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In Vitro and In Vivo Efficacies of the Azole SCH56592 against *Cryptococcus neoformans*

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Multiple isolates of *Cryptococcus neoformans* were tested to compare the in vitro activity of a new triazole, SCH56592, with those of amphotericin B, fluconazole, and itraconazole. MICs of each drug were determined, and minimum fungicidal concentrations of SCH56592 and amphotericin B were measured. MICs of SCH56592 were lower than those of amphotericin B and fluconazole but not those of itraconazole. Minimum fungicidal concentrations of SCH56592 were lower than those of amphotericin B. SCH56592 in the presence of human serum produces an in vitro fungicidal effect for *Cryptococcus neoformans*. The data indicate that SCH56592 might exert fungicidal as well as inhibitory properties in vivo. On the basis of these results, SCH56592 was evaluated with a rabbit model of experimental cryptococcal meningitis; SCH56592 treatment was compared with treatment with fluconazole. Despite no detectable drug concentrations in the cerebrospinal fluid, the activity of SCH56592 against *C. neoformans* infection was equivalent to that of fluconazole. SCH56592 has potent in vitro activity against *C. neoformans* and compares favorably to treatment with fluconazole for a central nervous system infection. SCH56592 should be studied for use in humans with cryptococcal infections.

Cryptococcosis is a worldwide infection that has dramatically increased in frequency as a result of the panepidemic of human immunodeficiency virus infection and an enlarging population of other immunocompromised people. Significant improvements in the management of cryptococcal meningitis have been made over the last decade. Amphotericin B, fluconazole, and amphotericin B in combination with flucytosine have all been carefully studied for efficacy in the treatment of cryptococcal meningitis with and without coexisting human immunodeficiency virus infection (1, 4, 16). Amphotericin B and flucytosine are successful for initial treatment, but fluconazole is required for long-term continuous suppressive therapy (2) or prophylaxis (15) in patients with AIDS. There is currently no regimen that is curative for a high percentage of patients having severe immunodepression. Also, there is concern that the increasing number of *Cryptococcus neoformans* strains relatively resistant to current therapies such as fluconazole (7, 17) may increase. Therefore, it is important to search for new compounds that will be rapidly and consistently fungicidal in the immunocompromised host.

In this study, a new triazole, SCH56592, was examined for its in vitro and in vivo activities against *C. neoformans*. SCH56592 was compared in vitro with amphotericin B, fluconazole, and itraconazole against a series of *C. neoformans* strains and found to be extremely potent and consistently fungicidal for all strains tested, including those for which fluconazole MICs were relatively high. On the basis of in vitro test results, an animal model of cryptococcal meningitis (8–11, 13, 14, 19) was used to determine the in vivo efficacy of SCH56592. The study design compared SCH56592 with fluconazole, an agent for which there is significant experience in the prophylaxis, suppression, and treatment of cryptococcosis in humans (2, 15, 16). In this experimental model, fluconazole and SCH56592 were found to have similar therapeutic activities.

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MATERIALS AND METHODS

Animals. New Zealand White rabbits (weight, 2 to 3 kg) were housed in separate cages and given rabbit chow (Purina) and water ad libitum. Intramuscular injections of 100 to 150 mg of ketamine (Ketaset; Bristol Laboratories, Syracuse, N.Y.) plus 15 to 25 mg of xylazine (Rompum; Mobay Corp., Shawnee, Kans.) were given for all invasive procedures. Animals were sacrificed with an intravenous injection of sodium pentobarbital (Letalis; Barber Veterinary Supply, Fayetteville, N.C.) at the termination of experiments.

Antifungal agents. SCH56592 (Schering-Plough Research Institute, Kenilworth, N.J.) was suspended in 0.4% methyl cellulose solution (per liter of H_2O: 4.0 g of methyl cellulose, 5.6 ml of Tween 80, and 9.0 g of NaCl) as follows. A stock suspension of 5,120 μg/ml for in vitro assays was prepared as 128.0 mg/25.0 ml of methyl cellulose solution in a volumetric flask. For in vivo testing, the SCH56592 suspension was prepared as 27.0 mg/ml (2.0 ml/2.8-kg animal) and 93.33 mg/ml (2.4 ml/2.8-kg animal) for the low-dose and high-dose experiments, respectively, and administered by oral gavage using a 3-in. (ca. 8-cm) gavage needle. Fluconazole 100-mg tablets (Pfizer-Roerig, New York, N.Y.) were scored and given orally in daily doses of 20 or 80 mg/kg of body weight.

Organisms. *C. neoformans* H99 is a clinical isolate which has been used in previous experiments (10). Six additional strains for susceptibility testing, including strains from both AIDS and non-AIDS patients, are clinical isolates from Duke University Medical Center; two strains each were obtained from J. R. Graybill (89-610 and 89-569) and M. R. McGinnis (N32 and N34); and one strain was obtained from M. Ghannoum (T-1).

In vitro susceptibility testing. The proposed method published by the National Committee for Clinical Laboratory Standards for yeast susceptibility testing was followed (6). This method specifies an inoculum of 10^3 CFU/ml in RPMI 1640 medium (Gibco) with MOPS [3-(*N*-morpholino)propanesulfonic acid] at 35°C and determination of end points at 72 h. These end points are defined as 80 and 100% growth reduction, compared with that in the control tube, by triazoles and amphotericin B, respectively. For minimum fungicidal concentrations of amphotericin B and SCH56592, 0.1-ml aliquots from tubes with growth inhibition were plated on Sabouraud agar plates and the lowest drug concentration that yielded three or fewer yeast colonies was recorded.

In vitro kill curve testing. Strain H99 was grown on Sabouraud agar for 48 h at 35°C and then suspended in 0.85% NaCl to the density of a McFarland no. 1 standard. Then 0.1 ml was transferred to 10.0 ml of RPMI 1640 broth and shaken at 250 rpm at 35°C for 24 h. Five milliliters was removed and diluted with RPMI 1640 medium to achieve 1×10^6 to 5×10^6 CFU/ml as measured with a spectrophotometer set to 530 nm. A further dilution of 1:100 in RPMI 1640 medium yielded 1×10^4 to 5×10^4 CFU/ml. To test the effect of serum, a separate test substituted human serum (single donor) in a volume that represented 5% of the broth volume. Culture vessels were incubated at 35°C on a shaker incubator at 250 rpm. Colony counts were determined at 0, 6, 12, 24, 48, 72, and 168 h by plating dilutions of single 0.1-ml samples from each vessel.

Antimicrobial assay. Concentrations of SCH56592 in sera and cerebrospinal fluid (CSF) were measured by high-pressure liquid chromatography at the Scher-

TABLE 1. MICs for 12 clinical strains of *C. neoformans*

Strain	MIC (μg/ml)[a] of:			
	Fluconazole	SCH56592[b]	Amphotericin B[b]	Itraconazole
H99	2.0	0.063	1.0	0.008
N34	1.0	0.063	0.5	—
N32	4.0	0.125	0.5	—
DUMC 100.91	2.0	0.125	1.0	0.008
DUMC 251.86	2.0	0.063	1.0	0.016
DUMC 136.89	4.0	0.125	0.25	0.008
DUMC 114.90	0.5	0.063	0.5	—
DUMC 140.92	4.0	0.125	1.0	0.031
89-569	1.0	0.125	1.0	—
89-610	16.0	0.25		
DUMC 133.95	16.0	0.25		
T-1	8.0	0.125		

[a] —, strain exhibited a strong inhibition by polyethylene glycol solvent; unable to interpret.
[b] Minimum fungicidal concentrations were within a twofold dilution of MICs for all strains of both SCH56592 and amphotericin B.

ing-Plough Research Institute. The limit of drug detection with the assay was 0.05 μg/ml, and all standards were made in pooled normal rabbit sera or CSF.

Production of cryptococcal meningitis. Beginning 1 day prior to inoculation and for the duration of the experiment, all animals received an intramuscular injection of cortisone acetate, either 2.5 mg/kg for the low-dose treatment or 5.0 mg/kg for the high-dose treatment (Merck Sharpe & Dohme, West Point, Pa.). Four-day-old cultures of *C. neoformans* (H99) on Sabouraud agar plates with chloramphenicol were suspended in 0.015 M phosphate-buffered saline (PBS), counted, and adjusted to 5×10^7 to 1×10^8 CFU/ml for the low-dose experiment and 1.3×10^9 CFU/ml for the high-dose experiment. Rabbits were sedated and inoculated intracisternally with 0.3 ml of the yeast suspension. On predetermined days following inoculation, intracisternal taps were performed and approximately 0.5 ml of CSF was aspirated. The CSF was diluted in PBS and cultured on Sabouraud agar with chloramphenicol. The results were expressed as $\log_{10}$ CFU per milliliter of CSF. In the low-dose experiment, CSF was aspirated on days 4, 7, 11, 14, and 18 after inoculation; in the high-dose experiment, CSF was aspirated on days 2, 4, 7, 10, 13, and 16.

Treatment regimens. Two treatment schedules were used. (i) Treatment was started on day 4 of infection and employed for 11 consecutive days with 20 mg of either SCH56592 or fluconazole per kg per day. (ii) Treatment with the higher dose of 80 mg of either SCH56592 or fluconazole per kg per day was started on day 2 of infection and continued for 15 consecutive days. There were four to nine rabbits in each group for each experiment.

Statistical methods. Analysis of variance was used to test for an overall difference in mean log concentration between drug groups at each time. Dunnett's one-sided multiple-comparison test was used to compare the mean log concentrations of each treatment with the control group. The overall slopes from linear regression of mean log concentration versus time were estimated (with 95% confidence limits).

RESULTS

The MICs of SCH56592 for all isolates from both AIDS and non-AIDS patients, including two strains of *C. neoformans* var. *gatti* (N32 and N34), were low (0.063 to 0.125 μg/ml) and relatively uniform (Table 1). The in vitro tests showed that yeasts were inhibited and killed at lower concentrations of SCH56592 than of amphotericin B and at concentrations at least 10-fold lower than those of fluconazole. For three strains for which MICs of fluconazole were relatively high (8 to 16 μg/ml), MICs of SCH56592 were low (Table 1). Time-kill curves for in vitro testing at 0.1, 1, and 10 μg of SCH56592 per ml showed fungicidal activity at the two higher concentrations of drug. This fungicidal activity was found after 54 h of drug exposure and appeared to be enhanced by the presence of serum in the medium.

Concentrations of SCH56592 in serum and CSF were measured to determine the amount of drug delivered to these body sites in treated animals. For both low and high doses of SCH56592, serum drug concentrations after several days of treatment were 20 to 100 times above the SCH56592 MIC for the most resistant *C. neoformans* strain found in vitro and remained at concentrations (trough levels) that were approximately 30 to 115 times the MIC for H99 throughout the entire 24-h period between doses. CSF was analyzed for the presence of SCH56592 at 2, 6, and 24 h after either high or low doses of the drug (Table 2). No drug was detected in the CSF of any of these animals (limit of assay detection, 0.05 μg/ml).

TABLE 2. SCH56592 in serum and CSF of rabbits with cryptococcal meningitis

Dose (mg/kg)	No. of rabbits	Day of treatment	Time (h) after last dose	Concn (μg/ml; mean ± SD)[a] in:	
				Serum	CSF
20	9	4	2	7.06 ± 2.13	<0.05
	7	5	24	1.96 ± 0.53	ND
80	5	9	2	18.24 ± 2.46	<0.05
	5	12	24	7.24 ± 2.82	ND
	5	15	6	ND	<0.05

[a] Limit of assay detection, 0.05 μg/ml. ND, not done.

Figure 1 shows that both fluconazole and SCH56592 at low and high doses reduced yeast counts in the CSF during the first and second weeks of treatment. In the low-dose treatment experiments the fall in CSF yeast counts was identical for SCH56592- and fluconazole-treated animals and similar to that in control animals. In these experiments the combination of several deaths in the control group, a lower initial CSF yeast count (between 10^3 and 10^4 CFU/ml for all animals), and a moderate dose of corticosteroid probably contributed to the finding that control rabbits had yeast counts similar to those in the treatment groups by the end of the experiment. Therefore, in experiments with high-dose azoles, higher doses of steroids and inocula were used. In these experiments, the high doses of each drug significantly reduced yeast counts compared with those after no treatment ($P < 0.05$). Both SCH56592 and fluconazole were similar in their rates of fungicidal activity within the CSF, although levels of SCH56592 were below our threshold of detection.

DISCUSSION

SCH56592 demonstrated potent in vitro activity against all strains of *C. neoformans* tested, including two strains of *C. neoformans* var. *gatti* (N32 and N34) as well as *C. neoformans* var. *neoformans* isolates from human immunodeficiency virus-seropositive patients. Furthermore, three isolates for which MICs of fluconazole were relatively high were found to be very susceptible to SCH56592 (Table 1). This spectrum of activity could become particularly useful if continuous exposure of severely immunocompromised hosts to fluconazole were to cause an increase in the number of fluconazole-resistant strains.

Pharmaceutical companies also strive for fungicidal activity in their in vitro screening protocols for novel antifungal compounds, although in vitro fungicidal activity has yet to be correlated with in vivo outcome. It is hypothesized that in vitro fungicidal activity is necessary for elimination of fungi from severely immunocompromised hosts. SCH56592 was fungicidal in vitro for all *C. neoformans* strains tested and on a weight basis was more fungicidal than amphotericin B. Results from kill curve testing suggested that the addition of serum to SCH56592 enhances killing of *C. neoformans*. This implies that

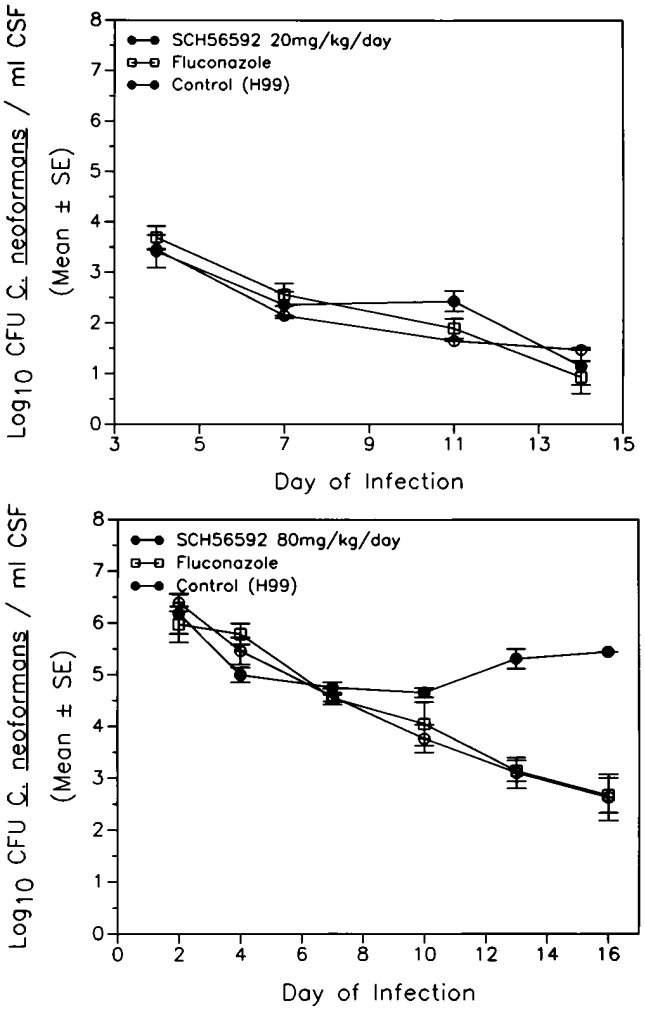

FIG. 1. Quantitative counts in CSF of immunosuppressed rabbits with cryptococcal meningitis and treatment with low-dose (top) and high-dose (bottom) SCH56592 versus fluconazole.

SCH56592 treatment could be potentiated within the host. Nassar et al. have recently discovered that there is a low-molecular-weight component of serum (<10,000 molecular weight) which can interact synergistically with fluconazole for killing *C. neoformans* in vitro (5). It is not certain that SCH56592 possesses this same potential. However, its fungicidal in vitro activity, particularly in the presence of serum, makes SCH56592 an attractive agent for studies in the treatment of cryptococcosis.

On the basis of these results, experiments were designed to study the effects of SCH56592 in an animal model of experimental cryptococcal meningitis. The animal model uses corticosteroid immunosuppression to produce a severe CSF leukopenia (10) which is relevant to many patients with cryptococcal meningitis. In this study, we attempted to compare the fungicidal activities of two drugs in the subarachnoid space over a defined period. Fluconazole was chosen as the comparative triazole because of significant experience with its use in treating and suppressing infection in humans and animals. In our experimental model and in human treatment trials, it has been noted that fluconazole treatment displays a delayed fungicidal response compared with that of amphotericin B (14).

The pharmacokinetics of SCH56592 for both the low-dose (20 mg/kg) and high-dose (80 mg/kg) regimens yielded serum drug concentrations that ranged from 30 to 115 times above the MIC, and high concentrations of drug persisted throughout the 24-h daily treatment periods. SCH56592 could not be detected in the CSF during meningitis in either the high- or low-dose regimen. Similar findings of undetectable drug concentrations in the CSF have been shown for itraconazole (11). In contrast, treatment with systemic fluconazole or the experimental azole SCH39304 produces high drug concentrations within the CSF whether or not there is meningitis (11, 14). These findings and their comparison with studies of bacterial meningitis suggest that itraconazole or SCH56592 is not likely to be active against infection at this site. However, direct comparisons of drug concentrations at the site have not always been predictive of treatment outcomes in fungal meningitis. For example, in humans and in this animal model of cryptococcal meningitis, CSF amphotericin B concentrations are well below the MICs for *C. neoformans* strains, yet amphotericin B remains the most potent fungicidal agent tested in our model (9). This suggests that amphotericin B has effects on immunological factors that can be important to recovery and/or that drug becomes localized in meningeal membranes or attaches to host cells that are carried to the site of infection, resulting in undetectable drug concentrations in aqueous specimens. For the lipophilic compound itraconazole, we have previously shown binding and accumulation in host cells (12), and thus the drug could be carried directly to the site of infection and not be measurable in the aqueous phase of the CSF. It has been shown that itraconazole can effectively treat cryptococcal meningitis both in humans (3, 18) and animals (11). Therefore, despite the apparently poor CSF pharmacokinetics of SCH56592, the outcome of treatment must still be measured.

Two treatment doses of SCH56592 and fluconazole were compared. In the lower-dose (20 mg/kg/day) study, no difference was found in the rate of yeast killing between the two triazoles. In this part of the experiment, some of the control animals died and others appeared to clear their infections. Therefore, the treatment regimens did not show a difference compared with no treatment. The experiment was repeated with higher doses of cortisone, inocula, and azoles. No difference in the killing of yeast in the CSF was found between the two treatment regimens, but there was significant killing of yeast in the CSF by both drugs compared with that in control animals. Analysis of these experiments suggests that at these two doses, SCH56592 and fluconazole have equivalent therapeutic activities in this animal model. There was consistent killing of *C. neoformans* over 2 weeks of treatment, but the subarachnoid spaces of most animals were not sterilized by the end of 2 weeks by either treatment regimen. Prior experience with amphotericin B and the combination of amphotericin B plus flucytosine found more rapid sterilization of CSF compared with that by fluconazole alone (8, 14). This finding of more rapid sterilization by amphotericin B and flucytosine has been confirmed in humans with cryptococcal meningitis (1, 16). We predict that SCH56592 will have a similar delay in CSF sterilization in humans compared with regimens containing amphotericin B. It is possible that the potent in vitro fungicidal activity of SCH56592 for *C. neoformans* is partially negated by its low concentration at the site of central nervous system infection. However, like itraconazole, it does have therapeutic activity in the CSF against this pathogen and will likely be effective in the treatment of cryptococcal infections in humans. The rabbit model of cryptococcal meningitis has been an accurate predictor of drug activity in humans. Thus, the study supports the further investigation of this triazole in the treatment of human cryptococcosis.

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