ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2000, p. 2327–2332 Vol. 44, No. 9
0066-4804/00/$04.00+0

Single-Dose AmBisome (Liposomal Amphotericin B) as Prophylaxis for Murine Systemic Candidiasis and Histoplasmosis

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Received 1 March 2000/Returned for modification 18 April 2000/Accepted 31 May 2000

AmBisome is a liposomal formulation of amphotericin B that has broad-spectrum antifungal activity and greatly reduced toxicity compared to the parent drug. In this study, amphotericin B deoxycholate (Fungizone) (1 mg/kg) and AmBisome (1 to 20 mg/kg) were tested as single-dose prophylactic agents in both immunocompetent and immunosuppressed C57BL/6 mice challenged with either *Candida albicans* or *Histoplasma capsulatum*. Prophylactic efficacy was based on survival and fungal burden in the target organ (kidneys or spleen). At 9 to 10 days after histoplasma challenge, 80 to 90% of both immunocompetent and immunosuppressed mice in the control and Fungizone groups had died. All AmBisome-treated mice survived, although in the AmBisome groups given 1 mg/kg, the mice became moribund by day 10 to 12. No spleen CFU were detected in the histoplasma-challenged mice given 10 or 20 mg of AmBisome per kg. By 23 to 24 days after histoplasma challenge, fungal growth and/or death had occurred in all immunosuppressed mice except for four mice receiving 20 mg of AmBisome per kg. There were still no detectable fungi in the spleens of immunocompetent mice given 10 or 20 mg of AmBisome per kg. In the *C. albicans* experiment at 7 days postchallenge, all animals in both untreated and treated groups were alive with culture-positive kidneys. The kidney fungal burdens in AmBisome groups given 5 to 20 mg/kg were at least 1 log unit lower than those in the Fungizone group and significantly lower than those in the untreated control group ($P < 0.05$). There was a trend toward decreasing fungal growth in the kidneys as the dose of AmBisome was increased. In conclusion, these results show that a single high dose of AmBisome (5 to 20 mg/kg) had prophylactic efficacy in immunocompetent and immunosuppressed murine *H. capsulatum* and *C. albicans* models.

Immunocompromised patients such as bone marrow and solid organ transplant patients, those with AIDS, or those with various types of leukemia or cancer undergoing chemotherapy are at high risk for developing opportunistic fungal infections (22). Also, the number of allogeneic bone marrow transplants continues to increase each year (14), and the incidence of fungal infections in these high-risk bone marrow transplant patients continues to increase (39). Despite advances in antifungal therapy, fungal infections in this high-risk group continue to be associated with a high mortality rate (44). Definitive diagnosis of a fungal infection in these high-risk patients has often not been possible due to the lack of sensitivity and specificity of culture and histological methods (17, 34). Although more rapid methods for diagnosing fungal infections are being developed (37), many fungal infections are diagnosed only at autopsy (7).

The present standard of care for high-risk febrile patients includes empiric antifungal therapy when the fever is unresponsive to 3 or 4 days of broad-spectrum antibacterial antibiotics (28). Since empiric antifungal therapy should also be broad spectrum, intravenous amphotericin B (0.5 to 0.6 mg/kg/day) has usually been administered in this setting (16). Despite this, breakthrough fungal infections have been reported even in patients receiving 0.6 to 1.0 mg of empiric amphotericin B per kg per day (6, 20).

To address the continuing problem of breakthrough fungal infections and the attendant high morbidity and mortality, many believe that prophylactic therapies might be a better approach for high-risk patients. Because of their low toxicity profiles, triazole antifungal drugs were investigated as prophylactic treatments in high-risk patients. Fluconazole was shown to significantly reduce systemic *Candida albicans* infections in bone marrow transplant patients, while the low incidence of mold infections in these studies remained unchanged (13, 32). However, in another study, oral fluconazole failed to reduce mortality or the need for systemic amphotericin B in patients receiving treatment for refractory acute myeloid leukemia (19). The oral solution of itraconazole has been evaluated for its prophylactic potential in several studies (25, 26; J. L. Harousseau, A. Stamatoullas, A. Dekker, B. DeBock, H. Bassaris, A. Fassas, L. Vazquez, W. Seifert, and K. De Beule, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. J-101, p. 480, 1998). Itraconazole was effective in preventing candida infections, and fewer deaths with proven fungal infections were observed in the itraconazole groups in all studies. Although the incidence of aspergillosis was low in these studies, proven deep aspergillus cases in the itraconazole treatment arms were reported in two of the three studies (25; Harousseau et al., 38th ICAAC).

Conventional amphotericin B has been used prophylactically (8), but the associated infusion-related complications, as well as longer-term nephrotoxic effects (23), usually outweigh the benefits of prophylactic administration of standard doses of amphotericin B. Thus, low-dose amphotericin B (LDAB) (0.1 to 0.25 mg/kg) has been examined as a safer broad-spectrum approach to prophylaxis. In two randomized, prospective, pla-

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cebo-controlled studies (27, 31), the results showed significantly improved survival in the LDAB groups, although this improved survival could not be attributed to prevention of fungal infections. Furthermore, LDAB did not significantly decrease the number of patients who were escalated to high-dose amphotericin B.

In recent years, several lipid formulations of amphotericin B with reduced nephrotoxicity have become commercially available, including Amphotec, an amphotericin B colloidal dispersion (4); Abelcet, an amphotericin B lipid complex (41); and AmBisome, a liposomal amphotericin B formulation (11). Compared to conventional amphotericin B, AmBisome has the best safety profile, having been given safely at doses as high as 15 mg/kg (T. J. Walsh, E. J. Anaissie, J. L. Goodman, P. Pappas, I. Bekersky, and D. N. Buell, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1640, p. 573, 1999). AmBisome also retains the broad spectrum of amphotericin B against both yeasts and molds, as indicated by its efficacy at 1 to 3 mg/kg for empiric therapy in febrile neutropenic patients (29, 42). Furthermore, experimental data from animals show that at higher doses, AmBisome distributes in high concentrations into many tissues of the body, including the lung, liver, spleen, kidney, bone marrow, and brain (9, 15, 30; A. Groll, N. Giri, C. Gonzalez, T. Sein, J. Bacher, S. Piscitelli, and T. Walsh, Abstr. 37th Intersci. Conf. Antimicrob. Agents Chemother., abstr. A-90, p. 19, 1997). Clinically, AmBisome has been investigated at doses of 1 or 2 mg/kg for prophylactic use in high-risk patients in several studies (18, 35, 36). The incidence of fungal infection was significantly reduced in liver transplant patients (36), but the other studies with bone marrow transplant patients were unable to demonstrate any significant reduction in suspected or proven fungal infections at these dose levels (18, 35).

Since AmBisome is much less toxic than conventional amphotericin B and preclinical data show that AmBisome continues to kill fungi in infected tissues for several weeks following cessation of therapy (1, 38), it was hypothesized that a single high dose of AmBisome could deliver adequate concentrations of amphotericin B in tissue to be effective prophylactically against fungal challenges in both immunocompetent and immunosuppressed animals. In the present study, AmBisome and amphotericin B deoxycholate (Fungizone) were compared as prophylactic agents in immunocompetent and immunosuppressed mice that were subsequently challenged with either *C. albicans* or *Histoplasma capsulatum*. The results of this study showed that one high dose of AmBisome (5 to 20 mg/kg) given 7 days prior to challenge was effective in inhibiting growth of *C. albicans* in the kidneys and preventing growth of *H. capsulatum* in the spleens of challenged mice.

MATERIALS AND METHODS

Fungal inocula. Three days prior to challenge, *C. albicans* (CP 39) was subcultured daily in Sabouraud dextrose broth, pelleted, and rinsed two times with 0.01 M phosphate-buffered saline, pH 7.2 (PBS). The final pellet was resuspended in 10 ml of PBS and the concentration of viable yeast (determined by 1% methylene blue staining) was adjusted with PBS to approximately 10^7 cells/ml for the immunocompetent mice or 10^6 cells/ml for the immunosuppressed mice.

H. capsulatum (ATCC 28122) was maintained in the yeast phase on Ham's modified medium (HMM) agar containing 16.6 g of Ham's F-12 nutrient mixture (Sigma Chemical Co., St. Louis, Mo.), 18.2 g of glucose, 1.0 g of glutamic acid, and 10 ml of 100× (35 mM) cystine stock per liter (pH 7.4). A suspension of *H. capsulatum* was prepared by washing an HMM agar slant with 5 ml of HMM broth and using 0.5 ml of the yeast suspension to inoculate 100 ml of HMM broth containing 0.2% ampicillin. After incubation in 5% CO_2 for 72 h at 35°C, the yeast was pelleted and rinsed twice with 30 ml of STM buffer (0.01 M KCl, 0.002 M $CaCl_2$, 0.0025 M $MgCl_2$, 0.05 M Trisma base, pH 7.4). The final pellet was resuspended in 30 ml of STM buffer and centrifuged at low speed for 2 min to remove yeast aggregates, and the top 15 to 20 ml of the supernatant was used for the yeast challenge. The concentration of viable yeast was adjusted with STM buffer to approximately 4×10^8 cells/ml for the immunocompetent mice or 8×10^7 cells/ml for the immunosuppressed mice.

Test substances. AmBisome (NeXstar Pharmaceuticals, Inc.), a lyophilized liposomal preparation of amphotericin B containing hydrogenated soybean phosphatidylcholine, cholesterol, distearoyl phosphatidylglycerol, and amphotericin B in a molar ratio of 2.0:1.0:0.8:0.4 (2), was reconstituted according to the manufacturer's instructions to give a 4-mg/ml solution of amphotericin B. Fungizone for injection (Bristol-Myers Squibb, Inc.), a lyophilized preparation of amphotericin B in sodium deoxycholate, was also reconstituted according to the manufacturer's instructions to give a 5-mg/ml solution of amphotericin B. Drug dilutions as needed for injection were prepared with 5% dextrose.

Challenge with *C. albicans* or *H. capsulatum*. Female C57BL/6N Tacf BR mice were used in these studies and had access to food and water ad libitum. Neutropenia was produced in the mice by an initial injection with 100 mg of cyclophosphamide (Sigma Chemical Co.) per kg 4 days prior to fungal challenge. Maintenance doses of 75 mg of cyclophosphamide per kg were given on the day of challenge and then every third day for the duration of the study. Single prophylactic intravenous doses of either AmBisome (1, 5, 10, or 20 mg/kg), Fungizone (1 mg/kg), or STM buffer (0.1 ml) (as a control) were given to the mice prior to fungal challenge. Experiments were repeated two or three times, with the time of prophylaxis varied from 2 to 7 days prior to challenge. On the day of *Candida* challenge (day 0), immunocompetent mice (8 weeks old; n = 8/group) were given intravenous injections of 10^6 yeast cells via the tail vein. Immunosuppressed mice (14 weeks old; n = 8/group) received 10^5 yeast cells. For the *Histoplasma* challenge, 5.1×10^7 yeast cells were injected into the immunocompetent mice (15 weeks old; n = 10/group), and 8×10^6 yeast cells were given to the immunosuppressed mice of the same age (n = 13/group). Mice were weighed immediately prior to fungal challenge and every 3 days throughout the experiment.

CFU determination. Seven days postchallenge with *C. albicans*, surviving mice (immunocompetent or immunosuppressed) were euthanized. Each animal's kidneys were removed and mechanically homogenized (Tissue Tearor; Biospec Products, Inc.) in 0.5 ml of PBS. Serial dilutions of the homogenates were plated in duplicate (0.1 ml) on Sabouraud dextrose agar plates and incubated at 30°C for 2 to 3 days to determine CFU per gram of kidneys. Immunocompetent mice challenged with *H. capsulatum* were euthanized on day 10 (n = 5/group, except for the Fungizone- and buffer-treated groups) or on day 24 for survivors from the groups treated with AmBisome at 5, 10, and 20 mg/kg (n = 5/group). Immunosuppressed mice were euthanized on day 9 postchallenge (n = 5/group, except for the Fungizone- and buffer treated groups), and the remaining surviving mice were sacrificed on day 23. Each animal's spleen was removed and mechanically homogenized in 0.5 ml of HMM broth containing 2% ampicillin. After serial dilution, 0.2-ml aliquots were plated in duplicate on HMM agar plates which were incubated for 10 to 14 days at 35°C with 5% CO_2 to determine CFU per gram of spleen.

Tissue preparation for HPLC analysis. One week after drug treatment, and prior to fungal challenge, three mice from each treatment group were sacrificed, and the kidneys from the mice to be challenged with candida or spleens from the mice to be challenged with histoplasma were removed for high-pressure liquid chromatography (HPLC) analysis of amphotericin B. The homogenization and extraction procedure of Mayhew et al. (24) was used for tissue sample preparation. An internal standard stock solution of *N*-acetyl amphotericin B (200 μl) and 2 ml of methanol were added to each weighed organ. After homogenization for 10 s, the samples were placed in a 50°C water bath for 15 min. After cooling, the samples were centrifuged for 10 min at 4,000 rpm (Sorvall SS34 rotor), and the supernatants were collected. The pellets were resuspended in 2 ml of methanol, heated again for 15 min at 50°C, and centrifuged, and the two supernatants were pooled and brought up to 5.0 ml with methanol. HPLC analysis was done using a 30-μl injection volume on an Ultrasphere C_{18}, 5-μm (4.6- by 250-mm) column, with a 1.5-ml/min flow rate, a 15-min run time, and detection at 405 nm. The mobile phase was methanol–acetonitrile–2.5 mM EDTA in a ratio of 50:20:30 (vol/vol/vol).

Statistical analysis. The data were analyzed using Sigma Stat statistical software (Jandel Scientific). Analyses of CFU-per-gram data were performed on $\log_{10}$-transformed data. The results were compared using one-way analysis of variance (12). When preliminary analysis of the results indicated the data were not normally distributed, the nonparametric Kruskall-Wallis one-way analysis of variance on ranks was used to compare data (12). Pairwise multiple comparisons were done using the Student-Newman-Keuls method when the data were normally distributed or with the Mann-Whitney rank sum test when data required a nonparametric test (21).

RESULTS

CFU determinations and survival. For most treatments, varying the time of prophylaxis between 2 and 7 days before challenge did not produce a statistically significant difference in response. When there were differences, the 7-day prophylaxis was in no case superior to a treatment given nearer to the time of challenge. However, since the 7-day experiments rep-

TABLE 1. CFU in spleen on day 9 or 10 postchallenge in mice treated prophylactically with Fungizone or AmBisome 7 days prior to challenge with *H. capsulatum*

Treatment	Dose (mg/kg)	Immunocompetent mice		Immunosuppressed mice	
		Mean $\log_{10}$ CFU/g of spleen ± SD	No. surviving/ total	Mean $\log_{10}$ CFU/g of spleen ± SD	No. surviving/ total
Control		ND[a,b]	1/10	ND[f]	2/10
Fungizone	1	ND[c]	1/10	ND[g]	1/10
AmBisome	1	4.81 ± 0.54[d]	10/10	4.96 ± 0.18	10/10
AmBisome	5	0.85[e]	10/10	3.49 ± 1.17	10/10
AmBisome	10	0	10/10	0	10/10
AmBisome	20	0	10/10	0	10/10

[a] ND, not determined.
[b] $\log_{10}$ CFU/gram of spleen in the sole survivor = 5.39.
[c] $\log_{10}$ CFU/gram of spleen in the sole survivor = 4.00.
[d] All 10 mice were sacrificed in moribund condition.
[e] Four out of five mice were culture negative. The value is for the single culture-positive animal.
[f] Bacterial contamination.
[g] $\log_{10}$ CFU/gram of spleen in the sole survivor = 5.13.

TABLE 2. Growth of *H. capsulatum* at day 23 or 24 postchallenge in mice treated prophylactically with AmBisome or Fungizone

Treatment	Dose (mg/kg)	Immunocompetent mice		Immunosuppressed mice	
		Mean $\log_{10}$ CFU/g of spleen	No. surviving/ total	Mean $\log_{10}$ CFU/g of spleen ± SD	No. surviving/ total
Control			0/5		0/5
Fungizone	1		0/5		0/5
AmBisome	1		ND[a,b]		0/5
AmBisome	5	2.40[c]	5/5	ND[d]	1/5
AmBisome	10	0	5/5	4.69 ± 0.08	3/5
AmBisome	20	0	5/5	1.36[c]	5/5

[a] ND, not determined.
[b] All mice were sacrificed in moribund condition on day 10.
[c] Four out of five mice were culture negative. The value is for the single culture-positive animal.
[d] Bacterial contamination.

resent the most stringent test of prophylaxis, only results from these studies are presented here.

(i) *H. capsulatum* challenge. Compared to results for the control groups, only prophylaxis with AmBisome resulted in lower fungal burdens in the spleens of either immunosuppressed or immunocompetent mice on day 9 or 10 (Table 1). Most of the animals in both the control groups and the Fungizone (1 mg/kg) groups did not survive through day 9 or 10, while there were no deaths in any of the AmBisome-treated groups. Among the survivors, the single prophylactic dose of 1 mg of AmBisome per kg was the least effective, with mean $\log_{10}$ CFU per gram of spleen of 4.81 and 4.96 for immunocompetent and immunosuppressed mice, respectively. When the AmBisome dose was increased to 5 mg/kg, four of five immunocompetent treated mice had no detectable CFU in their spleens on day 10. In comparison, all immunosuppressed mice given 5 mg of AmBisome per kg had positive spleen cultures, although the mean $\log_{10}$ CFU per gram of spleen for this group was reduced to 3.49. When the dose was increased to 10 or 20 mg/kg, there were no detectable fungi in either the immunocompetent or the immunosuppressed mice on day 10 or 9.

At about 3 weeks postchallenge, the fungal burden in the immunocompetent animals receiving 5, 10, or 20 mg of AmBisome per kg had not changed (Table 2). There was still no detectable fungus in 100% (10 or 20 mg/kg) and 80% (5 mg/kg) of the mice. In contrast, at a similar time point, the protection from infection in the immunosuppressed mice had diminished, with only 20% survival in the 5-mg/kg group and 60% survival in the 10-mg/kg group. At 20 mg/kg, most of the mice (80%) remained free of infection, but some infection was detected in one of the mice.

(ii) *C. albicans* challenge. At 7 days after fungal challenge, all mice in all experimental groups were still alive. However, microbiological analysis revealed that all animals had positive kidney cultures (Table 3). The mean $\log_{10}$ CFU per gram for both immunocompetent and immunosuppressed control groups averaged 6.0 to 6.2. Prophylaxis with 1 mg of Fungizone per kg significantly ($P < 0.05$) reduced the fungal burden regardless of immune status. Compared to 1 mg of Fungizone per kg, all AmBisome doses (1, 5, 10, or 20 mg/kg) produced significantly greater protection ($P < 0.05$) in the immunocompetent mice 7 days postchallenge. In immunosuppressed mice, AmBisome dosing at 20 mg/kg, but not 1 or 5 mg/kg, resulted in a significantly lower mean CFU in the kidneys ($P < 0.05$) than 1 mg of Fungizone per kg. In immunocompetent mice, there were significantly fewer CFU per gram of kidneys ($P < 0.05$) following treatment with 5 to 20 mg of AmBisome per kg compared to 1 mg of AmBisome per kg (Table 3). In immunosuppressed mice, only the 20-mg/kg AmBisome dose produced significantly fewer CFU per gram of kidney ($P < 0.05$) than 1 mg of AmBisome per kg. Since all of the surviving AmBisome-treated, candida-challenged mice had some yeast in their kidneys at 7 days postchallenge, follow-up studies of prolonged protection against fungal infection were not done.

Drug concentrations in tissue samples. Analysis of drug concentrations in tissue samples was done 7 days after the single prophylactic injection of either Fungizone or AmBisome to determine the concentration of amphotericin B in the target organ at the time of fungal challenge.

(i) Drug concentrations in spleens from immunosuppressed mice. Compared to treatment with 1 mg of Fungizone per kg, treatment with AmBisome (1, 5, 10, or 20 mg/kg) produced significantly ($P < 0.05$) higher splenic drug concentrations (Table 4). There were also significantly higher ($P < 0.05$) concentrations of amphotericin B in the spleens of mice given the higher doses (5, 10, or 20 mg/kg) of AmBisome compared to the lowest dose (1 mg/kg) of AmBisome. The data also showed that while animals given 5 or 10 mg of AmBisome per kg did not have significantly different drug concentrations in their spleens, the mice given 20 mg of AmBisome per kg had

TABLE 3. CFU in kidney on day 7 postchallenge in mice treated prophylactically with Fungizone or AmBisome 7 days prior to challenge with *C. albicans*

Treatment	Dose (mg/kg)	Immunocompetent mice		Immunosuppressed mice	
		Mean $\log_{10}$ CFU/g of kidney ± SD	No. surviving/ total	Mean $\log_{10}$ CFU/g of kidney ± SD	No. surviving/ total
Control		6.00 ± 0.44	5/5	6.22 ± 0.73	5/5
Fungizone	1	5.20 ± 0.73	5/5	4.18 ± 1.20	5/5
AmBisome	1	4.59 ± 0.48	5/5	3.22 ± 0.98	5/5
AmBisome	5	3.82 ± 0.24	5/5	3.46 ± 0.68	5/5
AmBisome	10	3.49 ± 0.33	5/5		
AmBisome	20	3.27 ± 0.44	5/5	2.67 ± 0.79	5/5

TABLE 4. Concentrations of amphotericin B in tissues of immunocompetent and immunosuppressed mice at the time of fungal challenge

Treatment	Dose (mg/kg)	μg/g of kidney (mean ± SD) in *C. albicans*-challenged mice		μg/g of spleen (mean ± SD) in *H. capsulatum*-challenged mice (immunosuppressed)
		Immunocompetent	Immunosuppressed	
Fungizone	1	1.33 ± 0.61	0.34 ± 0.08	5.0 ± 3.3
AmBisome	1	0.13 ± 0.22	0.49 ± 0.06	16.7 ± 6.8
AmBisome	5	0.63 ± 0.09	1.27 ± 0.34	39.5 ± 8.4
AmBisome	10	2.91 ± 1.53		56.2 ± 9.3
AmBisome	20	8.08 ± 0.38	10.51 ± 2.89	254 ± 107

significantly higher splenic drug concentrations. These observations may help to explain the sustained protection at 23 days postchallenge associated with the single 20-mg/kg AmBisome dose and the lack of sustained protection by the 5- or 10-mg/kg dose at the same time point in immunosuppressed mice.

(ii) Drug concentrations in kidneys of immunocompetent and immunosuppressed mice. The concentration of amphotericin B in the kidneys of mice given AmBisome at either 10 or 20 mg/kg (immunocompetent mice) or 5 or 20 mg/kg (immunosuppressed mice) was significantly higher than that observed in mice receiving 1 mg of Fungizone per kg ($P < 0.05$ for both) (Table 4). Comparable drug concentrations in the kidneys were observed in immunocompetent mice with 1 or 5 mg of AmBisome per kg and 1 mg of Fungizone per kg, while prophylaxis with 1 mg of AmBisome or Fungizone per kg in immunosuppressed mice resulted in comparable kidney drug concentrations. When comparing drug levels in mice given 5 mg of AmBisome per kg with those given 20 mg/kg, the relative increase in drug levels in the kidneys is greater than the increase in the administered dose. This result is consistent with the nonlinear pharmacokinetic behavior of AmBisome previously observed (40), which supports reticuloendothelial system saturation and redistribution of the drug to non-reticuloendothelial system tissues such as the kidneys.

DISCUSSION

AmBisome was investigated for prophylaxis in this study because of its broad-spectrum antifungal activity (11), its minimal acute and chronic toxicities (40; Walsh et al., 39th ICAAC), and its sustained bioavailability in tissues (3). The results of the present study showed that a single, prophylactic dose of 5 to 20 mg of AmBisome per kg given 1 week prior to challenge with either *C. albicans* or *H. capsulatum* in immunocompetent or immunosuppressed mice was effective in preventing or inhibiting fungal growth. The single, high dose of AmBisome was chosen for the study based on previous reports that demonstrated at least a 14-day maintenance of bioactive drug. Van Etten and coworkers (38) reported that in murine candidiasis, five daily doses of 7 mg of AmBisome per kg continued to decrease the kidney CFU by 2 log units when the respective data at 24 h and 14 days posttreatment were compared. Similarly, in another study, four treatments with 6 mg of AmBisome per kg in *H. capsulatum*-infected mice produced a 2-log-unit reduction in CFU in the spleen between 24 h and 14 days posttreatment (1). Thus, in the present study the detection of high amphotericin B levels in the kidneys and spleens 1 week after dosing and the inhibition of fungal growth following candida or histoplasma challenge confirm the observations of other investigators that AmBisome can deliver sufficient amounts of amphotericin B to the tissues that can remain bioactively available for extended periods.

In the present study, 9 or 10 days after histoplasma challenge, both 10- and 20-mg/kg AmBisome treatments completely prevented growth of the fungus in the spleens of either immunocompetent or immunosuppressed mice. Even at the lower dose of 5 mg/kg, 80% of the immunocompetent mice were protected from infection, although all of the immunosuppressed mice given 5 mg/kg had some infection in their spleens. When the fungal growth in the spleens was again assessed about 2 weeks later, AmBisome prophylaxis had been maintained in the immunocompetent mice given 5, 10, or 20 mg/kg, but fungal growth in the spleens of the immunosuppressed mice had now significantly increased. In the immunosuppressed animals, there were 20 and 60% survival in the 5- and 10-mg/kg treatment groups, respectively, with four of the five mice given 20 mg/kg still free of infection.

These results indicate that a single prophylactic AmBisome treatment did not completely eliminate the histoplasma from the spleens. The T-cell immune response in the immunocompetent mice was likely necessary to clear any residual histoplasma, since T lymphocytes are very important in the immunological response to *H. capsulatum* infection (43). Mice that continue to be immunosuppressed with cyclophosphamide were probably unable to clear the infection, since cyclophosphamide is reported to affect the later stages of cell division in the bone marrow, resulting in decreased numbers of granulocytic cells, monocytic cells, lymphoid cells, and myeloid blast cells (10).

In comparison, all candida-challenged immunosuppressed or immunocompetent mice given a single prophylactic dose of 5, 10, or 20 mg of AmBisome per kg had some fungi in their kidneys following challenge, although the CFU were significantly lower in the treated than in the untreated mice. The improved fungal clearance of histoplasma compared to candida-challenged mice may have been due to the differences in the amphotericin B concentrations in the target organs at the time of fungal challenge. At these dose levels, the drug concentrations in the spleens were approximately 20 to 60 times higher than those in the kidneys. Furthermore, there are reports of AmBisome and other liposomes being taken up by macrophages in vitro (5, 33). This is significant since *H. capsulatum* replicates in macrophages while *C. albicans* replicates extracellularly. Thus, organ and cellular localization of AmBisome would seem to favor increased localization of drug concentrations in close proximity to the *H. capsulatum*.

There was also a correlation between the concentration of AmBisome in tissue and the efficacy of a given prophylactic dose. As the dose of AmBisome was raised, the spleen drug concentrations increased, as well as the efficacy of the AmBisome for inhibiting histoplasma. This resulted in sustained inhibition of histoplasma replication in the spleens, particularly with 20 mg of AmBisome per kg. When the kidneys of prophylactically treated animals were evaluated for amphotericin B concentration, there was also a dose-dependent increase in drug levels in tissue. This correlated with an improvement in efficacy with increasing AmBisome doses, although the increase in efficacy was not as marked in candida-challenged mice as it was with histoplasma-challenged mice. This was probably because of the lower levels of drug in the kidneys compared to the spleens at all dose levels.

The prophylactic use of low doses of AmBisome has been investigated clinically by Tollemar and colleagues. In a randomized, prospective prophylactic trial with bone marrow transplant recipients (35), they showed that AmBisome at 1 mg/kg/day significantly reduced fungal colonization but that

suspected and proven fungal infections were not significantly reduced compared to those in the placebo-treated control group. However, in liver transplant recipients (36), AmBisome at 1 mg/kg/day completely prevented invasive fungal infections, while in the placebo-treated control group, 6 of 37 patients developed such infections ($P = 0.01$). With neutropenic patients, other investigators compared AmBisome given three times weekly at 2 mg/kg with a placebo (18). Significantly fewer patients in the AmBisome arm became colonized with fungus compared to the placebo arm (15 versus 35, respectively; $P = 0.05$), but this regimen did not lead to a significant reduction in fungal infection or in the requirement for systemic antifungal therapy.

The recommended therapeutic dose of AmBisome in the United States is 3 to 5 mg/kg, and higher daily dosing, up to 15 mg/kg, has been reported to be well tolerated (Walsh et al., 39th ICAAC). The clinical data combined with the results of the present preclinical study suggest that intermittent dosing with 5 to 20 mg of AmBisome per kg might be a safe and effective prophylactic treatment for both extracellular and intracellular fungal infections. The results of the clinical trials with LDAB prophylaxis and the improved safety and pharmacokinetics of amphotericin B when it is formulated as AmBisome provide a strong rationale for further investigating AmBisome at higher doses in a prophylactic setting. The presented preclinical studies with a single high AmBisome dose ranging from 5 to 20 mg/kg suggest that intermittent prophylactic dosing with higher levels of AmBisome in greater numbers of patients might provide statistically significant data to show efficacy with AmBisome in this setting.

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