ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1994, p. 1859–1861
0066-4804/94/$04.00+0

Vol. 38, No. 8

NOTES

Pneumocystis carinii Is Resistant to Imidazole Antifungal Agents

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Received 31 January 1994/Returned for modification 29 March 1994/Accepted 23 May 1994

Because *Pneumocystis carinii* is closely related to fungi, drugs useful for treating mycoses have been considered for use in the treatment of *P. carinii* pneumonia. Six antifungal imidazole drugs were tested for their activities against *P. carinii* in a short-term culture screen and in animals. None of the imidazoles tested was effective in inoculated infected rats, and only miconazole showed slight effects in culture at the high concentration of 10 μg/ml. Analysis of cell membranes from culture-grown *P. carinii* showed that ergosterol, the target for this class of antifungal agents, was absent, so that the lack of effect of these agents is rational.

Pneumocystis carinii is an important cause of morbidity and mortality in immunocompromised hosts (8). Although formerly thought to be a protozoan, it is now generally agreed that it is more closely related to fungi, but its exact position among the fungi is not clear. Some studies suggest that *P. carinii* is closely related to *Saccharomyces cerevisiae* (5, 10), while others have suggested a relationship to the filamentous fungi in the genera *Aspergillus* (4, 9) and *Rhodotorula* (11).

Since most fungal infections respond to therapy with imidazoles, a role for these compounds for use in the treatment of *P. carinii* pneumonia has been discussed. However, successful systemic antifungal agents inhibit the synthesis of ergosterol, the sterol common in most fungal cell membranes, and one report suggests that ergosterol is not present in *P. carinii* (6). Although the lack of clinical evidence for a response of *P. carinii* pneumonia to amphotericin B or fluconazole used for the treatment of other fungal infections suggests insensitivity of *P. carinii* to polyene or imidazole antimicrobial agents, direct evaluations of these compounds have been lacking.

Six imidazoles were tested in a short-term culture system with human embryonic lung fibroblast cells (WI-38 or MRC-5) with *P. carinii* from infected rat lung as described previously (1). Compound Sch39304 was supplied by Schering; etanidazole (Radinyl) was supplied by Roberts Pharmaceutical Corp.; and itraconazole, fluconazole, ketoconazole, and miconazole were from Schering-Plough; fluconazole was also obtained from Roerig-Pfizer. Host cells grown to confluency in minimum essential medium (MEM) with 10% fetal calf serum were used in cultures grown in 24-well tissue culture plates. They were inoculated to produce approximately 7×10^5 viable *P. carinii* trophozoites per ml in each well. The inoculum was from *P. carinii*-infected rat lung; counts of organisms stained with Giemsa or fluorescein diacetate-ethidium bromide were used to adjust the inoculum to the desired concentration.

Each drug concentration to be tested was incorporated into the medium in four wells on each of four plates. Each plate contained four wells without drug and four wells with drug diluent (1.0 and 0.1% dimethyl sulfoxide) that were inoculated with *P. carinii*; these wells served as positive growth controls. Experiments were discarded if the numbers of organisms in these wells failed to increase more than threefold over 7 days. Plates were incubated in a gaseous mixture of 5% O_2–10% CO_2–85% N_2 at 35°C.

The number of *P. carinii* organisms was counted as described previously (1). Two individuals counted trophozoites, cysts, and cells in 10 oil immersion fields of each slide. Data are expressed as the percentage of the control counts.

The development of infection in rats was by inoculation, as described previously (3). Rats from Harlan Sprague-Dawley (Indianapolis, Ind.) colony 202 were virus-free and *P. carinii*-free when they were shipped from the supplier. Female rats weighing between 120 and 140 g were given dexamethasone at 0.36 mg/kg of body weight in drinking water for 4 days prior to transtracheal inoculation or until lymphocytes were depleted by 50%. An inoculum from infected rat lung was prepared by adjusting the numbers of trophozoites so that there were at least 2×10^6 per ml. If examination of the Giemsa-stained preparations revealed contamination, the inoculum was discarded.

Animals were anesthetized by intramuscular injection of 0.2 ml of a solution containing ketamine hydrochloride (80 mg/ml), acepromazine (1.78 mg/ml), and atropine (0.38 mg/ml). Drugs were administered to rats that had developed *P. carinii* infection after having been transtracheally inoculated as described previously (2, 3). At 4 weeks postinoculation, the rats were assigned by weight to treatment groups so that approximately equal mean weights were achieved in all groups. There were at least 10 rats in each group. Rats were housed in open cages and were given water containing dexamethasone at 0.36 mg/kg/day and tetracycline at 0.5 g/liter. Animals received a normal diet (23% protein). The drug study included a group of untreated animals and a group of trimethoprim-sulfamethoxazole-treated (50/250 mg/kg/day) animals.

Treatments were with Sch39304, itraconazole, fluconazole, ketoconazole, and miconazole, each given by gavage at 40 mg/kg/day, and etanidazole, given intravenously at 500 mg/kg/day. The untreated control animals were given 25% ethanol by gavage; ethanol was the diluent for the drugs given by gavage.

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TABLE 1. Effects of imidazole antifungal agents on *P. carinii* in culture[a]

Drug	Dose (μg/ml)	% of control growth at day 10 (study 1)	% of control growth at day 7 (study 2)
Sch39304	10	102	137
Itraconazole	10	77	74
Fluconazole[b]	10	80	128
Ketoconazole[c]	10	45	84
Miconazole	10	24	42
Etanidazole	10	Not tested	73
Trimethoprim-sulfamethoxazole	50/250	10	10

[a] Each culture containing a test compound was compared with a control culture that received drug diluent only. The untreated controls for study 1 were 18.2 ± 4.1 organisms (mean ± standard error) at day 10; for study 2, controls were 7.4 ± 0.8 at day 7.

[b] Cultures containing fluconazole from Schering-Plough and fluconazole purchased from Roerig-Pfizer were tested side by side in a separate experiment; growth in the presence of these two fluconazole preparations was 102 and 94% of that in diluent control cultures at day 7, respectively.

[c] In other experiments, growth in a culture containing ketoconazole at 100 μg/ml was 64% of that of the control culture.

At the end of 3 weeks of therapy, animals were anesthetized as described above and were exsanguinated by cardiac puncture, their lungs were removed, and representative portions of the lungs were used to make impression smears. Four impression smears fixed in methanol were used for staining with Giemsa and modified methenamine-silver nitrate. Slides were blinded and examined microscopically by three examiners who scored them according to the following system, which is approximately logarithmic: >100 organisms per ×1,000 field, 5+; 11 to 100 per field, 4+; 1 to 10 per field, 3+; 2 to 9 in 10 fields, 2+; 1 in 10 or more fields, 1+; no organisms in 50 fields, 0. Data are expressed as means ± standard errors. Statistical analysis was by analysis of variance and the *t* test.

In culture, miconazole was slightly effective at 10 μg/ml, but the other drugs produced less than a 50% decline in growth relative to that of the controls in each of two studies (Table 1). Previously, we have selected compounds for testing in animals when the compounds were effective in culture at 1.0 μg/ml or less. With these antifungal agents, the in vitro culture tests suggested that the activities of these compounds were low. The drugs were therefore administered to animals at very high doses to detect any efficacy. Some of the doses selected were toxic, and the doses of both Sch39304 and itraconazole were cut to 20 mg/kg/day after 3 and 4 days, respectively. Even so, a number of animals were lost to drug toxicity. All animals included in the final evaluations completed 3 weeks of treatment. None of the compounds tested except for the combination of trimethoprim-sulfamethoxazole decreased the numbers of *P. carinii* organisms in rat lungs (Table 2). The infectivity scores for all of the imidazoles were similar to the score for the gavage control group (4.5 ± 0.1). Etanidazole administered intravenously produced no decrease in infectivity (4.4 ± 0.3) in comparison with that in the etanidazole control group (4.6 ± 0.1), although the dose was increased to 1,000 mg/kg/day after the first week. The inoculated rat model yields uniformly heavily infected animals, so that the activities of minimally effective compounds can be detected. In the present study, however, there was no indication of any drug effect even when toxic levels of drug were used.

To investigate the reason for the inactivities of these compounds, we examined the sterols of *P. carinii*. The in vitro cultivation of *P. carinii* on MRC-5 or WI-38 cell lines has provided sufficient quantities of organisms for lipid analysis; we chose cultured organisms because the sterol content could be compared with that reported previously (6) for *P. carinii* directly isolated from rats. *P. carinii* harvested from culture (60×10^6 to 90×10^6 trophozoites contaminated with 0.1×10^6 to 0.3×10^6 host cells) were extracted with chloroform and methanol by standard techniques by using butylated hydroxytoluene as an antioxidant (7). Silane derivatives of the total lipid extract and sterol standards were made, 5 μl of trimethylchlorosilane and 100 μl of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide were incubated with the lipid extract at 60°C for 5 to 15 min. Analysis was by gas chromatography-mass spectroscopy. The instrumentation included a Hewlett-Packard 5890 gas chromatograph equipped with an Ultra 1 column, a 5970B mass selective detector, and a 7673A autosampler. Thin-layer chromatography was done on silica gel G plates developed in one direction with chloroform-methanol-water (65:25:4, by volume) and at 90 degrees from the first direction with *N*-butanol–acetic acid–water (60:20:20).

Gas chromatography-mass spectroscopic analysis of *P. carinii* extracts revealed two peaks identified as sterols on the basis of an ion fragment with a mass of 129. The major sterol was identified as cholesterol on the basis of ion fragments with masses of 129, 275, 329, 353, 368, and 458 for the silane derivatives. In addition, this sterol, when cochromatographed with an ergosterol standard, gave a ratio of retention times of 1.197 ± 0.0021 ($n = 4$; each study was done with triplicate samples); authentic cholesterol compared with ergosterol gave a ratio of 1.203 ± 0.0012. Finally, the major sterol from *P. carinii* extracts cochromatographed with cholesterol in the

TABLE 2. Effects of imidazole antifungal agents in rat therapy model

Drug	Dose (mg/kg)	Route	No. of infected rats/ total no. of rats	Giemsa score	Silver score
Sch39304	40[a]	Gavage	6/6	4.3 ± 0.2	3.6 ± 0.2
Itraconazole	40[b]	Gavage	5/5	4.2 ± 0.2	3.7 ± 0.2
Fluconazole	40	Gavage	7/7	4.3 ± 0.2	3.6 ± 0.2
Miconazole	40	Gavage	4/4	4.4 ± 0.2	3.5 ± 0.2
Ketoconazole	40	Gavage	5/5	4.4 ± 0.2	3.5 ± 0.2
Etanidazole	500[c]	Intravenous	6/6	4.4 ± 0.3	3.7 ± 0.2
Trimethoprim-sulfamethoxazole	50/250	Oral	5/7	0.6 ± 0.3[d]	0.8 ± 0.2[d]
Gavage control		Gavage	9/9	4.5 ± 0.1	3.7 ± 0.2
Untreated control			8/8	4.6 ± 0.1	3.7 ± 0.2

[a] Dose reduced to 20 mg/kg daily after 3 days.

[b] Dose reduced to 20 mg/kg daily after 4 days.

[c] Dose increased to 1,000 mg/kg daily after 7 days.

[d] $P < 0.001$.

two-dimensional thin-layer system that resolved nine separate lipid components from the *P. carinii* extracts. The minor sterol component of *P. carinii* extracts was not conclusively identified, but it was not ergosterol. The ratio of retention times of the *P. carinii* minor sterol to a cholesterol standard was 1.269 ± 0.005, which was highly significantly different ($P < 0.0001$) from the ratio of ergosterol to the cholesterol standard (1.203 ± 0.003). No peaks which corresponded to ergosterol were detected in extracts of *P. carinii*. Extracts from *S. cerevisiae* run as controls did contain identifiable ergosterol in parallel experiments. These studies suggest that ergosterol is absent from cultured *P. carinii*, as has previously been observed for *P. carinii* harvested directly from rats (6). Despite many fungal characteristics proven by recombinant DNA techniques, *P. carinii* cannot be considered in all ways to be biochemically like other systemically pathogenic fungi. As would be predicted, because the main sterol present in *P. carinii* membranes is cholesterol rather than ergosterol, the organism is resistant to imidazole antifungal agents.

This work was supported in part by NIH grants NOI-AI-72647 and UOI-AI-25959-01 and by Roberts Pharmaceutical Corp. (Eatontown, N.J.).

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