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Comparative Activities of Bay n7133, ICI 153,066, and Ketoconazole in Murine Cryptococcosis

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Two new antifungal triazoles, BAY n7133 and ICI 153,066, were compared with ketoconazole in treatment of mice challenged intraperitoneally with *Cryptococcus neoformans*. At high challenge doses, thymus-containing normal mice had prolonged survival after treatment with BAY n7133. Athymic mice, which have severely deficient cell-mediated immunity, were not protected. At low challenge doses, athymic mice also had prolonged survival. Although BAY n7133 was protective, it was less effective than either ketoconazole or ICI 153,066. BAY n7133 was also less effective in mice challenged intracerebrally than in those challenged intraperitoneally. Both ketoconazole and ICI 153,066 prolonged survival and lowered cryptococcal spleen counts to a greater extent than did BAY n7133.

Cryptococcal meningitis has continued to have a high mortality (1). This has prompted clinicians to explore combination amphotericin B (AMB)-flucytosine chemotherapy and to develop new antifungal drugs such as ketoconazole (3). AMB-flucytosine treatment has reduced the toxicity of longer courses of AMB, but at the cost of flucytosine bone marrow toxicity (1). Ketoconazole appears to be effective in a very limited experience with extrameningeal disseminated cryptococcosis (3). However, clinical experience with cryptococcal meningitis is minimal. In the mouse model, ketoconazole markedly prolonged survival in cryptococcal meningitis. However, ketoconazole did not sterilize tissues of *Cryptococcus neoformans* (2, 5). Therefore, there remains a need for new agents that are effective in meningeal cryptococcosis. In the present studies of murine cryptococcosis, we evaluated BAY n7133, an investigational triazole with anticryptococcal activity. We also compared this drug with ketoconazole, the imidazole in clinical use, and with another investigational drug, ICI 153,066. The structures of these drugs are shown in Fig. 1.

MATERIALS AND METHODS

Drugs. Ketoconazole was obtained as the powder from Janssen Pharmaceutica Co., New Brunswick, N.J. For in vitro studies, the drug was dissolved at 10 mg/ml in 0.7 N HCl and then further diluted in water. For oral administration to mice, the powder was suspended in 0.3% Noble agar at a concentration adjusted to deliver the desired dose in 0.1 ml via a gavage feeding needle. Mice were treated twice daily between 8:00 and 9:00 a.m. and 4:00 and 5:00 p.m., except for weekends, when they were treated only once daily. BAY n7133 was obtained from George Arcieri, Miles Laboratories, Inc., West Haven, Conn. For in vitro studies, the drug was dissolved in 95% ethanol and then diluted in water. For oral administration to mice, the powder was dissolved in vegetable oil (Wesson brand) at a concentration adjusted to deliver the desired oral dose in a 0.1-ml volume. Mice were treated twice daily on the same schedule as with ketoconazole.

ICI 153,066 was obtained as the finely ground powder from John Ryley, ICI Pharmaceuticals Division, Birmingham, England. For in vitro studies, the powder was dissolved in dimethylsulfoxide (DMSO), 10 mg/ml, and further diluted in water. For oral administration to mice, the powder was initially dissolved in DMSO at a concentration adjusted to deliver the desired dose in a 0.1-ml volume once daily; in later studies we evaluated the drug suspended in agar in a similar fashion as ketoconazole.

AMB was obtained as the commercial desoxycholate preparation (Fungizone; E. R. Squibb and Sons, Princeton, N.J.). AMB was diluted in 5% dextrose to the appropriate dose in 0.1 ml. This was given by intraperitoneal (i.p.) injection on Monday, Wednesday, and Friday of each week.

Mice. For most studies, BALB/c mice of both sexes were used at approximately 5 weeks of age. They were either thymus-containing, normally furred (nu/+) heterozygotes, or athymic (nu/nu) homozygotes. These mice have been bred in our colony for a number of years and are free of mycoplasmal, viral, and protozoal pathogens. nu/+ mice weighed approximately 20 g when used, and nu/nu mice weighed approximately 15 g when used. Mice were caged in groups of five and given food and water ad libitum. Because of limitations in BALB/c mouse supplies, where indicated we

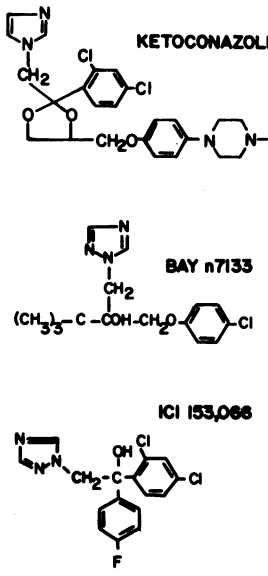

FIG. 1. Structure of ketoconazole, ICI 153,066, and BAY n7133.

also used ICR outbred mice (Timco, Houston, Tex.) weighing 15 to 20 g.

C. neoformans isolate 251 was used. We have used this isolate in prior studies (2). The fungus was maintained at 4°C on Sabouraud agar plates and inoculated into Sabouraud broth and incubated at 37°C for 24 to 48 h before use. Yeasts were washed to remove free capsular polysaccharide and then suspended in phosphate-buffered saline (pH 7.2). The number of yeasts in the suspension was counted on a hemocytometer, and this was checked by serial dilution colony counts.

Challenge and treatment. Mice were challenged i.p. with 0.1 ml of normal saline containing *C. neoformans* organisms. Three days were allowed for the infection to become well established after i.p. challenge, and then treatment was begun. In the case of intracerebral (i.c.) challenge, only 0.03 ml was used for challenge, and the challenge was given via 30-gauge needle in the midline with direct inoculation through the cranium, approximately 5 mm posterior to the eyes (2). Approximately 10% of the mice succumbed within 24 h after i.c. challenge, usually within the first few hours. These mice were not counted in survival. Because the infection kills much more rapidly after this route of challenge, mice were treated beginning 1 day after challenge. In one study of mice challenged i.c., combination AMB-BAY n7133 was compared with either drug given alone.

In several studies, groups of 5 to 10 mice were sacrificed 1 to 2 weeks after challenge to measure colony counts. Brains and spleens were removed aseptically, weighed, added to 1 ml of sterile water in a Ten Broeck ground glass homogenizer, and homogenized manually. Serial dilutions were cultured in duplicate 0.1-ml volumes.

In vitro susceptibility. Petri dishes (5-cm diameter) were inoculated with 40 ml of Sabouraud agar containing 10^5 CFU of *C. neoformans* per ml. Plates were stored for up to 1 week at 4°C before use. Before use, wells were formed with an 8-mm cork borer; 80 μl of Sabouraud broth containing various dilutions of drug was added to each well and allowed to diffuse at 4°C for 4 h. Where appropriate, control wells contained dilutions of agar or ethanol in water. Plates were incubated overnight at 37°C, and two diameters of duplicate wells were measured for the clear zone representing inhibition of growth. The well showing the smallest distinct zone was interpreted as the minimum inhibitory concentration (MIC).

Serum concentrations. Mice were given ICI 153,066 (25 mg/kg in agar) once daily, BAY n7133 (50 mg/kg in oil) twice daily, or ketoconazole (50 mg/kg as a powder suspension) twice daily. After the third dose, groups of two to seven mice were sacrificed by exsanguination at 1, 2, 4, and 8 h after the dose. Each drug was measured by bioassay of individual mice. The method used has been published previously (6). The *Candida pseudotropicalis* test organism was sensitive to all three drugs. We modified this to use standards for BAY n7133 initially dissolved in ethanol and ICI 153,066 initially dissolved in DMSO and then diluted into mouse serum. The minimum detectable concentrations were as follows: ketoconazole, 0.3 μg/ml; BAY n7133, 0.625 μg/ml; for ICI 153,066, 0.3 μg/ml. A DMSO control was also diluted into mouse serum.

Statistics. The Wilcoxon test for life table analysis was used to compare survival of various groups. The one-tailed rank sum test was used to compare tissue colony counts, as the distribution did not follow a normal pattern.

RESULTS

In vitro studies. With the agar well diffusion method described, the MICs were 0.625 μg/ml for ketoconazole, 10 μg/ml for BAY n7133, and 5 μg/ml for ICI 153,066. DMSO and ethanol, at the same concentrations used for the highest drug standard, did not produce zones of inhibition. Serum concentrations after three doses of drug are shown in Fig. 2. With ketoconazole, the mean peak concentration was 13 times above the MIC. For ICI 153,066, the peak serum concentration was approximately twice the MIC. With BAY n7133, peak concentration was in the same range as the MIC.

In vivo studies. In preliminary studies, we determined that 100 mg of BAY n7133 in oil per kg was toxic to mice, with death occurring after 2 to 3 days of twice-daily treatment. A lower dose of 75 mg/kg was well tolerated for 14 days. The remaining survival studies are summarized in Table 1.

Our initial studies were designed to determine whether BAY n7133 had any protective effect in mice challenged i.p. In study 1, thymus-containing mice were given a high challenge of 9×10^4 *C. neoformans* cells; mice treated with BAY n7133 had a prolonged survival compared with controls ($P < 0.01$). In contrast were the athymic mice. These mice have deficient cell-mediated immunity and are more susceptible to crypto-

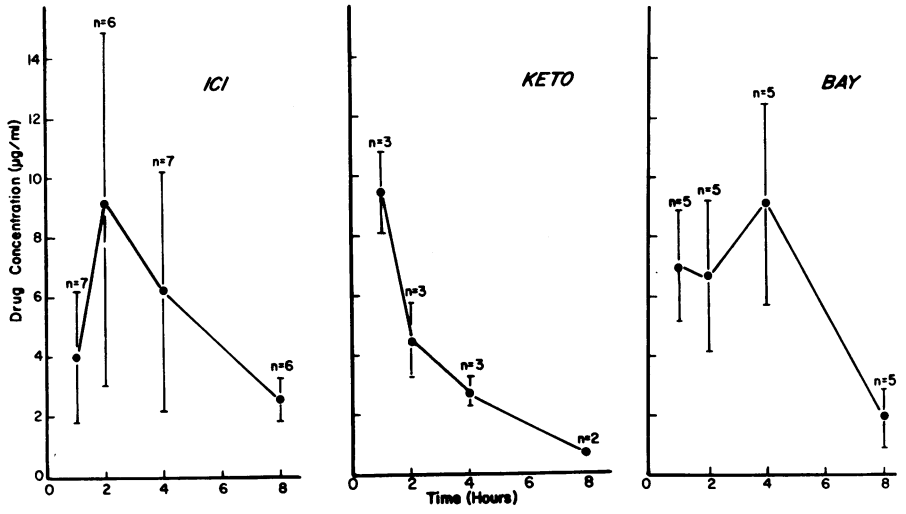

FIG. 2. Serum concentrations after oral administration of 25 mg of ICI 153,066 per kg (ICI), 50 mg of ketoconazole per kg (keto), and 50 mg of BAY n7133 per kg (BAY) to BALB/c mice. BAY was given in oil; the others were given as powders suspended in agar. Means ± standard deviations are given.

coccosis than normal mice; at this large challenge dose, they did not benefit from BAY n7133. In study 2, athymic mice were challenged i.p. with a lower dose of 1.5×10^3 *C. neoformans* cells and treated with BAY n7133 (50 mg/kg). At this lower challenge dose, the mice survived significantly longer than did oil-treated controls ($P < 0.05$). However, the benefit was a less than 1-week increase in survival.

Having shown a modest protective effect of BAY n7133 in study 3, we compared this drug with ketoconazole and ICI 153,066. A maximum treatment benefit was sought. Therefore, BALB/c thymus-containing mice were challenged i.p. with a lower dose of 10^4 *C. neoformans* cells. Treatment was begun 3 days later, with ICI 153,066 (25 mg/kg daily) delivered either in DMSO or as powder suspension in agar. Controls treated with DMSO or agar alone succumbed between 2 and 3 weeks after challenge. ICI 153,066, presented in DMSO or as powder, markedly prolonged survival, as did ketoconazole. A lower dose of 15 mg/kg also prolonged survival in nu/+ mice (data not shown). It was not as beneficial as the dose of 25 mg/kg. Because we were concerned that a dose of 75 mg/kg was very close to the toxic range, we lowered the dose of BAY n7133 to 65 mg/kg. BAY n7133 was also effective in prolonging survival.

We next determined whether treatment efficacy was similarly reflected in tissue counts. BALB/c thymus-containing mice were challenged i.p. with 10^5 *C. neoformans* cells and treated from day 3 through day 9. One day later (to allow time for drugs to be cleared) mice were sacrificed, and colony counts were made from spleen and brain tissues (Fig. 3). Despite the higher challenge, central nervous system dissemination proceeded slowly after i.p. challenge, and brain counts in many animals were below our threshold of 18 CFU/g. Brain counts were thus uninterpretable and are not shown. However, when compared with controls, spleen counts were significantly reduced ($P < 0.05$) in both ICI 153,066- and ketoconazole-treated mice, but not in mice treated with BAY n7133. None of the regimens sterilized the spleens.

We then evaluated the efficacy of these drugs in ICR mice challenged i.c. and treated beginning 1 day after challenge. In study 4, BAY n7133 was not protective, whereas prolonged survival ($P < 0.01$) was conferred by both ketoconazole and ICI 153,066 (Table 1). Ketoconazole- and ICI 153,066-treated mice eventually died, the last ones succumbing 2 months after challenge. There were no differences between the two drugs. Additionally, most of the mice succumbing were autopsied with brain cultures for cryptococci. High counts were present in all of these mice. The study was repeated, with similar results.

Study 5 was a search for possible beneficial interaction of AMB and BAY n7133 in mice challenged intracerebrally with 10^2 *C. neoformans* cells. Beginning 1 day after challenge, ICR mice were treated with AMB (0.5 mg/kg, three times weekly i.p.), BAY n7133 in oil, or both drugs. The controls received both 5% dextrose i.p. and oil orally. AMB improved survival from 0 to 50% out to 40 days after challenge. BAY n7133 did not prolong survival as a single drug

TABLE 1. Survival studies of murine cryptococcosis

Study no.	Mouse strain	Therapeutic regimen: Inoculum CFU	Inoculum Route	Compound[a] (vehicle)	Dose (mg/kg)	Frequency[c]	Therapeutic result: Days after challenge	No. of 40-day survivors/total	Mean days to death (range)	*P* versus control[b]
1	BALB/c	10^5	i.p.	Oil	0	bid	3 to 16	0/10	11 (8–18)	
				BAY (oil)	75	bid	3 to 16	0/10	14 (10–21)	<0.01
	BALB/c, athymic	10^5	i.p.	Oil	0	bid	3 to 13	0/10	9 (8–13)	
				BAY (oil)	75	bid	3 to 14	0/10	9 (7–14)	NS
2	BALB/c, athymic	10^3	i.p.	Oil	0	bid	1 to 16	0/10	26 (20–32)	
				BAY (oil)	75	bid	1 to 16	1/10	32 (25–40)	<0.05
3	BALB/c	10^4	i.p.	Agar	0	bid	3 to 23	0/10	21 (9–31)	
				ICI (agar)	25	qd	3 to 23	3/10	41 (35–>60)	<0.001
				BAY (oil)	65	bid	3 to 23	2/10	35 (24–55)	<0.01
				Keto (agar)	50	bid	3 to 23	5/10	37 (11–48)	<0.001
				DMSO	0	qd	3 to 18	0/10	21 (10–38)	
				ICI (DMSO)	25	qd	3 to 18	3/10	38 (29–43)	<0.001
4	ICR	10^2	i.c.	Oil	0	bid	2 to 14	0/8	9 (1–15)	
				Agar	0	bid	2 to 21	0/9	11 (7–21)	
				ICI (agar)	25	qd	2 to 22	3/9	33 (2–45)	<0.01
				BAY (oil)	65	bid	2 to 21	0/9	12 (2–21)	NS
				Keto (agar)	60	bid	2 to 22	3/9	26 (6–43)	<0.01
5	ICR	10^2	i.c.	Oil		bid	1 to 40	1/10	14 (6–47)	
				BAY (oil)	50	bid	1 to 40	2/10	19 (6–47)	NS
				AMB (water)	0.5	MWF	1 to 40	5/10	26 (8–47)	<0.01
				AMB + BAY (oil)	50	bid	1 to 40	3/10	28 (5–47)	<0.01
				AMB (water) + BAY	0.5	MWF				

[a] BAY, BAY ~7133; ICI, ICI 153,066.
[b] Wilcoxon test of life tables. NS, Not significant.
[c] bid, Twice a day; qd, once a day; MWF, Mondays, Wednesdays, and Fridays.

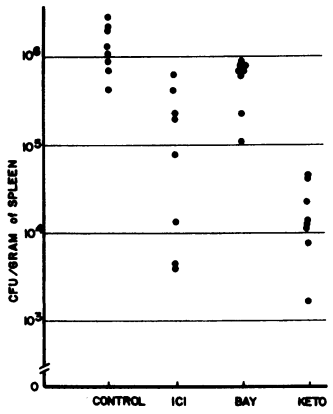

FIG. 3. Spleen counts of mice challenged i.p. with 10^5 *C. neoformans* cells and sacrificed 10 days after challenge. Mice were treated from day 3 through day 9 with either 0.3% agar (control), BAY n7133 (65 mg/kg, twice daily), ketoconazole (50 mg/kg, twice daily), or ICI 153,066 (25 mg/kg, once daily).

and did not further improve the survival benefit conferred by AMB.

DISCUSSION

In the present studies, we have confirmed that all three azole antifungal drugs were absorbed after oral administration. Serum concentrations varied considerably. Because parenterally administered forms were not available, we could not measure percent absorption. Therefore, we could not determine whether the broad range of concentration was due to impaired absorption or accelerated excretion or inactivation. However, serum peak concentrations exceeded the MIC of *C. neoformans* for that agent. Survival studies after i.p. challenge showed that BAY n7133, ICI 153,066, and ketoconazole all prolonged survival. Further, for both BAY n7133 and ICI 153,066, the prolonged survival was more apparent in thymus-containing mice than in athymic mice. This was consistent with earlier studies of AMB and flucytosine, in which we found that antifungal therapy was more effective in immunologically competent mice than in those with depressed cell-mediated immunity (4).

However, in two respects BAY n7133 appeared less effective than the other two drugs. First, the spleen counts of *C. neoformans* were significantly reduced by both ketoconazole and ICI 153,066, but not by BAY n7133. Second, BAY n7133 showed no benefit in mice challenged i.c.

In the case of tissue counts after i.p. challenge, the rate of dissemination to the central nervous system was slow, and we could not detect differences in brain counts among any groups. Although both ketoconazole and ICI 153,066 depressed spleen counts, neither agent sterilized the spleens. However, both were more effective than BAY n7133. It is reasonable to question whether higher doses or more prolonged treatment with BAY n7133 might have increased efficacy. However, the highest dose we used, 75 mg/kg, approached toxicity and could not have been further increased. Further, in unpublished studies we have found these doses to be effective in murine aspergillosis. Similarly, mice were treated for as long as 3 weeks with BAY n7133, to the point where treated mice were beginning to succumb. Further extension of treatment was unlikely to benefit the mice.

In the case of mice challenged i.c., there was no difference between control mice and those treated with BAY n7133, despite the initiation of treatment after only 1 day postchallenge. However, both ICI 153,066 and ketoconazole provided marked prolongation of survival. Again, none of the regimens sterilized the mouse brains, and all mice ultimately died with cryptococcosis. For ICI 153,066- and ketoconazole-treated mice, this was delayed up to 2 months after challenge. There were no differences in ketoconazole or ICI 153,066 recipients. The failure of BAY n7133 might be explained by poor penetration into mouse brain. In unpublished studies, we have found that BAY n7133 achieved peak brain concentrations of 3 to 5 μg/g 2 h after a dose of 50 mg/kg. This is less than the MIC of BAY n7133 for this isolate of *C. neoformans*. However with imidazole antifungal drug therapy, there is no proven correlation between in vitro MIC or tissue concentration and prolongation of survival. Nevertheless, high spleen counts of *C. neoformans* in BAY n7133 recipients did correlate with the reduced efficacy of BAY n7133.

It is important to emphasize that i.p. infection is not analogous to human cryptococcosis, in which infection occurs by the pulmonary route. However, it may not be totally inappropriate in that most patients are seen at a time well after dissemination has occurred. Also, we did reproduce two important correlates of human cryptococcosis. These are intracerebral disease with little evidence of extracranial infection and also infection of a host with severe depression of cell-mediated immunity. In previous studies we had found that ketoconazole prolonged survival in mice challenged i.c. (2). Although ketoconazole was again protective in i.c. challenged mice, BAY n7133 was not beneficial. In previous studies, athymic mice have provided a severe challenge to standard AMB-flucytosine therapy (4). Likewise here, athymic mice responded minimally to chemotherapy with ICI 153,066 or BAY n7133.

BAY n7133 may soon enter clinical trials in the United States. Although animal studies do not always predict the clinical applications of various agents, they could be helpful in suggesting where these investigations may or may not be fruitful. The above studies do not suggest that BAY n7133 will be a major addition to treatment of disseminated cryptococcosis. ICI 153,066 warrants further investigation.

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