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AmBisome: liposomal formulation, structure, mechanism of action and pre-clinical experience

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Amphotericin B is the treatment of choice for life-threatening systemic fungal infections such as candidosis and aspergillosis. To improve this drug's efficacy and reduce its acute and chronic toxicities, several lipid formulations of the drug have been developed, including AmBisome, a liposomal formulation of amphotericin B. The liposome is composed of high transition temperature phospholipids and cholesterol, designed to incorporate amphotericin B securely into the liposomal bilayer. AmBisome can bind to fungal cell walls, where the liposome is disrupted. The amphotericin B, after being released from the liposomes, is thought to transfer through the cell wall and bind to ergosterol in the fungal cell membrane. This mechanism of action of AmBisome results in its potent *in vitro* fungicidal activity while the integrity of the liposome is maintained in the presence of mammalian cells, for which it has minimal toxicity. In animal models, AmBisome is effective in treating both intracellular (leishmaniasis and histoplasmosis) and extracellular (candidosis and aspergillosis) systemic infections. Because of its low toxicity at the organ level, intravenous AmBisome can be safely delivered at markedly high doses of amphotericin B (1–30 mg/kg) for the treatment of systemic fungal infections. AmBisome has a circulating half-life of 5–24 h in animals, and in animal models appears to localize at sites of infection in the brain (cryptococcosis, aspergillosis, coccidioidomycosis), lungs (blastomycosis, paracoccidioidomycosis, aspergillosis) and kidneys (candidosis), delivering amphotericin B that remains bioavailable in tissues for several weeks following treatment.

Introduction

AmBisome is a liposomal formulation of amphotericin B in which the drug is strongly associated with the bilayer structure of small unilamellar liposomes. In addition to amphotericin B, AmBisome is composed of hydrogenated soy phosphatidylcholine, distearoyl phosphatidylglycerol and cholesterol. This report will summarize the rationale for the development of AmBisome's unique liposome formulation of amphotericin B, including how liposome properties affect the pharmacokinetics and mechanism of action of amphotericin B when it is incorporated into a liposome. In addition, the results of pre-clinical testing with AmBisome will be presented. Finally, the article will show how the pre-clinical results with AmBisome may offer some direction toward combating fungal diseases in the clinical setting.

Development and structure of AmBisome

The basic component of all clinically useful liposomes is the phospholipid molecule. Phospholipids are amphiphilic molecules composed of a three-carbon glycerol backbone, a polar, hydrophilic head group attached to the first glycerol carbon and two hydrophobic fatty acid side chains linked to the other two glycerol carbons (Figure 1). The polar head group may be positively charged, negatively charged or neutral. The fatty acid side chains may vary in length, and they may be saturated or unsaturated. When phospholipids are mixed with water, they spontaneously tend to arrange into bilayer structures. The polar head groups are on the outer surfaces of the bilayer and interact with water. The fatty acid side chains form the inner portion of the bilayer, and interact with the side chains of other phospholipid molecules to stabilize the membrane-

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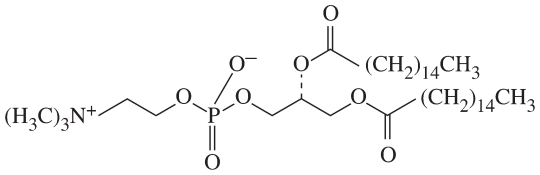

| -POLAR HEADGROUP- |-BACKBONE-|-FATTY ACID SIDECHAINS-|

Figure 1. Chemical structure of dipalmitoyl phosphatidylcholine, a typical phospholipid used to prepare liposomes.

like bilayers. In 1965, Bangham *et al.*[1] described closed bilayer structures that formed when phospholipids were mixed in water. This was the first description of liposomes.

Liposome stability is dependent on the nature of the phospholipid molecules that are used to form the liposome preparation. Pure phospholipid bilayers are known to undergo a transition from a gel to liquid crystalline state as temperature is increased. This transition occurs over a narrow range around the characteristic transition temperature, T_c. As shown in Table 1, the transition temperature of a given phospholipid is dependent on the fatty acid chain length and degree of unsaturation, as well as the nature of the head group.[2] Thus, dioleoylphosphatidylcholine, with one site of unsaturation in each of the 18-carbon side chains, has a T_c of −22°C, whereas distearoyl phosphatidylcholine, with no sites of unsaturation in the 18-carbon side chains, has a T_c of 55°C. Liposome bilayers tend to become more permeable at or above their transition temperature, and thus release their internal contents.[3] Cholesterol can be mixed with phospholipids, and liposomes that contain cholesterol are less prone to leaking their internal contents. Following these principles, liposomes formulated to act as drug carriers that are stable under physiological conditions should be prepared from phospholipids with T_cs above 37°C, and should have a high molar fraction of cholesterol as part of their composition.

AmBisome is composed of hydrogenated soy phosphatidylcholine and distearoyl phosphatidylglycerol. These components have high T_cs, and were used to make a formulation that would be stable at 37°C. In addition, it has been proposed that the negative charge on the distearoyl phosphatidylglycerol was able to interact with the positive amine group of the amphotericin B, forming an ionic complex in the bilayer. Cholesterol was also included in the AmBisome formulation to add stability[4] and help hold the amphotericin B in the liposome bilayer, since cholesterol binds with amphotericin B. Finally, AmBisome was designed as very rigid, small unilamellar liposomes with mean diameters <100 nm. Such small, rigid unilamellar liposomes are known to have long circulation times in the bloodstream, and may be sterilized by filtration through 0.2 μm pore membrane filters.

The precise nature of the interaction of amphotericin B with the lipid components of AmBisome is not known. However, Fujii *et al.*[5] presented experimental evidence showing that when the amphotericin B concentration in the lipid bilayer of AmBisome-like liposomes is increased above a critical concentration, the drug exists in an aggregated state. These workers also provided data that supported the formation of amphotericin B channels or pores across the lipid bilayer when the critical concentration of drug was reached. A model consistent with this experimental evidence was proposed in which amphotericin B was associated in a circular array of eight molecules, and in which two of these arrays form tail-to-tail dimers of 16 molecules of amphotericin B that can act as channels spanning the liposome bilayer of AmBisome. In this formation, amphotericin B remains firmly associated with the liposome structure while it is in circulation.

Toxicity of AmBisome

To determine the comparative toxicity of AmBisome and amphotericin B for mammalian cells *in vitro*, AmBisome was first incubated at 37°C for 2 h with washed human red blood cells. At concentrations up to 100 mg/L, AmBisome caused only 5% haemolysis compared with 92% haemolysis with amphotericin B at a concentration of 1 mg/L.[6] These results suggest that <1% of the amphotericin B was

Table 1. Transition temperatures (T_cs) for some phospholipids used to prepare liposomes

Phospholipid	Abbreviation	Chain length:double bonds	T_c (°C)
Dilauryloyl phosphatidylglycerol	DLPG	12:0	4
Dilauryloyl phosphatidylcholine	DLPC	12:0	−1.8
Dimyristoyl phosphatidylcholine	DMPC	14:0	23
Dipalmitoyl phosphatidylcholine	DPPC	16:0	41
Dipalmitoyl phosphatidylethanolamine	DPPE	16:0	60
Distearoyl phosphatidylglycerol	DSPG	18:0	55
Distearoyl phosphatidylcholine	DSPC	18:0	55
Dioleoyl phosphatidylcholine	DOPC	18:1	−22

released from AmBisome during the incubation. The rapid and extensive damage to red blood cells by amphotericin B, but not AmBisome, was also confirmed by measuring potassium release from rat red blood cells incubated with these two drug forms.[7]

Other *in vitro* studies evaluated the toxicity of AmBisome and amphotericin B for a variety of other cell types, such as primary Langerhans cells[8] and established canine kidney and murine macrophage cell lines.[9] AmBisome was found to be less toxic than amphotericin B based on morphometric analysis of cellular ultrastructure in Langerhans cells, and by several viability assays on the kidney and macrophage cell lines. The reduced *in vitro* toxicity of AmBisome toward kidney and macrophage cells may help explain the lower toxicity of AmBisome when it is administered intravenously.

The earliest toxicity testing of AmBisome *in vivo* was carried out in the C57BL/6 strain of mice, which is very sensitive to amphotericin B toxicity. In these mice, the intravenous dose of AmBisome that would cause 50% of the animals to die of toxic effects (LD_{50}) was found to be >175 mg/kg.[10] By comparison, conventional amphotericin B, has an LD_{50} of 2–3 mg/kg in this strain of mice. Thus, early in its development, it was apparent that AmBisome was significantly less toxic than conventional amphotericin B. Since that time, AmBisome has been evaluated for pre-clinical toxicology in rats,[11] rabbits[12] and dogs.[13] Results from these studies confirmed the AmBisome safety profile originally observed in mice.

Pharmacokinetics of AmBisome

The pre-clinical pharmacokinetics of AmBisome was studied in mice, rats, rabbits and dogs. As shown in Table 2, the pre-clinical pharmacokinetics of AmBisome demonstrated a non-linear clearance from plasma, presumably due to saturation of the reticuloendothelial system (RES). Thus, in the species studied, the C_{max} and $AUC_{0-\infty}$ of amphotericin B when given as AmBisome increased in a manner greater than the incremental increase in dose. The mean elimination half-life ($t_{½}$) had a range of 5–24 h depending on dose and species. In all species, both the volume of distribution (V_d) and the total clearance (CL) tended to decrease when dose was increased from 1 to 10 mg/kg. $AUC_{0-\infty}$ values for AmBisome are higher than for an equivalent dose of conventional amphotericin B, and in all cases increased disproportionately to the relative increase in dose. As discussed more fully below, these pharmacokinetic properties of AmBisome are consistent with the premise that the amphotericin B in AmBisome remains associated with the liposome structure while it is in circulation.

Mechanism of action

AmBisome can effectively inhibit the *in vivo* replication of a broad range of fungi in both immunocompetent and immunocompromised animals. To further elucidate the mechanism involved in AmBisome's therapeutic efficacy, sulphorhodamine, a red fluorescent dye, was used to label liposomes.[14] Groups of C57BL/6 mice were inoculated intravenously with *Candida albicans*, and 23 and 30 h after fungal challenge were treated intravenously with one of the following agents: sulphorhodamine-labelled AmBisome; sulphorhodamine-labelled liposomes without drug; free sulphorhodamine dye; or 10 mM succinate in 9% sucrose buffer. Approximately 17 h after the second liposome treatment, mice were killed and their kidneys removed, frozen and sectioned. Sequential sections were stained with either haematoxylin–eosin to demonstrate tissue morphology, Gomori methanamine silver (GMS) to visualize *Candida* in tissue, or were unstained for examination with the fluorescent microscope to detect the presence of the red fluorescent dye.

Table 2. Pre-clinical pharmacokinetic parameters of AmBisome

Species	Dose (mg/kg)	C_{max} (mg/L)	$AUC_{0-\infty}$ (μg·h/mL)	$t_{½}$ (h)	V_d (L/kg)	CL (mL/h/kg)
Mouse	1	8	36	17	0.68	28
	5	50	1081	24	0.16	4.6
Rat	1	7.2	64	9.5	0.21	16
	3	30.3	374	7.9	0.10	8.4
	9	141.3	1136	8	0.10	8
Rabbit	2.5	53	207	5.2	0.09	12
	5	132	838	5.5	0.05	5.3
	10	287	2223	7.7	0.05	4.2
Dog	1	1.9	11	9.3	0.96	79
	4	18	164	8.4	0.29	26
	8	72	986	11	0.14	10

With the GMS stain, the kidneys of mice treated with free sulphorhodamine dye showed areas of pseudohyphae and yeast cells due to the infection. When the same tissue was examined by fluorescence microscopy, there was red fluorescence permeating the tissue and bright red fluorescence outlining all the kidney cells. In comparison, control mice given only buffer showed dull red autofluorescence distributed throughout the infected tissue. In contrast to the dye- or buffer-treated mice, tissue from animals treated with sulphorhodamine-labelled empty (non-drug-containing) liposomes showed bright red fluorescence that appeared to be localized in areas of fungal growth. The tissues from fluorescent-AmBisome-treated mice also showed bright red fluorescence in the area of the fungal infection, but the extent of the infection in the mice given sulphorhodamine-labelled AmBisome had been reduced compared with the mice treated with fluorescently labelled empty liposomes. This observation was probably due to the fungal killing by AmBisome.

The results indicated that the entrapped fluorescent dye from both sulphorhodamine-labelled empty liposomes and sulphorhodamine-labelled AmBisome did not distribute evenly throughout the kidney tissue, but rather tended to localize near the areas of fungal infection. This observation contrasted with the observations in the kidneys of mice given the free dye, which showed dye distributed throughout the tissue. These data indicate that the liposome-entrapped dye remained associated with the liposomes, and that the liposomes with or without drug localized at the site of fungal infection in the kidneys. However, this experiment was unable to determine whether liposomes remained intact in the infected areas, or whether there was a direct interaction between the liposomes and the fungus.

Attempts were made to visualize intact liposomes on the surface of fungi by means of freeze-fracture electron microscopy. *Candida glabrata* cells were treated for up to 7 h with AmBisome, liposomes without drug or buffer, and then frozen and fractured with a freeze etch device. The preparations were coated with platinum and examined with an electron microscope. The results showed that intact AmBisome and liposomes without drug could be detected attached to the fungal cell wall, not the cell membrane. Buffer-treated yeast cells showed no such liposome-like structures associated with their cell walls or cell membranes. Since spherical vesicles of various sizes were present within the cytoplasm of all yeast cells, it was not possible to determine with certainty whether any intact liposomes were present in the cytoplasm of treated yeast cells.[6]

Another electron microscope technique was used to confirm that liposomes were attaching to the cell walls of fungi. AmBisome and liposomes without drug were formulated with a small amount of dipalmitoyl phosphatidylethanolamine that had been covalently bound to nanogold particles (*c.* 1.4 nm in diameter). Fungal cells including *C. glabrata*, *C. albicans* and *Aspergillus fumigatus* were treated with the gold-labelled AmBisome or liposomes without drug for up to 24 h. Following incubation, the treated fungi were fixed with 2.5% glutaraldehyde and the gold particles were enhanced with silver-enhancing reagent. Samples were stained with osmium tetroxide and uranyl acetate, embedded in Epon/Araldite and sectioned for examination by transmission electron microscopy.

The results showed that liposomal lipid (visualized as dense black, round to oval structures) from AmBisome was not only on the fungal cell wall surface, but also distributed throughout the damaged cytoplasm of the fungi after 14 h of incubation. The lipid could be seen penetrating through the fungal cell wall, reaching the fungal cell membrane and entering the fungal cytoplasm. Untreated fungi had a normal cytoplasmic appearance and were devoid of any dense, black structures. In comparison, lipid from the liposomes without drug could not be seen penetrating into the fungal cytoplasm even after prolonged incubation with these liposomes. Instead, a large amount of gold-labelled lipid from the liposomes without drug was attached to the outer fungal cell wall, not the fungal cell membrane.[15]

The data indicate that AmBisome and liposomes without drug, but with the same lipid composition as AmBisome, have an affinity for fungal cell walls. The lipid from AmBisome subsequently penetrates through the fungal cell wall and enters the fungal cytoplasm. The mechanism responsible for the disruption of AmBisome at the cell wall surface has not been elucidated. However, the data indicate that amphotericin B released from disrupted AmBisome damages the fungal cell membrane, producing sufficient alteration of the cell to allow the liposomes or portions of the disrupted liposomes to penetrate through the cell wall, cell membrane and distribute throughout the cytoplasm. Similar action of AmBisome is postulated to occur when the liposomal drug encounters fungi in infected host tissues.

Pre-clinical efficacy of AmBisome

The process of incorporating amphotericin B into the AmBisome formulation could possibly alter its spectrum of antifungal activity, either *in vitro* or *in vivo*. The *in vitro* experiments conducted to study this consisted of MIC and minimal fungicidal concentration (MFC) determinations against common fungal pathogens including *Candida*, *Cryptococcus*, *Aspergillus* and *Fusarium* spp. The results from both tube macrodilution and plate microdilution test methods confirmed that the MIC and MFC profile for AmBisome is similar to that of amphotericin B.[15,16] The MIC of AmBisome ranged from 0.05 to 2.5 mg/L as compared with 0.1 to 2.5 mg/L for amphotericin B. Thus, the incorporation of amphotericin B into the liposome bilayer of AmBisome has little or no inhibitory effect on its MICs *in vitro*.

The antifungal efficacy of AmBisome was also compared with amphotericin B in cultured Langerhans cells

infected with *C. glabrata*.[8] The *Candida*-infected cells were incubated with amphotericin B or AmBisome at 12.5 mg/L for up to 48 h. Both AmBisome and amphotericin B were equally effective after 48 h, reducing the amount of viable fungus by 5 logs. Therefore, AmBisome could effectively treat this intracellular infection. However, at this concentration, AmBisome was much less cytotoxic to the cultured Langerhans cells than amphotericin B.

AmBisome has been studied for efficacy in a number of animal models of both intracellular and extracellular infections. Several studies examined the efficacy of AmBisome against intracellular infections *in vivo*. A group in France compared AmBisome (six doses of 0.8, 5 and 50 mg/kg, iv) and conventional amphotericin B (six doses of 0.8 mg/kg, iv) to meglumine antimoniate (eleven doses of 200 mg/kg, ip) for efficacy against the parasitic disease leishmanaisis.[17] All treatments were completed on day 17, and on days 20, 60 and 120 the investigators examined animals from each group for the amount of *Leishmania infantum* promastigotes in the liver, spleen and lung. AmBisome at the two higher doses completely eradicated parasites from all tissues examined. Also, AmBisome 0.8 mg/kg eradicated parasites from lung, and was able to decrease the parasitic burden by at least 4–6 logs in the spleen and liver compared with untreated controls. Furthermore, AmBisome 0.8 mg/kg was more effective at reducing the parasitic burden in all tissues compared with either of the other treatments. The investigators suggested that the persistence of high levels of amphotericin B in tissues after administration of AmBisome could help explain its remarkable efficacy against this organism.

Histoplasma capsulatum is another intracellular fungus that has been found to be highly susceptible to AmBisome therapy in an immunosuppressed mouse model.[14] Low doses of Fungizone or AmBisome (four doses of 0.3 or 0.6 mg/kg) were compared, and a higher dose of AmBisome (four doses of 6 mg/kg) was also tested to determine whether a higher dose gave a better therapeutic response. One goal of the study was to determine whether several weeks after treatment, it would still be possible to keep the level of infection low. At the lower doses, neither Fungizone nor AmBisome were particularly effective. Twenty-four hours after the last lower dose treatments, cfu/g of spleen were reduced by *c.* 2 logs compared with untreated controls, but regrowth was evident after 14 days in all cases. However, the higher dose of AmBisome (6 mg/kg) dramatically reduced the cfu by 5 logs at 24 h post-treatment compared with the control. Furthermore, even though treatment had been terminated for 2 weeks, the cfu/g of spleen continued to decrease by a further 2 logs, indicating that the drug in the tissue remained bioactive even after therapy was discontinued.

The relationship between antifungal efficacy and tissue concentration of drug delivered by different formulations of amphotericin B was studied by Groll *et al.*[18] These investigators evaluated groups of uninfected and *C. albicans*-infected rabbits that were treated daily for 7 days with each of the three commercially available lipid formulations of amphotericin B, as well as the conventional deoxycholate form of the drug. The animals were examined for amphotericin B concentration in the plasma, cerebrospinal fluid and brain tissue 30 min after the seventh dose. The results showed quite clearly that the AmBisome-treated animals achieved significantly higher concentrations in the plasma of both the infected and uninfected groups compared with the other formulations. Virtually no drug (<0.1 mg/L) was found in the cerebrospinal fluid of any of the treatment groups. However, in the brain tissue itself, there was a significantly higher tissue concentration of amphotericin B in the AmBisome-treated groups than in groups receiving any of the other formulations. Interestingly, amphotericin B deoxycholate given at 1 mg/kg produced a significantly higher plasma concentration of drug and a slightly higher concentration of drug in the brain tissue than either amphotericin B colloidal dispersion (ABCD; Amphocil/Amphotec) or amphotericin B lipid complex (ABLC, Abelcet), the other two amphotericin B lipid formulations. In infected animals, all treatments significantly reduced the *C. albicans* cfu in the brain. However, only AmBisome and conventional amphotericin B were able to completely clear *C. albicans* from the brains of all animals in these groups. This study highlights important pharmacokinetic differences between the various lipid formulations of amphotericin B, and indicates that higher plasma concentrations of drug lead to higher brain concentrations, which in turn could be a reason for increased efficacy.

Several years ago, Albert *et al.*[19] described a mouse model of meningitis caused by *Cryptococcus neoformans*. The animals were treated with multiple doses of amphotericin B (0.3 mg/kg iv or 3 mg/kg ip) or AmBisome (1, 3, 20 or 30 mg/kg iv). Some animals were killed during the therapy, and culture results showed that 3 mg/kg amphotericin B was more effective than 3 mg/kg of AmBisome at lowering fungal cfu in the brain. However, when the full six-treatment regimen was completed, and the animals were killed 2 weeks after that, there was a 6 log increase in the number of *C. neoformans* cfu in the brains of mice treated with amphotericin B. In contrast, in the AmBisome 3 mg/kg group, the cfu dropped by 1 log, showing that AmBisome therapy was continuing to kill the fungi even after treatment was stopped. At the higher AmBisome doses of 20 and 30 mg/kg, 44% and 78% of mice, respectively, were culture negative in the brain for *Cryptococcus* on day 30. Thus, this earlier work also provided evidence of AmBisome effectiveness against brain infections.

Recently, Clemons *et al.*[20] presented a study on the use of intravenous AmBisome to treat coccidioidal meningitis in rabbits. Immunosuppressed rabbits were challenged intracisternally with *Coccidioides immitis.* Five days post-infection, groups of rabbits were treated with either fluconazole (nineteen doses of 80 mg/kg/day, po), AmBisome (15 mg/kg iv three times a week for 3 weeks), or ampho-

tericin B (1 mg/kg iv three times a week for 3 weeks). The control group received 5% glucose on a regimen identical to the AmBisome group. All animals treated with fluconazole, amphotericin B and AmBisome survived, whereas 75% of the controls died ($P < 0.0005$). The cfu in the spinal cord and brain were significantly reduced by all treatment regimens compared with surviving controls ($P < 0.01$–0.0005). However, the AmBisome-treated group had three- and 11-fold lower cfu in the brain and in the spinal cord, respectively, compared with the fluconazole group, and six- and 35-fold lower cfu, respectively, compared with the amphotericin B-treated group. The histopathological analysis rated brain lesions on a scale from 0 for normal to 6 for most severe. In the control animals, the average score was 6, compared with 2.7 for the fluconazole group and 0.88 for the AmBisome group. The brain lesions for the amphotericin B treatment group were not rated histopathologically. Finally, all the clinical signs of coccidioidal meningitis were dramatically reduced in the AmBisome treatment group. These studies showed that AmBisome was superior to either fluconazole or amphotericin B for the treatment of experimental coccidioidal meningitis. Since AmBisome can be given systemically at high doses, it may not be necessary to treat every day in order for it to be effective, as demonstrated in this coccidioidal meningitis study. In conclusion, this and the other animal studies reviewed above show that AmBisome may have an important role in treating infections in the brain, both because it can deliver effective concentrations of active drug, and because it is able to reduce or eradicate the fungal burden.

Another series of animal studies have evaluated AmBisome in fungal infections that localize primarily in the lungs. In one study, the treatment of invasive pulmonary aspergillosis with either AmBisome or amphotericin B was evaluated in a granulocytopenic rabbit model.[21] After the rabbits were inoculated intratracheally with *A. fumigatus* conidia, they subsequently developed pneumonia characterized by high mortality, culture-positive lung tissues and gross pulmonary haemorrhage. AmBisome was administered intravenously at doses of 1 mg/kg/day ($n = 10$), 5 mg/kg/day ($n = 5$) or 10 mg/kg/day ($n = 12$) for 10 consecutive days starting 1 day post-infection. One group of animals was treated with amphotericin B at a dose of 1 mg/kg/day ($n = 8$) for 10 consecutive days, and a control group consisted of infected, untreated animals ($n = 14$). In the three AmBisome-treated groups, survival ranged from 80% to 100%, and was significantly increased compared with rabbits treated with amphotericin B ($P < 0.01$). The efficacies of AmBisome 5 and 10 mg/kg and amphotericin B were equivalent in reducing the numbers of cfu of *Aspergillus* per gram of lung tissue. Pulmonary haemorrhage was also reduced significantly in all treatment groups, but the lesions were smaller and less conspicuous in rabbits treated with AmBisome at 5 mg/kg ($P < 0.001$) or 10 mg/kg ($P < 0.0001$) compared with amphotericin B ($P < 0.01$). This study concluded that AmBisome at 5 mg/kg was more efficacious than amphotericin B deoxycholate in the treatment of pulmonary aspergillosis, resulting in increased survival, reduced numbers of viable organisms, decreased tissue injury and the prevention of nephrotoxicity.

A study by Leenders *et al.*[22] compared the efficacy of AmBisome and amphotercin B in an unusual rat aspergillosis model. The rats were infected only in the left lung, and 40 h later they were treated with either amphotercin B 1 mg/kg/day or AmBisome 1 or 10 mg/kg/day for 10 consecutive days. Animals were monitored for survival, and 24 h after the final treatment, the fungal burden in the left lung, the right lung, and the liver and spleen were quantified. Both amphotericin B 1 mg/kg/day and AmBisome 10 mg/kg/day increased survival; however, only AmBisome 10 mg/kg/day was able to cause a significant reduction in cfu in the left lung ($P = 0.003$). Interestingly, dissemination to the right lung was reduced in both of the AmBisome treatment groups, while conventional amphotericin B was ineffective at preventing lung dissemination. Dissemination to the liver and spleen was reduced by all treatments, but statistically significant reductions were only observed in the AmBisome treatment groups (1 or 10 mg/kg/day). AmBisome 10 mg/kg/day completely prevented dissemination to the liver and spleen. These dissemination results again provide evidence that AmBisome is able to deliver highly bioavailable amphotericin B to lungs and other organs.

An efficacy comparison between Abelcet and AmBisome has been performed in a pulmonary aspergillosis model in mice.[23] Immunosuppressed mice were challenged intranasally with 8×10^4 *A. fumigatus* conidia. Groups of seven infected mice were treated intravenously with AmBisome 15 mg/kg, Abelcet 15 mg/kg, amphotericin B 1 mg/kg or 5% glucose. Treatments were given daily for 4 days beginning 2 h after challenge. As shown in Figure 2,

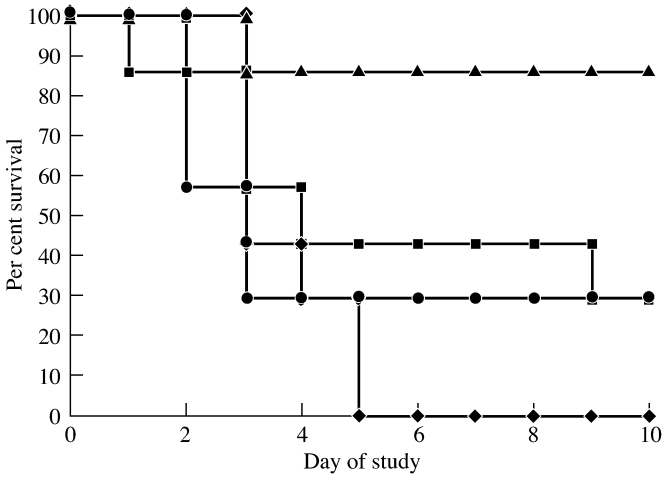

Figure 2. Survival of immunosuppressed DBA/2 mice challenged with 8×10^4 *A. fumigatus* conidia. Four daily treatments were administered beginning 2 h post-challenge. Treatments included: glucose (control, ◆), amphotericin B 1 mg/kg (Fungizone, ●), AmBisome 15 mg/kg (▲) or Abelcet 15 mg/kg (■).

Table 3. Effect of dosing regimen on AmBisome efficacy in the treatment of systemic candidosis in immunosuppressed mice

AmBisome dose (mg/kg)	Experiment days of treatment	Cumulative dose (mg/kg)	Log_{10} cfu/g kidney on day 62	
			median	range
D5W control	2, 4, 6, 13, 20	0	no survivors	–
20 +2.5	2, 4, 6 13, 20, 27, 34	70	4.08	2.63–4.95
20 +5	2, 4, 6 13, 20	70	3.74	2.16–4.69
20 +10	2, 4, 6 13	70	2.48	1.38–3.77
20 +20	2, 4, 6 13, 20, 27, 34	140	2.62	1.27–4.31

all of the control mice were dead by day 5. The survival rate for groups treated with either Abelcet or amphotericin B (Fungizone) was 29% on day 9 post-infection. However, the AmBisome treatment group had an 86% rate of survival.

To determine differences in drug disposition in this mouse study, amphotericin B levels in the lungs of both infected and uninfected animals were compared. Groups of mice were treated with AmBisome 1, 4, 8 or 12 mg/kg, or amphotericin B 1 mg/kg for 4 days and tissues were analysed for drug levels at 24 h after the last drug treatment. For any given dose level of AmBisome, the concentration of amphotericin B in the lungs of the uninfected animals was lower than the concentration in the lungs of the infected animals treated at the same dose level, and the concentrations in the lungs showed a dose-dependent response. These results indicate that drug does tend to accumulate preferentially at sites of infection, and may help explain AmBisome's efficacy at higher dose levels.

Animal studies have shown that AmBisome is also very effective in both treating fungal infections in the kidneys,[24,25] and in preventing fungal infections in this organ.[26] In the prophylactic study, it was reported that amphotericin B levels in the kidneys of AmBisome-treated mice (5, 10 or 20 mg/kg) ranged from 0.63 to 8.08 mg/kg 7 days after treatment. This information prompted an investigation to determine whether an AmBisome loading dose could be given to mice during the early part of their treatment regimen, after which dosing could be reduced without loss of efficacy.[27] In a mouse candidosis model, all groups of animals were treated with 20 mg/kg AmBisome on days 2, 4 and 6 as a loading dose. Subsequently, separate groups were treated once a week with 2.5 mg/kg for 4 weeks, 5 mg/kg for 2 weeks or with 10 mg/kg for just 1 week. The high-dose control group continued to be treated with 20 mg/kg once a week for 4 weeks. On day 62, *c.* 28 days after the final treatment, the cfu in kidney tissue for each group was determined. The results are shown in Table 3. Untreated animals had a median log_{10} cfu/g kidney of 7.5 on day 32 when killed in a moribund condition (data not shown). After the 20 mg/kg loading dose treatments, the group that received only one maintenance dose of 10 mg/kg had as low a level of cfu in kidney tissue as the group receiving 20 mg/kg weekly after the loading dose period. In addition, the group treated with a single maintenance dose of 10 mg/kg had fewer cfu in the kidneys than the other groups treated with the same total amount of AmBisome, but spread out over a longer time period of treatment using lower weekly doses of AmBisome. The results of this initial study do seem to suggest that a high loading dose regimen, followed by once or twice a week dosing, could be an effective AmBisome treatment regimen.

AmBisome prophylaxis

AmBisome has been shown to have a very good safety profile compared with conventional amphotericin B, and the animal studies described above indicate that AmBisome can deliver drug that remains bioavailable in tissues after treatment has concluded. Therefore, AmBisome would be an ideal candidate for prophylactic therapy in patients at high risk for fungal infections. To evaluate AmBisome in a prophylactic regimen, normal or neutropenic mice were treated with a single dose of AmBisome (1, 5, 10 or 20 mg/kg) or amphotericin B (1 mg/kg) 7 days before fungal challenge with either *C. albicans* or *H. capsulatum*.[27] Seven to 10 days after challenge, control and treatment groups were analysed for survival and *C. albicans* or *H. capsulatum* cfu in the kidneys or in the spleen, respectively.

In the *H. capsulatum* prophylaxis study, only 10–20% of control or amphotericin B (l mg/kg)-treated animals survived. AmBisome prophylaxis given as a single dose of

Table 4. Mean $\log_{10}$ cfu/g spleen ± S.D. on day 9 or 10 post-challenge in mice treated prophylactically with Fungizone or AmBisome 7 days before challenge with *H. capsulatum*

		Immunocompetent mice		Immunosuppressed mice	
Treatment	Dose (mg/kg)	mean $\log_{10}$ cfu/g spleen ± S.D.	survival	mean $\log_{10}$ cfu/g spleen ± S.D.	survival
D5W control	–	5.39	1/10	ND[a]	2/10
Fungizone	1	4	1/10	5.13	1/10
AmBisome	1	4.8 ± 0.5	10/10	5 ± 0.2	10/10
AmBisome	5	0.85[b]	10/10	3.5 ± 1.2	10/10
AmBisome	10	0	10/10	0	10/10
AmBisome	20	0	10/10	0	10/10

[a]Bacterial contamination.
[b]Value is for single culture-positive animal.

Table 5. Mean $\log_{10}$ cfu/g kidney ± S.D. on day 7 post-challenge in mice that were treated prophylactically with Fungizone or AmBisome 7 days before challenge with *C. albicans*

		Immunocompetent mice		Immunosuppressed mice	
Treatment	Dose (mg/kg)	mean $\log_{10}$ cfu/g kidney ± S.D.	survival	mean $\log_{10}$ cfu/g kidney ± S.D.	survival
D5W control	–	6 ± 0.4	5/5	6.2 ± 0.7	5/5
Fungizone	1	5.2 ± 0.7	5/5	4.2 ± 1.2	5/5
AmBisome	1	4.6 ± 0.5	5/5	3.2 ± 1	5/5
AmBisome	5	3.8 ± 0.2	5/5	3.5 ± 0.7	5/5
AmBisome	10	3.5 ± 0.3	5/5	ND[a]	ND[a]
AmBisome	20	3.3 ± 0.4	5/5	2.7 ± 0.8	5/5

[a]ND, not done.

1, 5, 10 or 20 mg/kg resulted in 100% survival at all dose levels. In addition, as shown in Table 4, AmBisome at 10 and 20 mg/kg completely prevented the infection from occurring in spleens of all challenged mice.

In the *C. albicans* prophylaxis study, all mice in the control and treatment groups survived to the end of the experiment. Amphotericin B (Fungizone) prophylaxis was somewhat effective in decreasing cfu in the kidneys as can be seen in Table 5, but all dose levels of AmBisome significantly reduced cfu in the kidneys ($P < 0.05$) compared with amphotericin B. However, no animal was completely protected from *C. albicans* growth in the kidneys. The difference in protection in the *C. albicans* study compared with the study with *H. capsulatum* may be due in part to the fact that the amphotericin B concentrations in the kidneys at the time of fungal challenge were considerably lower than in the spleen, the target organ for histoplasmosis. In addition, liposomes are known to be taken up by macrophages, and *H. capsulatum* replicates in macrophages while *C. albicans* replicates extracellularly. Thus, macrophage uptake of AmBisome would tend to increase drug concentration preferentially at the site of *H. capsulatum* replication.

AmBisome combination therapy

Recent experimental work has examined the activity of AmBisome in combination with azithromycin or terbinafine on *C. albicans*.[28,29] Terbinafine is an antifungal agent, but azithromycin is an antibacterial agent. However, a recent publication demonstrated that azithromycin, when used in combination with amphotericin B, reduced the MIC of the latter for a *Fusarium* species.[30] *In vitro* azithromycin alone had absolutely no effect on *C. albicans* (MIC > 320 mg/L). However, the MICs of AmBisome, or Fungizone with azithromycin were about one-quarter of the corresponding MICs without azithromycin. Terbinafine gave a similar pattern of MICs for *C. albicans* in combination with Fungizone or AmBisome. Terbinafine alone had an

Table 6. MICs of AmBisome, amphotericin B (Fungizone), terbinafine and azithromycin, alone and in combination, for *C. albicans*

Treatment	MIC (mg/L) for *C. albicans*
AmBisome	0.37
AmBisome + terbinafine (5 mg/L)	0.04
AmBisome + azithromycin (80 mg/L)	0.08
Amphotericin B	0.32
Amphotericin B + terbinafine (10 mg/L)	0.04
Amphotericin B + azithromycin (1 mg/L)	0.08
Terbinafine	>80
Azithromycin	>320

Table 7. Efficacy of AmBisome or amphotericin B (Fungizone) alone and in combination with terbinafine against *C. albicans* infection in immunosuppressed mice[a]

Treatment	Median $\log_{10}$ cfu/g kidney	Survival (%)
D5W control	6.71	4/4 (100)
Terbinafine (100 mg/kg)	6.31	4/6 (67)
AmBisome (1.5 mg/kg)	5.01	4/6 (67)
AmBisome (1.5 mg/kg) + terbinafine (100 mg/kg)	3.58	7/7 (100)
Amphotericin B (0.7 mg/kg)	4.59	7/7 (100)
Amphotericin B (0.7 mg/kg) + terbinafine (100 mg/kg)	3.85	6/7 (87)

[a]AmBisome or amphotericin B was administered intravenously on days 2, 3, 4 and 6 followed by oral terbinafine where indicated on days 4, 5, 6, 7, 8 and 9.

MIC in excess of 80 mg/L, but when it was combined with Fungizone or AmBisome, the MICs were dramatically reduced (see Table 6). Combination treatments were then tested *in vivo* in a mouse model of candidosis. Immunosuppressed mice were challenged with *C. albicans*, and AmBisome (1.5 or 3 mg/kg iv) or Fungizone (0.7 mg/kg iv) therapy was given on days 2, 3, 4 and 6. Terbinafine treatment at 100 mg/kg po followed on days 4–9. Survival was recorded until day 14, at which time cfu in kidney tissue were determined. The results in Table 7 show that without treatment, or with terbinafine alone, there were very large fungal burdens in the kidneys. Fungizone alone reduced the fungal burden by 2 logs compared with the controls, and Fungizone plus terbinafine reduced the fungal burden even further. Likewise, AmBisome efficacy at 1.5 mg/kg was definitely improved by the addition of terbinafine to the therapeutic regimen, reducing the fungal burden by 3 logs compared with controls.

Conclusion

AmBisome is a true liposomal formulation of amphotericin B with unique toxicological and pharmacokinetic properties. Pre-clinical studies have demonstrated that AmBisome retains the full spectrum of antifungal activity of conventional amphotericin B. In addition, recent animal studies with AmBisome suggest that new clinical applications, such as prophylactic treatment and combination therapy, may be feasible.

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