Characteristics of Lipid-Based Formulations That Influence Their Biological Behavior in the Plasma of Patients

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Patients with cancer and infectious diseases often have changes in the composition and concentration of their different blood components. These changes include variations in the composition and concentration of plasma lipoprotein lipids, in transfer rates of endogenous lipid between different lipoprotein classes, and in concentrations of phagocytic cells (i.e., monocytes and macrophages). It appears that the interaction of many lipid-based drug formulations with plasma lipoproteins and blood phagocytic cells may be responsible for the unpredictable pharmacokinetics and pharmacodynamics of these compounds when administered to patients with diseases. This review examines the potential mechanisms that may explain the biological behavior of lipid-based drug formulations used in the treatment of infectious diseases and cancer.

A number of very potent antimicrobial agents are already available for use in the treatment of infectious diseases; however, the therapeutic index of these compounds is extremely low. This is due to a lack of drug specificity. Approaches that improve delivery of antimicrobial agents to regions of disease and away from sites of potential toxicity are currently under investigation. To date, a variety of ways to improve specificity have been discovered, including regional infusion of free drug, intracavity (peritoneal, pleural, or cranial) administration of free drug, and use of macromolecular lipid-based formulations such as liposomes.

These lipid-based formulations have been extensively investigated as a means of selectively delivering drugs to specific tissues [1-9]. They are phospholipid vesicles consisting of aqueous compartments bounded by lipid bilayer membranes. Such delivery vesicles have several advantages, including the protection of encapsulated drugs or macromolecules from enzymatic degradation or immune reaction.

Furthermore, they have low inherent toxicity and can be conjugated with various targeting ligands to enhance target-cell binding. This procedure serves to reduce nonspecific toxicity of cytotoxic drugs, with a corresponding and preferable increase in toxicity against target cells [10-12]. Liposomes have been used as carriers for a wide variety of antitumor, immunosuppressive, and antimicrobial agents [10, 13-18].

Lipoproteins are aggregates of lipid and protein molecules that transport cholesteryl esters (CEs), triglyceride, and phospholipids through the vascular and extravascular body fluids. Great diversity of composition and physical properties is possible, particularly in diseased states. As such, the classification and definition of lipoproteins are particularly difficult. Each lipoprotein has a wide range of components, each with its own metabolic origin and fate. Lipoprotein components undergo complex metabolic interplay with receptors, with enzymes located on the lipoproteins that are attached to capillary endothelium, and with other circulating lipoproteins, both in the vascular compartment and within the tissue-fluid space [19, 20].

However, lipoproteins have a wider biological significance than simply in lipid transport; they are involved in a diversity of processes such as immune reactions, coagulation, and tissue repair [21-23]. It has been demonstrated that the interaction of hydrophobic compounds (drugs and liposomes) with plasma lipoproteins can modify their pharmacokinetics, tissue distribution, and pharmacological activity [24-27]. It is our contention that not only do the drugs significantly bound to lipoproteins become changed in terms of their pharmacokinetics and tissue distribution, but also the pharmacokinetics and tissue distribution of the lipid-based formulations themselves (independent of drug incorporation) are affected by their interaction with lipoproteins.

This review will examine the potential mechanisms that may explain the biological behavior of newly developed liposome-based drug formulations used in the treatment of infectious diseases.

Development of Liposomal Amphotericin B

Amphotericin B (AmB) is a polyene macrolide antibiotic extensively utilized in the treatment of systemic fungal infec-
In the early 1980s our laboratory incorporated AmB into liposomes consisting of dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) in a 7:3 molar ratio and a lipid-to-drug molar ratio of 4:1 [29, 30]. Typically this formulation contained 60%–80% lipid:AmB complexes (L-AmB), as later described by Janoff et al. [31, 32]. We have shown in both experimental and clinical studies that this formulation has less toxicity than conventional AmB and thus can be given in higher doses. However, to date, the mechanisms that decrease the renal cytotoxic effects of L-AmB are not fully understood.

Animal Studies

The effectiveness of free AmB (Fungizone, consisting of AmB and sodium deoxycholate; Bristol-Myers Squibb, Nutley, NJ) and that of L-AmB were tested against experimentally induced systemic candidiasis in mice [33, 34]. Mice were inoculated intravenously with a strain of Candida albicans isolated from a patient with systemic candidiasis. Two days following the inoculation, a severe infection was detected in the liver, spleen, and kidneys. All treatments were administered intravenously, starting 2 days after the inoculation with C. albicans. Empty liposomes did not affect the duration of survival. Multiple doses of free AmB (at its maximum tolerated dose) enhanced the mice's length of survival significantly; however, a similar regimen of L-AmB was by far superior to the one with free AmB and led to prolonged survival (>60 days) and to a 60% rate of cure (i.e., no histopathologic or microbiological evidence of infection). L-AmB injected as a single cumulative dose corresponding to the total dose of the other regimens led to a statistically significant enhancement in duration of survival in comparison with the result obtained with free AmB. These data demonstrate that L-AmB is far more active than free AmB in this model. The findings in experimentally induced candidiasis were later confirmed by others [35]. AmB incorporated into liposomes was also shown to be effective against experimentally induced histoplasmosis [36] and cryptococcosis [37]. L-AmB has also been shown to be effective against experimentally induced leishmaniasis in hamsters and nonhuman primates [38]. L-AmB was shown to be from 331 to 750 times more active than meglumine antimonate and from 2 to 5 times more active than free AmB in hamsters infected with Leishmania donovani. In squirrel monkeys infected with L. donovani, administration of L-AmB led to a 99% suppression of amastigotes in the liver.

These experiments with L-AmB suggest that the therapeutic index is increased by the better tolerance of high AmB dosages but that the efficacy of a given dose is similar or even slightly decreased with use of L-AmB. However, leishmaniasis appears to be an exception, since the efficacy of L-AmB is increased at a low unitary dosage.

Clinical Trials

Early clinical trials with L-AmB in patients with systemic mycoses that were refractory to free AmB and other antifungal agents were conducted at The University of Texas M. D. Anderson Cancer Center between 1983 and 1989 [39–41]. Though the dosage was modified according to each patient's tolerance, the standard regimen was administration of AmB (2 mg/kg body weight) daily for 3 days; when this was well tolerated (no fever, chills, or changes in kidney function), the dose was increased by 1 mg/kg every fourth dose, up to 5 mg/kg.

Then 5 mg/kg was infused once daily for 3 days until the patient had received a total of 75 mg/kg. The maximal single dose of AmB administered in those studies was 6 mg/kg. It is important to point out that the single maximum tolerated dose of AmB in mice ranged from 16 to 20 mg/kg and the active dose was 1 mg/kg, so a therapeutic blood concentration of 10–20 μg/mL was maintained in the animal.

All patients tolerated L-AmB well; mild fever and chills occurred in only a few. Potassium supplementation was required by most patients, particularly those who received doses of >2 mg/kg. Clinical improvement was observed in most patients during the first week of treatment, and no long-term renal, hepatic, or CNS toxic effects were observed. An additional study included 46 patients with cancer who had a variety of systemic fungal infections and were treated with L-AmB [42]. Twenty-one of these patients had disseminated candidiasis, 19 had aspergillosis, and the rest had a variety of other fungal infections. Forty patients failed to respond to conventional AmB therapy, and six were given L-AmB because therapy with conventional AmB resulted in severe side effects (e.g., nausea, vomiting, and hypokalemia).

Twenty-four patients responded completely and 22 patients did not respond at all. No short- or long-term toxic effects were observed. Acute side effects associated with conventional AmB therapy (e.g., fever, chills, and potassium loss) were infrequent and milder in patients given L-AmB than commonly observed in patients given conventional AmB. No chronic renal, hematologic, or CNS side effects were observed following therapy with L-AmB.

L-AmB is effective and less toxic than free AmB in the treatment of fungal infections caused by C. albicans and Aspergillus niger, even in patients with neutropenia. The administration of L-AmB allowed for antileukemic treatment despite the presence of an active fungal infection and chemotherapy-induced neutropenia, which usually compromises treatment of the fungal infection. L-AmB therapy is easier for patients than AmB therapy, in part because of lower fluid volumes and shorter intravenous infusion times, which enable continuation of antifungal treatment on an outpatient basis.

Recently, L-AmB has been reformulated and marketed as a new lipid complex of amphotericin B (amphotericin B lipid complex [ABLC]; Abelcet; The Liposome Company, Princeton, NJ). ABLC consists of amphotericin B complexed

Characteristics of Liposomes That Influence Their Behavior
with two lipids, DMPC and DMPG, in a drug-to-lipid molar ratio of 1:1; DMPC and DMPG are present in a molar ratio of 7:3. The complex is particulate in nature and ribbonlike. It has properties of particulate formulations and has exhibited a higher therapeutic index than that of AmB (Fungizone) itself, and with less toxicity, for certain systemic fungal infections in animals and humans [43–51].

Two other AmB lipidic formulations are also being prepared on a large scale and are available for clinical use. AmB colloidal dispersion (ABCD; Amphocil; Sequus Pharmaceuticals, Menlo Park, CA) is a stable complex of AmB and cholesteryl sulfate in a 1:1 molar ratio. ABCD [52] has equivalent antifungal activity but is less toxic than the commercially available form of AmB, AmB plus deoxycholate (Fungizone). In vitro studies have shown that the drug-lipid complex does not hemolyze erythrocytes and binds less to plasma lipoproteins than does the conventional form of AmB [53, 54].

Studies of healthy volunteers indicated that the drug disposition of ABCD was similar to that of Fungizone. Acute side effects of ABCD were comparable with those of AmB but occurred at doses of 1.5 mg/(kg·d) vs. 0.5–0.75 mg/(kg·d). The renal toxicity of ABCD is believed to be reduced because the AmB is bound as a cholesterol complex, so less “free” drug is available to interact with renal tubules [54].

AmBisome (Vestar, San Dimas, CA) [53, 55] is a liposomal small unilamellar vesicle formulation, supplied as a lyophilized powder, which must be reconstituted before intravenous infusion. It is the only liposomal AmB preparation currently licensed in the United Kingdom. The liposomes consist of hydrogenated soy phosphatidylcholine, cholesterol, distearoyl phosphatidylglycerol, alpha-tocopherol, sucrose, and disodium succinate hexahydrate. A starting dose of 1.0 mg/(kg·d) has been recommended, increasing to 3.0 mg/(kg·d), although doses up to 5.0 mg/(kg·d) have been used in compassionate studies when use of the conventional AmB preparation led to unacceptable toxic effects [55–57]. The highest concentrations of AmBisome are found in the liver and spleen; however, concentrations in the lung and kidney are highly inconsistent.

Phagocyte Transport of L-AmB

Liposomes are avidly taken up by phagocytes in the circulation and in tissues. We and others [58–60] have shown that liposomes are distributed in animals and humans in organs rich in mononuclear phagocyte system cells. We previously observed that liposome incorporation enhanced the delivery of AmB to Candida-infected organs in mice [61]. A potential exists, therefore, that monocytes/macrophages in peripheral blood may take up the drug-laden liposomes and transport them to the infected sites.

The in vitro uptake of AmB and L-AmB by murine peritoneal macrophages was studied by Mehta et al. [62]. Resident peritoneal macrophages were incubated at several time intervals with L-AmB or AmB in RPMI 1640 supplemented with 10% fetal calf serum. After each time interval, the supernatants were discarded and the monolayer was washed three times with warm PBS. The cells were then lysed, and radioactivity in the cell lysates was measured. Maximal uptake of L-AmB was observed after 8 hours, with a gradual decrease from 24 to 72 hours. No uptake of AmB was observed.

These data are in agreement with the findings of our work with murine peritoneal macrophages and human peripheral blood monocytes [63]. In both cases we observed that the macrophages’ liposome-uptake capacity was maximal at 8 hours, with a lipid-saturation capacity of 100 μg of lipid per million macrophages. However, recovery of the drug in organs rich in mononuclear phagocytes does not necessarily mean that the drug is taken up by phagocytes in vivo.

In the setting of neutropenia, phagocytic transport is less likely to play a major role. Furthermore, since resident macrophages are less sensitive to cytotoxic agents than are other cell types, we believe that in the neutropenic setting a different transport of AmB (in terms of plasma lipoproteins) may exist.

Lipoprotein Transport of L-AmB

Serum lipoproteins have been shown to influence the pharmacokinetics, tissue distribution, and pharmacological activity of a number of lipophilic compounds, including AmB [24, 64]. In humans, AmB’s large volume of distribution (4 L/kg) seems to be a result of its high accumulation in the kidney, liver, and lung tissues [65]. Injection of drug-free liposomes (DMPC:DMPG weight per weight [w/w] ratio, 7:3) into the human circulation has resulted in a large volume of distribution and a long terminal half-life [58]. When AmB was injected intravenously into mice, only 15% of the original dose could be accounted for, 10% in the lung and 5% in the liver [66].

Furthermore, pharmacokinetics studies in humans have shown AmB to have a long terminal half-life (15 days) and a very short distribution half-life [65]. Intravenous injection of AmB into animals has resulted in slow or sustained release of the drug and alterations of tissue kinetics and distribution [67]. It has been suggested that AmB’s pharmacokinetics may be a reflection of its slow release from a tissue or organ site due to the high-affinity binding of the drug to cholesterol in serum lipoproteins or cell membranes [26, 68–70].

Brajttburg and co-workers [26] examined the interactions of AmB with human serum lipoproteins in vitro in an attempt to understand these interactions and how they might affect the pharmacological behavior of AmB. Their studies showed AmB to be equally associated with high-density lipoprotein (HDL) and low-density lipoprotein (LDL) after 1 hour of incubation at 25°C [26]. Furthermore, AmB injected in LDL was toxic to rabbits: 70% of the rabbits died of a nontoxic dose of AmB (1.0 mg/kg), which implies that LDL association would increase the toxicity of AmB [69]. Data from further studies by Barwicz and co-workers support this observation [71].
The results we obtained demonstrated that changes in temperature and liposomal lipid composition affect the distribution of AmB in serum lipoproteins [72]. In brief, serum obtained from healthy volunteers was incubated with known concentrations of AmB or different liposomal formulations of AmB (1–100 µg/mL) at 37°C for various periods of time (5, 10, 20, 30, 45, and 60 minutes). At the end of each incubation period, serum was removed and separated into HDL and LDL fractions by affinity chromatography. AmB in each lipoprotein fraction was quantified by HPLC, and the lipoprotein content was assessed.

Equal distribution of AmB was found in the human serum lipoprotein fractions following 1 hour's incubation at 25°C (51.0% ± 6.0% in HDL fraction and 48.8% ± 7.0% in LDL fraction). In contrast, >90% of the concentration of AmB was found in the HDL fraction following 1 hour's incubation at 37°C. AmB was differentially distributed between HDL and LDL by apparent lipid-surface charge. AmB incorporated into liposomes composed of negatively charged lipids showed an HDL:LDL ratio of 9:1. Liposomes composed only of negatively charged lipids showed an HDL:LDL ratio of 1:1. Liposomes composed of only neutral and positively charged lipids showed an HDL:LDL ratio of 6:4.

Studies were subsequently conducted in which human serum was incubated with L-Amb (DMPC:DMPG w/w ratio of 7:3, with a DMPG:AmB molar ratio of 4:1) for 60 minutes at 37°C. The serum was separated into its lipoprotein fractions, and DMPG and AmB were quantified by HPLC; 90% of the drug and 80% of the lipid were found in the HDL fraction in a 3:1 molar ratio (DMPG:AmB), vs. a 6:1 molar ratio (DMPG:AmB) in the LDL fraction. These experiments further suggested that AmB and DMPG may cotransfer as an intact drug-lipid complex to serum lipoproteins.

The modification of the distribution of AmB to serum lipoproteins at 37°C may be related to the transition temperature of lipoproteins, which is between 27°C and 34°C [73, 74]. At the transition temperature, CEs within the lipoprotein core exist as an isotropic solution, while below this temperature they form disordered smectic liquid crystals [73, 74]. The core of HDL becomes more ordered at the higher temperature, thus making it easier for the AmB molecule to associate with it. This hypothesis is based on the assumption that AmB is incorporated into the lipophilic core of these lipoproteins.

DMPG as an anionic exogenous phospholipid may distribute into HDL (as opposed to LDL) and be partially responsible for the concurrent transport of AmB to HDL. Since HDL and LDL are not found in an equimolar ratio in human serum but at an LDL:HDL ratio of 6:1 [72], the data suggest that some mechanism besides random probability must drive this drug-liposome complex toward HDL rather than LDL. When L-Amb (DMPG:AmB molar ratio, 4:1) was incubated for 1 hour at 37°C in human serum, AmB and DMPG seemed to cotransfer to the serum lipoproteins [72]. These observations suggest that phospholipids with a negative charge may be responsible for the altered AmB-lipoprotein distribution patterns.

Furthermore, the DMPG:AmB molar ratio found in the lipoprotein fractions is similar to the initial molar ratio of the liposomes before incubation, which suggests that the drug-lipid association remains intact as the complex travels to HDL.

Recent work by Barwicz and co-workers has suggested that AmB complexation with LDL and very-low-density lipoproteins (VLDLs) may be responsible for the nephrotoxicity of AmB in vivo and that hindering this complex formation results in a decrease in such nephrotoxicity [71].

The rate at which AmB appears in the HDL fraction increases when AmB is incorporated in a liposome composed of neutral and negatively charged phospholipids at a pH level of 7.4. Morton and Zilversmit have also demonstrated that a highly purified lipid transfer protein (LTP) facilitates the transfer of CE, triglyceride, and phospholipids between lipoprotein classes [75, 76]. They demonstrated that LTP interacts with HDL, LDL, and VLDL, and the HDL-LTP interaction is the most likely to occur [75, 76].

This interaction is attributed to HDL's ability to attract negatively charged particles (as the negative charge density increases on the surface of the lipoprotein, the lipoprotein-LTP interaction is more prominent) as well as to LTP's tendency to associate with negatively charged particles [77]. The interaction between HDL and LTP appears to be reversible [75, 76]. In addition, work by Surewicz and co-workers has suggested the formation of thermally stable complexes between anionic phospholipids such as DMPG and apolipoprotein AI, one of the predominant protein components associated with HDL [78].

We have observed that when Fungizone (AmB and sodium deoxycholate) and sodium deoxycholate were incubated for 120 minutes at 37°C in delipidated human serum containing 0.64 µg of total protein per mL of LTP, the transfer of CE from HDL to LDL was not impaired (figure 1A) [79]. However, L-Amb at all concentrations containing >10 µg of AmB per mL significantly decreased the transfer of CE compared with that in controls [81]. Since AmB interacts with cholesterol and CE [80] and the transfer of CE between lipoproteins is regulated by LTP [75, 76], we conducted studies to determine (1) the influence of LTP on the distribution of AmB between HDL and LDL and (2) the influence of liposomal-lipid surface charge on LTP-regulated transfer of CE from HDL to LDL.

Our results demonstrated that the presence of LTP facilitates the transfer of AmB (incubated as Fungizone) between HDL and LDL. The addition of LTP resulted in increased distribution of AmB to the LDL fraction (table 1). Furthermore, the presence of Fungizone or sodium deoxycholate alone did not reduce the CE-transfer activity of LTP (figure 1A) [79]. These observations suggest that the redistribution of AmB from HDL to LDL may be regulated by LTP. Furthermore, previous investigators have suggested that AmB interacts with CE and cholesterol [80] upon incubation in human serum, thus supporting...
AmB-containing positively charged liposomes (DMPC:stearyl-
lanthine w/w ratio, 7:1) or negatively charged liposomes
(DMPC:DMPG w/w ratio, 7:3) decreased LTP-regulated trans-
fer of CE from HDL to LDL and therefore prevented the trans-
fer of AmB from HDL to LDL (figure 1B) [79].

These observations may be explained in part by the influence
of lipid surface charge on lipid transfer among lipoproteins.
Billheimer and Gaylor [81] observed the decrease in transfer of
CE between HDL and LDL in the presence of both DMPC
and DMPG liposomes. Those investigators found that phospha-
tidylglycerol increases the exchange of CE between HDL and
the liposome in the presence of LTP but not in the absence
of LTP. The presence of unsaturated acyl chains in the phos-
pholipid enhances exchange. However, neutral phospholipids, such
as sphingomyelin, drastically decrease cholesterol exchange
with the liposome.

The presence of positively and negatively charged phospho-
lipids in liposomes results in the reduction of LTP-mediated
transfer of CE from HDL to LDL; since AmB interacts with
CE, this finding may explain in part the lesser distribution of
AmB into LDL when AmB is incorporated into these lipo-

Figure 1. A, effect of AmB (AmB + sodium deoxycholate, — –), sodium deoxycholate (––), and liposomal AmB (●) on the
cholesteryl ester transfer from high-density lipoproteins (HDL) to
low-density lipoproteins (LDL). The asterisk indicates P < .05, vs.
AmB (AmB + deoxycholate) and deoxycholate alone. B, effect of
liposomal-lipid charge on the cholesteryl ester transfer from HDL to
LDL. Dimyristoyl phosphatidylcholine (DMPC [neutral], ––), di-
myristoyl phosphatidylglycerol (DMPG [negative], ––),
DMPC:DMPG wt/wt ratio of 7:3 (negative, ––), or DMPC:stearyl-
lanthine (SA) wt/wt ratio of 7:1 (positive, ––) liposomes were incu-
bated in human plasma for 60 minutes at 37°C. The asterisk indicates
P < .05, vs. DMPC:SA liposomes. Data in A and B are expressed
as mean ± SD (number of individual experiments = 6).

Biological Importance of AmB’s Association with Lipoproteins

Initial work by others has suggested that the renal toxicity
of AmB can be influenced by liposomal charge, phospholipid
acyl chain length, chain saturation, and the liposomal-lipid/
AmB ratio [30, 31, 82–84]. For example, AmB-containing
liposomes composed of phospholipids with unsaturated acyl
chains are as toxic as AmB to mammalian cells; however,
those composed of phospholipids with saturated acyl chains
are less toxic [30, 85]. Previous studies have demonstrated a
decrease in cytotoxicity of AmB when the drug is delivered
in the form of L-AmB to LLC PK1 cells (a pig kidney epithe-

telial cell line) [30, 86] and to primary cultures of rabbit prox-
imal tubule cells [87].

The mechanisms that result in the decreased renal cyto-
toxic effects of L-AmB are not fully understood. Krause and Juliano
[86] have suggested that the decrease in toxicity of L-AmB
compared with that of AmB is related to a selective transfer
of the drug from liposomes to fungal but not mammalian cell
membranes. This selective toxicity toward the fungal mem-
brane is probably regulated by physical characteristics of the
donor and of the target membrane [30]. Brajtburg and co-
workers demonstrated that AmB is highly bound to plasma
lipoproteins [26] and that AmB-induced cytotoxic effects on
mammalian RBCs but not on C. albicans cells decreased in
the presence of either HDL or LDL [88].

Previously, we demonstrated that HDL-associated AmB and
HDL-associated L-AmB are less toxic to LLC PK1 renal cells
than are AmB or LDL-associated AmB (figure 2) [89]. The
reduced toxicity of HDL-associated AmB may be explained
by the low level of expression of HDL receptors on LLC PK1

the hypothesis that it is AmB-associated CE that is being trans-
ferred from HDL to LDL by LTP.

Association of AmB with HDL increases when AmB is
incorporated in positively or negatively charged lipid carriers
[72]. However, we found that the addition of LTP facilitated
only a minimal transfer of AmB from HDL into LDL when
AmB was incorporated into liposomes composed of positively
charged phospholipids (data not shown) or negatively charged
ones (table 1) [79]. Furthermore, the presence of empty or
Table 1. Effect of lipid transfer protein (LTP) on the distribution of AmB and L-AmB in serum lipoproteins after 60 minutes of incubation in pooled human serum.

<table>
<thead>
<tr>
<th>Lipoprotein fraction</th>
<th>Percentage of initial AmB concentration</th>
<th>Percentage of initial L-AmB concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No LTP added</td>
<td>LTP (0.64 μg/mL) added</td>
</tr>
<tr>
<td>HDL</td>
<td>74 ± 0.5</td>
<td>48.6 ± 4.9*</td>
</tr>
<tr>
<td>LDL</td>
<td>22 ± 5.5</td>
<td>45.6 ± 4.4*</td>
</tr>
</tbody>
</table>

NOTE. Table is modified from [79]. Data are means ± SD (n = 6). AmB = amphotericin B; HDL = high-density lipoproteins; L-AmB = liposomal amphotericin B; LDL = low-density lipoproteins. * P < .05, vs. AmB with no LTP added.

cells [89]. The sustained toxicity observed with AmB alone in trypsinized cells may be related to a direct membrane effect. However, when AmB is associated with LDL, the toxicity is maintained, which suggests that both direct membrane-related and non-membrane-related toxicity may occur.

HDL- and LDL-associated AmB were equally toxic to fungal cells, which suggests that the presence of lipoproteins does not alter the antifungal activity of AmB. Such effects may be related to the liberation of monomeric AmB associated with lipoproteins or L-AmB, by fungal [31, 85] or endothelial-derived phospholipases [31, 85]. The low concentrations of unbound and water-soluble monomeric AmB present in L-AmB [82, 90, 91] may be sufficient for fungal toxicity but not adequate for forming AmB aggregates that are toxic to mammalian cells [85, 86]. AmB complexed with lipid is less toxic than the self-associated form of AmB in medium, but the monomeric form of AmB interacts with fungal cell membranes and is nontoxic against mammalian cell membranes, as shown by Bolard et al. [90].

Differences in Lipoprotein Concentration and Composition Alter the Plasma Pharmacokinetics and Distribution of L-AmB

Differences in the pharmacokinetics and tissue distribution of free AmB were demonstrated in healthy vs. hyperlipidemic rats with induced diabetes. In contrast, the pharmacokinetics and tissue distribution of L-AmB were unchanged in diabetic rats, which suggests an independence of this delivery mechanism from the diabetic disease state and endogenous triglyceride and cholesterol levels [24]. However, a limitation of this study was that we could not determine if changes in the pharmacokinetics and tissue distribution of AmB were a direct result of the plasma hyperlipidemia or other diabetes-associated physiological alterations (e.g., in blood flow, liver metabolism, and renal metabolism).

To determine if the pharmacokinetics and tissue distribution of AmB and L-AmB were altered in the setting of plasma dyslipidemia (hypercholesterolemia) independent of other physiological alterations, rats were administered a continuous infusion of Intralipid (Clinteck Nutrition, Deerfield, IL). Intralipid is a fatty acid/triglyceride emulsion administered intravenously as a nutritional supplement to debilitated patients. We found that continuous infusion of Intralipid in rats for 5 days resulted in an increase in the total serum cholesterol and HDL cholesterol concentrations but did not alter the LDL cholesterol or total serum triglyceride concentrations [92].

The influence of 5% Intralipid and 0.45% normal-saline infusions on the concentration in serum and distribution in tissue of AmB (Fungizone, consisting of AmB and sodium deoxycholate) and L-AmB in rats was compared (table 2) [93]. In animals receiving a continuous Intralipid infusion, concentrations of AmB in kidneys and lungs were significantly higher, but the concentration of AmB in serum was significantly lower in animals administered AmB than in those given L-AmB. In animals receiving a continuous normal-saline infusion, concentrations of AmB in kidneys and the spleen were significantly higher, but the concentration of AmB in serum was significantly lower in animals administered AmB than in those given L-AmB. These results suggest that the increase in total serum cholesterol and HDL cholesterol levels during the Intralipid infusion decreased the clearance of AmB from the bloodstream and decreased the L-AmB concentration in the kidney and lung.

Development of Liposomal Nystatin

Plasma Distribution of Free and Liposomal Nystatin

Unlike AmB, when free nystatin (figure 3A) dissolved in 1% methanol at a concentration of 20 μg/mL was incubated
in human plasma for 5 and 120 minutes at 37°C, >65% of the initial concentration was retained within the lipoprotein-deficient plasma fraction, which contains mainly aqueous plasma proteins (e.g., albumin and a-1 glycoprotein) (figure 3A). This plasma distribution pattern does not significantly change when nystatin is incorporated into liposomes composed of negatively charged and neutral phospholipids. The nystatin found in the lipoprotein fraction is mostly retained within the HDL fraction. However, equivalent concentrations of the drug per mg of lipoprotein-cholesterol were found in the HDL and LDL/VLDL plasma fractions when it was incubated in the plasma as free nystatin (figure 3B). Following 2 hours of incubation, the majority of the lipoprotein-associated nystatin appears within the HDL (figure 3B). These findings suggest that an alternative mechanism, independent of lipoprotein mass, may be involved in determining the plasma distribution of these compounds.

Pharmacokinetics of Liposomal Nystatin in HIV-Infected Patients

A phase I clinical trial was completed that studied the safety, tolerance, and pharmacokinetics of a single dose of liposomal nystatin in 17 patients with HIV infection [94]. Four patient groups were studied, each at one of the following dose levels: 0.25, 0.5, 0.75, and 1 mg/kg. Patients were hospitalized during the first day of the study and then followed up as outpatients daily for 1 week. None of the patients experienced any significant neurological, cardiac, pulmonary, or renal toxic effects at any of the dose levels studied.

Pharmacokinetic analysis (table 3) shows that after liposomal nystatin is administered, the drug appears to be distributed initially into the blood circulation and to be cleared within a terminal half-life of 5 hours. Furthermore, the kinetics of liposomal nystatin appear to be dose-independent in the range of 0.5 to 1 mg/kg; however, the area under the concentration-time curve appears to increase in direct proportion to the dose administered for doses ranging from 0.25 mg/kg to 0.75 mg/kg, which suggests that in this dose range there is no apparent saturation of drug-clearance mechanisms.

However, area under the concentration-time curve does not increase when patients are administered 0.75 mg/kg vs. 1.0 mg/kg, suggesting a saturation of drug clearance once a dose of ≥0.75 mg/kg is administered. In addition, an increase in terminal half-life as the dose increases further argues for a saturation in drug-clearance mechanisms. These results suggest that tissue exposure to liposomal nystatin increases with increasing doses of this drug, up to 0.75 mg/kg. With potentially enhanced tissue distribution, greater intracellular concentrations of nystatin may be achieved.

Development of Liposomal Annamycin

Anthraccline compounds, such as doxorubicin, are effective anticancer agents used in the treatment of human leukemia, lymphoma, breast carcinoma, and soft-tissue sarcoma [95–97]. However, their use has been limited by acute myelosuppression, chronic cardiotoxicity [98], and multidrug resistance [99, 100]. The emergence of such resistance has been linked to the overabundance of the cell membrane P-glycoprotein, which acts as an energy-dependent drug efflux pump [101].

Several approaches have been investigated to reduce the cardiotoxicity and to overcome the multidrug resistance associated with these compounds. The incorporation of doxorubicin into liposomes has resulted in decreased cardiotoxicity (compared with that of its free counterpart) without altering its efficacy [18, 102, 103]. Furthermore, a number of liposomal doxorubicin formulations have been reported to overcome multidrug resistance [104, 105]. An alternative way to overcome such resistance is the use of analogs that are not substrates for P-glycoprotein. Annamycin was synthesized with such properties in mind [106–108]. Annamycin is a lipophilic and non-

| Table 2. Tissue distribution and serum concentrations of AmB or L-AmB (1.0 mg/kg) in rats receiving continuous infusion of 0.45% normal saline (NS) or 5% Intralipid for 7 days. |
| Variable | 0.45% NS | 5% Intralipid | 0.45% NS | 5% Intralipid |
| Kidney | 735.1 ± 285.6 | 657.8 ± 210 | 298.2 ± 96.2<sup>†</sup> | 155.9 ± 56.3<sup>†</sup> |
| Liver | 468.2 ± 169.2 | 587 ± 305.5 | 398.0 ± 280.4 | 667.9 ± 665.3 |
| Lung | 1,906.4 ± 1,526.8 | 1,799.3 ± 1,605 | 695.3 ± 250.2 | 160.1 ± 46.2<sup>†</sup> |
| Spleen | 2,112.9 ± 403.4 | 2,242.5 ± 1,445 | 906.6 ± 52.6<sup>†</sup> | 1,489 ± 419.9<sup>†</sup> |

Serum concentration (ng/mL)

AmB | 70.01 ± 13.7 | 139.3 ± 15.4* | 298.2 ± 96.2<sup>†</sup> | 155.9 ± 56.3<sup>†</sup> |
| L-AmB | 183.7 ± 36.9<sup>†</sup> | 304.7 ± 20.3<sup>†</sup> |

NOTE. Table is modified from [93]. Data are means ± SD (n = 5). AmB = amphotericin B (Fungizone; amphotericin + sodium deoxycholate); L-AmB = liposomal amphotericin B.

* P < .05, vs. AmB + 0.45% NS infusion.
† P < .05, vs. AmB + 5% Intralipid infusion.
cross-resistant anthracycline antibiotic currently in clinical development as a liposomal formulation (L-annamycin) composed of DMPC and DMPG.

**Biological Activity of Liposomal Annamycin**

L-annamycin has shown markedly enhanced antitumor activity compared with that of doxorubicin in several in vivo tumor models, including human xenografts that express the MDR 1 phenotype [108]. Preliminary studies have demonstrated that annamycin or L-annamycin have a significantly higher cellular uptake compared to that of doxorubicin [107]. The incorporation of annamycin into liposomes prolongs the terminal serum half-life of annamycin compared to that of free annamycin and doxorubicin [106]. In addition, the incorporation of annamycin into these liposomes resulted in a markedly increased tumor uptake of annamycin as compared with uptake of free annamycin and doxorubicin [18]. However, an explanation for the altered pharmacokinetics, organ distribution, and pharmacodynamics of L-annamycin [106–108] has not been found.

**Interaction with Plasma Lipoproteins**

Our studies with AmB [24, 93] and another lipophilic compound, cyclosporine [109], suggest that the pharmacokinetics and tissue distribution of these compounds appear to be influenced by their interaction with plasma lipoproteins. Furthermore, the plasma lipoprotein distribution of AmB seems to be influenced by LTP [79]. To determine if these findings extend to other lipophilic compounds, we conducted studies to determine the plasma distribution of doxorubicin, annamycin, and L-annamycin [110, 111] and the influence of LTP on the plasma distribution of annamycin and L-annamycin [110].

Our results demonstrated that when annamycin was incorporated into liposomes composed of DMPC and DMPG, more than two-thirds of the initial annamycin concentration would distribute into the HDL fraction (table 4), and the distribution of annamycin and L-annamycin within human plasma was independent of LTP activity [110]. We further observed that elevation of plasma LDL-cholesterol or VLDL-triglyceride concentrations increased the amounts of

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>No. of patients</th>
<th>No. of minutes per half-life phase</th>
<th>Cpo (µg/mL)</th>
<th>AUC (µg/mL x min)</th>
<th>Vd (L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>4</td>
<td>9.7 ± 1</td>
<td>3.7 ± 0.3</td>
<td>452 ± 86</td>
<td>5.2 ± 0.7</td>
</tr>
<tr>
<td>0.50</td>
<td>4</td>
<td>12 ± 4.8</td>
<td>5.9 ± 0.8</td>
<td>732 ± 172</td>
<td>7.7 ± 1.5</td>
</tr>
<tr>
<td>0.75</td>
<td>4</td>
<td>10.2 ± 4.2</td>
<td>6.5 ± 0.5</td>
<td>1,274 ± 483</td>
<td>10 ± 0.8</td>
</tr>
<tr>
<td>1.0</td>
<td>4</td>
<td>7.3 ± 3</td>
<td>9.0 ± 0.9</td>
<td>1,263 ± 301</td>
<td>9.1 ± 1.6</td>
</tr>
</tbody>
</table>

**Table 3.** Pharmacokinetic analysis of single doses of liposomal nystatin injected intravenously into HIV-infected patients.

NOTE. Table is modified from [94]. Data are means ± SD. AUC = area under the concentration-time curve; Cpo = drug concentration at time 0; Vd = volume of distribution.
Table 4. Distribution of annamycin and liposomal annamycin at different concentrations within human plasma following incubation for 60 minutes at 37°C. Following incubation, plasma samples were assayed by fluorimetry for drug in each of the lipoprotein and lipoprotein-deficient plasma (LPDP) fractions.

<table>
<thead>
<tr>
<th>Drug compound, concentration (µg/mL)</th>
<th>LPDP Percentage of initial drug concentration, per indicated plasma fraction</th>
<th>HDL Percentage of initial drug concentration, per indicated plasma fraction</th>
<th>LDL Percentage of initial drug concentration, per indicated plasma fraction</th>
<th>VLDL Percentage of initial drug concentration, per indicated plasma fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annamycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>55.9 ± 8.1</td>
<td>9.1 ± 3.1</td>
<td>8.7 ± 4.4</td>
<td>17.8 ± 5.1</td>
</tr>
<tr>
<td>10</td>
<td>48.7 ± 2.9</td>
<td>14.1 ± 4.0</td>
<td>11.5 ± 1.1</td>
<td>29.0 ± 7.0</td>
</tr>
<tr>
<td>20</td>
<td>50.5 ± 3.6</td>
<td>11.2 ± 5.0</td>
<td>11.5 ± 1.7</td>
<td>22.1 ± 1.0</td>
</tr>
<tr>
<td>Liposomal annamycin*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10.8 ± 5.5</td>
<td>89.8 ± 11.0</td>
<td>1.1 ± 1.0</td>
<td>1.8 ± 1.4</td>
</tr>
<tr>
<td>10</td>
<td>10.9 ± 4.4</td>
<td>79.8 ± 7.1</td>
<td>5.4 ± 2.0</td>
<td>5.4 ± 2.2</td>
</tr>
<tr>
<td>20</td>
<td>15.4 ± 4.0</td>
<td>68.7 ± 4.1</td>
<td>9.0 ± 4.1</td>
<td>6.7 ± 5.2</td>
</tr>
</tbody>
</table>

NOTE. Table is modified from [110]. Data are means ± SD (n = 5). HDL = high-density lipoproteins, LDL = low-density lipoproteins; VLDL = very-low-density lipoproteins.

* Composed of phospholipids DMPC and DMPG (dimyristoyl phosphatidylcholine and dimyristoyl phosphatidylglycerol in a lipid:drug ratio of 15:1 (wt/wt).

† Percentage of initial drug concentration incubated.

Table 5. Distribution of annamycin (20 µg/mL) after 60 minutes of incubation at 37°C within human plasma enriched or preenriched with low-density lipoproteins (LDL) or very-low-density lipoproteins (VLDL). Annamycin either was incubated in plasma pretreated with LDL or VLDL 24 hours prior to the experiment or was incubated at the same time as LDL or VLDL for 60 minutes.

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Percentage of initial annamycin concentration, per indicated fraction</th>
<th>Percentage recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HDL + plasma component</td>
<td>LDL</td>
</tr>
<tr>
<td>Nontreated</td>
<td>62.9 ± 10.8</td>
<td>6.6 ± 4.5</td>
</tr>
<tr>
<td>LDL-enriched*</td>
<td>30.3 ± 5.3</td>
<td>64.5 ± 4.3</td>
</tr>
<tr>
<td>VLDL-enriched*</td>
<td>18.2 ± 6.9</td>
<td>12.8 ± 2.5</td>
</tr>
<tr>
<td>Preenriched with LDL*</td>
<td>50.9 ± 3.5</td>
<td>37.9 ± 8.7</td>
</tr>
<tr>
<td>Preenriched with VLDL*</td>
<td>48.9 ± 4.1</td>
<td>9.9 ± 0.7</td>
</tr>
</tbody>
</table>

NOTE. Table is modified from [111]. Data are means ± SD (n = 5). HDL = high-density lipoproteins.

* Percentage of initial annamycin concentration incubated.

† Percentage of initial annamycin concentration incubated.

§ Drug and LDL (1,288 µg of LDL-cholesterol per mL of plasma) were added to plasma at the same time.

II LDL (1,288 pg of LDL-cholesterol per mL of plasma) was added to plasma and incubated for 24 hours at 37°C prior to the experiment.

VLDL (300 pg of VLDL-triglyceride per mL of plasma) was added to plasma and incubated for 24 hours at 37°C prior to the experiment.

These results suggest that annamycin and L-annamycin lipoprotein distribution may not be regulated by a mechanism that involves LTP but by mass plasma lipoprotein levels.

Since many patients with cancer have hypertriglyceridemia, these findings may provide an explanation for the alterations in pharmacokinetics and pharmacodynamics seen with administration of L-annamycin.

Summary

Lipid-based drug formulations composed of DMPC and DMPG are distributed in plasma lipoproteins upon incubation.
in plasma. Changes in plasma triglyceride and cholesterol concentrations not only may alter plasma distribution of these formulations but also may have a bearing on their pharmacokinetics and therapeutic index. For example, to understand the therapeutic importance of AmB's association with plasma lipoproteins, the influence of lipoproteins on its antifungal activity and renal cytotoxicity was examined.

The antifungal activity of AmB and L-AmB was not altered in the presence of HDL or LDL. However, AmB was less nephrotoxic when associated with HDL but still remained equally as toxic as free AmB when associated with LDL [89]. This reduced nephrotoxicity appears to be related to the drug's decreased uptake by renal cells when it is associated with HDL, which may be the result of these cells' low expression of HDL receptors [89].

These findings suggest that the ratio of HDL-associated vs. LDL-associated polyenes is important in terms of its renal toxicity. Toxic effects on the kidney may be due to higher relative exposure of the drug to renal cells when given as free AmB (Fungizone) rather than as lipid-associated drug. It appears that in the case of L-AmB, because of its association with HDL, less drug is available to be targeted to sites of toxicity in the face of increased delivery to infected and inflammatory sites. The presence of negatively charged liposomes causes the reduction of LTP-mediated transfer of lipids between HDL and LDL and may be responsible for the predominant distribution of AmB into the HDL fraction.

The reduced renal toxicity associated with the administration of L-AmB is most likely related to its increased ratio of association to HDL vs. LDL, as compared with the ratio for Fungizone. The observed regulation of LTP thus may serve to modulate the transfer of lipoprotein-associated drugs.

A second important aspect of liposomal formulations is related to their availability at sites of infection. We have previously shown that AmB accumulated at sites of fungal infections [61] and inflammation [62]. These findings may be related to capillary leakage, phagocyte uptake and transport, or both. Once the particles loaded with the polyenes reach the infected sites, the drug may be exchanged from particle to fungal membranes or released from the particle following exposure to endothelial-derived phospholipases [31, 85].

Furthermore, lipid composition, lipid particle size, phospholipid fluidity, and net phospholipid charge play vital roles in determining the plasma distribution, pharmacokinetics, and pharmacological effects of lipid-based drug formulations. Regardless of the physical nature of these lipid-based compounds (either as disklike particles or lipid complexes), they all behave fundamentally as particles.

Understanding how variations in plasma lipid concentrations and physicochemical properties of lipid-based drug formulations affect their interactions with lipoproteins could help explain the observed changes in the pharmacokinetics and pharmacodynamics of liposomal polyenes following their administration into infected patients with plasma dyslipidemia.

References


Table 6. Distribution of liposomal annamycin (20 µg/mL) after 60 minutes of incubation at 37°C within human plasma enriched or preenriched with low-density lipoproteins (LDL) or very-low-density lipoproteins (VLDL). Liposomal annamycin either was incubated in plasma pretreated with LDL or VLDL. 24 hours prior to the experiment or was incubated at the same time as LDL or VLDL for 60 minutes.

<table>
<thead>
<tr>
<th>Plasma</th>
<th>HDL + plasma component</th>
<th>LDL</th>
<th>VLDL</th>
<th>Percentage recovery †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-treated</td>
<td>78.0 ± 3.3</td>
<td>12.9 ± 3.6</td>
<td>2.9 ± 0.5</td>
<td>93.8 ± 2.5</td>
</tr>
<tr>
<td>LDL-enriched</td>
<td>30.3 ± 5.3 ‡</td>
<td>74.0 ± 4.3‡</td>
<td>5.1 ± 2.4</td>
<td>109.4 ± 4.0‡</td>
</tr>
<tr>
<td>VLDL-enriched</td>
<td>32.8 ± 1.4‡</td>
<td>10.5 ± 4.5</td>
<td>60.1 ± 6.1‡</td>
<td>103.4 ± 4.0‡</td>
</tr>
<tr>
<td>Preenriched with LDL*</td>
<td>31.0 ± 5.3‡</td>
<td>52.9 ± 8.7‡</td>
<td>5.1 ± 2.5</td>
<td>89.0 ± 5.5</td>
</tr>
<tr>
<td>Preenriched with VLDL**</td>
<td>79.5 ± 12.8</td>
<td>12.5 ± 2.0</td>
<td>14.9 ± 7.2‡</td>
<td>106.9 ± 7.3‡</td>
</tr>
</tbody>
</table>

NOTE. Table is modified from [111]. Data are means ± SD (n = 5). HDL = high-density lipoproteins.

* Composed of phospholipids DMPC and DMPG (defined in text and table 4) in a lipid:drug ratio of 15:1 (wt/wt).
† Percentage of initial annamycin concentration incubated.
‡ Drug and LDL (1,288 µg of LDL-cholesterol per mL of plasma) were added to plasma at the same time.
§ P < .05, vs. nontreated plasma group.
∥ Drug and VLDL (300 µg of VLDL-triglyceride per mL of plasma) were added to plasma at the same time.
** LDL (1,288 µg of LDL-cholesterol per mL of plasma) was added to plasma and incubated for 24 hours at 37°C prior to the experiment.

** VLDL (300 µg of VLDL-triglyceride per mL of plasma) was added to plasma and incubated for 24 hours at 37°C prior to the experiment.


