Combined Therapy with Fluconazole and Flucytosine in Murine Cryptococcal Meningitis

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To assess the possible beneficial effects of combined therapy (fluconazole and flucytosine) in the treatment of cryptococcal meningitis in the immunocompromised host, we compared therapy with fluconazole and flucytosine, individually and combined, in the experimental murine model. BALB/c athymic (nu/nu) mice were infected intracerebrally with 150 to 300 CFU of Cryptococcus neoformans. In mortality studies, treatment was initiated 24 h postinfection and continued for 10 to 14 days with either fluconazole (1 to 15 mg/kg of body weight per day), flucytosine (60 to 120 mg/kg/8 h), both drugs, or 0.3% Noble agar (control). Combined therapy delayed mortality significantly when compared with controls and single-drug regimens. This was observed over a broad range of doses. Quantitative determinations of CFU in brain tissue demonstrated a significantly lower burden of C. neoformans in mice receiving combined therapy. The results indicate that combined therapy with fluconazole and flucytosine is superior to single-drug therapy.

Cryptococcal meningitis is one of the most common fungal infections in immunocompromised hosts, especially patients with AIDS (8, 10, 13). Depending on the definition used, the therapeutic failure rate in these patients is approximately 30 to 50% and the patients frequently have relapses posttreatment (9, 13, 22). Various treatment regimens for cryptococcal meningitis in non-AIDS patients have been evaluated, and to date, the most established scheme is combination chemotherapy with amphoterin B (AmB) and flucytosine (2, 9). However, nephrotoxicity and myelotoxicity are frequent complications (5, 20). The nephrotoxicity induced by AmB leads to a rise in serum flucytosine concentrations, resulting in myelosuppression. This is particularly a problem for AIDS patients, who are frequently already granulocytopenic when antifungal therapy is commenced.

New azole antymycotic agents have recently been introduced for treatment of cryptoccocal meningitis. The toxicities of these azole antymycotic agents occur less frequently and are less severe than those associated with AmB (12). If such agents are as effective in treatment as AmB plus flucytosine, they would be the preferred therapy. Fluconazole, a bis-triazole, is one of these new azole antymycotic agents with broad-spectrum activity and is now in clinical use. The compound penetrates well into cerebrospinal fluid and the central nervous system and appears to be especially promising against Cryptococcus neoformans (1, 15, 21). In a recent comparative therapy study between fluconazole and AmB in AIDS patients with cryptococcal meningitis, the success rate was approximately 50% for each regimen (7).

An additional recent study calls attention to the superiority of AmB plus flucytosine contrasted to fluconazole alone in the therapy of acute cryptococcal meningitis in AIDS patients (14). However, it is important to note that the number of patients in this study was small in experience because of complicating factors of their underlying disease and cannot be considered conclusive.

The frequent therapeutic failures plus the toxicities of current treatment regimens underline the need for new therapeutic approaches in cryptococcal meningitis. One such approach might be combination therapy with fluconazole and another antifungal agent. For the present model, we chose flucytosine as the second antifungal agent, because it is commonly used with AmB in treatment of cryptococcal meningitis and can be given orally. In addition, a regimen of fluconazole and flucytosine might be superior to either drug given alone and yet be less toxic than AmB and flucytosine.

MATERIALS AND METHODS

Animals. Specific pathogen-free BALB/c athymic (nu/nu) mice were raised under barrier conditions at Audie L. Murphy Hospital. Male and female mice at approximately 6 weeks of age and weighing 20 g were used for all experiments. They were housed in cages of five per group and had access to food and water ad libitum.

Organism. C. neoformans R 89-98, a clinical isolate, was maintained at 4°C on Sabouraud dextrose agar (SDA) slants until used. For each study, C. neoformans was cultured at 37°C for 72 h on SDA. Yeast cells were collected, washed twice with 0.9% NaCl, quantitated by using hemacytometer counts, and adjusted to the desired concentration in saline. The concentration of C. neoformans was confirmed by quantitative culture of 10-fold serial dilutions.

Induction of meningitis. For intracerebral challenge, mice were lightly anesthetized with methoxyflurane via inhalation. The cranium was swabbed with 70% ethanol. The inoculum, a challenge dose of between 150 to 300 CFU per mouse, was suspended in a tuberculin syringe. It was delivered through a 26.5-gauge needle in a volume of 60 μl per dose by a direct puncture at the cranium midline, approximately 6 mm posterior to the orbit. The injection caused less than 2% mortality at the time of challenge, and the few deaths occurring within 48 h of challenge were considered traumatic and were not included in the results.

Chemotherapy. Fluconazole (UK-49,858) lot R-9 was provided by Pfizer Central Research (Groton, Conn.). Flucytosine was obtained in the commercial form as Ancobon (250 mg; Hoffmann-La Roche) from the local pharmacy. The

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doses for both compounds were determined in milligrams per kilogram of body weight. The average weight of a mouse was 20 g. Both antifungal agents were suspended in 0.3% Noble agar and administered per os by gavage in a volume of 0.2 ml per dose. The following dosages for fluconazole used in experiments were given once daily: 1, 2.5, 5, 15, 20, and 80 mg/kg. Flucytosine was administered orally by gavage every 8 h in doses of 60, 80, 100, and 120 mg/kg. Control groups received 0.3% Noble agar, the drug vehicle, in a volume of 0.4 ml per dose once daily.

**Determination of antifungal efficacy. (i) Mortality studies.** At 24 h postinfection, mice were randomly assigned to treatment groups of 9 to 10 animals. Therapy began 1 day after infection and lasted for 10 to 14 consecutive days, with daily observation continuing posttreatment until day 30. The clinical response to antifungal therapy was measured by delay of mortality.

(ii) **Quantitative culture studies.** Mice were randomly assigned to groups of 5 to 9 animals 24 h postchallenge prior to initiation of antifungal therapy. Treatment was given for seven consecutive days. Animals were sacrificed by cervical dislocation at 48 h after the last treatment. The brains were removed and homogenized in 0.9% NaCl supplemented with 60 μg of amikacin per ml and 60 μg of piperacillin per ml. The homogenates were diluted (by serial 10-fold dilutions) to 10^{-8} in saline containing amikacin and piperacillin (each 60 μg/ml). A 0.1-ml quantity of the undiluted homogenate and dilutions was plated on SDA. Culture plates were incubated for 72 h at 37°C. CFU were then counted, and the number of CFU per g of tissue was calculated.

(iii) **In vitro studies.** MICs and minimal lethal concentrations (MLC) using experimental strain R 89-98t were determined for flucytosine and fluconazole by following procedures previously described (17).

**Drug levels in serum.** Serum fluconazole concentrations were determined by a gas-liquid chromatography assay (11) after single oral doses of 15, 5, 2.5, and 1 mg/kg to mice. Groups of three mice were anesthetized and sacrificed by exsanguination at 1, 2, 4, and 8 h postadministration. The sera from each group were pooled, and drug concentrations were measured. The bioassay procedure modified by Bodet et al. (3) was used to measure serum flucytosine concentrations after single oral doses of 60, 80, and 120 mg/kg. Groups of three to four mice were anesthetized and sacrificed by exsanguination at 1 and 3 h postadministration. The bioassay employed cannot reliably measure flucytosine levels of less than 20 μg/ml.

**Statistical analysis.** Mortality and quantitative culture studies were analyzed by Bonferroni t test and Tukey's studentized range test (18). A P value of less than 0.05 was considered significant.

### RESULTS

**In vitro studies.** MICs and MLCs of the fluconazole and flucytosine for the experimental strain of C. neoformans are presented in Table 1. The MIC and MLC of fluconazole given alone were at least 20-fold higher than the corresponding fluconazole values when combined with flucytosine, except for the 48-h MLCs (80 μg/ml), which were identical. By using a checkerboard titration method (17), the MIC of flucytosine decreased from 20 μg/ml when given alone to ≤1.25 μg/ml when given with fluconazole. Because flucytosine values were off the scale, it is difficult to precisely define synergism, e.g., a reduction in the MIC or MLC of each drug by 16-fold when used in combination contrasted to when each agent was used alone. However, flucytosine reduced the fluconazole concentration needed to inhibit the organism, as judged by MICs. For MLC at 24 h, the flucytosine concentration needed decreased by a factor of 2 when both drugs were given and that of fluconazole decreased by a factor of ≥64 when both drugs were given. The MLCs at 48 h were unevolvable. We observed maximum serum fluconazole concentrations after single oral doses of 1, 2.5, 5, and 15 mg/kg between the first and second hour, with the concentrations ranging from 0.82 (1 mg/kg) to 10.1 μg/ml (15 mg/kg). After single oral flucytosine doses of 60 and 80 mg/kg, flucytosine concentrations in serum were detected inconsistently after 1 h postadministration. The average level in serum of mice given 120 mg/kg was 29.78 μg/ml at 1 h postadministration. At 3 h, concentrations of all dosages of the drug in serum fell below the minimal detectable limit (20 μg/ml) of the bioassay procedure employed.

**In vivo mortality studies.** Intracerebral infection of athymic mice with 150 to 300 CFU of C. neoformans caused death of controls mice from days 7 to 15 postinfection. Groups of 9 to 10 animals were treated with each regimen in mortality studies. In Fig. 1, percent mortality is shown after oral treatment with fluconazole at 5 mg/kg/day, flucytosine at 80 mg/kg/8 h, or combined therapy for 14 days. All drug regimens delayed the time of death significantly compared with the controls although no compound provided total protection. There was no difference in mortality between fluconazole- and flucytosine-treated animals. Combined therapy with both compounds prolonged survival significantly compared with survival of the controls and groups receiving single-drug treatment (P < 0.05 by Tukey's studentized range test). In the next representative experiment (Fig. 2), when drug dosages were reduced (60 mg/kg/8 h for flucytosine and 2.5 mg/kg/day for fluconazole) and treatment was shortened to 10 days, similar results were obtained. Compared with control mice, all mice treated showed significantly delayed mortality. Flucytosine- and flucytosine-treated groups did not differ in survival time. Compared with mice in single-drug regimen groups, mice which had received both compounds demonstrated delayed mortality (P < 0.05). In the experiment shown in Fig. 3, mice were infected with 150 to 300 CFU per mouse and treatment was initiated 24 h postchallenge. Flucytosine was further reduced to 1 mg/kg/day, while flucytosine was maintained at 60 mg/kg/8 h. Therapy was again administered for 10 days. As in previous experiments, there was significantly delayed mortality of drug-fed groups compared with controls. At the lower dose, flucytosine recipients showed no difference in time of death compared with that of flucytosine or combined-treatment groups.

**Quantitative culture studies.** Tissue count data demon-

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**TABLE 1. In vitro susceptibility of C. neoformans isolate R 98-89 to fluconazole and flucytosine**

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<tr>
<th>Conc and sampling time</th>
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tissue when compared with the azole and fluconazole (Flu) group received 3 mg/kg/day of fluconazole (Flu). Flucytosine (5FC) group was given 80 mg/kg/8 h, the control group received 0.3% Noble agar, and the group given combination therapy received the concentrations just given. There were nine or ten mice per group. \( P < 0.05 \) (using Tukey’s studentized range test) when combined-treatment group compared with single-drug groups (fluconazole- and flucytosine-treated groups) and control group. \( P < 0.05 \) when single-drug regimen groups compared with control group.

The results of this study evaluating the combined effects of fluconazole and flucytosine in cryptococcal meningitis demonstrated that flucytosine at 60 to 120 mg/kg/8 h and fluconazole at 5 mg/kg/day, when administered in combination, significantly reduced the burden of \( C. \) neoformans in brain tissue when compared with single-compound-treated groups and controls. This outcome was confirmed with dosage of flucytosine at 60 mg/kg/8 h and fluconazole at 2.5 mg/kg/day. Fluconazole-treated mice showed significant median reduction of yeast counts \( (0.13 \times 10^9 \pm 0.08 \times 10^9) \) compared with those of flucytosine-treated mice \( (2.09 \times 10^9 \pm 1.96 \times 10^9) \) \( (P < 0.05 \) by Tukey’s studentized range test) but not with those of control mice \( (0.69 \times 10^9 \pm 0.51 \times 10^9) \). Flucytosine recipients did not differ from controls. Animals which had received combined therapy with both agents exhibited significant \( (0.007 \times 10^9 \pm 0.01 \times 10^9) \) reduction in the burden in brain tissue compared with those of single-drug treatment groups and controls \( (P < 0.05) \). In another study, the concentration of fluconazole was reduced to 1 mg/kg/day, while flucytosine was maintained at 60 mg/kg. No difference in tissue counts between controls \( (4.8 \times 10^7 \pm 2.58 \times 10^7) \) and single-therapy regimen groups \( (2.41 \times 10^7 \pm 14.6 \times 10^6) \). Fluconazole, \( 2.01 \times 10^7 \pm 1.54 \times 10^7 \) was achieved. Combination therapy, nevertheless, lowered the burden in brain tissue significantly \( (0.21 \times 10^7 \pm 0.19 \times 10^7) \) compared with those of control mice and flucytosine-treated mice \( (P < 0.05) \). Although no statistically significant difference was found between combination therapy and fluconazole, the median burden of \( C. \) neoformans in brain tissue was reduced at least by a factor of ten when animals were treated with combined therapy. In all studies, there was no indication of antagonism between the drugs.

**DISCUSSION**

The results of this study evaluating the combined effects of fluconazole and flucytosine in cryptococcal meningitis demonstrate that combined therapy of murine cryptococcal meningitis with fluconazole and flucytosine was superior to single treatment with either antifungal agent. The increased therapeutic success was confirmed by mortality and quantitative tissue culture studies. These differences were observed over a broad range of doses and are not an artifact of experimental design. Prior in vivo and in vitro studies have been conducted to investigate the use of combination therapy in systemic fungal infections. However, the results have been controversial. Some studies

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**FIG. 1.** Effect of single-drug and combined therapy with fluconazole and flucytosine after 14 days of treatment on percent mortality in athymic mice infected with 150 to 300 CFU of \( C. \) neoformans. The fluconazole (Flu) group received 3 mg/kg/day, the flucytosine (5FC) group was given 80 mg/kg/8 h, the control group received 0.3% Noble agar, and the group given combination therapy received the concentrations just given. There were nine or ten mice per group. \( P < 0.05 \) (using Tukey’s studentized range test) when combined-treatment group compared with single-drug groups (fluconazole- and flucytosine-treated groups) and control group. \( P < 0.05 \) when single-drug regimen groups compared with control group.

**FIG. 2.** Effect of single-drug and combined therapy with fluconazole (Flu) (2.5 mg/kg/day) and flucytosine (5FC) (60 mg/kg/8 h) after 10 days of treatment on percent mortality in murine cryptococcal meningitis. The control group received 0.3% Noble agar. \( P < 0.05 \) when combined-treatment group compared with groups given fluconazole and flucytosine individually and control group. \( P < 0.05 \) comparing single-drug regimen groups with control group. There were 10 animals per group.

**FIG. 3.** Efficacy of single-drug and combined therapy on percent mortality in murine cryptococcal meningitis after 10 days of treatment. Fluconazole (Flu) was given at 1 mg/kg/day; flucytosine (5FC) was administered at 60 mg/kg/8 h. \( P < 0.05 \) comparing combined-therapy group with control and flucytosine-treated group. \( P < 0.05 \) comparing fluconazole- and flucytosine-treated groups with control group. There was one survivor in combined-therapy group on day 30 postinfection. There were 10 animals per group.
showed no interaction, while others demonstrated antagonistic or beneficial interaction. In vitro studies by Brajtburg et al. (4) found neutral or antagonistic effects between AmB and ketoconazole in short-term experiments. However, potentiation of antifungal effects were observed in long-term experiments (48 h of exposure of C. albicans to antifungal agents). The study by Polak (16) evaluated combined therapy of fluconosine with intraconazole or fluconazole in a murine model of cryptococcosis than we employed. By using high doses of itraconazole, a synergistic effect was achieved with combined therapy. In contrast, the combination with fluconazole did not alter the results when treatment was given for 5 days and seemed to have antagonistic effects when treatment was given over a 10-day period. If azole antifungal agents are similar in their mode of action, then the results of Polak are internally contradictory and not readily explicable. Also, a study conducted by Craven and Graybill (6) suggested a positive (but not significant) trend of combined therapy with fluconosine and ketoconazole in experimental cryptococcal meningitis. Other investigators demonstrated antagonism of AmB and ketoconazole in a murine model of disseminated aspergillosis after pretreatment with the imidazole (19). Comparisons among each of those studies and with the present model are difficult because of differences in experimental designs, pathogens studied, drug concentrations, and regimens used.

Athymic mice (used in all experiments) are severely immunosuppressed and the absence of host resistance in these animals is to some extent comparable with the immune status of patients with AIDS. Cryptococcal meningitis is the most common systemic mycotic infection in AIDS patients (8). Fluconazole has been shown to be effective in the treatment of cryptococcal meningitis in these patients (7, 21). However, even after extended therapy, the persistence and recurrence rate of the infection is over 50%. The present model suggests a potential new approach for treatment of cryptococcal meningitis in AIDS patients. Combined fluconazole and fluconosine treatment may achieve an increase in therapeutic response with less toxicity than those of currently used regimens.

ACKNOWLEDGMENT

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REFERENCES


12. Kobayashi, G. S., S. Travis, and G. Medoff. 1986. Comparison of the in vitro and in vivo activity of the bis-triazole drug with intraconazole or ketoconazole in the present model are to some extent


