PNEUMOCANDINS FROM *Zalerion arboricola*

IV. BIOLOGICAL EVALUATION OF NATURAL AND SEMISYNTHETIC PNEUMOCANDINS FOR ACTIVITY AGAINST *Pneumocystis carinii* AND *Candida* SPECIES

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(Received for publication June 15, 1992)

A series of lipopeptide compounds co-produced during the fermentation of pneumocandin A_0 (L-671,329) and related semisynthetic compounds were evaluated *in vivo* against *Pneumocystis carinii* pneumonia and systemic candidiasis. In addition, they were tested *in vitro* against a panel of pathogenic *Candida* species and in a *Candida* membrane 1,3-β-D-glucan synthesis assay. The results of these studies demonstrate that pneumocandin A_0 and pneumocandin B_0 (L-688,786) are the most potent compounds when considering both antipneumocystis and anticandida activity. Other compounds in the series are selectively more potent against *P. carinii* or *Candida albicans* suggesting a diverging structure-activity relationship. Evaluation of these compounds for their ability to inhibit *C. albicans* 1,3-β-D-glucan synthesis *in vitro* demonstrates that they inhibit this process. A positive correlation between 1,3-β-D-glucan synthesis inhibition and *in vitro* antifungal activity was also demonstrated for some of the pneumocandins.

The pneumocandins, isolated as fermentation products produced by *Zalerion arboricola* ATCC 20868[1] and related semisynthetic derivatives,[2] were evaluated in murine models for acute *Pneumocystis carinii* pneumonia[3] and disseminated candidiasis infection[4] following the discovery that pneumocandin A_0 (L-671,329) was active against both pathogens.[3,5] Pneumocandins were also evaluated *in vitro* against a panel of clinically relevant pathogenic fungi and in a *Candida albicans* membrane 1,3-β-D-glucan synthesis assay, since the proposed mechanism of action of these compounds is alteration of the cell wall via the inhibition of 1,3-β-D-glucan synthesis.[6]

The pneumocandins are closely related to the echinocandins,[7] another family of lipopeptides with antifungal activity and are, therefore, compared here to two members of this class, tetrahydroechinocandin B and cilofungin.[8] Some echinocandins characteristically hemolyze human and mouse erythrocytes *in vitro* at therapeutically useful concentrations,[9] while pneumocandin A_0 (L-671,329) exhibits low hemolytic potential.[5] To determine the hemolytic potential of this class of compounds, all of the pneumocandins were evaluated in a red hemolysis assay.

The data generated in this study demonstrate that the pneumocandins are potent antifungal agents. This collection of closely related structures allows for the development of some initial structure-activity relationships and demonstrates that there is divergence in activity against *P. carinii* and *C. albicans*.

Materials and Methods

Compounds

All of the naturally occuring pneumocandins were isolated and purified by the Merck Natural Products

Isolation group. The semisynthetic 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. Trimethoprim and sulfamethoxazole were obtained from Phoenix Pharmaceuticals (St. Louis, MO). Pentamidine isethionate was obtained from Sigma (St. Louis, MO). Test compounds were solubilized in 10% DMSO. Amphotericin B (AMB) was prepared according to the manufacturer's instructions for Fungizone (E. R. Squibb).

Glucan Synthase Inhibition Assay

Buffer A: 10% glycerol (1.2 M) in phosphate buffered saline (PBS) containing phenylmethylsolfonyl fluoride (PMSF) (0.5 mM) and dithiothreitol (DTT) (1 mM). Buffer B: Same as buffer A, but without PMSF and DTT. Buffer C: DULBECCO's PBS, pH 7.0 (Gibco, Grand Island, NY) containing EDTA (5 mM) and DTT (50 mM). α-Amylase stock solution: The enzyme was obtained from Sigma Chemical Co., St. Louis, MO, supplied as a suspension in ammonium sulfate. This suspension (10 μl, 0.24 mg protein, equivalent to 264 units) was diluted with 115 μl buffer B. UDP-[6, ^{3}H]-glucose was obtained from Amersham Radiochemicals, Arlington Heights, IL, supplied at 250 μCi/250 μl (14.7 Ci/mmol); 5 μl of a 1 : 5 dilution (500,000 dpm) is equivalent to 77 pmol. GTP-γ-S was obtained from Sigma.

Candida albicans (MY 1208) cells were grown to early log phase (6~8 hours) in 500 ml of Sabouraud Dextrose broth (Difco) in a 2-liter baffled shake flask at 28°C at 150 rpm. The membrane-associated synthase system was prepared from protoplasts essentially by the method of TAFT *et al.*[10] The assay of ^{3}H-glucan was conducted as a modification of the method previously described by CABIB and KANG for *Saccharomyces cerevisiae*.[11] The total volume in wells of a microtiter plate designed for automated collection of the products of synthesis was 80 μl. The incubation system contained 6.6% DMSO, 125 mM Tris-HCl (pH 7.5), 0.25 mM DTT, 0.15 mM PMSF, 0.4 M glycerol, 0.75 mM EDTA, 0.25% BSA, 40 mM GTP-γ-S, 2.5 mM laminaribiose, 30 units/system of α-amylase, synthase (500 μg protein/ml assay system) contained in 15 μl and 25 μm UDP-glucose, the required concentration added in 10 μl including UDP-[6, ^{3}H]-glucose (500,000 dpm). To determine the inhibitiory effect of analogs, samples were added at 1.5 mM and serially diluted to a level of 0.04 μM. Compounds for evaluation were contained in 4 μl of 50% DMSO. During the assay, microtiter plates were covered with Parafilm and incubated for 60 minutes at 24°C with agitation on a Minimix stand (Fisher Scientific). The reaction was terminated by the addition of TCA (10%, 100 μl/well) and the resulting insoluble materials were collected on a glass filter mat in a Wallac (LKB) cell harvester, followed by automated wash cycles (water and 95% ethanol). The incorporated radiolabel per well was determined in a model 1205 Betaplate liquid scintillation counter (LKB, Wallac). The 1,3-β-D-glucan synthesis inhibitory concentration (IC_{50}) was defined as the concentration at which a compound inhibited 50% of the production of acid precipitable product.

Broth Microdilution Assay for Minimum Inhibitory and Fungicidal Determinations

Compounds were evaluated against a selected panel of clinically relevant fungi for the determination of antifungal spectrum and potency. Broth microdilution methodology was employed to determine minimum fungicidal concentrations (MFC) as described previously.[12] MFC values were determined by removing aliquots from each well and plating them on compound-free agar medium and defined as the lowest concentration of compounds showing less than 4 colonies.

Red Blood Cells (RBC) Lysis Assay

A 4%-suspension of freshly drawn whole heparinized human or CD-1 mouse blood was prepared by adding 2.0 ml of blood to 50 ml sterile 5% glucose. Compounds were solubilized in 10% DMSO at a concentration of 4.0 mg/ml and further diluted to a concentration of 0.5 μg/ml by the addition of sterile 5% glucose. Test compounds were dispensed into wells of microtiter plates and serially two-fold diluted in 5% glucose and then 38 μl of RBC suspension was added to each well to yield final test concentrations of 400 to 0.20 μg/ml. Plates were gently and incubated at room temperature and results were determined after 2 hours of incubation. Hemolysis of erythrocytes cells was indicated by complete or partial clearing (lysis). Minimum lytic concentration (MLC) is defined as the lowest concentration of a test compound which produces complete or partial lysis of erythrocytes.

The Target Organ Kidney Assay (TOKA)—Evaluation of *In Vivo* Efficacy Against *Candida albicans*

The Target Organ Kidney Assay (TOKA) used for evaluation of antifungal efficacy against *C. albicans* has been described in detail previously.[4] Briefly, *C. albicans* MY 1055 was administered iv in the tail veins DBA/2 mice, at an inoculum of 7.5×10^4 cells/mouse. Pneumocandins were solubilized in 10% DMSO and tested at various concentrations ranging from 1.25 to 10 mg/kg ip, twice-daily (b.i.d.) for 4 consecutive days. Five mice per group were sacrificed at 7 days after initiation of infection for determination of *C. albicans* colony forming units (cfu) per gram kidneys. Effective dose 90% (ED_{90}) estimates were derived to rank efficacy. The range of variability in the TOKA is within a two-fold dilution range around the ED_{90} value.

Rat Acute *Pneumocystis carinii* Pneumonia Model—Evaluation of *In Vivo* Efficacy Against *Pneumocystis carinii*

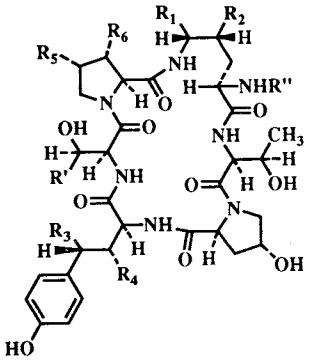
The dexamethasone-immunosuppressed rat model used in these studies has been described in detail elsewhere.[3] To evaluate the efficacy of the compounds in this model, rats immunosuppressed for six weeks were treated intraperitoneally or subcutaneously, b.i.d. for four days. All compounds were solubilized in 10% DMSO and injected in a 0.5 ml volume. Compounds were tested at the appropriate levels to establish an ED_{90}. Each test group contained 6 rats. On the morning following the last dose, rats were sacrificed and their lungs were removed and processed according to the procedure outlined previously.[3]

Results and Discussion

The *in vivo* ED_{90} values for the pneumocandins ranged from 0.15 mg/kg to >2.5 mg/kg against *P. carinii* and from 0.35 mg/kg to >6.00 mg/kg for *C. albicans* (Table 1). Pneumocandin B_0 was the best dual purpose compound in the series for the treatment of *P. carinii* and *C. albicans*. It also had the broadest anticandida spectrum and potency *in vitro* (Table 2). These were obvious differences in the structure-activity relationships (SAR) for the pneumocandin compounds against these two pathogens *in vivo*. The anticandida activity was depressed in compounds reduced at both R_1 and R_2 such as pneumocandin A_2, pneumocandin A_4 and pneumocandin B_2, while antipneumocystis activity was generally less sensitive to these changes. The anticandida activity was also depressed in pneumocandin C_0 when there was a 4-hydroxyproline rather than a 3-hyroxyproline (pneumocandin B_0). In contrast compound pneumocandin C_0 retained anti-pneumocystis activity which was comparable to pneumocandin B_0. The compounds which appeared to have reduced antipneumocystis activity were pneumocandin A_4, semisynthetic bis-reduced compounds L-691,936 and L-692,289 and mono-reduced L-706,577 indicating that the R_3 hydroxyl is important for potent antipneumocystis activity. In contrast reduction at R_3 had less influence on the anticandida activity.

The lack of an *in vitro* assay for *P. carinii* makes it difficult to determine if the SAR differences seen relative to anticandida activity with these compounds were due to intrinsic potency or pharmacokinetic differences between the mouse and the rat. Since *P. carinii* resides exclusively in the lungs and *C. albicans* efficacy determinations were conducted on kidney tissue, differences in tissue distribution of the compounds could account for divergent SAR's. Tissue distribution studies with radiolabeled pneumocandin B_0 have indicated that there was little difference in distribution of this compound in lungs relative to kidneys (R. HAJDU, unpublished data, not shown). However, pneumocandin B_0 is one of the few compounds with good efficacy against both pathogens and tissue distribution data is not available for other compounds which are selective for *C. albicans* or *P. carinii*. Evaluation of the whole body distribution of other pneumocandins would aid in determining if the difference is due to the concentration of compound in specific tissues. The divergence of SAR's is further exemplified by comparing the antipneumocystis activity of tetrahydroechinocandin B and cilofungin (Table 1). While tetrahydroechinocandin B was slightly more

Table 1. Structures of the pneumocandins and related compounds and corresponding biological data.



R″

A = 10,12-dimethylmyristoyl
B = stearoyl
C = *p*-(*n*-octyloxy)benzoyl

Compound	R_1	R_2	R_3	R_4	R_5	R_6	R′	R″	PCP ED_{90} (mg/kg)	TOKA ED_{90} (mg/kg)	MLC (μg/ml) man/mouse	1,3-β-D-glucan (μg/ml)
Pneumocandin A_0	OH	OH	OH	OH	CH_3	OH	H_2NCOCH_2	A	0.25	1.15	>400/>400	0.20
Pneumocandin A_2	H	H	OH	OH	CH_3	OH	H_2NCOCH_2	A	<1.25	>6.00	400/400	0.20
Pneumocandin A_4	H	H	H	H	CH_3	OH	H_2NCOCH_2	A	>2.50	>6.00	>400/>400	0.30
L-691,936	H	OH	H	OH	CH_3	OH	H_2NCOCH_2	A	>1.00	2.00	>400/>400	1.00
Pneumocandin B_0	OH	OH	OH	OH	H	OH	H_2NCOCH_2	A	0.15	0.35	>400/>400	0.07
Pneumocandin B_2	H	H	OH	OH	H	OH	H_2NCOCH_2	A	0.60	>6.00	>400/>400	
L-733,686	H	OH	OH	OH	H	OH	H_2NCOCH_2	A	0.15	>6.00	>400/>400	0.30
L-706,577	OH	OH	H	OH	H	OH	H_2NCOCH_2	A	>1.25	1.46	>400/200	0.04
L-692,289	H	OH	H	OH	H	OH	H_2NCOCH_2	A	1.25	1.66	>400/>400	0.07
Pneumocandin C_0	OH	OH	OH	OH	OH	H	H_2NCOCH_2	A	0.30	>6.00	200/200	0.50
Tetrahydroechinocandin B	OH	OH	OH	OH	CH_3	OH	H_3C	B	0.15	0.50	25/25	0.15
Cilofungin	OH	OH	OH	OH	CH_3	OH	H_3C	C	4.00	1.13	>400/>400	>1.00

PCP is the *P. carinii* pneumonia *in vivo* results, TOKA is the target organ kidney assay for *C. albicans in vivo*, MLC is the minimum lytic concentration with human or mouse erythrocytes and 1,3-β-D-glucan is the IC_{50} for glucan synthesis inhibition *in vitro*.

Table 2. Anticandida minimal fungicidal concentrations (MFC's) and 1,3-β-D-glucan synthesis IC_{50}'s for the pneumocandins and related analogs.

Compound	Minimum fungicidal concentration (MFC) μg/ml					1,3-β-D-glucan IC_{50} (μg/ml)
	Candida albicans			*C. tropicalis*	*C. parapsilosis*	
	MY 1055	MY 1028	MY 1750	MY 1012	MY 1010	
Pneumocandin A_0	0.125	0.5	0.125	0.5	2.0	0.20
Pneumocandin A_2	2.0	1.0	2.0	4.0	8.0	0.20
Pneumocandin A_4	0.5	0.5	0.5	2.0	2.0	0.30
L-691,936	1.0	0.5	0.25	1.0	4.0	1.00
Pneumocandin B_0	0.125	0.06	NR	0.06	2.0	0.07
Pneumocandin B_2	1.0	1.0	16.0	1.0	8.0	
L-733,686	2.0	0.25	4.0	5.0	0.5	0.30
L-706,577	0.25	0.125	0.5	0.06	4.0	0.04
L-692,289	0.25	0.25	NR	0.5	4.0	0.07
Pneumocandin C_0	2.0	2.0	4.0	1.0	32.0	0.50
Tetrahydroechinocandin B	0.5	0.5	0.25	2.0	4.0	0.15
Cilofungin	0.5	0.5	0.5	0.25	8.0	>1.00

The particular strain of each species of *Candida* is listed above the species name.

active than cilofungin against *C. albicans in vivo* there is a substantial difference in the activity of these two compounds against *P. carinii*. Furthermore the potent antipneumocystis activity of tetrahydroechinocandin B implies that the cilofungin side chain (*p*-(*n*-octyloxy)benzoyl) is responsible for the loss of this activity.

The 1,3-β-D-glucan IC_{50}'s did not correlate well with the *in vitro* anticandida activity and may be attributable to permeability differences of viable yeast cells. A 1,3-β-D-glucan synthesis assay, in which inhibition by the lipopeptides can be measured, has not been established for *P. carinii*.

Most of the pneumocandins were not hemolytic with the exception of pneumocandin A_2, L-706,577 and pneumocandin C_0 which were mildly hemolytic (200~400 μg/ml) relative to an MLC of 25 μg/ml for tetrahydroechinocandin B. Cilofungin was not hemolytic (>400 μg/ml) as previously reported and was originally selected from a series of semisynthetic echinocandins for further development in part due to the lack of hemolytic activity.[9]

In summary, the pneumocandins are potentially useful for both *Candida* and *Pneumocystis* infections, both of which are prevalent in the immunocompromised patient. The reduced hemolytic potential of pneumocandin A_0 (L-671,329) seems to be consistent throughout this group of compounds suggesting that these compounds may be safer than the other naturally occuring echinocandins. Although inhibition of 1,3-β-D-glucan synthesis is the proposed mechanism of action of these compounds this has not been demonstrated in the case of *P. carinii* other than reports of the assumed presence of 1,3-β-D-glucan polymer in the wall of the cysts.[13,14] The divergence in SAR and differences in potency against *C. albicans* and *P. carinii* suggests that there may be a difference in the target between in these two pathogens.

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