Optimizing efficacy of Amphotericin B through nanomodification

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Abstract: The polyene antibiotic Amphotericin B (AMB) is one of the first therapeutic agents to be marketed commercially as nanosized formulations in which the drug is associated with lipids as liposomes or complexes. In this way, its renal toxicity is reduced and its therapeutic index improved. This review summarizes the particular properties of AMB which justify this type of formulation and the early work leading up to their development. The clinical results obtained in the treatment of fungal infections are reviewed and their activity against leishmaniasis is also evoked. Some newer formulations of AMB, based on both lipids and polymers are described. In particular, their potential by the oral and pulmonary routes are discussed. Finally, the development of targeted systems to deliver the drug to specific cells and tissues is considered.
Keywords: Amphotericin B, liposomes, nanoparticles, micelles, *Candida*, *Aspergillus*

Introduction

The polyene antibiotic Amphotericin B (AMB) is one of the first therapeutic agents to be marketed commercially as nanosized formulations. This review will summarize the particular properties of AMB which justify this type of formulation and the early work leading up to their development. The clinical results obtained in the treatment of fungal infections will be reviewed. Finally, some newer formulations, in which the drug has been associated with polymers as well as lipids, and new directions in the use of AMB will be considered.

Properties of AMB and early work with AMB in liposomes

Properties of AMB

The antimicrobial properties of AMB, a macrolide extracted from *Streptomyces nodosus*, were first noted in the 1950s (Vandeputte et al 1955–1956) and the antibiotic arrived on the market in 1958 (Utz et al 1958–1959). The drug possesses a wide spectrum of activity, encompassing a large number of fungal species as well as protozoan parasites (*Leishmania* species) and amoebae (*Naegleria* species) (for a recent review, see Kleinberg 2006). More recently, it was found to have some activity against prion diseases (Hartsel and Weiland 2003; Mangé et al 2000). Despite its therapeutic importance, the physicochemical properties of AMB lead to some difficulties in its formulation and utilization, and solutions based on "nanotechnology" have been developed in response to these.

AMB (Figure 1) is an asymmetrical, cyclic molecule with one hydrophobic and one hydrophilic face, and an aminosugar (mycosamine) group. It has a very limited solubility profile, being almost completely insoluble in water, sparingly soluble in alcohols and soluble in organic solvents such as DMSO and DMF (Brittain 1994). In water, AMB aggregates, forming first dimers by apposition of two hydrophobic faces

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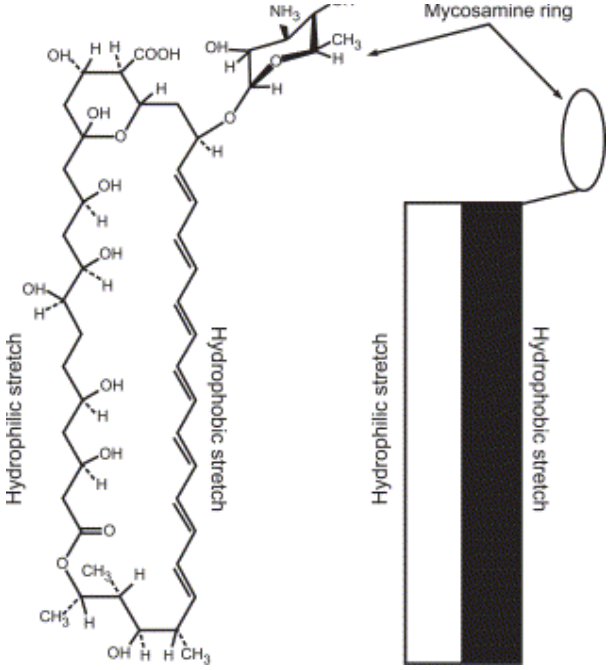

Figure 1 Structure of Amphotericin B.

followed by larger aggregates (Mazerski et al 1982). This insolubility in aqueous media also leads to low bioavailability of AMB by the oral route. Its use is therefore limited to intravenous infusion and local application. The conventional formulation of AMB (Fungizone®) is mixed micelles with the detergent sodium deoxycholate, and in this form it is the drug of choice for systemic infections with sensitive fungal species (Georgopapadakou and Walsh 1996). It is usually administered by slow perfusion diluted in 5% glucose. However, dose-limiting side-effects are frequent, the most severe being renal toxicity (Brezis et al 1984).

Both the therapeutic and toxic effects of AMB derive from its interaction with lipids, and in particular, membrane sterols. The antibiotic can form complexes with both ergosterol, the principal sterol in fungal cell membranes, and cholesterol in mammalian cell membranes. The result of this is the formation of pores leading to the leakage of electrolytes and other cell components (De Kruijff and Demel 1974). The selectivity for fungal cell membranes is the result of greater affinity for ergosterol than cholesterol, due to the presence of a double bond on carbon 22 in the former (Cybulska et al 1986).

Given the affinity of the antibiotic for biological membranes, incorporation of AMB into lipid-based nanosystems in order to improve its therapeutic index has been studied since the 1980s. Three of these systems are now commercially available.

Mechanisms of action

In 1996, Hartsel and Bolard reviewed the mechanisms by which the selectivity of AMB towards fungal cells can be improved by association with lipid systems. One mechanism is related to the molecular state of the AMB when it is released from the formulation. It has been observed that while both monomeric and self-aggregated AMB associate with ergosterol, only self-aggregated AMB forms pores in cholesterol-containing membranes. It follows that a formulation that can assure that AMB is released only as monomers will have an improved therapeutic index. On dilution in the plasma, AMB is rapidly released from Fungizone® in the aggregated form and toxicity to mammalian cells ensues. However, AMB can also bind to membrane phospholipids, so the relative affinity for cell and drug delivery system lipids may contribute to determining the reduction of toxicity. When AMB binds to cell membranes it has a pro-oxidative effect, and this may be as important as pore formation in generating cell damage (Bratjburg et al 1985).

Furthermore, the affinity of AMB for lipids means that it is readily incorporated into plasma lipoproteins, particularly low density lipoprotein (Bratjburg et al 1984). Receptor-mediated uptake of low density lipoprotein carrying AMB by renal epithelial cells is one mechanism of toxicity in this organ. Therefore, the rate of transfer between the drug delivery system and circulating lipoproteins will be another factor which determines the efficacy of the system (Legrand et al 1996).

AMB also has effects on the immune system, and in particular can modulate the functions of macrophages. For example, it stimulates production of cytokines such as interleukin 1 (Chia and McManus 1990) and tumor necrosis factor alpha (TNF-α) (Tokuda et al 1993), reactive oxygen intermediates (Wilson et al 1991) and nitric oxide (NO) (Herrmann et al 1994; Mozaffarian et al 1997), as well as chemotaxis and phagocytosis. These properties could contribute to the antimicrobial activity of AMB, but could also increase its toxicity, for example by causing fever and chills. Larabi et al (2001) compared the production of NO and TNF-α induced in non infected mouse peritoneal macrophages by different lipid formulations of AMB compared with free AMB, in association with co-stimulants. At equivalent AMB concentration, mediator production was always less with the lipid formulations than with the free drug, and the liposomal forms (eg, AmBisome®) reduced this more than lipid complexes (eg, Abelcet®). In macrophages infected with *Leishmania donovani*, AMB also contributed to stimulating NO and TNF-α production, but the concentrations at which

this occurred were much higher than those causing parasite killing (Larabi et al unpublished results), suggesting that in this case at least, the immunostimulating effects contribute more to the side-effects of AMB than to its antiparasitic activity.

Early liposomal formulations

The first study incorporating AMB into liposomes was performed by New et al in 1981. Their interest was in the antileishmanial properties of the antibiotic, and followed on from studies of encapsulated antimonial drugs. The fact that *Leishmania* parasites are located within phagocytic cells, and that liposomes are also preferentially accumulated by these cells made this approach particularly attractive (Heath et al 1984). However, the main effect of the liposomal formulation was to reduce the toxicity of AMB, allowing higher doses to be administered and thus increasing "efficacy". Soon afterwards, similar results were obtained in infections with *Cryptococcus* (Graybill et al 1982) and in histoplasmosis (Taylor et al 1982). However, the antimicrobial activity of AMB *per se* was not increased by encapsulation. During this period, the influence of the liposome composition, and size, on the activity of AMB was studied. In one study (Lopez-Berestein et al 1983), it was found that liposomes containing phospholipids alone were more efficient than liposomes containing sterols (cholesterol or ergosterol). One explanation for this could be that the strong binding of AMB to sterol prevents its release from the liposomes and its interaction with fungal cell membranes. A study by Szoka et al (1987) of a range of liposome sizes and compositions found that there was no correlation between the extent of reduction of toxicity against mouse macrophages in vitro and the reduction of lethality in vivo. In vivo, small sterol-containing liposomes were less toxic than larger ones, and liposomes without sterol but containing phospholipids which were in a "solid" (liquid crystal) state at physiological temperatures were less toxic than ones in which the phospholipids were in a fluid state. A large number of different AMB formulations were tested, leading to the commercialization of three of them. These have quite different physico-chemical structures (see below), but all reduce the toxicity of AMB compared with Fungizone®.

Commercial formulations of AMB

Three lipid-based formulations of AMB are at present licensed for clinical use. Their physico-chemical properties are listed in Table 1.

Physico-chemical properties

AmBisome® is the only true liposomal formulation of the three. It is composed of small, unilamellar vesicles composed of lipids which yield a very stable bilayer, in the gel state at physiological temperature. AMB is incorporated into this bilayer at 10 moles %. The size of the liposomes (about 80 nm) means that they have a long circulating half-life and a good penetration into tissues. The stable bilayer composition should reduce exchanges with lipoproteins and contribute to the very low toxicity of this formulation (Adler-Moore and Proffitt 2002).

Amphotec® (Amphocil™ in Europe and Amphotec® in the US) is composed of complexes between cholesteryl sulfate and AMB in equimolar proportions. These have the form of thin discs of about 120 nm in diameter. However, despite the small size, their circulation time is much less than that of AmBisome® and they deliver AMB rapidly to phagocytic cells (Guo 2001).

Abelcet® is composed of two synthetic phospholipids – dimyristoyl phosphatidylcholine and dimyristoyl phos-

Table 1 Commercial formulations of AMB

Name	Composition	AMB/lipid (mol%)	Charge	Form	Size (μm)	Reference
AmBisome®	Hydrogenated soy phosphatidylcholine:cholesterol: distearoylphosphatidylcholine: AMB 2:1:0.8:0.4	10	Negative	Small unilamellar liposomes	0.08	Adler-Moore and Proffitt 2002
Amphotec® (Amphocil®)	Cholestryl sulfate: AMB 1:1	50	Negative	Disc-like complexes	0.12	Guo 2001
Abelcet®	Dimyristoylphosphatidylcholine: Dimyristoylphosphatidylglycerol: AMB 7:3:10	50	Negative	Ribbon-like complexes	1–10	Janoff et al 1993

phatidylglycerol in a 7 to 3 molar ratio with an equimolar amount of AMB. These components assemble in ribbons of 1 to 10 micrometers in length. These larger objects are rapidly accumulated in the mononuclear phagocyte system (Janoff et al 1993).

Clinical studies in fungal infections

Systemic fungal infections, dominated by *Candida* and *Aspergillus* infections, remain the leading cause of infection-related mortality and morbidity in many populations of immunocompromised patients. Azoles are often recommended for *Candida* infection but the epidemiology of *Candida* infection has changed over the last few years. *C albicans* now comprises less than half of the isolates of candidemia worldwide (Eggimann et al 2003; Pappas et al 2003). The other half is represented by a variety of non-albicans species, for some of which the susceptibility to azoles, particularly fluconazole, is decreased. For *Aspergillus* and other less common moulds, the mortality rates are greater than 60% and even higher in patients with disseminated infection, although the extended-spectrum azoles represent a major advance as a first-line treatment (Herbrecht et al 2002). Therefore, there is a need for more effective antifungal drugs with a wide spectrum.

In this respect, AMB has the advantage of covering most of the fungal pathogens involved in human disease. However, the use of AMB formulated with deoxycholate (Fungizone®) has been limited by infusion-related side effects and cumulative nephrotoxicity which, in fine, actually increase overall healthcare expenses, despite its primarily low cost (Maertens et al 2001). For these reasons, and because other alternatives are now available, primary therapy with Fungizone® is more and more challenged by new antifungal therapies for use in many systemic mycoses, including moulds, such as *Aspergillus*. These new antifungal therapies include extended-spectrum triazoles, the echinocandins, and also lipid formulations of AMB, as described above (Herbrecht et al 2003). Among the lipophilic formulations of AMB commercially available, the majority of studies have been carried out with liposomal AMB (AmBisome®).

Clinical efficacy of liposomal AMB

Due to the paucity of diagnostic means for fungal infections and the poor prognosis of full-blown invasive fungal infections, clinicians use several strategies when faced with fungal infections. Antifungal drugs are given for demonstrated infections but in high-risk patients, they may be administered empirically, in the case of persistent fever despite appropriate antibacterials, or as prophylaxis, in every patient at risk of fungal infection whatever the clinical signs. The efficacy of liposomal AMB has been studied in these different settings, both in open and randomized studies. Liposomal AMB has also proved effective in the treatment of visceral leishmaniasis (see below).

Demonstrated infections

In full-blown fungal infections, initial open studies involving patients refractory to, or intolerant of, Fungizone® showed improvement or cure in 66% and 81% of patients with invasive aspergillosis and invasive candidiasis respectively, with the liposomal form (Ringden et al 1991). Two studies have suggested a superior efficacy of liposomal AMB compared to Fungizone® in probable or proven fungal invasive infections but were insufficient to give a definitive answer (Leenders et al 1997; Leenders et al 1998).

Another important issue is the dose to be administered. It was expected that the good tolerance profile would allow high liposomal AMB doses to be given and achieve better efficacy without increasing toxicity. In a randomized trial comparing two doses of liposomal AMB (1 versus 4mg/kg/day) for the primary treatment of invasive aspergillosis, an overall response rate of 55% was observed, regardless of the dose, with no difference in either arm (Ellis et al 1998). This substantial response rate demonstrates evidence of the efficacy of liposomal AMB in first-line therapy of invasive aspergillosis. To determine the appropriate daily dose for the initial treatment of invasive aspergillosis and other filamentous fungal infections in immunocompromised patients, a phase 3, multi-center, randomized, double-blind study of the safety and efficacy of an liposomal AMB loading dose regimen versus a standard liposomal AMB regimen was performed (Cornely 2005). The study compared a loading regimen of 10 mg/kg/day × 14d versus the standard regimen of 3 mg/kg/day for 14 days. The standard regimen had a favorable overall response rate of 50% and a 12-week survival rate of 72% comparable to those previously reported for voriconazole in a similarly designed trial (Herbrecht et al 2002). However, the high-dose regimen did not demonstrate any improvement in overall response or survival.

In an attempt to sum up the efficacy of lipid formulations of AMB, not all in the liposomal form, a meta-analysis of seven randomized studies was performed (Barrett et al 2003). This analysis did not show any difference in the response rate between the lipid formulations of AMB and Fungizone® but showed a decrease in mortality (OR = 0.72; 95% CI = 0.54 – 0.97).

The poor outcome of mould infections and the availability of several antifungal drugs of different classes have stimulated the evaluation of alternatives based on combinations of different antifungal drugs (Johnson et al 2004). Apart from the association of Fungizone® and 5-fluorocytosine which has been the recommended treatment for cryptococcal meningitis for a long time, the other associations, mainly between Fungizone® and azoles, raised several concerns about possible toxicities (Polak 1999). In vivo studies have shown encouraging results, for the associations of liposomal AMB with both voriconazole and echinocandins in models as different as rat models of invasive aspergillosis (Kirkpatrick et al 2006), a murine model of cerebral aspergillosis (Clemons et al 2005), or a murine model of *C. glabrata* systemic infection (Olson et al 2005). In humans, a few case reports and small series of benefic results of associations have been reported but none are randomized studies (Aliff et al 2003; Kontoyiannis et al 2003; Marr et al 2004).

Empirical therapy

In the setting of empirical therapy for persistent febrile neutropenia, comparative studies concluded that liposomal AMB is as effective as Fungizone® (Prentice et al 1997; Walsh et al 1999). A double-blind study compared the safety of liposomal AMB (3 or 5 mg/kg/day) and AMB lipid complex (5 mg/kg/day) (Wingard et al 2000). Neither of the two liposomal AMB dosages yielded a better outcome than AMB lipid complex.

Primary prophylaxis

Three randomized trials have assessed the efficacy of low doses of liposomal AMB as prophylaxis in bone marrow transplant recipients, without demonstrating any benefit (Kelsey et al 1999; Tollemar et al 1993a, 1993b). One study in liver transplant patients showed a significant decrease in invasive *Candida* spp. infections in the liposome-treated patients, compared to the placebo-treated patients but the 1-month survival was identical in both groups. However, long-term survival was increased in patients who received liposomal AMB (Tollemar et al 1995). Recently, a pharmacokinetic study of once-weekly high-dose liposomal AMB as fungal prophylaxis for immunocompromised children undergoing stem cell transplantation suggested that this dosage may provide useful protection against fungal infections (Mehta et al 2006).

Tolerability

Patients treated with liposomal AMB at 3 mg/kg/day had less infusion-related adverse events, needed less premedication, and had less nephrotoxicity than patients treated with Fungizone® at 0.6 mg/kg/day (Prentice et al 1997; Walsh et al 1999). The tolerability of high doses up to 7.5–15 mg/kg/day appeared satisfactory (Walsh et al 2001). These results justified comparing a liposomal AMB loading dose regimen versus a standard liposomal AMB regimen (Cornely 2005). Higher rates of hypokalemia and nephrotoxicity were seen compared with the standard dose regimen with no better efficacy. In neonates, high doses (5–7 mg/kg/day) of liposomal AMB for a median of 18 days seem to be much better tolerated than in adults (Juster-Reicher et al 2003). Compared with AmB lipid complex at 5 mg/kg/day, liposomal AMB at 3 and 5 mg/kg/day showed less infusion-related reactions and nephrotoxicity in febrile neutropenic patients (Wingard et al 2000). This study clearly indicates that liposomal AMB was better tolerated than AMB lipid complex.

Conclusion

Liposomal AMB has been shown to be at least as efficacious as Fungizone® and has a dramatically improved safety profile compared with the traditional form. The recommended dose is 3–5mg/kg/day for demonstrated fungal infection and 3 mg/kg/day for empirical therapy, and doses up to 3 mg/kg/day of liposomal AMB are well tolerated. Higher doses have not shown any therapeutic benefit in invasive aspergillosis whereas they increased renal toxicity. For some rare filamentous fungus infections such as those due to zygomycetes and *Fusarium* spp, the liposomal formulation may be considered as first-line therapy because of the absence of an effective alternative, although new azoles may be effective towards some of these fungi. The in vitro models and the experimental data in animals show that combination therapy may improve outcome, but these experimental results remain to be confirmed with clinical trials.

Lipid formulations of AMB in the treatment of leishmaniasis

Leishmaniasis is a family of protozoal infections transmitted by sand-fly bites, which affects about 12 million people in warm regions throughout the world. Visceral leishmaniasis, in which the parasite – *Leishmania donovani* in India and Bangladesh, *L. infantum* in the rest of Asia, Africa and Europe and *L. chagasi* in the Americas – develops within tissue macrophages in the liver, spleen and bone marrow, is the most serious manifestation (Herwaldt 1999). It is endemic in India, Bangladesh and Sudan where it represents a major public health problem and is also becoming increasingly prevalent as an opportunistic infection in Western countries,

among individuals who are infected with the HIV virus or are immunocompromised for other reasons. Cutaneous leishmaniasis, characterized by skin lesions, is more common but less serious. Muco-cutaneous and disseminated cutaneous manifestations also occur (Herwaldt 1999). Early treatment options were pentavalent antimonials and pentamidine, which have shown problems of toxicity and resistance (Murray, 2001). AMB was found to be an effective treatment for visceral leishmaniasis in the 1990s (Murray 2004; Singh and Sivakumar 2004). Lipid formulations of AMB are a particularly attractive alternative in this context because they are accumulated in the same cells as the parasite. Thus AmBisome® was approved by the FDA in 1997 (Meyerhoff 1999). Despite the reduced toxicity of the lipid formulations, their high cost is prohibitive in the zones in which visceral leishmaniasis is endemic. A comparative study by Sundar et al (2004) showed that although the higher doses that could be given with the lipid formulations reduced the total time for cure and therefore the cost of hospitalization, this only partly offset the high purchase price of the drug. On the other hand, in a European situation, when cost is not such a preponderant issue, lipid formulations of AMB have become the treatment of choice (Gradoni et al 2004).

Cutaneous leishmaniais can also be treated effectively with lipid formulations of AMB (Amato et al 2004, Yardley and Croft 2000). In this case, the smaller formulations (AmBisome®, Amphocil™) are the most effective, because of their small size.

Recently, a new drug, miltefosine (hexadecylphosphocholine) has been shown to be effective against visceral leishmaniasis by the oral route (Murray 2001; Sundar et al 2002). This is a definite breakthrough and shows an obvious advantage over the current formulations of AMB, which are administered intravenously. Associations of AMB and miltefosine may have some therapeutic advantage (Seifert and Croft 2006).

Other formulations of AMB and new trends in their administration

Lipid-based formulations

An adhoc solution to the problem of AMB toxicity is to mix Fungizone® with Intralipid®, a preparation for parenteral nutrition, which consists of an oil-in-water emulsion stabilized with lecithin. The AMB is complexed by the phospholipids on the surface of the oil globules and its toxicity is reduced compared with Fungizone® alone. However, this method does not give reproducible results (Tomii 2002).

More recently, AMB has also been mixed with another proprietary lipid emulsion formulation, Lipofundin®. AMB was added as a powder, and the use of a high-pressure homogenizer promoted its dissolution in the interfacial layer, according to the patented SolEmuls® technology. However, no toxicity data are available for this formulation (Müller et al 2004).

De novo emulsion formulations of AMB have also been described, for example, those studied by Egito and collaborators (1996a). The emulsion form reduced the toxicity of AMB considerably, compared with Fungizone®, although less than AmBisome®, and allowed higher doses to be given, allowing a better cure rate of infections with *Candida albicans*. Circular dichroism studies showed that the AMB remained in the monomeric form within the emulsions, over a wide range of dilutions (Egito et al 1996b).

Seki and co-workers have formulated AMB into nano-sized emulsions called "Lipid Nano Spheres" which attempt to imitate lipoproteins. These small particles (25–50 nm) reduced AMB toxicity compared with Fungizone®, and showed similar activity against *Candida albicans*. Like AmBisome®, these small particles showed reduced uptake by macrophages and persistence in the circulation (Fukui et al 2003).

Heat-treatment of AMB is a simple method of reducing toxicity. Heating the Fungizone® formulation to 70° C provokes the formation of superaggregates, as detected by spectrophotometric methods, rather than aggregates, which are less toxic to mammalian cells while retaining almost equivalent antifungal activity (Gaboriau et al 1997). Cryo-transmission electron microscopy revealed that while the native product was composed mainly of micelles of about 4 nm in diameter, with some threadlike aggregated micelles, the heated formulation contained much larger networks of about 300 nm (van Etten et al 2000).

Another approach to modulating the solubility, and therefore the toxicity of AMB was the use of ions from the Hofmeister series which alter water properties. While kosmotropes increased AMB aggregation, the chaotrophic ions thiocyanate and trichloroacetate were found to allow solubilization of AMB as monomers (Grijalba et al 2006).

Larabi et al developed a lipid complex system with a similar composition to Abelcet®, but prepared by a different method known as nanoprecipitation. This led to the formation of thin discs of about 250 nm in diameter, in which the lipids were probably in an interdigitated form rather than a bilayer (Larabi et al 2004a). This change in size and morphology reduced the toxicity, both towards macrophages in vitro and in vivo after both acute and chronic administration

to mice, compared to both Fungizone® and Abelcet® (Larabi et al 2003, Larabi et al 2004b). The activity of the complexes against visceral leishmaniasis in mice was higher than that of Abelcet® but not as high as that of AmBisome® (Larabi et al 2003). This illustrates the importance of nanosystem morphology in determining the biological effect.

Another disc-like formulation was developed by Lincopan et al (2005, 2006) using the cationic lipid dioctadecyldimethylammonium bromide (DODAB). This lipid formed bilayer fragments of about 65 nm in diameter with AMB at a low drug-to-lipid ratio. This formulation reduced nephrotoxicity and hepatotoxicity compared with Fungizone®, but spleen toxicity due to the cationic lipid was observed. At higher drug-to-lipid ratios, drug particles surrounded by a lipid bilayer are formed. The toxicity and therapeutic activity of these formulations have not yet been investigated.

Another group (Oda et al 2006) has tried to imitate lipoprotein particles as an original delivery system for AMB. The specific apolipoprotein from high density lipoprotein, ApoA-I, was added to mixtures of dimyristoylphosphatidylcholine, dimyristoylphosphatidylglycerol and AMB. After sonication and dialysis, a limpid preparation was obtained, consisting of disc-like particles of 8–10 nm in diameter. The circular dichroism spectra indicated that AMB was associated with lipid in the formulation. This preparation had much lower toxicity than Fungizone® in vitro and in vivo, which allowed higher doses to be given to mice, leading to effective treatment of *Candida albicans* infection. These systems also showed a good activity against *Leishmania major* in Balb/C mice, although no comparison with any other formulation was made (Nelson et al 2006).

Lipid cochleates are an interesting system for delivering AMB. These are formed mainly from phosphatidylserine and calcium, which associate by electrostatic interaction to form cylindrical structures consisting of a rolled-up bilayer. They are particularly appropriate for entrapping small hydrophobic or amphiphilic molecules like AMB (Zarif 2005). Like other lipid-based systems, AMB cochleates reduce the toxicity of the antibiotic. They are effective against murine candidasis and aspergillosis after i.p. and oral administration (Santangelo et al 2000; Delmas et al 2002).

Polymer-based formulations

AMB has been conjugated to a number of macromolecules with the aim of improving its solubility. Many of these have been derived from polysaccharides. For example, AMB has been conjugated to arabinogalactan (Ehrenfreund-Kleinman et al 2002). Polymers of about 30 kDa with about 20% of AMB by weight were obtained. The maximum tolerated dose of AMB was greatly increased by conjugation, while the antifungal activity against *Candida albicans* remained comparable to that of Fungizone®. A similar approach used dextran as the polysaccharide carrier. In particular, the preparation of a conjugate in which the free aldehyde groups were blocked showed very low toxicity towards mammalian cells while conserving antiparasitic activity (Sokolsky-Papkov et al 2006). AMB has also been conjugated to poly (ethylene glycol) (PEG). Attachment of AMB to a PEG of 40 kDa led to a highly water-soluble product which was only hydrolyzed slowly in rat plasma. It was 6 times less toxic than Fungizone® in rats and showed equal or superior antifungal activity (Conover et al 2003).

A number of groups have incorporated AMB into micelles prepared from amphiphilic polymers. Diblock copolymers consisting of poly (ethylene oxide) and poly (aspartic acid) substituted with various hydrophobic groups have been extensively studied in the laboratory of Kwon. In particular, poly (ethylene oxide)-block-poly (N-hexyl-L-aspartamide)-stearic acid ester micelles allow the antibiotic to be incorporated in its non aggregated form, as shown by spectrophotometric measurements and to be released in a sustained fashion. Such micelles show similar activity to Fungizone® in a mouse model of disseminated candidiasis (Adams and Kwon 2004). A similar system based on partially benzylated poly (aspartic acid) without a PEG block has been investigated by Yoo et al (2006). This polymer formed "nanoparticular" micelles of 20 nm in diameter, in which AMB aggregation was reduced compared with Fungizone®, as judged by its spectral properties. The acute toxicity in mice was reduced, as was damage to kidney cells after intravenous administration to rats, while the in vitro activity against *Candida albicans* was similar to that of Fungizone®. Vandermeulan et al (2006) have used poly (ethylene glycol)-block-poly (ε-caprolactone-co-trimethylenecarbonate) micelles to encapsulate AMB. These micelles are easy to prepare and although they reduce the antifungal activity they also reduce the amount of hemolysis.

There have been a few reports of nanoparticulate forms of AMB. A study by Venier-Julienne et al (1995) used AMB incorporated into poly (D, L-lactide-co-glycolide) nanoparticles. When their activity was tested against cultures of promastigotes of *L. donovani* within peritoneal macrophages, unloaded nanoparticles had a high an effect as loaded ones. This could be attributed to reactive oxygen intermediate generation following phagocytosis of the nanoparticles.

Espuelas et al attempted to incorporate AMB into poly (ε-caprolactone) nanoparticles (Espuelas et al 1998a; Espuelas et al 1998b). In fact, AMB was adsorbed onto the surface of the particles and was released on dilution, but despite this limitation, the acute toxicity of AMB in mice was reduced compared with Fungizone® (Espuelas et al 1997). During this work, it was noted that AMB also formed mixed micelles with the poloxamer 188 surfactant used to stabilize the nanoparticles (Espuelas et al 1998b). These micelles were found to have activity against clinical isolates of *Candida albicans* in vitro and also, interestingly, to reverse the resistance of *Leishmania donovani* parasites which had been rendered resistant to the drug by in vitro pressure, by a synergistic effect of AMB and the poloxamer (Espuelas et al 2000). However, the results obtained with *Candida albicans*-infected macrophages and in mice were disappointing, since the LD_{50} was increased compared with Fungizone® (Espuelas et al 2003).

More recently, AMB was incorporated into nanoparticles formed by a complex of two polysaccharides of opposing charge: chitosan and dextran sulfate. A high encapsulation rate for AMB was obtained, but spectral analysis showed that it was aggregated. A reduction in renal toxicity was observed but the large size of these particles (600–800 nm) suggests that they would only be useful for liver delivery (Tiyaboonchai et al 2006).

Microsphere formulations of AMB have also been tested for therapy of leishmaniasis. Albumin microspheres reduced the toxicity and increased the therapeutic efficiency of AMB against *Leishmania infantum* in hamsters. As might be expected, the microparticulate form increased drug accumulation in the liver and spleen (Sanchez-Brunete et al 2004). High doses of AMB administered in these particles deactivated expression of anti-inflammatory cytokines and increased pro-inflammatory ones, which probably contributed to the therapeutic effect (Rama Iniguez et al 2006). Different microsphere formulations were tested (Sanchez-Brunete et al 2005). Poly (lactide-co-glycolide) and polyanhydride microspheres were less effective than albumin ones in reducing liver and spleen parasite load, and albumin microspheres also induced a significant antibody response to parasite antigens.

Carbon nanotubes (CNT) have been attracting much attention lately as potential drug delivery systems. AMB has been linked covalently to functionalized CNT at the same time as fluorescein and uptake of the resulting particles into Jurkat cells was demonstrated (Wu et al 2005). Interestingly, the minimal inhibitory concentrations for several fungal species were reduced by this association, while "empty" CNT were without effect.

Administration of AMB by other routes: oral and pulmonary

One major disadvantage of AMB is its very low bioavailability by the oral route. This is essentially due to its very low solubility in aqueous media and its relatively high molecular weight (Dangi et al 1998). A number of different lipid-based systems have been used in attempts to improve the intestinal absorption of AMB. The presentation of the drug in the monomeric form could be expected to facilitate its dissolution and other components of the formulations may have absorption promoting effects.

Thus, ternary mixed micelle systems of AMB, deoxycholate and oleic acid, monoolein or soy lecithin were found to enhance the permeability of AMB in isolated intestinal loops (Dangi et al 1998). These systems have not been tested *in vivo*, however. Another system in which AMB was mixed with Peceol, a glyceride-rich vehicle for oral administration, also gave promising results, increasing lymphatic transport of AMB after oral administration to rats (Risovic et al 2004).

AMB cochleates (see above) have also proved to be efficient in the treatment of fungal infections by the oral route. In a model of Balb/C mice infected with *Candida albicans*, oral administration of these lipid particles was able to eradicate the infection from the lungs and prolong survival as effectively as Fungizone® given i.p. at a similar dose (Santangelo et al 2000). Efficacy was also demonstrated in a murine model of systemic aspergillosis (Delmas et al 2002).

Recently, AMB was associated with another lipidic antileishmanial agent, miltefosine (hexadecylphosphocholine or HePC). As described above, this molecule has been shown to be active by the oral route (Murray 2001; Sundar et al 2002) and has the interesting property of opening tight junctions in the Caco2 intestinal cell model (Ménez et al 2006a). Its alkylphospholipid structure suggested that it might be able to associate with AMB and in fact, spectroscopic studies showed that this can be the case (Ménez et al 2006b). However, rather than promoting absorption of AMB, the association led to a reduction of both cellular uptake and transepithelial transport in the Caco2 model (Ménez et al 2006b).

Kayser et al (2003) formulated a nanosuspension of AMB for administration by the oral route. The particles were prepared by high pressure homogenization of the drug with a mixture of surfactants. A reduction in parasite load in the liver was observed when the formulation was administered orally to mice infected with *Leishmania donovani*. This result

may be related to the presence in the formulation of Tween 80®, which is known to promote passage across biological membranes.

Pulmonary infections with *Aspergillus* spp. are a major clinical problem in immunocompromised patients. In consequence, there has been much interest in the use of aerosolized AMB formulations for treatment, and for prophylaxis in patients undergoing transplant surgery (Drew 2006). Animal studies have shown that liposomal formulations lead to higher concentrations of AMB in the lungs than Fungizone® (Ruijgrok et al 2000) or AMB directly solubilized in fluorocarbons (Vyas et al 2005). Clinical trials have shown that the lipid formulations are easier to aerosolize and better tolerated than Fungizone® (Perfect et al 2004; Drew 2006).

Specific targeting of AMB-loaded nanosystems

The studies described above have mainly taken advantage of the uptake of colloidal particles by the mononuclear phagocyte system and thus reach micro-organisms within these cells, or on the small size (for example AmBisome®) and surface properties which allow the carriers to remain in the circulation and reach other tissues in a non specific function. However, there are a few reports of attempts to deliver AMB to particular sites using nanosystems bearing specific targeting ligands. The ligands used have been sugars, antibodies or small peptides, as described in the following paragraphs.

Although the "natural" target of colloidal drug carriers is phagocytic cells, their uptake by macrophage, and particularly the Küpffer cells of the liver, can be greatly increased by modifying the surface with mannose residues which are recognized by the mannose-fucose receptor on these cells (Barratt and Schuber 1993). This strategy has been applied to the delivery of AMB to macrophages for treatment of leishmanias (Vyas et al 2000). Similarly, liposomes loaded with AMB and coated with mannan or pullanan, a glucose-containing polysaccharide have been administered to rats as an aerosol to target alveolar macrophages. Drug concentrations were higher than those delivered by unmodified liposomes and were sustained for 24h (Vyas et al 2005).

Galactose receptors are expressed by liver cells and some micro-organisms. Polylactide microspheres containing AMB have been functionalized with galactose residues and have been shown to bind to *Kluyveromyces bulgaricus* yeast cells (Kassab et al 2002). Heparin is a negatively charged polysaccharide with many interesting biological properties, including bioadhesion. Clemons et al (2001) encapsulated AMB within small (105 nm) hydrophilic nanoparticles bearing heparin at their surface. Their retention in the lungs was increased compared with Fungizone®, leading to a better therapeutic index against pulmonary blastomycosis in mice.

The coupling of antibodies to the surface of a liposome can theoretically give a delivery system targeted to a specific cell type. Small liposomes bearing a monoclonal antibody to *Cryptococcus neoformans* bound specifically to the yeast and, when administered intravenously to infected mice, prolonged survival longer than AMB in solution in dimethylsulfoxide/phosphate-buffered saline, non-targeted liposomes or liposomes targeted with an irrelevant antibody (Dromer et al 1990). However, antibody-bearing systems will still be accumulated within mononuclear phagocytes unless their surface is modified to avoid opsonization. Thus, the concept of sterically stabilized liposomes has emerged, in which the surface is covered with end-grafted PEG chains. Targeting can be achieved by coupling the antibody or other ligand to the distal end of a proportion of these chains (Mercadal et al 1999). Thus, sterically stabilized liposomes containing AMB and bearing an antibody specific for pulmonary endothelium at the end of the PEG chains have been prepared (Otsubo et al 1998). Accumulation of antibiotic in the lungs was observed, as opposed to its remaining in the blood in the case of non-targeted PEG-bearing liposomes or accumulating in the liver in the case of conventional liposomes. This was accompanied by increased efficacy against experimental aspergillosis in mice.

One smaller ligand which has been to direct AMB-loaded liposomes is the tetrapeptide tuftsin (Thr-Lys-Pro-Arg, Agrawal et al 2002), which binds to a specific receptor on macrophages. This peptide has the advantage of being both a targeting element promoting liver accumulation and a macrophage activator. The anti-leishmanial activity of the drug is thus reinforced by macrophage-mediated effects. Zhang et al (2003) have used a targeting strategy to deliver AMB across the blood-brain barrier. A peptide analogue of bradykinin, RMP-7, was coupled to PEG on sterically stabilized liposomes. This peptide interacts with the B2 receptor on brain capillary endothelial cells and increases the permeability of the vessels. By this means, AMB accumulation in the brain can be improved.

Conclusion

AMB is a good example of how an appropriate delivery system can improve the therapeutic index of a drug. The advantage of the commercial lipid-based formulations is principally that they reduce toxicity compared with the conventional formulation, allowing higher doses to be given. A

disadvantage is the high cost of these formulations, particularly with respect to their activity against parasitic diseases. Kleinberg (2006) has attempted to review the cost-effectiveness of these formulations compared with Fungizone®, taking into account all factors such as the time of hospitalization, in a North American setting, and concluded that in many cases, for example in the opportunistic infections in cancer patients, lipid formulations are a better choice than the conventional formulation. However, the cost of these formulations remains prohibitively high for the treatment of leishmaniasis in endemic areas (Sundar et al 2004).

Another disadvantage of AMB for mass treatment is its very low bioavailability by the oral route. Some studies reported above suggest that the use of nanosized formulations based on lipids or other amphiphilic molecules could be useful in overcoming this problem. Similarly, the delivery of drugs by the pulmonary route to combat respiratory infections is attracting much attention at the moment. New formulations of AMB could contribute in this area by increasing tolerability and ensuring delivery to the appropriate part of the lung. Another non parenteral route for which new AMB formulations could provide a therapeutic advance is in the eye, for example, in the treatment of fungal keratitis. Liposomal formulations have shown some advantages by this route, by reducing irritation and prolonging the residence time of drugs (Bochot et al 2000).

Finally, progress in the design of drug delivery systems has led to the development of carriers targeted to specific tissues and cells. Such technology applied to AMB would lead to a further increase in its therapeutic index. Therefore, as a result of innovative formulations, after almost 50 years on the market, AMB remains an extremely useful drug.

References

Adams M, Kwon GS. 2004. Spectroscopic investigation of the aggregation state of amphotericin B during loading, freeze-drying and reconstitution of polymeric micelles. *J Pharm Pharmaceut Sci*, 7(S1):1–6.

Adler-Moore JP, Proffitt R. 2002. AmBisome: liposomal formulation, structure, mechanism of action and pre-clinical experience. *J Antimicrob Ther*, 49(Suppl1):21–30.

Agrawal AJ, Agrawal A, Pal A, et al. 2002. Superior chemotherapeutic efficacy of Amphotericin B in tuftsin-bearing liposomes against *Leishmania donovani* infection in hamsters. *J Drug Target*, 10:41–5.

Aliff TB, Maslak PG, Jurcic JG, et al. 2003. Refractory Aspergillus pneumonia in patients with acute leukemia: successful therapy with combination caspofungin and liposomal amphotericin. *Cancer*, 97:1025–32.

Amato VS, Rabello A, Rotondo-Silva A, et al. 2004. Successful treatment of cutaneous leishmaniasis with lipid formulations of amphotericin B in two immunocompromised patients. *Acta Trop*, 92:127–32.

Barratt G, Schuber F. 1993. Targeting of liposomes with mannose terminated ligands. In Gregoriadis G (ed). Liposome Technology, Vol. III, 2nd ed. Boca Raton: CRC Press Inc. p 199–218.

Barrett JP, Vardulaki KA, Conlon C, et al. 2003. A systematic review of the antifungal effectiveness and tolerability of amphotericin B formulations. *Clin Ther*, 25:1295–320.

Bochot A, Couvreur P, Fattal E. 2000. Intravitreal administration of antisense oligonucleotides: potential of liposomal delivery. *Prog Retin Eye Res*, 19:131–47.

Bratjburg J, Elberg S, Bolard J, et al. 1984. Interaction of plasma proteins and lipoproteins with amphotericin B. *J Infect Dis*, 149:986–97.

Bratjburg J, Elberg S, Medoff J, et al. 1985. Involvement of oxidative damage in erythrocyte lysis induced by amphotericin B. *Antimicrob Agents Chemother*, 27:172–6.

Brezis M, Rosen P, Silva K. et al. 1984. Polyene toxicity in renal medulla: injury mediated by transport activity. *Science*, 224:66–8.

Brittain HG. 1994. Circular dichroism studies of the self-association of Amphotericin B. *Chirality*, 6:665–9.

Chia JK, McManus EJ. 1990. In vitro tumor necrosis factor induction assay for analysis of febrile toxicity associated with amphotericin B preparations. *Antimicrob Agents Chemother*, 34:906–8.

Clemons KV, Ranney DF, Stevens DA. 2001. A novel heparin-coated hydrophilic preparation of amphotericin B hydrosomes. *Curr Opin Investig Drugs*, 2:480–7.

Clemons KV, Espiritu M, Parmar R, et al. 2005. Comparative efficacies of conventional amphotericin B, liposomal amphotericin B (AmBisome), caspofungin, micafungin, and voriconazole alone and in combination against experimental murine central nervous system aspergillosis. *Antimicrob Agents Chemother*, 49:4867–75.

Conover CD, Zhao H, Longley CB, et al. 2003. Utility of poly(ethylene glycol) conjugation to create prodrugs of amphotericin B. *Bioconjugate Chem*, 14:661–6.

Cornely O. 2005. Ambiload. In: *Amer Soc Hematology*; Atlanta, GA.

Cybulska B, Herve M, Borowski E, et al. 1986. Effects of the polar head structure of polyene macrolide antifungal antibiotics on the mode of permeabilization of ergosterol- and cholsterol-containing lipidic vesicles studied by 31P-NMR. *Mol Pharmacol*, 29:293–8.

Dangi JS, Vyas SP, Dixit VK. 1998. Effect of various lipid-bile salt mixed micelles on the intestinal absorption of amphotericin B in rat. *Drug Devel Ind Pharm*, 24:631–5.

De Kruijff B, Demel RA. 1974. Polyene antibiotic-sterol interactions in membranes of *Acoleplasma laidlawii* cells and lecithin liposomes. 3. Molecular structure of the polyene antibiotic-cholestrol complexes. *Biochim Biophys Acta*, 339:57–70.

Delmas G, Park S, Chen ZW, et al. 2002. Efficacy of orally delivered cochleates containing amphotericin B in a murine model of aspergillosis. *Antimicrob Agents Chemother*, 46:2704–7.

Drew R. 2006. Potential role of aerosolized amphotericin B formulations in the prevention and adjunctive treatment of invasive fungal infections. *Int J Antimicrob Agents*, 27S:S36–44.

Dromer F, Barbet J, Bolard J, et al. 1990. Improvement of amphotericin B activity experimental crytococcosis by incorporation into specific immunoliposomes. *Antimicrob Agents Chemother*, 34:2055–60.

Eggimann P, Garbino J, Pittet D. 2003. Epidemiology of Candida species infections in critically ill non-immunosuppressed patients. *Lancet Infect Dis*, 3:685–702.

Ellis M, Spence D, de Pauw B, et al. 1998. An EORTC international multicenter randomized trial (EORTC number 19923) comparing two dosages of liposomal amphotericin B for treatment of invasive aspergillosis. *Clin Infect Dis*, 27:1406–12.

Egito EST, Appel M, Fessi H, et al. 1996a. In-vitro and in-vivo evaluation of a new amphotericin B emulsion-based delivery system. *J Antimicrob Chemother*, 38:485–97.

Egito EST, Fessi H, Appel M, et al. 1996b. A morphological study of an amphotericin B emulsion-based delivery system. *Int J Pharm*, 145:17–27.

Ehrenfreund-Kleinman T, Azzam T, Falk R, et al. 2002. Synthesis and characterization of novel water soluble amphotericin B-arabinogalactan conjugates. *Biomaterials*, 23:1327–35.

Espuelas MS, Legrand P, Chéron M, et al. 1998a. Interactions of amphotericin B with polymeric colloids. A spectroscopic study. *Colloids Surfaces B: Biointerfaces*, 11:141–51.

Espuelas MS, Legrand P, Chéron M, et al. 1998b. Interactions of amphotericin B with polymeric colloids. 2. Effect of poloxamer on the adsorption of amphotericin B onto poly(ε-caprolactone) nanospheres. *Colloids Surfaces B: Biointerfaces*, 11:203–12.

Espuelas MS, Legrand P, Loiseau PM, et al. 2000. In vitro reversion of amphotericin B resistance in *Leishmania donovani* by poloxamer 188. *Antimicrob Agents Chemother*, 44:2190–2.

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*, 10:593–8.

Espuelas MS, Legrand P, Campanero MA, et al. 2003. Polymeric carriers for amphotericin B: in vitro activity, toxicity and therapeutic efficacy against systemic candidiasis in neutropenic mice. *J Antimicrob Chemother*, 52:419–27.

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 commerical lipid-based formulations. *Int J Pharm*, 267:101–12.

Gaboriau F, Chéron M, Petit C, et al. 1997. Heat-induced superaggregation of amphotericin B reduces its in vitro toxicity: a new way to improve its therapeutic index. *Antimicrob Agents Chemother*, 41:2345–51.

Georgopapadakou NH, Walsh TJ. 1996. Antifungal agents: chemotherapeutic targets and immunologic strategies. *Antimicrob Agents Chemother*, 40:279–91.

Gradoni L, Gramiccia M, Scalone A. 2004 Change in human visceral leishmaniasis treatment in Italy: retrospective study of 630 patients. *Parassitologia*, 46:199–201.

Graybill JR, Craven PC, Taylor RL, et al. 1982. Treatment of murine Cryptococcus with liposome-associated amphotericin B. *J Infect Dis*, 145:748–52.

Grijalba MT, Chéron M, Borowski E, et al. 2006. Modulation of mpolyene antibiotics self-association by ions from the Hofmeister series. *Biochim Biophys Acta*, 1760:973–9.

Guo LSS. 2001. Amphotericin B colloidal dispersion: an improved antifungal therapy. *Adv Drug Deliv Rev*, 47:149–63.

Hartsel S, Bolard J. 1996. Amphotericin B: new life for an old drug. *TIPS*, 17:445–9.

Hartsel SC, Weiland TR. 2003. Amphotericin B binds to amyloid fibrils and delays their formation: a therapeutic mechanism. *Biochemistry*, 42:6228–33.

Heath S, Chance ML, New RR. 1984. Quantitative and ultrastructural studies on the uptake of drug loaded liposomes by mononuclear phagocytes infected with *Leishmania donovani*. *Mol Biochem Parasitol*, 12:49–60.

Herbrecht R, Denning DW, Patterson TF, et al. 2002. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. *N Engl J Med*, 347:408–15.

Herbrecht R, Natarajan-Ame S, Nivoix Y, et al. 2003. The lipid formulations of amphotericin B. *Expert Opin Pharmacother*, 4:1277–87.

Herrmann JL, Dubois N, Fourgeaud M, et al. 1994. Synergic inhibitory activity of amphotericin-B and gamma interferon against intracellular Cryptococcus neoformans in murine macrophages. *J Antimicrob Chemother*, 34:1051–8.

Herwaldt BL. 1999. Leishmaniasis. *Lancet*, 54: 1191–9.

Janoff AS, Perkins WR, Saletan SL, et al. 1993. Amphotericin B lipid complex (ABLC™): a molecular rationale for the attenuation of amphotericin B-related toxicites. *J Liposome Res*, 3:451–71.

Johnson MD, MacDougall C, Ostrosky-Zeichner L, et al. 2004. Combination antifungal therapy. *Antimicrob Agents Chemother*, 48:693–715.

Juster-Reicher A, Flidel-Rimon O, Amitay M, et al. 2003. High-dose liposomal amphotericin B in the therapy of systemic candidiasis in neonates. *Eur J Clin Microbiol Infect Dis*, 22:603–7.

Kassab R, Parrot-Lopez H, Fessi H, et al. 2002. Molecular recognition by *Kluyveromyces* of amphotericin B-loaded, galactose-targeted, poly(lactic acid) microspheres. *Bioorg Med Chem*, 10: 1767–75.

Kayser O, Olbrich C, Yardley V, et al. 2003. Formulation of amphotericin B as nanosuspension for oral administration. *Int J Pharm*, 254:73–5.

Kelsey SM, Goldman JM, McCann S, et al. 1999. Liposomal amphotericin (AmBisome) in the prophylaxis of fungal infections in neutropenic patients: a randomised, double-blind, placebo-controlled study. *Bone Marrow Transplant*, 23:163–8.

Kirkpatrick WR, Coco BJ, Patterson TF. 2006. Sequential or combination antifungal therapy with voriconazole and liposomal amphotericin B in a Guinea pig model of invasive aspergillosis. *Antimicrob Agents Chemother*, 50:1567–9.

Kleinberg M. 2006. What is the current and future status of conventional amphotericin B? *Int J Antimicrob Agents*, 27S:S12–16.

Kontoyiannis DP, Hachem R, Lewis RE, et al. 2003. Efficacy and toxicity of caspofungin in combination with liposomal amphotericin B as primary or salvage treatment of invasive aspergillosis in patients with hematologic malignancies. *Cancer*, 98:292–9.

Larabi M, Legrand P, Appel M, et al. 2001. Reduction of NO synthase expression and TNF alpha production in macrophages by amphotericin B lipid carriers. *Antimicrob Agents Chemother*, 45:553–62.

Larabi M, Yardley V, Loiseau PM, et al. 2003. Toxicity and antileishmanial activity of a new stable lipid suspension of amphotericin B. *Antimicrob Agents Chemother*, 47:3774–9.

Larabi M, Gulik A, Dedieu J-P, et al. 2004a. New lipid formulation of amphotericin B : spectral and microscopic analysis. *Biochim Biophys Acta*, 1664:172–81.

Larabi M, Pages N, Pons F, et al. 2004. Study of the toxicity of a new formulation of amphotericin B. *J Antimicrob Chemother,* 53:81–8.

Leenders AC, Reiss P, Portegies P, et al. 1997. Liposomal amphotericin B (AmBisome) compared with amphotericin B both followed by oral fluconazole in the treatment of AIDS-associated cryptococcal meningitis. *Aids*, 11:1463–71.

Leenders AC, Daenen S, Jansen RL, et al. 1998. Liposomal amphotericin B compared with amphotericin B deoxycholate in the treatment of documented and suspected neutropenia-associated invasive fungal infections. *Br J Haematol*, 103:205–12.

Legrand P, Vertut-Doï 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.

Lincopan N, Mamizuka EM, Carmona-Ribeiro AM, et al. 2005. Low nephrotoxicity of an effective amphotericin B formulation with cationic bilayer fragments. *J Antimicrob Chemother*, 55: 727–34.

Lincopan N, Borelli P, Fock R, et al. 2006. Toxicity of an effective amphotericin B formulation at high cationic lipid to drug molar ratio. *Exp Toxicol Pathol*, 58: 175–83.

Lopez-Berestein G, Mehta R, Hopfer R, et al. 1983. Effects of sterols on the therapeutic efficacy of liposomal amphotericin B in murine candidiasis. *Cancer Drug Deliv*, 1:37–42.

Maertens J, Vrebos M, Boogaerts M. 2001. Assessing risk factors for systemic fungal infections. *Eur J Cancer Care (Engl)*, 10:56–62.

Mangé A, Nishida N, Milhavet O, et al. 2000. Amphotericin B inhibits the generation of the scrapie isoform of the prion protein in infected cultures. *Virology*, 74:3135–40.

Marr KA, Boeckh M, Carter RA, et al. 2004. Combination antifungal therapy for invasive aspergillosis. *Clin Infect Di*s, 39:797–802.

Mazerski J, Bolard J, Borowski E. 1982. Self association of some polyene macrolide antibiotics in aqueous media. *Biochim Biophys Acta*, 719:11–17.

Mehta P, Vinks A, Filipovich A, et al. 2006. High-dose weekly AmBisome antifungal prophylaxis in pediatric patients undergoing hematopoietic stem cell transplantation: a pharmacokinetic study. *Biol Blood Marrow Transplant*, 12:235–40.

Ménez C, Buyse M, Chacun H, et al. 2006a. Modulation of intestinal barrier properties by miltefosine. *Biochem Pharmacol*, 71:486–96.

Ménez C, Buyse M, Besnard M, et al. 2006b. Interaction between miltefosine and amphotericin B: consequences for their activity towards intestinal epithelial cells and *L. donovani* promastigotes in vitro. *Antimicrob Agents Chemother*, 50:3793–800.

Mercadal M, Domingo JC, Petriz JC, et al. 1999. A novel strategy affords high-yield coupling of antibody to extremities of liposomal surface grafted PEG chains. *Biochim Biophys Acta*, 1418:232–8.

Meyerhoff A. 1999. U.S. Food and Drug Administration approval of AmBisome (liposomal amphotericin B) for treatment of visceral leishmaniasis. *Clin Infect Dis*, 28:42-8; discussion 49–51.

Mozaffarian N, Berman JW, Casadevall A. 1997. Enhancement of nitric oxide synthesis by macrophages represents an additional mechanism of action for amphotericin B. *Antimicrob Agents Chemother*, 41:1825–9.

Müller RH, Schmidt S, Buttle I, et al. 2004. SolEmuls® - novel technology for the formulation of i.v. emulsions with poorly soluble drugs. *Int J Pharm*, 269:293–302.

Murray HW. 2001. Clinical and experimental advances in treatment of visceral leishmaniasis. *Antimicrob Agents Chemother*, 45:2185–97.

Murray HW. 2004. Progress in the treatment of a neglected infectious disease: visceral leishmaniasis. *Expert Rev Anti-infect Ther*, 2:279–92.

Nelson KG, Bishop JV, Ryan RO, et al. 2006. Nanodisk-associated amphotericin B clears *Leishmania major* cutaneous infections in susceptible BALB/c mice. *Antimicrob Agents Chemother*, 50:1238–44.

New RR, Chance ML, Heath S. 1981. Antileishmanial activity of amphotericin and other antifungal agents entrapped in liposomes. *J Antimicrob Chemother*, 8:371–81.

Oda MN, Hargreaves PL, Beckstead JA, et al. 2006. Reconstituted high-density lipoprotein enriched with the polyene antibiotic amphotericin B. *J Lipid Res*, 47:260–7, errata p 1114.

Olson JA, Adler-Moore JP, Smith PJ, et al. 2005. Treatment of Candida glabrata infection in immunosuppressed mice by using a combination of liposomal amphotericin B with caspofungin or micafungin. *Antimicrob Agents Chemother*, 49:4895–902.

Otsubo T, Maruyama K, Maesaki S, et al. 1998. Long-circulating immunoliposomal amphotericin B against invasive pulmonary aspergillosis in mice. *Antimicrob Agents Chemother*, 42:40–4.

Pappas PG, Rex JH, Lee J, et al. 2003. A prospective observational study of candidemia: epidemiology, therapy, and influences on mortality in hospitalized adult and pediatric patients. *Clin Infect Dis*, 37:634–43.

Perfect JR, Dodds Ashley E, Drew R. 2004. Design of aerosolized amphotericin B formulations for prophylaxis trials among lung transplant recipients. *Clin Infect Dis*, 39(Suppl 4):S207–10.

Polak A. 1999. The past, present and future of antimycotic combination therapy. *Mycoses*, 42:355–70.

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. *Br J Haematol*, 98:711–18.

Rama Iniguez, S, Dea-Ayuela MA, Sánchez-Brunete JA, et al. 2006. Real-time reverse transcription-PCR quantification of cytokine mRNA expression in golden Syrian hamster infected with Leishmania infantum and treated with a new amphotericin B formulation. *Antimicrob Agents Chemother*, 50:1195–201.

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.

Risovic V, Sachs-Barrable K, Boyd M, et al. 2004. Potential mechanisms by which Peceol increases the gastrointestinal absorption of amphotericin B. *Drug Dev Ind Pharm*, 30:767–74.

Ruijgrok RJ, Fens MH, Bakker-Woudenberg IA, et al. 2005. Nebulization of four commercially available amphotericin B formulations in persistently granulocytopenic rats with invasive pulmonary aspergillosis: evidence of long-term biological activity. *J Pharm Pharmacol,* 57:1289–95.

Sánchez-Brunete JA, Dea MA, Rama S, et al. 2004. Treatment of experimental visceral leishmaniasis with amphotericin B in stable albumin microspheres. *Antimicrob Agents Chemother*, 48:3246–52.

Sánchez-Brunete JA, Dea MA, Rama S, et al. 2005. Influence of the vehicle on the properties and efficacy of microparticles containing amphotericin B. *J Drug Target*, 13:225–33.

Santangelo R, Paderu P, Delmas G, et al. 2000. Efficacy of oral cochleate-amphotericin B in a mouse model of systemic candidiasis. *Antimicrob Agents Chemother*, 44: 2356–60.

Seifert K, Croft SL. 2006. In vitro and in vivo interactions between miltefosine and other antileishmanial drugs. *Antimicrob Agents Chemother*, 50:73–9.

Singh S, Sivakumar R. 2004. Challenges and new discoveries in the treatment of leishmaniasis. *J Infect Chemother*, 10:307–15.

Sokolsky-Papkov M, Domb AJ, Golenser J. 2006. Impact of aldehyde content on amphotericin B-dextran imine conjugate toxicity. *Biomacromolecules*, 7:1529–35.

Sundar S, Mehta H, Suresh AV, et al. 2004. Amphotericin B treatment for Indian visceral leishmaniasis: conventional versus lipid formulations. *Clin Infect Dis*, 38:377–83.

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 Agents Chemother*, 31:421–9.

Taylor RL, Williams DM, Craven PC, et al. 1982. Amphotericin B in liposomes: a novel therapy for histoplasmosis. *Am Rev Respir Dis*, 125:610–11.

Tiyaboonchai W, Limpeanchob N. 2006. Formulation and characterization of amphotericin B-chitosan-dextran sulphate nanoparticles. *Int J Pharm*, doi:10.1016/j.ijpharm.2006.08.013

Tollemar J, Ringden O, Andersson S, et al. 1993a. Randomized double-blind study of liposomal amphotericin B (Ambisome) prophylaxis of invasive fungal infections in bone marrow transplant recipients. *Bone Marrow Transplant*, 12:577–82.

Tollemar J, Ringden O, Andersson S, et al. 1993b. Prophylactic use of liposomal amphotericin B (AmBisome) against fungal infections: a randomized trial in bone marrow transplant recipients. *Transplant Proc*, 25:1495–7.

Tollemar J, Hockerstedt K, Ericzon BG, et al. 1995. Prophylaxis with liposomal amphotericin B (AmBisome) prevents fungal infections in liver transplant recipients: long-term results of a randomized, placebo-controlled trial. *Transplant Proc*, 27:1195–8.

Tokuda Y, Tsuji M, Yamazaki M, et al. 1993. Augmentation of murine tumor necrosis factor production by amphotericin B in vitro and in vivo. *Antimicrob Agents Chemother*, 37:2228–30.

Tomii Y. 2002. Lipid formulation as a drug carrier for drug delivery. *Curr Pharmaceut Design*, 8:467–74.

Utz JP, Treger A, McCullough NB et al. 1958–1959. Amphotericin B: intravenous use in 21 patients with systemic fungal diseases. *Antibiot Annu*, 1958–1959:628–34.

Van Etten EWM, Van Vianen W, Roovers P, et al. 2000. Mild heating of Amphotericin B-desoxycholate: effects on ultrastructure, in vitro activity and toxicity, and therapeutic efficacy in severe candidiasis in leukopenic mice. *Antimicrob Agents Chemother*, 44:1598–603.

Vanderputte J, Wachtel JL, Stiller ET. 1955–1956. Amphotericins A and B, antifungal antibiotics produced by a streptomycete. II The isolation and properties of the crystalline amphotericins. *Antibiot Annu*, 1955–1956:587–91.

Vandermeulen G, Rouxhet L, Arien A, et al. 2006. Encapsulation of amphotericin B in poly(ethylene glycol) -block-poly(e-caprolactone-*co*-trimethylenecarbonate) polymeric micelles. *Int J Pharm*, 309:234–40.

Venier-Julienne MC, Vouldoukis I, Monjour L, et al. 1995. In vitro study of the anti-leishmanial activity of biodegradable nanoparticles. *J Drug Target*, 3:23–9.

Vyas SP, Katare YK, Mishra V, et al. 2000. Ligand directed macrophage targeting of amphotericin B loaded liposomes. *Int J Pharm*, 210:1–14.

Vyas SP, Quraishi S, Gupta S, et al. 2005. Aerosolized liposome-based delivery of amphotericin B to alveolar macrophages. *Int J Pharm*, 296:12–25.

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, Goodman JL, Pappas P, et al. 2001. Safety, tolerance, and pharmacokinetics of high-dose liposomal amphotericin B (AmBisome) in patients infected with Aspergillus species and other filamentous fungi: maximum tolerated dose study. *Antimicrob Agents Chemother*, 45:3487–96.

Wilson E, Thorson L, Speert DP. 1991. Enhancement of macrophage superoxide anion production by amphotericin B. *Antimicrob Agents Chemother*, 35:796–800.

Wingard JR, White MH, Anaissie E, et al. 2000. A randomized, double-blind comparative trial evaluating the safety of liposomal amphotericin B versus amphotericin B lipid complex in the empirical treatment of febrile neutropenia. L Amph/ABLC Collaborative Study Group. *Clin Infect Dis*, 31:1155–63.

Wu W, Wieckowski S, Pastorin G, et al. 2005. Targeted delivery of amphotericin B to cells by using functionalized carbon nanotubes. *Angew Chem Int Ed*, 44:6358–62.

Yardley V, Croft SL. 2000. A comparison of the activities of three amphotericin B lipid formulations against experimental visceral and cutaneous leishmaniasis. *Int J Antimicrob Agents*, 13:243–8.

Yoo BK, Jalil Miah MA, Lee E-S, et al. 2006. Reduced renal toxicity of nanoparticular amphotericin B micelles prepared with partially benzylated poly-L-aspartic acid. *Biol Pharm Bull*, 29:1700–5.

Zarif L. 2005. Drug delivery by lipid cochleates. *Meth Enzymol*, 391:314–29.

Zhang X, Xie J, Li S, et al. 2003. The study on brain targeting of the amphotericin B liposomes. *J Drug Target,* 11:117–22.