

Optimizing efficacy of amphotericin B through nanomodification

Suresh P Vyas
Swati Gupta

Drug Delivery Research Laboratory,
Department of Pharmaceutical
Sciences, Dr Hari Singh Gour
University, Sagar (M.P), India

Abstract: Fungal infections and leishmaniasis are an important cause of morbidity and mortality in immunocompromised patients. The macrolide polyene antibiotic amphotericin B (AmB) has long been recognized as a powerful fungicidal and leishmanicidal drug. A conventional intravenous dosage form of AmB, AmB- deoxycholate (Fungizone or D-AmB), is the most effective clinically available for treating fungal and parasitic (leishmaniasis) infections. However, the clinical efficacy of AmB is limited by its adverse effects mainly nephrotoxicity. Efforts to lower the toxicity are based on synthesis of AmB analogues such as AmB esters or preparation of AmB-lipid associations in the forms of liposomal AmB (L-AmB or AmBisome), AmB lipid complex (Abelcet or ABLC), AmB colloidal dispersion (Amphocil or ABCD), and intralipid AmB. These newer formulations are substantially more expensive, but allow patients to receive higher doses for longer periods of time with decreased renal toxicity than conventional AmB. Modifications of liposomal surface in order to avoid RES uptake, thus increased targetability has been attempted. Emulsomes and other nanoparticles are special carrier systems for intracellular localization in macrophage rich organs like liver and spleen. Injectable nano-carriers have important potential applications as in site-specific drug delivery.

Keywords: amphotericin B, fungal infections, leishmaniasis, nanocarriers

Introduction

Systemic fungal infections may randomly be divided into two wide categories: endemic diseases such as histoplasmosis, coccidioidomycosis, and blastomycosis; and opportunistic diseases such as cryptococcosis, aspergillosis, and candidosis, which occur almost entirely in patient with impaired host defenses. Antifungal therapy is based on several factors, such as the causative agent, the succession or incursion of the disease, and so on. Antifungal therapy may have to be administered empirically in febrile neutropenic patients who do not respond to treatment with antibacterial drugs (Medoff et al 1992). Antifungal agents are considerably fewer in number because of emergence of newer pathogenic fungi causing deep-seated mycosis. Clinically used major groups of antifungal agents are polyene antibiotics, azole derivatives, allylamines-thiocarbamates, morpholines and miscellaneous compounds such as 5-fluorocytosine and griseofulvin. Polyenes and azoles are most commonly used. Polyene antifungal agents used for the treatment of human diseases are amphotericin B (AmB) nystatin and natamycin. The only parenteral preparation with broad range of antifungal activity is AmB. Over the past several years, augmented efforts in both basic and clinical antifungal pharmacology have resulted in a number of exclusively new, reengineered or reconsidered compounds, which are at various stages of preclinical and early clinical development (Hay 1994; Georgopapadakou and Walsh 1996; Maesaki 2002).

Similarly, leishmaniasis causes high morbidity and mortality worldwide which is escalating due to its spread as a HIV-associated infection (Alvar et al 1997; Herwaldt 1999; Murray 1999). Due to serious side-effects of pentavalent antimonials (the first-line treatment) such as cardiac and renal toxicity and failures, and development of

Correspondence: Suresh P Vyas
Drug Delivery Research Laboratory,
Department of Pharmaceutical Sciences,
Dr Hari Singh Gour University,
Sagar (M.P) 470 003, India
Telefax: +917582 265525
E-mail: vyas_sp@rediffmail.com;
spvyas@sancharnet.in

resistant strains in prevalent areas many practitioners have turned to conventional AmB, a very active antifungal agent, for first-line therapy, which remains almost 100% effective (Pearson and Sousa 1996; Alvar et al 1997; Sereno et al 2000). Since the parasites are found only within reticulo-endothelial macrophages, the disease is preferably suited for drug delivery therapy. Therefore, the new AmB lipid-based formulations (AmBisome, Abelcet, and Amphocil) have been proposed for the treatment of visceral leishmaniasis (VL) (Davidson et al 1991; Berman et al 1992; Paul et al 1997). The results indicated that these AmB carriers were effective at lower doses with abridged toxicity as compared to the conventional AmB formulation. The US Food and Drug Administration approved L-AmB for the treatment of VL and highly recommended their use for resistant VL in immunocompromised patients (Meyerhoff 1999; Espuelas et al 2002).

Recently, drug delivery systems (DDS) have received substantial attention in the field of drug development. In DDS, pharmacological techniques are used to control pharmacokinetic properties (absorption, distribution, metabolism, and excretion) and to improve the efficacy and safety of a drug. Lipid formulations, such as liposomes and emulsion based carriers, are very highly predictable and are now explored in numerous directions, and several products have already been made commercially available (Tomii 2002).

In this review the enhancement of the efficacy of AmB is addressed using lipid-based nanocarriers and paying particular attention towards current commercial liposomal formulations.

Parent amphotericin B

AmB, a lipophilic polyene antifungal agent, was initially secluded from a strain of *Streptomyces nodosus* in 1956 by Gold et al (Gold et al 1956). It is an amphoteric compound composed of a hydrophilic polyhydroxyl chain along one side and a lipophilic polyene hydrocarbon chain on the other (Hoeprich 1992). AmB is poorly soluble in water (Storm and van Etten 1997). The drug became available commercially as Fungizone (Bristol-Myers-Squibb, USA) in 1960 as a colloidal suspension of AmB in which the bile salt deoxycholate was used as the solubilizing agent (Arikan and Rex 2001).

Role and mechanisms

The interaction of AmB with membrane sterol changes the membrane permeability, which in turn leads to cellular

dysfunction and eventually to cell destruction and death (Bolard et al 1991; Legrand et al 1992). AmB inhibits membrane enzymes like proton ATPase in fungal cells (Surarit and Shepherd 1987) and Na^+/K^+ -ATPase in mammalian cells (Vertut-Doi et al 1988) and this inhibitory activity depletes cellular energy reserves and reduces proliferative ability (Schindler et al 1993). Another possible mechanism by which membrane permeability changes occur is AmB-induced lipid peroxidation of cell membranes (Brajtburg et al 1985). Likewise, binding of AmB to low-density lipoprotein (LDL) and its consequent internalization modulate its toxicity (Brajtburg and Bolard 1996).

Pharmacology and adverse effects

Klepser et al obtained the time-kill curves for AmB against *Candida albicans* (Klepser et al 1997) and showed that AmB displays concentration-dependent fungicidal activity. Andes (Andes 1999) investigated the pharmacodynamics of AmB in neutropenic mice with disseminated candidiasis and showed non-linear kinetics, in vivo concentration-dependent killing, and prolonged concentration-dependent post-antibiotic effects (PAEs) of AmB. The efficacy of AmB is compromised by a high frequency of adverse effects, including fever, chills, nausea, vomiting, headache, and renal dysfunction with associated anemia, hypokalemia, and hypomagnesemia (Hiemenz and Walsh 1996).

Role of lipid formulations

There are three ways by which the therapeutic index of AmB might be improved: (i) increasing the selectivity of polyene-induced damage to fungal, as opposed to mammalian, cells; (ii) decreasing toxicity to host cells bearing LDL receptors; and (iii) decreasing toxicity for cells of the immune system, thereby protecting the immunostimulatory activity. Approaches designed to address these three issues involve the preparation of AmB-lipid associations. Therefore, there has been substantial exploration into the development of less toxic preparations of AmB. For the past decade, investigators have evaluated the use of colloidal dispersions and phospholipid vesicles known as liposomes as a targeted drug delivery systems for AmB. These efforts have led to the expansion of commercial preparations of phospholipid vesicles for therapeutic use such as AmBisome, ABLC, and ABCD (Hiemenz and Walsh 1996; Wong-Beringer et al. 1998). These preparations have been shown to be less toxic than AmB and to display altered pharmacokinetic properties because they are concentrated in the organs of the reticulo-endothelial

system, but not in the kidney where only low concentrations are achieved (Kretschmar et al 2001).

Selected characteristics of lipid formulations that have been studied thoroughly and are in clinical trials are summarized in Table 1.

Intermediaries of antifungal and antiparasitic activity

Macrophages may function as a reservoir of AmB for intracellular and extracellular antimicrobial action. Mehta et al (1997) conducted a study to investigate the role of macrophages in candidacidal activity of L-AmB. The results suggested that the improved candidacidal activity of L-AmB was possibly not due to activation of the macrophages. Instead, higher uptake and retention of L-AmB and its slow release from the macrophages led to its improved candidacidal activity. When lipids associated AmB is taken into macrophages (Legrand et al 1996; Mehta et al 1994) or monocytes, it may function to inhibit fungal or parasitic cells also present inside these cells or it may dissociate from the complex inside the phagocytic

cell and exit as free AmB to inhibit extracellular microbes (Figure 1). If the AmB-lipid bond is strong, AmB will dissociate slowly as a monomer. The monomer, then, would be active against fungal and parasitic cells and not toxic to mammalian cells.

Effects of lipid-based carrier constructs on AmB Binding to lipoproteins and its internalization

AmB binding to lipoproteins may persuade the ability of mammalian cells to internalize the drug. If the AmB-carrier bond is weak and labile, as is presented in Figure 2 then when the complex is diluted in blood, AmB will dissociate from the lipid carrier and bind to LDL, just as AmB in Fungizone does when it dissociates from deoxycholate. The LDL-AmB complex can be internalized into cells bearing LDL receptors, and toxic effects comparable to those observed with Fungizone will occur.

When the AmB-carrier complex is strong and inert, it remains intact after introduction into the bloodstream but can still bind lipoproteins. ABLC may bind to highdensity

Table 1 Characteristics of some lipid formulations under clinical trials.

AmB preparation	Composition (mol%), charge of phospholipids ^b	Shape and diam (µm)	Bioavailability compared with Fungizone	Clinical trial references
Fungizone	D-AmB (7:3), negative	Micelles, < 0.4		
Liposomes (L-AmB5, L-AmB10)	DMPC-DMPG-AmB (7:3:0.5,7:3:1), negative	Multilamellar vesicles + sheets, 1–6	Lower	Emminger et al 1994; Lopez-Berestein 1987; Lopez-Berestein et al 1987; Lopez-Berestein et al 1985; Lopez-Berestein and Juliano 1987; Ralph et al 1993
AmB-lipid complex (ABLC, Abelcet)	DMPC-DMPG-AmB (7:3:3), negative	Sheets, 1.6–11	Lower	de Marie et al 1994; Fromtling 1995; Janoff et al 1993
Ampholiposomes	EPC-CHOL-SA-AmB (4:3:1:0.5), positive	Oligolamellar vesicles, 0.2–0.3	Greater	Meunier et al 1988; Sculier et al 1989
AmBisome	HSPC-CHOL-DSPG-AmB (2:1:0.8:0.4), negative	Small unilamellar vesicles, 0.06	Greater	de Marie et al 1994
L-AmB	SPC-CHOL-AmB (7:3:1), neutral	Small unilamellar vesicles	Equal	Gokhale et al 1993; Gokhale et al 1993
AmB-colloidal dispersion (ABCD, Amphocil)	CS-AmB (1:1), negative	Discs, 0.12	Lower	de Marie et al 1994; Fromtling 1993; Guo and Working 1993; Stevens 1994

Adapted from Brajtburg and Bolard (1996).

Abbreviations: D, deoxycholate; CHOL, cholesterol; SA, stearylamine; HSPC, hydrogenated phosphatidylcholine; DSPG, distearoyl phosphatidylglycerol; CS, cholesteryl sulfate; DMPC and DMPG, dimyristoyl phosphatidylcholine and glycerol respectively.

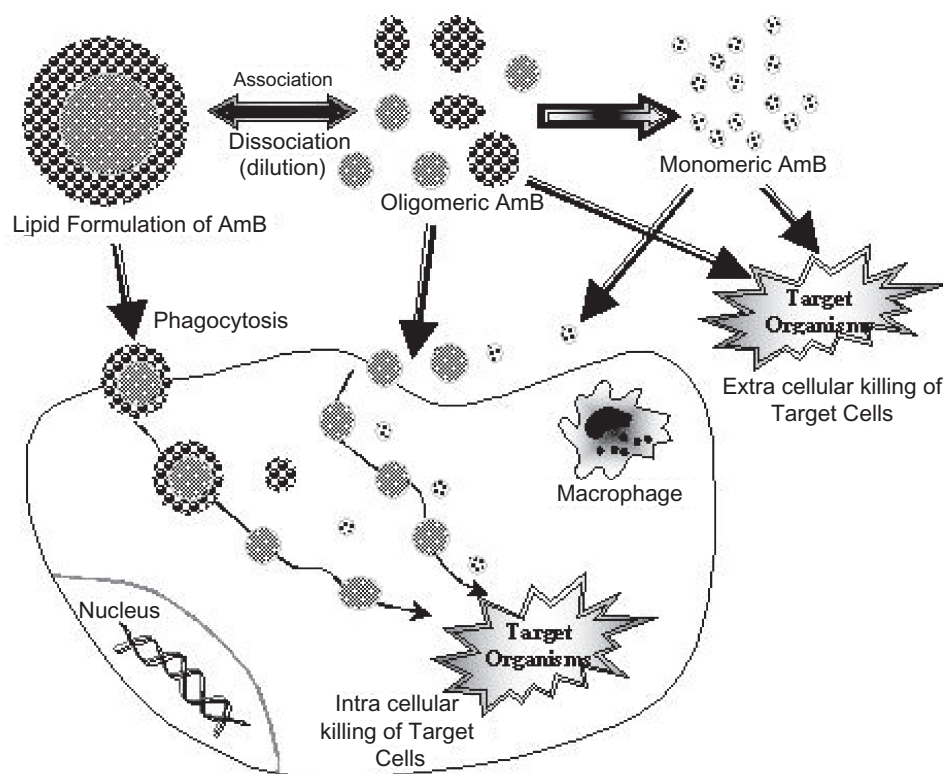


Figure 1 Several pathways by which lipid formulations of AmB are thought to reach fungal or parasitic cells.

lipoproteins (HDL) and remain in the bloodstream, lacking toxicity (Wasan et al 1994). On the other hand, neither ABCD (Guo and Working 1993) nor AmB incorporated into egg lecithin-bile salts mixed micelles (Brajtburg et al 1994) binds to lipoproteins, and both are relatively nontoxic (Brajtburg and Bolard 1996).

Liposomal amphotericin B [L-AmB]

In 1965, Bangham et al (1965) reported that a small closed vesicular structure, consisted of lipid bilayers could be formed when phospholipids are hydrated by the addition of water. These structures were first named as “smectic mesophases” by Bangham and later called ‘liposomes’ by Gerald Weissman (Ostro and Cullis 1989; Bangham 1992). In 1981, New et al. (New et al 1981) first examined the effects of L-AmB, using leishmania model and reported that L-AmB had a lower toxicity than AmB itself and the treatment with a higher dose of L-AmB could be feasible. Afterward, the validity of the L-AmB for mice histoplasmosis (Taylor et al 1982), cryptococcosis (Graybill et al 1982), and candidiasis (Lopez-Berestein et al 1983; Tremblay et al 1984) was assessed. In all cases, the L-AmB showed a lower toxicity than AmB to the host animals and thus could be administered at higher doses. Drugs incorporated in liposome

were also shown to distribute mainly to reticuloendothelial tissues including liver, spleen, and lung (Abra and Hunt 1981). Later, a clinical trial performed in cancer patients who co-developed fungal infection confirmed that the L-AmB showed a higher tolerance than AmB even in human (Lopez-Berestein et al 1985).

AmBisome

Early evaluations were performed using the MLV-type agents. In 1987, Szoka et al prepared the Small unilamellar vesicles (SCV) containing sterol and explored the effects of component substances of liposome and a size of the particle on the expression of toxicity (Szoka et al 1987). They concluded that the sterol including L-AmB was less toxic than that without sterol. They also reported that, when sterol was integrated, the smaller liposome was less toxic than the larger liposome and that, when sterol was excluded in contrast, the larger particle was less toxic than the smaller particle. Based on these findings, NeXtar Inc. succeeded in formulating the SUV type L-AmB (AmBisome). AmBisome has been licensed for use in Europe for over 5 years. It received FDA approval on 11th August 1997 for the treatment of patients with aspergillosis, candidiasis, and/or cryptococcal infections refractory or intolerant to AmB.

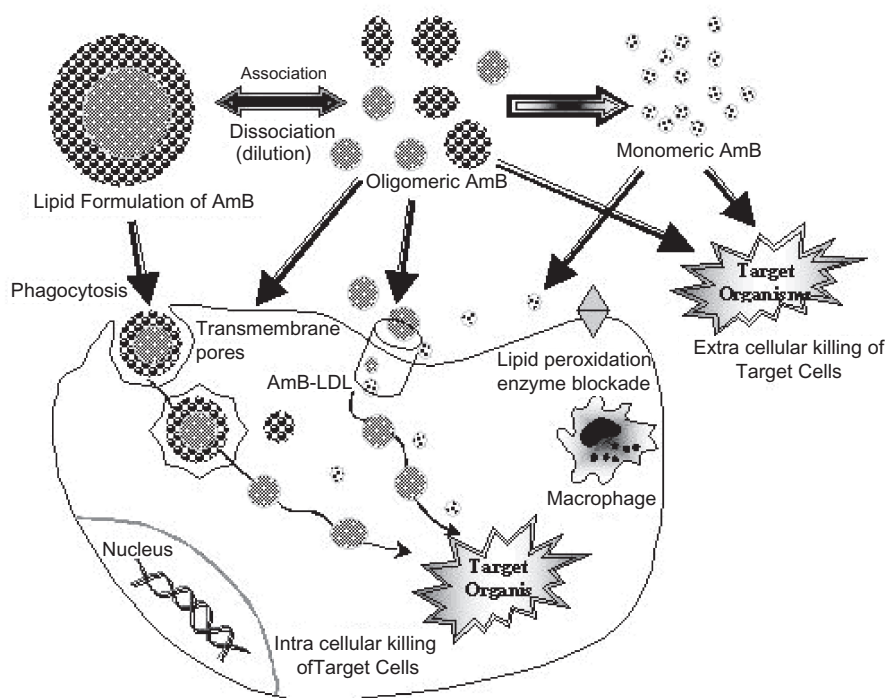


Figure 2 Several pathways by which the lipid formulations of AmB may reach mammalian cells.

General Properties

Among the new generation lipid-associated AmB formulations being developed throughout the world, the only true liposomal form of AmB is AmBisome. AmBisome is a suspension of small unilamellar liposomes in buffered 9% sucrose whose composition is HSPC (hydrogenated soya phosphatidyl choline)/Chol/DSPG/AmB (2:1:0.8:0.4 mole ratio). AmB is anchored tightly in the AmBisome bilayer due to favorable interactions of the macrolide with the surrounding lipids. DSPG probably interacts directly with AmB; cholesterol may also play a role. The exact nature of these interactions is not known, but the data are consistent with a barrel like structure formed by AmB molecules. Two barrels fit together tail to tail to span the lipid bilayer and form a pore that is permeable to ions and other solutes (Figure 3). The product is stored as a lyophilized powder that is reconstituted with the addition of water for injection followed by the few seconds of shaking to produce a slightly opalescent, yellow solution. In its lyophilized presentation, stored at 4°C, AmBisome has a shelf life in excess of 30 months (Schmidt et al 1998).

Mechanism of action

AmBisome has been tested in mammalian cell toxicity assay and has proved to be remarkable benign. Rat cell lysis assays are a measure of free (or readily available) AmB. Fungizone produced 92% lysis of rat cells in two hours at 37°C at a

drug concentration of 1 µg/ml. AmBisome produced only 5% lysis under the same conditions and time of incubation even at high concentration of 100 µg/ml (AmB equivalent). These data suggest that AmB is retained sufficiently tightly inside the AmBisome so that less than 1% of the drug is free (or loose enough to be transferred to mammalian cells) in buffer. Potentially the association of AmB with AmBisome is dependent on the concentration of liposomes, if there exist equilibrium between free and liposome bound drug. But, in buffer, even as low as 1 µg/ml, the drug remains exclusively with the liposome as evidenced by circular dichroism studies over a range of concentrations (Fujii 1996). In vitro studies in human and mouse serum show complete retention of AmB by AmBisome for 6–24 hours. For AmBisome in vivo there is evidence that AmB is largely retained by the liposome over several hours of circulation in mice (van Etten et al 1995). Certainly the drug is not available in a free or toxic form since the LD₅₀ of AmBisome is greater than 160 mg/kg in this species, as compared to 2.3 mg/kg for D-AmB.

There is evidence that AmBisome (and liposomes of the same composition without drug) can gain direct access to sites of fungal infection as intact structures probably because of leaky vasculature. The assumption has been made that with the prolong circulation life time seen for AmBisome, uptake into infected tissue and direct action of the liposomal drug may contribute to therapy (Adler-Moore et al 1993).

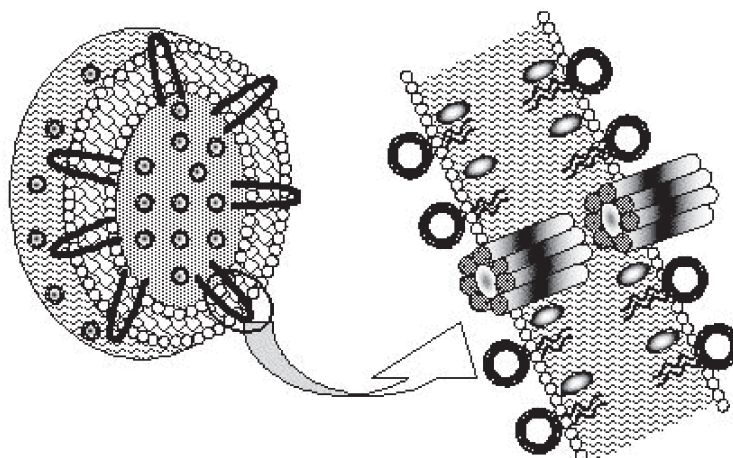


Figure 3 Proposed arrangement of AmB molecules (black) in the AmBisome bilayer. This structure accounts for the observation of rapid ion fluxes across the AmBisome bilayer in response to imposing a pH gradient from inside to outside. The individual AmB molecules form a "barrel" two of which fit tail-to-tail to form a pore spanning the bilayer. This structure is believed to contribute to the exceptional stability of AmBisome to loss of drug in buffer or plasma.

Indeed, AmBisome is highly active against cultured fungal species (Anaissie et al 1991), although the liposomal drug may be somewhat slower acting than D-AmB (van Etten et al 1995). The liposomes, with or without drug bind to fungal cells, and AmBisome (but not drug free liposomes) disintegrates slowly. Gold labeled lipids incorporated in AmBisome like liposomes can be located by electron microscopy (after silver enhancement). Initially intact liposomes are seen gathered around and bound to the cell wall of *Candida glabrata*. After 14 hours, incubation, gold labeled lipid is seen inside the cell membrane. The cell structure appears disrupted at this point, presumably due to action of AmB that accompanies breakup of the liposome (Adler-Moore 1994). While it appears feasible for AmBisome to act directly on systemic fungal infections, the quantitative contribution of intact liposomes to the success of systemic treatment with AmBisome needs further study. Macrophages, including kupffer cells of the liver and stationary macrophage in the spleen, are a major cellular site for uptake of AmBisome and other lipid-associated AmB preparations (Hartsel and Bolard 1996). It is likely that macrophages and possibly neutrophils play key roles as depots for AmB, although the details have not been elucidated (Schmidt et al 1998).

Pharmacology, efficacy- and toxicity during preclinical trials

Boswell et al (Boswell et al 1998) examined the single- and multiple-dose pharmacokinetics and toxicological profile of AmBisome in rats. Rats were administered AmBisome at doses of 1, 3, 9, and 20 mg/kg/day. Substantial plasma concentrations (380 and 500 µg/mL in females and males,

respectively) were attained after AmBisome therapy of 20 mg/kg for 30 days. The results suggested that, 100-fold higher plasma concentrations of AmB could be attained with AmBisome at doses up to 20 mg/kg/day as compared to conventional AmB. Unlike the conventional preparation, AmBisome at high doses resulted in slight nephrotoxicity but moderate hepatotoxicity. Another study showed that in brain tissue of noninfected rabbits, AmBisome attained mean tissue levels 4–7 times higher than that with D-AmB, ABCD, or ABLC. Conversely, none of the lipid formulations nor the conventional AmB can attain detectable levels in cerebrospinal fluid (CSF) in the absence of meningocerebral inflammation. Nevertheless, the high level of AmBisome attained in brain tissue is potentially promising for its future use in fungal infections of central nervous system (Groll et al 1997). The efficacy of D-AmB compared to those of the lipid formulations in murine cryptococcosis also showed that AmBisome was one of the most efficacious formulations (Clemons and Stevens 1998).

The results of in vitro experiments against common pathogens including *Candida*, *Cryptococcus*, *Aspergillus*, and *Fusarium* species from both tube macrodilution and plate microdilution test methods confirmed that the MIC and minimal fungicidal concentration (MFC) profile for AmBisome is similar to that of AmB (Adler-Moore and Proffitt 1998; Anaissie et al 1991). The MIC of AmBisome ranged from 0.05 to 2.5 mg/L as compared with 0.1 to 2.5 mg/L for AmB. Thus the integration of AmB into the liposome bilayer of AmBisome has little or no inhibitory effect on its MICs in vitro. The in vivo study conducted by Francis et al (1994) on neutropenic rabbits with pulmonary

aspergillosis designed to compare the clinical outcome with AmBisome at doses of 1, 5, and 10 mg/kg and conventional AmB at a dose of 1 mg/kg. All doses of AmBisome showed better survival than was seen with conventional AmB. Pulmonary haemorrhage was also reduced significantly in all treatment groups, but the lesions were smaller and less striking in rabbits treated with AmBisome at 5 mg/kg ($P < 0.001$) or 10 mg/kg ($P < 0.0001$) compared to AmB ($P < 0.01$). In conclusion, AmBisome at 5 mg/kg was more efficacious than D-AmB. The antifungal effectiveness of AmBisome was also compared with AmB in cultured Langerhans cells infected with *C. glabrata* (Sperry et al 1998). The *Candida* infected cells were incubated with AmB or AmBisome at 12.5 mg/L for up to 48 hours. Both AmBisome and AmB were found to be equally effective after 48 hours, reducing the amount of viable fungus by 5 logs. Nevertheless, AmBisome was much less cytotoxic to the cultured Langerhans cells than AmB at this concentration. Effectiveness of increasing doses of AmBisome (8 to 30 mg/kg/day) vs D-AmB (1 or 2 mg/kg/day) was also examined in neutropenic mice with hematogenous *C. lusitaniae* and *C. krusei* infection. Two of the infecting *C. lusitaniae* strains were resistant to AmB. Despite the fact that high doses of AmBisome were significantly more effective in infections due to AmB-susceptible isolates, there was no advantage of using AmBisome over the conventional preparation for infections due to AmB-resistant isolates (Karyotakis and Anaissie 1994).

AmBisome have proved to be an effective treatment for VL. In vitro, free AmB was 3–6 times more active than AmBisome against both *Leishmania major* promastigotes in culture and amastigotes in murine macrophages. In a BALB/c *L. major* model of cutaneous infection, AmBisome administered once a day on 6 alternate days by the intravenous route produced a dose-response effect between 6.25 and 50 mg/kg (Yardley and Croft 1997). The intracellular fungus that has been found to be highly susceptible to AmBisome therapy in an immunosuppressed mouse model is *Histoplasma capsulatum* (Adler-Moore 1994). Low doses of Fungizone or AmBisome (4 doses of 0.3 or 0.6 mg/kg) were compared, and a higher dose of AmBisome (4 doses of 6 mg/kg) was also tested to resolve whether a higher dose gave a better therapeutic response. At the lower doses neither Fungizone nor AmBisome were predominantly effective. Twenty-four hours after the last lower dose treatments, colony forming units (cfu)/g of spleen were abridged by c. 2 logs compared with untreated controls, but regrowth was evident after 14 days in all cases. Conversely the higher dose of AmBisome

(6 mg/kg) considerably reduced the cfu by 5 logs at 24 hours post-treatment compared with control.

Groll et al (2000) 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 AmB as well as with D-AmB and showed that the AmBisome treated animals attained considerably higher drug concentration in the plasma of both the infected and uninfected groups compared with other formulations. Practically no drug (< 0.1 mg/L) was found in the cerebrospinal fluid (CSF) of any of the treatment groups. Nevertheless, there was a considerably higher concentration of AmB in the brain tissue itself in the AmBisome-treated groups than in groups receiving any of the other formulations, which in turn could be a reason for increased efficacy. The tolerance and efficacy of Fungizone (6 doses of 0.8 mg/kg, i.v.) were compared with those of AmBisome (6 doses of 0.8, 5 and 50 mg/kg, i.v.) and meglumine antimoniate (11 doses of 200 mg/kg i.p.) in a BALB/c mice model of VL induced by *Leishmania infantum*. A dose range study showed that administration of AmBisome at the well-tolerated doses of 5 or 50 mg/kg of body weight completely eradicated the parasites from the liver, spleen and lungs. At 0.8 mg/kg, AmBisome proved more efficacious than Fungizone administered at the same dose and was capable to decrease the parasitic burden by at least 4–6 logs in the spleen and liver compared with untreated controls (Gangneux et al 1996).

Albert et al (1995) treated a mouse model of meningitis (caused by *Cryptococcus neoformans*) with multiple doses of AmB (0.3 mg/kg i.v. or 0.3 mg/kg i.p.) or AmBisome (1, 3, 20, or 30 mg/kg i.v.). Some animals were killed during the therapy, and culture results showed that 3 mg/kg AmB was more effective than 3 mg/kg of AmBisome for lowering fungal cfu in the brain. Nevertheless, when the animals were killed two weeks after the full six treatment regimen there was a 6 log increase in the number of *C. neoformans* cfu in the brains of mice treated with AmB. In contrast, in the AmBisome 3 mg/kg group, the cfu dropped by 1 log showing that AmBisome therapy was continue to kill the fungi even after treatment was stopped. In an efficacy study of AmBisome by Berman et al (1986), 99% of *Leishmania donovani* parasites were eliminated from the liver and spleen of infected hamsters by one administration of 1.5–11 mg of AmBisome per kg. A total of 98%–99% of hepatosplenic parasites were eliminated from squirrel monkeys by three administrations of 4 mg of AmBisome per kg. AmBisome was 170–750 times as active as antimony in hamsters, and approximately 60 times as active as antimony in monkeys. Recently Clemons et al (2000)

challenged the immunosuppressed rabbits intracisternally with *Coccidioides immitis*. Five days post-infection, groups of rabbits were treated with either fluconazole (19 doses of 80 mg/kg/day, p.o.), AmBisome (15 mg/kg i.v. 3 times a week for 3 weeks), AmB (1 mg/kg i.v. three times a week for 3 weeks), or 5% glucose (control). All animals treated with fluconazole, AmB and AmBisome were survived, whereas 75% of the controls were died ($P < 0.0005$). The AmBisome-treated group had 3- and 11- fold lower cfu in the brain and in the spinal cord, respectively, compared with the fluconazole group, and 6- and 35- fold lower cfu, respectively, compared with the AmB treated group and AmBisome was found to be superior to either fluconazole or AmB for the treatment of experimental coccidioidal meningitis.

In another study the efficacy of AmBisome (5 doses: 0.05, 0.1, 0.5, 0.8, and 3 mg/kg of body weight) was compared to that of Fungizone (4 doses: 0.05, 0.1, 0.5, and 0.8 mg/kg) in a BALB/c mice model of VL induced by *Leishmania infantum*. AmBisome was about 3 times more active than the conventional drug against both strains (strain 1 was obtained from an untreated patient, and strain 2 was obtained from a patient who had received 12.5 g of AmB over 3 years). Median effective doses (ED_{50}) of AmBisome were 0.054 (strain 1) and 0.194 (strain 2) mg/kg. ED_{50} of conventional AmB were 0.171 (strain 1) and 0.406 (strain 2) mg/kg. Determination of drug tissue levels, 3 days after the last drug administration, showed the drug accumulation in hepatic and splenic tissues much higher after administration of AmBisome than after conventional AmB. A lack of toxicity was noted in all groups treated with AmBisome (Paul et al 1997).

In a pulmonary aspergillosis model in mice, immunosuppressed mice were challenged intranasally with 8×10^4 *A. fumigatus* conidia (Olson et al 2000). Groups of seven infected mice were treated intravenously with AmBisome 15 mg/kg, Abelcet 15 mg/kg, AmB 1 mg/kg, or 5% glucose daily for 4 days beginning 2 hours after challenge. All of the control mice were dead by day 5. The survival rate for groups treated with either Abelcet or AmB (Fungizone) was 29% on day 9 post-infection. However, the AmBisome treatment group had 86% rate of survival. Leenders et al (Leenders et al 1996) compared the efficacy of AmBisome and the AmB in an unusual rat aspergillosis model. The rats were infected only in the left lung, and 40 h later they were treated with either AmB 1 mg/kg/day or AmBisome 1 or 10 mg/kg/day for 10 consecutive days. Both AmB 1 mg/kg/day and AmBisome 10 mg/kg/day increased survival; nevertheless, only AmBisome 10 mg/kg/day was

able to cause a significant diminution in cfu in the left lung ($P = 0.003$). Interestingly, distribution to the right lung was abridged in both of the AmBisome treatment groups, while conventional AmB was ineffective to prevent lung dissemination. Distribution 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 the distribution to the liver and spleen. Animal studies have revealed that AmBisome is also very effective in both treating and preventing fungal infections in the kidneys (Adler-Moore et al 1991; van Etten et al 1993; Garcia et al 2000). In the prophylactic study, AmB levels in the kidneys of AmBisome-treated mice (5, 10, or 20 mg/kg) were ranged from 0.63 to 8.08 mg/kg 7 days after treatment (Adler-Moore and Proffitt 2002).

Clinical efficacy and safety

AmBisome is much better endured than conventional AmB and is specified in the treatment of severe systemic fungal infections where patients fail to respond to AmB, are intolerant to its side-effect, or who have renal impairment prohibiting the use of conventional drug. AmBisome was first used clinically in 1987 when a heart transplant patient developed pulmonary aspergillosis, which due to nephrotoxicity could not be treated with conventional AmB (Katz et al 1990). After 34 days of treatment with AmBisome at 1 mg/kg/day, the infection was exterminated and no proof of recurrence was reported during a 16-month follow up period. Kidney function was also improved and acute side-effects such as fever and chills were not seen during therapy. Since then, AmBisome has been developed throughout the world and is currently licensed in more than 30 countries, including the US where it has sanctioned for empiric use (fever of unknown origin). In a controlled randomized trial, a short antifungal prophylaxis course of AmBisome was found to reduce the incidence of proven invasive fungal infections considerably during the first month following liver transplantation surgery. AmBisome was well tolerated, although backache, thrombocytopenia and renal function impairment were reported in a few patients (Tollemar et al 1995). Clinical studies on immunocompromised adult and pediatric patients with invasive fungal infections, primarily candidiasis and aspergillosis, were designed to evaluate the efficacy of AmBisome. The results obtained for AmBisome in these studies were promising and complete or partial response was seen. Specifically, the use of AmBisome in febrile neutropenic patients with suspected or confirmed invasive mycoses resolved the

fungal infection in 61% of the episodes. Treatment efficacy was 77% for aspergillosis (Mills et al 1994). Ringden et al (Ringden et al 1991) also reported a favorable eradication rate of 83% for *Candida* and 41% for *Aspergillus* infections in immunocompromised patients treated with AmBisome. AmBisome was also effective in pediatric patients with similar disorders. A report on the serum and pulmonary concentrations of AmBisome in a patient with acute liver transplant failure is also noteworthy. During follow-up of a patient with liver transplant failure and pulmonary aspergillosis, it was observed that peak and trough serum concentrations of AmB were increased, as were pulmonary concentrations of the drug (Heinemann et al 1997). The authors hypothesized that, in absence of a normally functioning liver tissue as a component of RES, the clearance function of the liver was diminished and that the clearance by the lung began to be important (Schmidt et al 1998).

The multicenter study by Meunier et al (1991) included 126 patients receiving 133 episodes of AmBisome treatment. The majority of these patients had failed previous conventional AmB therapy due to toxicity. AmBisome was administered for 21 days at an average daily dose of 2.1 mg/kg (range = 0.45–5 mg/kg). Hypokalaemia was the most common side-effect observed in 24 cases. In 17 episodes, creatinine was initially high, but returned to normal. Glutamylxaloacetate transaminase became elevated in 19 instances, and elevation in alkaline phosphatase was observed in 22 instances. Nevertheless there was no report of discontinuation of AmBisome therapy due to adverse side-effects. Thus, AmBisome was well tolerated even in severely ill patients. Walsh et al (1998) administered AmBisome to 36 febrile neutropenic patients for empirical antifungal therapy at doses of 1, 2.5, 5, or 7.5 mg/kg. No fungal infections were observed, suggesting that AmBisome was effective in preventing breakthrough fungal infections. A more recent report including 687 febrile neutropenic patients and comparing D-AmB with AmBisome as empirical therapeutic agents validated the previous data. It was again shown that AmBisome was as effective as the conventional drug and was associated with fewer breakthrough fungal infections and fewer toxic reactions (Walsh et al 1999).

Recently AmBisome safety was judged in a series of 187 transplant recipients. AmBisome was administered daily at dose levels between 1 and 4 mg/kg for a median of 11 days (range of 1–112 days). Side effects including allergic reaction, low back pain during infusion, dyspnea, low serum potassium, and nausea and vomiting ascribed to AmBisome therapy were observed in only 7% of

the cases and resulted in discontinuation of therapy in 6 cases. In this context, with patients receiving a variety of potentially toxic drugs, the AmBisome side-effect profile was mild and controllable in the vast majority of patients (Ringden et al 1994). Recent multicenter randomized trials compared D-AmB at 1 mg/kg/day to AmBisome at 1 and 3 mg/kg/day in adults (Prentice et al 1997) and children (Hann et al 1995) with febrile neutropenia unresponsive to broad spectrum antibiotics. A group of 193 adult patients was prospectively randomized into the three treatment groups. Fifty-two patients had confirmed mycosis, seven were not classifiable and the rest were stratified as having fever of unknown origin (FUO). The adult study showed significantly lower adverse events for the AmBisome groups. D-AmB showed 50% nephrotoxicity compared with 16% and 18% showed by AmBisome 1 and 3 mg/kg/day groups ($p = 0.001$). Also hypokalemia was considerably less in the AmBisome cohorts. A paediatric study created a similar picture, but differed in detail. Nephrotoxicity was lower in the AmBisome compared with D-AmB but the differences were not statistically significant. Considerable advantages were seen for AmBisome therapy in incidences of hypokalemia, treatment delay, and resolution of fever. Davidson et al measured the optimum dose and schedule for AmBisome treatment of VL. A group of 88 patients, mostly children was treated with 4 different dose regimens. Eighty-four patients were completely cured of their disease by the initial treatment course lasting 10 days (4 or 5 days daily treatment at 3 or 4 mg/kg/day and 1 follow up on day 10). Four relapsing children received an additional 10-day course of treatment at 3 mg/kg/day which cured them all (Davidson et al 1996). This study is outstanding not only for the short course treatment and high cure rate of VL patients, but also for the favorable safety profile (no significant adverse events) (Schmidt et al 1998).

The efficacy and safety of 3 regimens of AmBisome in the treatment of Indian VL were compared in a prospective open randomized trial. Thirty parasitologically confirmed patients were randomly divided into 3 equal treatment groups; group 1 received AmBisome 2 mg/kg on days 1, 2, 3, 4, 5, 6, and 10 (total dose 14 mg/kg); group 2 received AmBisome 2 mg/kg on days 1, 2, 3, 4, and 10 (total dose 10 mg/kg); group 3 received the same dosage on days 1, 5 and 10 (total dose 6 mg/kg). Clinical cure resulted in all patients by day 24. Haemoglobin, white blood cell count, body weight and serum albumin level improved on day 24 and became normal by day 180. No patient relapsed within 12 months of follow up (Thakur et al 1996).

Immunoliposomes

The current encouraging progress regarding lipid-based formulations of AmB is the development of novel liposomes with specific properties. One of these, “immunoliposomes”, contains fungus-specific antibodies on their surface which target them directly to the fungal cells. AmB coated with immunoliposomes abridged mortality appreciably in mice with invasive pulmonary aspergillosis as compared conventional L-AmB (100% vs 16.7% survival rate). AmB coated with immunoliposomes was also more effective than AmB integrated with long-circulating liposomes (100% vs 83.3% survival rate) (Otsubo et al 1998). Likewise, treatment of murine candidiasis and cryptococcosis with AmB integrated with immunoliposomes proved enhanced activity compared to that with conventional L-AmB (Belay et al 1991; Dromer et al 1990).

Long-circulating liposomes

The other novel delivery system, ‘long circulating liposomes’ are coated with polyethylene glycol (PEG), resulting in a sterically stabilized surface. Since the time period to reside in circulation is prolonged by the structural nature of long-circulating liposomes, more intact liposomes can get localized at the site of infection, thus enhancing the in vivo efficacy (Storm and van Etten 1997). In an experimental murine model of systemic candidiasis, AmB integrated with long-circulating liposomes (PEG-L-AmB) proved to be more effective than the conventional L-AmB (van Etten et al 1995; van Etten et al 1998). Nevertheless, intracellular antifungal activity of PEG-L-AmB assessed in *C. albicans* infected murine peritoneal macrophages was as low as that of conventional L-AmB, while it was higher for D-AmB (van Etten et al 1998).

Other lipid based nanomodifications

Lipid nanospheres

Studies on efficacies of NS-718, AmB encapsulated in lipid nanosphere are in progress. Lipid nanosphere is composed of equal amounts of egg lecithin and soybean oil. The carrier potentials of lipid nanosphere are characterized by lower uptake by the reticuloendothelial system and good distribution to the sites of inflammation. When equivalent dose of NS-718 or Fungizone were injected intravenously into rats, the plasma AmB level yielded by NS-718 was higher than Fungizone at all time up to 2 hours. In a tissue distribution study, the concentration in the liver after the

injection of NS-718 was lower than that of Fungizone. This characteristic of NS-718 to avoid uptaking by reticuloendothelial system (RES) is related to high plasma concentration of AmB. These results suggest that NS-718 have several unique characteristics different from other lipid formulations for the treatment of fungal infections (Seki et al 1994; Tomii 2002). In another study NS-718 was found to be more effective than D-AmB or L-AmB against clinical isolates of *C. albicans* and *Aspergillus fumigatus*. NS-718 was well tolerated and showed improved survival markedly at equivalent doses in treating pulmonary aspergillosis in rat. Increased activity was also supported by pharmacokinetic study (Kohno et al 1995).

Fukui et al investigated whether AmB retained its antifungal activity in NS-718 (Fukui et al 1996). Antifungal activity of NS-718 against *Candida albicans* was similar to that of AmB and Fungizone. However, the antifungal activity of L-AmB was decreased. Thus, NS-718 maintained the potent activity of AmB against fungal cell even though the AmB was incorporated into LNS particles. Hossain et al compared the direct cytotoxicity of NS-718 with that of Fungizone in human proximal tubule cells in vitro and showed decreased cytotoxicity of NS-718 (Hossain et al 2000). These results showed an increased selectivity between toxicity of NS-718 against mammalian cells and antifungal activity.

In vitro and In vivo antifungal efficacy of NS-718 was also studied in pulmonary cryptococcosis in mice. NS-718 was found to have better in vitro efficacy against clinical isolates of *Cryptococcus neoformans* than other AmB formulations, was well tolerated, and efficacy was much higher than that of D-AmB or L-AmB in treating pulmonary cryptococcosis in mice (Hossain et al 1998). In vivo antifungal efficacy of NS-718 was also studied in invasive pulmonary aspergillosis in rats (Otsubo et al 1999). The results showed that NS-718 was effective in treating pulmonary aspergillosis in rats, but equivalent doses of Fungizone and L-AmB were either lethally toxic or ineffective.

In a rat model of localized candidiasis, LNS-AmB significantly inhibited the growth of *C. albicans* in the pouch, whereas AmBisome did not, even though the AmB concentrations in the pouch were similar. This difference in antifungal activity between LNS-AmB and AmBisome was also found in vitro. That is, the antifungal activity of LNS-AmB against *C. albicans* was similar to that of Fungizone and dimethyl sulfoxide-solubilized AmB, while AmBisome showed weaker antifungal activity than did other formulations (Figure 4). In a mouse model of systemic candidiasis,

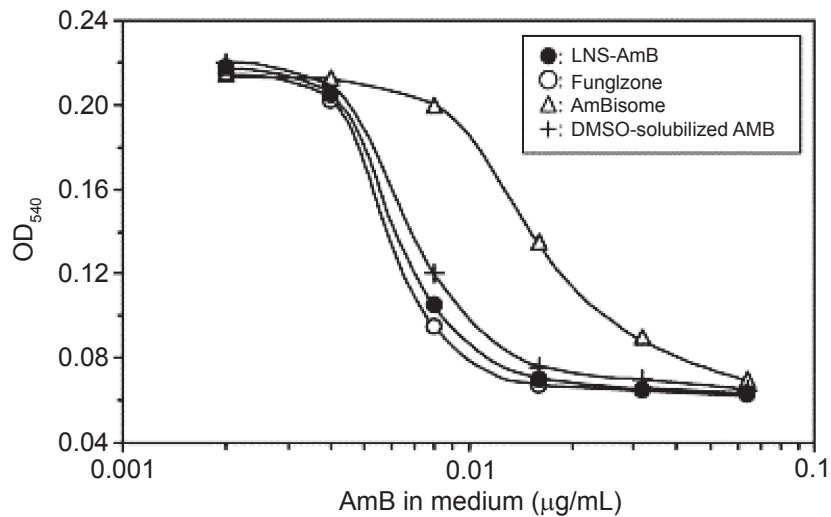


Figure 4 Antifungal activity of LNS-AmB, Fungizone, AmBisome, and DMSO-solubilized AmB in vitro. The growth inhibition of *C. albicans* was measured by the change in optical density at 540 nm in SD-MOPS broth after a 24-h incubation at 35°C. Results are the mean of two experiments. Adapted from Fukui et al (2003).

LNS-AmB (1.0 mg/kg) greatly improved the survival rate (Figure 5) and was therefore much more effective than AmBisome (8.0 mg/kg) ($P < 0.05$) or Fungizone (1.0 mg/kg) ($P < 0.01$) (Fukui et al 2003).

Cochleates

Cochleates are stable phospholipid-calcium precipitates comprised mainly of phosphatidylserine. The in vivo therapeutic efficacy of cochleates containing AmB (CAmB) administered orally was evaluated in a mouse model of systemic candidiasis. The fungal tissue burden in kidneys and lungs was assessed, and a dose-dependent reduction in

C. albicans from the kidneys was observed, with a maximum 3.5-log reduction in total cell counts at 2.5 mg/kg/day. However, complete clearance of the organism from the lungs, resulting in more than a 4-log reduction, was observed at the same dose. (Santangelo et al 2000).

In the study by Zarif et al (Zarif et al 2000) CAmB protect ICR mice infected with *C. albicans* when the agent is administered intraperitoneally at doses of as low as 0.1 mg/kg/day. In a tissue burden study, CAmB, Fungizone, and AmBisome were effective in the kidneys, but in the spleen CAmB was more potent than Fungizone at 1 mg/kg/day and was equivalent to AmBisome at 10 mg/kg/day.

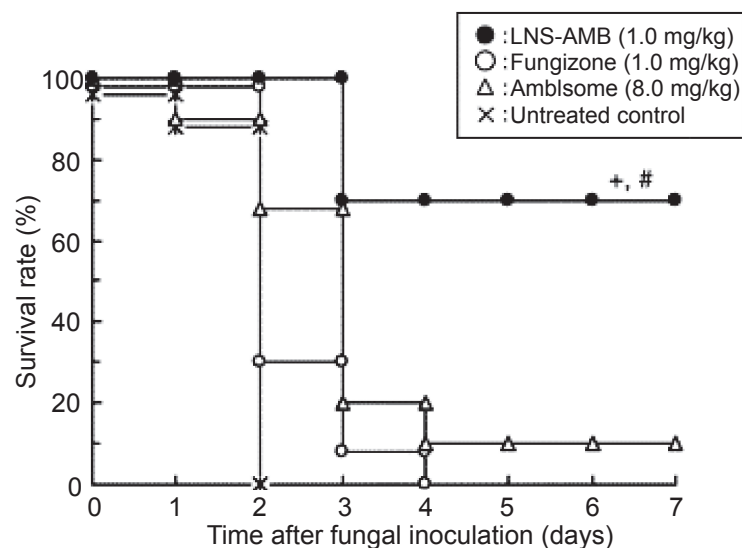


Figure 5 Survival of mice infected with *C. albicans* and treated with LNS-AmB, Fungizone, or AmBisome. Treatment was started 4 hours after fungal inoculation. +, $P < 0.05$ compared with AmBisome; #, $P < 0.01$ compared with Fungizone. Adapted from Fukui et al (2003).

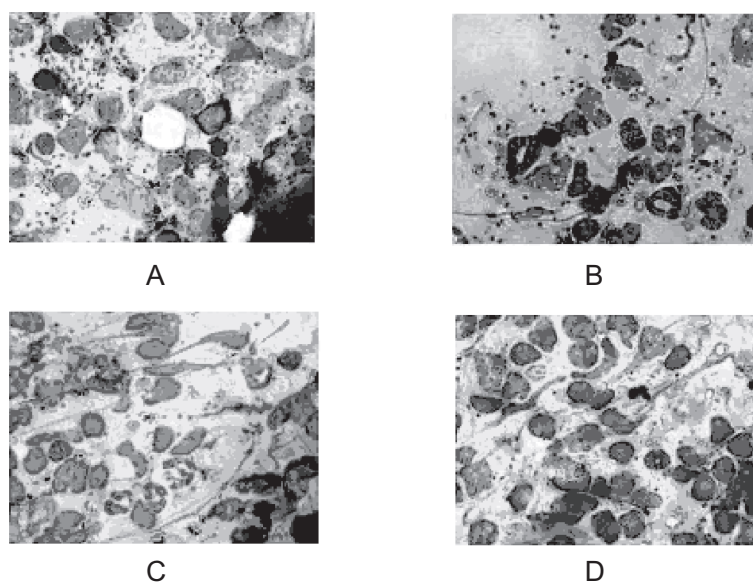


Figure 6 Photographs showing geimsa stained splenic smears of hamster treated with emulsomes and control formulations. A-untreated control group; B-Mycol (AmB for injection) treated group; C-TLEs or Trilaurin based emulsomes treated group; D-TSEs or Tristearin based emulsomes treated group.

Emulsome: a novel nano lipid particle

Emulsomes are a new generation colloidal carrier system in which the internal core is made of solid fats and triglycerides which is stabilized by high concentration of lecithins in the form of o/w emulsion (Amselem et al 1994). The effects of emulsomes, nanosize range lipid particles containing AmB (EAmB) were compared with the reference formulation Fungizone and with the commercial preparation AmBisome. Both Fungizone and EAmB had a minimum inhibitory concentration (MIC) of 0.039 $\mu\text{g/ml}$ against *C. albicans* ATCC10231, whereas the MIC for AmBisome was considerably higher (0.156 $\mu\text{g/ml}$). However, the yeasts were more rapidly killed by Fungizone than by EAmB in spite of similar MIC values. The killing of *C. albicans* was delayed when EAmB was used. In a tissue culture model and in mice, the incorporation of AmB into emulsomes resulted in a considerable reduction of toxicity in comparison with Fungizone. For comparison of the in vivo effect of the preparations, a mouse model of systemic infection with *C. albicans* was used. All preparations were able to reduce the fungal burden in the liver and kidneys in comparison with

control animals treated with isotonic saline. AmBisome was more efficient in the reduction of the fungal burden of the liver than EAmB and Fungizone, even when applied in a similar dosage of 1 mg/kg. In the kidneys, EAmB and Fungizone were slightly more effective than AmBisome. Therefore the incorporation of AmB into nanosize lipid particles was able to reduce toxicity without loss of efficiency (Kretschmar et al 2001).

In our laboratory we have developed and evaluated AmB loaded emulsomes for the treatment of VL. By virtue of solidified or semisolidified internal oily core it provided a better opportunity to load AmB in high concentration. In vivo studies on *L. donovani* infected hamsters showed better results for AmB emulsomes as compared to control (D-AmB, Mycol) (Figure 6).

The maximal percentage of parasite suppression (55.7%) was obtained with 0.5 mg/kg of AmB loaded trilaurin emulsomes (TLEs). Tristearin emulsomes (TSEs) showed 40.7% parasite suppression at the same dose whereas only 33.6% of parasite suppression was observed with relatively higher dose (1 mg/kg) of D-AmB or Mycol (Table 2).

Table 2 Activity of emulsome formulations against *L. donovani* in hamsters infected for 30 days

S.No	Formulation code	% drug entrapment	Dosage given (mg/kg)	% parasite suppression
1	Mycol	–	1 mg/kg	33.6%
2	TLEs	80.1	0.5 mg/kg	55.7%
3	TSEs	84.7	0.5 mg/kg	40.7%

Future directions

Fungal infections are on the rise worldwide, particularly as the population of immunocompromised patients continues to grow. By itself, AmB is an effective antifungal and anti-leishmanial agent, though it is highly toxic, particularly to the kidney. The goal of these lipid formulations of the AmB is to transport the drug throughout the body without exposing it to sensitive organs and tissues and then to deliver it in concentrated dosage to the target site. To a certain extent all the lipid formulations accomplish this goal. The maximum tolerable dose of AmB is about 1 mg/kg/day. However these lipid formulations allow physician to go up to 5 times the dose of AmB without increasing infusion related toxicities. All the lipid formulations of AmB demonstrate improved efficacy, primarily because of the higher administered dose, and reduced kidney toxicity, compared to AmB. As such the future of these lipid formulations is bright and it is apparent that these lipid based products will replace AmB as the mainstays in the treatment of systemic fungal infections and leishmaniasis.

Targeting AmB using the colloidal carrier systems, ie, liposomes, emulsomes, or nanospheres etc to the sites of infection could readily be utilized in terms of their industrial application as this can provide a better therapy mode for treatment of systemic fungal infections and leishmaniasis in comparison with currently available drug regimen in the market for these respective diseases. High loading efficiency and protracted release profile may further reduce the dose size and dose frequency. Further the easier ligation of surface specific ligands could enhance the target specificity and performance efficiency. Thus the drug AmB, which is well known for its effectiveness however, compromised due to its contraindicated manifestations, can safely be administered for effective cure of infective diseases. Nevertheless, these nanocarriers may provide curable disposition of systemic microbial infections. Moreover, the colloidal nature of these nanocarriers leads to their passive accumulation in pathogen harbouring or infected macrophages. More advances in nanotechnology will hopefully result in more efficient and less toxic AmB therapeutic regimens.

References

- Abra RM, Hunt CA. 1981. Liposome disposition in vivo. III. Dose and vesicle-size effects. *Biocim Biophys Acta*, 666:493–503.
- Adler-Moore JP, Chiang S, Satorius A, et al. 1991. Treatment of murine Candidosis and cryptococcosis with a unilamellar liposomal amphotericin B formulation (AmBisome). *J Antimicrob Chemother*, 28(Suppl B):63–71.
- Adler-Moore JP, Fujii G, Lee MJA, et al. 1993. In vitro and in vivo interactions of AmBisome with pathogenic fungi. *J Liposome Res*, 3:151–6.
- Adler-Moore JP, Proffitt RT. 1998. AmBisome: long circulating formulation of Amphotericin B. In Woodle MC and Storm G, eds. *Long Circulating Liposomes: Old drugs, New Therapeutics*. New York: Springer-Verlag. p. 185–206.
- Adler-Moore J, Proffitt RT. 2002. AmBisome: liposomal formulation, structure, mechanism of action and preclinical experience. *J Antimicrob Chemother*, 49 (Suppl S1):21–30.
- Adler-Moore JP. 1994. AmBisome targeting to fungal infections. *Bone marrow transplantation*, 14(Suppl 5):S3–S7.
- Albert MM, Stahl-Carroll L, Luther MF, et al. 1995. Comparison of liposomal amphotericin B to amphotericin B for treatment of murine cryptococcal meningitis. *J Myco Med*, 5:1–6.
- Alvar J, Canavate C, Gutierrez-Solar B, et al. 1997. Leishmania and human immunodeficiency virus co-infection: the first 10 years. *Clin Microbiol Rev*, 10:298–319.
- Amselem S, Yogev A, Zawoznik E, et al. 1994. Emulsomes, a novel drug delivery technology. *Proceedings of the International Symposium on Controlled Release of Bioactive Materials*, 21:1368–69.
- Anaissie E, Paetznick V, Proffitt R, et al. 1991. Comparison of the in vitro antifungal activity of free and liposome-encapsulated Amphotericin B. *Eur J Clin Microb Infect Dis*, 10:665–68.
- Andes D. 1999. In 39th Interscience Conference on Antimicrobial Agents and Chemotherapy: San Francisco, abstract no. 1002, p. 28.
- Arikan S, Rex JH. 2001. Lipid-based antifungal agents: current status. *Curr Pharm Des*, 7:393–415.
- Bangham AD, Standish MM, Watkins JC. 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J Mol Biol*, 13:238–52.
- Bangham AD. 1992. Liposomes: realizing their promise. *Hosp Pract (Off Ed)*, 27:51–6, 61–2.
- Belay T, Hospenthal DR, Rogers AL, et al. 1991. Evaluation of antibody-bearing liposomal amphotericin B in the treatment of systemic candidiasis in a neutropenic murine model. *J Med Vet Mycol*, 29:419–421.
- Berman JD, Hanson WL, Chapman WL, et al. 1986. Antileishmanial activity of liposome-encapsulated amphotericin B in hamsters and monkeys. *Antimicrob Agents Chemother*, 30:847–51.
- Berman JD, Ksionski G, Chapman WL, et al. 1992. Activity of amphotericin B cholesterol dispersion (Amphocil) in experimental visceral leishmaniasis. *Antimicrob Agents Chemother*, 36:1978–80.
- Bolard J, Legrand P, Heitz F, et al. 1991. One-sided action of amphotericin B on cholesterol-containing membranes is determined by its self-association in the medium. *Biochemistry*, 30:5707–15.
- Boswell GW, Bekersky I, Buell D, et al. 1998. Toxicological profile and pharmacokinetics of a unilamellar liposomal vesicle formulation of amphotericin B in rats. *Antimicrob Agents Chemother*, 42:263–68.
- Brajtburg J, Bolard J. 1996. Carrier effects on biological activity of amphotericin B. *Clin Microb Rev*, 9:512–31.
- Brajtburg J, Elberg S, Kobayashi GS, et al. 1994. Amphotericin B incorporated into egg lecithin-bile salt mixed micelles: molecular and cellular aspects relevant to therapeutic efficacy in experimental mycoses. *Antimicrob Agents Chemother*, 38:300–06.
- Brajtburg J, Elberg S, Schwartz DR, et al. 1985. Involvement of oxidative damage in erythrocyte lysis induced by amphotericin B. *Antimicrob Agents Chemother*, 27:172–76.
- Clemons KV, Howell KJ, Calderon L, et al. 2000. Efficacy of intravenous AmBisome against coccidioidal meningitis in rabbits. In *Abstracts of the Fortieth Interscience Conference on Antimicrobial Agents and Chemotherapy*, Toronto, Canada, Abstract 2120. Washington, DC: American Society for Microbiology. p. 396.
- Clemons KV, Stevens DA. 1998. Comparison of fungizone, Amphotec, AmBisome, and Abelcet for treatment of systemic murine cryptococcosis. *Antimicrob Agents Chemother*, 42:899–902.
- Davidson RN, Croft SL, Scott A, et al. 1991. Liposomal amphotericin B in drug-resistant visceral leishmaniasis. *Lancet*, 337:1061–62.

- Davidson RN, di Martino L, Gradoni L, et al. 1996. Short-course treatment of visceral leishmaniasis with liposomal amphotericin B (AmBisome). *Clin Infect Dis*, 6:938–43.
- de Marie S, Janknegt R, Bakker-Woudenberg IAJ. 1994. Clinical use of liposomal and lipid-complexed amphotericin B. *J Antimicrob Chemother*, 33:907–16.
- Dromer F, Barbet J, Bolard J, et al. 1990. Improvement of amphotericin B activity during experimental cryptococcosis by incorporation into specific immunoliposomes. *Antimicrob Agents Chemother*, 34:2055–60.
- Emminger W, Graninger W, Emminger-Schmidmeier W, et al. 1994. Tolerance of high doses of amphotericin B by infusion of a liposomal formulation in children with cancer. *Ann Hematol*, 68:27–31.
- Espuelas MS, Legrand P, Loiseau PM, et al. 2002. In vitro antileishmanial activity of amphotericin B loaded in poly(epsilon-caprolactone) nanospheres. *J Drug Target*, 10:593–99.
- Francis P, Lee JW, Hoffman A, et al. 1994. Efficacy of unilamellar liposomal amphotericin B in treatment of pulmonary aspergillosis in persistently granulocytopenic rabbits: the potential role of bronchoalveolar D-mannitol and serum galactomannan as markers of infection. *J Infect Dis*, 169:356–68.
- Fromtling RA. 1993. Amphotericin B cholesterol sulfate complex (colloidal dispersion). *Drugs Future*, 18:303–06.
- Fromtling RA. 1995. Amphotericin B lipid complex. *Drugs Future*, 20:129–34.
- Fujii G. 1996. Liposomal Amphotericin B (AmBisome): Realization of the drug delivery concept. *Vesicles*, 12: 491–526.
- Fukui H, Koike T, Nakagawa T, et al. 2003. Comparison of LNS-AmB, a novel low-dose formulation of amphotericin B with lipid nano-sphere (LNS), with commercial lipid-based formulations. *Int J Pharm*, 267:101–12.
- Fukui H, Koike T, Saheki A, et al. 1996. In 23rd International Symposium on Controlled Release of Bioactive Materials.
- Gangneux JP, Sulahian A, Garin YJ, et al. 1996. Therapy of visceral leishmaniasis due to *Leishmania infantum*: experimental assessment of efficacy of AmBisome. *Antimicrob Agents Chemother*, 40:1214–18.
- Garcia A, Adler-Moore JP, Proffitt RT. 2000. Single dose AmBisome (liposomal amphotericin B) as prophylaxis for murine systemic candidiasis and histoplasmosis. *Antimicrob Agents Chemother*, 44:2327–32.
- Georgopadakou NH, Walsh TJ. 1996. Antifungal agents: chemotherapeutic targets and immunologic strategies. *Antimicrob Agents Chemother*, 40:279–91.
- Gokhale PC, Barapatre RJ, Advani SH, et al. 1993. Pharmacokinetics and tolerance of liposomal amphotericin B in patients. *J Antimicrob Chemother*, 32:133–139.
- Gokhale PC, Barapatre RJ, Advani SH, et al. 1993. Successful treatment of disseminated candidiasis resistant to amphotericin B by liposomal amphotericin B: a case report. *J Cancer Res Clin Oncol*, 119:569–571.
- Gold W, Stout HA, Pagano JF, et al. 1956. Antibiotics Annual 1955–1956. In Welch H, Marti-Ibanez F. (eds). Medical Encyclopedia. New York. p 579–86.
- Graybill JR, Craven PC, Taylor RL, et al. 1982. Treatment of murine cryptococcosis with liposome-associated amphotericin B. *J Infect Dis*, 145:748–52.
- Groll A, Giri N, Gonzalez C, et al. 1997. In 37th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Canada, abstract A–90.
- Groll AH, Giri N, Petraitis V, et al. 2000. Comparative efficacy and distribution of lipid formulations of amphotericin B in experimental *Candida albicans* infections of the central nervous system. *J Infect Dis*, 182:274–82.
- Guo LS, Working PK. 1993. Complexes of amphotericin B and cholesteryl sulfate. *J Liposome Res*, 3:473–490.
- Hann IN, Stevens RF, Pinkerton CR. 1995. 2nd International Symposium on Febrile Neutropenia, Brussels.
- Hartel S, Bolard J. 1996. Amphotericin B: new life for an old drug. *Trends Pharmacol Sci*, 17:445–49.
- Hay RJ. 1994. Antifungal drugs on the horizon. *J Am Acad Dermatol*, 31:S82–85.
- Heinemann V, Bosse D, Jehn U, et al. 1997. Enhanced pulmonary accumulation of liposomal amphotericin B (AmBisome) in acute liver transplant failure. *J Antimicrob Chemother*, 40:295–97.
- Herwaldt BL. 1999. Leishmaniasis. *Lancet*, 354:1191–99.
- Hiemenz JW, Walsh TJ. 1996. Lipid formulations of amphotericin B: recent progress and future directions. *Clin Infect Dis*, 22(Suppl 2):S133–44.
- Hoepfich PD. 1992. Clinical use of amphotericin B and derivatives: lore, mystique, and fact. *Clin Infect Dis*, 14 (Suppl 1):S114–S9.
- Hossain MA, Maesaki S, Kakeya H, et al. 1998. Efficacy of NS-718, a novel lipid nanosphere-encapsulated amphotericin B, against *Cryptococcus neoformans*. *Antimicrob Agents Chemother*, 42:1722–25.
- Hossain MA, Maesaki S, Razaque MS, et al. 2000. Attenuation of nephrotoxicity by a novel lipid nanosphere (NS-718) incorporating amphotericin B. *J Antimicrob Chemother*, 46:263–8.
- Janoff AS, Perkins WR, Saletan SL, et al. 1993. Amphotericin B lipid complex (ABLCL). A molecular rationale for the attenuation of amphotericin B related toxicities. *J Liposome Res*, 3:451–71.
- Karyotakis NC, Anaissie EJ. 1994. Efficacy of escalating doses of liposomal amphotericin B (AmBisome) against hematogenous *Candida lusitanae* and *Candida krusei* infection in neutropenic mice. *Antimicrob Agents Chemother*, 38:2660–62.
- Katz NM, Pierce PF, Anzeck RA, et al. 1990. Liposomal amphotericin B for treatment of pulmonary aspergillosis in a heart transplant patient. *J Heart Transplant*, 9:14–17.
- Klepser ME, Wolfe EJ, Jones RN, et al. 1997. Antifungal pharmacodynamic characteristics of fluconazole and amphotericin B tested against *Candida albicans*. *Antimicrob Agents Chemother*, 41:1392–95.
- Kohno S, Otsubo T, Hara K. 1995. American Society for Microbiology, Washington D.C. Programs and abstracts of the 35th Interscience Conference on Antimicrobial agents and Chemotherapy, p. 131, Abstr F109.
- Kretschmar M, Amselem S, Zawoznik E, et al. 2001. Efficient treatment of murine systemic infection with *Candida albicans* using amphotericin B incorporated in nanosize range particles (emulsomes). *Mycoses*, 44:281–86.
- Leenders ACAP, de Marie S, ten Kate MT, et al. 1996. Liposomal amphotericin B (AmBisome) reduces dissemination of infection as compared with amphotericin B deoxycholate (Fungizone) in a rat model of pulmonary aspergillosis. *J Antimicrob Chemother*, 38:215–25.
- Legrand P, Romero EA, Cohen BE, et al. 1992. Effects of aggregation and solvent on the toxicity of amphotericin B to human erythrocytes. *Antimicrob. Agents Chemother*, 36:2518–22.
- Legrand P, Vertut-Doi A, Bolard J. 1996. Comparative internalization and recycling of different amphotericin B formulations by a macrophage-like cell line. *J Antimicrob Chemother*, 37:519–33.
- Lopez-Berestein G, Bodey GP, Frankel LS, et al. 1987. Treatment of hepatosplenic candidiasis with liposomal-amphotericin. B. *J Clin Oncol*, 5:310–17.
- Lopez-Berestein G, Fainstein V, Hopfer R, et al. 1985. Liposomal amphotericin B for the treatment of systemic fungal infections in patients with cancer: a preliminary study. *J Infect Dis*, 151:704–10.
- Lopez-Berestein G, Juliano RL. 1987. Application of liposomes to the delivery of antifungal agents. In Ostro MJ, ed. Liposomes. New York: Marcel Dekker. p. 253–276.
- Lopez-Berestein G, Metha R, Hopfer RL, et al. 1983. Treatment and prophylaxis of disseminated infection due to *Candida albicans* in mice with liposome-encapsulated amphotericin B. *J Infect Dis*, 147:939–45.
- Lopez-Berestein G. 1987. Liposomes as carriers of antimicrobial agents. *Antimicrob Agents Chemother*, 31:675–8.
- Maesaki S. 2002. Drug delivery system of anti-fungal and parasitic agents. *Curr Pharm Des*, 8:433–40.
- Medoff G, Dismukes WE, Pappagianis D, et al. 1992. Evaluation of new antifungal drugs for the treatment of systemic fungal infections. Infectious Diseases Society of America and the Food and Drug Administration. *Clin Infect Dis*, 15(Suppl 1):S274–281.

- Mehta RT, McQueen TJ, Keyhani A, et al. 1994. Phagocyte transport as mechanism for enhanced therapeutic activity of liposomal amphotericin B. *Chemotherapy*, 40:256–64.
- Mehta RT, Poddar S, Kalidas M, et al. 1997. Role of macrophages in the candidal activity of liposomal amphotericin B. *J Infect Dis*, 175:214–17.
- Meunier F, Prentice HG, Ringden O. 1991. Liposomal amphotericin B (AmBisome): safety data from a phase II/III clinical trial. *J Antimicrob Chemother*, 28 (Suppl B):83–91.
- Meunier F, Sculier JP, Coune A, et al. 1988. Amphotericin B encapsulated in liposomes administered to cancer patients. *Ann N Y Acad Sci*, 544:598–610.
- Meyerhoff A. 1999. US Food and Drug administration approval of AmBisome (liposomal amphotericin B) for treatment of visceral leishmaniasis. *Clin Infect Dis*, 28:49–51.
- Mills W, Chopra R, Linch DC, et al. 1994. Liposomal amphotericin B in the treatment of fungal infections in neutropenic patients: a single-centre experience of 133 episodes in 116 patients. *Br J Haematol*, 86:754–60.
- Murray HW. 1999. Kala-azar as an AIDS related opportunistic infection. *Aids Patient Care STDS*, 13:459–65.
- New RR, Chance ML, Heath S. 1981. Antileishmanial activity of amphotericin and other antifungal agents entrapped in liposomes. *J Antimicrob Chemother*, 8:371–81.
- Olson J, Huynh V, Bunch T, et al. 2000. Differences in efficacy and tissue drug concentrations following treatment of murine pulmonary aspergillosis with AmBisome or Abelcet. In Abstracts of the Fourteenth International Society for Human and Animal Mycoses, Buenos Aires, Argentina, Abstract 217, Zurich. International Society for Human and Animal Mycology, p. 228.
- Ostro MJ, Cullis PR. 1989. Use of liposomes as injectable–drug delivery systems. *Am J Hosp Pharm*, 46:1576–87.
- Otsubo T, Maesaki S, Hossain MA, et al. 1999. In vitro and in vivo activities of NS-718, a new lipid nanosphere incorporating amphotericin B, against *Aspergillus fumigatus*. *Antimicrob Agents Chemother*, 43:471–5.
- Otsubo T, Maruyama K, Maesaki S, et al. 1998. Long-circulating immunoliposomal amphotericin B against invasive pulmonary aspergillosis in mice. *Antimicrob Agents Chemother*, 41:40–4.
- Paul M, Durand R, Fessi H, et al. 1997. Activity of a new liposomal formulation of amphotericin B against two strains of *Leishmania infantum* in a murine model. *Antimicrob Agents Chemother*, 41:1731–34.
- Pearson RD, Sousa AD. 1996. Clinical spectrum of Leishmaniasis. *Clin Infect Dis*, 22:1–11.
- Prentice HG, Hann IM, Herbrecht R, et al. 1997. A randomized comparison of liposomal versus conventional amphotericin B for the treatment of pyrexia of unknown origin in neutropenic patients. *Brit J Haematol*, 98:711–18.
- Ralph ED, Barber KR, Grant CWM. 1993. Clinical experience with multilamellar liposomal amphotericin B in patients with proven and suspected fungal infections. *Scand. J Infect Dis*, 23:487–96.
- Ringden O, Andstrom E, Remberger M, et al. 1994. Safety of liposomal amphotericin B (AmBisome) in 187 transplant recipients treated with cyclosporin. *Bone Marrow Transplant*, 14(Suppl B):S10–S14.
- Ringden O, Meunier F, Tollemar J, et al. 1991. Efficacy of amphotericin B encapsulated in liposomes (AmBisome) in the treatment of invasive fungal infections in immunocompromised patients. *J Antimicrob Chemother*, 28(Suppl B):73–82.
- Santangelo R, Paderu P, Delmas G, et al. 2000. Efficacy of oral coxlate–amphotericin B in a mouse model of systemic candidiasis. *Antimicrob Agents Chemother*, 44:2356–60.
- Schindler JJ, Warren RP, Allen SD, et al. 1993. Immunological effects of amphotericin B and liposomal amphotericin B on splenocytes from immune–normal and immune–compromised mice. *Antimicrob Agents Chemother*, 37:2716–2721.
- Schmidt PG, Adler-Moore JP, Forssen EA, et al. 1998. Unilamellar liposomes for anticancer and antifungal therapy. In Lasic DD and Papahadjopoulos D. (eds). *Medical Applications of liposomes*. New York: Elsevier. p. 703–731.
- Sculier JP, Delcroix C, Brassinne C, et al. 1989. Pharmacokinetics of amphotericin B in patients receiving repeated intravenous high doses of amphotericin B entrapped into sonicated liposomes. *J Liposome Res*, 1:151–66.
- Seki J, Sasaki H, Doi M, Yoshikawa H, et al. 1994. Lipid Nano-Sphere (LNS), a protein-free analogue of lipoproteins, as a novel drug carrier for parenteral administration. IV. *J Control Release*, 28:352–53.
- Sereno D, Holzmuller P, Lemesre JL. 2000. Efficacy of second line drugs on antimonyl-resistant amastigotes of *Leishmania infantum*. *Acta Trop*, 74:25–31.
- Sperry PJ, Cua DJ, Wetzel S A, et al. 1998. Antimicrobial activity of AmBisome and nonliposomal amphotericin B following uptake of *Candida glabrata* by murine epidermal Langerhans cells. *Med Mycol*, 36:135–141.
- Stevens DA. 1994. Overview of amphotericin B colloidal dispersion (amphocil). *J Infect*, 28(S I):45–49.
- Storm G, van Etten E. 1997. Biopharmaceutical aspects of lipid formulations of amphotericin B. *Eur J Clin Microb and Infect Dis*, 16:64–73.
- Surarit R, Shepherd MG. 1987. The effects of azole and polyene antifungals on the plasma membrane enzymes of *Candida albicans*. *J Med Vet Mycol*, 25:403–13.
- Szoka FC, Milholland D, Barza M. 1987. Effect of lipid composition and liposome size on toxicity and in vitro fungicidal activity of liposome–intercalated amphotericin B. *Antimicrob Agent Chemother*, 31:421–9.
- Taylor RL, Williams DM, Craven PC, et al. 1982. Amphotericin B in liposomes: a novel therapy for histoplasmosis. *Am Rev Resp Dis*, 125:610–1.
- Thakur CP, Pandey AK, Sinha GP, et al. 1996. Comparison of three treatment regimens with liposomal amphotericin B (AmBisome) for visceral leishmaniasis in India: a randomized dose–finding study. *Trans Royal Soc Trop Med Hyg*, 90:319–22.
- Tollemar J, Hockerstedt K, Ericzon B-G, et al. 1995. Liposomal amphotericin B prevents invasive fungal infections in liver transplant recipients. A randomized, placebo–controlled study. *Transplantation*, 59:45–50.
- Tomii Y. 2002. Lipid formulation as a drug carrier for drug delivery. *Curr Pharm Des*, 8:467–74.
- Tremblay C, Barza M, Fiore C, et al. 1984. Efficacy of liposome–intercalated amphotericin B in the treatment of systemic candidiasis in mice. *Antimicrob Agent Chemother*, 26:170–3.
- van Etten EW, Otte–Lambillion M, van Vianen W, et al. 1995. Biodistribution of liposomal amphotericin B (AmBisome) and amphotericin B–desoxycholate (Fungizone) in uninfected immunocompetent mice and leucopenic mice infected with *Candida albicans*. *J Antimicrob Chemother*, 35:509–19.
- van Etten EW, Snijders SV, van Vianen W, et al. 1998. Superior efficacy of liposomal amphotericin B with prolonged circulation in blood in the treatment of severe candidiasis in leukopenic mice. *Antimicrob Agents Chemother*, 42:2431–33.
- van Etten EW, ten Kate MT, Stearne LE, et al. 1995. Amphotericin B liposomes with prolonged circulation in blood: in vitro antifungal activity, toxicity, and efficacy in systemic candidiasis in leukopenic mice. *Antimicrob Agents Chemother*, 39:1954–58.
- van Etten EW, van den Heuvel-de Groot C, Bakker–Woudenberg IA. 1993. Efficacies of amphotericin B–desoxycholate (Fungizone), liposomal amphotericin B (AmBisome) and fluconazole in the treatment of systemic candidosis in immunocompetent and leucopenic mice. *J Antimicrob Chemother*, 32:723–39.
- van Etten EW, van Vianen W, Hak J, et al. 1998. Activity of liposomal amphotericin B with prolonged circulation in blood versus those of AmBisome and fungizone against intracellular *Candida albicans* in murine peritoneal macrophages. *Antimicrob Agents Chemother*, 42:2437–39.

- Vertut-Doi A, Hannaert P, Bolard J. 1988. The polyene antibiotic amphotericin B inhibits the Na⁺/K⁺ pump of human erythrocytes. *Biochem Biophys Res Commun*, 157:692–697.
- Walsh TJ, Finberg RW, Arndt C, et al. 1999. Liposomal amphotericin B for empirical therapy in patients with persistent fever and neutropenia. National Institute of Allergy and Infectious Diseases Mycoses Study Group. *N Engl J Med*, 340:764–71.
- Walsh TJ, Yeldandi V, McEvoy M, et al. 1998. Safety, tolerance, and pharmacokinetics of a small unilamellar liposomal formulation of amphotericin B (AmBisome) in neutropenic patients. *Antimicrob Agents Chemother*, 42:2391–98.
- Wasan KM, Morton RE, Rosenblum MG, et al. 1994. Decreased toxicity of liposomal amphotericin B due to association of amphotericin B with high-density lipoproteins: role of lipid transfer protein. *J Pharm Sci*, 83:1006–10.
- Wong-Beringer A, Jacobs RA, Guglielmo BJ. 1998. Lipid formulations of amphotericin B: clinical efficacy and toxicities. *Clin Infect Dis*, 27:603–18.
- Yardley V, Croft SL. 1997. Activity of liposomal amphotericin B against experimental cutaneous leishmaniasis. *Antimicrob Agents Chemother*, 41:752–56.
- Zarif L, Graybill JR, Perlin D, et al. 2000. Antifungal activity of amphotericin B cochleates against *Candida albicans* infection in a mouse model. *Antimicrob Agents Chemother*, 44:1463–69.