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Antipneumocystis Activity of Water-Soluble Lipopeptide L-693,989 in Rats

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Water-soluble lipopeptide L-693,989 was evaluated for its antipneumocystis activity in rats. Rats from colonies with latent *Pneumocystis carinii* infections were immunosuppressed with dexamethasone for 6 weeks to facilitate the development of acute *P. carinii* pneumonia (PCP). After 6 weeks, the rats were maintained on dexamethasone and were treated twice daily for 4 days with various concentrations of L-693,989. At a dose of 0.15 mg/kg of body weight, the compound effectively eliminated 90% of the cysts in 4 days. Trophozoite forms of *P. carinii* were still present in these animals, as determined by using a *P. carinii*-specific DNA probe. A 3-week therapy study showed that the trophozoite load did not expand during treatment and that the trophozoites already present at the initiation of therapy appeared to persist. This may be a consequence of the stage specificity of the compound for cyst development and the severe immunosuppressive effects of dexamethasone on rats. When evaluated as a daily parenteral prophylactic agent, L-693,989 was effective in preventing the development of both *P. carinii* cysts and trophozoites, demonstrating its potential for use in prophylaxis and implying that the cyst stage of *P. carinii* is an obligatory step in trophozoite multiplication. The foamy exudate commonly associated with *P. carinii* infections was absent in the lungs of rats on prophylaxis. The compound was also evaluated via oral administration and was found to have a 90% effective dose of 32 mg/kg for therapy of acute infections and 5 mg/kg for daily prophylaxis.

Pneumocystis carinii pneumonia (PCP) is a life-threatening disease which occurs in immunocompromised patients, especially those afflicted with AIDS. Although agents are available for the treatment and prevention of PCP, there is an unusually high incidence of adverse reactions to these treatments, particularly in patients with AIDS (14). Consequently, safer agents for controlling this disease are needed. We recently reported a novel method for controlling *P. carinii* infections in rodents using 1,3-β-glucan synthesis inhibitors (12). The more potent of these inhibitors, the lipopeptide natural product L-671,329 (Fig. 1), rapidly eliminates *P. carinii* cysts in 4 days when it is used to treat immunosuppressed rats with acute PCP. In addition to the antipneumocystis activity, L-671,329 is also effective against *Candida* infections in animal models (2). The lack of a counterpart for 1,3-β-glucan synthesis in humans should allow for selective killing of *P. carinii* and *Candida* spp. A shortcoming of L-671,329 and similar lipopeptides is their insolubility in aqueous solution, limiting their potential use as intravenous agents. Although cosolvent systems have been used for some insoluble drugs in the past, many of these formulations may no longer be acceptable because of adverse reactions (4, 8, 10); thus, water-soluble compounds are much more desirable. Therefore, a semisynthetic water-soluble prodrug, L-693,989 (Fig. 1), was synthesized (1) from the natural product L-688,786 (an analog of L-671,329 [Fig. 1]) (13). The in vivo anticandidal (3) and antipneumocystis activities of L-693,989 were comparable to those of L-671,329 and L-688,786.

To determine the potential clinical use of L-693,989 in treating and preventing *P. carinii* infections, a variety of studies were conducted using the immunosuppressed rat model for PCP. Comparisons were made with the two most commonly used antipneumocystis agents, pentamidine isethionate and trimethoprim-sulfamethoxazole (TMP-SMX). Cilofungin, a related semisynthetic lipopeptide which was under development for and shown to be effective in humans against systemic (5) and esophageal (6) candidiasis, was also evaluated. Cilofungin is not water soluble and was administered to patients intravenously in 26% polyethylene glycol 300 (6). This vehicle was suspected of causing anion gap acidosis in several patients in one of the clinical trials with cilofungin (7).

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 were isolated by the Natural Products Isolation group. TMP and SMX were obtained from Phoenix Pharmaceuticals (St. Louis, Mo.). Pentamidine isethionate was obtained from Sigma (St. Louis, Mo.).

Efficacy studies. The dexamethasone immunosuppressed rat model used in these studies is similar to that described in detail elsewhere (9, 12). Briefly, male Sprague-Dawley rats (weight, 240 to 260 g) obtained from Sasco Laboratories (Omaha, Nebr.) were used in these studies. Rats were fed regular rodent chow (23% protein; Purina, St. Louis, Mo.) and were immunosuppressed with 2 mg of dexamethasone (Butler, Columbus, Ohio) per liter in the drinking water. Tetracycline (1 g/liter) was added to the drinking water to minimize bacterial infections. For studies of therapy of acute infections (acute therapy studies), rats previously immunosuppressed for 6 weeks were treated twice daily for 4 to 21 days by subcutaneous (s.c.) injection with various amounts of L-693,989 in a volume of 0.5 ml of sterile water. All animals remained on immunosuppression therapy with dex-

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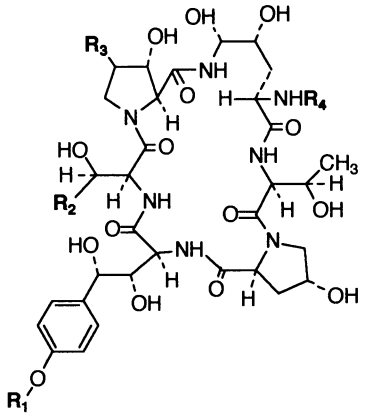

Compound	R_1	R_2	R_3	R_4	ED_{90} (mg/kg)
L-671,329	H	CH_2CONH_2	CH_3	10,12 dimethylmyristoyl	0.15
L-688,786	H	CH_2CONH_2	H	10,12 dimethylmyristoyl	0.08-0.15
L-693,989	PO_3Na	CH_2CONH_2	H	10,12 dimethylmyristoyl	0.15
Cilofungin	H	CH_3	CH_3	p-(n-octyloxy)benzoyl	≥5.00
Tetrahydroechinocandin B	H	CH_3	CH_3	stearoyl	0.08

FIG. 1. Structure of the lipopeptide antibiotics. L-671,329 and L-688,786 are natural products which possess potent antifungal activity against various species of *Candida*. They are produced and isolated from cultures of the fungus *Zalerion arboricola*. Their proposed mechanism of action is the inhibition of the synthesis of 1,3-β-glucan, a major constituent of many fungal cell walls (12). L-693,989 is the phosphate ester prodrug of L-688,786, and tetrahydroechinocandin B and cilofungin are semisynthetic derivatives of echinocandin B. The tetrahydroechinocandin B nucleus differs from the nuclei of the natural products from *Z. arboricola* by having a threonine rather than a hydroxyglutamine. The ED_{90}s represent the doses required to eliminate 90% of the cysts from the lungs of acutely infected rats after 4 days of therapy.

amethasone throughout the study. Three rats were sacrificed at the initiation of the study to confirm the presence of acute *P. carinii* pneumonia (see section on evaluation of lung tissue below). Two known antipneumocystis agents, pentamidine isethionate and TMP-SMX, were used as positive controls in the study. TMP-SMX was delivered in the drinking water ad libitum at a concentration of 0.2 g of TMP per liter and 1.0 g of SMX per liter. Pentamidine isethionate at a concentration of 10.0 mg/kg of body weight was injected intravenously in 0.25 ml of water via the tail vein once a day. Six animals per drug treatment group and vehicle control groups were sacrificed at various time points to determine the degree of infection. Routinely, some of the immunosuppressed rats die during immunosuppression as a result of being highly susceptible to a variety of opportunistic infections. All animals used in the study were housed and cared for under the guidelines set forth in the *NIH Guide for the Care and Use of Laboratory Animals* (16).

Dose titration experiments for treatment of acute PCP. Rapid clearance of cysts was observed after only 4 days of s.c. treatments with the lipopeptide compounds; subsequently, all acute therapy studies were conducted with only 4 days of twice-daily dosing. Dexamethasone-treated rats with acute PCP were injected s.c. with compounds at various concentrations to determine the effective dose for 90% cyst clearance (ED_{90}) relative to the ED_{90}s for vehicle controls. Each dose was given to six animals. Compounds included L-693,989, L-671,329, L-688,786, and two semisynthetic structurally related lipopeptides, tetrahydroechinocandin B and cilofungin (structures are given in Fig. 1). All compounds except L-693,989 were delivered in 10% dimethyl sulfoxide; L-693,989 was delivered in water. Cilofungin was also evaluated in a vehicle containing 26% polyethylene glycol 300, since this was the vehicle used in clinical trials (5).

Evaluation of lung tissue. All lung tissues were processed with a Brinkman homogenizer, and the quantitation of cysts in each lung was done as described previously (12), with the exception that all centrifugations were conducted at 1,700 × *g*. Quantitation of *P. carinii* trophozoites (nuclei) was achieved by hybridization of a radiolabeled, *P. carinii*-specific DNA probe with total DNA extracted from the lung tissues of individual rats (11). 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 methenamine silver. Hematoxylin-eosin-stained sections were examined to determine whether the foamy exudate commonly found with PCP was present. Slides that were counterstained with methanemine silver were examined for the degree of cyst accumulation.

Direct effect of L-693,989 on *P. carinii* cysts in vitro. To explore the effect of prodrug L-693,989 on cysts, freshly homogenized rat lung tissue from acutely infected animals was incubated for 24 h at 37°C in parent compound

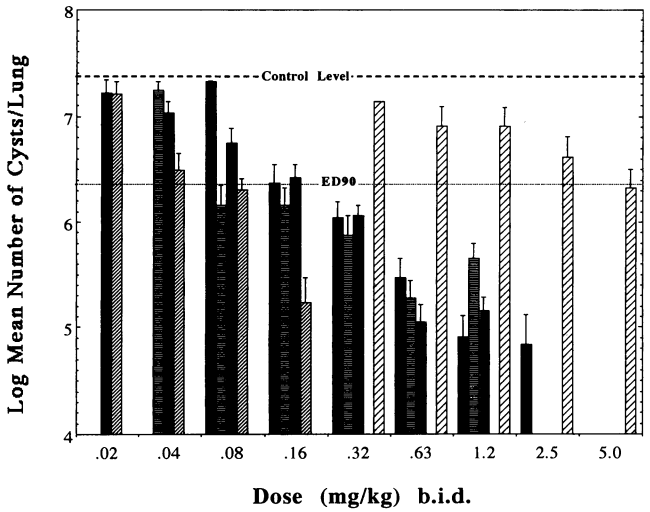

FIG. 2. Dose titrations for cyst clearance with various lipopeptide compounds. Each point represents 3 to 17 animals; the control line represents the mean of 68 control animals that were sham treated with the vehicle twice daily (b.i.d.). The mean number of cysts per rat lung in the control group was 7.39 ± 0.13. The ED_{90} represents a 90% reduction in the number of cysts in treated rats relative to the number of cysts in the control group. The error bars represent the standard error of the log mean. ■, L-671,329; ▤, L-688,786; ▩, L-693,989; ▨, tetrahydroechinocandin B; ▨, cilofungin.

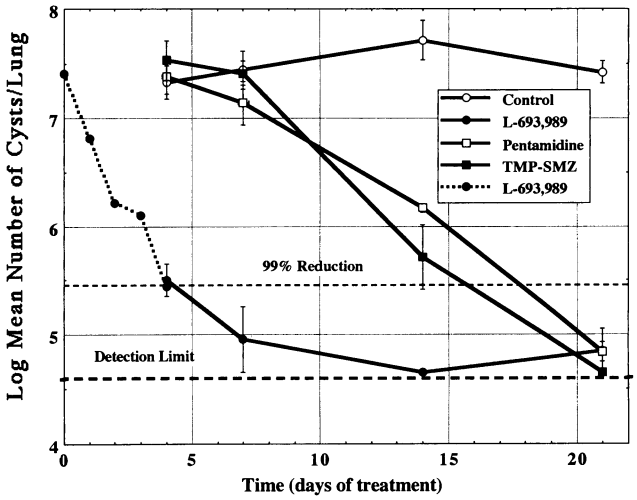

FIG. 3. Cyst clearance during treatment of acute PCP with L-693,989, TMP-SMX (SMZ), and pentamidine. Each point represents six animals. The error bars represent the standard errors of the log means. The dotted line represents the results of a short-term study for determination of the degree of cyst clearance after treatment with L-693,989 for periods of less than 4 days.

L-688,786 at a concentration of 5 mg/ml (10% dimethyl sulfoxide). The sample was then air dried on microscope slides, fixed in ether-sulfuric acid, and stained with toluidine blue (12). The sample was then examined microscopically and compared with slides containing the same homogenate incubated in vehicle alone.

s.c. prophylaxis. To explore the prophylactic potential of L-693,989, the compound (in sterile water) was injected s.c. at a dose of 1 mg/kg daily from the initiation of dexamethasone treatment and was continued through 6 weeks of immunosuppression. All animals were then sacrificed, and the degree of *P. carinii* infection was determined as described above. Cyst counts in the lungs of L-693,989-treated rats were compared with those in the lungs of vehicle control-treated rats. Twenty rats were used for each group.

Oral efficacy. Rats with acute PCP were given 0.5 ml of L-693,989 (in sterile water) by gavage so that concentrations ranging from 6.25 to 50 mg/kg twice daily for 4 days were delivered. After therapy, rats were sacrificed and their lung tissues were processed for quantitation of cysts (as described above) so that the number of cysts could be compared with those in vehicle control-treated rats.

An oral prophylaxis study was also conducted with L-693,989. Rats were dosed daily by gavage with 0.5 ml of L-693,989 in sterile water at concentrations ranging from 6.25 to 50 mg/kg during the entire 6-week immunosuppression period. After 6 weeks, the rats were sacrificed and the numbers of cysts were determined as described above.

RESULTS

Dose titration. The ED_{90}s of L-671,329, L-688,786, and L-693,989 were approximately 0.15 mg/kg (Fig. 1) when the compounds were administered s.c. twice daily for 4 days to treat acute PCP. In contrast, cilofungin was more than 25 times less active against *P. carinii*, with an ED_{90} of 3.0 mg/kg. Tetrahydroechinocandin B, which differs from cilofungin only at the fatty acid side chain, was similar in potency to L-693,989, with an ED_{90} of 0.08 mg/kg. The complete titration data are shown in Fig. 2. Similar results were seen with intravenous administration of L-693,989, L-688,786, and cilofungin in 26% polyethylene glycol 300 (data not shown).

Therapy. In the 3-week therapy study, there was rapid elimination of cysts after 4 days of treatment with L-693,989, which was in contrast to the results for TMP-SMX and pentamidine, which required more than 2 weeks to achieve the same degree of cyst elimination (Fig. 3). Gross signs of toxicity were not observed with L-693,989 or TMP-SMX;

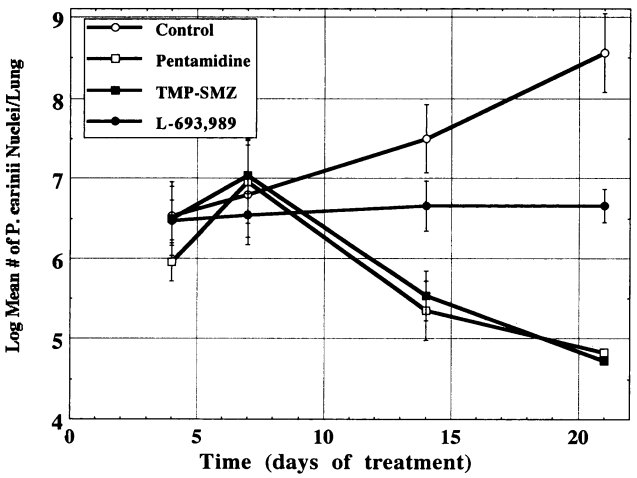

FIG. 4. Using a *P. carinii*-specific DNA probe, the total number of *P. carinii* nuclei per rat lung was determined after acute therapy with L-693,989, TMP-SMX (SMZ), or pentamidine. Each point represents the same six animals per group as in Fig. 3. The error bars represent the standard errors of the log means.

TABLE 1. Results of a prophylaxis study with daily injections of 1 mg of L-693,989 per kg during the initial 6 weeks of immunosuppression of the rats[a]

Treatment group	Log mean no. (% reduction) from:		Lung wt (g)
	Cysts	Nuclei	
Control	7.41 ± 0.17	7.60 ± 0.21	2.13 ± 0.16
L-693,989	4.69 ± 0.21 (99.8)[b]	4.87 ± 0.39 (99.8)[b]	1.76 ± 0.05

[a] The mean number of cysts per lung was determined microscopically; nuclei (trophozoites) were quantitated by using the *P. carinii*-specific DNA probe (11). The limit of detection was a log mean of 4.69. The statistics represent standard error of the log mean. Seven of the 10 rats in the L-693,989 prophylaxis group did not have detectable cysts and were scored as having 1 cyst in 20 microscopic fields. There were 11 rats in the control group, all of which were heavily infected with *P. carinii*.

[b] $P \leq 0.001$ (Student's t test).

rats treated with pentamidine isethionate were lethargic and exhibited necrosis at the injection site. Results of an additional study conducted to determine cyst clearance after treatment with L-693,989 for periods of less than 4 days are shown by the dotted line in Fig. 3. More than 75% of the cysts were eliminated after only 1 day of treatment, suggesting either the rapid turnover of cysts or the requirement for continuous renewal of cyst wall glucan. Overnight incubation of freshly isolated cysts in 5 mg of L-688,786 (the active parent of prodrug L-693,989) per ml had no effect on the cysts, and therefore it is unlikely that the compound has a cysticidal effect on *P. carinii*.

Trophozoite elimination (measured as nuclei) paralleled cyst clearance with both TMP-SMX and pentamidine treatment, while trophozoites persisted after treatment with L-693,989 for the same interval (Fig. 4). However, the number of *P. carinii* nuclei did not increase in the group treated with L-693,989, suggesting that progression of the infection was arrested.

s.c. prophylaxis. Daily prophylaxis with 1 mg of L-693,989 per kg administered s.c. effectively prevented the initial onset of PCP, as indicated by the minimal numbers of cysts and nuclei present in the lungs of treated rats relative to those in the lungs of controls (Table 1). The mean weight of the lungs of animals which were treated with L-693,989 was also lower than that of the lungs of the control rats, most likely because of the lack of edema, infiltrates, and *P. carinii* organisms, all of which were present in control animals with acute PCP. Histological examination of the lung tissues from these animals clearly demonstrated the lack of cysts (Fig. 5) and the foamy exudate (Fig. 6) commonly seen in the lung tissues of animals with acute PCP. There were initially 20 rats in each group, but after the 6 weeks of the study, there were 10 rats in the L-693,989 treatment group and 11 rats in the control group. The rats that died during the study, however, did not succumb to PCP but, rather, to other

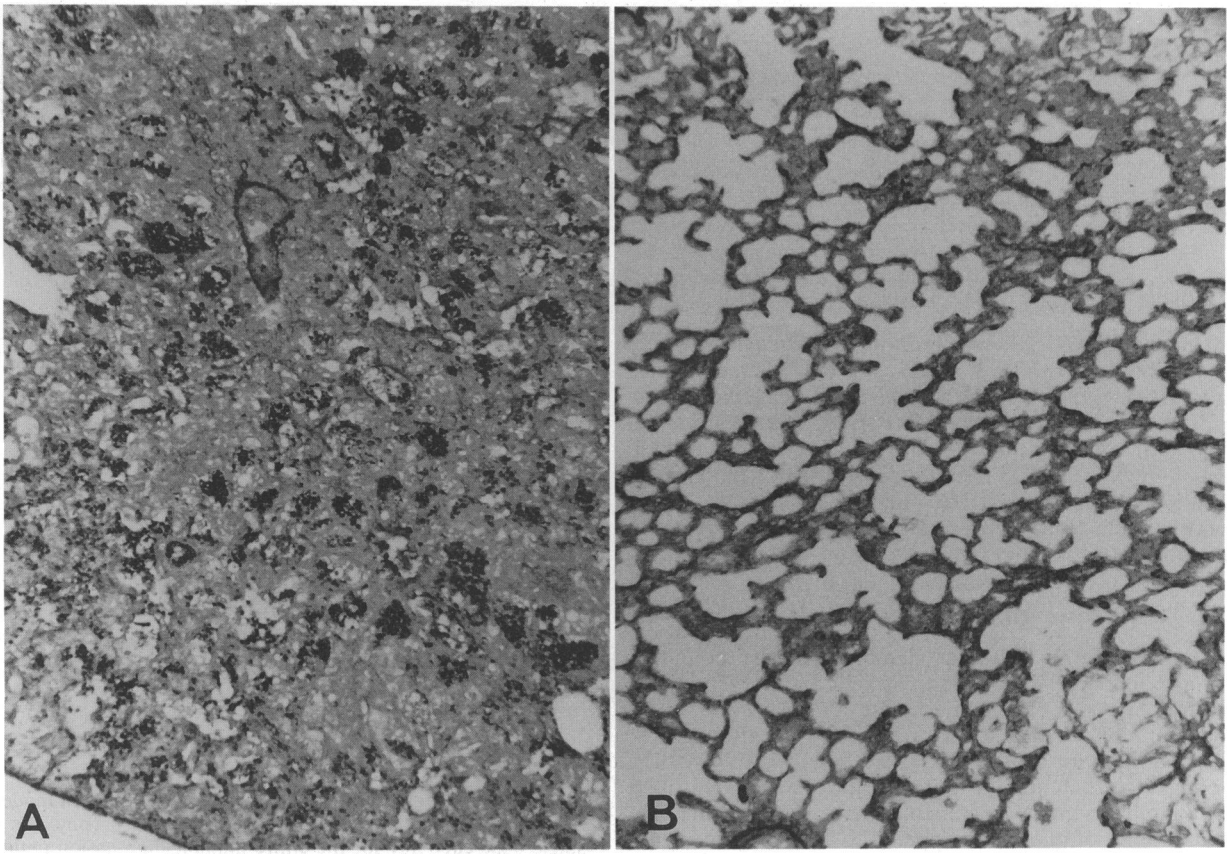

FIG. 5. Hematoxylin-eosin–methenamine silver-stained lung sections. (A) The lung of a control immunosuppressed rat after 6 weeks of dexamethasone treatment. Note the clusters of densely staining *P. carinii* cysts in many of the alveoli. (B) A section of lung from a rat treated prophylactically with L-693,989. No cysts are present, and the air spaces are clear because of the effective prevention of a *P. carinii* infection.

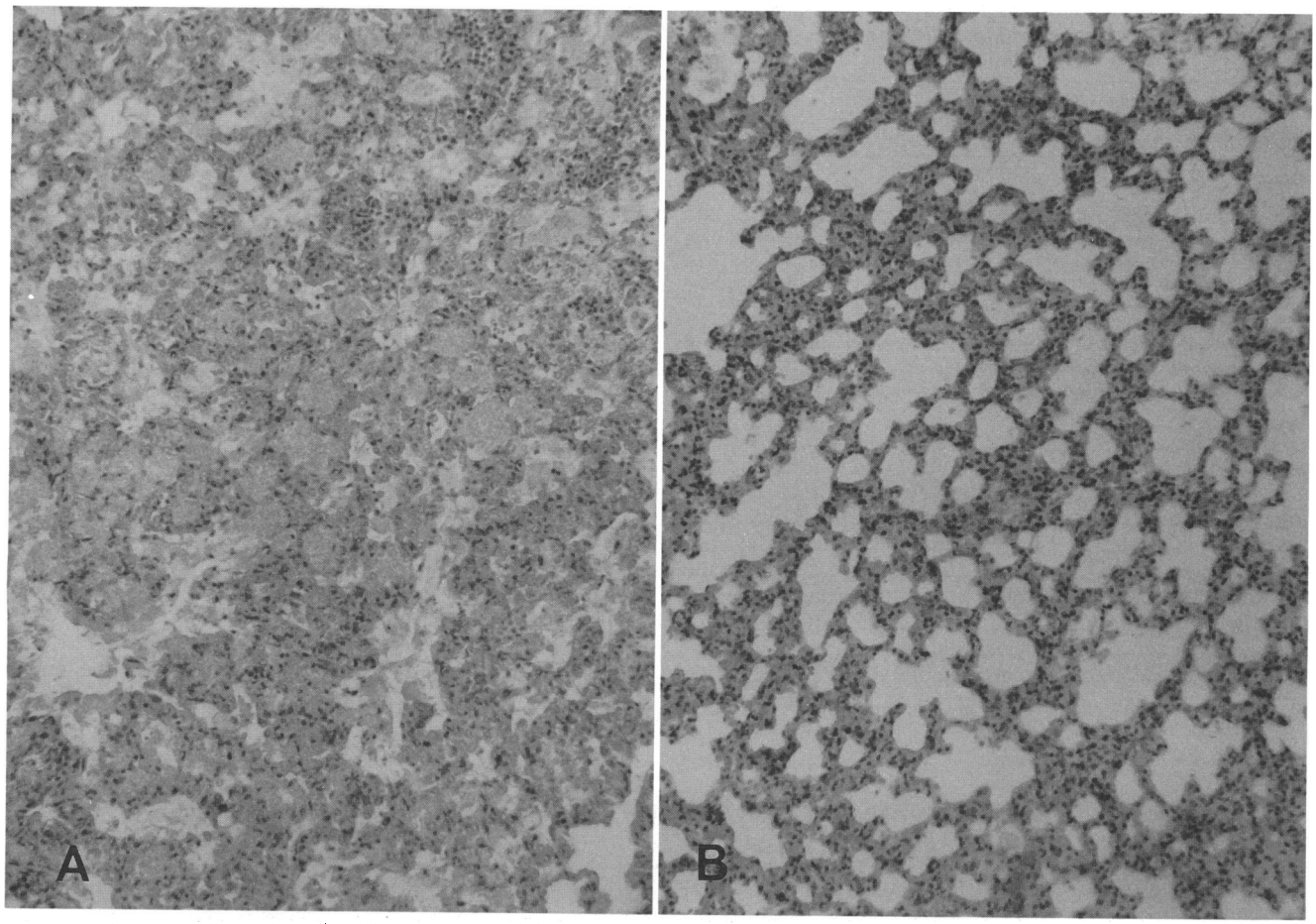

FIG. 6. Hematoxylin-eosin-stained lung sections. (A) The lung of a control immunosuppressed rat after 6 weeks of dexamethasone treatment. Note the gray staining exudate in most of the alveoli, which commonly occurs as a result of *P. carinii* infection. The nature of this material is unclear and it is thought to be a combination of organisms and infiltrates from the host. (B) A section of lung from a rat treated prophylactically with L-693,989. The air spaces are clear because of the effective prevention of a *P. carinii* infection.

opportunistic infections or as a result of extended steroid treatment.

Oral efficacy. L-693,989 was found to be efficacious when it was administered orally. The ED_{90} for cyst clearance was 32 mg/kg when L-693,989 was administered twice daily for 4 days for the treatment of acute *P. carinii* infections, while the ED_{90} for daily prophylaxis in dexamethasone-treated rats was 5 mg/kg (Fig. 7). The standard error for the groups was relatively low, demonstrating that the effect was consistent within each dosage group, especially at the more efficacious doses.

DISCUSSION

The results of these studies demonstrate the potential clinical use of L-693,989 for the treatment and prevention of *P. carinii* pneumonia. The compound is a potent inhibitor of cyst development, with an ED_{90} of 0.15 mg/kg after only 4 days of treatment for acute PCP. L-693,989 had activity that was equal to that of its parent, the natural product L-688,786, and 25 times greater than that of cilofungin. The reduced activity of cilofungin appears to be associated with its octyloxybenzoate side chain, since tetrahydroechinocandin B is much more potent and differs from cilofungin only by having a stearate side chain. It is also interesting that

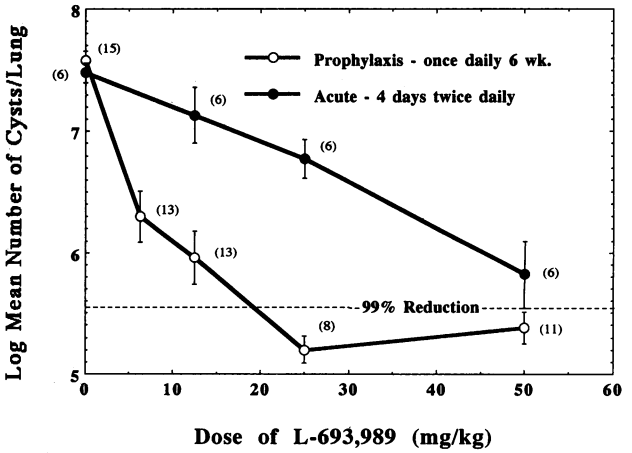

FIG. 7. An oral dose titration of L-693,989 in the rat model. The acute therapy was b.i.d. for 4 days, while the prophylaxis was a single dose each day for the entire immunosuppression period of 6 weeks. The bars represent the standard errors of the log means, and the numbers in parentheses represent the numbers of animals in the groups.

cilofungin was equally as potent as L-671,329 in the treatment of systemic candidiasis in a mouse model (1, 2), suggesting that the mechanism by which lipopeptides block the development of *P. carinii* cysts and *Candida* spp. may differ. However, the tissue distribution and the use of different rodent species for the two models could also account for the difference.

The cyst clearance obtained with L-693,989 was substantially more rapid than that obtained with the conventional antipneumocystis agents. While the clinical advantages of this can be determined only in patients with acute PCP, one would postulate that the rapid elimination of cysts from the alveoli would aid in restoring respiratory function. The number of trophozoites present in rats with acute PCP did not decrease during therapy with L-693,989, nor did it increase, suggesting that the trophozoite population may be incapable of expanding in the presence of the compound. This implies that the cyst stage is obligatory for trophozoite proliferation. Trophozoite clearance with TMP-SMX and pentamidine paralleled cyst clearance, indicating that these compounds are not cyst specific. The minimal change in nuclei number after 4 days of treatment with L-693,989 relative to that after 4 days of treatment with TMP-SMX and pentamidine (Fig. 4) suggests that the cysts contribute minimally, if at all, to the DNA probe counts, since all cysts were virtually eliminated by L-693,989 by that time point (Fig. 3). This may be attributable to our finding that only a small percentage of cysts have intracystic bodies (<10% [unpublished data]), or it may be due to a lack of disruption of the cyst forms when the DNA probe procedure is used (11).

Daily parenteral prophylaxis with 1 mg of L-693,989 per kg was very effective in preventing the development of cysts and trophozoite forms. The foamy inflammatory exudate commonly seen in human PCP was present in control rats but was absent from rats which received L-693,989 (Fig. 6). Further studies are needed to determine whether parenteral prophylaxis for PCP is feasible, although an oral agent for prophylaxis would be preferred. Despite its poor oral bioavailability in rats (≤1.0%), L-693,989 effectively prevented the initial onset of PCP with an ED_{90} of 5 mg/kg, which was over six times lower than the ED_{90} for treating acute PCP orally. Whether such a regimen would be effective in humans depends on the relative oral bioavailability of L-693,989 in humans and the intrinsic potency of L-693,989 against *P. carinii* infection in humans.

If it is applicable to humans, oral prophylaxis with L-693,989 would have clear advantages over oral prophylaxis with the drugs currently being used and may lack the side effects seen with the currently used agents because of the pathogen-specific mechanism of action of L-693,989. It would also eliminate the need for using aerosolized pentamidine, which is cumbersome and is suspected of causing disseminated *P. carinii* infections (15). Oral treatment of early stages of acute PCP with L-693,989 may be feasible with an increased treatment period beyond 4 days. However, a higher dose relative to that needed for prophylaxis would likely be required.

This study also adds new evidence with respect to the biology of *P. carinii*. The fact that trophozoites persist in acute therapy while neither stage develops during prophylaxis provides further evidence that the cyst form is an obligatory step in trophozoite proliferation. This may also explain the very limited in vitro growth of *P. carinii*. If the cyst form cannot develop in tissue culture, the number of trophozoites produced would be a function of the number of cysts isolated directly from lung tissue, which are thought to mature and release eight new trophozoites.

In conclusion, L-693,989 is a potentially useful agent for treating and preventing PCP. This, combined with its anticandidal activity (1, 3), makes it an attractive dual agent for immunocompromised patients. Its solubility in water allows for intravenous use, and it may also be useful as an oral prophylactic agent. The clinical impact of rapid cyst elimination by L-693,989 without rapid trophozoite elimination in treating acute PCP is not clear at this point. It is clear, however, that prophylaxis with L-693,989 prevents the initial onset of PCP.

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