayed potent anti-*A. fumigatus* activities in vivo (1, 2, 10). In order to define more clearly the potential antifungal activities of these novel semisynthetic derivatives, a comprehensive in vitro biological evaluation was undertaken.

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MATERIALS AND METHODS

Compounds. Semisynthetic pneumocandin analogs were produced in the Department of Synthetic Chemical Research, and pneumocandin Bo was isolated in the Department of Natural Products Chemistry (both departments are at Merck Research Laboratories, Rahway, N.J.). These pneumocandins were shown by high-performance liquid chromatography to be >95% pure. L-705589, L-731373, L-733560, nikkomycin Z (NCZ; Cutter Laboratories, Berkeley, Calif.), and fluconazole (FCZ; Pfizer Central Research, Groton, Conn.) were formulated in sterile distilled water. Amphotericin B (AMB; Fungizone; E. R. Squibb & Sons, Inc. Princeton, N.J.) was formulated according to the manufacturer's instructions. Pneumocandin Bo, ketoconazole (KTZ; Janssen, Beerse, Belgium), and flucytosine (5-FC; PCR Research Chemicals, Gainesville, Fla.) were formulated in 5% dimethyl sulfoxide (Fisher, Fairlawn, N.J.).

Organisms. The antifungal agents were evaluated against a large battery of clinical isolates from the Merck Clinical Culture Collection. They included *Candida* spp., *Cryptococcus neoformans*, *A. fumigatus*, *Aspergillus flavus*, and *Aspergillus niger* isolates. AMB-, 5-FC-, FCZ-, and KTZ-resistant fungal isolates were graciously provided by M. Rinaldi, Mycology Reference Laboratory, San Antonio, Tex.

Antifungal susceptibility assays. Antifungal susceptibility assays were performed by the broth macrodilution method against *Candida* spp. and *C. neoformans* strains to determine the MICs and minimum fungicidal concentrations (MFCs) of the pneumocandins and AMB. The method was adapted from the tentative standard reference method (document M27-P), as recommended by the Subcommittee on Antifungal Susceptibility Testing, National Committee for Clinical Laboratory Standards (NCCLS) (31), to a modified broth microdilution method reported previously (16). The inoculum (1×10^3 to 5×10^3 CFU/ml) was standardized with a spectrophotometer (optical density at 530 nm). RPMI 1640 medium with L-glutamine, without sodium bicarbonate (Whittaker Bioproducts, Boston, Mass.), and buffered with 0.165 M (34.54 g/liter) MOPS (morpholinepropanesulfonic acid) buffer (pH 7.0 at 35°C; Sigma, St. Louis, Mo.) was used.

Test compounds were prepared as concentrated stock solutions, diluted in RPMI 1640 medium, and tested at concentrations ranging from 128 to 0.06 μg/ml. For studies with serum the compounds were diluted in 0 to 50% fresh pooled human or mouse sera, and then RPMI 1640 medium was added prior to inoculation. The MIC was defined as the lowest concentration of compound that completely inhibited visible growth (absence of detectable turbidity). The MICs were recorded after 24 h of incubation at 35 to 37°C. After the MICs were recorded, the microtiter plates were shaken and an MIC-2000 inoculator (Dynatech) was used to transfer a 1.5-μl sample from each well of the microtiter plate

to a single-reservoir plate containing 10 ml of Sabouraud dextrose agar (BBL, Cockeysville, Md.), and these were incubated for 24 h (or 48 h for *Cryptococcus* spp.) at 35 to 37°C. The MFC was defined as the lowest concentration of compound at which growth of fewer than four colonies occurred. The MICs and MFCs were determined for all *Candida* species and *C. neoformans* strains, while only MICs were determined for *Aspergillus* species.

The susceptibilities of the *A. fumigatus* strains were determined by a disk diffusion method on potato dextrose agar (PDA; Difco) adapted from the methods described in *Antibiotics in Laboratory Medicine* (3). Briefly, 10 ml of PDA was seeded with 10^6 CFU of *A. fumigatus* spores, and the mixture was poured into petri dishes. Paper disks impregnated with the pneumocandin compounds at concentrations ranging from 128 to 0.06 μg/ml were then placed on the agar surface. The plates were incubated for 24 h at 35°C, zones of inhibition were measured, and the critical concentration (CC) of each compound was determined. The CC is a theoretical calculated concentration at the edge of the zone of inhibition and represents a measure of the susceptibility of a test organism, similar but not identical to the MIC, as measured by dilution techniques that involve somewhat different test conditions. The CC is expressed by the following general formula: $\ln m' = \ln m_0(X^2/4DT_0)$, where $\ln m'$ is the natural logarithm of the critical concentration, $\ln m_0$ is the natural logarithm of the concentration of drug applied to the agar surface, X^2 is the square of the distance between the reservoir and the edge of the zone of inhibition, D is the diffusion coefficient for the antimicrobial agent under study, in the test medium, and at the temperature of the test system, and T_0 is the critical time at which point the position of the zone of inhibition is determined.

Geometric mean MICs and MFCs and the MICs and MFCs of the compounds necessary to inhibit and kill 50% and 90% of the *Candida* and *Cryptococcus* isolates tested were determined, while only the CCs and MICs were determined for *Aspergillus* species.

Resistance induction. The potential for resistance development was determined by recording the MICs and MFCs after each of 20 serial transfers for *C. albicans* MY1055 when it was incubated in the presence of subinhibitory concentrations of L-733560. The test was conducted as described above for the microdilution method, except these experiments were performed in 10 ml of YNBD by the macrodilution methods recommended by NCCLS. Nonpassaged *C. albicans* and *C. albicans* at the final (20th) passage were tested by the broth microdilution methodology as described above.

Drug combination studies. Drug combination testing was performed by the broth microdilution checkerboard method to evaluate combinations of L-705589, L-731373, or L-733560 with AMB and L-733560 with FCZ or NCZ against *C. albicans*, *C. neoformans*, and *A. fumigatus* isolates (27). The in vitro interactions were calculated algebraically and were interpreted as synergistic, indifferent, or antagonistic, depending on whether the antifungal activity of the combination was greater than, equivalent to, or less than the activities of the individual agents alone, respectively. For each combination, the fractional fungicidal concentrations (FFCs) and the fractional inhibitory concentration (FICs) for *Aspergillus* spp. were calculated as follows: the FFC or FIC of agent A = the MFC or MIC of agent A in combination/the MFC or MIC of agent A alone and the FFC or FIC of agent B = the MFC or MIC of agent B in combination/the MFC or MIC of agent B alone. The summation of the FFC or FIC index (εFFC or FIC) for each combination was calculated as follows: εFFC or FIC = FFC or FIC of agent A + FFC or FIC of agent B. The results were interpreted as follows: synergism, εFFC or FIC ≤ 0.5; indifference, εFFC or FIC $= 0.5 < X \leq 4$; antagonism, εFFC or FIC ≥ 4.

Erythrocyte hemolysis assay. A microtiter erythrocyte hemolysis assay was used to determine the potential of pneumocandin analogs to lyse human or mouse erythrocytes. A suspension of freshly drawn heparinized human or CD-1 mouse (Charles River, Wilmington, Mass.) whole blood (2 ml) was added to 50 ml of sterile 5% glucose. A 4-mg/ml stock drug suspension was diluted by the addition of 0.2 ml of stock drug suspension to 1.4 ml of sterile 5% glucose. Test solutions were dispensed into microtiter wells and were serially diluted in 5% glucose to yield final test concentrations of 400 to 0.20 μg/ml. Finally, 38 μl of the erythrocyte suspension was added to each well. Hemolysis of erythrocytes was indicated by complete or partial clearing (hemolysis) and was defined as the minimum lytic concentration (MLC) of a test compound after 2 h at room temperature.

RESULTS

The MFC data in Table 1 show the in vitro susceptibilities of clinical fungal isolates to the pneumocandins and AMB. The MICs (data not shown) of all compounds did not differ more than twofold from the MFCs. In general, L-731373 and L-733560 were more potent than L-705589 against the *Candida* spp. L-733560 was less active than L-705589 against *Candida tropicalis* isolates, and L-731373 was less potent than L-705589 against *Candida guilliermondii* isolates. When comparing geometric mean MFCs, L-731373 and L-733560 were more active than AMB and L-705589 against *C. albicans*, *C. tropicalis*, *Candida lusitaniae*, *Candida krusei*, *Candida pseudotropicalis*, and *Candida glabrata* isolates. Against *C. guilliermondii* and *Candida parapsilosis* isolates, L-733560 had less than twofold lower activity than AMB. AMB was more effective than the semisynthetic pneumocandin analogs against *C. neoformans* isolates, although L-733560 did show slight activity (geometric mean MFC, 16 μg/ml).

Pneumocandin analogs did not show activity in vitro against the *A. fumigatus* isolates when they were tested by the conventional broth microdilution methodology (MICs, >128 μg/ml). However, when a disk diffusion susceptibility assay was used, these compounds and AMB formed zones of inhibition on agar seeded with *A. fumigatus* strains. The CC ranges of pneumocandin Bo, L-705589, L-731373, L-733560, and AMB were 0.03 to 0.05, 0.06 to 0.09, 0.06 to 0.08, 0.04 to 0.27, and 0.04 to 0.43 μg/ml, respectively. Agar dilution CCs were determined in PDA with an inoculum of 1.2×10^5 *A. fumigatus* CFU/ml representing the range of CCs for isolates MF5668, CLY315, CLY522, and CLY523. CCs were determined by the method of Acar and Goldstein (3). These data indicate that the CCs of L-705589 and L-731373 were comparable and that the CCs of L-733560 were slightly greater than those of the other pneumocandins. Against *A. fumigatus* MF5668, the CC of AMB was more than threefold greater than those of the pneumocandins (0.43 μg/ml), while against the other *A. fumigatus* isolates the CCs of AMB were nearly equivalent (0.04 to 0.13 μg/ml).

The in vitro susceptibilities of fungi resistant to the pneumocandins are given in Table 2. L-731373 and L-733560 were more effective than L-705589 and AMB against AMB-, 5-FC-, and FCZ-resistant *C. albicans* organisms and FCZ-resistant *C. tropicalis* organisms, with MFCs ranging between 0.06 and 0.5 μg/ml. L-705589, L-731373, and L-733560 were also more potent than AMB and pneumocandin Bo against AMB-resistant *C. lusitaniae* isolates (MFCs, 0.50 to 2 μg/ml), but all were equipotent against 5-FC-resistant *C. glabrata* isolates (MFCs, 0.50 to 2 μg/ml). As was seen with the susceptible isolates, the 5-FC- and FCZ-resistant *C. neoformans* isolates were 16- to 32-fold more susceptible to AMB than to L-731373 or L-733560.

Repeated exposure of *C. albicans* MY1055 to subinhibitory concentrations of L-733560 did not significantly alter the MICs (selecting for resistance). The final MIC of 0.004 μg/ml did not change significantly from the initial MIC of 0.008 μg/ml, even after 20 passages. The original *C. albicans* isolate and the isolate obtained after 20 passages were examined microscopically, and no morphological changes were apparent, nor did the MICs or MFCs change when they were assayed by the broth microdilution method.

As shown in Fig. 1, pneumocandin Bo and its semisynthetic analogs were fungicidal, killing 99% of the *C. albicans* isolates within 3 to 5 h at 1× their predetermined MFC. This was based on the initial susceptibility testing results for the isolates obtained by the methodology recommended by NCCLS. This supports glucan synthesis inhibition as a target of fungicidal agents. At 1× its MFC, AMB was also fungicidal, but FCZ was fungistatic (Fig. 1). Killing rates by pneumocandin Bo and analogs were slower than those by AMB, but they were progressive and prolonged beyond 9 h after a 1-h lag period. Rates of killing after the lag period were similar for pneumocandins Bo, L-705589, and L-731373, while L-733560 at 0.25× and 4× its MFC showed a more rapid rate of killing between 3 and 4 h after the beginning of exposure to the organism (0.25× and 4× the MFC; data not shown).

Human or mouse serum did not significantly affect (fourfold or greater increase in the MFC) the susceptibility of *C. albicans* to the semisynthetic pneumocandin analogs L-705589,

TABLE 1. MFCs of pneumocandin Bo, L-705589, L-731373, L-733560, and AMB for 200 clinical fungal isolates[a]

Organism (no. of isolates tested)	Compound	MFC (μg/ml)[b]			
		Range	50%	90%	Geometric mean
Candida albicans (40)	Pneumocandin Bo	0.12–0.50	0.12	0.25	0.17
	L-705589	0.06–0.50	0.25	0.50	0.27
	L-731373	≤0.06–0.25	≤0.06	≤0.06	0.06
	L-733560	0.03–0.5	0.125	0.50	0.13
	AMB	0.25–1	0.50	1	0.50
Candida tropicalis (20)	Pneumocandin Bo	0.12–0.50	0.25	0.50	0.30
	L-705589	≤0.12–0.50	0.25	0.50	0.19
	L-731373	≤0.06	≤0.06	≤0.06	0.06
	L-733560	0.12–0.50	0.12	0.25	0.26
	AMB	0.25–1	0.25	0.50	0.41
Candida parapsilosis (20)	Pneumocandin Bo	1–4	2	4	2.1
	L-705589	2–4	2	4	2.46
	L-731373	0.50–1	1	1	0.80
	L-733560	0.25–1	0.50	0.50	0.50
	AMB	0.25–1	0.50	1	0.60
Candida lusitaniae (20)	Pneumocandin Bo	0.25–2	1	2	1
	L-705589	1–2	2	2	1.55
	L-731373	≤0.06–0.50	0.12	0.50	0.14
	L-733560	0.06–0.50	0.50	0.50	0.33
	AMB	0.50–1	1	1	0.80
Candida guilliermondii (20)	Pneumocandin Bo	4–128	16	64	22.6
	L-705589	1–4	1	4	1.52
	L-731373	0.50–16	2	16	1.8
	L-733560	0.50–2	1	2	0.96
	AMB	0.25–1	0.50	1	0.50
Candida krusei (20)	Pneumocandin Bo	1–2	2	2	1.90
	L-705589	2–8	4	8	4.30
	L-731373	0.12–0.50	0.25	0.25	0.26
	L-733560	0.25–1	0.25	0.50	0.32
	AMB	0.50–1	1	1	0.80
Candida pseudotropicalis (20)	Pneumocandin Bo	0.50–4	1	2	1.3
	L-705589	0.25–1	0.50	1	0.57
	L-731373	≤0.06–2	0.12	1	0.20
	L-733560	≤0.06–2	0.25	1	0.24
	AMB	0.50–2	1	1	1
Candida glabrata	Pneumocandin Bo	0.25–2	1	2	1.2
	L-705589	1–2	2	2	1.57
	L-731373	≤0.06–0.50	0.25	0.25	0.15
	L-733560	0.12–0.50	0.25	0.50	0.32
	AMB	0.50–1	0.50	0.50	0.50
Cryptococcus neoformans (20)	Pneumocandin Bo	0.50–>64	32	>64	52.0
	L-705589	32–64	64	64	53.3
	L-731373	NT[c]	NT	NT	NT
	L-733560	8–32	16	32	16.0
	AMB	0.50–2	1	2	0.70
Aspergillus fumigatus (16)	Pneumocandin Bo	>128	>128	>128	>128
	L-705589	NT	NT	NT	NT
	L-731373	>128	>128	>128	>128
	L-733560	>128	>128	>128	>128
	AMB	1–2	2	2	1.68
Aspergillus flavus (12)	Pneumocandin Bo	>128	>128	>128	>128
	L-705589	NT	NT	NT	NT
	L-731373	>128	>128	>128	>128
	L-733560	>128	>128	>128	>128
	AMB	1–4	2	2	1.88

Continued on following page

TABLE 1—*Continued*

Organism (no. of isolates tested)	Compound	MFC (μg/ml)[b]			
		Range	50%	90%	Geometric mean
Aspergillus niger (19)	Pneumocandin Bo	128–>128	>128	>128	>128
	L-705589	NT	NT	NT	NT
	L-731373	16–>128	128	128	110.60
	L-733560	64–>128	128	>128	>128
	AMB	0.5–2	1	1	1.19

[a] The method was adapted from the tentative standard reference method (document M27-P) recommended by the Committee on Antifungal Susceptibility Testing, NCCLS (31), to a modified broth microdilution method reported previously (16).
[b] 50% and 90%, MFCs at which 50 and 90% of strains are inhibited, respectively.
[c] NT, not tested.

L-731373, or AMB in RPMI 1640 medium (Table 3). However, both human and mouse sera had a significant effect on the susceptibility of *C. albicans* to pneumocandin Bo. The MFCs of pneumocandin Bo as the concentrations of human or mouse serum reached 50% were 64- to 128-fold higher than those in 0% serum. MFCs of L-733560 in serum increased slightly compared with those of pneumocandin Bo and were at least two- to eightfold higher when the compound was tested with human or mouse serum.

The results of drug combination studies showed that AMB combined with pneumocandin Bo or its analogs exhibited indifferent to additive activity against *C. albicans*, *C. neoformans*, and *A. fumigatus* strains (FFCs or FICs, 0.67 to 1.14). Importantly, AMB combined with L-733560 or L-705589 showed additive to synergistic effects against *C. neoformans* or *A. fumigatus* strains (FFCs or FICs, 0.32 to 0.98). When FCZ or NKZ was combined with L-733560 for testing against *C. albicans*, *C. neoformans*, and *A. fumigatus* strains (FFCs or FICs, 0.57 to 1.07), their activities appeared either indifferent or additive, especially when L-733560 was combined with FCZ against *C. neoformans* (FFCs, 0.57 to 0.58) or when L-733560 was combined with NCZ against *C. albicans* (FFCs, 0.60 to 0.84).

The semisynthetic pneumocandin analogs L-705589, L-731373, and L-733560 were relatively nonhemolytic (compared with AMB) against human or mouse erythrocytes in an unwashed erythrocyte hemolysis assay. The MLCs of the semisynthetic pneumocandins ranged from 200 to >400 μg/ml against human or mouse erythrocytes. The MLCs of the positive hemolysis control compound AMB were 3.13 μg/ml for mouse erythrocytes and 25 μg/ml for human erythrocytes, while the negative hemolysis control compound 5-FC was not hemolytic against either human or mouse erythrocytes (MLCs, >400 μg/ml).

DISCUSSION

L-705589, L-731373, and L-733560 represent a new generation of semisynthetic amine derivatives of pneumocandin Bo. These compounds were found to be significantly more potent than narrow-spectrum pneumocandin Bo and its analogs against clinically relevant *Candida* isolates susceptible and resistant to antifungal agents. Because of the recent widespread emergence of antifungal resistance, especially to FCZ (11, 22, 35), it is imperative that a chemotherapeutic agent that is effective against these fungi resistant to antifungal agents be developed. The activities of the semisynthetic compounds against susceptible isolates and a variety of drug-resistant isolates were equivalent, suggesting their potential utility against these emerging pathogens. In addition, the pneumocandin analogs did not exhibit resistance induction in vitro. Furthermore, naturally occurring echinocandin-resistant, virulent fungal isolates have not been described as yet.

In an in vitro *C. albicans* 1,3-β-D-glucan synthase assay, the semisynthetic analogs were 70- to 100-fold more potent than pneumocandin Bo, with 50% inhibitory concentrations ranging between 1 and 10 μM (6, 7, 12). Importantly, pneumocandin analogs showed demonstrable fungicidal activity. The cell wall 1,3-β-D-glucan-inhibitory pneumocandins were fungicidal in growth inhibition assays, supporting the premise that glucan synthesis inhibition is a fungicidal target. For the semisynthetic compounds there was an obvious lag period that lasted approximately 1 h, indicating that fungal growth and metabolism are required for killing to occur. Also, the rates of killing and time

TABLE 2. In vitro susceptibilities of resistant yeast isolates to pneumocandin Bo, L-705589, L-731373, L-733560, and AMB[a]

Organism	Agent to which organism is resistant	MFC (μg/ml)				
		AMB	Pneumocandin Bo	L-705589	L-731373	L-733560
C. albicans (1)[b]	AMB	8	2	1	0.25	0.50
C. albicans (3)	5-FC	0.50–1	0.50–2	0.25–0.50	≤0.06–0.125	≤0.06–0.125
C. albicans (7)	FCZ	0.50–1	0.50–2	0.25–2	≤0.06–0.50	0.06–0.50
C. tropicalis (1)	FCZ	1	0.50	0.50	0.125	0.25
C. lusitaniae (2)	AMB	8	8	0.50–2	0.5–2	1
C. glabrata (1)	5-FC	1	2	1	0.5	1
C. neoformans (1)	5-FC	1	64	64	32	16
C. neoformans (7)	FCZ	0.50–1	32–64	64	32	16–32

[a] The method was adapted from the tentative standard reference method (document M27-P) recommended by the Committee on Antifungal Susceptibility Testing, NCCLS (31), to a modified broth microdilution method reported previously (16).
[b] Values in parentheses indicate the number of isolates tested.

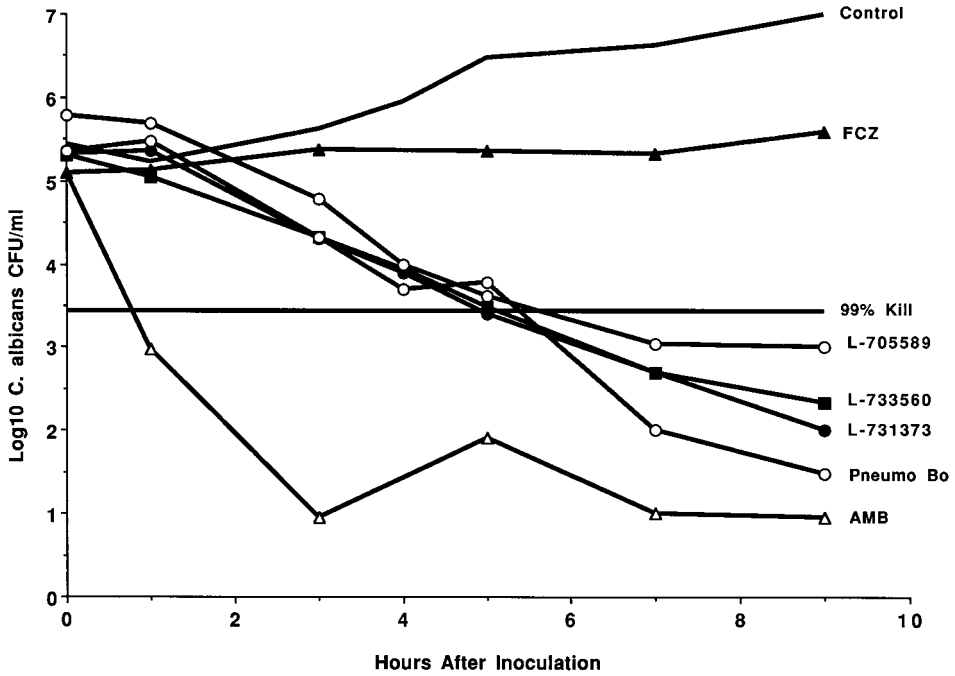

FIG. 1. Growth inhibition of *C. albicans* MY1055 by pneumocandins, FCZ, and AMB at 1× the MFC.

to killing for *C. albicans* were not highly concentration dependent, as has been described previously by us and others with pneumocandins Ao and Bo and other echinocandins (6, 7, 18). The rates of killing and time to killing with pneumocandins were significantly longer compared with those with AMB, which does not require cell growth for activity, but this observation did not appear to affect their comparative activities in vivo. AMB and the pneumocandins are equally efficacious against disseminated *C. albicans* and *A. fumigatus* infections in mouse models (1, 2, 8).

Some differences in the susceptibility of *C. albicans* MY1055 to pneumocandin Bo and L-733,560 were noted when susceptibilities were assessed with human and mouse sera in different media (the data obtained with other media are not shown). The relevance of this finding remains uncertain until more elaborate serum-binding-kinetic studies are performed, and especially since both compounds were found to be highly efficacious in vivo.

Semisynthetic pneumocandins have been demonstrated to have a favorable spectrum of antifungal activity, especially against the clinically more prevalent fungal diseases (i.e., candidiasis and aspergillosis). Although there is no standard in vitro susceptibility assay for assessing the activities of compounds against *P. carinii*, the pneumocandins proved to be highly potent and effective therapeutically against *P. carinii* pneumonia expressed in immunosuppressed rats (36). The potencies of the semisynthetic pneumocandins against *P. carinii* were determined to be approximately 10-fold greater than that of pneumocandin Bo.

The in vitro activities of the pneumocandins against *A. fumigatus* isolates were not apparent by the standard broth dilution methodology, even though potent in vivo efficacy against

TABLE 3. Effects of human or mouse serum on susceptibility of *C. albicans* MY1055 to pneumocandin Bo, semisynthetic analogs, and AMB[a]

Test medium and compound	MFC (μg/ml) in serum at:					
	0%	10%	20%	30%	40%	50%
RPMI 1640 medium plus human serum						
AMB	0.25	0.25	0.25	0.25	0.25	0.5
Pneumocandin Bo	0.25	2	4	4	8	16
L-705589	0.125	0.125	0.125	0.25	0.25	0.25
L-731373	0.06	0.06	0.125	0.125	0.125	0.25
L-733560	≤0.06	≤0.06	≤0.06	0.125	0.125	0.5
RPMI 1640 medium plus mouse serum						
AMB	0.25	0.25	0.25	0.25	0.25	0.5
Pneumocandin Bo	0.125	2	4	8	8	16
L-705589	0.125	0.125	0.125	0.125	0.25	0.25
L-731373	0.06	0.06	0.06	0.06	0.125	0.125
L-733560	≤0.06	≤0.06	≤0.06	0.125	0.25	0.25

[a] The method was adapted from the tentative standard reference method (document M27-P) recommended by the Committee on Antifungal Susceptibility Testing, NCCLS (31), to a modified broth microdilution method reported previously (16).

A. fumigatus in rodents was demonstrated (1, 2, 10). It has generally been established that *Aspergillus* species are not susceptible to the echinocandin class of antifungal agents in standard broth dilution susceptibility assays (9, 15, 23). Kurtz et al. (28) previously demonstrated that pneumocandins can produce profound morphological changes in *Aspergillus* hyphae. Highly sensitive bioassay systems for echinocandins have been reported (18). Those systems use inhibition of *Aspergillus* growth on agar (18). In the present study we also used the disk diffusion method as a means of determining the susceptibilities of *A. fumigatus* isolates to the semisynthetic compounds. The in vivo anti-*A. fumigatus* efficacy of L-731373 in a mouse model of disseminated aspergillosis was found to be significantly less than the efficacy of either L-705589 or L-733560 (1, 2), so a positive correlation to in vitro activity determined by disk diffusion methods was not established. However, the distribution, pharmacokinetic, or metabolic properties of L-731373 may have contributed to its inferior activity observed in vivo.

Only slight activity of the semisynthetic analogs was apparent against *C. neoformans* isolates. It has been postulated that *C. neoformans* may possess 1,6-β-glucan or other non-1,3-β-D-glucans (i.e., α-1,3, α-1,6) in its cell wall, thus explaining its relative resistance to pneumocandins (21, 24, 33, 39). On the other hand, variable responses against *C. neoformans* isolates may also involve penetration or access of the compound to the target, the metabolic state of the yeast in broth culture, or undefined resistance mechanisms.

It was also encouraging that in drug combination studies AMB, FCZ, or NCZ combinations with pneumocandin L-733,560 were not antagonistic against *Candida*, *Cryptococcus*, or *Aspergillus* isolates. In fact, additive effects against certain isolates were found. This may have substantial clinical significance, first, in situations in which patients may already be receiving an antifungal drug before the initiation of pneumocandin therapy; second, in expanding the spectrum of activity to possibly include anti-*Cryptococcus* activity; and lastly, in potentially increasing the therapeutic index through synergistic action and dose reduction of individual components, e.g., reduction of the AMB concentration.

Pneumocandin analogs were relatively nonhemolytic against human or mouse erythrocytes, which suggests that erythrocyte hemolysis should not be a mechanistic factor for dose-limiting toxicity in vivo. AMB is highly hemolytic and exhibits severe toxic manifestations clinically, but its dose-limiting toxicity may not be due solely to its hemolytic effects. Finally, since β-glucan is a selective target that is present only in fungal cell walls and not in mammalian cells, its mode of action rules out the possibility of mechanism-based toxicity in the mammalian host.

The studies described here, combined with the preclinical efficacy studies performed in animal models thus far, support the further evaluation of pneumocandins as developmental antifungal candidates possessing several potential advantages over the already marketed azoles and polyene agents.

ACKNOWLEDGMENTS

We thank M. Hammond, J. Balkovec, R. Black, A. Bouffard, and J. Dropinski from the Department of Synthetic Chemical Research and R. Schwartz from the Department of Natural Products Chemistry for providing pneumocandin compounds and chemical support.

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