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Effect of Fluconazole on Fungicidal Activity of Flucytosine in Murine Cryptococcal Meningitis

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Both animal and in vitro studies have demonstrated that combinations of flucytosine with amphotericin B and with fluconazole have significantly improved activity against cryptococcal meningitis compared with the activity of each drug used alone. However, very few dose levels of these agents have been tested in combination. This study evaluated the efficacy of fluconazole plus flucytosine in a murine model of cryptococcal meningitis over a broad range of dose combinations (fluconazole, 0 to 40 μg/g of body weight per day; flucytosine, 0 to 200 μg/g/day). Both drugs were dissolved in drinking water, with treatment on days 2 to 11. In this highly reproducible model, fluconazole had a dramatic effect on the fungicidal activity of flucytosine. Flucytosine at dose levels of as much as 200 μg/g/day alone or in combination with low doses of fluconazole had minimal fungicidal activity, whereas in combination with fluconazole at 24 to 40 μg/g/day, flucytosine showed fungicidal activity in the range of 45 to 65% of the animals treated at doses of 40 to 100 μg/g/day. This striking effect of fluconazole is consistent with the results of both in vitro and clinical studies. In the clinic, the use of flucytosine is often limited by severe toxicity, while toxicity is rarely observed with fluconazole. These results suggest that when flucytosine is given with higher doses of fluconazole, the maximum therapeutic effect of the former in the clinic may be observed at dose levels that are far less than the doses commonly employed (150 μg/g daily).

A variety of therapeutic approaches to the treatment of cryptococcal meningitis have been tried, most of which have had limited success. For example, among patients with AIDS-related cryptococcal meningitis treated with amphotericin B or fluconazole for 10 weeks, approximately one-third resolve their meningitis, one-third have active meningitis still present, and nearly one-third die (3, 18). In patients without AIDS, results have also been disappointing, with fewer than 50% responding to amphotericin B (2). Combining flucytosine with fluconazole or amphotericin B results in improved rates of success, usually in the range of 55 to 65% for patients with and without HIV coinfection (2, 11, 12, 15). However, even these modest improvements in the rates of success are associated with significant associated drug toxicity, since nearly two-thirds of the patients reported troubling side effects, of which half were described as intolerable (12, 15, 19). Nevertheless, treatment with combinations of antifungal agents has given promising results and offers the most immediate opportunity for significant improvement in therapeutic effectiveness.

Both animal and in vitro studies have demonstrated that the combinations of flucytosine with amphotericin B and with fluconazole have significantly improved antifungal activity compared with the activity of each drug used alone (1, 18). However, very few dose levels of these agents were tested in combination. More effective approaches which can identify safe combinations with the potential for increased activity are needed. The present study was designed to evaluate the activity of fluconazole plus flucytosine in a murine model of cryptococcal meningitis over a broad range of dose combinations and to determine a range of dose combinations with promising activity.

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MATERIALS AND METHODS

Animal protocol. Pathogen-free BALB/c male mice, approximately 6 weeks of age and weighing 20 to 25 g, were used in all experiments. The animals were housed in isolation cages (four per cage) and were given free access to food and water. The mice were briefly anesthetized with CO_2 narcosis, weighed individually, and challenged intracerebrally with approximately 300 CFU of *Cryptococcus neoformans* cells in a volume of 0.06 ml, which was delivered through a 27-gauge needle by direct puncture through the cranial vault approximately 6 mm posterior to the orbit. The animal protocol was approved by the University Institutional Animal Care and Use Committee.

Chemotherapy. Mice were randomly assigned to treatment groups (cages) 48 h after intracerebral challenge, and treatment was initiated with the assigned concentrations of fluconazole and flucytosine dissolved in the sole source of drinking water. Drug concentrations were calculated on the basis of the weights of the animals in each treatment cage, measured daily water intake, and assigned drug doses. Treatment was continued for 11 days, with the water supply and added drugs changed every 4th day. The dose levels of fluconazole and flucytosine tested were from 0 to 40 μg/g of body weight per day in 4-μg/g increments and from 0 to 200 μg/g/day in 20-μg/g increments, respectively. In general, two cages with four animals each were treated at each dose combination tested.

Mycologic procedures. The *C. neoformans* isolate was obtained from the laboratory of J. Richard Graybill (isolate 89-127) and was maintained on Sabouraud dextrose agar at 4°C. On the evening prior to infection of the mice, the isolate was grown on brain heart infusion agar at 37°C. The organisms were washed with normal pyrogen-free saline before suspension in saline. The concentrations of organisms injected into the mice were confirmed by serial 10-fold dilutions of the initial suspension and by counting the numbers of CFU on plates prepared from 0.06 ml of the suspension ejected from the inoculation syringe just before and after inoculation of the mice from each cage.

For measurement of brain fungal burden, animals were sacrificed 24 h after cessation of treatment and the brains were removed, weighed, and homogenized in 2.0 ml of normal saline. Duplicate 10-fold serial dilutions of the whole brain homogenate were prepared for quantitative counts of CFU. A 0.1-ml aliquot from each dilution was plated on Sabouraud dextrose agar and incubated at 37°C for 72 to 96 h, and the numbers of CFU were recorded. For animals sacrificed to determine if the brain had become sterile, the entire brain homogenate was placed in a tube containing brain heart infusion broth, incubated at 37°C, and observed for 96 h to determine the presence or absence of growth. If growth was

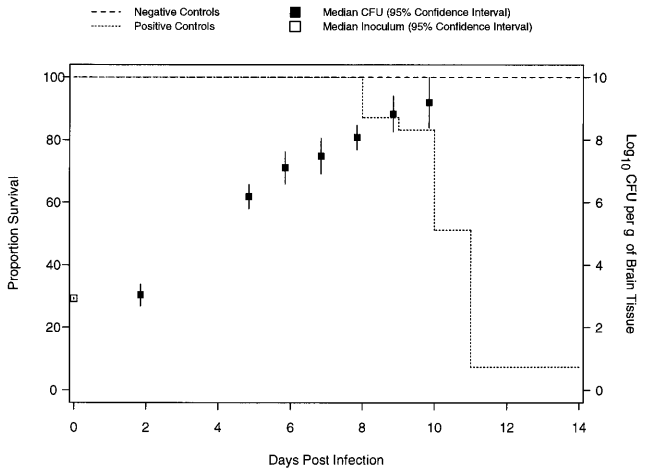

FIG. 1. Kaplan-Meier survival curves for untreated negative- and positive-control animals by day postinfection and the median numbers of CFU of *C. neoformans* per gram of brain tissue (vertical bars, 95% CIs) by day postinfection.

observed, an aliquot was plated on Sabouraud dextrose agar to confirm the presence or absence of *C. neoformans.*

Measurements of efficacy. Treatment activity was measured by the following endpoints: survival, weight loss, and sterilization of brain tissue. Survival in the following two forms was considered: firstly, as duration of survival time, and secondly, as the proportion of animals treated at each dose combination which were alive at the end of the 14-day experiment. Weight change for each animal was based on weight at the time of sacrifice relative to the initial weight recorded at the time of infection.

In each experiment, groups of three to four control mice were sacrificed on selected days following infection to confirm that comparable levels of fungal burden were achieved in each experiment. In one experiment, untreated control animals were monitored up to day 14 for survival; animals exhibiting signs of distress were sacrificed, and the day following the date of sacrifice was considered the date of death.

Statistical analysis. The primary objective of these experiments was to evaluate the dependence of observed measures of efficacy (survival, weight loss, and proportion of whole brain tissue cultures that were sterile) on dose levels of fluconazole and flucytosine in combination. A local nonparametric regression (loess) method was used to fit and visualize the response surface for each measure of efficacy (5, 6). Local linear regression was employed to fit the fluconazole dose response, and local quadratic regression was used to fit the flucytosine dose response. Loess regression is a statistical method for fitting and visualizing general underlying patterns of multivariate data. This method is robust, in that it does not require specifying the nature of the association between the measure of efficacy and dose levels. The fitted surface at each point depends only on nearby (local) observations (defined as 40% of the neighboring data points); thus, the patterns of association are not forced to be the same over the entire range of doses. This method is resistant, meaning that the fitted surface is not distorted by localized misbehavior (4, 9). Observations which do not follow the local pattern or which have undue influence on the fit are identified for closer scrutiny. The relative contributions of potentially explanatory variables to the loess fit were assessed by robust analysis of variance (5, 6). Confidence intervals (CIs) for the fitted surface were based on pointwise estimates and were calculated at the 95% level.

The duration of survival for untreated controls was estimated by the Kaplan-Meier method (10). CIs for the proportion surviving up to a time point were estimated by Greenwood's formula according to the method described by Link (7, 13). Differences in the duration of survival between groups were tested by using the log rank statistic (8). Animals surviving until the scheduled day of sacrifice were considered alive, with survival time censored at that day, whereas animals dying or found moribund prior to the scheduled day of sacrifice were considered dead, with survival time equal to the day of death or the day following sacrifice, respectively. Analysis of survival for treated animals was based on the proportion of animals alive at the end of the 14-day experiment for each dose combination separately within each experiment. Analysis of the sterility of brain tissue for treated animals was based on the proportion of animals having sterile whole brain homogenate cultures for each dose combination separately within each experiment. Descriptive statistics were based on medians and robust 95% CIs (9). All statistical analyses were performed by using the S-Plus statistical environment (14, 20–23). Because of the exploratory nature of these analyses, only P values of less than 0.001 were considered significant.

RESULTS

All animals that were challenged with nonviable *C. neoformans* (negative controls) survived for 14 days (Fig. 1) and maintained stable weight (median weight change relative to baseline weight, 0% [95% CI, −1.5 to 1.5]). Water consumption among these negative-control animals was relatively constant across dose levels of fluconazole and flucytosine at approximately 3.5 ml per animal per day (median, 3.44 ml; 95% CI, 3.36 to 3.52). Among untreated control animals challenged with viable organisms (positive controls) and observed for survival, 93% died by day 11 (95% CI, 53 to 99%), with a median survival of 9 days ($P < 0.00001$ [comparing durations of survival for positive versus negative controls]; Fig. 1). Mice tolerated as much as 7 days of untreated infection, resulting in 10^8 CFU/g of brain tissue, before exhibiting signs of distress or dying.

Survival for treated mice. Figure 2 shows a three-dimensional plot of the loess-fitted response surface for the proportion of animals surviving to day 14 by dose levels of fluconazole and flucytosine. In this figure, the fitted proportion of animals alive at day 14 for a particular dose combination is represented by the height of the surface above the point on the fluconazole-flucytosine plane which corresponds to the dose levels for that combination. The relative contribution of fluconazole to the loess fit of the association between the proportion alive and flucytosine is highly significant ($P < 0.00001$). When used alone, ≥170 μg of flucytosine or ≥24 μg of fluconazole per g per day was required for 100% survival to day 14, while in combination, flucytosine at 80 μg/g/day plus fluconazole at 16 μg/g/day was sufficient for 100% survival. Thus, by combining these two drugs, 100% survival could be achieved with a dose of flucytosine which was 50% lower than that required for flucytosine alone and with a dose of fluconazole that was 30% lower than that required for fluconazole alone.

Weight change for treated mice. Figure 3 shows the loess-fitted response surface for the relative percent weight changes from baseline by dose combinations of fluconazole and flucytosine. In this figure, the fitted weight change at a particular dose combination is represented by the height of the surface above the point on the fluconazole-flucytosine plane which

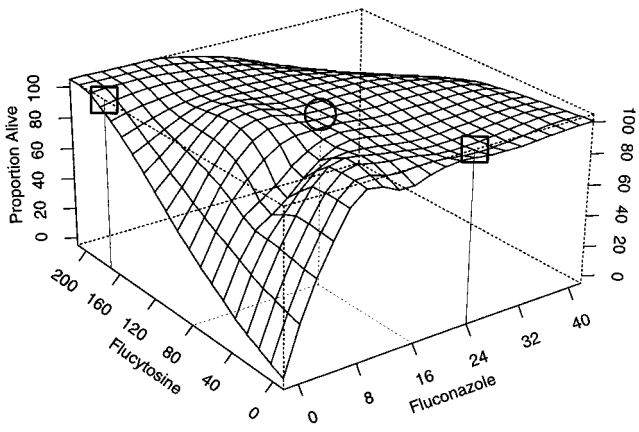

FIG. 2. Response surface showing the loess fit of the association between proportion of animals alive at day 14 and the dose levels of fluconazole and flucytosine. The square on the left marks the point above which 100% survival was observed with flucytosine alone at ≥170 μg/g/day; the square on the right marks the point above which 100% survival was observed with fluconazole alone at ≥24 μg/g/day; the circle in the middle marks the point at which 100% survival was observed with the combination of fluconazole (16 μg/g/day) and flucytosine (80 μg/g/day).

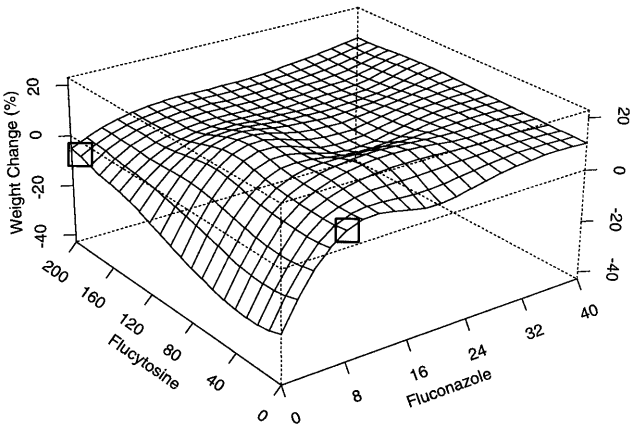

FIG. 3. Response surface showing the loess fit of the association between percent weight change and the dose levels of fluconazole and flucytosine. The squares on the left and right, respectively, mark the points at which the maximum percent weight change observed were >5% when flucytosine was used alone (200 μg/g/day) and >5% when fluconazole was used alone (8 μg/g/day).

corresponds to the dose levels for that combination. Fluconazole alone at doses of greater than 8 μg/g/day resulted in stable weight. Flucytosine alone at doses of as much as 200 μg/g/day did not protect against weight loss, and there was little incremental protection to weight loss when flucytosine was added to fluconazole ($P = 0.89$).

Sterilization of brain tissue for treated mice. The response surface shown in Fig. 4 provides an overview of the association between fungicidal activity and dose levels of fluconazole and flucytosine. To examine the influence of fluconazole on the flucytosine dose-response curves, Fig. 5 shows the flucytosine dose-response curves for selected fluconazole dose levels increasing from 0 to 40 μg/g/day. These flucytosine dose-response curves show a marked change in shape over the range of fluconazole dosing. Flucytosine had little or no ability to render the brains of these animals sterile in the absence of fluconazole. However, when flucytosine was combined with fluconazole at doses of 24 μg/g/day or higher, the proportion of animals with sterile brain tissue increased with increasing doses of flucytosine up to the range of 80 to 100 μg/g/day. As doses of flucytosine were increased above 100 μg/g/day, the proportion of animals with sterile brain tissue actually decreased. Furthermore, as the dose level of fluconazole increased above 16 μg/g/day, equivalent or better rates of response were observed at lower doses of flucytosine.

DISCUSSION

This study evaluated the activity of flucytosine in combination with fluconazole over a wide range of dose levels by using a murine model of cryptococcal meningitis. In this model, fluconazole had a consistent and dramatic effect on the fungicidal activity of flucytosine over a broad range of fluconazole and flucytosine dose levels. Flucytosine at dose levels of up to 200 μg/g/day had no fungicidal activity, whereas in combination with fluconazole at 24 to 40 μg/g/day, flucytosine sterilized the brains in 45 to 65% of the animals treated at doses of 40 to 100 μg/g/day. However, the converse was not true. As doses of fluconazole were increased across a threshold of 16 μg/g, increasing proportions of whole brain cultures became sterile. As flucytosine was added to fluconazole, incremental improvements in animal survival and the proportion of animals with sterile whole brain cultures increased. At very high doses of flucytosine, there was a decrease in animal survival and the proportion of animals with sterile brain cultures declined, which was possibly a result of the toxic side effects of flucytosine, which include its known bone marrow-suppressive effects.

This striking effect of fluconazole on the fungicidal activity of flucytosine against cryptococcus has also been observed in in vitro studies (16). For 90% of the 50 clinical isolates tested, the MIC of flucytosine alone which reduced growth by 80% was 16 μg/ml, whereas when it was combined with fluconazole, the MIC of flucytosine at which 90% of the isolates were inhibited was 1 μg/ml. Furthermore, in combination with fluconazole, the MICs of flucytosine were ≤8 μg/ml for 100% of the isolates, which are levels well below the maximum which can be achieved in clinical situations.

The impact of fluconazole on the flucytosine dose-response curve demonstrated in our experiments is also consistent with the results of clinical trials which evaluated flucytosine alone and in combination with fluconazole. Flucytosine alone at 150 μg/g/day had minimal activity in humans (11), while flucytosine at 150 μg/g/day in combination with fluconazole at 400 mg/day in humans showed modest activity (12). For a 60-kg patient, 400 mg of fluconazole per day would correspond to a dose of <7 μg/g/day, whereas the dose range of fluconazole at which increased fungicidal activity was observed in our experiments was 24 to 40 μg/g/day in combination with flucytosine. This higher dose range of fluconazole corresponds to 1,400 to 2,400 mg/day in clinical practice. Fluconazole doses of as much as 2,000 mg/day have been administered in combination with flucytosine at 150 μg/g/day in the clinic with no unacceptable side effects (15). While drug concentrations were not measured in these experiments, peak concentrations of fluconazole and flucytosine in serum 30 min after gavage dosing across the dose ranges employed in this trial produced values of fluconazole and flucytosine, respectively, in the ranges of 1 to 10 and 5 to 30 μg/ml (17a).

In the present report, we have taken advantage of the availability of new statistical methods to estimate and visualize the dose-response surfaces for fungicidal activity, survival, and weight loss. By evaluating these easily determined endpoints and by considering a wide range of dose combinations at widely spaced dose levels of each drug alone and in combination, we were able to explore the contribution of each drug. Instead of selecting a specific dose combination to be used in subsequent

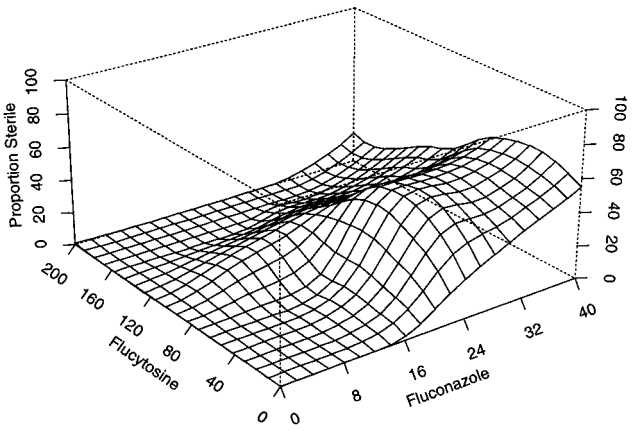

FIG. 4. Response surface showing the loess fit of the association between the proportion of animals with sterile whole brain tissue cultures and the dose levels of fluconazole and flucytosine.

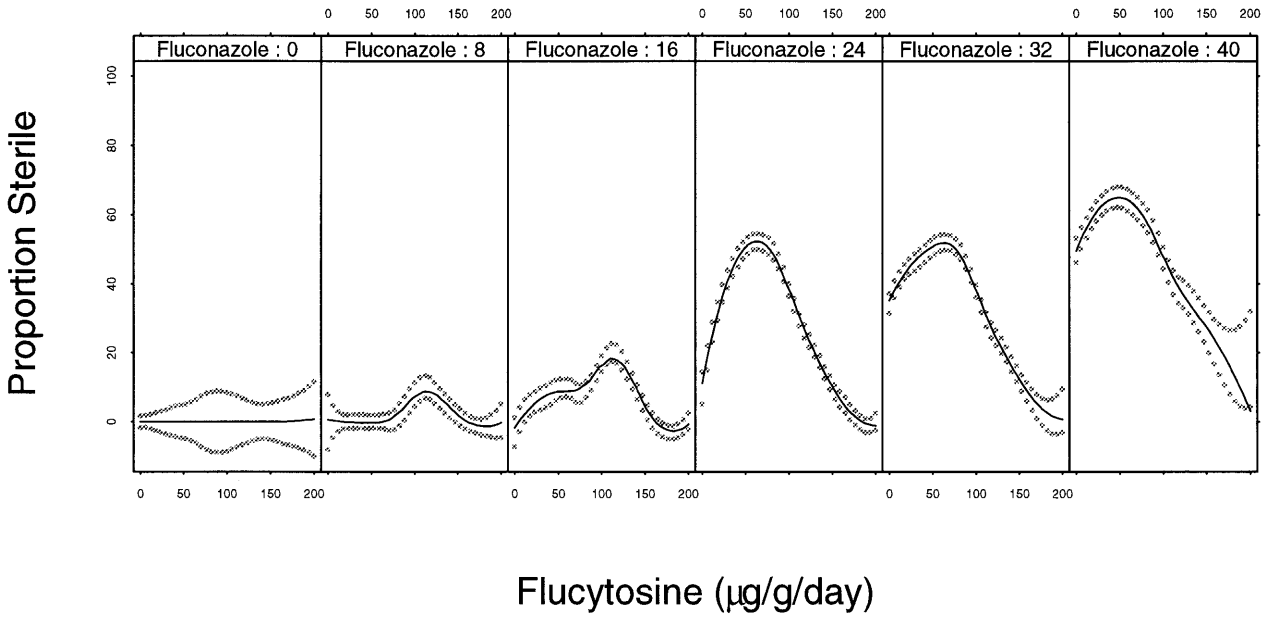

FIG. 5. Two-dimensional slices from the response surface shown in Fig. 4 showing the effect of dose level on the flucytosine dose-response curve (solid lines). Broken lines, upper and lower limits of the 95% pointwise CIs.

human clinical testing, these results identify a range of dose levels of fluconazole and flucytosine that, in combination, define a region with the most promising fungicidal activity. The identification of this region provides more useful information for guiding subsequent experiments and clinical trials than statistical summaries based on statistically significant differences between specific dose combinations and controls or estimation of specific dose combinations, e.g., the 50% lethal dose.

Conclusions. Fluconazole had a dramatic effect on the antifungal activity of flucytosine. When used alone, flucytosine at doses of as much as 200 μg/g/day was not able to render the animals' brains sterile after 11 days of treatment. However, the combination of fluconazole with flucytosine rendered more than 50% of the animals' brain cultures sterile at clinically achievable doses of both drugs. In clinical practice, the use of flucytosine is often limited by severe toxicity, while toxicity is rarely observed with fluconazole. The ability to achieve equivalent or improved fungicidal activity with lower doses of flucytosine when it is combined with higher doses of fluconazole is consistent with the effect of fluconazole on the in vitro inhibitory action of flucytosine reported by Nguyen and colleagues (17). The results of our experiments suggest that when flucytosine is given with higher doses of fluconazole, the maximum therapeutic effect of the former may be observed at dose levels that are far less than doses commonly employed (150 μg/g daily) (2, 11, 12, 15, 19).

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