

New Semisynthetic Pneumocandins with Improved Efficacies against *Pneumocystis carinii* in the Rat

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A new series of semisynthetic, water-soluble pneumocandin analogs has been found to be extremely potent against *Pneumocystis carinii* in an immunocompromised-rat model. These compounds are 5 to 10 times more potent than the parent natural product, pneumocandin B₀ (L-688,786) (R. E. Schwartz et al., *J. Antibiot.* 45:1853–1866, 1992), and >100 times more potent than cilofungin. One compound in particular, L-733,560, had a 90% effective dose against *P. carinii* cysts of 0.01 mg/kg of body weight when delivered parenterally (subcutaneously, twice daily for 4 days). This compound was also effective when given orally for the treatment and prevention of *P. carinii* pneumonia. For treating acute *P. carinii* pneumonia, oral doses of 2.2 mg/kg twice daily for 4 days were required to eliminate 90% of the cysts. A once-daily oral prophylactic dose of 2.2 mg/kg prevented cyst development, and a dose of 6.2 mg/kg prevented any development of *P. carinii* organisms (cysts and trophozoites), as determined through the use of a *P. carinii*-specific DNA probe (P. A. Liberator et al., *J. Clin. Microbiol.* 30:2968–2974, 1992). These results demonstrate that the antipneumocystis activities of the pneumocandins can be significantly improved through synthetic modification. Several of these compounds are also extremely effective against candidiasis (K. Bartizal et al., *Antimicrob. Agents Chemother.* 39:1070–1076, 1995) and aspergillosis (G. K. Abruzzo et al., *Antimicrob. Agents Chemother.* 39:860–894, 1995) in murine models, making them attractive as broad-spectrum antifungal agents.

Among the most promising classes of compounds for the potential treatment and prevention of systemic fungal infections and *Pneumocystis carinii* pneumonia (PCP) are the echinocandins and the closely related pneumocandins (1, 3, 4, 11, 15). These compounds are believed to function by inhibiting the biosynthesis of β -1,3-glucan, a major component of the cell walls of many fungi and of *P. carinii* cyst walls (13). Many of the echinocandins have potent in vivo efficacy against *Candida albicans* (3) and *P. carinii* (15) in murine models. The semisynthetic echinocandin cilofungin has also been shown to effectively treat candidiasis in humans (6, 7, 10). Despite the significant efficacies of compounds in this class, the poor water solubility of compounds in this class has hindered their development for clinical use. Clinical trials with cilofungin for intravenous use in humans were abandoned because of side effects attributable to the cosolvent (9, 10).

The lack of water solubility of this class was overcome by the development of pneumocandin prodrugs such as L-693,989 (2) and, more recently, by the addition of charged amino groups to the peptide core of pneumocandin B₀ (L-688,786), resulting in water-soluble, nonprodrug compounds which are substantially more efficacious (5). This additional potency has also resulted in an expanded spectrum for these drugs, which have been shown to have potent activity against *Aspergillus fumigatus* in murine models (1). Prior to this, only modest in vivo anti-*Aspergillus* activity had been reported with extremely high doses of cilofungin (8). The broadened spectrum of these pneumocandins with amino substitutions, which encompasses both *Aspergillus* spp. and *Candida* spp., may, for the first time,

make echinocandins useful for empiric treatment of systemic fungal infections.

These amino compounds are also substantially more efficacious against *P. carinii* and may represent a new class of agents for the safe, effective control of this organism in immunocompromised hosts. This study demonstrates the efficacies of these compounds as both prophylactic and therapeutic agents in the immunocompromised-rat model for PCP when administered either parenterally or orally. The efficacies of these compounds are also compared with those of pneumocandin B₀, L-693,989, and cilofungin.

MATERIALS AND METHODS

Compounds. All of the lipopeptides used for these studies were synthesized by the Merck Synthetic Chemical Research Group from natural products produced in the Department of Fermentation Microbiology and isolated by the Natural Products Isolation Group.

Glucan synthesis inhibition assay. *C. albicans* (MY1208) cells were grown at 28°C to early log phase (6 to 8 h) in 500 ml of Sabouraud dextrose broth (Difco) in a 2-liter baffled shake flask. The membrane-associated synthase system was prepared from protoplasts by the method of Taft et al. (19). The [³H]glucan assay was conducted as previously reported (16). To determine the inhibitory effects of analogs, samples were serially diluted from the concentration providing complete inhibition down to levels lacking inhibitory activity. The 1,3- β -D-glucan synthesis 50% inhibitory concentration (IC₅₀) was defined as the concentration at which a compound inhibited 50% of the production of acid-precipitable product.

Efficacy studies. The dexamethasone-immunosuppressed-rat model used in these studies is described elsewhere (17). Briefly, male Sprague-Dawley rats weighing 240 to 260 g (Sasco Laboratories, Omaha, Nebr.) were used in these studies. The rats were fed 23% protein rodent chow (Purina, St. Louis, Mo.) and were immunosuppressed with 2 mg of dexamethasone (Butler, Columbus, Ohio) per liter of their drinking water. Tetracycline (1 g/liter) was added to the drinking water to minimize bacterial infections. For acute therapy studies, rats previously immunosuppressed for 6 weeks were treated twice daily (b.i.d.) for 4 days with specified amounts of a compound in sterile water, either by subcutaneous injection with 0.25 ml of a compound or by oral gavage with 0.5 ml of a compound. All animals remained on immunosuppressive therapy with dexamethasone throughout the study. Three rats were sacrificed at the initiation of a study to microscopically confirm the presence of acute PCP. Routinely, some of the rats die during immunosuppression as a result of being highly susceptible to a variety

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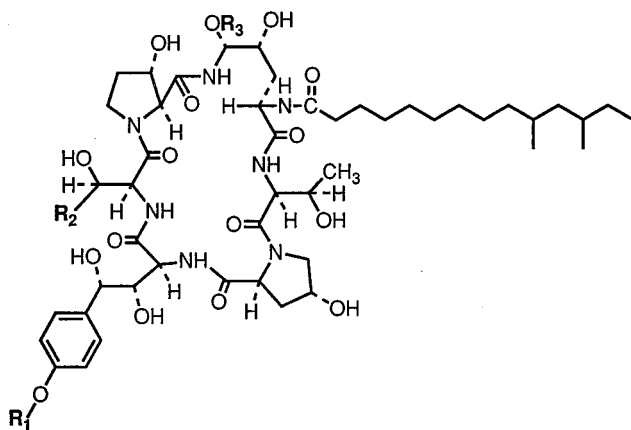


FIG. 1. Structure of the lipopeptide antibiotics. L-688,786 is the natural product parent of all of the semisynthetic pneumocandins in Table 1 and is commonly referred to as pneumocandin B₀. It is produced and isolated from cultures of the fungus *Zalerion arboricola* (18). L-693,989 is the phosphate ester prodrug of L-688,786 (2). The remaining three compounds are water-soluble pneumocandins (nonprodrug) with modifications of L-688,786 at the R2 (L-731,373), R3 (L-705,589), or R2 and R3 (L-733,560) positions. Data on the efficacies of the lipopeptide antibiotics are shown in Table 1.

of opportunistic infections. All animals used in the study were housed and cared for under the guidelines set forth in the *Guide for the Care and Use of Laboratory Animals* (12a).

Evaluation of lung tissue. All lung tissues were processed with a Brinkmann homogenizer, and the quantitation of cysts per lung was performed as described previously (17). The quantitation of *P. carinii* trophozoites (nuclei) was achieved by hybridization of a radiolabelled *P. carinii*-specific DNA probe with total DNA extracted from individual rat lung tissues (12). Histological sections of lung tissue from animals in the parenteral-prophylaxis study were also prepared. The lung tissue was fixed in 10% formalin, embedded in paraffin, sectioned, stained with hematoxylin-eosin, and in some cases counterstained with methanamine silver. Hematoxylin-eosin-stained sections were examined to determine if the foamy exudate commonly associated with PCP was present. The slides counterstained with methanamine silver were examined for degrees of cyst accumulation.

Dose titration experiments for treatment of acute PCP. Dexamethasone-treated rats with acute PCP were injected subcutaneously with various concentrations of compounds to determine the effective dose for 90% cyst clearance (ED₉₀) relative to that of vehicle controls. Each dosing group contained six animals. The compounds included L-693,989, cilofungin, L-733,560, L-705,589, and L-731,373 (Fig. 1 and Table 1). All compounds were delivered in sterile water with the exception of cilofungin, which was delivered in 10% dimethyl sulfoxide.

Efficacies of compounds given orally. Rats with acute PCP received treatment by oral gavage b.i.d. for 4 days with 0.5 ml of a compound in sterile water to deliver concentrations ranging from 2.5 to 50 mg/kg of body weight. After therapy, the rats were sacrificed and their lung tissues were processed for the quantitation of cysts and trophozoites for comparison with those of rats given vehicle controls.

Oral prophylaxis. Rats were dosed daily by gavage with 0.5 ml of a compound in sterile water at concentrations ranging from 0.75 to 6.25 mg/kg during the entire 6-week immunosuppression period. After 6 weeks, the rats were sacrificed

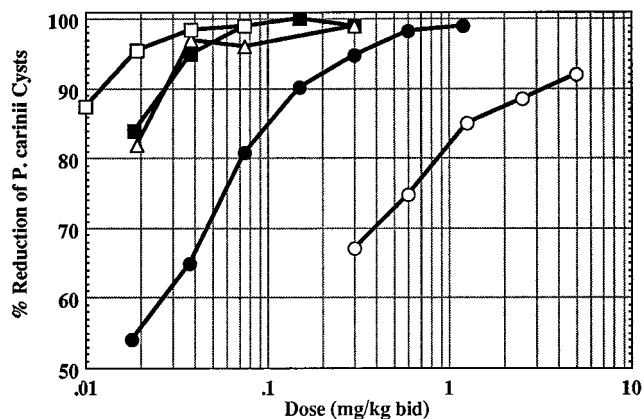


FIG. 2. Comparative titration curves for parenteral therapy of acute *P. carinii* infection in the immunosuppressed-rat model. All points are the means of data for four to six rats. All compounds (L-693,989 [●], L-705,589 [■], L-731,373 [△], L-733,560 [□], and cilofungin [○]) were administered b.i.d. for 4 days. With the exception of cilofungin, which was solubilized in 10% dimethyl sulfoxide, all compounds were administered in water. The percent reduction of cysts was calculated relative to a log₁₀ mean cyst load of 7.55 ± 0.20 (mean ± standard error) for control animals given a vehicle.

and the numbers of cysts and trophozoites were determined by visual assessment by light microscopy and with the *P. carinii*-specific DNA probe, respectively.

RESULTS

Glucan synthesis inhibition assay. The IC₅₀s for the pneumocandin analogs in the *C. albicans* membrane glucan synthesis assay are shown in Table 1. There was a direct correlation between the IC₅₀s of these compounds in the *C. albicans* glucan assay and their relative potencies against both *C. albicans* and *P. carinii*. However, this was not the case for *A. fumigatus*, for which L-731,373 was ineffective despite an IC₅₀ of 10 nM in the *C. albicans* membrane assay. There is clearly a difference in the efficacies of analogs against *A. fumigatus*, and in this series of compounds the aminoethyl ether substitution at R2 is required to achieve significant activity (1, 5).

Dose titration. The parenteral ED₉₀s (Table 1) for L-705,589 and L-731,373 in the immunosuppressed-rat model were both approximately 0.03 mg/kg when the compounds were administered subcutaneously b.i.d. for 4 days for treatment of acute PCP. L-733,560 was threefold more potent, with an ED₉₀ of 0.01 mg/kg. In contrast, L-693,989 was approximately 15 times less active (ED₉₀ of 0.15 mg/kg) and cilofungin was approximately 300 times less active (ED₉₀ of 3.0 mg/kg) than L-733,560. The complete titration data are shown in Fig. 2.

TABLE 1. Structures and efficacies of the lipopeptide antibiotics

Compound	Modification(s) at:			ED ₉₀ (mg/kg) for:			IC ₅₀ (nM) for β-glucan
	R1	R2	R3	<i>P. carinii</i>	<i>C. albicans</i> ^a	<i>A. fumigatus</i> ^b	
L-688,786	H	CH ₂ CONH ₂	H	0.15	1.45	ND ^c	70
L-693,989	PO ₃ HNa	CH ₂ CONH ₂	H	0.15	1.50	>20.00	NA ^d
L-731,373	H	(CH ₂) ₂ NH ₂	H	0.03	0.03	>20.00	10
L-705,589	H	CH ₂ CONH ₂	(CH ₂) ₂ NH ₂	0.03	0.05	0.12	11
L-733,560	H	(CH ₂) ₂ NH ₂	(CH ₂) ₂ NH ₂	0.01	0.01	0.47	1

^a ED₉₀s in an in vivo model with DBA/2 mice, as reported by Bartizal et al. (4).

^b ED₉₀s in a mouse survival model, as reported by Abruzzo et al. (1).

^c ND, not done.

^d NA, not active, >1 μM.

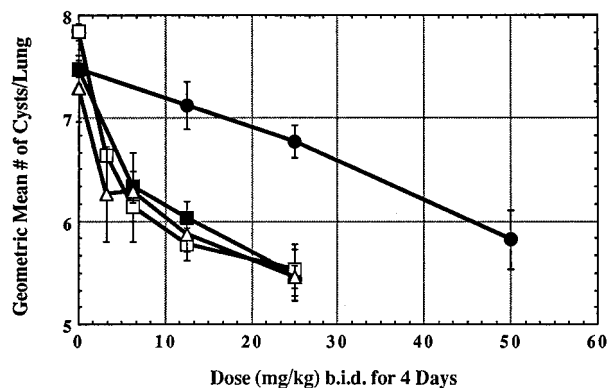


FIG. 3. Comparative titration curves for oral therapy of acute *P. carinii* infection in the immunosuppressed-rat model. All points are the means of data for four to six rats. All compounds (L-693,989 [●], L-705,589 [■], L-731,373 [△], and L-733,560 [□]) were administered in water b.i.d. for 4 days. All data are the \log_{10} mean numbers of cysts \pm the standard errors (shown with error bars).

Efficacies of compounds given orally and oral prophylaxis.

All compounds tested were found to be efficacious when administered orally for 4 days b.i.d. to treat acute *P. carinii* infections (Fig. 3). The ED_{90} s for cyst clearance were 2.2 mg/kg for L-733,560, 4.0 mg/kg for L-731,373, 5.0 mg/kg for L-705,589, and 32.0 mg/kg for L-693,989.

The ED_{90} for daily prophylaxis in dexamethasone-treated rats was 2.2 mg/kg for L-733,560 (Fig. 4). The standard error for the groups was relatively small, demonstrating that the effect was consistent within each dosage group, especially at the more efficacious doses. The *P. carinii*-specific DNA probe (Fig. 4) showed that L-733,560 at an oral dose of 6.25 mg/kg efficiently prevented proliferation of both *P. carinii* cysts and trophozoites (nuclei) (limit of detection, \log_{10} 4.69). The effects of orally administered cilofungin were not evaluated in these studies.

Histological examination of the lung tissues from the animals on daily prophylaxis clearly demonstrated a lack of the cysts, trophozoites, and foamy exudate commonly seen in lung tissues of infected animals with acute PCP. Similar histological

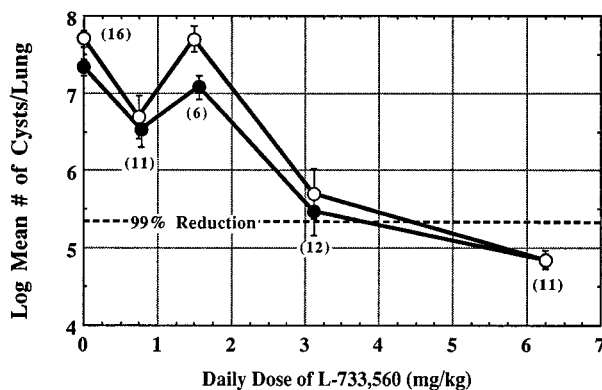


FIG. 4. Oral prophylaxis dose titration of L-733,560 in the rat model. A single oral dose was given each day for the entire immunosuppression period of 6 weeks. The bars represent standard errors of the \log_{10} means, and the numbers of animals in the groups are in parentheses. The limit of detection was \log_{10} 4.69. Five rats in the 6.25-mg/kg group and seven rats in the 3.12-mg/kg group had no detectable cysts and were scored as having one cyst in 20 microscopic fields. All animals in the control group had detectable cyst levels. ●, visual cyst count; ○, DNA probe trophozoite count.

effects have been shown previously for subcutaneous prophylaxis with L-693,989 (17).

DISCUSSION

Modification of pneumocandin B₀ at the R2 position by the conversion of the hydroxyglutamine to a hydroxyornithine (L-731,373) or the addition of an aminoethyl ether at the R3 position (L-705,589) increases the antipneumocystis activities of the compounds by fourfold. These modifications combined (as in L-733,560) were synergistic, resulting in a 10-fold improvement in potency against *P. carinii*. Results from previous studies (1, 4) show that the activity against *C. albicans* was enhanced 30- to 50-fold with the individual modifications of L-688,786 (L-705,589 and L-731,373) and 200-fold with both modifications (L-733,560) (Table 1). In contrast, the aminoethyl ether at R2 is critical for the activity against *A. fumigatus*, resulting in a >500-fold improvement in potency, while the modification at R3 resulted in only minimal additional activity (1). These results demonstrate that the structure-activity relationship for each organism may be unique for this class, as has been previously proposed (15). However, it may also be, in part, due to the differences in the animal models used for demonstrating the efficacies against each organism.

In addition to the 10-fold improvement in parenteral activity of L-733,560 over that of L-688,786 against *P. carinii*, there is a >50-fold improvement when L-733,560 is used as an oral prophylactic agent. This, combined with the impressive efficacies of these compounds against *C. albicans* and *A. fumigatus*, demonstrates the potential for the use of these compounds as broad-spectrum prophylactic agents in immunocompromised patients. These compounds should provide a safer alternative to amphotericin B, which is rarely used for antifungal prophylaxis because of its toxicity and which lacks efficacy against *P. carinii*. Of course, the potential use of any of these compounds for oral prophylaxis would depend on their bioavailability in humans.

It has also been demonstrated previously that L-693,989 can be used at microgram doses via aerosol prophylaxis to prevent PCP in rats (14). The pneumocandins with amino substitutions would be expected to be 5 to 10 times more effective as aerosols than L-693,989, which was fully effective at preventing PCP at a daily dose of 0.7 μ g per lung or a weekly dose of 77.9 μ g per lung (14). The improved spectrum of activity of these compounds could also aid in preventing the colonization of *Aspergillus* spp. in the lung, which is the common source for the development of systemic aspergillosis.

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