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Successful Treatment of *Pneumocystis carinii* Pneumonia in Mice with Benanomicin A (ME1451)

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Benanomicin A (BNM-A) has antimycotic activities via binding to mannan in the cell walls of fungi. Anti-*Pneumocystis carinii* activity of the agent was examined in the *P. carinii*-infected BALB/c *nu*/*nu* female mouse model because *P. carinii* also possesses mannan in the membranes. The infected mice were treated with intraperitoneal injections of six doses of BNM-A (1, 2.5, 5, 10, 30, and 100 mg/kg of body weight), 4 mg of pentamidine isethionate per kg, 100 mg of sulfamethoxazole per kg combined with 20 mg of trimethoprim per kg (co-trimoxazole), or saline for 21 days. Each dosage group consisted of 10 mice. During treatment, five mice in the control group (saline) died, whereas 8 to 10 mice in all treatment groups survived. Almost the same efficacies were obtained for the groups treated with 5 mg or more and 10 mg or more of BNM-A per kg regarding the weight and number, respectively, of cysts found in the lungs as were obtained for the groups treated with pentamidine isethionate and co-trimoxazole. Overall, a dose of 10 mg of BNM-A per kg was effective against *P. carinii* pneumonia infection in the mice. Thus, BNM-A is a good candidate for a novel treatment for *P. carinii* pneumonia as a compound with a new mechanism of action against *P. carinii*.

As *Pneumocystis carinii* pneumonia is the most prevalent and serious AIDS-related opportunistic infection in developed countries (6), the number of cases of *P. carinii* pneumonia has increased in association with the increase in the number of AIDS cases since the mid-1980s (14). Although the screening of anti-*P. carinii* compounds has been hampered by the difficulty of cultivating the organism in vitro, several new agents are being extensively evaluated and some are now available to treat the disease clinically (12, 13). Among these, oral trimethoprim-sulfamethoxazole (co-trimoxazole) and intravenous pentamidine are highly effective and are accepted as standard therapies (12, 13), with the efficacies of the two agents being equivalent (11, 15, 22). However, they are often poorly tolerated (15) and are known to cause severe skin reactions (9) in some cases. Thus, the morbidity resulting from pneumonia remains substantial. Therefore, we should continue to look for more effective and less toxic new agents for better management of the disease. Recently, several compounds with different mechanisms of action, including topoisomerase (3) and β-1,3-glucan synthetase (16), have been developed in in vitro and in vivo rat models on the basis of knowledge of specific enzymes.

DNA sequences encoding rRNA genes revealed that *P. carinii* may be a fungus rather than a protozoan (4) and that the cell wall of the cyst form of the organism is similar to the cell wall of some yeasts which contain high levels of β-1,3-glucan. In fact, antifungal compounds, β-1,3-glucan synthesis inhibitors, were reported to have anti-*P. carinii* activity in a rat model (16). Furthermore, the major protein components of *P. carinii* in both cysts and trophozoites are mannose-rich glycoproteins (18), and macrophage mannose receptor can bind the organism (5). On the basis of these findings, we examined the anti-*P. carinii* activity of benanomicin A (BNM-A), which was developed as an antifungal agent (17) which acts via binding to mannan of the cell walls of fungi (24), in a *P. carinii*-infected mouse model.

MATERIALS AND METHODS

Sources of compounds. BNM-A is produced by *Actinomadura* sp. strain MH193-16F4, isolated as a red powder, and readily soluble in water as its sodium salt (17). It belongs to a new family of benzonaphthacene antifungal antibiotics (Fig. 1).

***P. carinii*-infected mouse model.** Specific-pathogen-free (SPF) female nude mice (BALB/c *nu*/*nu*; 5 weeks old, weighing about 16.0 g) were purchased from Japan SLC Co., Ltd. (Shizuoka, Japan). The *P. carinii* derived from mice used in this study was isolated in the Animal Facility of Kyoto University (10). *P. carinii*-infected nude mice were kept in an isolator, which is a plastic unit with laminar air flow and HEPA filtration, and given autoclaved water, food, and bedding to exclude infections from other pathogens. New SPF nude mice were added to the same isolator every month to ensure a *P. carinii*-infected mouse colony by airborne transmission (10). In our airborne transmission system, *P. carinii* was detected in the lungs of SPF mice at week 6 and the weights of the mice increased, to 20 to 21 g, until week 10 after coisolation. After 10 weeks, the weights decreased gradually in association with the progression of *P. carinii* pneumonia. Half of the infected mice died within 20 weeks after coisolation if no anti-*P. carinii* agents were given. To produce the total of 100 *P. carinii*-infected mice used in this study, a group of 10 SPF nude female mice was kept in each of 10 isolators with the *P. carinii*-infected mice for 17 weeks. Before starting the experiment, two mice were selected arbitrarily and sacrificed after exposure to ether gas to confirm the infection by microscopic examinations by Grocott staining. The mean number of cysts per field (magnification, ×400) for the two mice was 36 as counted by the method described below. At week 17 (i.e., at the start of this study), the mean weight of all mice was 18 g. At this point, the mice were divided into nine groups of 10 mice each, in nine isolators, so that the mean weight of the mice in each group was nearly 18 g.

Administration of agents. Groups of ten *P. carinii*-infected mice were treated

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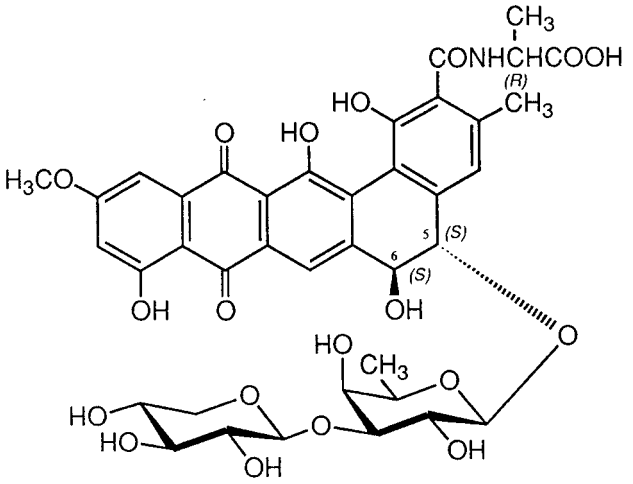

FIG. 1. Structure of BNM-A. Molecular weight, 849.

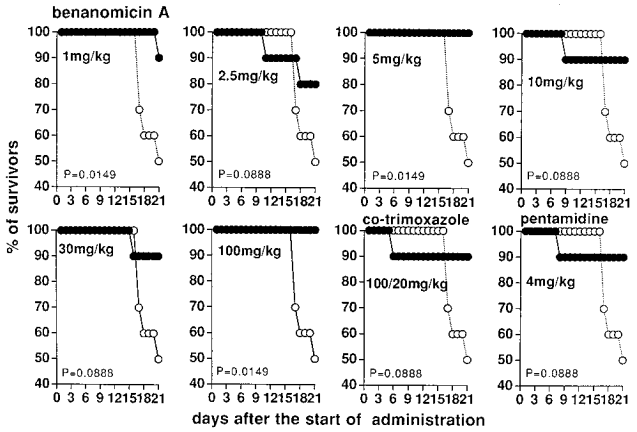

FIG. 2. Effects of BNM-A on survival of *P. carinii*-infected mice. Closed and open circles indicate treated and control mice, respectively. *P* values were obtained by the Kaplan-Meier method.

with either six doses of BNM-A (1, 2.5, 5, 10, 30, and 100 mg/kg), 4 mg of pentamidine isethionate per kg, or 100 mg of sulfamethoxazole per kg combined with 20 mg of trimethoprim per kg by intraperitoneal injection once a day for 21 days. Ten other mice, the control group, were treated with saline in the same manner for the same duration. Weights were checked every 3 days during treatment. Mice that died during treatment were autopsied to examine the cause of death. All surviving mice were sacrificed after exposure to ether gas on day 22. The lungs were removed immediately, weighed, and fixed in 10% formalin in phosphate-buffered solution (pH 7.4).

Evaluation of lung tissue. After fixation, the lungs were embedded in paraffin and sectioned. Then, one section each from five lobes of the lungs (the upper, middle, and lower lobes in the right lung and the upper and lower lobes in the left lung) were stained by Grocott staining. The number of cysts in five microscopic fields (×400) per section was counted. That is, cysts from a total of 25 microscopic fields per mouse were counted and averaged. Two examiners counted the number of cysts in the same specimens of the control group, with the result that differences in the counts varied only within 5% (means ± standard deviations, 78.4 ± 47.9 and 77.5 ± 46.6). Then, counts of cysts in the treated groups were performed in a blinded manner by one examiner.

Statistical analyses. Differences in the survival rates between the treatment groups and the control group were analyzed by the Kaplan-Meier method. One-way analysis of variance with pairwise comparison according to the Bonferroni method was used to compare changes in weight during treatment, ratios of lung weight to body weight at sacrifice, and numbers of cysts in the lung sections for the treatment groups and the control group.

RESULTS

Survival during treatment. The effects of BNM-A on the survival of *P. carinii*-infected mice are shown in Fig. 2. In the control group, three mice died at day 16, one died at day 17, and one died at day 21. Thus, the survival rate was 50% at completion of the study. Furthermore, all of the surviving mice were cyanotic (data not shown). In contrast, only one mouse died in the group treated with 1 mg of BNM-A per kg, and two died in the group treated with 2.5 mg of BNM-A per kg. However, most of the surviving mice in these groups were cyanotic (data not shown). When mice were treated with 5 mg or more of BNM-A per kg, or with co-trimoxazole or pentamidine, only one mouse or no mice died during treatment and no cyanosis was seen in any surviving mice. Since the mice in the control group had started to die at day 16, no dose-dependent effect on survival was observed for the control group and the treated groups when evaluated by the Kaplan-Meier method (Fig. 2). There were no causes of death other than *P. carinii* pneumonia for mice that died during treatment.

Weight during treatment. Changes of weight during treatment are shown in Fig. 3. In the control group, the mean weight of the surviving mice kept decreasing during the study period. In all treated groups except that treated with co-trimoxazole, the mean weight continued decreasing until day 9. However, the mean weight then started to increase in the treated groups except for that treated with 1 mg of BNM-A per kg. The gains in weight of mice in groups treated with 5 mg or more of BNM-A per kg and with pentamidine were significant ($P < 0.05$). However, the mean weight in each group treated with BNM-A did not return to the weight recorded at the start of treatment. Among BNM-A-treated groups, the mean weight at day 21 was the highest in the group treated with 10 mg/kg (Table 1). In the co-trimoxazole-treated group, the mean weight of the mice did not decrease after starting treatment and was significantly ($P < 0.05$) higher at day 11 or later when compared with the weight of mice in the control group. Judging from the weight changes, treatment with 1 and 2.5 mg of BNM-A per kg was not effective.

Lung-to-body weight ratio. As *P. carinii* pneumonia develops, the lung weight increases but body weight decreases. Thus, the ratio of lung weight to body weight can be a marker to monitor the severity of the pneumonia. The mean lung and body weights and the ratio for each group at the end of the

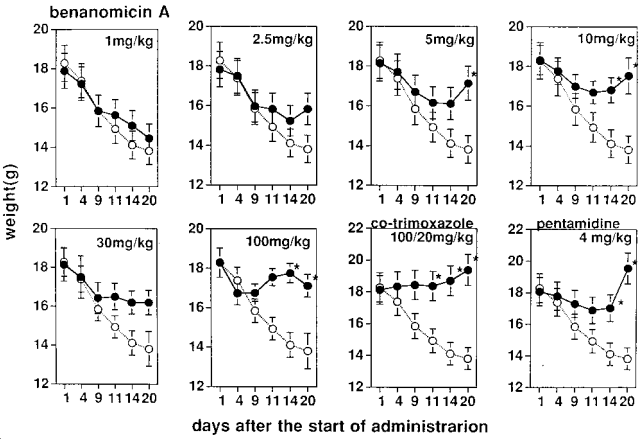

FIG. 3. Changes of weight during treatment with BNM-A. Closed and open circles indicate treated and control mice, respectively. Asterisks indicate weights which were significant ($P < 0.05$) compared with those for the control group, determined by one-way analysis of variance with pairwise comparison according to the Bonferroni method.

TABLE 1. Ratio of lung weight to body weight of *P. carinii*-infected mice at day 22

Treatment	Weight		Lung wt/body wt (%) (mean ± SD)	No. of survivors/ total no. of mice
	Lung (g) (mean ± SD)[a]	Body (g) (mean ± SD)		
BNM-A (mg/kg)				
1	0.46 ± 0.69	14.79 ± 1.92	3.07 ± 0.37	9/10
2.5	0.45 ± 0.08	15.41 ± 2.11	3.00 ± 0.76	9/10
5	0.45 ± 0.10	17.39 ± 2.94	2.70 ± 0.97	10/10
10	0.39 ± 0.06	17.48 ± 1.50	2.26 ± 0.38	9/10
30	0.34 ± 0.12	16.52 ± 1.95	2.12 ± 0.80*[b]	9/10
100	0.27 ± 0.07	16.85 ± 2.19	1.60 ± 0.62*	10/10
Co-trimoxazole[b]	0.36 ± 0.11	19.54 ± 1.71	1.85 ± 0.60*	9/10
Pentamidine[c]	0.36 ± 0.06	18.39 ± 2.17	1.99 ± 0.47*	9/10
Control (saline)	0.44 ± 0.09	14.32 ± 1.72	3.16 ± 0.72	5/10

[a] SD, standard deviation.
[b] *, the ratio for each group is significantly ($P < 0.05$) smaller than that for the control group by one-way analysis of variance with pairwise comparison according to the Bonferroni method.
[c] 100 mg of sulfamethoxazole per kg combined with 20 mg of trimethoprim per kg.
[d] 4 mg of pentamidine isethionate per kg.

study are listed in Table 1. As expected, mean lung weights showed a dose-dependent decrease in response to treatment with BNM-A. In contrast, mean body weights increased. Consequently, the ratio of lung weight to body weight decreased dose dependently with BNM-A treatment and was significantly smaller for groups treated with 30 and 100 mg of BNM-A per kg, co-trimoxazole, and pentamidine than for the control group.

Efficacy of BNM-A for reducing the number of *P. carinii* cysts in the lung. The mean numbers of cysts remaining in the lungs upon completion of treatment are shown in Fig. 4 (two mice that died at days 17 and 21 in the control group were included in this evaluation). A dose-dependent effect of BNM-A on the number of cysts was observed. The mean number was significantly ($P < 0.05$) reduced for the groups treated with 5 mg or more of BNM-A per kg, co-trimoxazole, and pentamidine when compared with that of the control group. The histological sections of lung tissue from each group are shown in Fig. 5. As shown in Fig. 5, large masses of *P. carinii* cells were seen filling the alveolar cavities in the control mice, whereas histological improvement was obtained dose dependently with BNM-A. The alveoli are clearly visible in sections from mice treated with doses of 5 mg or more of BNM-A per kg, co-trimoxazole, and pentamidine.

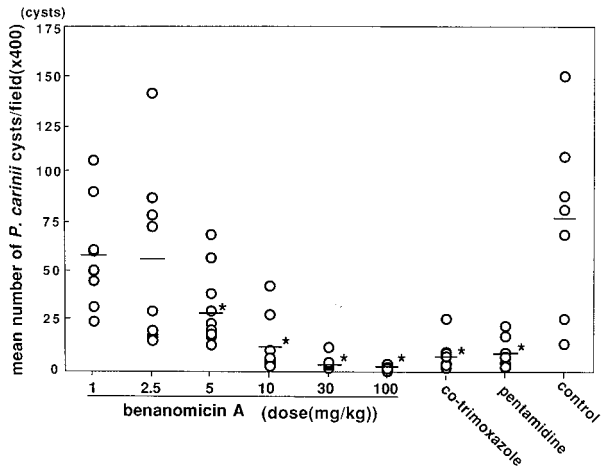

FIG. 4. Efficacy of BNM-A for eliminating *P. carinii* cysts from the lung. The number of *P. carinii* cysts was counted after lung sections were stained by Grocott staining. Each circle indicates the mean number of cysts per field (magnification, ×400) for each mouse. The bars indicate the means. The mean number of cysts per lung field (magnification, ×400) in the control group was 77.5 ± 46.6 (mean ± standard deviation). In contrast, the mean numbers (± standard deviations) for the groups treated with 1, 2.5, 5, 10, 30, and 100 mg of BNM-A per kg were 58.7 ± 26.2, 54.0 ± 44.0, 31.4 ± 18.8, 12.2 ± 14.5, 4.5 ± 3.1, and 2.0 ± 1.0, respectively, and those for the groups treated with co-trimoxazole and pentamidine were 8.6 ± 7.5 and 8.9 ± 7.2, respectively. Asterisks indicate means which were significant ($P < 0.05$) compared with that of the control group, by one-way analysis of variance with pairwise comparison according to the Bonferroni method. Co-trimoxazole, 100 mg of sulfamethoxazole per kg combined with 20 mg of trimethoprim per kg. Pentamidine, 4 mg of pentamidine isethionate per kg.

DISCUSSION

We successfully treated nude mice infected with *P. carinii* with BNM-A, co-trimoxazole, and pentamidine. A *P. carinii*-infected rat model has been widely used for examining the efficacy of experimental drugs (3, 8, 16, 19, 20). Although this system produces *P. carinii* pneumonia in immunosuppressed rats 10 weeks earlier than our system, dexamethazone and antibiotics have to be used concomitantly throughout the study. Therefore, the influence of these accessory agents and drug-to-drug interactions can not be excluded. Our system, in contrast, can examine the effects of a single agent. Because there are, so far, no reliable in vitro culture systems for *P. carinii*, animal models must be used to examine the efficacy of anti-*P. carinii* agents. Although *P. carinii* cells have been recently shown to be propagated in a short-term cell culture using human embryonic lung fibroblasts (2, 21), further improvement of the system is needed before reducing the use of animals in the screening of anti-*P. carinii* agents. Since BNM-A has very low bioavailability, it must be used as an intravenous drug if it will be used in humans. Further screening, using an in vitro system, of related compounds which could be administered orally is desired.

BNM-A was first discovered from an actinomycete (17) and is now being developed as a broad-spectrum antifungal agent (23). According to preliminary studies investigating the mechanism of antifungal action of the drug, a shift in an absorption peak of BNM-A at 500 to 515 nm in the presence of both α- and β-mannans was observed by spectrophotometric analysis and remarkable downfield shifts of chromophoric signals of BNM-A were observed by NMR analysis upon the addition of mannan, indicating that the drug binds to mannan in the cell walls of fungi (24). This information about the mode of anti-

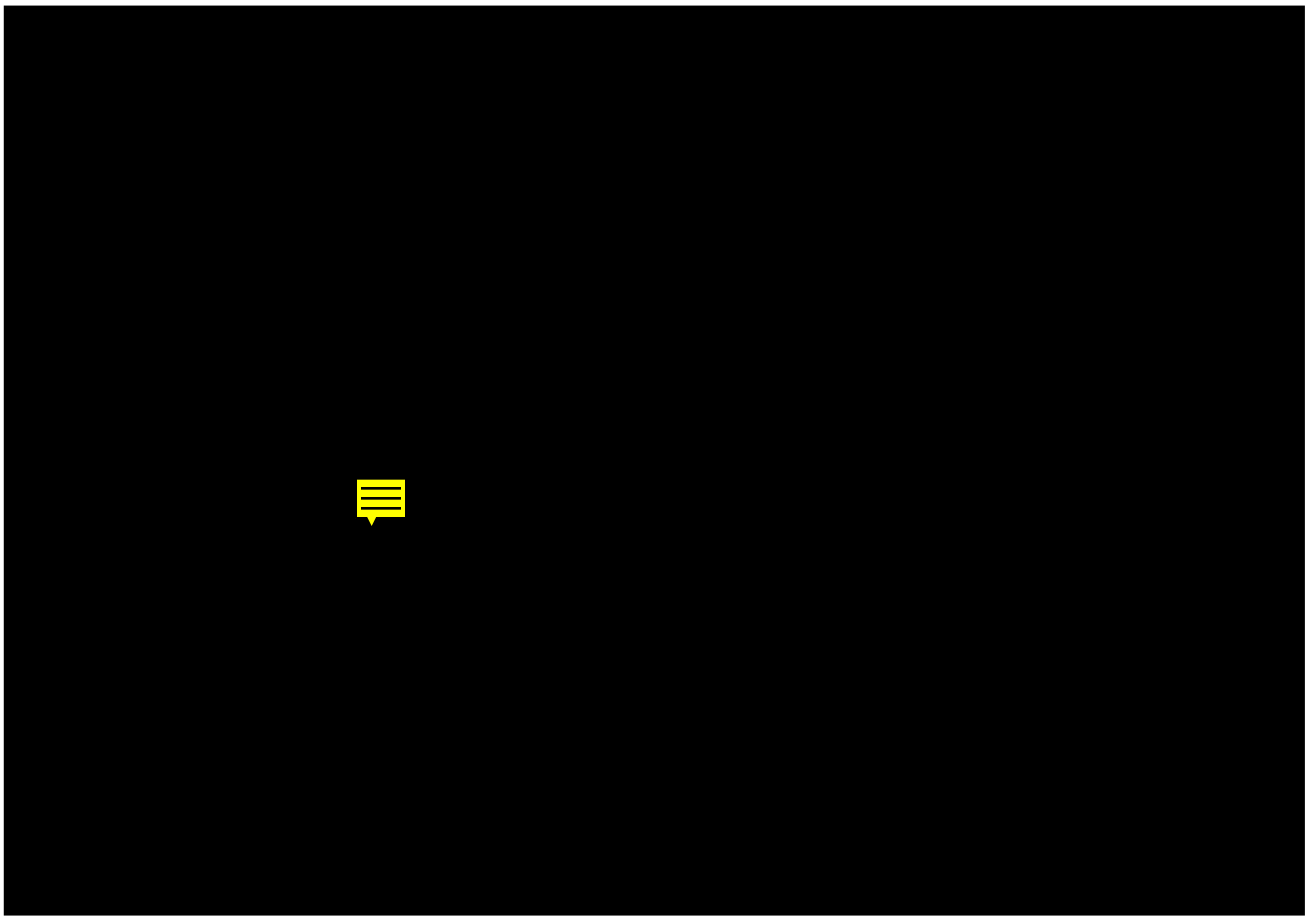

FIG. 5. Histological sections of lung tissue. Grocott staining (magnification, ×400).

fungal action of BNM-A prompted us to examine the anti-*P. carinii* activity of the agent, because the membranes of the organism, in both cyst and trophozoite forms, also contain mannans (18). As we presumed before the study, BNM-A had anti-*P. carinii* activity. As little as 5 mg of BNM-A per kg led to an increase in weight, eliminated the cysts, and caused histological improvements in *P. carinii*-infected mice. Furthermore, almost the same efficacy as that of co-trimoxazole or pentamidine could be obtained with 10 mg of BNM-A per kg. However, at the completion of the study, the mean weights of the mice treated with 30 mg or more of BNM-A per kg were lower than those treated with 5 or 10 mg/kg, despite the dose-dependent efficacy obtained regarding elimination of the cysts. This weight loss might be a result of a minor adverse reaction of the agent (e.g., appetite loss). Overall, a dose of 10 mg of BNM-A per kg was effective, as well as safe, in the treatment of *P. carinii* pneumonia in mice. Furthermore, according to the results of the general toxicity test, a dose of 10 mg/kg proved to be well tolerated by other animals, including rats, dogs, and monkeys as determined by examination of blood and urine biochemistry and histologial examination of every organ. The 50% lethal doses of BNM-A in mice, rats, dogs, and monkeys were 600 to 900, 600 to 1,200, 600 to 1,200, and 300 to 600 mg/kg, respectively (Meiji Seika Kaisha, Ltd.; unpublished data). These results provide encouragement that a clinical study with humans might be possible in the near future.

Patients with AIDS and *P. carinii* pneumonia often suffer from oral candidiasis, especially when they are treated with an adjunctive corticosteroid, as recommended recently (12, 13). In such cases, BNM-A treatment may be very beneficial, because the agent also has an antifungal activity (23). Although BNM-A was reported to have anti-HIV activity in vitro (7), whether or not such efficacy can be expected in vivo is still unclear.

There were several reports on anti-*P. carinii* compounds with different mechanisms of action, such as dihydrofolate reductase inhibitor (1, 8, 20), topoisomerase inhibitor (3), and β-1,3-glucan synthetase inhibitor (16). BNM-A appears to resemble β-glucan inhibitors in some of its properties. However, BNM-A did not inhibit (1,3)-β-glucan synthesis of *Aspergillus fumigatus*, even though it killed the organism (unpublished data). Although the detailed mechanism of the anti-*P. carinii* activity of BNM-A was not examined in this study, the selective binding of BNM-A to mannans in the cell walls of *P. carinii* can be expected, according to the study on fungi (24). Thus, BNM-A is a promising candidate for treatment of *P. carinii* pneumonia or, at least, a lead compound with a neoteric mechanism of action against *P. carinii.*

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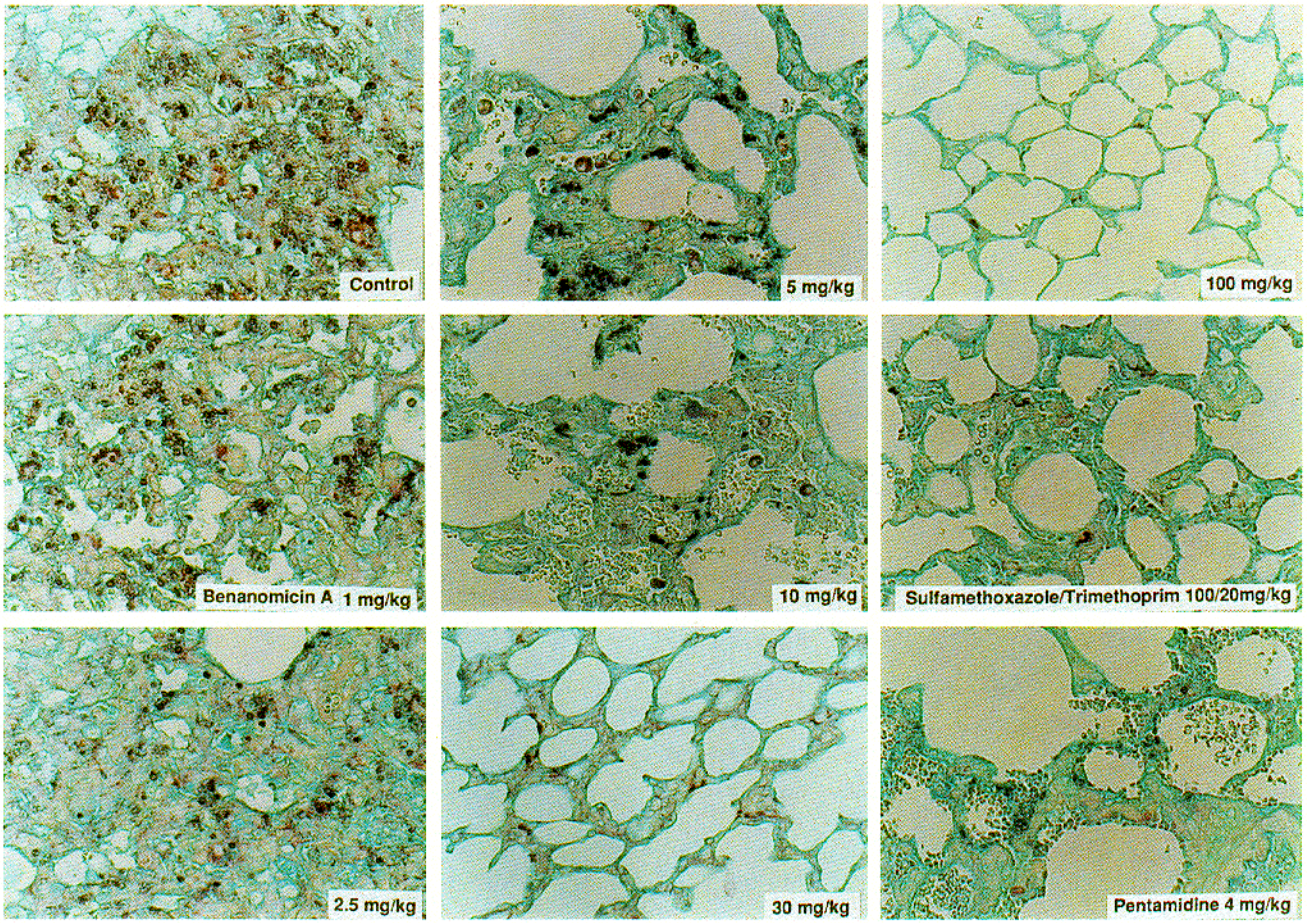
Control
5 mg/kg
100 mg/kg
Benanomicin A 1 mg/kg
10 mg/kg
Sulfamethoxazole/Trimethoprim 100/20mg/kg
2.5 mg/kg
30 mg/kg
Pentamidine 4 mg/kg