

Carrier Effects on Biological Activity of Amphotericin B

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INTRODUCTION

Disseminated fungal infections are on the increase (58), and the therapeutic difficulties encountered in their management have prompted investigators and clinicians to be more creative in the development of antifungal drugs. Contributing to this increase in fungal infections is the increased number of patients with AIDS, many of whom are surviving longer with supportive therapy, and increased numbers of transplant patients as well as cancer patients undergoing aggressive chemotherapy (57). Amphotericin B (AmB) (Fig. 1) has been used in the therapy of fungal infections for more than 30 years, and, despite the development of new antifungal drugs, it remains the drug of choice for the treatment of severe systemic fungal disease (70). AmB is also well established as an alternative drug in the treatment of visceral leishmaniasis (11, 54).

The major drawback to the use of AmB is its insolubility in water. To become biologically active, it must be solubilized in an aqueous milieu, and the manner in which that is done, as well as the route by which it is administered to humans or experimental animals, determines its effectiveness (17). The formulation licensed for routine clinical use, which is produced and marketed by Bristol-Myers Squibb, Co., as Fungizone, is a mixture of AmB with a detergent, deoxycholate, in a phosphate buffer. Under these conditions, AmB forms a colloidal dispersion suitable for intravenous administration.

Fungizone has the broadest spectrum of activity of any available antifungal agent and is fungicidal when tested in vitro. Therapeutic doses of Fungizone, however, often cause severe side effects, including pain at the site of injection, fevers, chills, nausea, vomiting, electrolyte abnormalities, and nephrotoxicity (44). The harmful side effects increase with increasing dosage; therefore, the amount of AmB that can be administered safely is limited. Dosage is an even greater limiting factor in the treatment of immunocompromised patients, including those with AIDS or cancer patients undergoing intensive cytoreductive therapy (96). To compound the difficulties, some of these patients are infected with fungal strains somewhat resistant to AmB (157).

Several strategies, including modification of the AmB molecule and changes in delivery systems, have been used to improve the therapeutic effectiveness of AmB and reduce its toxicity. For example, semisynthetic derivatives prepared by substitution of one or both of the functional amino or carboxyl groups were attempted. Of these derivatives, the methyl ester of AmB has been investigated most extensively (163), but other compounds are under study as well (166; see references 22 and 168 for reviews). Several derivatives of AmB which were modified solely at the C-13 hemiketal position have been prepared (182). Modifications in delivery have involved the administration of AmB in combination with other antifungal drugs in attempts to identify synergistic interactions and have also involved the delivery of AmB directly to the target organ by aerosolization or intranasal deposition (reviewed in reference 170). Perhaps the most promising approach has involved modification of the physical state of AmB, and the first reports of greater efficacy of liposomal AmB compared with Fungizone appeared more than a decade ago (128, 148). Since then, numerous lipid formulations of AmB, which, in general, have all had improved therapeutic value with less toxicity than the parent compound, have been developed and studied.

Three novel delivery systems have progressed from the laboratory to advanced clinical trials and are commercially available (reviewed in references 12, 24, 57, 69, 71, 89, and 140). All three formulations are expensive, and their relative efficacy is unknown since they have never been compared in a systematic

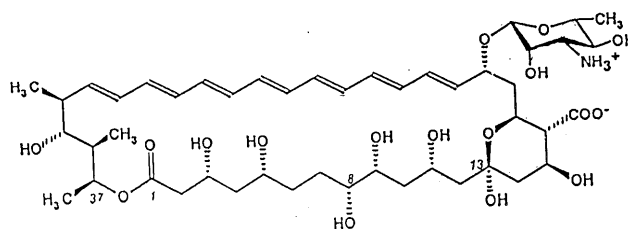


FIG. 1. Structure of AmB.

manner. In vitro investigations designed to correlate the physical characteristics of the various formulations with their biological activity have resulted in some correlations (reviewed in reference 101), but the cellular basis of therapeutic effectiveness has not been determined.

In this review, we present current concepts of the molecular basis of the biological activity of lipid-based AmB formulations with the express purpose of addressing the issues surrounding the relationship of carrier effects to efficacy in vitro and in vivo and the role played by various animal models in comparisons of Fungizone with lipid-associated AmB in vivo. Finally, data gathered from recent clinical studies are reviewed.

THERAPEUTIC MECHANISMS OF AmB

Binding of AmB to Cell Membranes and Cell Damage

AmB is more damaging to fungal than mammalian cells, but the reason(s) for this selective activity is unknown. To broach the subject, we must ask the following questions. (i) How does AmB mediate the damage to cells? (ii) Why is AmB more active against fungal than mammalian cells? (iii) How can the damaging effects of AmB be made more selective for fungal cells?

The sites of potential attack of AmB on fungal and mammalian cells are illustrated in Fig. 2. In both instances, the most often considered attack site is the cell membrane. To hypothesize about the interaction of AmB with the cell membrane, one must first understand the nature of Fungizone when added to an aqueous solution. In water, the AmB partly dissociates from the deoxycholate and the "free" AmB forms a mixture of water-soluble monomers and oligomers with insoluble aggregates of various aggregation states (122). A similar mixture is also found when AmB is dissolved in an organic solvent such as dimethyl sulfoxide or dimethylformamide before addition of water. The proportions of these different chemical species and their state of aggregation depend upon many factors, including the method of mixing, the temperature of mixing, and, most importantly, the concentration of AmB. The various chemical species may interact with membrane sterol in different ways to evoke changes in membrane permeability (28, 122). The membrane changes then lead to cellular dysfunction and eventually to cell destruction and death. It is possible that fungal and *Leishmania* spp. cells are more susceptible to the monomeric form of AmB than are host cells.

The greater potential for AmB to damage fungal and parasitic cells than mammalian cells probably relates to the type of sterol incorporated into their membranes. Ergosterol is found in fungal cell membranes, ergosterol or ergosterol precursors are found in parasitic cells, and cholesterol is found in mammalian cells. The interaction of AmB with membranes containing different sterols is described in more detail in the section on molecular aspects of biological activity, below. Included in Fig. 2 is an illustration of the inhibitory function of AmB on mem-

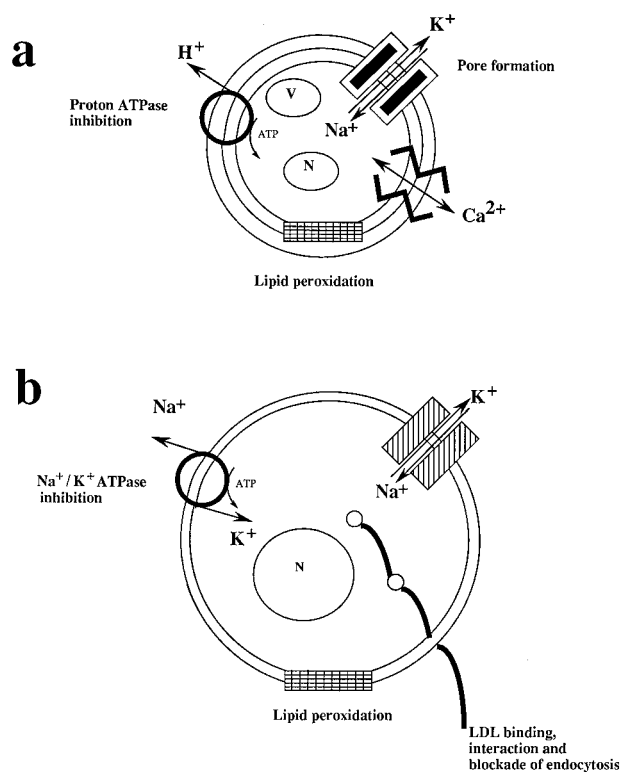


FIG. 2. Possible sites of AmB attack against fungal cells (a) and mammalian cells (b).

brane enzymes: proton ATPase in fungal cells (176) and Na^+ / K^+ -ATPase in mammalian cells (192). Inhibiting these enzymes would deplete cellular energy reserves and reduce proliferative ability (169).

AmB-induced lipid peroxidation of cell membranes, resulting in a corresponding increase in fragility, has been proposed as a possible mechanism by which membrane permeability changes occur as well (33). AmB-induced leakage of Ca^{2+} has been demonstrated in ergosterol-containing vesicles (161), but a cause-and-effect relationship between this and fungicidal activity has not been shown.

Binding of AmB to Lipoproteins and Its Internalization

To fully understand the mechanism of AmB-induced toxicity, one must also consider the binding of AmB to lipoproteins and internalization of the AmB-lipoprotein complex. It was shown recently that internalization of the drug into Chinese hamster ovary (CHO) cells in the presence of serum occurred by endocytosis. As a consequence, the possibility exists that fusion between endosomes and lysosomes was blocked (193). Low-density lipoprotein (LDL) receptors may mediate attachment and ingestion, since AmB-LDL complexes were processed in human fibroblasts by the LDL receptor pathway to the same extent as was LDL alone, i.e., when it was not complexed to AmB (125). Furthermore, AmB associated with LDL remains toxic for LLCPK1 renal cells, but removing the high-affinity LDL receptors from these cells decreases toxicity (199).

Investigators performing studies in vitro have often ignored the fact that AmB binds to serum lipoproteins (30, 197), making the amount of free AmB in plasma much smaller than would be expected from the dose injected and greatly decreasing the initiation of membrane permeability, as shown by mea-

surements of K^+ release from mammalian cells in the presence or absence of serum (193). On the other hand, most of the in vitro studies have been performed with erythrocytes or with mammalian cells with LDL receptors but in the absence of serum. Under these conditions, the only mechanisms that would be detected are those affecting plasma membrane permeability.

These in vitro observations of the toxicity of AmB, which suggest binding of the drug to LDL and LDL receptors, could explain two in vivo observations, namely, the increase in the toxicity of AmB-LDL complexes administered to rabbits (114) and the inhibition of the AmB-LDL interaction in conjunction with decreased toxicity in mice if AmB was bound to surfactants before administration (14). Recognition of the fact that the binding of AmB to LDL and its subsequent internalization modulate toxicity should serve as the impetus for the design of AmB derivatives and lipid formulations with decreased AmB-LDL binding.

Immunostimulatory Effects

The immunomodulatory effects of AmB have been recognized for many years, and its immunomodulatory activity, along with that of other antifungal drugs, was reviewed recently (202). Although there are often conflicting data, it would appear that AmB is immunostimulatory predominantly within an appropriate dose range. For example, AmB enhances the immune response in most common inbred mouse strains (reviewed in reference 23). Since AmB is known to stimulate the immune system under the appropriate conditions, it has even been suggested that AmB might be used prophylactically against fungal infections (189). It has been shown repeatedly that in vitro, AmB has stimulatory effects on fungal and mammalian cells, including cells of the immune system (reference 37 and references therein). In contrast, it has been reported that AmB suppressed both humoral and cell-mediated immunity, as well as macrophage activation. The simplest explanation for these contradictory results is that a dose-dependent toxicity for mammalian cells precludes the demonstration of any immunostimulatory effects.

DESCRIPTION OF THE DELIVERY SYSTEMS OF AmB

The preceding discussion suggests 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 are described in the next sections. They involve the preparation of AmB-lipid associations.

AmB-Lipid Formulations

The lipid formulations of AmB are prepared with either phospholipids or detergents, and the resultant associations fall roughly into two categories, liposomes and mixed micelles, respectively. Liposomes consist of one or more concentric phospholipid bilayers separated by aqueous compartments. The properties of liposomes differ depending on the composition of the lipids constituting the membrane. Micelles, on the other hand, are colloid particles formed by an aggregation of detergent molecules. Liposomal structure is dependent upon the appropriate ratio of AmB to phospholipids and the structures can break down in the presence of high ratios of AmB to phospholipids. In liposomes, AmB is actually inserted into the

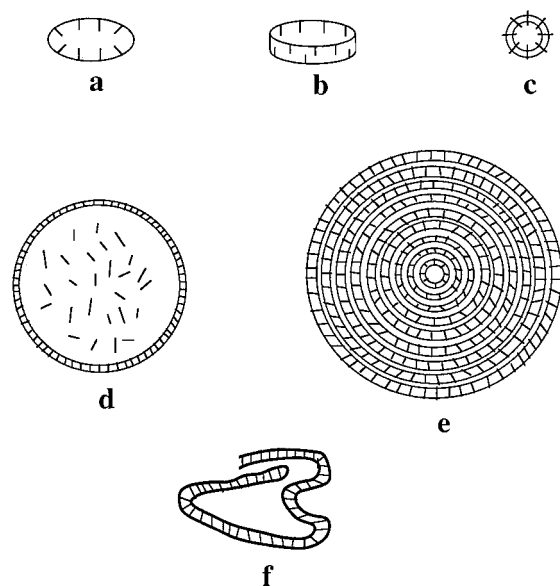


FIG. 3. Schematic representation of different lipid formulations of AmB. (a) Fungizone (deoxycholate-AmB mixed micelles); (b) ABCD or Amphocil (cholesteryl sulfate-AmB mixed micelles); (c) AmBisome (AmB-containing SUV); (d) AmB-containing LUV; (e) L-AmpB (AmB-containing multilamellar vesicles); (f) ABLC, ribbon-like formations (DMPC-DMPG-AmB, 4.6:2.3:3). Symbols: —, AmB molecule; O, membranes of various lipid compositions.

lipid bilayer of the structure; it is not solubilized in the aqueous chamber of the liposome. Fungizone, an AmB-deoxycholate complex (deoxycholate being an ionic surfactant), was the first micellar preparation formulated. It became commercially available in 1958. Since that time, formulations with nonionic surfactant esters of sucrose and polyoxyethylene glycol have been prepared and studied.

Data derived from investigations of the first liposomal formulations of AmB designed for therapy were published by

New et al. in 1981 (148). They prepared liposomes by sonication of a mixture of AmB and various lecithins and then tested them in a leishmanial model. Their study was followed by numerous others in which liposomal preparations containing various phospholipids and sterols were investigated. Liposomes currently under study are prepared by shaking mixtures by hand or by sonication with a tungsten probe or in a bath sonicator or by a mixture of hand shaking and sonication. AmB can be added before or after the sonication step. A number of different formulations have been developed, but studies of their activity have been limited to in vitro observations (see reference 181 for a review up to 1992).

Schematic lipid formulations are illustrated in Fig. 3, and selected characteristics of lipid formulations that have been studied thoroughly and are in clinical trials are summarized in Table 1. Four lipid-based preparations, namely, liposomal AmB with multilamellar or unilamellar vesicles, AmB colloidal dispersion (ABCD), AmB-cholesteryl sulfate complex, and AmB lipid complex (ABLC), have been studied. We have been studying mixed micelles containing egg yolk phosphatidylcholine (EPC) and deoxycholate or glycocholate, termed Edam and Egam, respectively (31, 35). Emulsions of soya oil and EPC which decrease the in vitro toxicity of AmB have also been prepared (112) and are being tested in clinical trials as described below (41, 147).

Nature of the AmB-Lipid Bond

AmB-detergents. The interactions of AmB with detergents, in particular that of AmB with deoxycholate as found in Fungizone, have been well characterized by electron spin resonance and quasi-elastic light scattering (118) and light scattering and electronic absorption (36, 178). Fungizone has an AmB-to-deoxycholate ratio of 1:2. In early studies (118), AmB-deoxycholate systems appeared to consist of aggregates of AmB-deoxycholate mixed micelles coexisting with pure deoxycholate micelles. The AmB-deoxycholate system was not in true equilibrium under any of the conditions studied. Dilution led to disappearance of the deoxycholate micelles and contin-

TABLE 1. Characteristics of some lipid formulations under clinical trial^a

AmB preparation	Composition (mol%), charge of phospholipids ^b	Shape and diam (μm)	Bioavailability compared with Fungizone	Clinical trial references
Fungizone	DOC-AmB (7:3), negative	Micelles, <0.4		
Liposomes (L-AmpB5, L-AmpB10)	DMPC-DMPG-AmB (7:3:0.5, 7:3:1), negative	Multilamellar vesicles + sheets, 1–6	Lower	63, 126–128, 132, 159
AmB-lipid complex (ABLC, Abelcet)	DMPC-DMPG-AmB (7:3:3), negative	Sheets, 1.6–11	Lower	57, 69, 99
Ampholiposomes	EPC-CHOL-SA-AmB (4:3:1:0.5), positive	Oligolamellar vesicles, 0.2–0.3	Greater	141, 171
AmBisome	HSPC-CHOL-DSPG-AmB (2:1:0.8:0.4), negative	Small unilamellar vesicles, 0.06	Greater	3, 57
L-AmpB	SPC-CHOL-AmB (7:3:1), neutral	Small unilamellar vesicles	Equal	75, 76
AmB-colloidal dispersion (ABCD, Amphocil)	CS-AmB (1:1), negative	Discs, 0.12	Lower	57, 68, 85, 173

^a Adapted from reference 24 with permission of the publisher.

^b Abbreviations: DOC, deoxycholate; CHOL, cholesterol; SA, stearylamine; HSPC, hydrogenated phosphatidylcholine; DSPG, distearoyl phosphatidylglycerol; CS, cholesteryl sulfate.

uous loss of deoxycholate from the AmB-deoxycholate aggregates; these events were accompanied by an increase in size and a decrease in solubility of the aggregates. The rate of aggregation was increased by 3 orders of magnitude when the deoxycholate concentration was reduced from 20 to 1 mM.

In more recent studies (36, 178), it was shown that if the deoxycholate concentration was subcritical for micellar formation, mixed micelles with AmB were formed first as a result of the penetration of the deoxycholate molecules into the AmB micelles. This is the case with Fungizone, as was described above. At higher concentrations of the surfactant molecules, the micellar structure was completely disrupted and AmB is found as monomers bound to deoxycholate. When the concentration of the surfactant was increased even further, micelles of the surfactant molecules built up. Two other nonionic surfactants, laurylsucrose (178) and MYRJ 59, a polyoxyethylene glycol derivative of stearic acid (180), induced similar results.

AmB-phospholipids. The nature of the AmB-phospholipid complex varies considerably with the liposomal system under consideration. Thus, there is no unique bond between AmB and the vectors (21, 111). For example, not only does entrapment of the drug result in "encapsulation," i.e., incorporation into the aqueous internal compartment, but also, since AmB is strongly hydrophobic, it binds directly to the lipid bilayer as well. With small unilamellar vesicles (SUV), the latter possibility is predominant since their aqueous compartment is relatively small compared with the lipid volume. In contrast, in multilamellar vesicles (MLV), a significant amount of AmB can be encapsulated in the aqueous compartments between the different constituent leaflets. Large unilamellar vesicles (LUV), on the other hand, have physical structures intermediate between those of SUV and MLV. A second variable adding to the complexity of the interaction between AmB and phospholipids is that AmB changes its conformation upon binding to liposomes. The conformation of the bound species depends on the curvature of the vesicles and is therefore not the same for SUV and LUV (26). Furthermore, the conformation of AmB varies when the drug is in the presence of saturated (dimyristoyl phosphatidylcholine [DMPC], dipalmitoyl phosphatidylcholine [DPPC], etc.) versus unsaturated (EPC, soya phosphatidylcholine [SPC], etc.) phospholipids and when the temperature is altered to allow phase transition. The AmB-to-lipid ratio is also important. New spectra were observed when sterols were incorporated into bilayers. Circular dichroism enabled the complexity of these interactions to be deciphered.

Incorporation of AmB into liposomes may modify the structure of the lipids themselves. At low AmB-to-phospholipid ratios (<1:10), sterol-free DPPC and DMPC SUV fuse or aggregate. The properties of DMPC-dimyristoyl phosphatidylglycerol (DMPG) MLV have also been studied carefully by differential scanning calorimetry, freeze-fracture electron microscopy, electron spin resonance, and circular dichroism. Hand-shaken MLV composed of DMPC-DMPG (7:3) containing 0 to 25 mol% AmB are predominantly bilayer in nature, although considerable disruption was observed at high AmB concentrations (78, 86, 104). There is a tendency toward drug-lipid separation, which leads to AmB self-association and separation into a rigid phase within the membrane, but the properties of the lipid matrix are almost unmodified. A 1:1 stoichiometry of AmB to phospholipid has been proposed in the domain of relative enrichment (60). Some liposome heterogeneity is observed. These preparations seem thermodynamically stable, in contrast to preparations of the same composition prepared in a bath-type sonicator. With formulations prepared in the bath-type sonicator, predominantly ribbon-like

structures form for AmB molar ratios of 5 to 25 mol%. These ribbon-like structures are actually collapsed and aggregated membranes, existing as interdigitated bilayers (98, 154). At lower AmB ratios, the formulations contain intact liposomes in which AmB, probably located at the membrane interface, is in monomeric form, similar to that seen in the presence of DMPC SUV or LUV (25, 135, 142). The AmB-to-phospholipid ratio in the ribbon-like structures prepared by sonication, as well as in the hand-shaken preparations, is thought to be 1:1.

Thermodynamic Stability

It is assumed that the lipid portion of the liposomes is not therapeutically active. The AmB molecules transferred from the liposomes to cells through the aqueous medium or by direct contact of the liposomes with cell membranes induce K^+ leakage and subsequent harmful events. Therefore, the thermodynamic (stability versus instability) and kinetic parameters of the AmB-lipid complex are the essential characteristics that must be determined. The basic characteristics of the complex will determine the conditions for the release of AmB from the vector when diluted in plasma, the ability of the complexes to bind to lipoproteins, and the possible dissociation of the complexes in phagolysosomes of macrophages or lysosomes of other mammalian cells. Unfortunately, most investigators have focused only on the physical state of the AmB or the lipid in stock solutions, which are at concentrations 100- to 1,000-fold higher than those achievable in plasma. The techniques used to analyze AmB binding to lipid include centrifugation on sucrose gradients, electronic absorption, circular dichroism, and energy transfer to a fluorescent probe.

A systematic analysis of the amount of AmB bound to phospholipids to determine various AmB-to-phospholipid ratios or AmB concentrations has been done only with SUV under conditions where AmB was added to the mixture after the liposomes had formed (106, 108). A pilot study with LUV was also performed (142). The ratio between bound AmB and total AmB depended upon the nature of the lipids. At an AmB concentration around $\leq 10^{-7}$ M, all of the AmB is bound, but as the concentration of AmB increases, unbound AmB can be detected. The length of the fatty acid chains on the phospholipids, their degree of saturation, and the physical state of the membranes (gel or liquid crystalline) are also important parameters. Ratios of AmB to phospholipid approaching 1:1 are obtained with EPC and DPPC SUV, whereas less desirable ratios are obtained with DMPC, DSPC, and DBPC SUV. It is important to note that cholesterol incorporation into the SUV bilayers strongly decreases AmB binding (106, 174). This result, however, was demonstrated only with SUV at high AmB-to-lipid ratios (>10:2), and not with LUV.

The concentration of AmB in humans is generally lower than 5 μ M. It is at this concentration or below, therefore, that the thermodynamic stability of the lipid-based formulations should be tested. The amount of free AmB released from the formulations is easily determined by UV-visible absorption spectrophotometry. Below 1 μ M, free AmB is predominantly monomeric, with a specific absorption at 409 nm. In Fig. 4, percentages of free AmB released by some formulations when diluted to 0.5 μ M and then incubated for 1 h at 37°C are shown (124). The AmB in Fungizone is totally uncomplexed under these conditions, whereas the AmB in AmBisome, as well as that in Egam and Edam (data not shown), appears to remain bound to the lipid components.

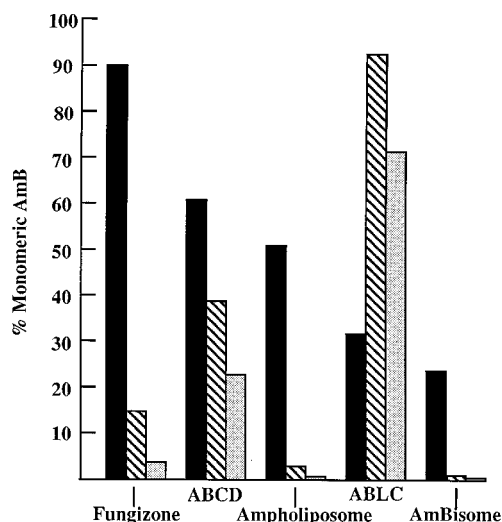


FIG. 4. Percent monomeric AmB released from various formulations upon dilution of an AmB preparation to 0.5 μM in phosphate-buffered saline and incubation for 1 h at 37°C (■); AmB taken up by J774 cells when incubated with a 10 μM AmB preparation and incubated for 1 h at 37°C (▨) or released from J774 cells 1 h after a 2-h incubation with a 10 μM AmB preparation (▩).

Chemical Stability

The importance of determining and maintaining the chemical stability of AmB cannot be overstated. The chemical stability of AmB can be determined in two ways, i.e., by determining its bioactivity and by measuring its concentration by high-performance liquid chromatography (HPLC). Fungizone, assayed for bioactivity, was rapidly degraded in water at 80°C or in synthetic liquid growth medium at 37°C (15). Similarly, it was inactive after being stored in water at 2°C for 48 h in the dark. In contrast, storage at 4°C in 5% glucose protected it from degradation, as measured by HPLC (112, 121, 143). It was stable when solubilized in serum as well but not when dissolved in dimethyl sulfoxide. In the latter instance, >60% of the drug was lost after 6 months (61). HPLC detection of AmB is problematic, however, because it is not known to what extent the methodology permits the detection of a chemical modification of the drug affecting not the polyene part of the molecule but, for instance, the lactone-mycosamine bond.

The binding of AmB to cholesterol in lipoproteins protected it from the loss of anti-*Candida* activity (30). Similarly, the incorporation of AmB into the newer lipid formulations has resulted in better chemical stability. AmB entrapped into liposomes prepared by sonication (ampholiposomes) was stable for 1 year at 4 or 20°C (90). The incorporation of AmB in egg lecithin-bile salt mixed micelles protected its anti-*Candida* activity when the drug was exposed to light (31). The possible effect of the degradation of AmB on its cellular activities is discussed below. The relevance of carrier effects of AmB stability observed *in vitro* to *in vivo* toxic and therapeutic activities is not clear, however.

MOLECULAR ASPECTS OF BIOLOGICAL ACTIVITY

The interaction of AmB with model membranes leads to their increased permeability to monovalent cations. As indicated above, this perturbation, which has been studied to a much greater extent than the internalization of the drug into cells, is often considered to be the primary anticellular activity of the antibiotic. The leakage of cations is generally assumed to

result from the formation of transmembrane AmB channels (21). Details of the mechanism of channel formation have been provided by recent studies, and three models based on these details have been proposed (88).

Model A is based on the fact that the interaction of AmB with sterols in membranes has been demonstrated with ergosterol but not with cholesterol. Toxicity for ergosterol-containing membranes has been observed with low concentrations of AmB, concentrations at which the AmB is completely monomeric. Monomeric AmB would be able to associate with ergosterol in the membrane, and this would then lead to channel formation. On the other hand, it has been shown that AmB induces leakage of K^+ through cholesterol-containing membranes only beyond a certain threshold of concentration, which corresponds to the formation of self-associated water-soluble species of AmB (28, 122). Oligomers are formed by head-to-tail association, which results in complexes sufficiently long to span the membrane and induce toxicity (28, 122). The association of monomers with ergosterol, as would occur in fungal cells, is illustrated in Fig. 5a. The model based on dimer formation and the ability of the dimer to span the cholesterol-containing mammalian membrane is shown in Fig. 5b. Since the degree of aggregation of AmB affects its interactions with membranes and since the carrier affects the relative proportions of monomeric and aggregated forms, the carrier is critical for determining whether ergosterol- or cholesterol-containing membranes will be affected the most.

Model B involves the formation across the cell membrane of aqueous or nonaqueous channels, whose relative preponderance depends on time and AmB concentration. The nonaqueous channels, with a predominantly ionic channel characteristic and modeled as preexisting or "prepore" aggregates, insert from solution and are short-lived, at least in the presence of ergosterol. These channels form readily above an AmB concentration of 0.5 μM , whereas predominantly aqueous channels form below that concentration. Nonaqueous channels may convert to aqueous channels over time. The aqueous channels permit relatively uncontrolled flow of water, urea, and glucose (50, 51). Thus, dependence on the time and stage of the effect are important concepts in this model. Differences related to the presence of cholesterol or ergosterol may lie in the greater ease with which aqueous channels form in ergosterol-containing membranes. Changing the aggregation state of AmB by altering the carrier would significantly alter the selectivity of AmB against cells.

A third model (Fig. 5c) is based on destruction of the integrity of the membrane by insertion of monomers, dimers, tetramers, or other preformed AmB aggregates (possibly in association with sterol) into the membrane bilayer to form membrane-spanning ion-conducting defects or nonbilayer phases (88). These changes would be similar to, but perhaps less drastic than, those caused by a typical amphiphilic detergent. The channels might also evolve with time as the AmB-AmB or AmB-lipid and sterol interactions develop.

Regardless of the model, the incorporation of AmB into lipid formulations decreases the amount of free AmB in solution, a desirable effect if selectivity is the goal. It is generally assumed that lipid formulations of AmB are not directly active on membranes, unlike free AmB. A general decrease in the activity of lipid-associated AmB over AmB alone was shown by measuring the AmB-induced leakage of K^+ from cholesterol- or ergosterol-containing EPC vesicles when AmB prepared in dimethyl sulfoxide was compared with AmB-DPPC SUV (190). AmB alone induced a higher degree of K^+ leakage from both types of vesicles, whereas the lipid formulation of AmB induced more leakage from the ergosterol-containing vesicles.

A similar difference in selectivity was observed with ergosterol- or cholesterol-containing *Mycoplasma* cells (191). Since the affinity of AmB to DPPC vesicles is greater than the affinity to EPC vesicles (106), these data support a role for stability of the complex in AmB sterol selectivity.

IN VITRO STUDIES

Damage to Mammalian and Fungal Cells

More than 20 years ago, several groups of investigators reported that the addition of sterols to a mixture of AmB and cells protected the cells from the damaging effects of AmB (reviewed in reference 115). Therefore, with the discovery that AmB incorporated into liposome or lipid complexes was less toxic to animals than was Fungizone but at the same time was equally therapeutic (134), experiments were designed to compare various toxic and antifungal activities of AmB alone and ABLC in vitro. Studies on carrier effects were extended to detergents other than deoxycholate (82, 165, 179) and to various lipids added to AmB-cell suspensions, e.g., lipoproteins (22), triglycerides (172), preformed phospholipid vesicles (190, 191), preformed mixed micelles of egg lecithin and glycocholate (32), and lecithin-stabilized triglyceride emulsions (117). The techniques used for measuring damage in these in vitro assays can be grouped into short- and long-term assays (101). Each type of assay is detailed below.

Short-term comparative assays. Erythrocytes and, more recently, tubular renal cells have been used as a convenient model for measurement of cell death (hemolysis) or decrease in the ability of the cell to retain K^+ against a concentration gradient. *Saccharomyces cerevisiae* (115, 117), *Candida albicans* (118), and *Cryptococcus neoformans* (107) were used most often as representative fungal cells. K^+ leakage, cell viability, and lysis were usually compared after a 1- to 3-h incubation period with the antibiotic. By using these short-term assays, the increase in selectivity of AmB-lipid mixtures over AmB alone was demonstrated. AmB in the presence of preformed liposomes (101, 106, 107), mixed micelles of egg lecithin and glycocholate (32), AmB incorporated into liposomes before addition to the assay (137), and stable nonliposomal lipid structures (99) all resulted in retention of the antifungal activity and reduction or abolition of toxicity.

Two hypotheses have been proposed for the lipid-induced increase in selectivity of AmB for fungal, as opposed to mammalian, cell membranes. First, it has been hypothesized that the decreased toxicity of AmB incorporated into liposomes and the retention of its antifungal activity occur because of the selective transfer of the drug to fungal cells (104, 137). This assumption led to a search for the biological basis of specificity. Perkins et al. (154) proposed that the secretion of the enzyme lipase by fungal cells enabled the escape of free AmB from ABLC, thus increasing selectivity for the fungal cell. It has not been possible to extend this observation to other AmB formulations to date. In addition, however, AmBisome has been shown to bind to the cell wall of *Candida glabrata* (2, 3), a necessary first step if free AmB is to escape into the fungal cell or create changes in the cell membrane.

The second hypothesis relates to model A presented in Fig. 5, and its premise is based on the idea that only free AmB damages cells (27, 105–107). In this model, the formation of channels traversing membranes requires more AmB molecules when cholesterol is present in membranes than when ergosterol is there. By extrapolation to biologically intact membranes tested in cellular assays, only self-associated molecules of AmB can damage mammalian cells, whose membranes con-

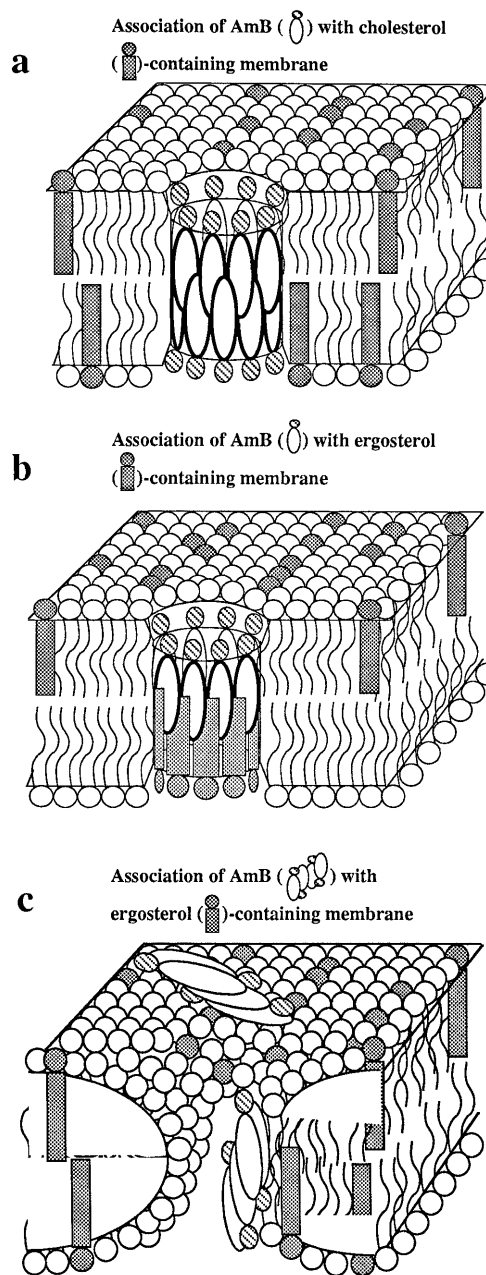


FIG. 5. Models illustrating the possibilities for the formation of channels by AmB interacting with sterol-containing membranes. (a) Model A, cholesterol-containing membrane; (b) model B, ergosterol-containing membrane; (c) AmB aggregates inserting into ergosterol- or cholesterol-containing membrane. The head-to-tail assembly of the AmB molecules in membranes and the complexation of ergosterol to AmB in a single leaflet have not yet been demonstrated experimentally.

tain cholesterol, whereas both self-associated and monomeric AmB can damage fungal or parasitic cells, whose membranes contain ergosterol or ergosterol precursors. This is a logical explanation for the findings in numerous short-term experiments (reviewed in reference 97).

Since only free (unbound) AmB is active against cells, the damaging action of an AmB formulation depends on the ability of AmB to dissociate from complexes. The affinity between AmB and deoxycholate in Fungizone is so weak that at the

concentrations used for in vitro cellular studies, AmB dissociates from deoxycholate completely. Therefore, when Fungizone was compared with AmB formulated in an organic solvent, there was no difference in activity. The affinity constants of lipid-based AmB formulations, however, were higher than that of Fungizone. AmB dissociation from lipids depends on concentration; i.e., in concentrated solution, the amount of AmB dissociated as self-associated molecules is small to negligible whereas the amount of AmB dissociated as a monomeric species in a dilute preparation approaches the amount observed in Fungizone. Since fungal cells (more susceptible to the effects of AmB) are assayed in dilute preparations and mammalian cells (more resistant to the effects of AmB) are assayed in more concentrated preparations, ABLC assayed under the same conditions as Fungizone would be expected to, and does, have decreased toxicity but retain antifungal activity. It should be noted that since the anticellular activity of AmB decreased with increased stability of ABLC, a very high stability would be expected to decrease the activity of AmB against both mammalian and fungal cells. This assumption was supported by results of studies measuring the activity of preparations of AmB mixed with liposomes containing saturated or unsaturated phosphatidylcholine (106) or increased AmB-to-lipid ratios (100); however, results of similar studies in which AmB was incorporated into stable lipid complexes varied. When ABLC was studied, an increase in the AmB-to-lipid ratio decreased its hemolytic activity, but no decrease in anti-*Candida* activity was noted at any ratio (99). In any case, the in vitro studies have uniformly resulted in observations of increased cellular selectivity for any AmB-vector combination.

Long-term assays. (i) Mammalian cells. Fungizone and liposomal AmB have been compared in long-term assays with kidney epithelial cell cultures as an in vitro model (116). Marked differences were noted between acute (2-h) and chronic toxicity of liposomal AmB. Liposomal AmB reduced acute toxicity as measured by an inhibition of glucose transport or protein synthesis. Moreover, the uptake of α -methylglucopyranoside was reduced in liposomal AmB-treated cells compared with that in cells treated with Fungizone. Doses of liposomal AmB that were without antifungal effect at 2 h had complete antifungal activity at 48 h.

(ii) Leishmanicidal activity. Time-dependent differences were also noted when the in vitro activities of Fungizone and AmB formulated with egg lecithin and bile salts were evaluated against *Leishmania mexicana* promastigotes (162). At doses of Fungizone causing immediate lysis, AmB formulations were not active. However, at higher doses, they induced a delayed but complete inhibition of cell growth. The time necessary for the development of leishmanicidal activity could be ascribed to the rate of delivery of free, monomeric AmB.

(iii) Fungal cells. The correlation of fungal cell susceptibility and therapeutic effectiveness for various drugs relies on standardized measurements of MIC or minimum lethal concentration (MLC) (155). In vitro assays are often included with in vivo animal studies of various AmB formulations (see, e.g., reference 188). In long-term assays, the antifungal activity of Fungizone and lipid-based AmB formulations is seen to depend upon the concentration of the drug, the stability of the formulation, the fungal strain being tested, and the incubation period (160).

All concentrations of AmB below and including 0.8 $\mu\text{g/ml}$, when added to preformed liposomes, had greater antifungal activity than did AmB alone, as determined by MLC assays after 18 h of incubation (92). Moreover, when Fungizone at 0.1 or 0.25 $\mu\text{g/ml}$ was dissolved in either glucose or Intralipid, a lipid solution widely used for parenteral nutrition, fungicidal

activity was noted in cultures treated with Fungizone-Intralipid but not with Fungizone in glucose (41). In contrast, ABCD and Fungizone had equivalent MICs in vitro (0.5 $\mu\text{g/ml}$) whereas the minimal fungicidal concentration (MFC) of Fungizone (1.0 $\mu\text{g/ml}$) was lower than that of ABCD (2.0 $\mu\text{g/ml}$) (95).

Different lipid formulations vary with respect to their effectiveness when compared with AmB alone. For example, Mehta et al. (137) found that the decrease in viability of fungal cells incubated for 18 h with AmB in an organic solvent was comparable to that of cells with AmB incorporated by coprecipitation into MLV but that AmB mixed with preformed liposomes was fungicidal at a lower concentration. In a 6-h assay (187), the minimum concentration of AmB required for 99.9% killing of *C. albicans* was 0.2 mg/liter when added as Fungizone and 12.8 mg/liter as AmBisome, whereas the concentrations of pegylated liposome AmB and laboratory-prepared nonpegylated AmB liposomes were intermediate between the two extremes (187).

Results of comparisons between Fungizone and various lipid formulations with respect to selected species of yeasts have resulted in conflicting data. For example, when Hopfer et al. (92) compared Fungizone and liposomal AmB against 19 yeast strains representing five different genera, the MICs of liposomal AmB surpassed those of Fungizone after a 24-h incubation period. The kinetics for killing by various concentrations of Fungizone were similar for virtually all *C. albicans* and *C. tropicalis* cells, but two of four isolates of *Cryptococcus neoformans* and one each of four isolates of *C. glabrata* and *C. parapsilosis* had strain-specific kinetics. In contrast to the above, liposomal AmB was much less effective than Fungizone in killing *Cryptococcus neoformans* cells (159). Hanson and Stevens (87) obtained similar MIC and MFC ranges in vitro when testing ABCD and Fungizone against 41 isolates of 15 different fungal species, both primary pathogens and opportunistic fungi. The number of isolates for which the ABCD MICs and MFCs were lower was the same as the number for which the Fungizone MICs and MFCs were lower. Differences between species were noted, especially for opportunistic organisms. Fourfold or greater decreases in ABCD with respect to Fungizone activity were seen in less than one-third of comparisons, and there were large increases in approximately 1/10. When AmBisome and Fungizone were assayed against 104 pathogenic yeasts, no strain differences were noted (10). The MICs determined at 24 h were, on average, twofold lower for AmBisome than Fungizone. Since there were differences in absolute MICs from experiment to experiment, it was suggested that such differences were the result of differences in the potency of various lots used to prepare the AmBisome. The MICs of AmBisome and Fungizone for *C. lusitanae* and *C. krusei* were identical (110). When Pahls and Schaffner compared the activity of AmBisome and Fungizone against *C. albicans* (four isolates), they noted that AmBisome was fungicidal at concentrations four to eight times lower than was Fungizone (150).

In most investigations in which Fungizone and lipid-associated AmB were compared, Fungizone was shown to kill more rapidly even if the activity after 24 h was equivalent. This effect was shown for *C. albicans*, for example, with AmB encapsulated in MLV (159), AmBisome (188), and ampholiposomes (90).

From these in vitro studies, it is clear that ABLC was more effective on some occasions and less effective on others than was Fungizone. Several possible explanations exist for the differences observed. First, since AmBisome and presumably other lipid complexes have been shown to bind to at least one yeast cell (2), the ability of various complexes to actually bind

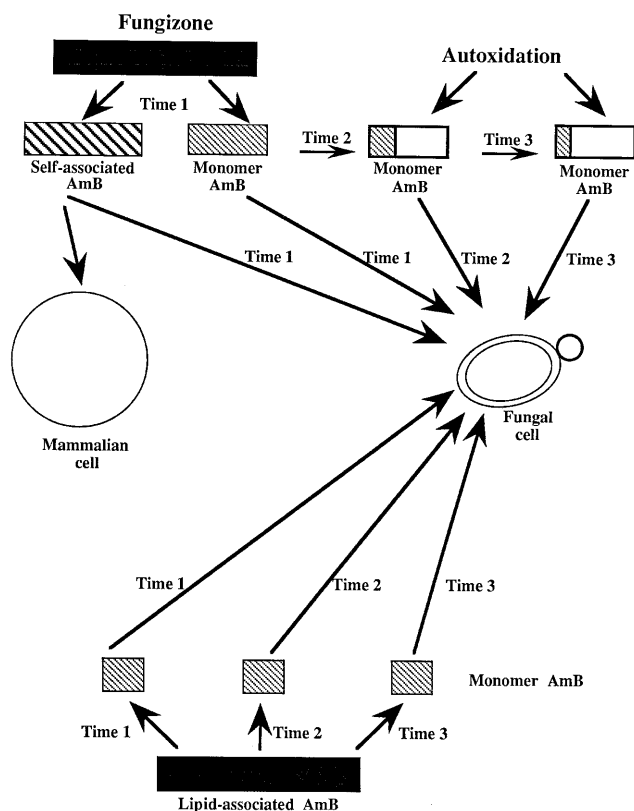


FIG. 6. Proposed origin for the greater long-term antifungal activity of some lipid formulations of AmB when compared with Fungizone. Monomeric AmB released as a result of the rapid dissociation of Fungizone is progressively decomposed by autoxidation. In contrast, AmB in lipid formulations is protected against lipid autoxidation; thus, small amounts of intact monomeric AmB are progressively released to act on fungal cells.

to the wall may govern their effectiveness. Moreover, in addition to the lipid facilitating binding, it may facilitate cell penetration (92), as has been proposed for AmB associated with a detergent (36). Yet another possibility is that the growth rates of various species of fungi may cause the fungi to respond differently to different formulations.

Several groups have considered the possibility that AmB has to be released from the ABLC before it can produce its antifungal effect. While this is a plausible explanation for complexes that are less active than Fungizone, it does not explain the data for complexes that have greater activity. Heymans et al. (90) suggested that slow release of the AmB may protect it from decomposition. As discussed above, AmB complexed with lipids is chemically and biologically more stable. It is assumed that in the absence of lipids, AmB which has dissociated from deoxycholate is decomposed by autoxidation, whereas in its complexed state, it is protected from oxidation and released slowly as a biologically active molecule.

In Fig. 6 we illustrate the concept of the effect of time on the production of biologically active AmB *in vitro* when presented to cells as Fungizone or ABLC. During the early incubation period, AmB dissociates from deoxycholate as a mixture of oligomeric and monomeric species, the former of which are toxic to mammalian cells and both of which are toxic to fungal cells. Dissociation of AmB from the lipid complex, on the other hand, is slow, only the monomeric form is released, and it has fungicidal activity. Overall, there would be less demonstrable antifungal activity because less AmB would be released at any

one time. As the incubation period proceeds, monomeric AmB gradually decomposes by autoxidation and the antifungal activity of AmB that has dissociated from Fungizone decreases, whereas there is a continuous supply of monomeric AmB being released from the ABLC. The gradual decomposition of AmB dissociated from Fungizone and the gradual dissociation of monomeric AmB from lipid complexes, considered along with variable susceptibility of fungal species to the drug, probably lead to the variability seen in the experimental studies.

It is curious that the effect of time on the relative effectiveness of Fungizone and lipid-associated AmB is seldom observed with mammalian cells. It is possible that serum in the cell culture medium protects AmB from degradation. In addition, the concentrations of Fungizone used in mammalian cell cultures were higher than those used with fungal cells. Therefore, Fungizone may have decomposed faster in fungal assays because the kinetics of autoxidation increase with dilution, which has significant effects on the deaggregation of AmB (119).

Cells of the Immune System

Carrier-induced decrease in AmB toxicity. The observations that association of AmB with lipids decreases toxicity for kidney cell-derived lines and human erythrocytes also apply to cells of the immune system. The proliferation of T cells, for example, was significantly less inhibited by AmB incorporated in liposomes than by Fungizone (20). Likewise, ABLC stimulated oxidative activity and integrin expression of polymorphonuclear neutrophils to a greater extent than did Fungizone, and ABLC was more effective at decreasing polymorphonuclear neutrophil motility as well. However, when compared with Fungizone, lipid complexing decreased AmB activation of human polymorphonuclear neutrophils (175). High concentrations of Fungizone, concentrations much higher than those required for clinical effectiveness, inhibited mouse splenocyte proliferation, whereas high concentrations of AmBisome had no effect. The authors concluded that since AmBisome had no effect on the immunologic activity of splenocytes and would therefore not depress further an already depressed immune system, it could be a promising therapy for the patient whose immune system was compromised (169).

The lack of effect on immune system cells of AmBisome at high concentrations may explain the decrease in toxic phenomena observed *in vivo* when this form of the drug is tested. The decrease in toxicity might then allow for the demonstration of the more positive effects of AmB on the immune system. For example, low concentrations of free AmB completely inhibited the serum induction of transglutaminase and production of superoxide anion by murine macrophages whereas encapsulation of the drug within liposomes protected the cells from these adverse effects (138). In an animal model of experimental candidiasis, liposomal AmB was more efficient than Fungizone in both treatment and prophylaxis, and it was assumed that an increase in the normal host defenses contributed to the therapeutic efficacy (131, 134).

Mediators of antifungal activity. Macrophages may function as a reservoir of AmB for intracellular and extracellular antimicrobial action. When AmB associated with lipids is taken into macrophages (123, 139) 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 (Fig. 7). 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.

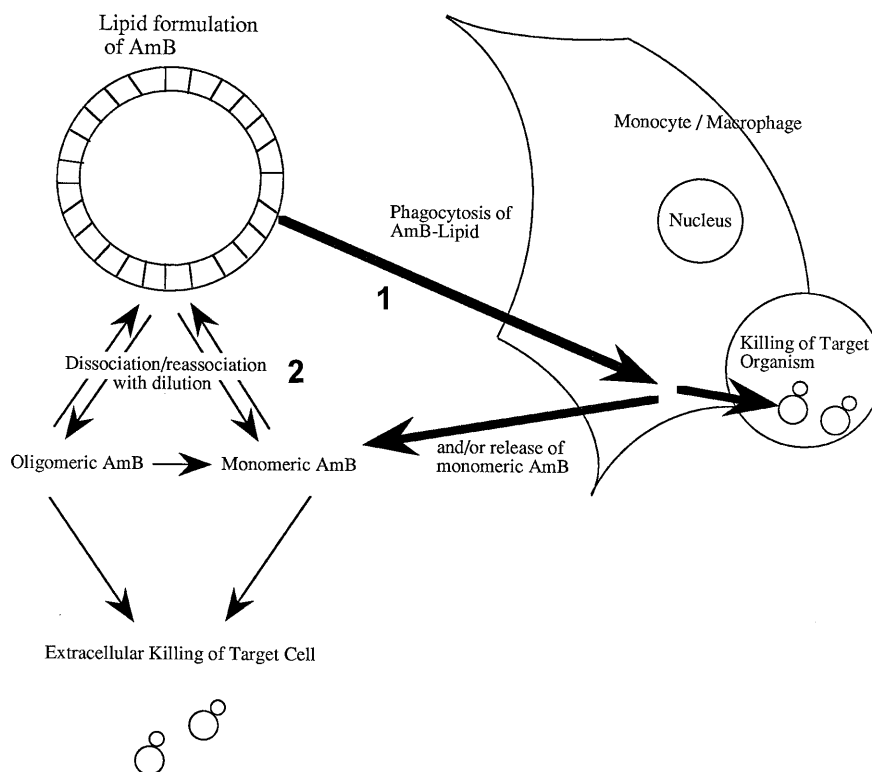


FIG. 7. Models of several pathways by which lipid formulations of AmB are thought to reach fungal cells. (Step 1) The lipid formulation of AmB may be phagocytized by macrophages, wherein it dissociates, and the AmB kills the intracellular fungal target, or dissociation of the complex may occur inside the cell with release of monomeric AmB to the external milieu. The possible binding of the lipid formulation directly to the cell is not illustrated in this diagram. (Step 2) The lipid formulation may dissociate upon dilution with medium. Adapted from reference 24 with permission of the publisher.

Carrier Effects on AmB Binding to Lipoproteins and Its Internalization

Free AmB binds to lipoproteins, and this binding may influence the ability of mammalian cells to internalize the drug. The manner in which a carrier modulates AmB binding to lipoprotein and its internalization depends on two characteristics of the AmB-carrier complex, namely, its stability and its lability. Consider, for example, two possible characteristic combinations: weak and labile or strong and inert.

If the AmB-carrier bond is weak and labile, as is presented in Fig. 8, 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. The toxicity of the ABLC in this instance is therefore related to its thermodynamic stability, which in turn regulates the rate of dissociation of free AmB from the complex.

When the AmB-carrier complex is strong and inert, it remains intact after introduction into the bloodstream but can still bind lipoproteins. Wasan and Lopez-Berenstein (198) have demonstrated that an AmB-DMPG complex was derived from AmB-DLPG-DMPG MLV bound to serum lipoproteins. The toxicity of an ABLC bound to serum lipoprotein may differ from that of AmB alone. However, AmB alone binds preferentially to LDL and can be internalized into cells expressing LDL receptors, whereas ABLC may bind to high-density lipoproteins (HDL) and remain in the bloodstream, lacking toxicity (199). Toxicity in this instance is related to the

specificity of the AmB-lipid formulation interaction with lipoproteins and its distribution.

On the other hand, neither ABCD (85) nor AmB incorporated into egg lecithin-bile salts mixed micelles (31) bound to lipoproteins, and both were relatively nontoxic. How, therefore, do they dissociate to free AmB for fungicidal activity? The most logical explanation is that the complexes are phagocytized without the intervention of LDL receptors and AmB is subsequently released slowly from the cell into the medium (123).

PHARMACOKINETICS AND TISSUE DISTRIBUTION OF AmB INCORPORATED INTO DELIVERY SYSTEMS

Detailed comparative data on the pharmacokinetics and tissue distribution of liposomal and lipid formulations of AmB have been reviewed by Janknegt et al. (97). Such studies were done after either a single dose of drug or sequential daily treatments. HPLC or bioassay (the latter being less sensitive) was usually used to determine AmB concentrations in plasma and tissue. The total AmB concentration was determined by both procedures, but essential information about drug distribution among delivery systems, lipoproteins, cells, and freely circulating drug was not provided. Single-dose studies are generally done at a dose of 1 mg/kg of body weight, with higher doses of AmB incorporated into delivery systems being studied for comparison. With regard to AmB levels in plasma, there has been only one study with healthy volunteers, in which ABCD produced a peak AmB concentration of 2 $\mu\text{g/ml}$, which

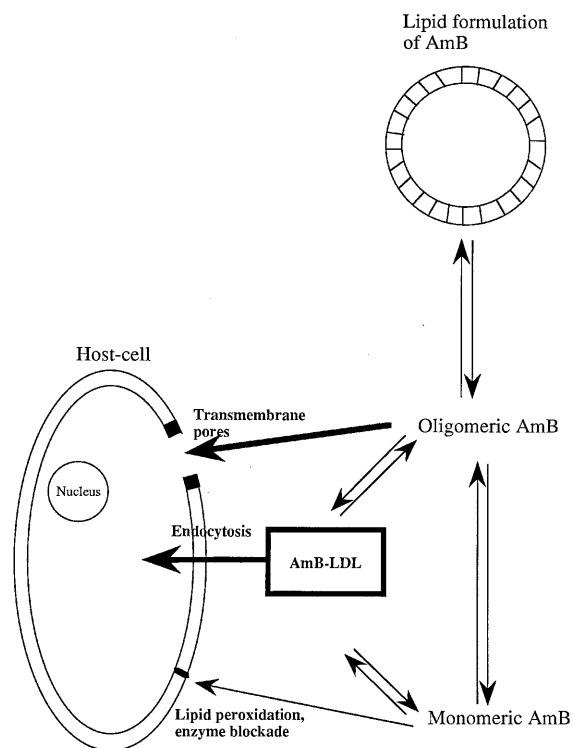


FIG. 8. Models of several pathways by which the lipid formulations of AmB may reach mammalian cells. Adapted from reference 24 with permission of the publisher.

decreased after 10 h to 0.1 $\mu\text{g/ml}$ and remained there for 400 h (167).

Pharmacokinetic studies with naive animals or animals infected with fungi have been numerous. For example, compared with Fungizone, the levels of AmB in plasma were lower when AmB was administered in the following lipid-associated delivery systems: DMPC-DMPG-AmB in healthy rats (200), ABCD in healthy rats (65, 84), and ABLC in infected mice. However, when higher doses of ABCD were administered, AmB levels similar to or higher than those achieved with Fungizone resulted within 24 h. Higher levels of free AmB were obtained in diabetic rats as well when liposomal AmB was tested (200). Remarkably high levels of free AmB were detected in rats after treatment with AmBisome (118 μM with an elimination half-life of 7.56 h) (158) or phosphatidylcholine-cholesterol (PC-CHOL) SUV (75).

In many instances, substantial increases in free AmB concentrations in tissues were detected with lipid formulations. ABCD (65, 86), ABLC (43), and PC-CHOL SUV (77) all resulted in increased concentrations of AmB in the liver. Almost 100% of the AmB in the administered dose of ABCD was recovered in the liver 30 min later but only 39.6% of the AmB associated with Fungizone was recovered. A moderate increase in the concentration in the liver was detected with EPC-phosphatidylethanolamine (PE)-CHOL SUV (4). In contrast, DMPC-DMPG MLV (130, 200), EPC-tocopherol succinate (TS) SUV, EPC-TS-CHOL SUV (185), and Fungizone all resulted in similar levels of free AmB in the livers of uninfected mice and rats. Healthy rats given DMPC-DMPG MLV had 45-fold-higher levels of free AmB in their lungs than did those treated with Fungizone, but those treated with ABCD and ABLC actually had lower levels of free AmB. Infection with *C.*

albicans caused mice to have 10-fold-higher concentrations of free AmB when treated with DMPC-DMPG MLV, EPC-PE-CHOL SUV, or Fungizone than did their comparable uninfected controls (4, 131). Significant increases in the concentration of free AmB in lungs were also observed with PC-CHOL SUV (77) and EPC-PE-CHOL SUV (4). When cholesterol was added to the vesicles, EPC SUV or EPC-phosphatidylserine (PS) SUV stimulated increased concentrations of free AmB in various tissues in healthy or infected mice but the addition of cholesterol to EPC-SA SUV had no modifying effect (4).

AmB concentrations resulting from the daily administration of Fungizone seldom exceed 2.5 $\mu\text{g/ml}$. Even lower peak concentrations in serum were obtained with ABLC (109, 196). In contrast, quite high concentrations of AmB (26 to 60 $\mu\text{g/ml}$) were reached in cancer patients treated with ampholiposomes (171). In this instance, AmB distribution followed a nonlinear bicompartamental model incorporating a liposome-free drug subsystem. On the other hand, when small unilamellar liposomes made of soya PC and cholesterol were used in a molar ratio of 1:3, the pharmacokinetic parameters were similar to those observed with Fungizone (75). AmB concentrations elevated over those achievable with Fungizone were also observed in rabbits given AmBisome (120, 182) but not in dogs given ABCD (64). Moreover, in dogs, steady-state levels were reached more rapidly with Fungizone than with ABCD.

Levels of AmB in the tissues of cancer patients treated with Fungizone have been reported in at least three studies (40, 42, 52). The concentrations of AmB varied with the tissues tested and with the total dose of Fungizone administered. Higher levels of AmB were detected in the liver and spleen than in the lungs and kidneys. In fact, most of the AmB administered was found in the liver. The absolute levels depended on the method of measurement and increased in the following order: bioassay of aqueous homogenate, bioassay of ethanol extract, and HPLC of ethanol extract. In one study in which Fungizone and ampholiposomes were compared in cancer patients (53), the tissue distribution of AmB was not significantly different between the two preparations, despite major differences in the pharmacokinetics. AmBisome administration actually resulted in significantly decreased concentrations of AmB in the lungs of solid-organ and bone marrow transplant recipients (184).

When equivalent concentrations of ABLC and Fungizone, 1 mg/kg, were given to mice, the concentrations of AmB detected in the liver and spleen were higher in the mice that received ABLC. Mice and rats given the same dose of AmBisome or Fungizone also had higher levels of AmB in their livers and spleens if given AmBisome but lower levels in the kidneys and lungs (43). Rabbits given 5 mg of AmBisome per kg had 10-fold-higher levels of AmB in their livers and spleens than did a similar group treated with Fungizone (120). The time taken for washout of AmB from the tissue after treatment, however, was very similar to its plasma elimination half-life (64, 65). Rats (84, 196) and dogs (64) also had higher levels of AmB in their livers in response to ABCD than in response to Fungizone. As AmB levels in the plasma decreased, the levels in the liver and spleen increased (158). When AmB liposomes prepared with different sizes, charges, and fatty acyl chains were compared, considerable variability was observed and no particular pattern of tissue distribution could be noted in healthy mice (152). Thus, two extreme conditions appear to exist. In the first, liver and other tissues function as reservoirs of drug for the plasma, and in the second, plasma functions as a reservoir for tissues. The two extremes are represented by ABCD and AmBisome, respectively.

ANIMAL STUDIES

Toxicity to Animal Cells versus Toxicity to Uninfected Animals

An early report (137) that liposomal AmB, which is known to be less toxic for mice *in vivo* than is Fungizone, was also less toxic for mammalian cells *in vitro* led to the idea that the toxicity of a particular formulation *in vitro* might serve as a predictor of its toxicity *in vivo*. Thus, in many studies, the carrier effects on the toxicity of AmB against cells *in vitro* were measured as a decrease in AmB-induced permeability or lysis of erythrocytes or as a decrease in toxicity to cultured animal cells. As summarized below, if a formulation is less toxic than Fungizone *in vivo*, it is also less toxic to animal cells *in vitro*, but lowered toxicity *in vitro* does not always predict lowered toxicity *in vivo*.

First, we consider the formulations that were less toxic *in vivo* and for which there was also decreased toxicity *in vitro*. All three commercial lipid-based formulations have 50% lethal doses (LD_{50}) higher than Fungizone: Amphocil, 18-fold higher; ABLC, 23-fold higher; and AmBisome, 87-fold higher (194). These formulations also have lower toxicity to animal cells *in vitro* than does Fungizone. Examples of less toxic formulations include a cholesteryl sulfate-AmB complex similar to Amphocil (84), ABLC (3, 98), a liposomal preparation of AmB (156), and AmB associated with triglycerides (172). An increase in AmB-to-lipid molar ratios (98) of liposomes prepared with saturated PC as compared with unsaturated PC (177) displayed decreased toxicity both *in vivo* and *in vitro*. Increasing the AmB-to-lipid ratio and using saturated phospholipids decrease the rate of AmB dissociation from complexes (4, 106). Thus, selected characteristics of a formulation which were indispensable for lowering toxicity *in vivo* resulted in decreased toxicity *in vitro*.

Not all AmB formulations that were less toxic than Fungizone *in vitro* were also less toxic *in vivo*, however. Esters of sucrose decreased AmB toxicity to mammalian cells *in vitro* but decreased toxicity to mice *in vivo* only in a very narrow range of AmB concentrations (83). Likewise, palmitoyl manose protected cultured mammalian cells from AmB toxicity *in vitro* whereas it reduced the acute phase of toxicity but had no impact on survival over a 3-day period *in vivo* (165). No consistent correlations were seen between detergent effects on the deaggregation of AmB and the survival of mice (13) or between toxicity to cells *in vitro* or to animals *in vivo* (180). The effects of preformed liposomes on AmB toxicity were also not predictable. In rabbits, liposomes lowered some toxicity; for example, the glomerular filtration rate and ion tubular permeability were affected much less, but there was still an increase in the excretion of *N*-acetyl- β -D-glucosaminase (103).

As with any *in vivo*-*in vitro* comparisons, comparison of the results derived from animal cells *in vitro* and those derived from animal models *in vivo* suffers because the complexity of the *in vivo* situation is many orders of magnitude greater than that of the *in vitro* system. At least three factors can modify the distribution of AmB *in vivo* and affect its efficacy, namely, its ability to bind to selected molecules found in plasma, its ability to bind to tissues and organs, and the ability of vesicles of differing sizes to traverse the endothelium.

As noted above, AmB formulations are administered intravenously and are therefore suspended in blood. Some of the AmB-lipid formulations bind to lipoproteins in blood. Moreover, erythrocytes interact with AmB. The affinities of different formulations to blood components are highly variable and thus unpredictable. The binding and internalization of AmB complexes to liver macrophages, as described in a previous section,

may be very important (139). AmB-DMPC MLV and ABCD are internalized to a much greater extent than is the AmB in Fungizone, whereas AmBisome and amphotiposomes are internalized to a lesser extent (123). The importance of macrophages for therapeutic efficacy and toxicity of liposomal AmB has been shown in mice infected with *Aspergillus* spp. and depleted of functional phagocytic cells by treatment with dichloromethylene diphosphonate (146). Finally, the sinusoidal endothelium acts as a filter by allowing access to parenchymal cells via a $\pm 0.1\text{-}\mu\text{m}$ fenestration. This opening is large enough for small particles such as AmBisome but too small for larger vesicles. Different formulations would have different levels of access to mammalian cells in infected organs and would therefore be expected to have different degrees of toxicity.

Experimental Therapy

No formulation of AmB prepared with a detergent or with preformed liposomes has ever been reported to be more therapeutic than Fungizone. Improved therapy in experimental parasitic infections was first noted, however, with formulations of AmB in which the drug was incorporated into liposomes by coprecipitation with lipids (148). This principle was then applied to treatment of experimental fungal infections (133, 134). Liposomal AmB prepared in this way is more stable and less toxic than when prepared with detergents or with preformed liposomes.

Even more stable and less toxic lipid-associated complexes of AmB, in the form of ABLC, ABCD, AmB mixed micelles, and Egam and Edam, were introduced later for experimental therapy. Liposomal AmB and ABLC have been tested in various animal models which differ in many aspects, such as the type of animal, the immunologic status of the animal (immunocompetent versus immunosuppressed), the fungal pathogen, the route of challenge and the dose of organism, and the therapeutic regimen. No large-scale studies have been performed on the comparative efficacy of the many different formulations tested independently against Fungizone.

In general, lipid-based AmB formulations are less toxic in uninfected animals than is Fungizone, and in infected animals their maximum tolerated doses, i.e., the highest dose that does not induce acute toxicity, are higher. An increase in the tolerated dose, however, does not *de facto* translate into increases in LD_{50} (181). In fact, infected, ill animals are more susceptible to AmB toxicity than are uninfected animals. The increase in efficacy results because the less toxic formulations can be administered in higher doses. It must be remembered, however, that the association of AmB with lipids in some formulations actually decreases the antifungal activity when compared with Fungizone.

Selected studies of the therapeutic efficacy of various lipid-based formulations of AmB with Fungizone are summarized below under disease categories. Effectiveness ratings are provided as "comparable," "worse" (Fungizone was more effective), or "better" (the test compound was more effective than Fungizone). These categories are also used in Table 2, in which the data discussed below are summarized.

Aspergillosis. Taken as a whole, lipid-based formulations were an improvement over Fungizone, although not necessarily at equivalent doses and not necessarily when both survival and culture of organs were assessed in the same model. In mice, AmB SUV (4) and ABLC (43) were more effective in prolonging the life of infected animals and decreasing fungal counts in the lungs (4) or the lungs and kidneys (43). The data in rabbit models have been variable. AmBisome, for example, was better than Fungizone in prolonging survival, but at equal

TABLE 2. Comparisons of various AmB-lipid based formulations with Fungizone at equal doses in animal models

AmB lipid-based formulation	Aspergillosis			Blastomycosis			Candidiasis		
	Efficacy ^a	Model	Reference	Efficacy	Model	Reference	Efficacy	Model	Reference(s)
ABCD	Worse	Rabbits	7				Comparable	Mice	85
ABCD-like	Worse	Rabbits	151						
AmB SUV	Better	Mice	4				Better Comparable Worse/better	Mice Mice Mice	93, 94 145, 185 77
AmB MLV							Comparable	Mice	128, 131, 134
ABLC	Better	Mice	43	Worse	Mice	45	Worse Comparable	Rabbits Mice	153 43, 144
AmBisome	Worse	Rabbits	67	Worse	Mice	48	Worse Comparable	Mice Mice	110, 150, 188 1
MLV lecithin, ergosterol							Worse	Mice	5
Egg lecithin mixed micelles							Better	Mice	35
AmB lipid dispersion							Comparable	Mice	38, 113

^a With respect to Fungizone.

doses (1 mg/kg) it was less effective at sterilizing tissue (67). If the dose of AmBisome was increased 5- or 10-fold, however, it was equally effective in reducing fungal burden. AmB-cholesteryl sulfate complex, similar to ABCD, was less effective than Fungizone in sterilizing the liver and kidneys of immunosuppressed rabbits (151), and ABCD itself, at doses equivalent to those of Fungizone, was also less effective in prolonging the survival of rabbits (7). However, 5 mg of ABCD per kg was as effective as 1 mg of Fungizone per kg (7).

Blastomycosis. When ABLC and Fungizone were compared at the same dose in a murine model of blastomycosis, ABLC was less effective (45). Fungizone caused a greater reduction in fungal burden in the lungs of infected mice, and the total numbers of survivors, as well as the total numbers of survivors that were fungus free, were greater for Fungizone.

Candidiasis. When the studies involving candidiasis in experimental animal models were reviewed, lipid-based formulations were comparable to Fungizone in about half of the studies (1, 85, 128, 131, 134, 145, 185) and were worse than Fungizone in the other half (5, 35, 56, 94, 113, 133, 150, 153). In no instances were they better. In general, the AmB SUV and AmB MLV formulations produced comparable data (128, 131, 134, 145, 185) whereas most other preparations, e.g., egg lecithin mixed micelles (35), AmB lipid dispersion (56, 113), liposomes with lecithin and ergosterol (5), AmBisome (15, 185), and ABLC (153), produced worse results.

There are logical explanations for some of the "worse" data. For example, liposomes containing ergosterol would probably be expected to be ineffective (133). When comparable doses in leukopenic mice are compared, the maximum tolerable dose of Fungizone will be quite small, and a comparable dose of a lipid-based formulation would be expected to be less effective. In another study (1), it is difficult to compare the results because Fungizone was administered by the intraperitoneal route and AmBisome was administered intravenously. In summary, although the studies of candidiasis in various animal models have not shown that the lipid-based formulations are better than Fungizone, in a number of studies the lipid-based formu-

lations have elicited comparable data. Considering that the lipid-based formulations are considerably less toxic at the same concentration as Fungizone, the use of the lipid-based preparations would be preferable.

Coccidioidomycosis. ABCD was not as effective as Fungizone on a milligram-per-kilogram basis when tested against *Coccidioides immitis* (46, 49). Efficacy was determined by culture of spleens, livers, and lungs of surviving mice.

Cryptococcosis. The lipid formulations tested for cryptococcosis in experimental models have included ABCD (95), ABLC (43, 153), liposomes made with lecithin and ergosterol (81), and egg lecithin mixed micelles (37). In all cases, the lipid-based formulations were either comparable to Fungizone or less effective. In some cases, the drug was comparable when survival was evaluated but worse when various organs were cultured (95).

Histoplasmosis. Positively charged SUV were significantly more effective in prolonging the survival of mice infected with *H. capsulatum* (183). AmBisome was compared with Fungizone for the treatment of disseminated murine histoplasmosis in athymic mice (80). The drugs were equally effective in prolonging survival as well as reducing fungal burden in the kidneys and spleen. Comparable data were obtained when the drugs were compared at doses up to 1 mg/kg administered intravenously and also at higher doses when Fungizone was administered intraperitoneally and AmBisome was administered intravenously.

Visceral leishmaniasis. In general, lipid-based formulations have proven to be consistently better than Fungizone in treating experimental leishmaniasis in both murine and hamster models (18, 19, 54, 55, 148). For example, AmB incorporated in MLV was two to four times more active than Fungizone against hepatic and splenic parasites in a hamster model (18) and ABCD was 15 times more effective in hamsters infected with low doses of *Leishmania donovani* and 4 times more effective if the dose of parasites was increased to produce heavily infected animals. In the one instance in which a com-

TABLE 2—Continued

Coccidioidomycosis			Cryptococcosis			Histoplasmosis			Visceral leishmaniasis		
Efficacy	Model	Reference	Efficacy	Model	Reference(s)	Efficacy	Model	Reference	Efficacy	Model	Reference(s)
Worse	Mice	46	Comparable	Mice	85, 95				Better	Hamsters	19
			Better	Mice	59				Better	Mice	148
									Better	Hamsters	18
									Comparable	Monkeys	18
Comparable	Mice	8	Worse	Mice	43						
Worse	Mice	47	Worse	Rabbits	153						
			Comparable	Mice	1	Comparable	Mice	80	Better	Mice	54, 55
			Comparable	Mice	81						
			Worse	Mice	35						
			Comparable	Mice	102						

parison was made in a primate, however, the lipid-based formulation was slightly less effective than Fungizone (18).

Conclusion. What can be learned from these experiments? First, at equivalent concentrations, the various lipid-based formulations were often better than Fungizone when tested in the parasitic infection visceral leishmaniasis. This may relate to the intracellular nature of the parasite. For example, AmBisome was inactive against free-living promastigotes of *L. donovani*, but equal to or better than Fungizone in macrophages in vitro or in experimental infection in vivo, respectively (54). Thus, the effectiveness of the lipid-based formulations may be related to the relative proportion of organisms within macrophages.

Second, at equivalent concentrations of AmB, Fungizone was often more effective in fungal infections than were the lipid-based formulations. These results can be explained by the thermodynamic stability of the lipid-based preparations; the small amount of free AmB that dissociates from the complexes impairs their antifungal effectiveness. However, since AmB is released from these complexes slowly, the complexes are much less toxic than free AmB, and much larger doses of the complexes can be administered, increasing their therapeutic value (80). Thus, the slow dissociation of free AmB from the lipid-based complexes puts the lipid-based complexes at a disadvantage when the severity of the infection is increased (73), when there is a decrease in the susceptibility of the causative agent to AmB (110), and when the interval selected for a particular assay is short (153).

However, other factors may come into play to make the lipid-based formulations more attractive as alternatives to Fungizone. The lipid-based formulations are clearly less toxic, and since their physicochemical characteristics are different, their pharmacokinetics and comparative effectiveness in various tissues may differ in long-term assays. In a murine model of systemic candidiasis, van Etten et al. (187) compared sterically stabilized and unstabilized liposomes and concluded that the stabilized liposomes remained in the circulation for a longer interval and that their toxicity was reduced but their antifungal activity was retained.

The above data suggest two hypotheses. First, the therapeutic potential of a lipid-based formulation depends on its direct antifungal activity in comparison with that of Fungizone. When the pathogen is not highly susceptible to AmB, the lower levels of free AmB that dissociate from the stable lipid-based complexes may not be sufficient for antimicrobial activity. Our recent study on the comparative therapeutic effect of Fungizone and Egam in mice infected with resistant strains of *Candida* spp. supports this hypothesis (62). Second, since the effectiveness of lipid-based formulations appears to be influenced by the stability of the ABLC, changes in the AmB-to-lipid ratio may enhance the effectiveness of the preparations. For example, we have shown that an increase in the AmB-to-lipid ratio in Egam and Edam increases stability and decreases antifungal activity. While differences in the effectiveness of preparations containing varied AmB-to-lipid ratios were not obvious in a murine model of candidiasis, they were very important in model of cryptococcosis (34). At lower doses, formulations with lower ratios of AmB to lipid were more effective at prolonging survival than were formulations with higher ratios.

Other Therapeutic Approaches

Specific targeting. The activity of ABLC in vivo may be improved by incorporating antibodies into the liposomal membranes, thus targeting the liposomes to fungal cells. Hospenthal et al. (93, 94) were the first to report success by incorporating polyclonal anti-*Candida* antibodies into liposomes for the treatment of experimental candidiasis. More recently, these data were confirmed in a neutropenic murine model (16). Immunotargeting of liposomal AmB has been shown to be successful in cryptococcosis as well. Dromer et al. (59) conjugated anti-cryptococcal antibodies to liposomes and used them successfully to treat experimental cryptococcosis. The immunoglobulin-bearing liposomes were more effective when survival was assessed than was liposomal AmB without the immunoglobulin and Fungizone.

Aerosolized liposomal AmB. Since many of the more serious fungal diseases develop in the lungs, an approach whereby the drug is deposited directly in the lungs might be advantageous. It would provide delivery of the AmB to the lung tissue *per se*, but the drug would probably also get into the systemic circulation from that position. In fact, aerosolized AmB alone has been shown to be effective for prophylaxis and therapy in a rat model of aspergillosis (170). A logical extension of this study, then, would be to administer an AmB-lipid-based formulation in this manner. Gilbert et al. (72, 73) did just that in experimental models of candidiasis and cryptococcosis. The treatment significantly reduced the number of *Candida* organisms in the kidneys and increased the mean time of survival and percent survival of mice. In a model of pulmonary aspergillosis, aerosolized AmBisome at the same concentration as Fungizone resulted in AmB concentrations in the lungs that were eight times higher than those derived from Fungizone (6). The authors proposed that the higher concentrations of AmB in the lungs resulted from increased retention of the complex.

Combination Therapy

The observations that liposomal AmB is less toxic *in vitro* and *in vivo* than Fungizone yet retains antifungal activity led to the suggestion that a combination of the lipid-based formulations with other drugs might provide synergistic activity. Indeed, synergistic activity was demonstrated *in vitro* for liposomal AmB combined with free or liposomal gramicidin (91). This combination has never been used in human trials, however. It has been suggested by others (186) that some patient populations might benefit from combination therapy with AmBisome and 5-fluorocytosine rather than Fungizone and 5-fluorocytosine.

CLINICAL TRIALS

Three types of lipid-based AmB formulations are now under intensive clinical investigation. They involve the incorporation of AmB into structures which can be described as liposomes (ampholiposomes, AmBisome, L-AmpB), sheets (ABLC, Abelcet), or discs (ABCD, Amphocil).

These lipid-based formulations differ in size, structure, shape, lipid composition, and molar AmB content (Table 1). Their physicochemical differences determine their thermodynamic stability, the distribution of AmB between the lipid formulation and lipoproteins, and their tissue distribution, levels in blood, uptake by macrophages, and penetration to the site of infection. Despite all these differences, however, the formulations have one common feature when compared with Fungizone; they are all less toxic than Fungizone to mammalian cells, to animals, and to humans. There are several excellent reviews in which clinical data have been summarized. Their references are given below.

Noncommercially Developed Lipid-Based Formulations

Multilamellar liposomal AmB (L-AmpB 5, L-AmpB 10). The first of the lipid-based formulations to be used clinically was the multilamellar liposomal AmB. Because of the positive treatment and prophylactic data that were obtained in a murine model of candidiasis (130, 134), clinical trials were begun with this formulation in 1983. Thus, AmB MLV was used for the treatment of systemic mycoses in patients with cancer whose infections had failed to respond to Fungizone. The preparation was less toxic and more effective than Fungizone (128). AmB MLV was also shown to have potential in the treatment of hepatosplenic candidiasis which was unresponsive

to Fungizone (127), as well as in the treatment of one patient with rhinocerebral mucormycosis (66). The early studies with this formulation were summarized in the late 1980s. More recently, Ralph et al. (159) investigated the use of AmB MLV formulations as a routine alternative to Fungizone. The MLV preparations proved to be useful as a direct replacement for Fungizone at the same dosage in patients commonly seen in an infectious disease service.

Small unilamellar liposomal AmB (ampholiposomes, L-AmpB). The second approach to liposomal antifungal drug delivery was based on the studies of a group in Belgium who had administered water-insoluble antimetabolic compounds to cancer patients by using SUV prepared from SPC and cholesterol. Patients tolerated these preparations well, and it was hypothesized that AmB incorporated into similar vesicles might provide a suitable, less toxic, alternative to Fungizone. Therefore, cancer patients with fungal infections were given AmB incorporated in small sonicated unilamellar vesicles composed of egg yolk lecithin, cholesterol, and stearylamine in a molar ratio of 4:3:1. This formulation, named ampholiposomes, was better tolerated and had a better therapeutic index than Fungizone (141, 171). Moreover, it was stable in storage for 1 year (90). It was never, however, developed for commercial use.

A similar preparation, consisting of small unilamellar sonicated vesicles of soya lecithin, was shown to be effective in a cancer patient with chronic disseminated candidiasis that was refractory to Fungizone (76).

AmB-Intralipid. In addition to the lipid-based formulations discussed above, studies have been performed with AmB formulated with Intralipid. Intralipid is a commercially available nutritional fluid, containing 20% lipid, which is administered intravenously. Early reports of the therapeutic efficacy of AmB-Intralipid emulsions in murine candidiasis (112) have been followed by reports of efficacy in murine cryptococcosis (102) and by retrospective studies of human clinical data (38, 39). Fungizone is often administered through the same catheter as Intralipid. When comparisons were done between Fungizone diluted with 5% glucose or infused with Intralipid, the Fungizone infused with Intralipid was tolerated better and was less nephrotoxic. The two modes of administration have been compared in human immunodeficiency virus-infected patients with candidiasis as well (41, 147); while renal toxicity was reduced with the Intralipid administration, the efficacy was the same for the two preparations. A caveat must be considered when preparing AmB in fat emulsions, however, since Washington et al. (201) determined that the addition of Fungizone to a preformed carrier fat emulsion resulted in precipitation of the drug. Clearly, additional studies are needed to characterize colloidal drug delivery systems.

Commercially Developed Lipid-Based Formulations of AmB

AmBisome. A formulation of AmB incorporated into small sonicated liposomes consisting of hydrogenated SPC, cholesterol, distearoyl phosphatidylglycerol, and AmB, named AmBisome, was developed by Vestar, Inc. It is currently marketed by NeXstar Pharmaceutical. AmBisome was introduced to the European market in 1989 and is now available in 18 countries. It is the most widely investigated lipid-based formulation of AmB (74). It has proven to be highly promising thus far in clinical trials (reviewed in references 3 and 57). Its less toxic profile than that of Fungizone led to its approval for general use. The data that have accumulated indicate that AmBisome is well tolerated by patients at risk, including children with cancer (63) and transplant patients (164). It is also effective in

the treatment of leishmaniasis (3, 12, 55). AmBisome therefore appears to be a very viable alternative to Fungizone for the treatment of both fungal and parasitic infections.

AmB-lipid complex. ABLC, marketed in Europe as Abelcet, is the first commercially produced nonliposomal ABLC. This nonliposomal preparation contains MLV with a 7:3 molar ratio of PC and DMPG, with AmB concentrations greater than those in the AmB MLV preparations on which ABLC was modeled. ABLC has been given to more than 450 healthy volunteers and patients, and data from more than 100 individuals have been reported (reviewed in references 57, 69, and 99). Limited success has been reported for the treatment of cryptococcosis in AIDS patients, as well as coccidioidomycosis (57). As with the other lipid-based formulations, the key advantage of ABLC is significantly reduced toxicity with the retention of its therapeutic potential (69). Phase III clinical trials are in progress to evaluate the safety and efficacy of ABLC against a number of fungal and parasitic diseases. ABLC received marketing approval in the United Kingdom in 1995 (74).

AmB colloidal dispersion. ABCD is the second nonliposomal AmB-lipid complex to be developed commercially. The basis for its development was the observation by Szoka et al. (177) that there was a significant decrease in toxicity when AmB was administered in combination with pure cholesterol sulfate carrier in a molar ratio of 1:1. Patients with life-threatening mycoses who had failed to tolerate or respond to conventional Fungizone therapy tolerated aggressive therapy with this formulation (173). The commercial formulation, ABCD, given the trademark Amphocil, was developed by Liposome Technology, Inc. The tolerance and efficacy of this formulation have been assessed in two clinical trials (86). It was well tolerated at doses higher than the usual doses of Fungizone. Furthermore, when administered to bone marrow transplant patients, ABCD did not induce alterations in renal function (9). Its safety and efficacy in clinical trials have been summarized previously (57, 68, 85, 173). Amphocil was approved for marketing in the United Kingdom in 1994.

CONCLUDING REMARKS

AmB has remarkable antifungal properties, but scientists and physicians still do not know how to use it to the best advantage for the patient. It is difficult to understand why Fungizone, the formulation with deoxycholate, remains the "gold standard." Deoxycholate is one of the most toxic bile salts (136, 195), and its toxicity probably contributes to the overall toxicity observed with Fungizone treatment. The use of a nontoxic bile salt such as glycocholate might well result in a less toxic formulation. Even today, more than 10 years after the promising debut of lipid-associated AmB formulations, Fungizone is still routinely used in clinics all over the world. A significant drawback to the newer, commercial, less toxic lipid-based formulations is their cost. They are very expensive, and no comparative trials have been performed to determine which one of the products might be advantageous over the others.

Furthermore, there is still no clear understanding of the relationship between physicochemical properties and pharmacokinetics or between pharmacokinetics and therapeutic activity. It is logical to assume that different pharmacokinetic properties result in clinically relevant differences in efficacy and tolerance (57), but the link has not been proven. The concentration of free AmB in serum and its relationship to outcome are not even known (79). Data acquired in studies involving specific animal models suggest that the relative efficacy of lipid-associated AmB depends on the model used. Further-

more, the data may not be transferable to humans because the pathogenesis and pharmacology in humans may be quite different from those in the animal used in the model. In vitro studies of dose-response relationships for toxic and antifungal activities of free and lipid-associated AmB also have limited applicability, because the outcome is strongly influenced by the in vitro conditions, which may not in any way approximate in vivo conditions.

Perhaps the single most important recommendation to be made is that comparative clinical trials must be performed so that informed decisions can be made about which of the lipid-based formulations is best. The use of these formulations should not be influenced by marketing techniques but should be based on sound experimental data. As stated by Glaser (74), "... without head-to-head trials, the fight for market share will largely depend on which product is the first to the market and on which product's marketing campaign is the most successful in manipulating physician preferences."

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