ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1991, p. 1334–1337
0066-4804/91/071334-04$02.00/0

Vol. 35, No. 7

Synergy between Cilofungin and Amphotericin B in a Murine Model of Candidiasis

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Received 7 December 1990/Accepted 10 April 1991

The efficacies of cilofungin and amphotericin B separately and together in mice with disseminated candidiasis were studied. Male CD-1 mice (age, 5 weeks) were infected intravenously with 3×10^5 CFU of *Candida albicans*. At 4 days postinfection, intraperitoneal therapy was initiated and was continued for 14 days. Therapy groups included those given cilofungin at 6.25 or 62.5 mg/kg/day (given twice daily), amphotericin B at 0.625 mg/kg/day (given once daily), cilofungin at 6.25 mg/kg/day plus amphotericin B, and cilofungin at 62.5 mg/kg/day plus amphotericin B. Mice were observed through 30 days postinfection. All infected untreated mice died of infection between days 6 and 18. Eighty-five percent of mice receiving cilofungin at 6.25 mg/kg/day died between days 13 and 30. All other mice survived. Quantitative determination of the number of CFU of *C. albicans* in the spleens and kidneys of all survivors revealed that mice that had received both drugs had lower residual burdens of *C. albicans*. All mice treated with cilofungin at 62.5 mg/kg/day plus amphotericin B had sterile spleens, whereas 42 to 58% of mice given cilofungin or amphotericin B monotherapy had sterile spleens. All kidneys were infected in mice which had received cilofungin at 62.5 mg/kg/day or amphotericin B. Neither organ was infected in 17% of each group receiving combination therapy with cilofungin and amphotericin B. The number of CFU in the kidneys of mice treated with cilofungin at 62.5 mg/kg/day plus amphotericin B was lower than those cultured from mice treated with cilofungin at 62.5 mg/kg/day ($P < 0.001$, Mann-Whitney) or amhotericin B ($P < 0.05$). Modest synergy was noted in inhibition of the *C. albicans* isolate in vitro. Pharmacokinetic studies showed elevated levels of cilofungin but not amphotericin B in sera of mice treated with combined therapy compared with those in mice given monotherapy. No overt toxicity was evident with any regimen. The mechanism of increased efficacy may be altered cilofungin distribution, excretion, or metabolism; antifungal synergy; or both. These results indicate that concurrent cilofungin-amphotericin B therapy has synergistic or additive efficacy in vivo.

The development of new classes of antifungal agents is an important mission for the future. A possible class of antifungal agents to be developed is cell wall synthesis inhibitors. Cilofungin, an inhibitor of β-1,3-glucan synthesis, has been the most widely studied of such agents to date (2, 4, 6, 8, 9, 12, 14). The demonstrated spectrum of activity of this drug is restricted to a few candidal species and, rarely, other genera (2, 4, 6, 8, 9, 14). The present study was undertaken to investigate the potential interaction of cilofungin with amphotericin B, a polyene drug which has been the one used most extensively to treat clinical candidal infections. A model was used in which the efficacy and toxicity of each drug have been defined previously (12). In the previous studies, each drug prolonged survival, but neither drug was curative when it was used alone.

(This study was presented in part at the 29th Interscience Conference on Antimicrobial Agents and Chemotherapy [7].)

MATERIALS AND METHODS

Inoculum. *Candida albicans* isolate 5, which has been used in previous studies of systemic candidosis (11, 12, 16), was used in this study. To prepare the infection inoculum, a loopful of organisms was taken from underwater storage at room temperature, streaked onto Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) slants, and incubated at 35°C for 24 h. The harvest from the slant cultures was subcultured overnight in Yeast Nitrogen Broth (Difco) with 0.5% glucose at 35°C on a gyratory shaker, washed in saline, and diluted in saline to 1.5×10^6 CFU/ml.

Inoculation of mice. Male CD-1 mice (age, 5 weeks; average weight, 23 g; Charles River Breeding Laboratories, Portage, Mich.) were used in this model, as described previously (11, 12, 16). Mice were given 0.2 ml of the inoculum suspension in a lateral tail vein. Randomized groups of 12 mice each were housed at 6 mice to a cage and were provided sterilized food and acidified water ad libitum.

Drugs. Cilofungin (Eli Lilly & Co., Indianapolis, Ind.) was provided as a powder. Stock solutions for therapy studies were made by dissolving powder in 33% polyethylene glycol 300 (Sigma Chemical Co., St. Louis, Mo.). Further dilutions were made in 33% polyethylene glycol 300. Stocks were stored for ≤1 week at 4°C.

Cilofungin for in vitro studies was prepared at a concentration of 1 mg/ml in 50% methanol–50% 0.04 M phosphate buffer (pH 6.0) for bioassays and 50% methanol–50% culture medium for susceptibility testing. Stocks were stored for ≤1 week at 4°C.

Amphotericin B (E. R. Squibb & Sons, Inc., Princeton, N.J.), which was provided as lyophilized cakes containing 50 mg of amphotericin B and 41 mg of sodium deoxycholate buffered with 20.2 mg of sodium phosphate, was reconstituted with sterile water, diluted in sterile water, and stored in the dark at −20°C.

In vivo studies. Therapy was initiated at 4 days postinfec-

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tion and was continued for 14 days. All drugs were delivered by intraperitoneal injection. Because preliminary pharmacokinetic studies have shown that serum cilofungin concentrations peak at approximately 1 h postdose in mice (4), cilofungin was given in divided doses twice each day (12). Cilofungin regimens of 62.5 or 6.25 mg/kg/day, whose efficacies and safety have been studied previously (12), were given in 0.1-ml injections. Amphotericin B at 0.625 mg/kg/day, a dose selected because of results obtained previously (12), was given in 0.2-ml injections once daily. Mice receiving both drugs were given each drug in separate injections. One group of infected mice received no injections. Because previous studies have demonstrated that the survival of infected mice given the diluent of either drug is no different from that of infected mice receiving no injections (12), groups given the diluents were not included in this study.

Deaths were recorded daily through 30 days postinfection. All surviving mice were killed by cervical dislocation and necropsies were performed. Previous studies (12) showed that the organs with residual infection in survivors were the spleens and kidneys. These were removed and homogenized in 5 ml of sterile saline with a Tissumizer (Tekmar Co., Cincinnati, Ohio). Dilutions of homogenates were cultured on blood agar plates (Becton-Dickinson, Irvine, Calif.) and incubated at 35°C for 48 h. The colonies that appeared on the plates were counted, and organ burdens of residual infection were expressed as $\log_{10}$ CFU per organ.

Pharmacokinetic studies. Sera from two mice were pooled at each time point studied. Once it was collected, serum was immediately stored at −20°C. Cilofungin concentrations were measured by bioassay as described in detail elsewhere (4), with the exceptions that the inoculum used was 4×10^5 conidia per ml and the phosphate buffer used for dilutions was 0.04 M. When samples containing both drugs were assayed, the amphotericin B zones were clearly visible through the agar and were always inside the zones made by cilofungin. The sizes of the zones of inhibition made by cilofungin were not different with or without amphotericin B. Thus, amphotericin B did not interfere with the measurement of cilofungin.

Amphotericin B was measured by bioassay as described previously (17). Because the assay organism selected, *Candida pseudotropicalis* isolate SA, is resistant to cilofungin, no zones of inhibition were produced by cilofungin. Cilofungin also did not affect the amphotericin B assay; amphotericin B zones were not different with or without added cilofungin. The lower limits of detection of the bioassays were 0.6 μg/ml for cilofungin and 0.6 μg/ml for amphotericin B.

In vitro susceptibility testing. MICs (8) and minimal fungicidal concentrations (10) were determined by broth macrodilution and subculture onto sheep blood agar plates as described previously. For drug interaction studies, a checkerboard-style assay by the same broth macrodilution technique as was used for the MIC determination was performed, as described previously (15).

Statistics. Differences in cumulative mortalities were analyzed by the Wilcoxon rank sum test (13). Differences in organ burdens were analyzed by the Mann Whitney U test (13). To test for the effects of multiple comparisons, analysis of variance and Student Newman-Keuls tests were applied (5). Sterilization rates were compared by the Fisher exact test (13). Values of $P \leq 0.05$ were considered significant.

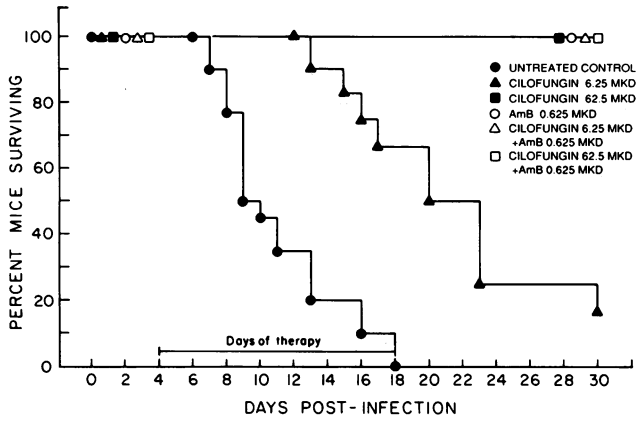

FIG. 1. Survival of mice challenged intravenously with *C. albicans* and untreated or treated with the indicated regimens. MKD, mg/kg/day; AmB, amphotericin B.

RESULTS

Survival. The infection was 100% lethal, with all untreated mice dying at 6 to 18 days postinfection (Fig. 1). Prolongation of survival was significant ($P < 0.001$) in mice given cilofungin at 6.25 mg/kg/day, but 85% of the mice died by the end of the experiment. Groups of mice treated with cilofungin at 62.5 mg/kg/day, amphotericin B at 0.625 mg/kg/day, a combination of both regimens, or cilofungin at 6.25 mg/kg/day plus amphotericin B at 0.625 mg/kg/day all had 100% survival ($P < 0.001$).

Sterilization of infection. The results (Table 1) indicate that all mice given monotherapy with cilofungin or amphotericin B were infected in one or both of the principal target organs. In the regimens combining amphotericin B with cilofungin at either 6.25 mg/kg/day (2 of 12 mice, 17%) or 62.5 mg/kg/day (2 of 12 mice, 17%), there were animals with no evident residual infection.

Therapy regimens that allowed infected mice to survive formed the basis of the quantitative comparisons. With respect to the kidneys, no monotherapy regimen sterilized any kidneys, whereas either combined regimen cleared infection from the kidneys of 2 of the 12 animals in each group. The residual renal infection was significantly ($P < 0.005$) less in amphotericin B-treated mice than in those treated with cilofungin at 62.5 mg/kg/day, and the addition of cilofungin at 6.25 mg/kg/day to amphotericin B did not improve the amphotericin B results. However, the efficacy of the combination of cilofungin at 62.5 mg/kg/day plus amphotericin B was significantly superior to that of the amphotericin B regimen alone ($P < 0.05$) or to that of the cilofungin regimen alone ($P < 0.001$) in reducing renal infection.

Cilofungin at 62.5 mg/kg/day sterilized 5 of 12 spleens and amphotericin B sterilized 7 of 12 spleens, but amphotericin B plus cilofungin at 6.25 mg/kg/day sterilized 9 of 12 spleens and amphotericin B plus cilofungin at 62.5 mg/kg/day sterilized the spleens of all animals (superior to amphotericin B alone [$P < 0.003$] or cilofungin at 62.5 mg/kg/day alone [$P < 0.02$]). Likewise, the residual infectious burden in the spleens of amphotericin B-treated animals was less than that in mice receiving cilofungin at 62.5 mg/kg/day, and the combination of amphotericin B with cilofungin at 6.25 mg/kg/day produced a smaller burden than that produced by amphotericin B alone, but neither comparison was signifi-

TABLE 1. Recovery of *C. albicans* from the organs of surviving mice

Therapy group (dose [mg/kg/day])	No. of mice alive/total no.	No. of mice with sterile spleen and kidney	No. of mice with the following sterile organ (mean, range $\log_{10}$ CFU/organ)[a]:	
			Spleen	Kidney
No drug	0/20			
Cilofungin				
(6.25)	2/12	0	1 (0.7, 0–1.0)	0 (6.7, 6.4–6.9)
(62.5)	12/12	0	5 (1.9,[b,c,d] 0–2.7)	0 (6.0,[e,f] 4.2–6.5)
Amphotericin B (0.625)	12/12	0	7 (1.2,[b,g,h] 0–2.1)	0 (5.4,[e,i,j] 1.3–5.9)
Cilofungin (6.25) + amphotericin B (0.625)	12/12	2	9 (0.97,[c,g] 0–1.7)	2 (5.8,[i] 0–6.7)
Cilofungin (62.5) + amphotericin B (0.625)	12/12	2	12 (0,[d,h] 0)	2 (4.8,[f,j] 0–5.8)

[a] Superscript letter(s) appearing in two groups indicates the *P* value generated when those two groups were compared.
[b] $P > 0.05$.
[c] $P = 0.05$.
[d] $P < 0.01$.
[e] $P < 0.005$.
[f] $P < 0.001$.
[g] $P > 0.05$.
[h] $P = 0.05$.
[i] $P > 0.05$.
[j] $P < 0.05$.

cant. However, the combination of cilofungin at 62.5 mg/kg/day with amphotericin B was significantly ($P = 0.05$) superior to amphotericin B alone and cilofungin at 62.5 mg/kg/day alone ($P < 0.01$).

Because several comparisons between groups were made, a more conservative method of significance testing on the quantitative burden data was also applied. To adjust for multiple comparisons, an analysis of variance test was performed on the logarithmically transformed data, and the Student Newman-Keuls multiple test was applied. By this test, the cilofungin (62.5 mg/kg/day)–amphotericin B regimen was superior ($P < 0.05$) to either component alone with respect to residual kidney infection but not to spleen infection.

In vitro drug interaction. In pursuit of a possible explanation of these results, further studies were undertaken. The MICs of drugs for the isolate used in the in vivo studies were 0.6 μg/ml for cilofungin and 0.5 μg/ml for amphotericin B, and the minimum fungicidal concentrations were 5.0 and 2.0 μg/ml, respectively. When inhibition was assayed in a checkerboard-style titration, modest synergy was demonstrated. In the presence of 0.25 μg of amphotericin B per ml (one-half the MIC), the isolate was inhibited if cilofungin tested in this titration at any concentration (0.04 to 40 μg/ml) was also present.

Pharmacokinetics of cilofungin after acute or chronic administration. In this study, cilofungin was given twice daily. One group of uninfected mice was given one dose of 31.25 mg of drug per kg of body weight, which was equal to one of the daily doses of the higher-dose cilofungin regimen (Fig. 2). The peak concentration that we found, 46 μg/ml, was present at 30 min postdose. Only 4.6 μg/ml was present at 2 h postdose. The estimated initial half-life was thus approximately 30 min. Additional groups of uninfected mice were given this regimen, with or without amphotericin B, for 14 days, as in the therapeutic experiments (Fig. 3). The curve of concentrations in serum after the last dose in mice given cilofungin alone appeared to have a later peak and longer initial half-life when compared with those found in the single-dose curve. Mice given the combined regimen chronically (Fig. 3) appeared to have markedly higher serum cilofungin concentrations than the latter group did, particularly at 1, 1.5, and 2 h postdose. In our study, the peak value of the combined regimen was 52 μg/ml (1.5 h). The values at 1 and 2 h postdose were considerably higher than those seen at these times with either acute or chronic administration of cilofungin monotherapy. Serum amphotericin B concentrations could not be detected at any of the times shown in Fig. 2 and 3 for mice given 0.625 mg of amphotericin B per kg acutely (single dose), chronically, or chronically with cilofungin at 62.5 mg/kg/day. No side effects were evident in the examination, behavior, or appetite of these mice given either or both of these regimens acutely or chronically.

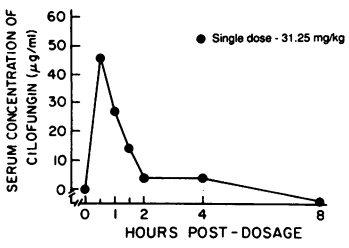

FIG. 2. Serum cilofungin concentrations after a single administration.

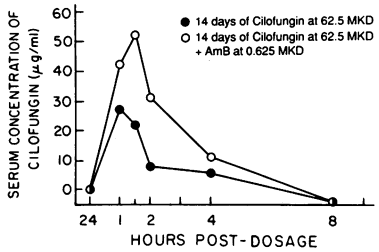

FIG. 3. Serum cilofungin concentrations after chronic administration, with and without concurrent amphotericin B. MKD, mg/kg/day; AmB, amphotericin B.

DISCUSSION

Results of this study demonstrate a lack of antagonism and an additive or synergistic effect (these terms are difficult to define in the in vivo situation) of cilofungin and amphotericin B in vivo. A positive interaction between glucan synthetase enzyme inhibitors and polyene drugs (which have a completely different mechanism of action that involves the cell membrane, intracellular oxidation, or both) may point to a future pathway for development of therapy against serious candidal infections and any other infections caused by susceptible organisms. If the enzyme inhibitor were to be similar to cilofungin in its property of having a narrow spectrum (2, 4, 6, 8, 9, 14), it is also of potential interest to the clinician to know that the use of both drugs, which may be necessary in some settings, would not result in the loss of activity because of antagonism.

The mechanisms of the cooperative effect demonstrated here could be a result of the synergy demonstrated in vitro with this isolate, the pharmacokinetic interactions, or both. The pharmacokinetic interactions suggest that concurrent amphotericin B therapy may alter cilofungin distribution, excretion, or metabolism. Cilofungin appears to be excreted largely by the biliary route (3). There is much uncertainty about the metabolic disposition of amphotericin B, but there is considerable evidence that the renal and biliary routes are minor excretory pathways (1). Although amphotericin B may cause renal toxicity, from what is known about cilofungin metabolism, it appears unlikely that impaired renal excretion of cilofungin is the mechanism of the altered cilofungin concentrations.

Results of a previous study (2) suggested that amphotericin B-cilofungin synergy in vitro is uncommon, but the method used to assay synergy was markedly different from that used by us. Perhaps the largest of the several differences is that the earlier study did not use a full checkerboard-type comparison but, rather, what would be analogous to two rows of such a comparison (at one-fourth the MIC of each drug). The applicability of our in vivo findings to other isolates of *Candida* or to other cell wall inhibitors requires further study.

REFERENCES

1. **Benson, J. M., and M. C. Nahata.** 1988. Clinical use of systemic antifungal agents. Clin. Pharm. **7**:424–438.
2. **Bulo, A. N., S. F. Bradley, and C. A. Kauffman.** 1988. The effect of cilofungin (LY 121019) in combination with amphotericin B or flucytosine against *Candida* species. Mycoses **32**:151–157.
3. **Eli Lilly & Co.** Cilofungin investigator's brochure. Eli Lilly & Co., Indianapolis, Ind.
4. **Galgiani, J. N., S. H. Sun, K. V. Clemons, and D. A. Stevens.** 1990. Activity of cilofungin against *Coccidioides immitis*: differential *in vitro* effects on mycelia and spherules correlated with *in vivo* studies. J. Infect. Dis. **162**:944–948.
5. **Glantz, S. A., and B. K. Slinker.** 1990. Primer of applied regression and analysis of variance, p. 300–302. McGraw-Hill Book Co., New York.
6. **Gordee, R. S., D. Zeckner, L. F. Ellis, A. L. Thakkar, and L. C. Howard.** 1984. *In vitro* and *in vivo* anti-*Candida* activity and toxicology of LY 121019. J. Antibiot. **37**:1054–1065.
7. **Hanson, L. H., A. M. Perlman, K. V. Clemons, and D. A. Stevens.** 1989. Program Abst. 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 818.
8. **Hanson, L. H., and D. A. Stevens.** 1989. Evaluation of cilofungin, a lipopeptide antifungal agent, in vitro against fungi isolated from clinical specimens. Antimicrob. Agents Chemother. **33**: 1391–1392.
9. **Hobbs, M., J. Perfect, and D. Durack.** 1988. Evaluation of in vitro antifungal activity of LY 121019. Eur. J. Clin. Microbiol. **7**:77–80.
10. **Lefler, E., and D. A. Stevens.** 1984. Inhibition and killing of *Candida albicans* in vitro by five imidazoles in clinical use. Antimicrob. Agents Chemother. **25**:450–454.
11. **Lefler, E., and D. A. Stevens.** 1985. New azole compounds: vibunazole (Bay n7133) and Bay l9139, compared with ketoconazole in the therapy of systemic candidosis and in pharmacokinetic studies, in mice. J. Antimicrob. Chemother. **15**:69–75.
12. **Morrison, C. J., and D. A. Stevens.** 1990. Comparative effects of cilofungin and amphotericin B on experimental murine candidiasis. Antimicrob. Agents Chemother. **34**:746–750.
13. **Sokal, R. R., and F. J. Rohlf.** 1981. Biometry, 2nd ed., p. 432–435. W. H. Freeman & Co., San Francisco.
14. **Spitzer, E. D., S. J. Travis, and G. S. Kobayashi.** 1988. Comparative in vitro activity of LY 121019 and amphotericin B against clinical isolates of *Candida* species. Eur. J. Clin. Microbiol. **7**:80–81.
15. **Stevens, D. A., and P. T. Vo.** 1982. Synergistic interaction of trimethoprim and sulfamethoxazole on *Paracoccidioides brasiliensis*. Antimicrob. Agents Chemother. **21**:852–854.
16. **Stiller, R. L., J. E. Bennett, H. J. Scholer, M. Wall, A. Polak, and D. A. Stevens.** 1983. Correlation of *in vitro* susceptibility test results with *in vivo* response: flucytosine therapy in a systemic candidiasis model. J. Infect. Dis. **147**:1070–1077.
17. **Tucker, R. M., P. L. Williams, E. A. Arathoon, and D. A. Stevens.** 1988. Treatment of mycoses with itraconazole. Ann. N. Y. Acad. Sci. **544**:451–470.