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In Vitro and In Vivo Activities of Sch 39304, Fluconazole, and Amphotericin B against *Histoplasma capsulatum*

G. S. KOBAYASHI,[1]* SHARON J. TRAVIS,[1] MICHAEL G. RINALDI,[2] AND G. MEDOFF[1]

Division of Infectious Diseases, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri 63110,[1] and Department of Pathology, University of Texas Health Science Center, San Antonio, Texas 78284[2]

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The antifungal activities of amphotericin B and two triazoles, Sch 39304 and fluconazole, were tested against *Histoplasma capsulatum*. In this study Sch 39304 compared favorably with amphotericin B in treating histoplasmosis in normal and leukopenic mice, whereas fluconazole was much less active. The differences in the efficacies of the triazoles appeared to be due to differences in their pharmacokinetics and the dosage schedule that was used. For amphotericin B there was a good correlation between in vitro and in vivo efficacy, but this was not true of the triazole derivatives. These results further demonstrate that, with the methods used in this study, in vitro susceptibility testing of triazoles may not be predictive of in vivo activity against isolates of *H. capsulatum*.

Amphotericin B (AmB) is the most effective agent for treating systemic mycotic diseases; however, toxicity and problems associated with its administration lessen its attractiveness. We previously showed that the nontoxic triazole derivative fluconazole (FLU) given orally was as effective as AmB given intraperitoneally (i.p.) in reducing the mortality of immunocompetent (11) and immunosuppressed (12) mice infected with *Histoplasma capsulatum*. The recent development of a new oral active triazole derivative, Sch 39304, which has improved pharmacokinetics in animals and humans (C. Lin, H. Kim, A. Lapiguera, D. Loebenberg, G. H. Miller, and S. Symchowicz, Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 163, 1988; W. Kramer, H. Kim, S. Symchowicz, G. Perentesis, M. Affrime, and C. Lin, 28th ICAAC, abstr. no. 165, 1988), prompted us to compare it with FLU.

MATERIALS AND METHODS

Organisms. Clinical isolates of *H. capsulatum* (A, B, C, D, E, F, G, and 3) were obtained from the Barnes Hospital Mycology Laboratory (St. Louis, Mo.). Strains G217B (ATCC 26032), G222B (ATCC 26034), G184B (ATCC 26028), and G186B (ATCC 26030) were obtained from the American Type Culture Collection (Rockville, Md.). All isolates were converted to the yeast-phase morphology by culturing them in 2% glucose–1% yeast extract (GYE) broth incubated at 37°C. Cultures were transferred to fresh medium every 5 days.

Preparation of inocula for susceptibility testing and animal infections. Clinical isolates of *H. capsulatum* were grown to the mid-log phase in GYE broth that was maintained at 37°C with constant agitation. Cell suspensions for inocula were quantitated with a hemacytometer, and viable units were determined by colony counts on GYE agar incubated at 30°C.

Compounds. Micronized Sch 39304 (inventory, 870294001; batch, 20428-105) was provided by David Loebenberg of Schering Plough Corp. (Bloomfield, N.J.). FLU (lot no. R-9) was provided by Anthony K. Knirsch (Pfizer Inc., Groton, Conn.). AmB (Fungizone; control no. 7J97498; E. R. Squibb & Sons, Princeton, N.J.) was purchased. Stock suspensions of Sch 39304 and FLU were suspended in sterile distilled water, and AmB was made up in 5% glucose just prior to use.

Susceptibility studies. Antifungal susceptibility studies were conducted with unbuffered Sabouraud dextrose broth (pH 5.6) dispensed into microdilution plates by previously described procedures (25). Susceptibility studies were also done in buffered Sabouraud dextrose broth (pH 7.0) by the same procedure (25). The MIC was defined as the lowest concentration of drug that inhibited multiplication of the yeasts, as determined by the absence of turbidity. Readings were taken when visible growth was detected in control wells (96 to 120 h).

Animals. Female CF_1 mice (age, 6 to 8 weeks; average weight, 23 g) were purchased from Charles River Mouse Farms (Wilmington, Mass.). Prior to experimentation, the mice were acclimatized to the laboratory environment for 1 week. They were housed in groups of five mice per cage and were fed and given water ad libitum.

Experimental therapy. As described previously (10–12), *H. capsulatum* G217B was used to establish our murine model of histoplasmosis. Immunocompetent mice were injected via the tail vein with 5×10^6 yeast cells per mouse. Treatment of mice was begun 24 h after infection. Mice were given graded doses of Sch 39304 or FLU by gavage once a day for a total of 6 consecutive days. The infected mice treated with AmB were given the drug by the i.p. route every other day for a total of six injections. The animals were observed daily for 28 days after the start of therapy, and the number of survivors was recorded.

In the studies evaluating the effect of immunosuppression on the treatment regimens, CF_1 mice were initially rendered leukopenic by a modification of the procedure of Cryz et al. (2). Four successive i.p. injections of 100 mg of cyclophosphamide in 0.2 ml of phosphate-buffered saline (pH 7.4) were given 6, 4, 2, and 0 days before intravenous infection with *H. capsulatum* (12). Leukopenia was quantitated by collecting a sample of blood from the tail vein into a leukocyte pipette and diluting it immediately with 9 volumes of leukocyte diluent followed by counting in a hemacytometer (1). Within 1 h following the final treatment with cyclophosphamide, the mice were infected with 4.5×10^5 to 6.4×10^5 yeasts of *H. capsulatum* G217B. Antifungal regimens were begun 24 h

* Corresponding author.

TABLE 1. Susceptibilities of *H. capsulatum* isolates to AmB, FLU, and Sch 39304

Isolate	MIC (μg/ml)[a]		
	AmB	FLU	Sch 39304
A	0.66 (0.39–0.78)	10.2 (7.8–15)	10.2 (7.8–15)
B	0.98 (0.78–1.56)	15 (15)	15 (15)
C	0.91 (0.39–1.56)	4.17 (3.9–7.8)	4.17 (3.9–7.8)
D	0.49 (0.39–0.78)	>1,000 (>1,000)	>1,000 (>1,000)
E	0.33 (0.2–0.39)	17.93 (7.8–31)	17.93 (7.8–31)
F	1.04 (0.78–1.56)	>1,000 (>1,000)	>1,000 (>1,000)
G217B	0.65 (0.39–0.78)	0.78 (0.78)	0.78 (0.78)
G222B	0.46 (0.39–0.78)	10.2 (7.8–15)	10.2 (7.8–15)
G184B	0.52 (0.39–0.78)	36.33 (15–63)	36.33 (15–63)
G186B	0.65 (0.39–0.78)	20.33 (15–31)	20.33 (15–31)
G	0.58 (0.39–0.78)	23 (15–31)	23 (15–31)
3	0.30 (0.2–0.39)	2.95 (2–3.9)	2.95 (2–3.9)

[a] Values are averages of three tests. Values in parentheses are the minimum and maximum values from three tests.

after mice were infected, and evaluations were performed as described above for immunocompetent animals.

A comparison of the efficacy of Sch 39304, FLU, and AmB in treating mice infected with an isolate of *H. capsulatum* that exhibited in vitro resistance (≥1,000 μg/ml) to the triazole derivatives was also conducted. Groups of mice were infected with 5×10^6 yeasts of the F strain of *H. capsulatum*, and therapy was begun 24 h after infection in the manner described above.

All therapy experiments were repeated at least three times, and there were either 5 or 10 mice per treatment group. At the termination of the experiments, surviving mice were necropsied and their spleens were removed. Each spleen was minced and cultured on agar (Mycosel; BBL Microbiological Systems, Cockeysville, Md.) and incubated at 25°C. The cultures were examined daily for growth of *H. capsulatum*, and cultures showing no growth were kept for 4 weeks before they were discarded.

Procedure for assay of triazoles in serum. Groups of five uninfected CF_1 mice were given a single or six daily oral treatments of either Sch 39304 or FLU at a dose of 20 mg/kg per day and were bled 24 h after the final treatment. Assay of FLU in serum was done by the megabore column gas-liquid chromatographic method described by Harris et al. (8) by using UK 54,373 [1-fluoro-2-(2,4-difluorophenyl)1,3-bis(1*H*-1,24-triazole-1-yl)-2-propanol] as the internal standard. Levels of Sch 39304 in serum were determined by a megabore column gas-liquid chromatographic procedure described by Lin et al. (28th ICAAC, abstr. no. 163, 1988).

Statistical comparisons. Therapeutic efficacies were compared by Student's *t* test, and significance was defined as $P < 0.05$.

RESULTS

In vitro susceptibility studies. The MICs of Sch 39304 and FLU determined in unbuffered Sabouraud dextrose broth (pH 5.6) against the same isolates of *H. capsulatum* were identical and ranged from 2.95 to ≥1,000 μg/ml. Susceptibilities of Sch 39304, FLU, and AmB determined in buffered Sabouraud dextrose broth (pH 7.0) yielded the same rank order for each of the isolates, although the MICs differed slightly (data not shown). In either unbuffered or buffered Sabouraud dextrose broth, isolates D and F grew as well as the control at all concentrations of Sch 39304 and FLU tested and appeared to be resistant to the triazoles by this in vitro assay (Table 1). In either buffered or unbuffered medium, the MIC of AmB against the different isolates ranged from 0.30 to 1.09 μg/ml (Table 1) and exhibited sharp endpoints.

Experimental therapy. Untreated CF_1 mice injected with 5×10^6 yeasts of *H. capsulatum* G217B were all dead by 10 days after infection (Fig. 1a). At doses of AmB ranging from 2.5 to 10 mg/kg per day, all of the infected mice survived for at least 28 days postinfection. At doses of 1.25 and 0.62 mg of AmB per kg, there were 80 and 40% survivors, respectively.

Ninety-five percent of infected animals treated with 20 or 10 mg of Sch 39304 per kg per day survived, and at doses of 5 mg/kg per day there were 90% survivors (Fig. 1b). At doses of 2.5 and 1.25 mg/kg per day, the percentage of survivors decreased to 30 and 15%, respectively.

One hundred percent of the animals died 8 to 11 days after infection and treatment with doses of FLU ranging from 1.25 to 5.0 mg/kg per day. The percentage of animals that survived after treatment with 10 and 20 mg of FLU per kg per day was only 7.5 and 15%, respectively (Fig. 1c).

Mice that were made leukopenic by treatment with cyclo-

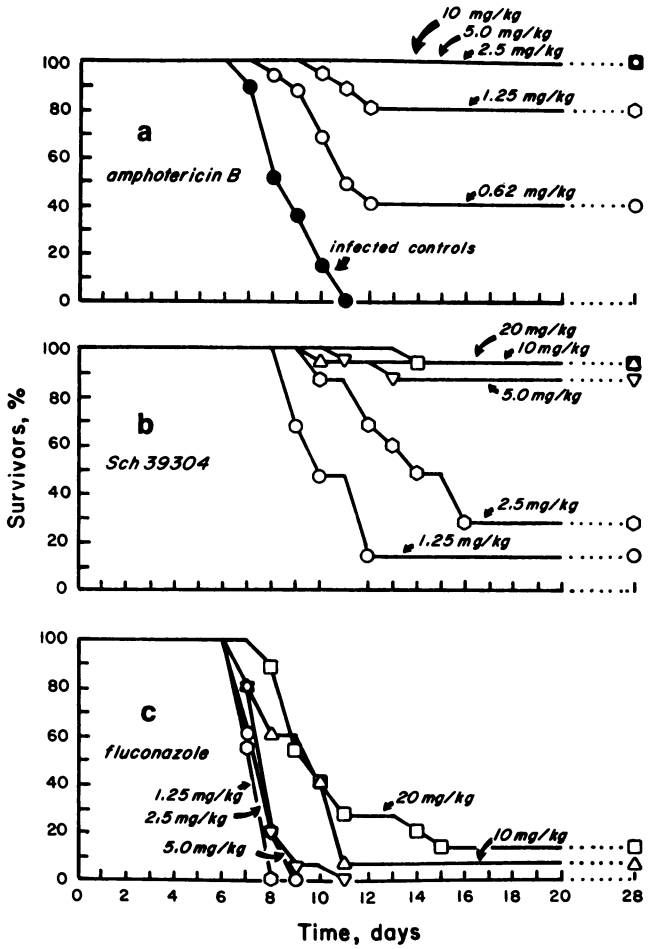

FIG. 1. Survival curves of normal CF_1 mice intravenously infected with *H. capsulatum* G217B and treated i.p. with the indicated dosages of AmB on alternate days for a total of six treatments (a) or orally with the indicated dosages once a day for 6 consecutive days with either Sch 39304 (b) or FLU (c). Antifungal treatments were begun 24 h after mice were infected. Solid circles (a) are infected and sham-treated controls.

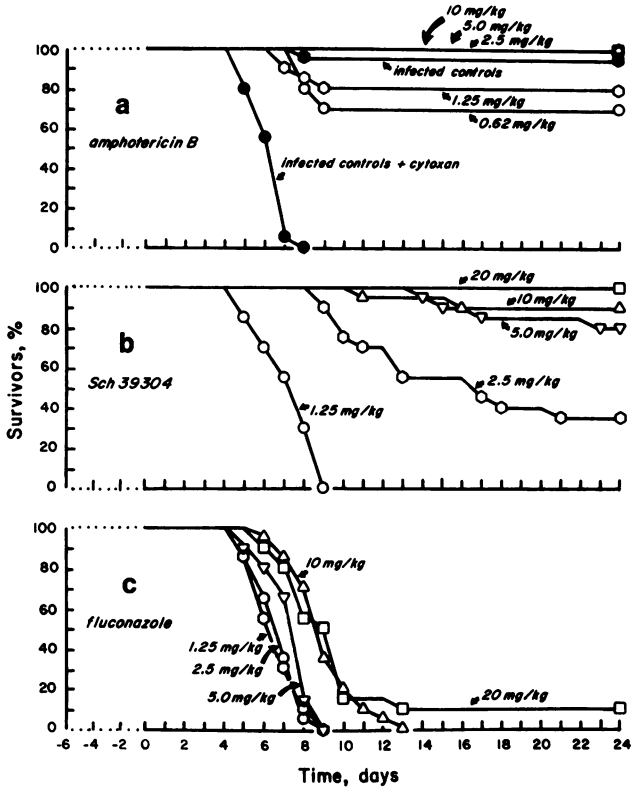

FIG. 2. Survival curves of cyclophosphamide-induced leukopenic CF_1 mice infected with *H. capsulatum* G217B and treated i.p. with the indicated dosages of AmB on alternate days for a total of six treatments (a) or orally with the indicated dosages once a day for 6 consecutive days with either Sch 39304 (b) or FLU (c). Antifungal treatments were started 24 h after mice were injected. Solid circles (a) are infected and sham-treated controls.

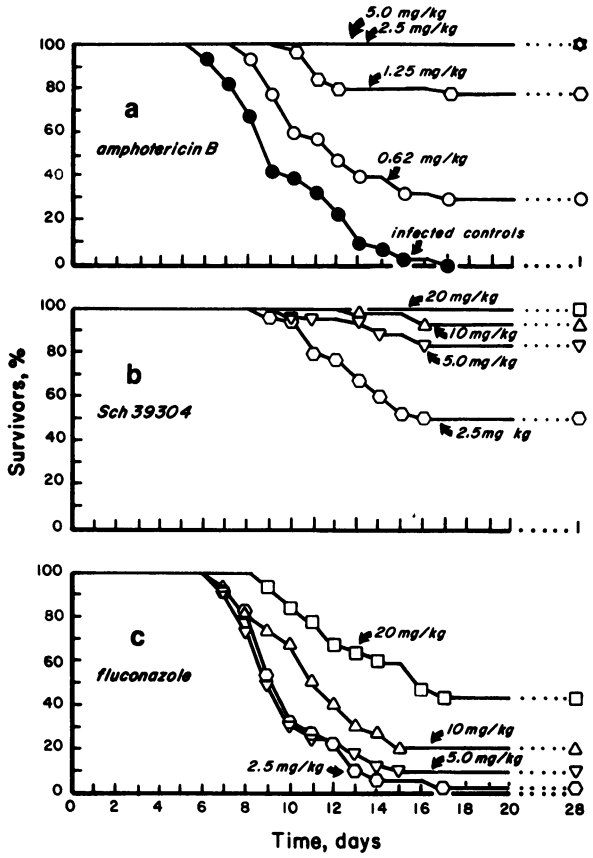

FIG. 3. Survival curves of normal CF_1 mice intravenously infected with *H. capsulatum* F (which exhibited in vitro resistance to Sch 39304 and FLU) and treated i.p. with the indicated dosages of AmB on alternate days for a total of six treatments (a) or orally with the indicated dosages once a day for 6 consecutive days with either Sch 39304 (b) or FLU (c). Antifungal treatments were begun 24 h after mice were infected. Solid circles (a) are infected and sham-treated controls.

phosphamide had less than 250 leukocytes per mm^3, and the count remained at this level for at least 4 days after the last dose of drug was administrated. The leukopenic mice were more susceptible to infection, as evidenced by the approximate 10-fold reduction in the inoculum which killed 100% of the mice (from 5×10^6 to 5×10^5 yeasts of *H. capsulatum* G217B). Leukopenic control animals died more rapidly even with the lower inoculum than normal infected mice did; 100% of the leukopenic mice were dead by day 6 after infection, whereas 100% of infected normal mice were dead by day 11 after infection. Despite the increased susceptibility of the leukopenic mice to *H. capsulatum* G217B, both AmB and Sch 39304 were still very effective in treating the infected animals (Fig. 2a and b), whereas FLU, at the dosages and schedule used in this study, was, again, not effective (Fig. 2c).

Pharmacokinetics of Sch 39304 and FLU in mice. The level of FLU in serum 24 h after one oral dose of 20 mg/kg was 0.19 μg/ml, whereas for Sch 39304 it was 0.75 μg/ml. At 24 h after six daily 20-mg/kg doses of FLU, the level in serum was 0.48 μg/ml, while for Sch 39304 the level in serum was 1.65 μg/ml.

Therapy of mice infected with *H. capsulatum* F, which exhibited in vitro resistance against the triazoles. Strain F, which was resistant to at least 1,000 μg of Sch 39304 and FLU per ml (Table 1), was less virulent for normal CF_1 mice than *H. capsulatum* G217B was. At equivalent infecting doses, strain F required 17 days after infection to achieve 100% death (Fig. 3a), as compared with 10 days for strain G217B (Fig. 1a). By using the regimen described above, treatment with 5 and 2.5 mg of AmB per kg per day resulted in 100% survival (Fig. 3a). At doses of 1.25 and 0.62 mg/kg given under the same regimen, 73.3 and 26.7% survivals, respectively, were achieved. These dose-response results were similar to those obtained with strain G217B.

All of the mice that received 20 mg of Sch 39304 per kg per day survived (Fig. 3b). Survival rates for groups of mice that were given 10, 5.0, and 2.5 mg/kg per day were 93.3, 83.3, and 50%, respectively. By using this drug regimen, FLU was also effective, leading to 43.3, 20, 10, and 3.3% survivals at doses of 20, 10, 5, and 2.5 mg/kg per day, respectively, for 6 consecutive days (Fig. 3c). In vitro susceptibility studies indicated that strain F was resistant to Sch 39304 and FLU compared with strain G217B (Table 1). However, at comparable dosages, both triazoles were effective in treating mice infected with either the susceptible G217B or F strains (Fig. 1 and 3). It is apparent from the results of these experiments that despite in vitro resistance to the triazoles, infections with *H. capsulatum* F responded to treatment regimens with these agents, which resulted in levels in blood far below the MICs of each drug. As with the mice infected with *H. capsulatum* G217B, Sch 39304 was more effective than FLU.

DISCUSSION

AmB has been the most effective agent for the treatment of life-threatening histoplasmosis (28). This was confirmed in our animal studies (Fig. 1a, 2a, and 3a). However, AmB has solubility problems, must be given parenterally, and is toxic. The discovery of the antifungal activity of the azole derivatives provided a large number of compounds that can be used as alternatives to AmB (4, 22). The derivatives with an imidazole nucleus represented the first major group of antifungal agents that were found to be active. Unfortunately, problems with solubility, toxicity, potency, and spectrum of activity have limited their usefulness. Antifungal derivatives with the triazole nucleus appear to be more promising. They are less toxic and have a broader spectrum of activity. FLU, an oral triazole antifungal agent, is soluble in water; has a high degree of bioavailability in humans; penetrates well into cerebrospinal fluid (17, 22, 23); is excreted unchanged in urine (17); and has been effective in treating experimental cryptococcosis (3, 16, 18, 27), coccidioidomycosis (7), histoplasmosis (6, 11, 12, 19), blastomycosis (13), candidiasis (9, 18, 20, 21, 23, 26), and aspergillosis (27).

As the number of antifungal agents increases, the requests for correlative in vitro susceptibility studies to predict clinical outcome have also increased. Unfortunately, there have been several problems associated with azole susceptibility testing (6, 11, 12, 15, 21, 24) and fungal susceptibility tests in general. In our study some of the isolates of *H. capsulatum* did not have sharp endpoints and exhibited discernible degrees of growth with increasing concentrations of the triazole derivatives. Odds (14) described this phenomenon and termed it "tailing" and ascribed it to the pH and composition of the test medium. In our study tailing was observed in unbuffered medium at pH 5.6 and buffered medium at pH 7.0. A recent review (5) discussed contributing factors such as preparation of standardized inocula, the pH of the medium, and the lack of sharp endpoints.

Sch 39304, a new oral triazole derivative, was as effective as AmB given parenterally in treating disseminated murine histoplasmosis at all doses tested ($P < 0.05$). Furthermore, Sch 39304 was significantly more active than FLU, to which it is structurally related, in treating normal and leukopenic mice infected with *H. capsulatum* ($P < 0.05$). We previously reported that FLU given orally was as effective as AmB given i.p. in treating histoplasmosis in mice (11, 12). The present in vivo results show that despite identical in vitro MICs, the same doses of Sch 39304 and FLU resulted in markedly different survival rates of mice infected with the same strain of *H. capsulatum*. In both normal and leukopenic infected mice, Sch 39304 was much more effective than FLU. We believe that the discrepancy between the in vitro and in vivo results with Sch 39304 and FLU may be partially explained by the differences in the pharmacokinetics between the two agents. While the half-life ($t_{1/2}$) for Sch 39304 in mice is 5.5 h compared with 5.1 h for FLU, the area under the curve extrapolated to infinity (AUC) for Sch 39304 is 187 μg · h/ml compared with 106 μg · h/ml for FLU (Lin et al., 28th ICAAC, abstr. no. 163, 1988). We further showed that at 24 h after a single oral dose of 20 mg/kg there was four times more Sch 39304 than FLU in serum and that after six daily doses of the triazoles given once each day, this difference was maintained (0.48 μg/ml for FLU and 1.65 μg/ml for Sch 39304).

In the present study, FLU was not as effective as AmB in treating murine histoplasmosis, as compared with our previous observations (11, 12). The apparent discrepancy can be attributed to the difference in the therapeutic regimen. In the previous studies, FLU was given twice a day, once in the morning and once in the afternoon, for 6 consecutive days, whereas in the present study FLU and Sch 39304 were given once daily for 6 days. Our data indicate that because of its pharmacokinetics, Sch 39304 may be a more desirable agent for treatment of histoplasmosis than FLU. The $t_{1/2}$ values and AUCs of Sch 39304 in humans are two times those of FLU (Kramer et al., 28th ICAAC, abstr. no. 165, 1988; unpublished data). It is apparent from the results of these experiments that despite in vitro resistance to the triazoles, infections with *H. capsulatum* F responded to treatment regimens with these agents, which resulted in levels in blood far below the MICs of each drug. As with the mice infected with *H. capsulatum* G217B, Sch 39304 was more effective than FLU. However, none of the drugs, including AmB, cured the surviving animals, since *H. capsulatum* was cultured from all spleens at the end of the experimental periods of observation.

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