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Efficacies of Four Antifungal Agents in Experimental Murine Sporotrichosis

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Four antifungal agents, amphotericin B, SDZ 89-485, itraconazole, and terbinafine, were tested for efficacy in a murine model of systemic sporotrichosis. Survival in the groups treated with amphotericin B, SDZ 89-485, and itraconazole was significantly better than that of control infected mice. However, these agents did not wholly protect the infected mice, as tail and paw lesions, whole-body radiographs, and positive cultures from survivors showed evidence of dissemination. Terbinafine-treated mice had the same or poorer survival as control infected mice, despite documented drug absorption.

Sporothrix schenckii causes lymphocutaneous and extracutaneous diseases in humans. Lymphocutaneous infection, the most common form of the disease, presents as granulomatous nodular lesions in the skin, subcutaneous tissue, and regional lymphatics after traumatic inoculation of the fungus into the host. Iodides have been successfully used to treat this form of infection since 1903 (2), but hypersensitivity reactions and intolerable side effects have limited their usefulness in some patients. Local heat has also been effective in some cases (14). Extracutaneous infection is rare (8) and more difficult to treat (18). Although amphotericin B has been used for therapy, it has a high failure rate (18) and high drug toxicity. Other agents, such as flucytosine (1, 17) and ketoconazole (3, 6), have been less than optimal in the treatment of experimental and clinical sporotrichosis infections. Better agents to treat these infections are needed.

Three new antifungal agents show good activity in vitro against *S. schenckii*. Terbinafine, an allylamine that is undergoing clinical trials for the treatment of dermatophytosis, had MICs of 0.1 to 0.4 μg/ml (9). Itraconazole, a triazole, was found to have MICs ranging from 0.1 to 1 μg/ml against 12 strains of *S. schenckii* (16). Preliminary results in the treatment of lymphocutaneous sporotrichosis with itraconazole have been encouraging (10), but we are not aware of any reports in which this drug was evaluated in human extracutaneous sporotrichosis. Another experimental triazole which is in its preclinical stage of development by Sandoz Forschungsinstitut, SDZ 89-485, reportedly had in vitro activity against *S. schenckii* (M. Schaude and H. Mieth, Rev. Iber. Micol. 5(Suppl. 1):88, 1988). These data stimulated interest in examining the efficacy of these agents in a murine model of systemic sporotrichosis.

MATERIALS AND METHODS

S. schenckii. *S. schenckii* B-3435, kindly donated by J. Kwon-Chung (National Institutes of Health, Bethesda, Md.), was grown to the yeast phase on blood-glucose-cysteine agar at 37°C for 14 days. Yeast cells were harvested, washed three times in phosphate-buffered saline (PBS) (pH 7.4) containing 6.7 mM phosphate (Quality Biologicals, Inc., Gaithersburg, Md.), and resuspended in PBS to yield a final concentration of 4×10^7/ml after enumeration with a hemacytometer.

Antifungal agents. Four antifungal agents were tested for their efficacy in this murine model. Amphotericin B (Fungizone, NDL 0003-0437-30 control 4C712; E. R. Squibb & Sons, Princeton, N.J.) containing Desoxycholate was dissolved in distilled water at a concentration of 0.9 mg/ml, divided into aliquots in vials, and frozen at −70°C. SDZ 89-485 (supplied by H. Mieth; lot 85903; Sandoz Forschungsinstitut, Vienna, Austria) was suspended in 0.2% Tween 20–10% dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, Mo.) in distilled water at 5 mg/ml as suggested by the manufacturer. Itraconazole (R51211 lot A 4201; Janssen, Beerse, Belgium) was dissolved in polyethylene glycol 200 (Sigma) by heating to yield final concentrations of 8 and 2 mg/ml.

Since there are no published data about the absorption of terbinafine in mice, we tried several different solvents prior to using it in our sporotrichosis model with the other antifungal agents. Terbinafine (SF 86-327; lot 80903; Sandoz Forschungsinstitut) was kindly provided by H. Mieth. This drug was soluble in water at concentrations up to 1 mg/ml and yielded a fine suspension at higher concentrations upon vigorous vortexing. The drug could be dissolved in 100% ethanol at concentrations up to 50 mg/ml but produced visible precipitates when diluted in 10% ethanol in water. The drug yielded a fine suspension in 1% Tween 20 (Fisher Scientific Co., Fairlawn, N.J.)–5% DMSO. In pilot studies on the absorption of terbinafine, female BALB/c mice (Charles River Breeding Laboratories, Inc., Frederick, Md.) weighing 18 to 20 g were gavaged with a 0.1-ml volume of drug in the solvents indicated in Table 1. To assess the effect of a systemic infection on drug metabolism, we inoculated mice with *S. schenckii* or PBS as described below and administered the drug daily for 23 consecutive days. Heparinized blood was taken from the retro-orbital plexus, and plasma from mice in each group was pooled and stored at −20°C until assayed. Blood (0.1 ml) was obtained from all of the mice in each group at the times shown in Table 1. Bioassay was performed as previously described (7), with the lower limit of detection being 0.16 to 0.31 μg/ml; inhibition zones in agar at concentrations below this limit were indistinct. Drug standards were prepared in a final concentration of 99% human serum, which provided zone sizes

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TABLE 1. Summary of drug levels in plasma from mice given terbinafine by gavage

Expt	Infected	Drug dose (mg/kg)	No. of mice	Treatment day	Solvent	Drug level (μg/ml) at:				AUC_{0-4}[a] (μg · h/ml)
						0.5 h	1 h	2 h	4 h	
1	No	50	3	1	Water	ND[b]	0.3	0.4	<0.16	
	No	12.5	3	1	Water	ND	<0.16	<0.16	<0.16	
	No	3.1	3	1	Water	ND	<0.16	<0.16	<0.16	
2	No	90	3	1	Ethanol (100%)	ND	<0.16	0.3	<0.16	
	No	30	3	1	Ethanol (30%)	ND	<0.16	<0.16	<0.16	
	No	10	3	1	Ethanol (10%)	ND	<0.16	<0.16	<0.16	
3	No	50	3	1	Ethanol (20%)	0.8	0.7	<0.16	<0.16	
	No	50	3	1	Tween 20–DMSO	0.6	0.7	0.6	0.7	2.43
4	No	200	8	1	Tween 20–DMSO	2.4	1.8	1.3	1.0	5.50
	Yes	200	10	1	Tween 20–DMSO	2.3	2.5	1.9	1.1	6.98
	No	50	7	1	Tween 20–DMSO	1.0	0.6	0.3	0.2	1.60
	Yes	50	10	1	Tween 20–DMSO	1.0	0.7	0.3	0.2	1.68
	No	200	7[c]	23	Tween 20–DMSO	1.1	1.2	0.8	0.4	3.05
	No	50	7	23	Tween 20–DMSO	0.8	1.0	0.3	0.2	1.80

[a] AUC_{0-4}, Area under the concentration-time curve from 0 to 4 h.
[b] ND, Not done.
[c] One of the original eight mice died on day 5 of therapy after gavage.

nearly identical to those obtained with mouse serum. All specimens were assayed in duplicate.

For the comparison of the various antifungal agents in our murine model of sporotrichosis, terbinafine was suspended in 1% Tween 20–5% DMSO in distilled water to yield 10 and 40 mg/ml.

Murine model of systemic sporotrichosis. A total of 130 normal, virus- and mycoplasma-free BALB/c mice aged 4.25 months and weighing 20.0 g were given water and food ad libitum and housed at room temperature in groups of 10 infected mice to evaluate antifungal agents and solvents or groups of 5 normal mice to determine drug toxicity. For the infected groups, each mouse received an injection of 4×10^6 *S. schenckii* cells in 0.1 ml of PBS via the lateral tail vein. For the normal groups, each mouse received a sham injection of 0.1 ml of PBS via the lateral tail vein. Treatments with drugs or solvents were begun 3 days after inoculation with *S. schenckii* and were done daily for 28 days. Amphotericin B was given as an intraperitoneal injection of 0.1 ml, while the remaining three drugs or solvents were administered by gavage of 0.1 or 0.2 ml. All mice were observed daily for appearance, ataxia, and mortality during treatment and for an additional 7 days after treatment. Dead mice were autopsied to determine the extent of infection or trauma caused by gavage or injection. At the end of the experiment, a mouse was selected from each group for whole-body X rays, and all surviving mice were weighed, sacrificed, and autopsied. Organ cultures of brain, liver, and spleen were each made in triplicate on 2% glucose–1% neopeptone agar and kept at 30°C for up to 4 weeks to detect *S. schenckii* growth. An animal was deemed culture positive if a single colony of *S. schenckii* grew from at least one culture of the three organs tested. An organ was eliminated from evaluation if all cultures from the same organ were contaminated during the 4 weeks of incubation. However, none of the mice had all three organs eliminated by contamination. Three mice were considered culture negative based upon results from only two organs, and two mice were considered culture negative based upon results from only one organ.

Statistical analysis. A two-tailed Wilcoxon test for multiple comparisons against a control group (13) was performed with the Bonferroni correction factor (5). All survival data were censored on day 38 of the experiment. The level of significance was taken to be 0.007 for the comparison of mice in the seven drug treatment groups with control infected mice and 0.017 for the comparison of mice in the three solvent treatment groups with control mice.

RESULTS

The results of our pilot experiments on the absorption of terbinafine in mice with three different solvents are shown in Table 1. Mice receiving drug suspended in water had barely detectable terbinafine levels in plasma; the bioassay was repeated twice on the same specimens, with the same results. Drug levels in plasma did not improve when ethanol was used as the solvent, and the mice receiving the highest dose became markedly lethargic for a prolonged period (4 to 6 h), consistent with the high dose of ethanol. Mice receiving terbinafine in 1% Tween 20–5% DMSO in water had higher sustained levels. In experiment 4, infection with *S. schenckii* did not influence the area under the curve for up to 4 h after the first dose of terbinafine. There was no apparent accumulation of bioactive drug during prolonged drug use, as has been reported for humans (7). Therefore, 1% Tween 20–5% DMSO in water was chosen as the solvent for terbinafine in the current murine sporotrichosis model.

This murine model of systemic sporotrichosis usually results in mortality about 3 weeks after inoculation with *S. schenckii* B-3435, as tested by prior infectivity experiments with an intravenous injection ranging from 4×10^6 to 1×10^8 yeast cells per mouse (data not shown). Figure 1 shows the cumulative survival data for all groups of infected mice for the duration of the experiment. Mice given oral gavage with the solvents alone were not statistically different from control infected mice. Mice treated with amphotericin B, SDZ 89-485, and itraconazole had statistically better survival than control infected mice ($P < 0.007$). Compared with control untreated mice, mice treated with terbinafine at 50 mg/kg (body weight) had statistically poorer survival, and mice given terbinafine at 200 mg/kg had the same survival. Groups of five uninfected mice receiving amphotericin B and the

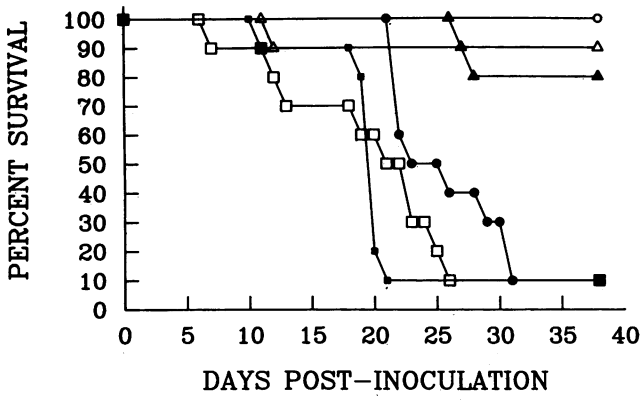

FIG. 1. Cumulative survival of eight groups of mice with systemic *S. schenckii* infection. Symbols: ●, control; ○, amphotericin B at 4.5 mg/kg and SDZ 89-485 at 25 and 50 mg/kg; ▲, itraconazole at 20 mg/kg; △, itraconazole at 80 mg/kg; ■, terbinafine at 50 mg/kg; □, terbinafine at 200 mg/kg.

higher doses of SDZ 89-485, itraconazole, and terbinafine were used to assess drug toxicity. All mice in the groups receiving amphotericin B and SDZ 89-485 survived. However, one of the five mice given itraconazole and three of the five mice treated with terbinafine died during the course of the experiment, owing to gavage trauma, as determined by autopsy.

Some of the clinical characteristics and culture data from the surviving mice of each of the infected groups are summarized in Table 2. During the course of the experiment, the control infected mice demonstrated decreased preening and matted fur. The one surviving mouse in the control infected group had nodular lesions on the tail and paws along with bone and joint destruction of the tail and distal extremities documented by whole-body X rays. At autopsy, this animal had pleural, pericardial, and peritoneal studding with white nodules but normal viscera. These postmortem findings were also noted in the other mice from this control infected group, which died during the study. Cultures of brain, liver, and spleen from the sole survivor of the control infected group were all positive for *S. schenckii*.

TABLE 2. Characteristic lesions and culture results in surviving mice sacrificed 38 days after inoculation with *S. schenckii*[a]

Treatment	Drug dose (mg/kg)	No. of survivors	Body wt[b] (g)	Positive culture from any site[c]	Visible abnormality[d]	
					Tail or paw	Ataxia
None		1	18.9	1	1	0
Amphotericin B	4.5	10	21.1 (0.2)	4	3	0
SDZ 89-485	25	10	20.1 (0.7)	9	10	4
	50	10	21.0 (0.5)	9	9	0
Itraconazole	20	8	19.0 (1.0)	8	8	3
	80	9	20.0 (0.5)	8	9	2
Terbinafine	50	1	21.9	1	1	0
	200	1	18.1	1	1	0

[a] There were 10 mice per group.
[b] Body weight was that of survivors on the day of sacrifice, reported as the mean (standard error) when more than one mouse survived.
[c] Reported as the number of survivors with at least one positive organ culture.
[d] Reported as the number of survivors with any tail or paw lesion or ataxia on the day of sacrifice.

Mice in the two groups treated with terbinafine had no better survival than control infected mice. Poor survival was not due to lack of drug absorption, since a bioassay of pooled sera from the two surviving uninfected mice receiving terbinafine at 200 mg/kg daily for 28 days revealed a concentration of 2.5 μg/ml. This drug level determined 1 h postdose was comparable to the concentration obtained 1 h after a single dose of 200 mg/kg (Table 1). Although other times after dose 28 were not studied, these mice did not have the suggestion of accelerated metabolism seen after 23 doses of 200 mg/kg (Table 1, experiment 4). Both survivors at the two terbinafine doses had exudative or necrotic tail lesions, and the mouse receiving 200 mg/kg also had nodular paw lesions. These abnormalities were corroborated by osteolytic changes on X rays. At autopsy, white nodules were present on the serosal surfaces, as had been seen in the other terbinafine-treated animals, which died during the course of the experiment. Cultures of all organs were positive for the mouse treated with the higher dose, while the spleen was positive in the mouse treated with the lower dose.

Itraconazole was tested in our murine model at doses of 20 and 80 mg/kg, which resulted in 80 and 90% survival of the infected mice, respectively. All surviving mice had tail abnormalities which were manifested as nodular to necrotic lesions in those treated with 20 mg/kg and as papules in the majority of those treated with 80 mg/kg. Nodular paw lesions were present in about half of the treated mice. Ataxia was noted in three of the survivors in the lower-dose group and in two of those in the higher-dose group. However, four uninfected mice treated with itraconazole at 80 mg/kg survived and showed no evidence of ataxia or other untoward effects, suggesting that ataxia was not likely to be a manifestation of drug toxicity. Serosal studding with nodular lesions was also present in all infected survivors which were autopsied. In addition, the majority of survivors from the itraconazole treatment groups had positive cultures. Of note is that brain cultures were positive in seven and four surviving mice receiving 20 and 80 mg/kg, respectively.

The triazole SDZ 89-485 used at 25 and 50 mg/kg resulted in the complete survival of the mice in these groups. However, tail lesions were seen in the majority as nodular or crusted lesions at the lower dose or papules at the higher dose. Osteolytic changes were also noted on the X rays for one mouse selected from each group. A few surviving mice also had nodular lesions on the paws. Ataxia was noted in four of the mice treated with 25 mg/kg but not in the infected or uninfected mice treated with 50 mg/kg, suggesting that ataxia was unlikely to be a manifestation of drug toxicity. At postmortem examination, serosal studding with white nodules was again seen in all mice sacrificed on the last day of the experiment. Organ cultures were positive for *S. schenckii* in the majority of the animals. Brain cultures were positive in six and seven surviving mice receiving 25 and 50 mg/kg, respectively.

Intraperitoneal treatment with amphotericin B at 4.5 mg/kg resulted in 100% survival of all mice tested. Although three mice had tail lesions, no ataxia or paw lesions were seen in any of the survivors. Whole-body X rays of a single mouse from this group revealed no bone or joint abnormalities. At autopsy, all serosal surfaces and viscera appeared grossly normal. Brain cultures were positive in only two mice; liver or spleen cultures were positive in a minority of mice.

DISCUSSION

Sporotrichosis remains a difficult infection to treat despite the use of iodides for the lymphocutaneous form and amphotericin B for the extracutaneous form. Three new agents, SDZ 89-485, itraconazole, and terbinafine, have demonstrated promising in vitro activity against *S. schenckii* and have not been previously compared in an experimental model of systemic sporotrichosis.

In our murine model of systemic sporotrichosis, tail and paw lesions with bone and joint destruction were evident, as previously described (1). In addition, ataxia or positive brain cultures were noted in most of the surviving mice receiving the triazoles itraconazole and SDZ 89-485. Control uninfected mice receiving the higher doses of the triazoles did not manifest ataxia or other adverse effects during the course of the evaluation, suggesting that the ataxia was more likely due to the *S. schenckii* infection than to drug toxicity. Gross hydrocephalus was evident in only one autopsied mouse, which had received 20 mg of itraconazole per kg; however, positive brain cultures were noted in mice treated with each of the antifungal agents tested, especially in those mice treated with itraconazole and SDZ 89-485. Disseminated disease in the brains of experimentally infected animals has not been previously reported and should be considered in future work with *S. schenckii*. However, meningitis caused by sporotrichosis is an extremely rare clinical disease in humans, with 15 cases noted in the literature (12).

In our model, amphotericin B was the most effective drug in prolonging survival and reducing the number of clinical lesions and positive organ cultures. Tsubura and Schwarz previously reported that amphotericin B at 10 mg/kg given intraperitoneally daily for 14 days reduced mortality by 76% and positive liver and spleen cultures by 60 to 80% in mice infected intravenously with *S. schenckii* (15). Although they mentioned that this dose of amphotericin B resulted in some drug toxicity, no further details were given. The amphotericin B dose of 4.5 mg/kg used in our study had no apparent adverse effects in the animals, and this group of mice appeared grossly normal, with appropriate weight gain at the end of our study.

The new triazole, SDZ 89-485, used at 25 and 50 mg/kg resulted in complete survival of all infected mice. However, tail lesions were present in the majority of mice in both groups. Osteolytic changes were seen on X rays taken from one mouse selected from each of the treatment groups. In addition, cultures of brain, liver, and spleen were positive for *S. schenckii* in most of the surviving mice. When subcutaneous chambers in mice were inoculated with *S. schenckii* (11), SDZ 89-485 demonstrated 50 and 95% effective doses of 2.98 and 7.34 mg/kg, respectively (M. Schaude, G. Petranyi, J. G. Meingassner, H. Ackerbauer, and H. Mieth, Rev. Iber. Micol. 5(Suppl. 1):88, 1988).

Itraconazole has been tested in a variety of superficial and deep mycoses (16). In our model with doses of 20 and 80 mg/kg, survival was prolonged, but tail and paw lesions and positive organ cultures were evident in the majority of the survivors. In a previous study with guinea pigs inoculated intratesticularly with *S. schenckii*, itraconazole prevented dissemination and cured 30 to 100% of animals receiving 10 to 40 mg/kg (16). However, itraconazole was begun on the day of inoculation, a time when infection or dissemination was not fully established. Cultures were taken only of the liver and both testicles. Clinical lesions typical of sporotrichosis such as those of the tail, paw, and bones were not mentioned. This experimental infection may have been easier to treat than disseminated sporotrichosis.

In our model, terbinafine-treated groups had mortality the same as or worse than that of control infected mice. Although in vitro activity showed promise, in vivo data demonstrated no efficacy for this systemic infection. Poor survival was not likely to be related to a lack of absorption, since pooled sera from uninfected animals receiving 200 mg of terbinafine per kg had a peak drug level similar to that in prior experiments. Dixon and Polak also reported no beneficial in vivo activity when terbinafine was tested at oral doses of 25 to 100 mg/kg in 5% methylcellulose solvent for experimental phaeohyphomycosis of the central nervous system in mice (4). However, no drug levels were reported to document drug absorption.

Of the four antifungal agents tested in our model, amphotericin B remains the most effective, with the fewest visible lesions and fewest positive cultures. The triazoles itraconazole and SDZ 89-485 increased survival but did not adequately protect the mice from disseminated disease in brain, bones, and viscera. The allylamine terbinafine offered no reduction in mortality or dissemination of this infection, despite documented levels of drug in plasma.

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