Treatment of Experimental Cryptococcal Meningitis with Fluconazole: Impact of Dose and Addition of Flucytosine on Mycologic and Pathophysiologic Outcome

Marinka Kartalija, Keith Kaye,* Jay H. Tureen, Qingxiang Liu, Martin G. Tauerber, Berkley R. Elliott, and Merle A. Sande

Infectious Diseases Laboratory, San Francisco General Hospital; Departments of Medicine and Pediatrics, University of California, San Francisco, California

Fluconazole is effective in the therapy of cryptococcal meningitis in patients with AIDS. The optimal dosage of fluconazole and the impact of combination with flucytosine are not known. In this study, rabbits with experimental cryptococcal meningitis were given fluconazole at low, intermediate, or high dose or in combination with a low or intermediate dose of flucytosine. Serial cerebrospinal fluid (CSF) examinations showed that all three doses of fluconazole and low-dose fluconazole in combination with intermediate-dose flucytosine were effective in reducing CSF cryptococcal titer, lactate, white blood cell count, and cryptococcal antigen (CRAG) titers. The intermediate and high doses of fluconazole reduced CSF fungal (P < .05) and CRAG (P < .001) titers earlier than low-dose fluconazole alone or in combination with flucytosine. Only the highest dose of fluconazole reduced brain edema after 7 days. In this model of cryptococcal meningitis, there was evidence of a dose response with fluconazole but no in vivo synergism with flucytosine.

Cryptococcal meningitis is one of the most common and serious manifestations of AIDS. Between 6% and 10% of patients with AIDS will develop central nervous system infection with Cryptococcus neoformans [1-4]. Despite antifungal therapy, the mortality approaches 20% under optimal conditions and remains as high as 50% in recent studies in Africa [5].

Amphotericin B has been the drug of choice for this disease for many years, but its use may be limited by toxicity, and administration is extremely difficult in developing countries, where a majority of the AIDS cases are found. Fluconazole, a relatively new triazole that can be administered orally, has increased activity against cryptococci and attains high cerebrospinal fluid (CSF) concentrations (~70% of simultaneous serum levels) compared with ketoconazole, the prototype triazole. In initial trials in AIDS patients with cryptococcal meningitis, fluconazole compared favorably with amphotericin B. However, mortality during the first 2 weeks of therapy was higher with fluconazole, and most authorities recommend initiating treatment with amphotericin B and switching to fluconazole after 10-14 days or when the patient has responded clinically [6, 7]. This approach may be impractical in developing nations, and more rapidly effective oral therapies are therefore needed.

The present studies were designed to test the relative efficacy of increasing doses of fluconazole compared with a standard dose and of fluconazole combined with a low or intermediate dose of flucytosine in a rabbit model of cryptococcal meningitis. Clinical, mycologic, and cytochemical end points of treatment were determined over the first week of therapy.

**Materials and Methods**

**Infected organism.** The organism used in these studies was C. neoformans H99 isolated from the CSF of a patient with cryptococcal meningitis (provided by J. Perfect, Duke University, Durham, NC). The inoculum was prepared by growing the organism on inhibitory mold agar plates (PML Microbiologicals, Tualatin, OR) for 4 days at room temperature. Then colonies were harvested and suspended in 0.015 M PBS to yield an absorbance previously determined to give a concentration of ~5 × 10^7 cfu/mL.

Sensitivity was tested by microtiter dilution according to M-27T National Committee for Clinical Laboratory Standards standard procedures. The MIC of fluconazole to the organism was 2.0 µg/mL at 48 and 72 h of incubation. The MIC of flucytosine was 2.0 µg/mL after 48 and 72 h of incubation (both sensitivities determined by M. G. Rinaldi, Fungus Testing Laboratory, University of Texas, San Antonio).

**Model of meningitis.** A modification of the previously described model of experimental cryptococcal meningitis was used [8]. New Zealand White rabbits (2-3 kg) were given cortisone acetate (4 mg/kg intramuscularly [im]) for the entire experiment to induce immunosuppression. One day after the start of treatment with cortisone, rabbits were anesthetized (acepromazine, 0.5-1 mg/kg, Pro-mace, Aveco, Fort Dodge, IA; ketamine, 30-50 mg/kg, Ketaset, Bristol Laboratories, Syracuse, NY; and xylazine, 2-5 mg/kg im, Gemini, Rugby Laboratories, Rockville, NY), and infected by intracisternal injection of ~2 × 10^7 cfu of C. neoformans in 0.4 mL of 0.015 M PBS, pH 7.4. Antifungal treatment was started 4 days after infection and continued for a total of 7 days (11 days after infection),...
at which time rabbits were euthanized by an intravenous overdose of pentobarbital (150 mg/kg intravenously [iv]).

**Experimental end points.** At days 0, 4, and 7 of treatment, CSF samples were taken by cisternal aspiration. Fungal titer were determined by culturing 10 mL of 10-fold serial dilutions of CSF on blood agar plates kept at room temperature for 4 days. Sterility was defined by lack of growth following plating of 50 mL of undiluted CSF on blood agar plates (level of detectability was therefore 20 cfu/mL of CSF [log10 cfu/mL = 1.3]). Titer of cryptococcal antigen (CRAG) were determined in 2-fold serial dilutions of CSF by latex particle agglutination using a commercial kit and following the instructions of the manufacturer (Meridian Diagnostics, Cincinnati). CSF lactate was measured using an automatic lactate/glucose analyzer (YSI 2300 G/L; YSI, Yellow Springs, OH). CSF white blood cells (WBC) were counted in a Neubaur hemocytometer. The remainder of the CSF was immediately centrifuged at 8000 g for 5 min and stored at −70°C. Serum and CSF fluconazole and fluycytosine concentrations were measured by high-performance liquid chromatography (HPLC) (fluconazole) or by bioassay (flucytosine) by the Fungus Testing Laboratory (San Antonio). Brain edema was determined by weighing gross specimens in both wet and dry phases as previously described [9].

**Experimental drugs and study design.** Rabbits were randomly assigned to 1 of the indicated treatment groups (see below). The three doses of fluconazole were chosen to achieve serum and CSF concentrations similar to those achieved in humans with a dose of 100, 400, or 800–1600 mg. Similarly, flucytosine doses reflected low to intermediate dosages used in humans. The 5 treatment regimens were low-dose fluconazole, 5 mg/kg/day subcutaneously (sc) as a single injection (fluconazole-5); intermediate-dose fluconazole, 20 mg/kg/day sc as a single injection (fluconazole-20); high-dose fluconazole, 40 mg/kg/day sc as a single injection (fluconazole-40); fluconazole-5 with either 100 mg/kg/day flucytosine sc in two daily doses (fluconazole-5 + flucytosine-100) or 200 mg/kg/day flucytosine sc in two daily doses (fluconazole-5 + flucytosine-200).

Flucytosine powder (Pfizer, New York) was dissolved in sterile saline with gentle heating to solubilize the drug before treatment. Flucytosine was provided by Hoffmann-La Roche (Basel, Switzerland) as a solution of 10 mg/mL. All treatment regimens lasted 7 days, starting 4 days after infection.

Experiments were done in groups of ~15 animals. Each group included several untreated infected control animals and animals assigned to at least two different treatment regimens. Results in control animals did not significantly change over the duration of the studies. Data from all animals were combined for analysis, and the different treatment groups were compared with each other.

**Statistical analysis.** All data are expressed as mean ± SD. For variables that showed a normal distribution (e.g., CSF lactate, titer, brain edema), groups were compared by one-way analysis of variance, followed by Newman-Keuls test corrected for multiple comparison. For variables not normally distributed (e.g., CSF WBC, CRAG), groups were compared by Mann-Whitney rank sum test corrected for multiple comparison.

**Results**

**Characterization of the rabbit model of cryptococcal meningitis.** At 4 days after infection, animals had signs of meningitis with mild irritability or decreased activity. CSF examination showed pleocytosis, increased lactate concentrations, elevated CRAG titers, and C. neoformans concentration of 10^4–10^5 cfu/mL (table 1, figure 1).

**CSF and serum drug concentrations.** Peak CSF and serum fluconazole and flucytosine concentrations were obtained 1 h (serum) and 1.5 h (CSF) after sc drug administration. Samples for trough measurements (fluconazole only) were obtained ~24 h after the last dose (table 2). There was a significant difference between groups for peak measurements and between the fluconazole-5 group and fluconazole-20 and -40 groups for trough concentrations. Drug penetration of fluconazole resulted in a CSF concentration of 60.3%–64.3% of serum concentration, consistent with previously reported information [10].

**Effect of treatment on cryptococcal titers and CRAG concentration in CSF.** After 4 days of treatment, only rabbits treated with fluconazole-20 or -40 had CSF cryptococcal titers that were significantly lower than those in controls. By 7 days, all treatment groups had cryptococcal titers significantly lower than those of untreated controls. While titers were lower in the fluconazole-20 and -40 groups than in the fluconazole-5 group, this difference was not statistically significant. Treatment with fluconazole-5 + flucytosine-100 was significantly less effective than treatment with the medium (20 mg/kg) or high (40 mg/kg) doses of flucytosine (figure 1).

The titer of CRAG in CSF continued to increase during the 11-day course of untreated infection, whereas CRAG titers dropped in all treated groups over the 7 days of therapy. On day 4 of therapy, only animals treated with fluconazole-20 and -40 or with fluconazole-5 + flucytosine-200 had significantly lower CSF levels of CRAG than did untreated animals. By day 7, all treatment groups, with the exception of the fluconazole-5 + flucytosine-100, had significantly lower CSF CRAG titers than untreated controls (table 1).

**Pathophysiologic parameters.** CSF lactate concentrations increased progressively in untreated controls during the study period. All treated groups showed significantly lower lactate concentrations compared with controls on days 4 and 7 of therapy, without significant difference between treatment groups. Similarly, WBC counts were elevated in all groups 4 days after infection and increased further in untreated controls while remaining stable or decreasing in the treated groups. After 7 days of therapy, all treatment groups except those treated with fluconazole-5 + flucytosine-100 had significantly fewer CSF WBCs than did controls (table 1).

Untreated control rabbits had increases in brain water content compared with untreated cortisone-treated rabbits. Of all treatment regimens, only high-dose fluconazole (fluconazole-40) resulted in rabbits having brain water content similar to that in untreated controls, while brain water content in the other treatment groups was comparable to that in infected untreated controls (table 1). Differences between infected controls and either uninfected controls or animals treated with fluconazole-
Table 1. Central nervous system parameters in experimental cryptococcal meningitis: response to therapy.

<table>
<thead>
<tr>
<th>Day of therapy</th>
<th>0</th>
<th>4</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CRAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>985 ± 2008 (30)</td>
<td>2575 ± 2637 (29)* [+161]</td>
<td>2969 ± 2430 (28)* [+201]</td>
</tr>
<tr>
<td>Low-flu</td>
<td>1120 ± 1652 (27)</td>
<td>1201 ± 1872 (26) [+7]</td>
<td>835 ± 1812 (27) [-25]</td>
</tr>
<tr>
<td>Mid-flu</td>
<td>992 ± 1266 (10)</td>
<td>545 ± 612 (11) [-45]</td>
<td>280 ± 323 (10) [-72]</td>
</tr>
<tr>
<td>Hi-flu</td>
<td>1102 ± 1251 (12)</td>
<td>475 ± 638 (11) [-57]</td>
<td>252 ± 337 (10) [-77]</td>
</tr>
<tr>
<td>Low-flu + 5FC100</td>
<td>2901 ± 2747 (12)*</td>
<td>1203 ± 639 (10) [-58]</td>
<td>1208 ± 1454 (12) [-58]</td>
</tr>
<tr>
<td>Low-flu + 5FC200</td>
<td>363 ± 210 (12)*</td>
<td>272 ± 205 (12)* [-25]</td>
<td>104 ± 83 (11)* [-71]</td>
</tr>
<tr>
<td>Lactate (mM/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.9 ± 0.8 (36)</td>
<td>6.8 ± 2.8 (36)**</td>
<td>8.4 ± 3.8 (30)**</td>
</tr>
<tr>
<td>Low-flu</td>
<td>3.2 ± 0.7 (36)</td>
<td>5.3 ± 1.6 (36)</td>
<td>4.4 ± 1.2 (30)</td>
</tr>
<tr>
<td>Mid-flu</td>
<td>2.9 ± 0.6 (11)</td>
<td>4.4 ± 1.8 (11)</td>
<td>3.8 ± 1.8 (10)</td>
</tr>
<tr>
<td>Hi-flu</td>
<td>3.5 ± 1.6 (12)</td>
<td>4.5 ± 2.1 (12)</td>
<td>3.4 ± 1.0 (11)</td>
</tr>
<tr>
<td>Low-flu + 5FC100</td>
<td>2.7 ± 0.5 (13)</td>
<td>4.4 ± 1.2 (13)</td>
<td>4.1 ± 1.6 (12)</td>
</tr>
<tr>
<td>Low-flu + 5FC200</td>
<td>2.8 ± 0.4 (18)</td>
<td>4.1 ± 0.9 (18)</td>
<td>3.9 ± 1.0 (13)</td>
</tr>
<tr>
<td>WBC (×10^6/mm^3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>273 ± 274 (35)</td>
<td>988 ± 1060 (29)</td>
<td>1468 ± 1822 (23)*</td>
</tr>
<tr>
<td>Low-flu</td>
<td>313 ± 326 (36)</td>
<td>631 ± 522 (36)</td>
<td>380 ± 259 (31)</td>
</tr>
<tr>
<td>Mid-flu</td>
<td>265 ± 321 (11)</td>
<td>346 ± 328 (11)</td>
<td>224 ± 218 (11)</td>
</tr>
<tr>
<td>Hi-flu</td>
<td>233 ± 397 (12)</td>
<td>241 ± 135 (10)</td>
<td>217 ± 283 (10)</td>
</tr>
<tr>
<td>Low-flu + 5FC100</td>
<td>269 ± 189 (13)</td>
<td>253 ± 214 (13)</td>
<td>493 ± 496 (12)</td>
</tr>
<tr>
<td>Low-flu + 5FC200</td>
<td>275 ± 189 (18)</td>
<td>353 ± 251 (18)</td>
<td>177 ± 77 (13)</td>
</tr>
<tr>
<td>Brain edema (g of H_2O/g of dry tissue)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (uninfected)</td>
<td>3.86 ± 0.07 (10)*</td>
<td>3.97 ± 0.11 (28)</td>
<td>3.91 ± 0.14 (10)</td>
</tr>
<tr>
<td>Control (infected)</td>
<td>3.97 ± 0.11 (28)</td>
<td>3.97 ± 0.11 (28)</td>
<td>3.97 ± 0.11 (28)</td>
</tr>
<tr>
<td>Low-flu</td>
<td>3.95 ± 0.14 (30)</td>
<td>3.95 ± 0.14 (30)</td>
<td>3.95 ± 0.14 (30)</td>
</tr>
<tr>
<td>Mid-flu</td>
<td>4.01 ± 0.13 (10)</td>
<td>4.01 ± 0.13 (10)</td>
<td>4.01 ± 0.13 (10)</td>
</tr>
<tr>
<td>Hi-flu</td>
<td>3.88 ± 0.10 (12)**</td>
<td>3.88 ± 0.10 (12)**</td>
<td>3.88 ± 0.10 (12)**</td>
</tr>
<tr>
<td>Low-flu + 5FC100</td>
<td>3.99 ± 0.22 (14)</td>
<td>3.99 ± 0.22 (14)</td>
<td>3.99 ± 0.22 (14)</td>
</tr>
<tr>
<td>Low-flu + 5FC200</td>
<td>3.99 ± 0.13 (10)</td>
<td>3.99 ± 0.13 (10)</td>
<td>3.99 ± 0.13 (10)</td>
</tr>
</tbody>
</table>

NOTE. Data are mean ± SD (n) (% change). CRAG = cryptococcal antigen; WBC = white blood cells; low-, mid-, and hi-flu = 5, 20, and 40 mg/kg/day fluconazole; 5FC100 and 5FC200 = 100 and 200 mg/kg/day flucytosine. All comparisons were by Mann-Whitney rank sum test, unless otherwise indicated, corrected for multiple comparisons.

* P < .05 for control vs. mid-flu, hi-flu, and low-flu + 5FC200.
† P < .05 for controls vs. all treatment groups except low-flu + 5FC100.
‡ P < .05 for control vs. low-flu + 5FC100.
§ P < .05 vs. all other groups.
¶ P < .05 for direct comparison with low-flu + 5FC100 by Student’s t test with Bonferroni correction.
‖ P < .05 vs. low-flu + 5FC100.
** P < .05 for controls vs. all other groups (analysis of variance, Newman-Keuls).
†† P < .05 for control vs. all treatment groups except low-flu + 5FC100.
‡‡ P < .01 for direct comparison of uninfected and infected control by Student’s t test.
§§ P < .03 for direct comparison of infected control and hi-flu by Student’s t test.

40 were significant (P < .05) when the statistical analysis was limited to these 3 groups.

Discussion

The number of AIDS patients developing cryptococcal meningitis remains high and is especially important in developing nations, where the difficulty of iv administration of amphotericin B limits treatment options for this infection. Fluconazole has proven effective in controlled trials [6, 11] at relatively low dosages (200–400 mg/day), but mortality during the first 2 weeks of treatment is higher than with amphotericin B.

It is therefore important to determine whether higher doses of fluconazole or the addition of flucytosine might improve chances of survival, presumably by increasing the rapidity with which the fungal infection in the CSF is controlled. The time-honored method of testing such a hypothesis is first to compare these new therapeutic options with the previously tested dosage in a well-standardized animal model of the infection, achieving serum and CSF drug concentrations that are relevant to human infection [12]. Although the fluconazole treatment regimens...
resulted in peak serum concentrations higher than those achieved with human equivalent doses, the half-life of fluconazole in the rabbit is significantly shorter than in humans (9 vs. 31 h) [13]. The three fluconazole treatment regimens had areas under the curve comparable to those obtained with human dosages of 100, 400, and 1200–1600 mg/day for low-, middle-, and high-dosage (fluconazole-5, -20, and -40) groups, respectively [14-16].

In this study, we examined two issues. First, we tested whether increasing the dose of fluconazole to achieve CSF levels much higher than those obtained with standard dosages might produce a more rapid therapeutic response. Second, we examined whether the addition of flucytosine to fluconazole resulted in improvement in either microbiologic or pathophysiologic end points. Use of either high-dose fluconazole or combination therapy with fluconazole and flucytosine are treatment alternatives that have not yet been adequately studied in human trials, although both have been tried in small studies [11, 17].

The present study included measurement of a number of microbiologic and pathophysiologic end points to give a relatively complete picture of therapeutic efficacy. These included quantitative cultures of CSF, CSF CRAG concentrations, and measurements of pathophysiologic parameters, which included CSF lactate concentration, WBC counts, and measurement of brain edema at completion of treatment. Brain edema, which may lead to intracranial hypertension, was increased in this model, as was CSF lactate, a marker for anaerobic brain metabolism [18]. In clinical studies of patients with cryptococcal meningitis, adverse prognostic factors have included abnormal mental status at presentation, high CRAG concentration in CSF, CSF leukocyte count <20 WBC/mm$^3$, positive extraneural cultures, intracranial hypertension, and hyponatremia [3, 4, 6, 19, 20]. Furthermore, it has been suggested that the increased mortality during the early phase of therapy with fluconazole is related to a relatively slow antifungal effect of the drug compared with that of amphotericin B.

The low dose of fluconazole (5 mg/kg/day) tested in rabbits with cryptococcal meningitis resulted in serum and CSF drug concentrations equivalent to levels obtained with ~100 mg/ day in humans. The medium dose (20 mg/kg/day) gave serum concentrations comparable to those achieved with standard human therapy (400 mg/day). The highest dose (40 mg/kg/day) resulted in much higher serum and CSF levels than do standard dosages in humans, comparable to 1200–1600 mg/day.

The effect of fluconazole therapy on the microbiologic parameters of cryptococcal meningitis was obvious; all groups receiving treatment had reduction in viable cryptococci and reduced CSF CRAG; however, fluconazole dosages equivalent to human dosages of 400 or 1200–1600 mg/day outperformed the low-dose regimen. Importantly, quantitative cryptococcal titers and CRAG titers were reduced more rapidly in the medi-
um- and high-dose–treated animals, with a trend favoring the highest dose over the intermediate dose. This indicates that there is a dose response between the concentration of fluconazole and its antifungal effects and that, therefore, high doses of fluconazole may potentially overcome the apparent inferiority of lower doses of the drug relative to amphotericin B in the early phase of treatment.

Addition of flucytosine to low-dose fluconazole failed to result in a consistent improvement of any experimental parameters over that observed with low-dose fluconazole alone and was thus less effective than increasing the dose of fluconazole. With the lower dose of flucytosine, there was a trend toward reduced antifungal activity, suggesting a possible antagonism at low concentrations of flucytosine. This adverse effect was not present when higher doses of flucytosine more comparable to those achieved in humans were examined, and this combination may have had some beneficial effect on CSF CRAG titers, but not fungal titers, compared with low-dose fluconazole alone. It is possible that our failure to demonstrate a clear benefit with flucytosine may have been due to a limitation of our model. Attempts to give higher doses of flucytosine resulted in increased mortality (data not shown), which may be a function of relative intolerance of this species to flucytosine.

Other experimental models of cryptococcal disease have demonstrated a more consistent additive effect when the two drugs were combined [21]. Larsen et al. [22] have reported results of a nonrandomized trial that appear to show benefit of addition of flucytosine to conventional dose fluconazole in AIDS patients with cryptococcal meningitis. In that study, a larger proportion of patients had negative CSF cultures at 10 weeks compared with results of previous studies of fluconazole alone. Of note, however, 28% of patients had to have flucytosine discontinued because of toxicity, and an additional 15% had to have the dose reduced. The apparent antagonism we observed with fluconazole combined with flucytosine at low doses underscores the importance of ensuring adequate serum and CSF concentrations of flucytosine if the dose is reduced for toxicity.

To some extent, pathophysiologic parameters of the disease showed trends similar to those seen with the microbial parameters. CSF lactate concentrations, which have been shown in several models of bacterial meningitis to reflect severity of disease [9, 18], were significantly lower in all treatment groups than in controls and appeared more profoundly affected by the higher doses of fluconazole, even though this trend did not reach statistical significance, in part due to the multiple experimental groups. Perhaps most interesting was the reduction in brain edema in animals treated with the highest dose of fluconazole, while no such effect was found in any of the other treatment groups.

It is tempting to speculate that a more rapid antifungal effect achieved with very high doses of fluconazole has beneficial effects on pathophysiologic abnormalities in the brain and may, therefore, provide a survival benefit early in therapy, while fluconazole in standard to lower doses proved to be inferior to amphotericin B in treating AIDS patients with cryptococcal meningitis. A recent report demonstrated that high-dose fluconazole therapy was well tolerated up to doses of 1600 mg/day [15], and preliminary data suggest that high-dose fluconazole therapy (800–1200 mg/day) combined with flucytosine is superior to conventional therapy in terms of time to sterilization and clinical response [23]. The present experimental study adds additional support to these human studies and argues that high-dose fluconazole therapy for cryptococcal meningitis should be formally tested in randomized clinical trials.

References
17. Haubrich RH, Haghighat D, Bozzette SA, Tilles J, McCutchan JA, the California Collaborative Treatment Group. High-dose fluconazole for