Comparative Chemotherapeutic Activity of Amphotericin B and Amphotericin B Methyl Ester

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The comparative efficacy of amphotericin B and amphotericin B methyl ester (AME) against experimental histoplasmosis, blastomycosis, cryptococcosis, and candidosis in mice was assessed by determining the effect of daily intraperitoneal therapy on 21-day survival and persistence of organisms in internal organs. AME, like amphotericin B, was effective against each of the experimental infections, but the efficacy was lower than the parent compound. For *Histoplasma* and *Blastomyces* infections the mean effective dose (ED₅₀) of amphotericin B was 0.3 mg/kg, whereas the corresponding values for AME, respectively, were 2.4 and 2.8 mg/kg. For *Cryptococcus* infection the ED₅₀ for amphotericin B was 0.2 mg/kg compared with 2.0 mg/kg for AME. The ED₅₀ of amphotericin B for *Candida* infection was lower than 0.05 mg/kg and the value of AME was between 0.5 to 0.05 mg/kg. The colony counts from internal organs of the surviving animals after the therapeutic regimens were compatible with the data on survival.

Amphotericin B, a polyene antibiotic, is most frequently used for the treatment of systemic mycoses. In view of its extreme insolubility, the drug is administered in a dispersed form, using sodium desoxycholate as the dispersing agent (Fungizone, E. R. Squibb & Sons, New Brunswick, N.J.). However, the chemotherapy of systemic mycoses is seriously hampered due to toxicity of amphotericin B (3, 6, 13, 14). The toxicity, in part, is attributed to its insolubility in water and body fluids.

Recently, amphotericin B methyl ester hydrochloride (AME), a water-soluble derivative of amphotericin B, has been prepared by Mechlin-ski and Schaffner (9). AME formed micelles in water but the degree of dispersion was significantly higher than that of Fungizone (1). The water-soluble derivative has been shown to be less toxic than the parent compound (5), but possesses a significant antifungal activity in vitro against a variety of pathogenic and potentially pathogenic fungi (1, 4, 9). The water solubility and wide spectrum of antifungal activity of AME in vitro warrant evaluation of its chemotherapeutic activity against systemic fungal infections.

The experiments reported here were designed to study the comparative therapeutic activity of AME and amphotericin B against experimental histoplasmosis, blastomycosis, cryptococcosis, and candidosis in mice.

MATERIALS AND METHODS

Animals. White, male CFI mice weighing 14 to 16 g were obtained from Carworth Farms, New City, N. Y. The mice were divided randomly into groups of 10, housed in metal cages, and given mouse pellets and water ad libitum.

Organisms. Four pathogenic fungi, *Histoplasma capsulatum* (G-217B), *Blastomyces dermatitidis* (BD-1), *Cryptococcus neoformans* (Cp-8), and *Candida albicans* (C-1), were used to produce experimental infections. All organisms were obtained from subcultures of primary human isolates and were maintained on Difco brain heart infusion agar slants supplemented with 1.0% glucose and 0.1% L-cysteine hydrochloride. Cultures were stored at 4 C and transferred every 3 to 4 weeks.

Preparation of inocula. Growth harvested after 48 h on brain heart infusion agar slants was suspended in 10 ml of sterile saline, and 2 ml of this suspension was inoculated into flasks containing 50 ml of a liquid synthetic medium (12). The flasks inoculated with *H. capsulatum* and *B. dermatitidis* were incubated for 36 h, and those with *C. neoformans* and *C. albicans* for 24 h, at 37 C in a gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) with a shaking speed of 150 rpm.

The cultures were harvested, washed twice with cold, sterile 0.9% NaCl, and centrifuged in a refrigerated Sorvall centrifuge at 30 × g for 0.5 to 1 min to remove aggregated yeast cells. The suspension was standardized turbidimetrically with use of a standard curve and diluted to contain the desired number of viable cells. The viability was determined by plate count as well as by vital staining with Janus green B

(11). All of the cell suspensions from the time of harvesting until the inoculation of animals were kept in an ice bath.

Experimental infections. A total of 220 mice in groups of 20 were used in each experiment. Mice were inoculated intravenously by 8×10^6 cells of *H. capsulatum*, 0.5×10^6 of *B. dermatitidis*, 0.5×10^6 of *C. neoformans*, and 0.4×10^5 of *C. albicans*.

Antibiotic therapy. Amphotericin B in the form of Fungizone and its methyl ester hydrochloride (AME) were evaluated for each fungal infection. Fungizone was a gift from E. R. Squibb & Sons and the AME was prepared in our laboratory. The dosages for both antibiotics were 10, 5, 1, 0.5, and 0.05 mg/kg per day. The antibiotics were prepared fresh daily, diluted with 5% sterile dextrose to yield the desired concentration in 0.5 ml. Animals were infected in the morning and treatment was started in the afternoon. Both antibiotics were administered intraperitoneally, once a day, for 21 days. The control groups received 0.5 ml of 5% sterile dextrose solution.

Examination of animals. Mice were observed daily for 21 days, and deaths were recorded. At the end of the experiment, up to 10 surviving mice from each group were autopsied, and pieces of brain, spleen, right lung, liver, and right kidney of mice infected with *Histoplasma*, *Blastomyces*, and *Cryptococcus* were cultured on brain heart infusion agar plates. In *Candida* infection only brain and right kidney were cultured. The plates were incubated at room temperature for 4 weeks.

Histopathology. Portions of the internal organs were preserved in 10% buffered formol saline. The tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin by Gomori's methenamine-silver nitrate technique (2). In addition, sections from mice infected with *Cryptococcus* were stained with mucicarmine (8).

RESULTS

The comparative efficacy of amphotericin B and AME against experimental histoplasmosis, blastomycosis, cryptococcosis, and candidosis in mice was assessed by determining the effect of daily intraperitoneal therapy on 21-day survival and persistence of organisms in internal organs. The results for efficacy against *H.*

capsulatum infection are presented in Fig. 1 and Table 1. Mice infected with 8×10^6 yeast cells of *H. capsulatum* showed 95% mortality at 21 days with a median survival time of 9 days. Amphotericin B was effective at levels of 10, 5, 1, and 0.5 mg/kg, protecting 90 to 100% of the treated mice, but was ineffective at 0.05 mg. AME was effective at 10 and 5 mg/kg, protecting 95 to 100% of infected animals. Minimal efficacy of AME was apparent at 1 mg/kg with 20% survival and no activity was observed at the lower dosages. Amphotericin B sterilized brain, lungs, liver, spleen, and kidneys of all mice treated with 10, 5, and 1 mg/kg of the drug.

FIG. 1. Percentage survival of mice infected intravenously with 8×10^6 yeast cells of *H. capsulatum* after daily intraperitoneal treatment with amphotericin B and AME for 21 days. Amphotericin B (F) is represented by broken lines, and AME by solid lines.

TABLE 1. Isolation of *H. capsulatum* from internal organs of mice after antibiotic therapy of experimental infection

Dosage (mg/kg)	Fungizone					AME				
	Brain	Spleen	Lung	Liver	Kidney	Brain	Spleen	Lung	Liver	Kidney
10	—	—	—	—	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—	—	—
1	—	—	—	—	—	0/4 ^a	0/4	1/4	0/4	2/4
0.5	0/10	6/10	6/10	5/10	2/10	NS ^b	NS	NS	NS	NS
0.05	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

^a Number of positive cultures/number of animals examined.

^b NS, No survivors.

However, AME sterilized all organs at 10 and 5 mg/kg and majority of organs at the 1-mg level.

Mice infected with 0.5×10^6 yeast cells of *B. dermatitidis* showed 95% mortality at 21 days (Fig. 2). Amphotericin B at doses of 10, 5, 1, and 0.5 mg/kg protected 95 to 100% of the infected mice, but no protection was seen at 0.05 mg. Similar levels of protection were observed at 10 and 5 mg of AME per kg, but the drug was ineffective at lower doses. Amphotericin B at levels of 10 and 5 mg/kg cleared all organs of the treated mice, but viable organisms were recovered from lungs at 1- and 0.5-mg doses (Table 2). Similarly, AME cleared the organs of mice treated at 10- and 5-mg/kg levels and the few surviving mice at 1 mg.

FIG. 2. Percentage survival of mice infected intravenously with 0.5×10^6 yeast cells of *B. dermatitidis* after daily intraperitoneal treatment with amphotericin B (F) and AME for 21 days. Amphotericin B (F) is represented by broken lines, and AME by solid lines.

Mice infected with 0.5×10^6 yeast cells of *C. neoformans* showed 90% mortality at 21 days (Fig. 3). Amphotericin B protected 90 to 100% of the mice treated with 10, 5, 1, and 0.5 mg/kg and 35% of the mice treated with 0.05 mg. AME was effective at 10 and 5 mg/kg, protecting 95 to 100% of the treated animals. However, AME at the 1-mg/kg level protected only 50% of the treated animals and was ineffective at 0.5- and 0.05-mg levels. Neither amphotericin B nor AME treatment could sterilize internal organs of all the mice infected with *C. neoformans* (Table 3). Amphotericin B sterilized the internal organs of 60 to 90% of mice treated with doses varying from 0.5 to 10 mg/kg, but all animals that survived after treatment with 0.05 mg of the drug yielded positive cultures. AME

FIG. 3. Percentage survival of mice infected intravenously with 0.5×10^6 yeast cells of *C. neoformans* after daily intraperitoneal treatment with amphotericin B and for 21 days. Amphotericin B (F) is represented by broken lines, and AME by solid lines.

TABLE 2. Isolation of *B. dermatitidis* from internal organs of mice after antibiotic therapy of experimental infection

Dosage (mg/kg)	Fungizone					AME				
	Brain	Spleen	Lung	Liver	Kidney	Brain	Spleen	Lung	Liver	Kidney
10	—	—	—	—	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—	—	—
1	—	—	3/10 ^a	—	—	0/2	0/2	1/2	0/2	0/2
0.5	—	—	8/10	—	—	NS ^b	NS	NS	NS	NS
0.05	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

^a Number of positive cultures/number of animals examined.

^b NS, No survivors.

TABLE 3. Isolation of *C. neoformans* from internal organs of mice after antibiotic therapy of experimental infection

Dosage (mg/kg)	Fungizone					AME				
	Brain	Spleen	Lung	Liver	Kidney	Brain	Spleen	Lung	Liver	Kidney
10	—	—	1/10 ^a	2/10	2/10	9/10	4/10	6/10	6/10	5/10
5	3/10	—	1/10	—	—	10/10	4/10	7/10	7/10	5/10
1	1/10	—	1/10	—	—	10/10	9/10	10/10	10/10	9/10
0.5	4/10	1/10	1/10	1/10	—	1/1	1/1	1/1	1/1	1/1
0.05	7/7	7/7	7/7	7/7	7/7	1/1	1/1	1/1	1/1	1/1

^a Number of positive cultures/number of animals examined.TABLE 4. Isolation of *C. albicans* from internal organs of mice after antibiotic therapy of experimental infection

Dosage (mg/kg)	Fungizone		AME	
	Brain	Kidney	Brain	Kidney
10	—	—	—	—
5	—	—	—	—
1	1/5 ^a	3/5	3/5	3/5
0.5	1/5	3/5	3/5	5/5
0.05	3/5	4/5	3/3	3/3

^a Number of positive cultures/number of animals examined.

was less effective than amphotericin B in clearing the internal organs of viable *Cryptococcus*. More than half the animals that received 10 mg of AME per kg harbored viable organisms in the internal organs and the incidence of positive cultures increased with decreasing dosage.

Mice injected with 0.4×10^5 yeast cells of *C. albicans* showed 75% mortality at 21 days (Fig. 4). Amphotericin B at doses ranging from 0.05 to 10 mg/kg protected 90 to 100% of the infected mice. Similar levels of protection were obtained in mice treated with 0.5 to 10 mg of AME per kg, but the drug was ineffective at the 0.05-mg level, where 30% of the treated mice survived as compared with 25% survival of infected controls. In chemotherapy of *Candida* infection, only brain and kidney were cultured, since these organs have been recognized as the target organs in mice after experimental infection. At 5- and 10-mg/kg levels, both Fungizone and AME cleared brain and kidney of the infected mice (Table 4). At lower levels both drugs were ineffective in sterilizing the internal organs. In Fig. 1-4, the results on survival of mice after 7 and 14 days of treatment also are presented. These data conform with the relative efficacy of the two antibiotics observed at 21 days. Additional trials with each infection yielded similar results on the chemotherapeutic activity of the two antibiotics.

FIG. 4. Percentage survival of mice infected intravenously with 0.4×10^5 yeast cells of *C. albicans* after daily intraperitoneal treatment with amphotericin B (F) and AME for 21 days. Amphotericin B (F) is represented by broken lines, and AME by solid lines.

A somewhat more precise evaluation can be obtained by comparing the amounts of each drug required to protect 50% of the infected mice after 21 days of treatment (mean effective dose [ED_{50}]). The estimated ED_{50} of amphotericin B and AME for *H. capsulatum*, *B. dermatitidis*, *C. neoformans*, and *C. albicans* infections in mice are presented in Table 5. AME was approximately one-tenth as effective as Fungizone in the chemotherapy of experimental histoplasmosis, blastomycosis, and cryptococcosis. For *Histoplasma* and *Blastomyces* infection, the ED_{50} of amphotericin B was 0.3 mg/kg and the corresponding dosages for AME were 2.4 and 2.8 mg/kg. The ED_{50} of amphotericin B for *Cryptococcus* infection was 0.2 mg/kg as compared with 2.0 mg/kg for AME. In *Candida* infection, the ED_{50} of amphotericin B was lower than 0.05 mg/kg,

TABLE 5. *Estimated ED₅₀ values of amphotericin B and AME against four fungal infections*

Infection with:	Intraperitoneal treatment (mg/kg/day)	% Surviving 21 days		Estimated ED ₅₀ (mg/kg) dosage (21 days)	
		Amphotericin B	AME	Amphotericin B	AME
<i>H. capsulatum</i>	10.0	95	100	0.3	2.4
	5.0	100	95		
	1.0	100	20		
	0.5	90	0		
	0.05	0	0		
<i>B. dermatitidis</i>	10.0	95	95	0.3	2.8
	5.0	95	95		
	1.0	100	10		
	0.5	95	0		
	0.05	0	5		
<i>C. neoformans</i>	10.0	100	100	0.2	2.0
	5.0	95	95		
	1.0	100	50		
	0.5	90	5		
	0.05	35	5		
<i>C. albicans</i>	10.0	95	95	<0.05	<0.5 >0.05
	5.0	90	95		
	1.0	100	100		
	0.5	100	95		
	0.05	95	30		

whereas ED₅₀ for AME was between 0.5 and 0.05 mg.

Histopathology. Internal organs from all the surviving mice after 21 days of treatment, that were used for the isolation of organisms, were examined histologically. No histological changes indicative of fungal infections were detected in brains, spleens, lung, livers, and kidneys of the mice treated with 10 and 5 mg/kg of either antibiotic after experimental infections with *H. capsulatum*, *B. dermatitidis*, or *C. albicans*. Even at lower levels (1 and 0.5 mg/kg), the internal organs of approximately 80% of these mice were free from detectable lesions. The lesions were characteristic of fungal infections and in most cases fungi were demonstrated by special staining. There were no significant differences in the characteristics and distribution of these lesions in mice treated with amphotericin B or AME. In *Cryptococcus* infection, the most consistent lesions in the form of focal meningoencephalitis with the presence of mucicarmine-positive cryptococcal cells were seen in brains of mice treated with as high as 10 mg of either antibiotic per kg. These lesions, however, were more severe and more frequently seen in the animals treated with AME, indicating lower efficacy of the drug.

Another interesting difference was seen in the degree of kidney damage in the animals treated with the two antibiotics. Mice treated with 0.5

mg of amphotericin B per kg for 21 days showed degenerative changes in the renal tubules which became more pronounced with increasing dosages. At the 10-mg/kg level, the tubular degeneration was more extensive and occasionally was associated with formation of hyaline casts in the medullary region. In contrast, only minimal tubular degeneration was seen in few animals given 10 mg of AME, per kg and no evidence of renal damage was detected at lower levels.

DISCUSSION

The results presented demonstrate significant chemotherapeutic activity of AME against experimental histoplasmosis, blastomycosis, cryptococcosis, and candidosis. However, the antifungal activity of AME was slightly lower than that of the parent compound. On the basis of ED₅₀ values, AME was one-fifth as active against *Candida* infection and one-tenth as active as amphotericin B against other systemic fungal infections. With the exception of *Candida*, these results are consistent with our earlier observations on in vitro antifungal activity of the two antibiotics (4).

Despite the similar in vitro activity of the two antibiotics against *C. albicans*, the chemotherapeutic activity of AME against experimental candidosis was lower than amphotericin B. The reason for this discrepancy is not clear, but it

could be the reflection of rapid excretion of AME associated with its greater water solubility and diffusibility. The observation (D. P. Bonner, W. Mechliniski, and C. P. Schaffner, *J. Antibiot.*, in press) that AME in true aqueous solution, particularly at acid pH, is more unstable than amphotericin B may also be a contributing factor.

Isolation of organisms from mice surviving at the end of 21 days of treatment also indicates that, against experimental candidosis, the efficacy of AME was closer to that of amphotericin B than against other systemic fungal infections. Both AME and amphotericin B at a dosage of 5 mg/kg eliminated *C. albicans* from brain and kidneys of the infected mice. With *C. neoformans* infection, neither antibiotic, at 10 mg/kg, sterilized the internal organs of all infected animals, illustrating the tenacity of cryptococcosis. However, with *H. capsulatum* and *B. dermatitidis* infections, AME was less effective than amphotericin B, since higher levels of the former were required to sterilize the internal organs of the infected animals.

Keim et al. (5) have studied the acute toxicity of amphotericin B and AME for mice and dogs. In mice, AME was one-twenty fifth and one-fifteenth as toxic as amphotericin B by the intravenous and intraperitoneal routes of administration, respectively. In dogs, AME was at most only one-eighth as nephrotoxic as amphotericin B after a single intravenous administration. In the present study, the histopathological examinations of the kidneys of infected mice surviving 21 days after daily intraperitoneal administration of the two antibiotics suggest that AME is approximately one-tenth as nephrotoxic as amphotericin B. These studies indicate that AME has a significant safety advantage over amphotericin B.

The lower efficacy of AME as compared with amphotericin B may be due to the lower stability of AME in aqueous solutions. Since these studies were performed, conditions for improving the stability of AME have been described (Bonner et al., *J. Antibiot.*, in press). Further chemotherapeutic testing utilizing the conditions for improved stability might reveal a higher degree of efficacy for AME than here reported.

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