ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1991, p. 1721–1725
0066-4804/91/091721-05$02.00/0

Vol. 35, No. 9

Treatment of Murine Cryptococcal Meningitis with an SCH 39304-Amphotericin B Combination

MARGARET M. ALBERT,[1] JOHN R. GRAYBILL,[1]* AND MICHAEL G. RINALDI[2]

Department of Medicine and Research, Audie L. Murphy Memorial Veterans Hospital,[1] and Department of Pathology, University of Texas Health Science Center,[2] San Antonio, Texas 78284

Received 4 February 1991/Accepted 24 June 1991

Cryptococcal meningitis was induced in BALB/c mice by intracerebral infection with *Cryptococcus neoformans*. Drug therapy was initiated 1 day later, with mice receiving amphotericin B (AMB), SCH 39304, combination therapy, or no drug therapy (controls). Most, but not all, combinations showed additive benefits, significantly prolonging survival and reducing organism counts in tissues compared with those in controls and groups which received the drugs independently. Optimum protection was obtained when a single dose of 10 mg of AMB per kg of body weight was combined with a fairly narrow SCH 39304 dose range. AMB antagonism did not occur with any regimen tested. AMB-azole combinations may be reasonable alternatives for patients who fail standard cryptococcosis therapeutic regimens.

One of the most common life-threatening infections in patients with AIDS is cryptococcal meningitis (8, 14). Thirty percent of these patients fail to respond to the traditional drug combination of amphotericin B (AMB) and flucytosine (25), and therapy must be stopped in many patients because of the toxicity and severe side effects of the drugs (8, 24). Additionally, 50 to 65% of patients have relapses after an apparent initial therapeutic success. This has convinced many investigators that long-term and even lifelong suppressive antifungal therapy is necessary (1, 14). With no current way of predicting which patients will have relapses, maintenance therapy is recommended for all patients (2). With the marked increase in systemic mycoses, there has been a corresponding increase in the number and kinds of antifungal agents used to treat them.

In this study, we evaluated combination therapy with AMB and the new triazole SCH 39304 in murine cryptococcal meningitis. We sought to determine whether the efficacy of the combination is superior to the efficacy of each drug administered alone. Additionally, we compared the efficacy of one large dose of AMB followed by SCH 39304 therapy with that of multiple, smaller doses of AMB combined with SCH 39304 to determine whether the efficacy of AMB could be maintained but the duration of therapy could be shortened. Finally, we wished to determine whether pretreatment of mice with SCH 39304 would antagonize the effects of AMB.

MATERIALS AND METHODS

Animals. Six-week-old BALB/c, heterozygous (*nu*/+) mice were obtained from the colony at our institution. Mice were maintained at five mice per bonneted cage and had access to food and water ad libitum.

Pathogen. Clinical isolate 89–98 of *Cryptococcus neoformans* was used to challenge the mice. Three days prior to each trial, *C. neoformans* was subcultured onto Sabouraud dextrose agar plates and incubated at 37°C.

Challenge. Organisms were washed three times in sterile 0.9% saline, counted on a hemacytometer, and diluted to the desired count. Anesthetized mice (methoxyflurane; Metophane; Pitman-Moore, Washington Crossing, N.J.) were inoculated intracranially (direct, midline, 6 mm posterior to the orbit) with 0.06 ml per mouse. The total CFU per mouse ranged from 131 to 378. Counts were verified by serial dilution culture. Mortality from the infection procedure was less than 1%. Data for mice that died within 24 h of challenge were not included in the results.

Drugs. SCH 39304 (batch 88-39304-X-1) was obtained as a powder (Schering Pharmaceutical Corp., Bloomfield, N.J.) and was suspended in 0.3% Noble agar, and a homogeneous suspension was produced. Sonication was not required. Lyophilized AMB (Fungizone; E. R. Squibb and Sons, Princeton, N.J.) was reconstituted with sterile double-distilled water. Further dilutions were made with 5% glucose in water. AMB was protected from light at all points during processing. Drugs were administered in 0.2-ml volumes.

Statistics. The Kruskal-Wallis nonparametric test was used for comparison, with $P < 0.05$ considered significant.

Treatment. (i) Survival trials. At 24 h postchallenge, mice were randomized into treatment groups and drug therapy was initiated. Each group was made up of 10 mice. Except where noted, mice were treated for 10 days and followed through day 28. In treatment group 1, controls, mice were treated daily with 0.3% Noble agar orally and 5% glucose intraperitoneally (i.p.) at the same frequency at which AMB was administered within the same trial. In treatment group 2, SCH 39304 was administered once daily by gavage at doses of between 0.1 and 30.0 mg/kg of body weight. In single-dose trials (treatment group 3), AMB was administered i.p. once only, with doses ranging from 4 to 10 mg/kg of body weight. In multiple-dose trials, AMB was given i.p. three times a week at doses of 0.5 or 3.0 mg/kg of body weight. As combination therapy (treatment group 4), mice received both drugs at the same doses as those given to mice in the single-drug treatment groups.

(ii) Tissue burden studies. At 24 h postchallenge, mice were randomized into groups of five to six mice each and treatment was initiated. Brain tissue burden was determined at 42 h, 5 days, and 9 days postinfection. Controls were treated once with 5% glucose i.p. Mice received 0.3% Noble agar daily by gavage. The number of doses for the different

* Corresponding author.

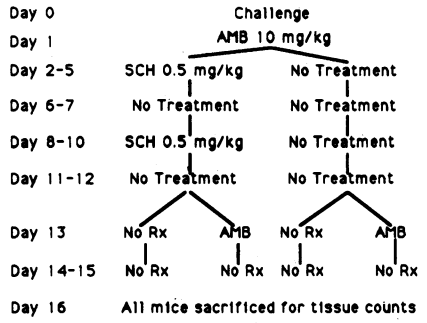

FIG. 1. Determination of antagonism of SCH 39304 on a second dose of AMB. Sch, SCH 39304 at 0.5 mg/kg/day; AMB, AMB at 10 mg/kg; No RX, no treatment, no drug therapy administered.

durations of treatment was as follows: 42-h study, one dose; 5-day study, three doses; and 9-day study, seven doses. In treatment group 2, AMB was administered i.p. in a single dose of 10 mg/kg of body weight. In treatment group 3, mice received SCH 39304 orally, at a dose of 0.5 mg/kg of body weight daily for 7 days. The SCH 39304 group existed only in the 9-day study. In treatment group 4, combination therapy, mice received AMB i.p. in a single dose of 10 mg/kg of body weight and SCH 39304 orally at a dose of 0.5 mg/kg of body weight; for the 42-h study, one dose was given; for the 5-day study, three doses were given; and for the 9-day study, seven doses were given.

A small tissue burden study was also performed to determine whether SCH 39304 would antagonize a second dose of AMB (Fig. 1). Mice received 0.5 mg of SCH 39304 per kg/day between single doses of 10 mg of AMB per kg. Tissue burden counts in these mice were compared with the counts of a parallel control group which received no treatment between AMB doses.

In all tissue burden studies, brains were removed 18 h after the final treatment, weighed, and homogenized in 2 ml of sterile saline; and 0.1 ml of serial dilutions was plated onto Sabouraud dextrose agar. Plates were counted after 3 days of incubation at 37°C.

Drug concentrations in serum. Two groups of 16 mice each were used to determine the drug concentrations in serum. A single dose of SCH 39304 at 0.50 mg/kg of body weight was administered to one group of mice. The other group received AMB at a dose of 10 mg/kg of body weight. Blood was collected, via cardiac puncture, from four anesthetized mice of each group at 1, 2, 8, and 24 h post drug administration. The concentrations of SCH 39304 in serum were determined by a gas-liquid chromatographic assay by using a slightly modified version of one described previously (6). AMB concentrations were determined by bioassay in antibiotic medium (21). The lowest concentration detectable by gas-liquid chromatography was 0.1 μg/ml, and the lowest concentration detectable by bioassay was 0.14 μg/ml.

MIC and MLCs. The MICs and minimum lethal concentrations (MLCs) of the two drugs were determined by a previously described broth macrodilution test (18).

RESULTS

As expected, MICs and MLCs of AMB were lower than those of SCH 39304. The MIC of AMB at 24 and 48 h was ≤0.14 μg/ml, whereas the MICs of SCH 39304 at 24 and 48 h were 40.0 and >80.0 μg/ml, respectively. MLCs showed a similar pattern, with the MLC of AMB at 24 and 48 h being ≤0.14 and ≤2.31 μg/ml, respectively. The MLC of SCH 39304 was >80.0 μg/ml at both time intervals.

TABLE 1. Survival studies for AMB administered in multiple doses

Treatment group (dose)[a]	Mean survival (days) for mice treated in[b]:		
	Trial 1	Trial 2	Trial 3
Controls	9.5 (8–12)	11.3 (9–15)	13.9 (10–19)
SCH 39304			
0.25			15.6 (14–19)
0.50		19.5 (17–22)[c]	
30.00	25.5 (22–36)[d]		
AMB			
0.50		14.5 (11–19)[e]	15.5 (12–27)
3.00	18.9 (15–20)[e]		
Combination			
SCH 39304 (0.25) + AMB (0.5)			18.2 (15–24)[c]
SCH 39304 (0.50) + AMB (0.5)		18.4 (16–21)[e]	
SCH 39304 (30.0) + AMB (3.0)	27.6 (24–36)[d]		

[a] SCH 0.25, 0.50, and 30.0, SCH 39304 at doses of 0.25, 0.50, and 30.0 mg/kg/day, respectively; AMB 0.5 and 3.0, AMB at doses of 0.5 and 3.0 mg/kg three times weekly, respectively.

[b] Each mouse in trials 1 to 3 was challenged with 131 to 330 CFU of *C. neoformans*; survival ranges are indicated in parentheses; There were 9 or 10 mice per group.

[c] $P < 0.05$ compared with all other groups.

[d] $P < 0.05$ compared with controls and AMB-treated mice.

[e] $P < 0.05$ compared with controls.

Levels of the drugs in serum were determined at 1, 2, 8, and 24 h after administration of a single dose of SCH 39304 at 0.5 mg/kg of body weight or AMB at 10 mg/kg of body weight. The peak level of AMB in serum was reached 2 h after dosing, with a mean ± standard error of the mean of 1.94 ± 0.11 μg/ml. SCH 39304 reached levels of 0.37 ± 0.036 μg/ml at 1 h posttreatment, with prolonged high levels of drug remaining throughout the 24-h test period.

Multiple AMB dose survival studies. Table 1 shows results of three survival trials done with *nu*/+ mice. In all trials, AMB was given three times per week at doses of 0.5 or 3.0 mg/kg of body weight (the weekly cumulative doses were 1.5 or 9 mg/kg of body weight, respectively).

In the first trial, mice were challenged with 213 CFU per mouse. The combination therapy group, as well as the groups which received each drug independently, survived significantly longer than controls did. SCH 39304 and the combination significantly prolonged survival over that of AMB alone, with several mice in these groups surviving well past the 28-day follow-up period; however, all mice eventually died. The combination did not show superiority over SCH 39304 at the high dose (30 mg/kg) used in this trial.

In the second trial, mice were challenged with 330 CFU per mouse and were treated with lower doses of both SCH 39304 and AMB. All drugs provided prolongation of survival in these mice over that in controls; SCH 39304 was superior to AMB and the combination.

In trial 3, mice were challenged with 131 CFU per mouse. Only mice which received the combination of SCH 39304 and AMB showed prolonged survival (4 to 5 days mean prolongation of survival) over controls.

Single AMB dose survival studies. The results of four

TABLE 2. Survival studies for AMB administered in a single large dose

Treatment group (dose)[a]	Mean survival (days) for mice treated in[b]:			
	Trial 4	Trial 5	Trial 6	Trial 7
Controls	10.8 (8–14)	10.1 (8–12)	10.3 (8–14)	12.8 (8–20)
SCH 39304				
0.10				11.2 (7–17)
0.25			16.0 (13–17)[c]	
0.50		13.9 (11–18)[c]		
1.00	18.4 (17–21)[c]			
AMB				
4.0				15.1 (11–21)
5.0			15.1 (9–22)[c]	
10.0	16.7 (11–23)[c]	16.8 (12–21)[d]		
Combination				
SCH 39304 (0.10) + AMB (4.0)				14.8 (11–18)
SCH 39304 (0.25) + AMB (5.0)			16.9 (14–19)[c]	
SCH 39304 (0.50) + AMB (10.0)		20.6 (18–23)[e]		
SCH 39304 (1.00) + AMB (10.0)	21.6 (19–26)[f]			

[a] SCH 0.10, 0.25, 0.50, and 1.00, SCH 39304 at doses of 0.10, 0.25, 0.50, and 1.00 mg/kg/day, respectively; AMB 4.0, 5.0, and 10.0, AMB at doses of 4.0, 5.0, and 10.0 mg/kg given as a single dose on the first day of drug therapy.
[b] Each mouse in trials 4 to 7 was challenged with 197 to 378 CFU of *C. neoformans*; survival ranges are indicated in parentheses. Trials 4, 6 and 7 had 8 to 10 mice per group. Trial 5 had 19 to 20 mice per group.
[c] $P < 0.05$ compared with controls.
[d] $P < 0.05$ compared with controls and SCH 39304-treated mice.
[e] $P < 0.05$ compared with all other groups.
[f] $P < 0.05$ compared with controls and AMB-treated mice.

survival trials done with *nu*/+ mice are summarized in Table 2. In all trials, AMB was administered only once, at 24 h postchallenge, at doses of 4, 5, or 10 mg/kg of body weight.

In trial 4, mice were challenged with 197 CFU per mouse. Mice in the SCH 39304 and AMB groups outlived controls, but there was no significant difference between SCH 39304 and AMB. Mice in the combination group lived significantly longer than controls and AMB-treated mice but not SCH 39304-treated mice.

In trial 5, mice were challenged with 275 CFU per mouse. As in the fourth trial, all groups receiving drugs showed prolonged survival over that in controls. AMB modestly prolonged survival over that provided by SCH 39304, but the best survival benefit was conferred by the combination.

In trial 6, mice were challenged with 378 CFU per mouse. There were no significant differences between SCH 39304, AMB, or the combination, although all provided a survival benefit in treated mice over that in controls.

In the seventh trial, the challenge was 207 CFU per mouse. There were no significant differences in survival between controls and any of the treatment groups.

Tissue burden studies. Table 3 summarizes the results of the 42-h and 5- and 9-day tissue burden studies. Because of an error, the inocula in these studies were quite high, with mice in the 42-h and 5-day study receiving 1,260 CFU per mouse and those in the 9-day study receiving 4,800 CFU per mouse. Nevertheless, AMB and SCH 39304 decreased the fungal burden, with the mice that received the combination having a significantly lower fungal population compared with those in controls and in those that received each drug alone.

In our small antagonism study, quantitative cultures reflected no significant difference between colony counts in

TABLE 3. Colony counts in brain tissue of mice postchallenge

Treatment group (dose)[a]	Mean colony count (CFU/g) in brain from mice sacrificed at[b]:		
	18 h[c]	4 days[c]	8 days[d]
Controls	1.7×10^3 (5.3×10^2–3.4×10^3)	1.4×10^7 (6.3×10^6–2.4×10^7)	2.8×10^{10} (2.4×10^8–7.9×10^{10})
SCH 39304 (0.5)	ND[e]	ND	2.5×10^9 (4.9×10^7–7.5×10^9)
AMB (10.0)	2.7×10^2[f] (0–9.7×10^2)	1.8×10^4[f] (2.2×10^3–7.5×10^4)	4.3×10^7[g] (7.8×10^6–8.6×10^7)
Combination of SCH 39304 (0.5) + AMB (10.0)	4.8×10^2[f] (0–1.3×10^3)	2.4×10^3[i] (4.1×10^2–8.2×10^3)	8.8×10^5[h] (9.3×10^4–2×10^6)

[a] SCH 0.5, SCH 39304 at a dose of 0.5 mg/kg of body weight; AMB 10, AMB at a dose of 10 mg/kg of body weight.
[b] There were five to six mice per group; ranges are indicated in parentheses.
[c] Each mouse was challenged with 1.3×10^3 CFU of *C. neoformans*.
[d] Each mouse was challenged with 4.8×10^3 CFU of *C. neoformans*.
[e] ND, not done.
[f] $P < 0.05$ compared with controls.
[g] $P < 0.05$ compared with controls and SCH 39304- and combination-treated mice.
[h] $P < 0.05$ compared with controls and SCH 39304- and AMB-treated mice.
[i] $P < 0.05$ compared with controls and AMB-treated mice.

mice that were treated with SCH 39304 and those that were untreated between AMB doses.

DISCUSSION

The results of this study suggest that AMB and SCH 39304 interact favorably against murine cryptococcosis. Optimum protection was obtained when a single dose of 10 mg of AMB per kg was used and was combined with a fairly narrow SCH 39304 dose range.

The in vitro MIC and MLC results appear to indicate that AMB is superior to SCH 39304, with lower concentrations of AMB being required to inhibit and kill the fungus. However, the correlation between in vitro and in vivo results is uncertain at best (17, 30).

There are at least three possible reasons why the regimen which combined a single dose of 10 mg of AMB per kg with daily doses of 0.5 mg of SCH 39304 per kg was superior to the other combinations tested in this study. First, the tissue burden studies showed a rapid, significant drop in the fungal population after administration of the single dose of 10 mg of AMB per kg. At 42 h, there was no significant difference between the AMB- and AMB-SCH 39304-treated groups. However, by day 5 and through day 9, the AMB-SCH 39304-treated group had significantly lower colony counts than did the control or AMB-treated group. Thus, SCH 39304 appeared to consolidate the AMB dose. These results suggest that the protective effects of this combination results from sequential activity, not synergism, and are consistent with evidence that polyene antifungal agents take effect more rapidly than do the antifungal azoles (3, 19). It is plausible that the large dose of AMB reduced the fungal burden to such a point that the remaining fungi were easily managed by low SCH 39304 doses.

Second, a study on *Candida albicans* performed by Sokol-Anderson et al. (22) showed that a single, large dose of AMB produces a minimal increase in catalase activity, whereas multiple doses significantly increase activity. It is proposed that catalase neutralizes the reactive forms of oxygen within fungi, thus rendering one of AMB's lytic and lethal mechanisms ineffective (11, 22, 23). A similar catalase increase in *C. neoformans* could explain the superior efficacy obtained by using the single dose of 10 mg of AMB per kg compared with those obtained by using multiple doses.

Third, there is in vitro evidence that the fungicidal effects of AMB are dose dependent (7). Although we did not determine fungicidal effects in this study, our results support a dose association, with the efficacy of a single AMB dose at 10 mg/kg of body weight being clearly superior to single doses of 4 or 5 mg/kg of body weight.

In prior experiments in which AMB and azoles were combined, the results were mixed. Studies of murine cryptococcosis performed by Graybill et al. (4) and Polak et al. (15) showed a therapeutic advantage to concurrent therapy with AMB and ketoconazole. However, a study of murine aspergillosis by Schaffner and Frick (20) showed AMB antagonism when mice were pretreated with ketoconazole. On the basis of the results of those studies, two possibilities exist. First, AMB antagonism may be fungus specific, because antagonism has been demonstrated only in *Aspergillus* species (15, 20). Second, the sequence of exposure to the drugs may be vital to the outcome. It is proposed that azole suppression of cytochrome P-450 activity blocks the demethylation pathway which converts 14-α-methylsterol to ergosterol and alters the primary sterol content in fungal cell membranes (26, 27). Thus, azoles could deprive AMB of its ergosterol binding site and, hence, some of its damaging and lethal effects on fungi (5, 28). Results of our small antagonism study indicate that AMB antagonism, by azole pretreatment, does not occur in murine cryptococcosis. However, true confirmation will require additional studies with larger numbers of animals.

An AMB-azole combination regimen similar to the one evaluated in this study may be an alternative for patients who fail traditional drug regimens against cryptococcosis. The abbreviated high-dose course of AMB would allow the use of this highly effective drug for the treatment of fungal infections, including mycoses of the central nervous system (12), with a potential decrease in toxicity. We experienced no acute deaths after administration of AMB at 10 mg/kg. However, this dose could not feasibly be administered to humans. Equivalent levels might be obtained in humans by using liposomal AMB, which has been found to be less toxic and more easily administered than free AMB (9, 10, 13). Doses of 3 mg/kg of body weight per day have been successfully administered to humans (9), and doses as high as 5 mg/kg of body weight given three times per week are being administered to select patients, with no severe side effects to date (3a).

An additional advantage of the combination may be elimination of the use of AMB for maintenance therapy. AMB is problematic because of the high incidence of life-threatening bacterial infections associated with the intravenous devices required for its administration (16). The longer half-life, oral dosing, and minimal toxicity associated with the azoles make them better candidates for maintenance therapy. Unfortunately, SCH 39304 may not be one of those candidates. After many promising animal and clinical studies (17, 21a, 29), SCH 39304 has been found to be oncogenic in animals, and the clinical status of the drug is pending. SCH 39304 is being provided on a compassionate-use basis, and in the future it may be administered as an orphan drug to those patients who fail therapy with other antifungal drug regimens.

ACKNOWLEDGMENTS

This work was supported by Schering Pharmaceutical Corp.

We gratefully acknowledge the technical assistance of Angelique J. Marquis, Annette W. Fothergill, Dianna A. McGough, and the staff of the Veterinary Medical Unit at Audie L. Murphy Memorial Veterans Hospital.

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