ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1997, p. 1120–1123 Vol. 41, No. 5
0066-4804/97/$04.00+0


# Combination Therapy with Fluconazole and Flucytosine in the Murine Model of Cryptococcal Meningitis

M. HONG NGUYEN,[1,2]* LAURA K. NAJVAR,[3] CHRISTINE Y. YU,[1] AND JOHN R. GRAYBILL[3]

*University of Florida College of Medicine[1] and the Gainesville VA Medical Center,[2] Gainesville, Florida, and University of Texas Health Sciences Center and Audie Murphy Veterans Hospital, San Antonio, Texas[3]*

Received 4 November 1996/Returned for modification 21 January 1997/Accepted 20 February 1997

**This study elucidates the role of combined fluconazole and flucytosine as therapy for cryptococcosis in the murine model of meningitis. Three strains of *Cryptococcus neoformans* for which the range of fluconazole MICs was wide—2 μg/ml (susceptible strain), 8 μg/ml (moderately susceptible strain), and 32 μg/ml (resistant strain)—were used for infection. One day postinfection, the mice were randomized into eight treatment groups: placebo; flucytosine (40 mg/kg of body weight/day); fluconazole at 3 mg/kg/day (low dosage), 10 mg/kg/day (moderate dosage), or 20 mg/kg/day (high dosage); and combined flucytosine and fluconazole at low, moderate, or high doses of fluconazole. Three major findings were demonstrated: (i) correlation between the MICs for the isolates and the in vivo effectiveness of fluconazole as assessed by the reduction in cryptococcal brain burden, (ii) a dose-response curve (a higher dose of fluconazole was significantly more efficacious than a lower dose [$P < 0.001$]), and (iii) synergism between fluconazole and flucytosine (therapy with a combination of fluconazole and flucytosine was superior to therapy with either agent alone [$P < 0.01$]).**

*Cryptococcus neoformans* is an important pathogen in immunocompromised hosts, especially in patients with AIDS, a transplant, or a malignancy. Morbidity and mortality remain high, despite therapy with amphotericin B or fluconazole (9). The combination of amphotericin B and flucytosine provided the highest rate of clinical success in one study (6). This combination, however, is frequently associated with toxic side effects, and the use of amphotericin B requires close laboratory monitoring, with long-term catheters for vascular access. Evaluation of new approaches to treating cryptococcal meningitis is therefore needed to ameliorate the outcome, minimize the side effects of the drugs, and shorten the hospital stay.

The combination of fluconazole and flucytosine appeared to be very promising in vitro, with synergism documented for the majority of the combinations tested (8). Previous studies assessing the efficacy of the combination of fluconazole and flucytosine were limited in that only one isolate was tested; thus, the information could not be extrapolated to isolates for which the MICs were different from the MIC for that organism (1). Furthermore, the correlation between fluconazole MIC and the in vivo response was not documented. The latter issue is becoming very important given recent antifungal susceptibility testing standardization and the emergence of fluconazole resistance among the *Candida* species. To date, the prevalence of cryptococcal resistance has not been determined, though the emergence of fluconazole-resistant *Cryptococcus* during prolonged usage of fluconazole has been reported (4, 11). In a survey of susceptibility patterns of 50 cryptococcal isolates from human immunodeficiency virus (HIV)-infected patients submitted to the Fungus Testing Laboratory, the fluconazole MIC for 30% was found to be ≥8 μg/ml (8). This finding is disturbing, since it might limit the use of fluconazole in the future.

Given these concerns, we studied the effect of combination therapy of fluconazole and flucytosine on *C. neoformans*, using the murine model of cryptococcal meningitis; the range of fluconazole MICs for all the cryptococcal isolates studied was wide.

## MATERIALS AND METHODS

**Organism.** Three strains of *C. neoformans* recovered from HIV-infected patients were studied.

**In vitro susceptibility testing.** Testing of susceptibility to fluconazole and flucytosine was performed by a broth macrodilution method by adhering to the National Committee for Clinical Laboratory Standards protocol (7). Combination testing was performed by a macrobroth checkerboard method. Details of this procedure have been described elsewhere (8).

**Mice.** Outbred ICR mice (Harlan Sprague-Dawley, Indianapolis, Ind.), 6 weeks old and weighing 20 g, were used in this experiment.

**Induction of meningitis.** After anesthetization of mice with methoxyflurane by inhalation, the mouse cranium was swabbed with 75% ethanol. The inoculum, approximately 400 CFU/mouse, was delivered with a 27.5-gauge needle in a volume of 60 μl by direct puncture into the cranium at midline.

**Therapy.** Immediately postinfection, the mice were randomized into groups of 10 as follows: no therapy (control); fluconazole at 3 mg/kg of body weight/day (Flu 3), 10 mg/kg/day (Flu 10), and 20 mg/kg/day (Flu 20); flucytosine at 40 mg/kg/day; fluconazole at 3 mg/kg/day and flucytosine at 40 mg/kg/day (Combo 3); fluconazole at 10 mg/kg/day and flucytosine at 40 mg/kg/day (Combo 10); and fluconazole at 20 mg/kg/day and flucytosine at 40 mg/kg/day (Combo 20). Fluconazole was administered by gavage in two divided doses. Flucytosine was given in drinking water, with the volume of water consumption estimated to be 4.5 ml/mouse per day.

Therapy began 24 h postinfection and lasted for 10 consecutive days. The mice were sacrificed by cervical dislocation at day 11 postinfection. Their brains were removed, weighed, and homogenized in 0.9% NaCl supplemented with amikacin and piperacillin. The homogenates were serially diluted 10-fold. Portions (0.1 ml each) of the undiluted homogenate and dilutions were plated onto Sabouraud dextrose agar. Culture plates were incubated at 37°C for 72 h. CFU per milliliter were counted, and the number of CFU per gram of brain tissue was then calculated.

**Statistical analysis.** For comparison of drug efficacy between various therapeutic groups, the data were logarithmically transformed to natural log to approximate a normal distribution prior to statistical analysis. The therapeutic groups were then compared with the Newman-Keuls test corrected for multiple comparisons.

## RESULTS

**In vitro susceptibility testing.** The range of fluconazole MICs for the three *C. neoformans* isolates selected was wide: 2 μg/ml (susceptible), 8 μg/ml (moderately susceptible), and 32

* Corresponding author. Mailing address: University of Florida College of Medicine, Department of Medicine, Division of Infectious Disease, P.O. Box 100277, JHMHC, 1600 S.W. Archer Rd., Gainesville, FL 32610. Phone: (352) 379-4027. Fax: (904) 379-4015. E-mail: nguyen.med@shands.ufl.edu.

TABLE 1. In vitro susceptibility testing of three *C. neoformans* isolates

| Isolate no. | MIC (μg/ml) of[a]: | | | | Mode of interaction between Flu and 5-FC[b] |
|---|---|---|---|---|---|
| | Flu tested alone | 5-FC tested alone | Flu tested in combination | 5-FC tested in combination | |
| 94-1190 | 2 | 4 | 2 | ≤0.125 | Indifferent |
| 92-2049 | 8 | 2 | 2 | ≤0.125 | Synergistic |
| 94-2413 | 32 | 2 | 4 | 0.25 | Synergistic |

[a] Flu, fluconazole; 5-FC, flucytosine.
[b] Defined according to fractional inhibitory index (3): <0.5, synergistic; 1 to 2, indifferent.

μg/ml (resistant) (Table 1). Flucytosine MICs for all three isolates were between 2 and 4 μg/ml. After flucytosine was combined with fluconazole, flucytosine MICs for all three isolates were reduced by at least fourfold (Table 1). After fluconazole was combined with flucytosine, fluconazole MICs for isolates 92-2049 and 94-2413 were reduced by four- and eightfold, respectively; the mode of interaction between the two agents was therefore defined as synergistic. The fluconazole MIC for isolate 94-1190 did not change with the addition of flucytosine; the mode of interaction was therefore defined as indifferent.

**Dose-response curve. (i) Single-drug therapy.** Flu 3, Flu 10, and Flu 20 reduced the *C. neoformans* brain burdens of mice infected with isolate 94-1190 by 3.6, 4.9, and 12.6 $\log_e$ units, respectively, compared with controls. The brain burden of Flu 20-treated mice was significantly lower than those of Flu 3-treated ($P = 0.0003$) and Flu 10-treated ($P = 0.0003$) mice (Tables 2 and 3).

Similarly, a stepwise reduction in brain burden of isolate 92-2049 was seen with increased doses of fluconazole (Tables 2 and 3). Although the degree of reduction was not as remarkable as for the mice infected with isolate 94-1190, the reduction in brain burden for Flu 20-treated mice compared with those for Flu 3-treated ($P = 0.0003$) and Flu 10-treated ($P = 0.03$) mice was still significant (Table 3).

In contrast to results for isolates 94-1190 and 92-2049, there was only a 1-$\log_e$ unit reduction of brain burden of isolate 94-2413 in fluconazole-treated mice compared with controls. There was no significant difference in the reduction of brain burden with increased doses of fluconazole ($P = 0.9$).

**(ii) Single-drug versus combination therapy.** For all three isolates, the addition of flucytosine to Flu 3 did not significantly reduce cryptococcal brain burden compared with that in mice treated with Flu 3 alone (Table 4).

For isolate 94-1190, Flu 20 was very effective; a reduction of 12.6 $\log_e$ units was achieved compared with controls. We were not able to show an additional effect when flucytosine was added to fluconazole (Table 2).

For isolate 92-2049, the addition of flucytosine to Flu 10 or Flu 20 significantly reduced brain burdens by 2.8 and 3.6 $\log_e$ units, respectively, in comparison with those for the Flu 10-treated ($P = 0.001$) and Flu 20-treated ($P = 0.001$) groups.

For isolate 94-2413, although there was no significant difference in brain burden between the control group and the groups treated with either flucytosine or fluconazole alone, the addition of flucytosine to fluconazole reduced brain burden by 3.4 $\log_e$ units in comparison with that for the Flu 10-treated group ($P = 0.002$) and by 3.5 $\log_e$ units in comparison with that for the Flu 20-treated group ($P = 0.001$) (Table 4).

## DISCUSSION

Amphotericin B and fluconazole are current acceptable therapies for cryptococcal meningitis, but the morbidity rate remains high. Flucytosine has activity against *C. neoformans* in vitro, but its use as a sole agent has been limited by the development of resistance to this drug. The combination of amphotericin B and flucytosine yields a better outcome (2, 6), but this approach is often limited by toxicity; amphotericin B induces renal insufficiency leading to the accumulation of flucytosine, which in turn is toxic for bone marrow.

There are several reasons why combined fluconazole and flucytosine might provide a superior approach to the therapy of cryptococcal meningitis. Fluconazole might inhibit the in vivo development of flucytosine resistance, thereby conserving the potent activity of flucytosine against *C. neoformans*. Furthermore, the sequential mode of action between these two agents makes synergistic interaction theoretically possible: fluconazole acts by damaging the fungal cell membrane, which facilitates the in vitro uptake of flucytosine. Indeed, the synergistic interaction between these two agents has been demonstrated in vitro (8).

The in vitro finding that combined fluconazole and flucytosine are significantly more active than either drug alone was confirmed in this murine model (Table 4). More importantly, the combination therapy was efficacious even in cases where monotherapy with either fluconazole or flucytosine was ineffective. For example, neither fluconazole nor flucytosine was active against an isolate for which the fluconazole MIC was 32 μg/ml (isolate 94-2413). When flucytosine was added to fluconazole, however, a 3.5-$\log_e$ unit reduction in cryptococcal brain burden was noted. To our knowledge, this is the first study to demonstrate that combined fluconazole and flucytosine are effective against a fluconazole-resistant cryptococcal isolate.

The above-described finding illustrates one appealing aspect of combination therapy: its ability to potentiate the efficacy of individual agents. This potentiation is particularly valuable in the management of relatively resistant organisms. Our finding sharply contrasts with that of Kartalija et al., who did not find any beneficial effect when flucytosine was added to fluconazole (5). There are potential reasons for the differences in these findings. In the study of Kartalija et al., only a low dosage of fluconazole (5 mg/kg/day) was tested in combination with flucytosine; higher dosages of fluconazole were not tested. Similar

TABLE 2. Mean logarithmic presentation of *C. neoformans* brain burden in infected mice according to specific treatment regimens

| Isolate no. | $\log_e$ CFU/g (mean ± SD) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | 5-FC[a] | Flu 3 | Combo 3 | Flu 10 | Combo 10 | Flu 20 | Combo 20 |
| 94-1190 | 18.5 ± 1.6 | 17.2 ± 2.8 | 14.9 ± 2.6 | 14.6 ± 2.6 | 13.6 ± 1.1 | 11.9 ± 1.8 | 5.8 ± 0.6 | 5.9 ± 0.9 |
| 92-2049 | 16.1 ± 0.5 | 15.2 ± 1.1 | 15.5 ± 0.8 | 13.6 ± 1.4 | 15.0 ± 0.8 | 12.1 ± 1.0 | 13.7 ± 1.2 | 10.1 ± 1.3 |
| 94-2413 | 13.1 ± 0.9 | 13.2 ± 1.5 | 11.8 ± 1.4 | 13.3 ± 1.0 | 11.9 ± 1.3 | 8.5 ± 1.9 | 12.1 ± 1.9 | 8.6 ± 1.9 |

[a] 5-FC, flucytosine.

TABLE 3. Logarithmic reduction of *C. neoformans* brain burden in fluconazole-treated mice compared with controls[a]

| Treatment group | Reduction in brain burden of isolate no.: | | |
|---|---|---|---|
| | 94-1190 | 92-2049 | 94-2413 |
| Flu 3 | 3.6[b] | 0.6[b] | 1.3 |
| Flu 10 | 4.9[c] | 1.1[d] | 1.1 |
| Flu 20 | 12.6 | 5.4 | 1 |

[a] As described in Materials and Methods, cryptococcal brain burden data were logarithmically transformed to natural log to approximate a normal distribution prior to statistical analysis.
[b] $P = 0.0003$ for Flu 20 versus Flu 3 values.
[c] $P = 0.0003$ for Flu 20 versus Flu 10 values.
[d] $P = 0.03$ for Flu 20 versus Flu 10 values.

to Kartalija et al., we also did not find any beneficial effect of flucytosine when this drug was added to a low dosage of fluconazole (3 mg/kg/day). When flucytosine was added to higher dosages of fluconazole (10 and 20 mg/kg/day), however, we were able to show a significant reduction of brain burden in comparison with those for groups treated with fluconazole alone; this effect was seen for all three isolates tested. Another potential reason for the differences between the findings of the two studies is that flucytosine was given twice daily in the study of Kartalija et al. Flucytosine's half-life is relatively short; therefore, administration twice daily might simply not be frequent enough to show a sustained flucytosine effect. To overcome this limitation in our study, flucytosine was given in drinking water.

In vitro study has demonstrated that fluconazole enhances the antifungal activity of flucytosine by reducing its MIC to a level significantly below the one in cerebrospinal fluid (8). Our present study corroborates this in vitro finding. Flucytosine at a low dosage (40 mg/kg/day) was ineffective when given alone. Its effect, however, was potentiated when flucytosine was combined with fluconazole; the result was a significant reduction in the cryptococcal brain burden of mice treated with a combination of the two agents compared with those of mice treated with either agent alone over a range of triazole doses. This finding illustrates the other advantage of combination therapy: the ability to reduce the amount of drug required for therapy and therefore to reduce dose-related toxicity. Reduction of the flucytosine dose might alleviate the side effects associated with flucytosine, since these are often dose related.

Another notable finding from our study is that the MICs of fluconazole correlated with in vivo outcome: the isolate for which the MIC was 2 μg/ml responded steadily to fluconazole, the isolate for which the MIC was 16 μg/ml responded moderately, and the isolate for which the MIC was 32 μg/ml did not respond to fluconazole. To our knowledge, this report is the first to address the correlation between the outcome for cryptococcal infection and in vitro MIC determined by the proposed standard for susceptibility testing (7). Our result implies that in vitro susceptibility testing may be useful in the evaluation of fluconazole therapy for cryptococcal meningitis. For isolates for which MICs were low (≤2 μg/ml), fluconazole was very effective in eradicating *Cryptococcus* from the brain.

It is interesting that the interaction between fluconazole and flucytosine demonstrated in vitro predicted the in vivo outcome. The in vitro synergistic interaction for isolates 92-2049 and 94-2413 translated into a significant reduction in brain burden when flucytosine was added to fluconazole therapy. On the other hand, the in vitro indifferent interaction for isolate 94-1190 translated into only a marginal and insignificant change in brain burden.

Our study also showed that higher dosages of fluconazole (20 mg/kg/day) were more effective than lower dosages for both highly susceptible (isolate 94-1190 [fluconazole MIC = 2 μg/ml]) and moderately susceptible (isolate 92-2049 [fluconazole MIC = 8 μg/ml]) isolates. Kartalija et al., using a rabbit model of cryptococcal meningitis, also found that high dosages (20 or 40 mg/kg/day) were superior to low dosages of fluconazole (5). Our finding and that of Kartalija et al. might explain the suboptimal result obtained with fluconazole in the treatment of cryptococcal meningitis in HIV-infected patients in a previous study (9); only 200 mg of fluconazole per day was used in that study. It is theoretically possible that since only 70 to 80% of serum fluconazole penetrates the cerebrospinal fluid, a higher dose should be used to achieve an adequate therapeutic level in the central nervous system. The relatively benign side effects of fluconazole make the use of a higher dosage possible.

Also important in this study was the difference in cryptococcal brain burden among the control mice infected with the susceptible, moderately susceptible, and resistant isolates (Table 2). The highest and lowest brain burdens were seen in the mice infected with susceptible and resistant isolates, respectively. This finding implies that the in vivo growth rate was higher for the susceptible than for the resistant isolates. Growth rate at 37°C has been linked with virulence. Our observation confirms that of Velez et al., who have shown that fluconazole-susceptible isolates are more virulent in mice than fluconazole-resistant isolates (10).

In conclusion, our study suggests that fluconazole susceptibility testing may be indicated if this drug is to be used for the therapy of cryptococcal meningitis. Moreover, the fluconazole dose-response relationship demonstrated in this study suggests that a dosage higher than is currently recommended (400 mg/kg/day) should be contemplated, especially early in therapy. Finally, the combination of fluconazole and flucytosine was superior to either drug alone in this murine model. Of note was that even a fluconazole-resistant cryptococcal isolate may respond to combined therapy with fluconazole and flucytosine. Clinical trials are being performed to elucidate the value of this combination in comparison with that of combined amphotericin B and flucytosine in the therapy of cryptococcal meningitis.

TABLE 4. Logarithmic reduction of *C. neoformans* brain burden in combination-treated mice compared with mice treated with fluconazole alone[a]

| Treatment group | Reduction in brain burden of isolate no.: | | |
|---|---|---|---|
| | 94-1190 | 92-2049 | 94-2413 |
| Combo 3 | 0.3 | 1.8 | −1.5 |
| Combo 10 | 1.7 | 2.8[b] | 3.4[c] |
| Combo 20 | 0.1 | 3.6[b] | 3.5[b] |

[a] As described in Materials and Methods, cryptococcal brain burden data were logarithmically transformed to natural log to approximate a normal distribution prior to statistical analysis.
[b] $P = 0.001$ (statistical difference in brain burden between combination-treated mice and fluconazole-treated mice).
[c] $P = 0.002$ (statistical difference in brain burden between combination-treated mice and fluconazole-treated mice).

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