Amphotericin B: new life for an old drug

Scott Hartsel and Jacques Bolard

Interest in amphotericin B has undergone a renaissance of sorts over the past few years despite the advent of the newer less-toxic azole antifungal drugs. This is, in part, owing to the unfortunate increase in fungal diseases worldwide. It is also, however, owing to the reduction of toxicity via innovative liposomal delivery systems, better understanding of drug mechanism and distribution and a surprising expansion of the antibiotic spectrum of amphotericin B to include select virus, parasite and possibly prion infections. In this article, **Scott Hartsel and Jacques Bolard** summarize the recent leaps in pharmaceutics, spectrum and molecular mechanistic knowledge of this surprising molecule.

By modern pharmacological standards, the antifungal agent amphotericin B is a very old drug. For nearly 40 years it has been the major antifungal drug for serious infections. Certainly, it rivals the venerable antibacterial penicillin G for its long-lived importance in its therapeutic niche and has proven far more resistant to microbial adaptability. Amphotericin B is still the drug of choice for many serious systemic fungal infections, which owing to AIDS and improved organ transplant immunosuppression drugs are becoming tragically frequent in immune-compromised individuals.

Amphotericin B, an amphipathic polyene macrolide, is a natural product derived from *Streptomyces nodosus*. The usual formulation of amphotericin B, a micellar deoxycholate complex (Fungizone), is highly toxic to patients, often causing decreased renal function, anaphylaxis, chills, high fever, nausea, phlebitis, anorexia and a host of other unpleasant effects. This array of untoward effects coupled with long therapeutic regimes nearly negates its usefulness in all but the most life-threatening systemic fungal infections[1–4].

However, remarkable advances in lipid-associated or liposomal drug delivery formulations have drastically reduced the toxicity of this drug toward humans while retaining its powerful antifungal action. In addition, a host of new and unexpected biological activities of amphotericin B and its derivatives is causing more researchers than ever before to look at this drug with a fresh eye.

The mechanism of action of amphotericin B seems deceptively clear. It produces membrane disruptions in the target cells and the subsequent leakage of ions and small molecules can kill or damage these cells[5]. The reason for its fungal selectivity seems to lie in the greater affinity of amphotericin B for the ergosterol-containir membranes of fungi versus the cholesterol-rich man malian cell membranes. The acute and chronic toxici toward humans, however, is ample evidence that th selectivity is not absolute. While these facts are we substantiated, the detailed molecular mechanisms fo amphotericin B action and toxicity are more comple than previously appreciated.

New delivery systems increase amphotericin B therapeutic index

Table 1 summarizes the properties of some of the mo clinically promising liposomal formulations and the cu rent standard, Fungizone, although over 30 more hav been tested. Many of these have demonstrated good eff cacy against fungi and reduced human toxicity as con pared to Fungizone (Refs 6, 7). Three of these formu lations, AmBisome, Abelcet (ABLC) and Amphoc (ABCD), are being sold in Europe and all will probabl be commercially available in the USA soon (onl Abelcet is, at present, FDA approved). The mech nism(s) for the reduced toxicity, however, are no entirely clear from model system studies.

There are major differences in the physical an pharmaceutical properties of these different liposom preparations (Table 1) and there seems to be no singl feature, even surface charge, which distinguishes th effective from the ineffective. None of these preparatior really conform to the popular conception of liposom delivery systems as encapsulations of drugs. Rathe these formulations are aggregations of drug and lipi with little, if any, amphotericin B entrapped as a solutio inside. Some, e.g. ABCD, do not even contain close vesicles. The reasons for the success of these system must involve the favourable disposition and release c drug. The heterogeneity of the physical states an structures suggests that there is more than one way t accomplish this[8,9].

In vitro studies have shown that, most of the tim amphotericin B in lipid formulations retains all or part c its antifungal activity, whereas its toxicity is greatl reduced or abolished. Two hypotheses have bee formulated for the origin of this increased *in vitro* selec tivity (Fig. 1). According to the first hypothesis, selectiv transfer of the drug occurs to fungal but not to mam malian cells[10,11]. The second hypothesis is based on the notion that only free (unbound to the lipid carrier amphotericin B damages cells[12,13] and that amphoterici B is gradually released from a liposomal formulatio with increased dilution. Fungi, being more sensitive t amphotericin B than mammalian cells, are susceptible at the lower range of total amphotericin B concentrations, a range where amphotericin B is totally dissociated from deoxycholate in Fungizone or from the carrier in the other formulations. In contrast, mammalian cells are affected only by higher free-amphotericin B concentrations, which are never attained with the liposomal formulations, in contrast to Fungizone.

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Table 1. Lipid-associated pharmaceutical preparations of amphotericin B (AmB)

AmB preparation	Composition	Mole ratio	Net charge	Physical state	Size (μm)
Fungizone	DOC/AmB	7:3	Negative	Micelles	<0.4
AmB–lipid complex (ABLC, Abelcet)	DMPC/DMPG/AmB	7:3:3	Negative	Sheets	1.6–11
Ampholiposome	EPC/Chol/SA/AmB	4:3:1:0.5	Positive	Oligolamellar vesicles	0.2–0.3
AmBisome	HSPC/Chol/DSPG/AmB	2:1:0.8:0.4	Negative	Small unilamellar vesicles	0.06
AmB–colloidal dispersion (ABCD, Amphocil)	CS/AmB	1:1	Negative	Discs	0.12

Chol, cholesterol; CS, cholesteryl sulphate; DMPC, dimyristoyl phosphatidylcholine; DMPG, dimyristoyl phosphatidylglycerol; DOC, deoxycholate; DSPG, distearoyl phosphatidylcholine; EPC, egg yolk phosphatidylcholine; HSPC, hydrogenated phosphatidylcholine; SA, stearylamine; SPC, soya phosphatidylcholine.

The fact that, *in vivo*, equally effective amphotericin B preparations may associate almost entirely with serum lipoproteins or be engulfed quickly by macrophages leads one to question the relevance of *in vitro* toxicity–efficacy studies not incorporating these features (the vast majority). In fact, the thermodynamic stability of the formulations also plays a role in the differential binding of amphotericin B from various formulations, to lipoproteins. We shall see below that amphotericin B toxicity may take its origin in the binding of amphotericin B to low density lipoproteins (LDL) and the subsequent internalization of the drug through LDL receptors. As a consequence, more stable amphotericin B-carrier complexes result in smaller amounts of amphotericin B bound to LDL and thus lower toxicity as compared with that of Fungizone. In addition, intact amphotericin B formulations may or may not bind to other lipoproteins. ABLC binds selectively to high density lipoproteins (HDL), whereas ABCD does not bind to lipoproteins. Another important observation is that amphotericin B lipid formulations may be engulfed by macrophages[14] (Fig. 2), which may subsequently release free amphotericin B. Macrophages can, therefore, be considered as amphotericin B reservoirs from which amphotericin B is slowly released. Furthermore, intracellular fungi or parasites present in the macrophages (e.g. *Leishmania* or *Candida*) can be directly attacked. The extent of uptake depends on the formulation: ABLC and AmBisome are taken up strongly, Amphocil poorly.

The extrapolation of the results obtained on animal cells to a whole animal system is not straightforward because the complexity of the system is increased. Added to the role of macrophages and lipoproteins described above is the distribution in the tissues and organs and the role of filter of the sinusoidal endothelium.

A broadening spectrum

While improved delivery systems have dominated amphotericin B research recently, some of the most interesting and surprising recent work has involved expanding the therapeutic role of amphotericin B and its related derivatives to diseases other than fungal infections.

An expansion of the therapeutic role of amphotericin B was noted as early as 1960 (Ref. 15) as a treatment for leishmaniasis, an insect-borne protozoal infection. Since then, numerous reports of the toxicity of amphotericin B towards the leishmanial organism have been published and amphotericin B is used as a second-tier therapeutic agent for *Leishmania brasiliensis* and *L. mexicana*. Amphotericin B may be especially promising for T-cell deficient individuals[16]. It is probable that the selectivity against *Leishmania* is determined by the presence of the ergosterol precursors episterol and 5-dehydroepisterol in their membranes, an unusual situation outside of the fungal kingdom. The recent reclassification of the putative protozoan *Pneumocystis carinii* as a fungus may lead to continued evaluation of amphotericin B for this problematic infection of AIDS patients[17].

Especially exciting have been reports of the antiviral, specifically anti-HIV properties of amphotericin B and its derivatives. Specifically, amphotericin B, amphotericin B methyl ester, a more soluble amphotericin B derivative, MS8209, and the related antibiotic nystatin have been shown to inhibit cellular infection by HIV *in vitro*[18–21]. The mechanism of this unexpected effect is uncertain but it is possible that these derivatives can selectively bind to HIV membranes because of the 2.5-fold higher cholesterol:lipid ratio of HIV virions as compared with the host[22]. More recently, it has been shown that MS8209 blocks virus–host fusion or uptake but does not effect cell–cell fusion[21]. It is further suggested that the effect is exerted, in part, upon a specific viral membrane glycoprotein and may not be membrane channel related[21]. Because many HIV patients undergo antibiotic therapy for persistent fungal infections, this possible action against HIV by amphotericin merits serious consideration and could confer a double benefit, as well as potentially complicating the interpretation of clinical trials of other anti-HIV drugs.

Equally surprising from a mechanistic standpoint are reports of amphotericin B as an antiprion agent. Currently no therapy exists for these enigmatic infective protein agents because they do not seem to have metabolic processes which can be targeted. Amphotericin B and

MS8209 have been shown to significantly delay the onset of the encephalopathy symptoms of scrapie in the hamster and mouse models, but do not prevent eventual neurodegeneration[23,24]. It is difficult to imagine the mechanism by which amphotericin B inhibits this pathogenesis but it is suggested that it may interact directly with the normal PrPC (cellular isoform of prion protein) brain protein and delay its interaction with the scrapie agent or its conversion into PrPSc (scrapie isoform), the pathological protease resistant protein which accumulates in the brain[23]. This accumulation is linked to the spongiform encephalopathy associated with this disease. The effect may be more complicated because even when infected hamsters have similar amounts of PrPSc after eight weeks of infection, amphotericin B-treated animals show no symptoms while untreated animals are already symptomatic[24]. Amphotericin B has been tested for effect on two persons suffering from the most common possible prion disease of humans, Creutzfeldt–Jakob disease, but the results were not promising, possibly owing to the advanced stage of the disease[25].

In addition to the above unexpected effects, amphotericin B may enhance resistance to microbial infections. In 1986 it was noted that amphotericin B enhanced resistance to bacteria (*Listeria* and *Staphylococcus* species) and parasites (*Schistosoma mansoni*) as well as fungi in murine disease models[26]. These results do not indicate direct toxicity but rather immune stimulation as the amphotericin B concentrations were sublethal for these organisms. Conflicting results have hampered research in this area but it now seems that at low amphotericin B concentrations (similar to therapeutic levels) there is a measurable general enhancement of the host resistance to infectious agents (for a review see Ref. 27). Recent comparative work has demonstrated in animal disease models that amphotericin B shows a consistent immune stimulatory response while azole drugs, the other important class of antifungals, show either neutral or immunosuppressive activity[28]. The mechanism of this immunostimulation is uncertain but tumour necrosis factor α production is stimulated in macrophages by amphotericin B in a manner reminiscent of LPS stimulation. This interesting effect is worth further investigation, especially as patients with fungal infections are often susceptible to multiple infectious agents and, as with the anti-HIV effect, a bonus activity would be a boon. In summary, the antimicrobial, antiprion and antiviral effects of amphotericin B are interesting but a full realization of the promise of lower toxicity indicated by liposomal preparations would be necessary before amphotericin B could be considered as a supplement to traditional antibiotics and antiviral agents.

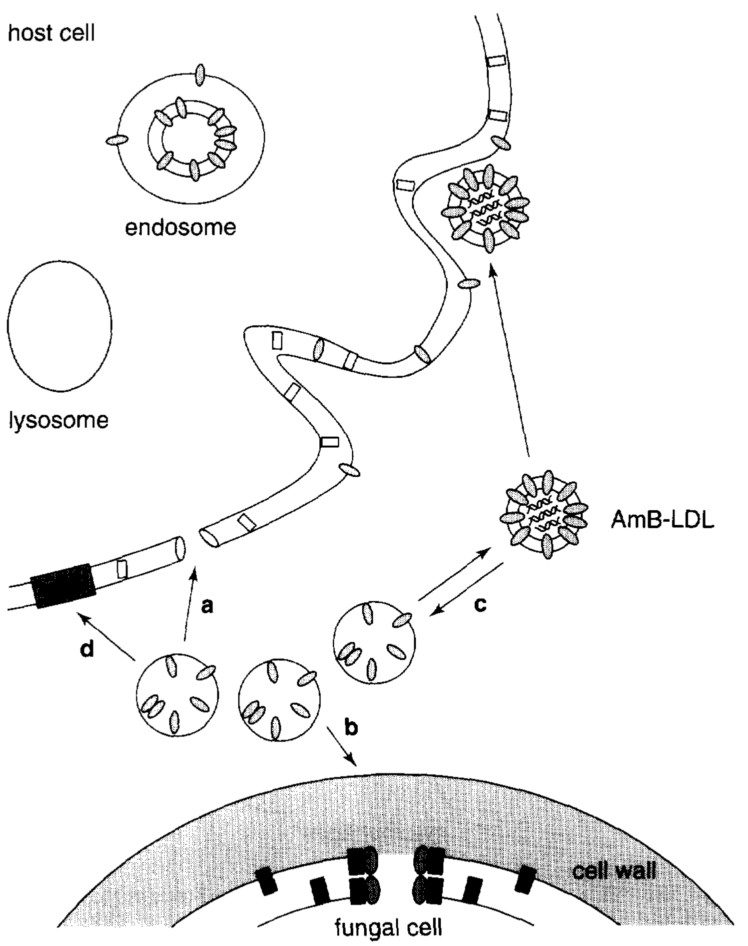

Fig. 1. Mechanisms of anticellular action of amphotericin B (AmB): the formation of transmembrane pores. **a:** With mammalian cells this formation only occurs for AmB concentrations above the threshold of drug self-association, whereas **b:** with fungal cells it may occur much below the threshold. Strong evidence of sterol–AmB complexation is only indicated for ergosterol-containing membranes. Endocytosis through LDL (low density lipoprotein) receptors. **c:** AmB bound to LDL is internalized. **d:** Lipid peroxidation makes membranes more fragile. 0, Amphotericin B; □, cholesterol; ■, ergosterol.

A new look at the mechanism

An elegant and widely reproduced model of amphotericin pores shows amphotericin B molecules inserted in the bilayer, with polar -OH groups in the centre, arranged like barrel staves sandwiched alternately with sterol molecules. This ground-breaking model was already outlined by 1974 when the fluid mosaic model of the membrane was just beginning to be embraced by the scientific community[29–31]. Today, this barrel stave structure is a paradigm for multiple subunit ion channel models and has served as the inspiration for a recent cottage industry of synthetic organic channel analogues. However, it seems now that amphotericin B has multiple cell disrupting structures and mechanisms, some of which may be as important as this well-known sterol pore mechanism[32]. Studies on model membranes have shown that, contrary to popular belief, sterols are not even necessary for channel formation and amphotericin B may interact specifically with phospholipids to induce non-bilayer or interdigitated lipid phases and defects associated with channel formation[33]. The importance of these structures and specific lipid interactions to antifungal activity and selectivity is unknown. The type of amphotericin B channel formed, or even whether one forms at all, has been shown to be critically dependent

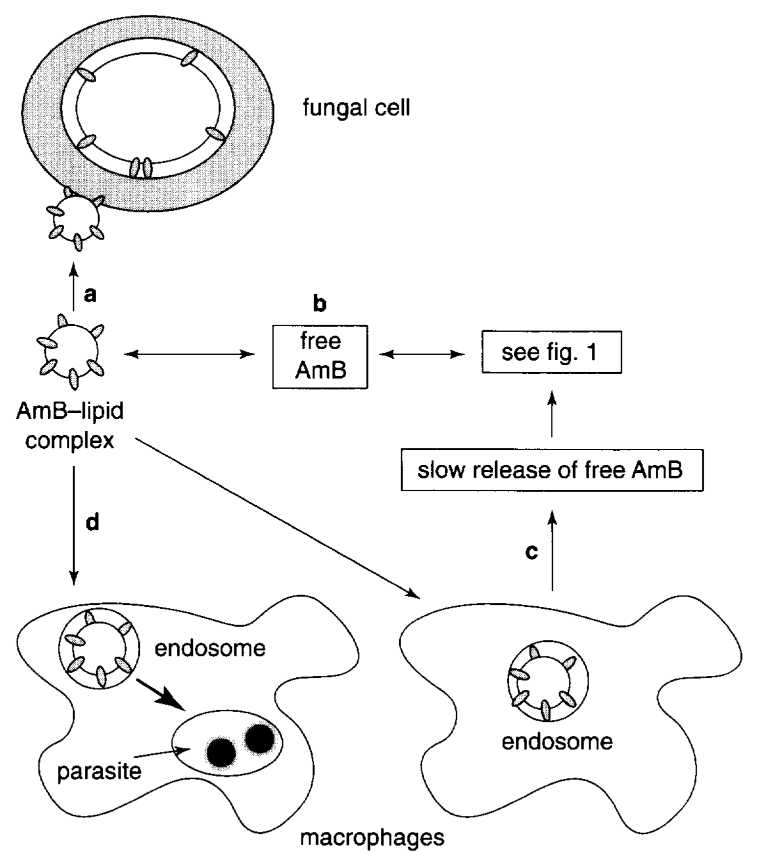

Fig. 2. Mechanisms of action of liposomal amphotericin B (AmB). AmB-containing formulations may **a:** adhere to fungal cells, or **b:** release free AmB upon dilution. They also may be internalized by macrophages. In the latter case AmB can be **c:** released progressively from them, or **d:** directly kill parasites inside them. Note, however, that if it is generally true that AmB inhibits endosome–lysosome fusion, then AmB must be transferred to phagolysosomes containing parasites by a mechanism other than fusion. It may be that, at a suitable concentration, AmB can be internalized and kill the parasite but not block the fusion. 0, Amphotericin B.

upon the antibiotic concentration[34] and the level of solution self-association[35–37], associations with other molecules and numerous additional factors unrelated to the target cell membrane composition. It may even be that amphotericin B-induced oxidative damage to cells is as important a factor in its toxicity as channel formation[38].

Most studies have neglected the fact that amphotericin B is administered intravenously and binds to serum components, in particular lipoproteins[39,40]. Under these conditions the amount of free amphotericin B in plasma available to form transmembrane channels is small. It was recently shown that, in the presence of serum, the interaction of amphotericin B with host-cells is not limited to plasma membrane binding: internalization of the drug in CHO cells occurs by endocytosis, possibly resulting in the blockade of the fusion between endosomes and lysosomes (Fig. 2)[41]. LDL receptors are thought to play a role in this uptake. This *in vitro* mechanism for the toxicity of amphotericin B, implying binding of the drug to LDL and LDL receptors, could explain two *in vivo* observations: (1) if amphotericin B/LDL is administered to rabbits, toxicity is increased[42]; and (2) a correlation is observed between the inhibition of the amphotericin B–lipoprotein interaction and the decreased toxicity for mice of amphotericin B bound to surfactants[43].

Because the vast majority of the studies upon amphotericin B toxicity have been performed on erythrocytes with no LDL receptors, or on mammalian cells with LDL receptors but in the absence of serum, the only mechanisms that could be demonstrated were mechanisms affecting the plasma membrane permeability. Intracellular toxicity of the drug resulting from its internalization through LDL receptors after binding to serum lipoproteins has been ignored. Consideration of this mechanism opens totally new directions for the design of amphotericin B derivatives or lipid formulations[44] focusing, for instance, on decreasing the drug binding to LDL, as compared with HDL.

Concluding remarks and future challenges

Notwithstanding its age, amphotericin B still has plenty of life. Basic research is correlating the role of self-association, lipid association, distribution in serum, uptake by macrophages and tissues, and mechanism with the efficacy and toxicity of this drug. Several different liposomal formulations have drastically improved the therapeutic index, though head-to-head comparative studies among these have yet to be done. Expanding and exploring the use of amphotericin B as a new potential antiviral, antiprion or antiparasitic agent now seems more realistic owing to this lower toxicity.

Despite these successes, it should be emphasized that liposomal systems are not the sole hope for increasing the therapeutic index of amphotericin B. Numerous chemical derivatives deserve additional attention[45]. A simple derivative, amphotericin B methyl ester, has a much higher therapeutic index than amphotericin B (Ref. 46). This drug initially developed a reputation as a neurotoxin[47] but subsequent studies suggest that this is an undeserved stigma[48] and that this drug or analogues are due for a serious re-evaluation. Some other derivatives, e.g. MS8209 (Ref. 21) and *N*-fructosyl-AME (*N*-methyl-*N*-D-fructo-pyranosyl amphotericin B methyl ester) (Ref. 49), have recently shown promising results. Another 'low tech' alternative to current liposomal preparations is the dosing of Fungizone in conjunction with Intralipid, a parenteral lipid nutritional supplement[50]. However, recent clinical studies have shown that while this change in delivery strategy can reduce immediate toxicity it does not change the maximum tolerated dose or long-term toxicity[51].

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Chemical name

MS8209: 1-deoxy-1-amino-4,6-*O*-benzylidene-D-fructosyl-amphotericin B

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