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Aerosolized L-693,989 for *Pneumocystis carinii* Prophylaxis in Rats

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Water-soluble pneumocandin L-693,989, a potent antipneumocystis agent in the rat model for *Pneumocystis carinii* pneumonia (PCP), inhibits *P. carinii* cyst development and effectively prevents the development of PCP when used as a prophylactic agent (D. M. Schmatz, M. A. Powles, D. C. McFadden, L. Pittarelli, J. Balkovec, M. Hammond, R. Zambias, P. Liberator, and J. Anderson, Antimicrob. Agents Chemother. 36:1964–1970, 1992). However, because of limited oral bioavailability, this compound would likely be restricted to parenteral use in humans. As an alternative, the aerosol delivery of L-693,989 was explored to determine the dosing regimen required to prevent the onset of PCP. Rats with latent *P. carinii* infections were immunosuppressed continuously with dexamethasone to promote the onset of PCP. During the 6-week immunosuppression period, L-693,989 was delivered to rats as a nebulized solution (volume median diameter of 3.8 μm) via a nose exposure inhalation chamber. The efficiency of aerosol delivery to the lungs and the rate of clearance were determined by using radiolabelled compound. It was found that a daily dose of 0.7 μg of L-693,989 per lung or a weekly dose of 77.9 μg/lung effectively prevented the development of *P. carinii* cysts and trophozoites as well as the associated pneumonia commonly seen in rats with acute *P. carinii* infections. These results demonstrate that L-693,989 is potentially useful as an aerosol prophylactic agent for PCP.

Pneumocystis carinii is the most common cause of pneumonia in AIDS patients and remains one of the major causes of death. Before the appearance of AIDS, *P. carinii* infections occurred primarily in children undergoing chemotherapy for leukemia and was effectively controlled, in most cases, with oral doses of trimethoprim-sulfamethoxazole. However, AIDS patients have an unusually high incidence of adverse reactions to this treatment, which has resulted in the use of pentamidine isethionate, a less desirable, toxic compound which was used parenterally prior to the use of trimethoprim-sulfamethoxazole. Pentamidine isethionate's toxicity combined with its lack of oral bioavailability limited the compound's use to parenteral therapy of acute *P. carinii* pneumonia (PCP).

To reduce the systemic toxicity problems associated with pentamidine and to encompass prophylactic applications, an aerosol formulation and a nebulized delivery system were developed. Aerosol delivery of pentamidine results in high, sustained drug concentrations in the lung, with minimal systemic drug uptake and associated toxicity (5). Aerosolized pentamidine is currently used in clinics on a monthly or biweekly basis for high-risk patients with AIDS. However, there has been an increased incidence of disseminated *P. carinii* (12, 13, 17, 19), as well as reports of PCP confined to the upper lobes of the lung (9), thought to be associated with the use of aerosolized pentamidine. The successful use of aerosolized pentamidine for the prevention of PCP, despite the dissemination and lung distribution issues, demonstrates that it is feasible to consider aerosol prophylaxis with other agents.

A new class of antipneumocystis agents, the pneumocandins, has recently been identified. These compounds are thought to work by inhibiting the synthesis of β-1,3-glucan, a component of the *P. carinii* cyst wall and of the cell wall of fungal pathogens such as *Candida* and *Aspergillus* species (18). The lack of β-1,3-glucan in humans makes this class of compounds attractive for the development of selective antipneumocystis or antifungal agents for clinical use.

Initially, the pneumocandins could be administered only in a cosolvent system because of their poor water solubility. However, chemical modification of the pneumocandins into prodrugs has resulted in compounds, such as L-693,989, which are very water soluble (>250 mg/ml) (3). This compound is efficiently converted back to parent pneumocandin B_0, L-688,786, in rats following intravenous injection and has improved pharmacokinetics, presumably due to a slower clearance rate (8). L-693,989 is fully effective in preventing the onset of PCP in the rat model at ≤1 mg/kg/day when injected subcutaneously (15). Unfortunately, the pneumocandins are not sufficiently bioavailable to warrant their use for oral prophylaxis.

The present study was conducted with aerosolized L-693,989 to explore whether the compound was efficacious via this route and to determine the dosing parameters required to achieve complete prevention of the disease in the rat model.

MATERIALS AND METHODS

Compound. The synthesis of L-693,989 [L-proline, (4*R*,5*R*)-*N*(2)-(10,12-dimethyl-1-oxotetradecyl)-4,5-dihydroxy-L-ornithyl-L-threonyl-*trans*-4-hydroxyl-L-prolyl-(*S*)-4-hydroxy-4-(4-phosphonooxyphenyl)-L-threonyl-3-hydroxy-L-glutaminyl-3-hydroxy-, cyclic (6.FWDARW.1)-peptide, *trans*-, monosodium salt] has been described elsewhere (3). ^{3}H-radiolabelled L-693,989 was prepared by Merck's Animal and Exploratory Drug Metabolism Department.

Preparation and characterization of solution for nebulization. The solution stability of L-693,989 in normal saline at various concentrations was assessed at room temperature. Aliquots were analyzed periodically over 24 h by high-pressure liquid chromatography. Assays were run on a Nucleosil C_{18}

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TABLE 1. Aerosol deposition study of L-693,989 with normal rats

L-693,989 concn (%)	Time (min) in chamber	Amt ± SD (μg) (%) of compound in lungs at:		
		0 h	24 h	48 h
1.00	87	267.7 ± 50.5 (100)	143.1 ± 22.9 (53.5)	112.6 ± 24.0 (42.0)
0.10	75	22.6 ± 4.8 (100)	9.7 ± 2.0 (42.9)	7.2 ± 1.2 (31.8)
0.01	71	2.2 ± 0.32 (100)	0.9 ± 0.2 (40.9)	0.6 ± 0.1 (27.2)

column in a mobile phase of 66% aqueous 0.01 M sodium tetraborate–0.05 M $CaCl_2$ followed by 34% acetonitrile. The droplet size of nebulized solutions of L-693,989 emitted from a Retec jet nebulizer operated at 8 liters/min was determined by dynamic laser light scattering with a Malvern 2600C droplet and particle sizer.

Dosing chamber and drug delivery. All aerosol studies were conducted at BioResearch Laboratories Ltd. (Senneville, Quebec, Canada) and were performed in accordance with U.S. Food and Drug Administration Good Laboratory Practice regulations (CFR part 58).

Cylindrical stainless-steel nose-only inhalation chambers were used in this study. A stainless-steel cylinder was placed in the center of each chamber so that the animals were breathing from a 1-in. (2.54 cm) annular zone around the periphery of the chamber. The body of each chamber had 60 separate ports in three separate rows, into which the conical front section of a polycarbonate restraint cone was inserted. The top section of each inhalation chamber had an opening for air inlet into which L-693,989 or saline control was introduced. The bottom section of each chamber had a corresponding air extraction port and a drain valve for cleaning. Each chamber was contained within a separate ventilated walk-in fume hood and was operated under slight negative pressure to prevent outward leakage of the test or control atmosphere. The restraint cones were tapered to fit approximately the shape of the animal's head and to prevent the animal from turning around in the cone. The cone containing the animal was fastened to the chamber with the nose portion of the cone protruding through a gasket into the chamber, allowing the animal to breathe the test atmosphere without otherwise coming into contact with the atmosphere. During an acclimation period of 1 week, the animals were introduced to the restraint cones for increasing periods of time.

Airflow through the chambers was set at 23 liters/min. This flow maintains a chamber environment of 20 to 24°C and at least 19% oxygen. The test atmosphere was generated by using a Retec nebulizer supplied with compressed air. Chamber drug concentration was controlled by varying the concentration of the drug in solution. Animals were subjected to nose-only exposure for a duration of 75 min, either daily or weekly, for a period of 6 weeks.

Determination of doses. Prior to the efficacy study, the amount of L-693,989 delivered to the lungs as an aerosol was determined. Solutions containing 1, 0.1, and 0.01% L-693,989 in normal saline were prepared and radiolabelled with ^{3}H-L-693,989 so that each solution nebulized delivered approximately 0.5 mCi/lung/animal during the inhalation exposure. Groups of 30 rats (Sprague-Dawley; Charles River Canada, St.-Constant, Quebec, Canada) were exposed to each aerosolized dose of compound, and animals were sacrificed either immediately after exposure, 24 h postdosing, or 48 h postdosing. The animals were subjected to single nose-only exposure for durations of 87, 75, and 71 min for the 1, 0.1, and 0.01% groups, respectively. Chamber filters placed at an unoccupied exposure opening collected aerosolized drug delivered during dosing. Following exposure, filters were removed, dried, and counted to determine the amount of radioactivity delivered. At appropriate times, animals were euthanized by exsanguination following intraperitoneal injection of sodium pentobarbitol. The lungs were weighed and processed immediately after collection. Lungs were homogenized, and duplicate aliquots were transferred to scintillation vials. Aliquots were solubilized in 2 ml of BTS-450 tissue solubilizer (Beckman) and mixed with 10 ml of acidified Ready-Gel liquid scintillation fluid (Beckman), and the radioactivity was measured. The total amount of radioactive compound in the lungs (disintegrations per minute) was calculated on the basis of the concentration of radioactivity (disintegrations per minute per gram) measured in the tissue aliquots and the total weight (in grams) of the tissue.

Animal model. An immunosuppressed rat model, similar to that described in detail elsewhere, was used in these studies (6, 15). Briefly, male Sprague-Dawley rats (Sasco Laboratories, Omaha, Neb.) weighing approximately 200 g were fed 23% protein rodent chow (Purina, St. Louis, Mo.) and were immunosuppressed with 2 mg of dexamethasone per liter added to the drinking water. Tetracycline (1 g/liter) was added to the water to minimize bacterial infections. All animals remained on immunosuppressive therapy throughout the 6 weeks of the study. Food and water were supplied ad libitum, except during the time the rats were being dosed. The rats were distributed into eight groups of 30 animals per group. The groups included a vehicle control for daily and weekly treatments; high-,

TABLE 2. Aerosol study of L-693,989 for daily prophylaxis of PCP

Inhalation achieved dose (μg/lung)	% reduction of trophozoites (nuclei)[a]	% reduction of cysts[b]	Lung wt (g) (± SD)	Lung/body wt ratio (%)	*n*
None (control)	NA[c] (7.51 ± 0.15)	NA (7.41 ± 0.09)	1.72 ± 0.40	1.23 ± 0.29	27
0.7	99[d] (5.50 ± 0.14)	>99[d] (4.74 ± 0.04)	1.26 ± 0.12[d]	0.91 ± 0.11[d]	26
8.1	>99[d] (5.22 ± 0.14)	>99[d] (4.69 ± 0.03)	1.24 ± 0.13[d]	0.89 ± 0.09[d]	29
88.5	99[d] (5.20 ± 0.16)	>99[d] (4.68 ± 0.02)	1.26 ± 0.11[d]	0.92 ± 0.10[d]	26

[a] Values in parentheses are log 10 mean numbers of nuclei per lung ± standard deviations. The limit of detection is 4.70.
[b] Values in parentheses are log mean numbers of cysts per lung ± standard deviations. The limit of detection is 4.66.
[c] NA, not applicable.
[d] $P < 0.001$.

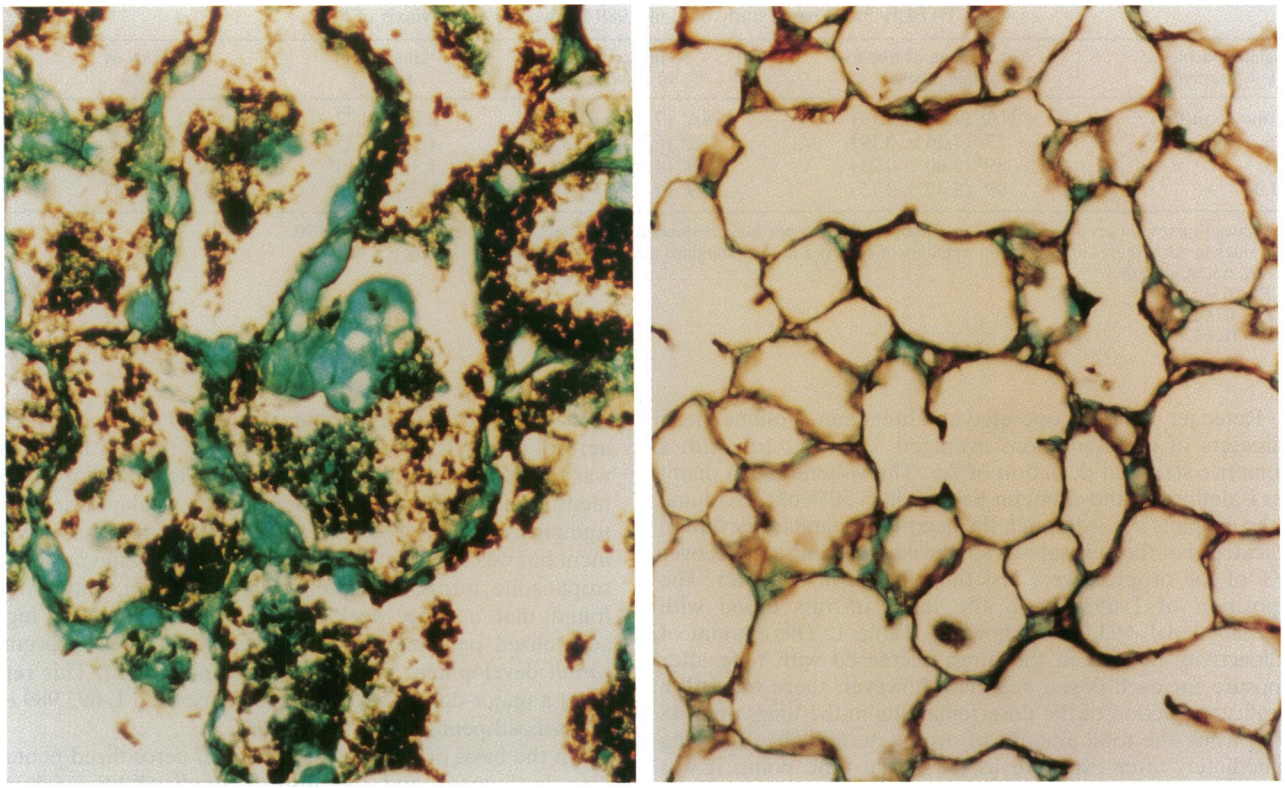

FIG. 1. Histology of lungs treated with L-693,989 versus that of untreated control lungs. The leftmost panel shows a hematoxylin-and-eosin–Gomori methenamine-silver-stained lung section from a rat in the vehicle control group undergoing daily prophylaxis. The rightmost panel shows a stained section from a rat treated with L-693,989 from the 1-μg/lung daily prophylaxis group. Magnification, ×2,700.

medium-, and low-dose daily dosed groups; and high-, medium-, and low-dose weekly dosed groups. The target doses for the high-, medium-, and low-dose groups were 100, 10, and 1 μg of L-693,989 per lung, respectively.

Evaluation of lung tissue. At the end of the treatment period, the animals fasted overnight and were sacrificed by exsanguination following anesthesia by intraperitoneal injection of sodium pentobarbitol (2 ml/kg). (Only animals that had completed treatment were included in evaluation and data analysis.) The lungs with trachea were dissected free of fat and weighed. The right lobes were infused with and retained in neutral buffered 10% formalin for histopathological evaluation. Sections were stained with hematoxylin and eosin and Gomori methenamine-silver stain. The left lobes were homogenized with a Brinkman homogenizer, and quantitation of cysts was done as described previously (15), with the exception that all centrifugations were conducted at 1,700 × *g*. Twenty fields of cysts were counted, and the total number of organisms per rat was determined as a function of the surface area on the slide, the volume of the applied sample, and the total volume of the processed homogenate. Quantitation of *P. carinii* trophozoites (nuclei) was achieved by hybridization with a radiolabelled *P. carinii*-specific DNA probe (10). Statistical evaluation of differences in the mean response to each L-693,989 dose in comparison with response to the vehicle control was accomplished by using the Wilcoxon rank sum test and the Bonferroni multiple-comparison procedure.

RESULTS

Drug deposition. Solutions of L-693,989 in normal saline had pH values of 4.0 to 4.5 and were chemically and physically stable for at least 8 h at room temperature. Droplet size analysis of nebulized solutions indicated that a 400-fold change in L-693,989 concentration (0.01 to 4.0%) had no significant effect on the droplet size distribution of aerosols produced by

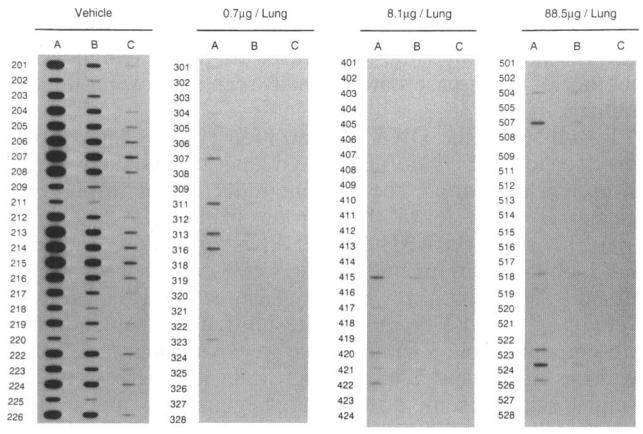

FIG. 2. Efficacy of daily prophylactic aerosolized L-693,989 as measured with a *P. carinii*-specific DNA hybridization probe. Animals from each of the four treatment groups (vehicle control and 88.5-, 8.1-, and 0.7-μg/lung groups) are represented in the respective panels. Each row, as indicated by the numbers in the vertical column at the left of the panels, represents an individual animal in that treatment group. Genomic DNA, prepared from the lungs of each animal, was immobilized on a nylon membrane in three dilutions: 25 μg for columns A, 2.5 μg for columns B, and 0.25 μg for columns C. Hybridization and quantitation are described in Materials and Methods.

TABLE 3. Aerosol study of L-693,989 for weekly prophylaxis of PCP

Inhalation-achieved dose (μg/lung)	% reduction of trophozoites (nuclei)[a]	% reduction of cysts[b]	Lung wt (g) (± SD)	Lung/body wt ratio (%)	n
None (control)	NA[c] (7.48 ± 0.19)	NA[c] (7.62 ± 0.10)	2.24 ± 0.72	1.72 ± 0.59	21
0.6	27 (7.26 ± 0.19)	82[d] (6.88 ± 0.18)	1.59 ± 0.35[e]	1.21 ± 0.32[f]	22
7.5	89[d] (5.60 ± 0.23)	>99[d] (4.95 ± 0.09)	1.31 ± 0.13[d]	0.95 ± 0.11[d]	22
77.9	99[d] (5.47 ± 0.15)	>99[d] (4.70 ± 0.04)	1.25 ± 0.09[d]	0.92 ± 0.11[d]	22

[a] Values in parentheses are log 10 mean numbers of nuclei per lung ± standard deviations. The limit of detection is 4.70.
[b] Values in parentheses are log mean numbers of cysts per lung ± standard deviations. The limit of detection is 4.66.
[c] NA, not applicable.
[d] $P < 0.001$.
[e] $P = 0.002$.
[f] $P = 0.005$.

the Retec jet nebulizer operated at 8 liters/min. Volume mean diameters of these solutions averaged 3.8 ± 0.2 μm with a geometric standard deviation of 2.5. The volume mean diameter is defined as the diameter below which 50% of the volume of the droplets lies. Although the geometric standard deviation indicated that the aerosol was somewhat heterodispersed, over 90% of the droplets were less than 10 μm in diameter. The deposition of L-693,989 in the lungs of rats dosed with radiolabelled L-693,989 is shown in Table 1. The amount of radioactivity present in the lungs decreased with time after exposure for each dosing regimen; however, there was still a significant fraction of the dose remaining in the lungs after 48 h. By using the total amount of drug determined in the lung immediately after exposure as the initial time point, it was found that the percentages of the dose remaining after 48 h were 42, 32, and 27% for the three treatment groups, respectively.

Daily prophylaxis. The target dose for the low-, medium-, and high-dose groups for the daily prophylaxis regimen were 1, 10, and 100 μg/lung, respectively. The actual achieved doses were 0.7, 8.1, and 88.5 μg/lung, as measured by the amount of drug collected on chamber filters. The results presented in Table 2 indicate that even at a dose of 0.7 μg/lung, L-693,989 was effective in preventing *P. carinii* cyst and trophozoite (nucleus) development. The significantly lower lung weights of the drug-treated animals are indicative of lack of inflammation and pathology normally associated with PCP. The histology of lungs treated daily (Fig. 1) with 0.7 μg/lung exemplifies the absence of the disease state normally seen in *P. carinii*-infected animals.

Results from the DNA hybridization (Fig. 2) show little if any detectable *P. carinii* signal from lungs treated daily with all three levels of drug. The percent reduction of trophozoites (nuclei) is ≥99% for all three groups (Table 2).

Weekly prophylaxis. The target doses for the low-, medium-, and high-dose groups undergoing weekly prophylaxis were 1, 10, and 100 μg/lung, respectively. The actual achieved doses were 0.6, 7.5, and 77.9 μg/lung. Results from cyst quantitation, presented in Table 3, indicate that weekly dosing was effective at doses of 7.5 and 77.9 μg/lung, while the increase in lung weights suggests some disease state beginning to appear at the 7.5-μg dose. The 77.9-μg dose has very little signal at the high level of DNA (representing a 98.7% reduction in trophozoites) (Table 3), while the 7.5-μg dose elicits an 89% reduction. The 0.6-μg dose was less effective, as evidenced by the intermediate lung weights and only a 27% reduction in trophozoites.

DISCUSSION

This study demonstrates that aerosol administration of L-693,989 is an effective method for preventing the development of PCP in the immunosuppressed rat model. Daily aerosol prophylaxis with the lowest dose tested (0.7 μg/lung) was fully effective at preventing cyst and trophozoite development. Similar efficacy was observed at a weekly dose of 77.9 μg/lung. A weekly dose of 7.5 μg/lung prevented cyst development but was not fully effective, as indicated by the presence of trophozoite forms. Using a similar rat model, Girard et al. found that a thrice-weekly dose of >4.8 or ≤8.6 mg of aerosolized pentamidine per kg was required to prevent *P. carinii* development in 100% of the animals (7). This represents a major dosing advantage of aerosolized L-693,989 over aerosolized pentamidine isethionate.

On the basis of the proven efficacy of aerosolized pentamidine in humans and the historic predictability of human efficacy by the rat model, it is likely that aerosolized L-693,989 would be very effective clinically. Since the projected dose of L-693,989 is substantially lower than that of pentamidine and since there were no indications of toxicity in rats at the highest daily dose tested, it is conceivable that dosing could be adjusted so that adequate levels of L-693,989 reach the upper lobes of the lung, where it appears to be difficult to attain efficacious levels of pentamidine (1, 9, 11, 16). Since there is no known counterpart to β-1,3-glucan synthesis in humans, there are no obvious reasons to suspect mechanism-based toxicity problems with this class of compounds.

A concern with aerosol delivery of compounds for preventing PCP is the potential for dissemination of the disease to other organs. Although pentamidine toxicity has been reduced by aerosol delivery, the lack of significant drug levels in the blood following administration may account for the increasing incidence of disseminated PCP (13, 17, 19). Quantitation of drug levels in blood following aerosol delivery for any antipneumocystis agent would be a useful exercise in projecting the potential for disseminated disease. At present, these studies have not been conducted with L-693,989.

Since the completion of this study, pneumocandins that are 10 times more potent against *P. carinii* (14) and 100 times more potent against *Candida albicans* in vivo (4) have been developed. It is likely that these compounds will be superior to L-693,989 as an aerosol. Many of these compounds also possess potent activity against *Aspergillus fumigatus* in a mouse survival model (2). As a result, these compounds may be useful for aerosol prophylaxis against *Aspergillus* infections, since these infections are also contracted via the respiratory route and begin by colonization of the lung.

In conclusion, aerosol prophylaxis with pneumocandins and related echinocandins may prove useful in the prevention of several opportunistic infections in immunocompromised patients. The superior potency and safety of these compounds, if

results obtained with rats are directly applicable to humans, would represent a major advance in the prophylaxis for PCP.

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