Combination Therapy with Amphotericin B and Fluconazole against Invasive Candidiasis in Neutropenic-Mouse and Infective-Endocarditis Rabbit Models

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Although there are an increasing number of new antifungal agents available, the morbidity and mortality due to invasive mycoses remain high. The high rates of polyene toxicities and the development of azole resistance have raised the issue of using antifungal agents of these classes in combination, despite theoretical concerns regarding antagonism between such agents. This study was designed to evaluate the *in vivo* efficacy of combined therapy with amphotericin B and fluconazole against *Candida albicans*. Two distinct animal models were used in this study: a neutropenic-mouse model of hematogenously disseminated candidiasis and the infective-endocarditis rabbit model. Treatment efficacy was assessed by determining reductions in mortality as well as decreases in tissue fungal densities. In the neutropenic-mouse model, amphotericin B, as well as combination therapy, significantly prolonged survival compared to untreated controls ($P < 10^{-5}$ and $P = 0.001$, respectively). The fungal densities in the kidneys of neutropenic mice were significantly reduced with either amphotericin B monotherapy or amphotericin B-fluconazole combined therapy compared to those of controls ($P < 10^{-6}$). Fluconazole monotherapy also reduced fungal densities in the kidneys; however, this decrease was not statistically significant ($P = 0.17$). In contrast, treatment with either fluconazole alone or combined with amphotericin B (but not amphotericin B monotherapy) significantly decreased fungal densities in the brain ($P = 0.025$). In the rabbit endocarditis model, amphotericin B monotherapy or combined therapy significantly decreased fungal densities in cardiac vegetations ($P < 0.01$ versus the controls). Although no significant antagonism was seen when fluconazole was given in combination with amphotericin B, combination therapy did not augment the antifungal activity of amphotericin B.

Despite the recent introduction of an increasing number of antifungal agents, the morbidity and mortality due to deep mycoses are still unacceptably high (1). The emergence of resistance to azole antifungals and of systemic toxicity to polyene agents (e.g., amphotericin B [AmphB]) has raised the issue of using such antifungals in combination to optimize therapeutic outcome. Antifungal combinations may increase the magnitude and rate of microbial killing *in vivo*, shorten the total duration of therapy, prevent the emergence of drug resistance, expand the spectrum of activity, and decrease drug-related toxicities by allowing the use of lower doses of antifungals (11). To date, AmphB has been the standard therapy for invasive *Candida* infections; however, this agent's high frequency of renal toxicity has limited its use (4, 14, 16). Fluconazole (FLU) is much less toxic than AmphB and has been widely used for the therapy and prevention of mucosal candidiasis in patients with AIDS (4, 12). However, frequent relapses and the emergence of azole resistance have raised concerns regarding the use of FLU in treating selected invasive candidal infections (12).

The clinical use of azoles and polyenes in combination is a controversial issue. Azoles primarily inhibit fungal sterol biosynthesis, while polyenes act by binding to such sterols and

creating pores in the target fungal membrane (10). Therefore, these two drug classes theoretically may antagonize each other. In our previous studies, we have shown that combinations of AmphB and FLU exhibit enhanced antifungal effects *in vitro* against *Candida albicans* over a wide range of drug concentrations (7). In this study, to confirm our *in vitro* findings, a neutropenic-mouse model and the infective-endocarditis (IE) rabbit model were used to examine the potential for *in vivo* synergy of AmphB and FLU in combination.

MATERIALS AND METHODS

Antifungals. AmphB was obtained commercially from Pharma-Tek Inc., Huntington, N.Y. A fresh AmphB stock solution (1 mg/ml) was prepared in sterile distilled water every 3 days and stored at 4°C in darkness. FLU was obtained as powder from Pfizer Inc., New York, N.Y. Fresh FLU stock solutions (1 mg/ml) were prepared according to the manufacturer's recommendations.

Microorganism and growth. *C. albicans* (OY-2-76), used to establish hematogenously disseminated candidiasis in the neutropenic-mouse model, was obtained from a patient with AIDS and oropharyngeal candidiasis (12). Although this isolate was associated with a noninvasive mucosal infection clinically, in pilot studies in our laboratory it caused widespread hematogenous candidiasis analogous to isolates obtained from patients with hematogenously disseminated candidiasis. Furthermore, this isolate has been extensively characterized in our laboratory for its growth and other physiological parameters. *C. albicans* ATCC 36082, used in the IE model, was obtained from the American Type Culture Collection (Rockville, Md.). The latter strain was selected because it was used in our previous studies employing the IE model and, therefore, is well characterized (21). Both isolates were passaged through BALB/c mice (Harlan-Sprague-Dawley, Inc., San Diego, Calif.) by intravascular injection to maximize virulence. The passaged isolates were grown in Sabouraud dextrose broth (Difco Laboratories, Detroit, Mich.) and were stored as frozen stock cultures. Prior to each experiment, yeast cells were passaged three times in fresh Sabouraud dextrose broth, harvested after the third passage, washed twice with sterile normal saline (NS)

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(0.89%, wt/vol), counted with a hemocytometer, and diluted to the desired concentrations with NS. Hemocytometer counts were routinely verified by quantitative cultures on agar.

MICs of FLU and AmphB against the two candidal strains used in this study were determined with a microdilution format recommended by the National Committee for Clinical Laboratory Standards (M27-T methodology) (9). Minimum fungicidal concentrations (MFCs) were determined by culturing a loopful (0.01 ml) from wells showing no visible growth. MFC is defined as the lowest antifungal concentration yielding no CFUs following 48 h of incubation at 37°C. For strain 36082 MIC and MFC values for FLU were 0.5 and >64 µg/ml, and for AmphB they were 0.25 and 0.25 µg/ml. For strain OY-2-76 MIC and MFC values for FLU were 0.25 and >64 µg/ml, and for AmphB they were 0.25 and 0.25 µg/ml.

Neutropenic-mouse model. BALB/c mice (male, 11 weeks old, and 20 to 25 g each) were obtained from Harlan-Sprague-Dawley. Mice were allowed to acclimatize for at least 2 days prior to each experiment. In order to prevent bacterial infections, cephalixin (Medirex Inc., Pine Brook, N.J.) was dispensed in the drinking water (1 µg/ml final concentration).

Cyclophosphamide, obtained commercially from Pharmacia Inc. (Columbus, Ohio), was administered by intraperitoneal (i.p.) injections (150 mg/kg of body weight on day 1 and 100 mg/kg on day 4), as modified from the protocol of Van 't Wout et al. (20). On day 5, 40 µl of blood was collected from each mouse by cardiac puncture. The collected blood was diluted with 20 ml of ISOTONE II solution (Coulter Inc., Hialeah, Fla.), and the erythrocytes were lysed with ZAP-OGLOBIN II (Coulter Inc.). The leukocyte content of the lysate was measured with a Coulter Counter (model ZF). The leukocyte counts were also confirmed by hemocytometer counts. A total dosage of 250 mg/kg (150 mg/kg on day 1 and 100 mg/kg on day 4) was found to be optimal for establishing the desired neutropenia (<500 cells/mm³) in pilot experiments in our laboratory.

On the day following the last cyclophosphamide treatment, neutropenic mice were infected with 0.5 ml of yeast cell suspension via the lateral tail vein. Six different inocula (1×10^3 , 5×10^3 , 1×10^4 , 5×10^4 , 1×10^5 , and 5×10^5 CFU/mouse) were used to establish the optimal challenge inoculum (i.e., to induce disseminated candidiasis in >95% of the mice [95% infective dose] without significant mortality). All neutropenic mice infected with an inoculum larger than 10^3 CFU/mouse died within 3 days after infection, while mice infected with 10^3 cells showed a 20% survival rate over an 8-day period. Animals infected with an inoculum greater than 5×10^4 all died hours after infection. Therefore, an inoculum of 10^3 CFU/mouse was considered optimal and was used for establishing candidal infections in this model.

Infected neutropenic mice were treated with antifungals i.p. once daily for 7 days, starting 4 h postinfection. The mice were randomly divided into four groups: untreated control, AmphB (0.75 mg/kg/day) treated, FLU (30 mg/kg/day) treated, and AmphB (0.75 mg/kg/day) plus FLU (30 mg/kg/day) treated. These selected antifungal dose regimens were designed to achieve serum drug levels which exceeded the *in vitro* MICs of both antifungal agents for the infecting *Candida* strain (4, 19). The expected serum FLU level in mice following treatment with a dose of 30 mg/kg/day is 25 µg/ml (7a). Based on these data, treating with this dose will result in supra-MIC levels for the infecting strain for the entire dosing interval.

Mice were sacrificed 1 day after the last treatment (day 8 postinfection) by cervical dislocation. The brains and kidneys were removed aseptically and weighed, and each organ was homogenized in 5 ml of NS. The homogenates were serially diluted and plated in Sabouraud dextrose agar (Difco) supplemented with 50 µg of chloramphenicol/ml to suppress bacterial contamination (United States Biochemical Corp., Cleveland, Ohio). The plates were incubated for 48 h at 35°C. Organ fungal densities were quantified as log₁₀ CFU per gram of tissue. Based on the tissue weight, a specific log₁₀ CFU per gram value was assigned for culture-negative lesions. For example, a tissue weighing 0.01 g was assigned a value of 2 log₁₀ CFU/g as the lower limit of detection.

Rabbit IE model. The rabbit model of *C. albicans* endocarditis used in this study has been described previously (21). Briefly, female New Zealand White rabbits weighing ca. 2.5 kg were used. Animals were caged separately and given food and water ad libitum. Rabbits were anesthetized by intramuscular injections of ketamine hydrochloride (Ketaset; Aveco Co., Inc., Fort Dodge, Iowa) at 35 mg/kg of body weight and xylazine (Rompun; Mobay Corp., Shawnee, Kans.) at 1.5 mg/kg. Sterile vegetations were induced on the aortic valve following transcardiotid-to-left-ventricular catheterization with a sterile polyethylene catheter (internal diameter, 0.86 mm; Becton Dickinson and Co., Parsippany, N.J.); the catheter remained in place for the duration of the study. At 48 h postcatheterization, *C. albicans* endocarditis was induced by injection of ca. 2×10^7 CFU intravenously via the marginal ear vein. This inoculum represents the 95% infective dose for this *Candida* strain in the model (21).

At 24 h postinfection, the rabbits were randomly divided into four groups: untreated control, AmphB (1 mg/kg/day intravenously [i.v.]) treated, FLU (100 mg/kg/day i.p.) treated, and treated with a combination of AmphB (1 mg/kg/day, i.v.) and FLU (100 mg/kg/day i.p.). Antifungal treatments were continued for either 7 or 14 days. Rabbits were sacrificed 24 h after the last antifungal dose to minimize antimicrobial carryover effects. The dose regimens were selected on the basis of several previous efficacy studies with AmphB and FLU monotherapy (21, 22). Following an i.p. dose of 100 mg of FLU/ml, the expected mean serum FLU level in the rabbit endocarditis model is 102 µg/ml (standard deviation, 32 µg/ml)

FIG. 1. Survival of neutropenic mice infected with *C. albicans* (10^3 CFU/mouse). *, $P < 0.001$ versus controls.

(21), a level more than 100 times higher than the MIC for the infecting *Candida* strain (36082). We have previously documented a very modest therapeutic effect of i.p. administered FLU in experimental *Candida* endocarditis (21, 22). This slow anticandidal effect *in vivo* by FLU monotherapy in this model allows for the detection of potential synergistic effects with other antifungals.

The rationale for initiating therapy earlier postinfection in the neutropenic-mouse model (4 h) versus the rabbit endocarditis model (24 h postinfection) relates to the relatively rapid onset of mortality in untreated mice with disseminated candidiasis versus the slower onset of mortality in untreated rabbits with *Candida* endocarditis.

On the assigned sacrifice day, rabbits were euthanized with 200 mg of pentobarbital sodium (Abbott Laboratories, North Chicago, Ill.) administered by rapid i.v. injection. Only animals with catheters in the proper transaortic valve position and macroscopic aortic valve vegetation were analyzed in this study. Cardiac vegetations from individual animals were removed, pooled, weighed, homogenized in 1 ml of NS, serially diluted, and quantitatively cultured on yeast potassium dextrose agar at 35°C for 8 h, and the mean log₁₀ CFUs per gram of vegetation ($\pm$ the standard deviation) were calculated. Similarly to the murine model, a specific log₁₀ CFU per gram value was assigned for culture-negative lesions based on the vegetation weight.

Statistics. Survival data for the neutropenic-mouse model of disseminated candidiasis between therapy groups were statistically compared by the Wilcoxon survival analysis, as lethality is the common outcome in this model. There were no differences in survival data in the IE model between untreated controls and drug-treated animals, since this is a nonlethal model of chronic candidal infection. Quantitative fungal densities in the therapy groups were compared statistically by the Kruskal-Wallis test with Tukey post hoc correction for multiple comparisons. Significance was defined as P being < 0.05.

RESULTS

Neutropenic-mouse model. Figure 1 summarizes the survival of the *C. albicans*-infected neutropenic mice. Treatment with AmphB or a combination of AmphB and FLU significantly prolonged the survival of animals compared to that of untreated controls ($P < 0.00001$ and $P = 0.001$, respectively). No significant differences in survival were observed between the groups receiving combined therapy and monotherapy with either AmphB or FLU ($P = 0.32$ and 0.13 , respectively). Although animals treated with FLU had a higher survival rate than the controls (43 versus 15%), this difference was not statistically significant ($P = 0.38$).

TABLE 1. Concentration of *Candida* in the kidneys and brains of neutropenic mice^a

Treatment group	Kidney (n)	Brain (n)
Control	5.80 ± 0.44 (8)	2.65 ± 1.26 (8)
AmphB	1.00 ± 0.53 ^b (18)	1.37 ± 0.60 (18)
FLU	2.63 ± 0.33 (9)	1.00 ± 0.74 ^b (9)
Combined	1.00 ± 0.98 ^b (12)	1.00 ± 0.42 ^b (12)

^a Mean log₁₀ CFU per gram (± standard deviation) after 7 days of treatment.^b *P* < 0.01 versus controls.

Table 1 summarizes the fungal densities of *Candida* in the kidneys and brains of infected neutropenic mice. AmphB monotherapy or AmphB combined with FLU yielded significant reductions in fungal densities in kidneys compared to those of untreated controls (*P* < 0.01). FLU monotherapy caused a substantial reduction in fungal densities in the kidneys compared to those of untreated controls. However, this reduction in CFUs was not statistically significant (*P* = 0.17). In contrast, treatment with FLU either alone or combined with AmphB caused significant reductions in *Candida* densities in the brain (*P* < 0.01 versus controls), while monotherapy with AmphB did not (*P* = 0.22). As shown in Table 1, the addition of FLU did not augment the activity of AmphB.

Rabbit IE model. Mean vegetation fungal densities from day 7 and day 14 of therapy are provided in Table 2. AmphB alone was significantly more effective at eradicating *Candida* from cardiac vegetations than FLU was. Addition of FLU to AmphB did not contribute further to the in vivo efficacy of AmphB. Importantly, no in vivo antagonism, but rather an indifferent effect, was observed between AmphB and FLU in this model.

DISCUSSION

Although use of combined antifungal therapy, particularly AmphB plus FLU, is an appealing alternative to monotherapy to improve the treatment outcome in invasive *Candida* infections, this issue remains controversial (17). This controversy is based on the specific modes of action of the azoles (e.g., FLU) and polyenes (e.g., AmphB). AmphB is believed to mediate its fungicidal effects by binding to target membrane sterols, while FLU acts by interfering with the synthesis of these same macromolecules. Therefore, theoretically, using these two agents concurrently may lead to antagonism.

In a previous study, we demonstrated that AmphB-FLU combinations had additive effects against *C. albicans* in vitro over a wide range of clinically relevant drug concentrations (7). Of critical importance, no in vitro antagonism was observed in combinations employing these two agents. To confirm these findings in vivo, we chose two distinct animal models: a neu-

tropenic-mouse model of hematogenously disseminated candidiasis and a rabbit model of IE. The murine model was selected because neutropenia is a significant risk factor for the development of hematogenously disseminated candidiasis (10) and adversely affects clinical outcome. The IE model was chosen because it is a rigorous test of antifungal efficacy. This latter model involves a large challenge inoculum for induction and results in high fungal densities at the site of infection (vegetations on traumatized cardiac valves), and the infection site is relatively devoid of neutrophils (localized neutropenia) (2), placing the entire burden for efficacy on the antifungal agent(s).

Our present study showed that the survival of neutropenic mice with disseminated candidiasis was prolonged when animals were treated with a combination of AmphB and FLU. Additionally, both AmphB monotherapy and AmphB-FLU therapy were more efficacious than FLU alone in clearing *Candida* from the kidneys. In contrast, FLU monotherapy and AmphB-FLU combined therapy were each more effective than AmphB in reducing the number of yeast cells in the brain. This latter finding may reflect the high penetrability of FLU into the central nervous system (cerebrospinal fluid) with or without inflammation (8, 13). Similarly, AmphB monotherapy and AmphB-FLU combined therapy were highly effective in reducing *Candida* densities in cardiac vegetations in the rabbit IE model.

Data from both models show that in general AmphB is more active than FLU. Furthermore, FLU alone or in combination with AmphB did not contribute further to the efficacy of AmphB. Although the survival of mice with disseminated candidiasis receiving combined therapy was somewhat lower than that of those receiving AmphB monotherapy, this effect did not reach statistical significance. Similar findings were reported by George and colleagues studying combination therapy in experimental invasive aspergillosis. These authors showed that, although no antagonism was seen when FLU was given prophylactically or therapeutically in combination with AmphB, combination therapy did not augment the antifungal activity of AmphB (6). Sugar et al. (18), using a murine model of acute invasive candidiasis, showed that combined therapy was superior to FLU alone, in an additive fashion, with no evidence of in vivo antagonism. In the same study, using a model of subacute invasive candidiasis, these investigators demonstrated that AmphB-FLU combinations exhibited an indifferent in vivo interaction without evidence of antagonism, similar to the findings in this study (18). However, despite the apparent lack of antagonism found in the current study, we recognize that the number of animals used may not be adequate to statistically exclude antagonism.

The lack of in vivo antagonism between AmphB and FLU demonstrated in this study and those of others (6, 17) may be explained in part by recent investigations of the mechanisms of action of AmphB and FLU. Bolard and Milhaud (3) suggested that the antifungal activity of AmphB does not solely depend on binding to ergosterol and poration of the fungal membrane. AmphB also appears to induce leakage of cytoplasmic material from the fungal cells without direct participation of ergosterol binding. Furthermore, the susceptibility of fungal cells to AmphB depends not only on the ergosterol content but also on the total phospholipid composition of the fungal membrane (3). The saturation of fatty acyl chains as it relates to lipid peroxidation may play a key role in the interaction of AmphB with the fungal membrane (3). In addition, we recently showed that FLU only partially inhibits ergosterol synthesis (44% inhibition when *C. albicans* was grown in the presence of one-half MIC), suggesting that residual membrane ergosterol is present in FLU-treated cells, providing an ample target for AmphB bind-

TABLE 2. Concentration of *Candida* in cardiac vegetations of rabbits^a

Treatment group	Mean colony count after treatment for:	
	7 Days (n)	14 Days (n)
Control	6.65 ± 0.37 (8)	6.61 ± 0.10 (7)
AmphB	4.34 ± 0.06 ^b (13)	4.36 ± 1.80 ^b (12)
FLU	6.23 ± 0.40 (9)	6.32 ± 0.54 (10)
Combined	5.10 ± 2.10 ^c (12)	4.90 ± 1.80 ^c (13)

^a Mean log₁₀ CFU per gram (± standard deviation).^b *P* < 0.001 versus controls on the respective sacrifice date.^c *P* < 0.005 versus controls and FLU-treated animals on the respective sacrifice date; not significant versus AmphB-treated animals alone.

ing (15). Collectively, these findings provide a rational theoretical basis for the lack of antagonism when these two agents are used concurrently. Of importance, clinical trials are under way for evaluating the treatment efficacy of AmphB and FLU combinations in cryptococcal meningitis and in disseminated candidiasis. The outcomes of these clinical trials will determine the utility of combined AmphB-FLU therapy in treating invasive human mycoses.

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