ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1991, p. 1329–1333
0066-4804/91/071329-05$02.00/0

Vol. 35, No. 7

Efficacy of Cilofungin Alone and in Combination with Amphotericin B in a Murine Model of Disseminated Aspergillosis

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Received 17 September 1990/Accepted 10 April 1991

Cilofungin, amphotericin B, and a combination of the two drugs were compared in a model of aspergillosis in immunocompetent mice in three experiments. Cilofungin was equivalent to amphotericin B in preventing death and eradicating cerebral aspergillosis, but it did not sterilize the kidneys. This is the first demonstration of the in vivo activity of cilofungin against any fungus other than *Candida albicans*. The mortality with combination therapy was higher than those with amphotericin B alone ($P = 0.003$) and cilofungin alone ($P = 0.054$), as was weight loss after infection, indicating antagonism between cilofungin and amphotericin B in this model. The mechanisms of action and antagonism remain to be explained.

Cilofungin is the first of a new class of antifungal agents, the lipopeptides, to come to clinical trials. It is highly active against some *Candida* spp., especially *C. albicans* and *C. tropicalis* (4, 5, 10, 11, 14). Activity in vitro against *Aspergillus* spp. is negligible (5). Its activity resides in its ability to noncompetitively bind (1,3)-β-D-glucan synthase (17), an enzyme necessary for cell wall synthesis.

In view of its proposed clinical use, the treatment of serious candidal infections, we were interested in any interference it might have with the activity of amphotericin B. *Candida* and *Aspergillus* infections are found in many similar host groups, for example, patients with leukemia and transplant patients, and any antagonism of amphotericin B by cilofungin in treating *Aspergillus* infections would have potentially serious consequences. Such an adverse interaction of ketoconazole on amphotericin B efficacy has been documented in vivo and in vitro (15). We therefore undertook an in vivo investigation of any amphotericin B-cilofungin interaction in a murine model of disseminated aspergillosis.

MATERIALS AND METHODS

Inoculum. The isolate used for this study is a typical *Aspergillus fumigatus* isolate cultured from a pulmonary lesion in a corticosteroid-treated patient from Los Angeles, Calif. It was isolated in 1986 and is designated 10AF/86/10. Its DNA type is 3, 9, A (*Sal*I); 5, 13, B (*Xho*I) (1). It grows at 50°C and produces large zones on an elastase medium (7). In vitro susceptibility tests were done as described previously (2).

The inocula were prepared by culturing the organism on potato dextrose agar for 10 days at 35°C. Conidia were collected in sterile 0.9% saline containing 0.01% Tween 80 and frozen in several 1- to 4-ml samples containing 10% dimethyl sulfoxide at −70°C. The viability of one of the samples was determined by 10-fold dilutions in 0.9% saline containing Tween, subculturing it onto sheep blood agar plates, and counting the colonies. Five days prior to mouse infection, a dilution of frozen sample was made in 0.9% saline to approximate the desired inoculum, and a further viability determination was made on this dilution, as described above. The vial containing the inoculum was stored at 4°C until the day of infection (always a Monday). Further adjustment of the inoculum (usually slight) was made, if necessary, just prior to mouse infection.

Mice. Virus-free female CD-1 mice (age, 5 weeks) were purchased from Charles River Breeding Laboratories, Wilmington, Mass. Only mice weighing 22 to 24.9 g 3 days prior to the experiment were used. Mice were housed in cages of five or six apiece and were given acidified water and sterilized food ad libitum. In experiments 2 and 3, groups of mice were weighed immediately after infection and again 3 days later. In all experiments, control groups consisted of 10 mice. In experiments 1 and 2, treated groups contained 12 mice, and in experiment 3, treated groups contained 15 mice.

Mouse infection. Mice were infected by tail vein injection of 0.15 ml of inoculum. The inoculum for experiments 1 and 2 was 5.5×10^7 conidia per ml, and for experiment 3 it was 7.0×10^7 conidia per ml, as determined by viability counts.

Drugs. Amphotericin B, as a colloidal suspension in deoxycholate (Fungizone; Squibb), was given in 5% glucose. Cilofungin was dissolved in 33% polyethylene glycol 300 (PEG). Both 33% PEG and deoxycholate in 5% glucose were also prepared. Amphotericin B was prepared so that each mouse received 3.3 mg/kg of body weight per dose on the basis of the mean weight of the mice 3 days before infection. Likewise, cilofungin was given as 62.5 mg/kg/day in two daily doses in experiments 1 and 2. In experiment 3, 93.8 mg/kg/day as three daily doses was given for the first 2 days, followed by 62.5 mg/kg/day in two daily doses.

Preliminary experiments with different amphotericin B doses ranging from 1 to 7.5 mg/kg indicated that 3.3 mg/kg was the most efficacious nontoxic dose in this model. The cilofungin dose was determined on the basis of previous data in a *Candida* infection model (5); this dose was efficacious and nontoxic.

Treatment. In experiments 1 and 2, treatment was started 24 h after infection; in experiment 3, it was started 18 h after infection. Treatment groups were assigned randomly by cage

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after infection. All drugs were given intraperitoneally in 0.1 ml. Cilofungin or PEG was given twice a day at 8:00 a.m. and 6:00 p.m. (or, in experiment 3, at 8-h intervals for the first 2 days) for 11 days. Amphotericin B was given on Tuesday, Wednesday, and Friday of the first week and Monday, Wednesday, and Friday of the second week in experiments 1 and 2. In experiment 3, an additional (seventh) dose was given on Thursday, day 3. Seven days of observation followed the end of treatment.

Cultures and histopathological examination. Cultures were not obtained from mice that died during the 18-day experiment, but a few that died early underwent an autopsy. Tissues were fixed in 10% formalin, and slides were stained with Gomori-methenamine silver and counterstained with hematoxylin and eosin. Organ involvement was quantitated microscopically by using the following classifications: severe, multiple areas of involvement, each with a large mass of hyphae; moderate, several areas of involvement with small collections of hyphae; mild, at least two areas of involvement with several hyphae; slight, a single hypha in at least two noncontiguous areas of the same organ.

In mice that survived to day 18, cerebral or ocular disease was noted by careful inspection prior to sacrifice. Mice that circled, that had head tilt, or that had clear-cut paralysis of one or more limbs were deemed to have cerebral disease. Then they were killed by cervical dislocation and subjected to autopsy. Samples of brains and both kidneys were cultured. Preliminary experiments indicated that few, if any, lungs, livers, or spleens were culture positive on day 18. The kidneys were removed, weighed, and then placed in 5 ml of sterile saline; the brains were placed in 5 ml of sterile saline. These organs were homogenized in a tissue grinder for 15 to 30 s, three and two 10-fold dilutions were made of kidneys and brains, respectively, and 0.5 ml of each dilution was distributed to Mycosel (BBL, Cockeysville, Md.) plates. Plates were incubated at 35°C for 2 days and colonies were counted.

Statistical analysis. Mortality was compared by the Wilcoxon rank sum test and Student's *t* test. For comparison of different treatment groups in the three experiments together, an analysis of variance was done by using Minitab (Minitab Data Analysis Software, Philadelphia, Pa.). Culture results were compared by the Fisher exact test for the frequency of organ culture positivity and by the Wilcoxon rank sum test for kidney colony counts. In the latter analyses, death was assumed to represent a worse outcome than survival with any fungal organ burden; organs from animals that died prior to sacrifice were thus assigned values equal to the highest control kidney counts in experiment 1.

RESULTS

The in vitro studies yielded a MIC and a minimum fungicidal concentration against amphotericin B of 2.0 μg/ml (plus or minus one twofold dilution in repeated assays) and against cilofungin of >50 μg/ml.

In experiment 1, three control groups were included: untreated, PEG-treated only, and deoxycholate-treated only. The mortality curve for each group is shown in Fig. 1. No statistical difference between the control groups was noted, and so the untreated and deoxycholate-treated controls were omitted from the subsequent two experiments. Overall, the mortality in the three control groups combined was 87%, and all surviving mice in these groups were small and cachectic. In experiments 2 and 3, the PEG-treated control group mortality was 100% in both experiments, with

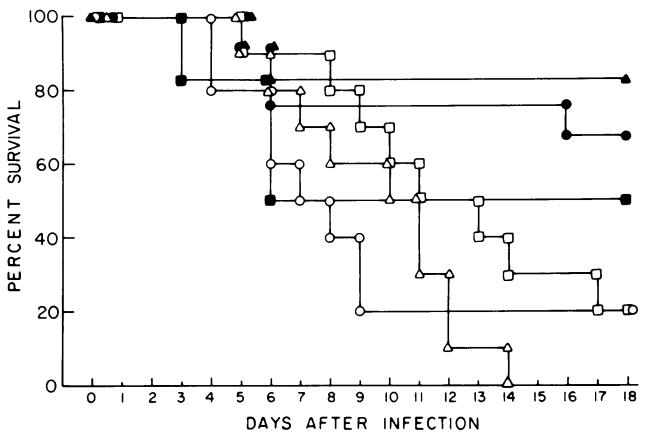

FIG. 1. Cumulative mortality of mice in treatment and control groups in experiment 1. Each control group consisted of 10 animals, and each treatment group consisted of 12 animals. Treatment was initiated 24 h after infection and continued until day 11. ▲, cilofungin, 62.5 mg/kg/day; ●, amphotericin B, 3.3 mg/kg; ■, amphotericin B and cilofungin; □, control; ○, deoxycholate control; △, PEG control.

90% mortality by day 10 in experiment 2 and 90% mortality by day 6 in experiment 3 (25% greater inoculum). Survival of the cilofungin- or amphotericin B-treated group was significantly greater than that of the PEG-treated group in all three experiments (P = 0.007 to <0.0001).

Figure 1 depicts the mortality curves in experiment 1. Of considerable surprise to us, given the available in vitro data, was the remarkable survival of the cilofungin-treated mice in all three experiments: 83, 83, and 80% in experiments 1, 2, and 3, respectively. This compared favorably with the amphotericin B-treated mice (67, 100, and 93%, respectively) and the mice treated with the combination of the two drugs (50, 67, and 73%, respectively).

It is notable that the earliest deaths were in the combination-treated group, even before any control deaths in experiments 1 and 3. This higher mortality in the combination-treated group was true for all three experiments, but compared with the amphotericin B-treated group, it was only statistically significant in experiment 2 (P = 0.02 by Student's *t* test). We therefore pooled the mortality data for all three experiments (they were considered separate blocks of the same experiment) and analyzed the mortality by analysis of variance. There was an overall reduction in survival time of 3.39 days in the combination-treated group compared with that in the amphotericin B-treated group, representing a reduction of 20%. This reduction was statistically significant (P = 0.003). The cilofungin-treated group had a slightly higher mortality than that of the combination-treated group, which did not quite reach statistical significance (P = 0.054). There was no difference between the amphotericin B-treated and the cilofungin-treated groups (P = 0.34).

From experiment 1, six mice that died early underwent autopsy, their organs were examined histologically, and the approximate hyphal burden in the different organs was quantitated. These mice were selected because the earliest deaths occurred in the combination-treated group, and synergistic toxicity was thought to be possible. The three combination-treated mice had quantitatively larger fungal burdens or more organs involved, as determined microscopically and macroscopically, than two deoxycholate-treated

TABLE 1. Culture, kidney weight, and clinical results of survivors in experiments 1 to 3

Expt and group	Mean kidney wt (g)	No. of kidney pairs positive/no. of mice in group	Mean ± SE kidney colony counts of organs cultured	No. of survivors with clinical cerebral disease/no. in group	No. of brains culture positive/no. of mice in group	Mean ± SE brain colony counts
Expt 1						
Controls	0.454	4/4	3,185 ± 1,586	ND[a]	3/4	322 ± 292
Cilofungin	0.402	10/10	397 ± 130[b]	2/10	1/10	0.2 ± 0.2
Amphotericin B	0.353	1/8	2.5 ± 2.5[c]	2/8	0/8	0 ± 0
Combination	0.367	1/6	74 ± 30[d]	3/6	1/6	0.34 ± 0.34
Expt 2						
Cilofungin	0.447	9/10	198 ± 63.6[e]	1/10	0/10	0 ± 0
Amphotericin B	0.361	4/12	545 ± 496[f]	5/12	0/12	0 ± 0
Combination	0.393	4/10	25 ± 15.7	3/10	2/10	2.2 ± 2.0
Expt 3						
Cilofungin	0.401	12/12	303 ± 80.4[g]	3/12	2/12	35.7 ± 34.9
Amphotericin B	0.381	3/14	4.6 ± 4.3[h]	3/14	1/14	0.14 ± 0.14
Combination	0.397	4/11	29.3 ± 18.1	3/11	2/11	13.6 ± 12.7

[a] ND, not determined.
[b] $P = 0.0001$ compared with controls; $P = 0.14$ compared with the amphotericin B-treated group; $P = 1.0$ compared with the combination-treated group.
[c] $P = 0.002$ compared with controls; $P = 0.4$ compared with the combination-treated group.
[d] $P = 0.028$ compared with controls.
[e] $P = 0.024$ compared with the amphotericin B-treated group; $P = 0.033$ compared with the combination-treated group.
[f] $P = 0.56$ compared with the combination-treated group.
[g] $P < 0.0001$ compared with the amphotericin B-treated group; $P = 0.047$ compared with the combination-treated group.
[h] $P = 0.12$ compared with the combination-treated group.

control mice and one cilofungin-treated mouse did. These data suggest primary antagonism of the antifungal activity of amphotericin B by cilofungin. There was no histological evidence to suggest toxicity.

Signs consistent with cerebral aspergillosis were noted prior to death (Table 1), although cultures were sometimes negative. Endophthalmitis was seen in three mice in experiments 1 and 2. At necropsy all mice in all experiments had enlarged spleens. In experiment 1, 3 of 4 control mice had macroscopically abnormal kidneys, as did 4 of 10 cilofungin-treated mice, 0 of 8 amphotericin B-treated mice, and 1 of 6 combination-treated mice. A similar frequency of macroscopic renal abnormality was found in the other experiments.

Culture results for the survivors in the three experiments are given in Table 1. In all three experiments, there was a statistically significantly lower frequency of culture-positive kidneys in either the amphotericin B-treated or combination-treated groups compared with the frequency in controls (P values varied from 0.03 to 0.003). In all three experiments, there was a significantly lower frequency of culture-positive kidneys in either the amphotericin B-treated or combination-treated groups compared with the frequency in cilofungin-treated groups (P values varied from 0.03 to 0.00005), but in no experiment were there significant differences between the amphotericin B-treated and combination-treated groups. In experiment 1, both the amphotericin B-treated and cilofungin-treated groups had a significantly lower frequency ($P < 0.05$) of culture-positive brains compared with that in controls. This was not true of the combination-treated group. There were no significant differences in the frequency of culture-positive brains between any treatment group in any of the three experiments.

Many significant differences in renal colony counts among treatment groups were found; these are given in the footnotes to Table 1. There were no significant differences in brain colony counts between treatment groups in any of the three experiments.

The weight loss noted in experiment 1 was documented in experiments 2 and 3 (Table 2). In both experiments, weight decline was greatest in the control and combination-treated groups. Comparison between the two control groups showed a greater weight loss in experiment 3 (25% higher inoculum), but even this was just less than the dramatic weight loss in the combination-treated group in experiment 2.

DISCUSSION

The data presented here indicate that cilofungin has considerable activity against *Aspergillus* spp. in vivo. In the most dramatic example, experiment 3, the inoculum gave 90% mortality by day 6 in control mice compared with only a 7% mortality over 18 days in the cilofungin-treated animals. The activity of cilofungin was seen in three separate experiments. Comparison with amphotericin B yielded no significant differences in mortality. The apparent discrepancy between experiments 1 and 2 in amphotericin B mor-

TABLE 2. Weight loss in different experimental groups after infection

Expt and group	Mean wt (g):		Loss
	Immediately after infection	3 days after infection	
Expt 2			
PEG controls	24.1	21.3	−2.8
Cilofungin	24.1	22.2	−1.9
Amphotericin B	24.5	23.9	−0.6
Combination	24.7	20.5	−4.2
Expt 3			
PEG controls	23.5	19.5	−4.0
Cilofungin	24.2	22.4	−1.8
Amphotericin B	24.2	22.4	−1.8
Combination	23.8	20.7	−3.0

tality, 33 versus 0%, was partly offset by a higher frequency in experiment 2 of cerebral disease (5 of 12 versus 2 of 8) and renal disease (4 of 12 versus 1 of 8) and higher mean colony counts in the kidneys of mice in experiment 2 (545 versus 2.5). This suggests that the actual inoculum was slightly less in experiment 2 or that some other factor allowed the mice to survive to day 18, with more severe disease in survivors than in mice in experiment 1. This is the first demonstration of the in vivo activity of cilofungin against any fungus other than *C. albicans*.

The data presented here also suggest that there is antagonism between amphotericin B and cilofungin in this model. The data that indicate this was, first, earlier and that there were more deaths in the combination-treated group compared with those in each of the treatment groups. There was a reduction in survival time of 20% overall in the combination-treated groups compared with that in the amphotericin B-treated group. Second, weight loss was greatest in the combination-treated group among all three treatment groups. There was also a slightly higher frequency of both renal and cerebral disease, determined by culture of kidney and brain specimens, in combination-treated survivors to day 18 compared with that in amphotericin B-treated mice. Renal colony counts in two of three experiments and brain colony counts in all three experiments were higher in the combination-treated mice compared with those in amphotericin B-treated mice. Histological examination of mice that died earlier suggested a higher fungal burden in combination-treated mice. The data therefore suggest, but are far from conclusive, that primary antifungal antagonism of amphotericin B by cilofungin is the reason for the antagonism that was seen. Confirmation might be obtained by quantitative culturing at set intervals early after the start of therapy, prior to death.

The activity of cilofungin against *Candida* spp. appears to reside in the inhibition of cell wall synthesis by noncompetitive binding to 1,3-β-glucan synthase. β-Glucan synthase enzymes are present in various *Aspergillus* spp., including *A. nidulans* (9), *A. niger* (18), *A. vesicolor* (6), and *A. terreus* (12). Both 1,3-β-glucanases (6, 18) and 1,4-β-glucanases (8, 18) and α-glucanases (8) have been identified in *Aspergillus* spp., but the α-glucanases may be involved only in conidiation and fructification (19). No published data have indicated any activity of cilofungin on *Aspergillus* spp., and for that reason, no data on the mechanism of action of cilofungin on aspergilli have been published. Cilofungin has, however, been shown to inhibit (1,3)-β-D-glucan synthase activity in another filamentous but nonpathogenic fungus, namely, *Neurospora crassa* (16). In the light of its mode of activity against *N. crassa* and *Candida* spp., inhibition of 1,3-β-glucan synthase in *A. fumigatus* is likely.

It is of particular interest that cilofungin appeared to be at least as efficacious as amphotericin B in sterilizing cerebral aspergillosis. The frequency of cerebral aspergillosis in our mice just prior to death (judged by clinical features) was higher than that demonstrated by culture. Cerebral aspergillosis in humans carries a particularly poor prognosis; only eight survivors in whom treatment was evaluable are noted in the literature (3). However, enthusiasm for cilofungin in this setting must be tempered, because amphotericin B was at least equally successful, and the latter drug usually fails to clear infection in patients.

The lack of renal sterilization by cilofungin is also notable and of interest. The half-life of cilofungin is short: 1.3 h in one study (13). The lack of renal sterilization that was seen may reflect less than optimal dose and/or dosing intervals or some intrinsic problem with the clearing of renal aspergillosis. That the mice survived and that the brains were mostly sterilized suggest that the former two possibilities are less likely than the third. However, in a model of disseminated candidiasis with prominent renal involvement, sterilization of the kidneys was not achieved in any cilofungin-treated mice when the same dosage schedule used in experiments 1 and 2 of this study was used (11), although amphotericin B was also generally ineffective (11).

In conclusion, the activity of cilofungin against *Aspergillus* spp. in vivo is noteworthy. The apparent lack of in vitro activity remains to be explained. Possibly, its spectrum of activity against other fungi will be extended by other in vivo studies. The addition of another agent with activity against aspergillosis may be of considerable value in the treatment of patients with this frequently life-threatening mycosis.

ACKNOWLEDGMENTS

We are indebted to Linda H. Hanson, Alon M. Perlman, Raymond L. Azzi, Karl V. Clemons, and Maura Moscardi-Bacchi for assistance and to W. Byron Brown for statistical help in the analysis of variance. We are grateful to Maureen Cervelli for typing the manuscript.

This study was supported in part by a grant from Eli Lilly & Co., Indianapolis, Ind.

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