Preferential Distribution of Amphotericin B Lipid Complex into Human HDL_3 Is a Consequence of High Density Lipoprotein Coat Lipid Content

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Abstract □ The purpose of this study was to determine the plasma lipoprotein (LP) distribution of amphotericin B (AmpB) and amphotericin B lipid complex [ABLC; Abelcet composed of dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG)] and define the relationship between LP lipid concentration and composition and the distribution of AmpB and ABLC in human plasma with varying total and lipoprotein cholesterol and triglycerides. AmpB and ABLC at a concentration of 20 μg amphotericin B/mL were incubated in plasma obtained from different human subjects ($n = 7$) for 60 min at 37 °C. Following these incubations plasma samples were separated into their high-density lipoprotein (HDL), triglyceride-rich lipoprotein (TRL; which contains very low-density lipoproteins and chylomicrons), low-density lipoprotein (LDL), and lipoprotein-deficient (LPDP) fractions by density-gradient ultracentrifugation (UC) and each fraction was assayed for AmpB using high-pressure liquid chromatography (HPLC). The HDL fraction was further separated into its HDL_3 and HDL_2 subclasses by UC and assayed for AmpB using HPLC. Separation of HDL into its subclasses was confirmed by gel electrophoresis. To assess the influence of modified lipoprotein concentrations and lipid composition on the plasma distribution of AmpB and ABLC, these compounds were incubated in plasmas from human subjects with varying total and lipoprotein lipid concentrations. In addition, to demonstrate that alterations in HDL lipid composition influence the plasma distribution of ABLC, ABLC (20 μg amphotericin B/mL) was incubated in plasma pretreated with dithionitrobenzoate (DTNB, a compound which inhibits lecithin:cholesterol acyltransferase conversion of HDL_3 free cholesterol to esterified cholesterol) 18 h prior to the experiment or in untreated plasma for 60 min at 37 °C. Total plasma and lipoprotein cholesterol (TC), free cholesterol (fC), esterified cholesterol (CE), triglyceride (TG), phospholipid (PL), and protein (TP) concentrations in each human sample were determined by enzymatic assays. When AmpB was incubated in human plasmas of varying lipid concentrations, the majority of the drug was recovered in the LPDP fraction. However, the majority of AmpB was recovered in the HDL_3 fraction following the incubation of ABLC. Differences in lipid coat content (fC and PL) carried by HDL influenced the distribution of ABLC within plasma of different human subjects. These findings were confirmed by the DTNB treatment experiments. These findings suggest that the association of AmpB with DMPC and DMPG to form drug–lipid complexes modifies the plasma distribution of the AmpB. In addition, the distribution of ABLC among plasma lipoproteins of different human subjects is defined by the HDL lipid coat content and is possibly an important consideration when evaluating the pharmacokinetics, toxicity, and activity of these compounds following administration to humans with differing plasma lipid concentrations.

Introduction

Amphotericin B (AmpB) remains one of the most effective and widely used agents in the treatment of systemic fungal infections including *Candida albicans* and *Histoplasma capsulatum*.[1] The clinical use of AmpB has been limited by dose-dependent nephrotoxicity, which may result in as much as a 60% reduction in the glomerular filtration rate.[1,2] The fungal cytotoxicity of AmpB is related to its amphiphilic structure, which facilitates binding to cell membrane sterols, thereby disrupting membrane integrity, and to its preferential binding to ergosterol in fungal membranes versus cholesterol in mammalian membranes.[3,4] However, when AmpB is formulated into a amphotericin B lipid complex (ABLC) it has been shown to be well-tolerated in doses up to 5 mg/kg of AmpB/kg of body weight and has been effective in patients that failed to respond to conventional AmpB therapy.[5,6]

Plasma lipoproteins are macromolecules of lipid and protein that transport polar and nonpolar lipids through the vascular and extravascular body fluids.[7−9] However, it is well-known that plasma lipoprotein profiles vary considerably between different animal species.[7,8] In addition, disease states can significantly influence plasma lipoprotein profiles, possibly resulting in altered therapeutic outcomes. Current research has shown that lipoprotein association of drug compounds can significantly influence not only the pharmacological and pharmacokinetics of the drug but also the relative toxicity.[7,8]

There is growing evidence that suggests increases in serum cholesterol concentrations increase the renal toxicity of AmpB. Koldin and co-workers demonstrated elevated AmpB-induced nephrotoxicity when AmpB bound to low-density lipoproteins (LDL) was administered to hypercholesterolemic rabbit's compared to AmpB alone.[10] Recent work by our lab suggests that increases in cholesterol, specifically LDL cholesterol levels, modify the disposition and renal toxicity of AmpB alone when administered to hypercholesterolemic rabbits.[11] However, the pharmacokinetics and renal toxicity of ABLC were independent of elevations in total and LDL cholesterol levels.[11] Lopez-Berestein et al. observed that when AmpB was administered to patients with leukemia who exhibited lower serum cholesterol concentrations, AmpB-induced renal toxicity was decreased.[12] Chabot and co-workers observed no measurable renal toxicity when AmpB was administered to cancer patients who exhibited hypocholesterolemia.[13] We have further reported that patients with a higher percentage of AmpB recovered within the serum LDL fraction are more susceptible to AmpB-induced kidney toxicity.[14]

A number of recent studies have suggested that increases in the association of AmpB with serum LDL enhanced the

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ability of AmpB to damage cells. Verlut-Doi et al. demonstrated that internalization of AmpB into Chinese hamster ovary cells in the presence of serum occurred by endocytosis.[15] Kreiger further reported that the cellular uptake of AmpB was a result of LDL-receptor mediated endocytosis.[16] We have reported that LDL-bound AmpB was as toxic to kidney cells as unbound AmpB.[17] However, HDL-bound AmpB was less toxic to these cells than either unbound or LDL-bound AmpB.[17] Furthermore, when the number of LDL receptors expressed on these cells were reduced, LDL-bound AmpB was less toxic to these cells than unbound AmpB,[17] suggesting that LDL receptors may play an important role in promoting the renal toxic affects of AmpB. Taken together, these studies[11,17] suggest an alternative hypothesis on how the ABLC formulation may decrease AmpB-induced renal toxicity than simply maintaining drug in the circulation in a complexed form effectively reducing the free concentration available to exert toxicity and/or by the rapid removal of the lipid complex from the circulation, thus reducing kidney tissue exposure.[5,6]

Our laboratory has recently observed that when ABLC was incubated in human plasma for 5−120 min at 37 °C, the majority of drug was recovered in the HDL fraction.[8] These findings are similar to what was observed with liposomal nystatin and annamycin.[8] A rationale for these results may be related to the similar lipid composition of these formulations [i.e., they all contain dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG)]. We have further observed that the DMPG component of these lipid-based formulations predominantly distributes into HDL because of its interaction with the protein components of HDL (apolipoproteins AI and AII).[8] Since ABLC is composed of the same phospholipids as liposomal nystatin and annamycin, the increased distribution of AmpB into the HDL fraction when formulated into these lipid complexes may also be a result of the attraction of DMPG for apolipoproteins AI and AII.[8,18] This rationale is further substantiated by recent findings which demonstrated that as the amount of HDL protein decreased, the percent of nystatin recovered within the HDL fraction proportionally decreased following the incubation of liposomal nystatin.[19]

The studies presented in this paper determine the plasma lipoprotein distribution of AmpB and ABLC following incubation in plasma from different human subjects with varying total and lipoprotein lipid concentrations and address the role of lipoprotein lipid and protein content on the distribution of AmpB to plasma lipoproteins.

Materials and Methods

Chemicals, Lipids, and Plasma—AmpB formulated as a micelle clear liquid (Fungizone; Bristol Myers Squibb, Princeton, NJ) and as a lipid complex, i.e., amphotericin B lipid complex (ABLC; Abelcet; The Liposome Company Inc., Princeton, NJ), were purchased from Vancouver General Hospital, Department of Pharmaceutical Sciences. The British Columbia Red Cross provided human plasma from seven different human volunteers not known to have preexisting hyperlipidemia. Organic solvents (methanol, etc.) were purchased from Fisher Canada. Ultracentrifugation supplies (i.e., centrifuge tubes, density gradient solutions) were purchased from Beckman Canada. Lipid and protein analysis kits were purchased from Sigma Chemical (St. Louis, MO). Free cholesterol and phospholipid analysis kits were purchased from Boehringer Mannheim (Germany).

Heterogeneity of Human Plasma Lipoprotein Profiles—Blood collected from healthy human volunteers (Screened by British Columbia Red Cross) was placed in drug-free glass test tubes, which contained 0.05M EDTA and centrifuged using a tabletop centrifuge for 10 min at 2,000 rpm; plasma was stored at −20 °C until used in the study.

Lipoprotein Separation—*Lipoprotein Separation by Step-Gradient Ultracentrifugation*—For the separation of lipoprotein plasma components by step-gradient ultracentrifugation, sodium bromide density solutions were carefully layered on top of the plasma samples in order of highest to lowest density. The density of the plasma samples was initially altered such that it had the greatest density of all layers in the gradient. The samples were ultracentrifuged overnight, and separation of the lipoprotein fractions was accomplished in a single spin. Each distinct layer was removed and separated for further analysis.

(i) Treatment of Plasma with AmpB or ABLC—To an ultraclear centrifuge tube (Beckman Instruments, Inc., Palo Alto, CA) was added 2.88 or 3 mL of human plasma for sample ($n = 3$ for AmpB and $n = 3$ for ABLC) or standard curve ($n = 6$) purposes, respectively. The contents of all tubes were prewarmed to 37 °C. To the samples tubes was added either 12 μL of an AmpB solution (5 mg/mL) or 12 μL of an ABLC suspension (5 mg/mL). The final concentration of AmpB in human plasma for both AmpB and ABLC formulations was 20 μg/mL. Immediately after the addition of AmpB or ABLC, the samples were returned to 37 °C and incubated for 60 min, whereupon they were removed and cooled on ice for 30 min.

(ii) Separation of Lipoprotein Constituent—Briefly, solutions of all densities (density [δ] = 1.006, 1.063, and 1.21 g/mL) were stored at 4 °C prior to the layering of the gradient. To the previously cooled plasma used for standard curves and the plasma samples was added 1.02 g of accurately weighted sodium bromide (0.34 g of sodium bromide per 1.0 mL of plasma) in order to modify the density of the plasma to approximately 1.25 g/mL.[19] Once the sodium bromide had dissolved into the plasma, 2.8 mL of the highest density sodium bromide ($\delta = 1.21$ g/mL) was carefully layered on top of the plasma. By using the same volume of 2.8 mL, the next highest sodium bromide solution ($\delta = 1.063$ g/mL) was layered on top of the sample, followed by 2.8 mL of the lowest density solution ($\delta = 1.006$ g/mL). The ultracentrifuge tubes were balanced, placed into individual titanium buckets, and capped. The buckets were placed into their respective positions on a SW 41 Ti swinging bucket rotor (Beckman Instruments, Inc.) and centrifuged at 40 000 rpm for 18 h at a temperature of 15 °C in a Beckman L8−80M Ultracentrifuge (Beckman Instruments, Inc.) Upon completion of the run, the ultracentrifuge tubes were carefully removed from the titanium buckets. Each tube showed four visibly distinct regions represented by the triglyceride rich lipoprotein (TRL, which includes very low-density lipoproteins and chylomicrons), LDL, HDL, and lipoprotein-deficient plasma (LPDP) fractions. Subsequently, each of the layers was removed with a Pasteur pipet from the top to the bottom layer, respectively, and the volume of each of the fractions was measured. Fractions removed for standard curve purposes were pooled together. All sample and pooled standard curve fractions were transferred to clean test tubes, covered, and stored at 4 °C until further analysis.

(iii) Control—LPDP was used as the control medium. The LPDP was acquired by the technique of step-gradient ultracentrifugation as described above. The LPDP was dialyzed (molecular weight cutoff 1000) against a 0.9% sodium chloride solution for 24 h at a temperature of 4 °C and was then used as the control medium for the incubation of AmpB or ABLC. Preparation of the samples with AmpB or ABLC as well as separation of the constituents was carried out by techniques identical to those described above for step-gradient ultracentrifugation. After ultracentrifugation, the removal of each "lipoprotein" fraction was estimated by comparison to previously separated lipoprotein fractions (i.e., removal of the layer from where TRL would normally reside was based on previous separation of actual plasma in which the layers were visible). The removal of the nonlipoprotein fractions in the control medium was estimated as being close to the placement of their separated lipoprotein counterparts in plasma.

HDL_2/HDL_3 Separation by Step-Gradient Ultracentrifugation—Plasma was separated into its TRL/LDL, HDL_2, HDL_3, and LPDP fractions by an ultracentrifugation method modified from the published methods of Groot et al.[20]

(i) Treatment of Plasma with AmpB or ABLC—To an ultraclear centrifuge tube (Beckman Instruments, Inc., Palo Alto, CA) was added 1.64 or 1.7 mL of human plasma for sample ($n = 3$ for AmpB and $n = 3$ for ABLC) or standard curve ($n = 6$) purposes, respectively. The contents of all tubes were prewarmed to 37 °C. To the samples tubes was added either 6.4 μL of an AmpB solution (5 mg/mL) or 6.4 μL of an ABLC suspension (5 mg/mL). The final

concentration of AmpB in human plasma for both AmpB and ABLC formulations was 20 μg/mL. Immediately after the addition of AmpB or ABLC, the samples were returned to 37 °C and incubated for 60 min, whereupon they were removed and cooled on ice for 30 min.

(ii) Separation of Lipoprotein Constituent—Briefly, solutions of all densities (δ = 1.00, 1.19, and 1.25 g/mL) were stored at 4 °C prior to the layering of the gradient. To the previously cooled plasma used for standard curves and the plasma samples was added 0.936 g of accurately weighed sodium bromide in order to modify the density of the plasma to approximately 1.40 g/mL.[20] Once the sodium bromide had dissolved into the plasma, 1.70 mL of the highest density sodium bromide (δ = 1.25 g/mL) was carefully layered on top of the plasma. By using the volume of 6.4 mL, the next highest sodium bromide solution (δ = 1.19 g/mL) was layered on top of the sample, followed by 1.70 mL of the lowest-density solution (δ = 1.00 g/mL). The ultracentrifuge tubes were balanced, placed into individual titanium buckets, and capped. The buckets were placed into their respective positions on a SW 41 Ti swinging bucket rotor (Beckman Instruments, Inc.) and centrifuged at 40 000 rpm for 21 h at a temperature of 15 °C in a Beckman L8-80M Ultracentrifuge (Beckman Instruments, Inc.) Upon completion of the run, the ultracentrifuge tubes were carefully removed from the titanium buckets. Each of the layers was removed from the top to the bottom layer with the following volumes; TRL/LDL, 0.5 mL; HDL_2, 2.4 mL; HDL_3, 4.8 mL; and LPDP/bottom, 4.3 mL. Fractions removed for standard curve purposes were pooled together. All sample and pooled standard curve fractions were transferred to clean test tubes, covered, and stored at 4 °C until further analysis.

(iii) Control—LPDP was used as the control medium. The LPDP was acquired by the technique of step-gradient ultracentrifugation as described above for HDL_2/HDL_3. The LPDP was dialyzed as previously described and then used as the control medium for the incubation of AmpB or ABLC. Preparation of the samples with AmpB or ABLC as well as separation of the constituents was carried out by techniques identical to those described above for HDL_2/HDL_3 step-gradient ultracentrifugation.

HDL_2/HDL_3 Separation by Gel Electrophoresis—Polyacrylamide gradient gels with a linear gradient of 4−30% of acrylamide were used in the separation of HDL_2 and HDL_3 as previously described.[21] The electrophoresis buffer contained TRIS base (90 mM), boric acid (80 mM), EDTA (3 mM), and sodium azide (3 mM) at pH 8.35. The gradient gels were preconditioned in electrophoresis buffer for 20 min at 125 V and 10° C in an electrophoresis chamber. Ten microliters of a sample containing HDL and sucrose (40%) and bromphenyl blue (0.05%) was applied to the gel for 20 min at 15 V, 30 min at 70 V, and then 24 h at 125 V at 10 °C. Calibration protein standards containing 2.5 μg of thyroglobulin, ferritin, catalase, lactate dehydrogenase, and bovine serum albumin were applied concurrently to other wells of the gel. Following electrophoresis, gels were stained for 1.5 h with Coomassie G-250 (0.04%) in methanol and acetic acid. The gels were than destained in 10% acetic acid and 30% methanol until the background was clear.

AmpB Quantification—AmpB was extracted from each lipoprotein and lipoprotein-deficient fraction by a liquid−liquid extraction method previously described.[22] Immediately prior to analysis, the residue was reconstituted with methanol and injected onto a high-pressure liquid chromatography (HPLC) column. The drug level was analyzed against external calibration curves by HPLC.

TRL, LDL, and LPDP Standard Curve Preparation—To a series of test tubes labeled 0, 0.039, 0.078, 0.156, 0.3125, 0.626, 1.25, 2.5, 5, 10 μg/mL was added 0.5 mL of pooled standard curve fraction, and to a test tube labeled 20 μg/mL was added 0.96 mL of the same pooled fraction. A 4 μL aliquot of either an AmpB solution (5 mg/mL) or an ABLC suspension (5 mg/mL) was then added to the test tube labeled 20 μg/mL, and the test tube was vortexed for 10 s. From the 20 μg/mL test tube, a 0.5 mL aliquot of this mixture was subsequently transferred to the test tube labeled 10 μg/mL. This procedure of serial dilution was carried out for the remaining tubes used for the standard curve.

(i) HDL Standard Curve Preparation—The procedure for the preparation of a HDL standard curve is the same as described above with one minor adjustment. To all tubes in the standard curve was added 1.0 mL of pooled standard curve fraction. To the tube labeled 20 μg/mL was added 1.92 mL of pooled standard curve fraction. An 8 μL aliquot of either an AmpB solution (5 mg/mL) or an ABLC suspension (5 mg/mL) was then added to the test tube labeled 20 μg/mL, and the procedure was carried out as described above.

(ii) Determination of AmpB Content within the Separated Lipoprotein Fraction: TRL and LDL—To an appropriately labeled test tube was added a 0.5 mL aliquot of sample. To these sample tubes as well as the tubes used for the standard curve was added 3 mL of dichloromethane. The mixture was vortexed for 10 s, and all samples were dried under a steady stream of nitrogen at ambient temperature. Once the sample was dried, the AmpB was extracted from the residue with a single methanol wash. Extraction efficiency was determined to be greater than 90% (data not shown). Briefly, a 3.0 mL aliquot of methanol was added to the residue, and the mixture was vortexed for 20 s before being completely dried under a steady stream of nitrogen at ambient temperature. Immediately prior to analysis, the residue was reconstituted with 0.5 mL of methanol and was injected onto the column.

(iii) Determination of AmpB Content within the Separated Lipoprotein Fraction: HDL—To an appropriately labeled test tube was added a 1.0 mL aliquot of sample. The procedure described above was then followed until the last step, when the residue was reconstituted with 0.250 mL of methanol prior to injection onto the column.

(iv) Determination of AmpB Content within the Separated Lipoprotein Fraction: LPDP—To an appropriately labeled test tube was added a 0.5 mL aliquot of sample. To these sample tubes as well as the tubes used for the standard curve was added 3 mL of dichloromethane. The mixture was vortexed for 10s, and all samples were dried under a steady stream of nitrogen at ambient temperature. Once the sample was dried, the AmpB was extracted from the residue with a series of methanol washes. Briefly, a 3.0 mL aliquot of methanol was added to the residue and the mixture was vortexed for 20 s. All test tubes were then centrifuged at 1200*g* for 2 min at 15 °C. The supernatant was transferred to a clean test tube, and the procedure was repeated an additional two times with 2.0 mL of methanol. The supernatant from each of the three extraction steps was pooled with the previous supernatant to provide a final volume of approximately 7 mL of methanol. This pooled methanol was then dried to completion under a steady stream of nitrogen at ambient temperature. Immediately prior to analysis, the residue was reconstituted with 0.5 mL of methanol and was injected onto the column.

HPLC Apparatus—The HPLC system consisted of a Waters 600 Controller interfaced to a Waters 717_{plus} autosampler and a Waters 486 tunable absorbency detector. The detector was set at an UV absorbency wavelength of 405 nm and an absorbency sensitivity of 0.05 absorbency units-full scale. All results were recorded on a Waters 746 Data Module Integrator (Waters Corp., Milford, MA). Samples (100 μL volume) were injected onto a Zorbax SB-C18 column (4.6 × 150 mm; 5 μm particle size), prefitted with a Zorbax Reliance SB-C18 guard column (4.6 × 12.5 mm; 5 μm particle size) (Rockland Technologies, Inc.). Chromatographic separation was carried out at ambient temperature. The mobile phase employed an isocratic flow and consisted of sodium acetate (10 mM) with acetonitrile (70/30 v/v) at a flow rate of 1.5 mL/min. The sensitivity of this assay was 50 ng/mL with an intraday CV of 5−8% (data not shown).

Lipid and Protein Content Analysis of Lipoprotein and Lipoprotein-Deficient Plasma Fractions—Total plasma and lipoprotein triglycerides, cholesterol (free and cholesteryl ester), phospholipids (phosphatidylcholine), and protein concentrations of the human plasma were determined by enzymatic assays purchased from Sigma Diagnostics (St. Louis, MO) and Boehringer Mannheim (Laval, QUE, Canada).

Experimental Design—To assess the distribution of AmpB and ABLC within human plasma, AmpB and ABLC at 20 μg of drug/mL of plasma were incubated in human plasma obtained from seven different human subjects for 60 min at 37 °C. Following incubation, the plasma samples were placed on ice to prevent redistribution of drug within the plasma samples as previously described.[19,22] The plasma was then partitioned into its lipoprotein and lipoprotein-deficient plasma fractions: TRL, consisting of VLDL and chylomicrons; LDL, consisting of intermediate-density lipoproteins and LDLs; HDL, consisting of all subclasses of HDL; and LPDP, consisting of albumin and α-1-glycoprotein by step-gradient ultracentrifugation. Each lipoprotein fraction was analyzed for cholesterol (total, esterified, and unesterified), triglyc-

Table 1—Total and Lipoprotein Plasma Lipid and Protein Concentrations in Samples from Seven Different Human Subjects[a]

plasma sample	TRL[b]			LDL			HDL			total[c]		
	C	TG	P	C	TG	P	C	TG	P	C	TG	P
patient I	11.6	32.1	7.0	36.8	14.3	19.2	23.3	20.1	128.9	77.6	83.1	4406.5
	(0.1)	(2.4)	(0.9)	(1.1)	(2.1)	(0.3)	(0.7)	(0.4)	(15.9)	(3.0)	(2.4)	(262.5)
patient II	45.5	53.5	25.8	16.9	102.3	79.3	26.5	63.9	318.9	106.4	233.7	4616.6
	(2.6)	(3.0)	(3.7)	(3.7)	(3.0)	(2.1)	(2.9)	(2.4)	(18.0)	(5.7)	(10.1)	(165.0)
patient III	54.7	175.1	39.1	41.6	26.2	34.1	23.0	28.8	305.8	125.9	250.5	4564.2
	(0.9)	(11.9)	(1.6)	(2.4)	(1.7)	(3.9)	(1.0)	(1.1)	(60.5)	(1.9)	(10.8)	(341.7)
patient IV	18.3	20.7	6.0	66.7	18.6	28.2	58.5	51.9	239.7	144.3	114.3	4728.0
	(1.8)	(1.6)	(2.2)	(3.6)	(1.6)	(1.4)	(7.4)	(19.2)	(38.8)	(5.6)	(14.4)	(317.6)
patient V	70.0	157.8	49.1	68.4	34.0	98.1	56.4	24.5	215.9	206.8	227.4	4963.5
	(3.3)	(7.3)	(1.5)	(5.0)	(5.2)	(3.1)	(10.7)	(5.8)	(6.4)	(7.0)	(8.6)	(311.5)
patient VI	39.0	31.3	10.8	94.8	20.6	35.5	65.8	35.0	215.7	211.7	110.3	4750.0
	(1.5)	(1.0)	(1.7)	(4.5)	(1.8)	(5.6)	(4.5)	(3.1)	(24.8)	(13.6)	(8.1)	(506.7)
patient VII	47.1	144.3	74.6	102.2	46.9	55.8	59.7	43.9	175.1	219.6	258.2	4749.2
	(3.5)	(2.8)	(8.9)	(5.4)	(3.9)	(6.7)	(10.1)	(1.6)	(11.3)	(16.2)	(4.3)	(164.4)

[a] Data are expressed as mean ± standard deviations (n=6 replicates). [b] Units in mg/dL. [c] Total values represent all plasma lipoprotein and lipoprotein-deficient fractions (which includes α-1 glycoprotein and albumin). Abbreviations: TRL, triglyceride-rich lipoproteins (including chylomicrons and very low-density lipoproteins); LDL, low-density lipoproteins; HDL, high-density lipoproteins; C, cholesterol (esterified and unesterified); TG, triglycerides; P, protein.

Table 2—Plasma Lipoprotein and Lipoprotein-Deficient Distribution of Amphotericin (AmpB) and Amphotericin B Lipid Complex (ABLC) (20 μG/mL) Following Incubation for 60 min at 37 °C in Samples from Seven Different Human Subjects[a]

plasma sample	TRL[b]		LDL		HDL		LPDP	
	AmpB	ABLC	AmpB	ABLC	AmpB	ABLC	AmpB	ABLC
patient I	12.2	0.9	5.7	2.0	7.0	79.4	66.8	11.6
	(0.8)	(0.1)	(0.2)	(0.04)	(0.1)	(0.2)	(10.2)	(1.2)
patient II	2.3	ND	2.5	1.3	12.3	61.4	72.9	25.0
	(0.3)		(0.8)	(0.04)	(1.5)	(2.2)	(1.6)	(1.7)
patient III	1.5	0.2	1.6	ND	7.0	89.7	89.8	2.7
	(0.2)	(0.2)	(0.2)		(1.0)	(1.8)	(1.0)	(0.2)
patient IV	4.2	ND	4.7	ND	4.5	86.6	80.4	4.5
	(0.5)		(0.7)		(0.2)	(3.9)	(6.6)	(0.2)
patient V	4.0	0.3	1.3	0.5	3.3	73.3	89.5	19.4
	(0.03)	(0.3)	(0.02)	(0.4)	(0.9)	(3.0)	(3.8)	(1.8)
patient VI	0.5	ND	2.2	0.5	7.3	50.0	77.4	42.6
	(0.1)		(0.5)	(0.5)	(1.3)	(8.4)	(8.7)	(4.3)
patient VII	10.6	0.8	1.6	1.0	1.6	71.3	69.1	22.4
	(0.4)	(0.01)	(0.2)	(0.1)	(0.3)	(0.1)	(2.8)	(3.6)

[a] Data are expressed as mean ± standard deviations (n = 3 replicates). [b] Percent of initial AmpB or ABLC concentration incubated. Abbreviations: TRL, triglyceride-rich lipoproteins (including chylomicrons and very low-density lipoproteins); LDL, low-density lipoproteins; HDL, high-density lipoproteins; LPDP, lipoprotein-deficient plasma fraction (which includes α-1, glycoprotein and albumin); AmpB, amphotericin B; ABLC, amphotericin B lipid complex; ND, non detectable. Percent of drug recovery for incubations in all patients ranges from 82.9 to 99.8%.

eride, phospholipids, protein, and AmpB concentration. Since the majority of AmpB was not recovered in the HDL fraction following the incubation of free AmpB in plasma (Table 2), all additional experiments pertaining to HDL were done with only ABLC.

To determine which subfractions of HDL AmpB is recovered from, AmpB (as a negative control) and ABLC at 20 μg of drug/mL of plasma were incubated in human plasma for 60 min at 37 °C. Following incubation the plasma was separated into its HDL_2 and HDL_3 fractions by ultracentrifugation and each fraction was assayed for AmpB by HPLC. Separation of HDL into its different subfractions was confirmed by gel electrophoresis (Figure 1).

To further assess the influence of HDL lipid composition and structure on the plasma distribution of ABLC, ABLC at 20 μg of drug/mL of plasma was incubated in human plasma pretreated for 18 h with a lecithin:cholesterol acyltransferase (LCAT) inhibitor, dithionitrobenzoate (DTNB; 15 mM), as previously described[24] for 60 min at 37 °C. Following incubation the plasma was separated into its TRL, LDL, HDL, and LPDP fractions by ultracentrifugation and each fraction was assayed for AmpB by HPLC (Figure 2). DTNB is a sulfhydryl inhibitor that prevents the esterification of cholesterol to cholesteryl ester,[23] resulting in an elevation of HDL_3 coat lipid concentrations (fC + PL) (Table 6). In a second DTNB treatment experiment following the incubation of ABLC in plasma, the plasma was separated into its HDL_2 and HDL_3 fractions by ultracentrifugation and each fraction was assayed for AmpB by HPLC (Table 6).

Table 3—Correlational Analysis Comparing the Amount of AmpB Recovered to Lipid and Protein Contents and Compositions of the Separated Lipoprotein and Lipoprotein-Deficient Plasma Fractions Following the Incubation of ABLC (20 μg/mL) for 60 min at 37 °C in Plasmas from Seven Different Human Subjects[a]

component or ratio	*r* value			
	TRL–AmpB*	LDL–AmpB**	HDL–AmpB	LPDP–AmpB
TC	–0.26	–0.33	–0.37	NA
CE				NA
fC	0.02	0.25	–0.66	NA
TG	0.33	0.57	–0.16	NA
PL	–0.15	–0.17	–0.66	NA
TP	0.01	–0.15	0.04	
Core lipid content (CE + TG)	0.21	–0.14	0.27	NA
Coat lipid content (fC + PL)	–0.12	–0.01	–0.78[c]	NA
TC:TP ratio	–0.62	–0.21	–0.42	NA
TG:TP ratio	0.71	0.21	0.23	NA
PL:TP ratio	–0.60	0.03	0.03	NA
TG:TC ratio	0.75	0.33	–0.04	NA

[a] Calculations are based on the Pearson correlation coefficient values with significance. Abbreviations: ABLC, amphotericin B lipid complex; TRL, triglyceride-rich lipoproteins (which includes very low-density lipoproteins and chylomicrons); LDL, low-density lipoproteins; HDL, high-density lipoproteins; LPDP, lipoprotein-deficient plasma (which includes α-1-glycoprotein and albumin); TC, total cholesterol; CE, cholesteryl ester; fC, free cholesterol; TG, total triglycerides; PL, phospholipid; TP, total protein. [b] NA, not applicable; analysis was not performed due to a lack of sufficient data. [c] $P < 0.05$. *n = 4; three patients had nondetectable AmpB concentrations. **n = 5; two patients had nondetectable AmpB concentrations.

Data and Statistical Analysis—Correlation coefficients between the amount of AmpB recovered within the TRL, HDL, and LDL plasma fractions and the amount of fC, CE, TC, TG, and TP within these fractions and plasma lipoprotein composition were determined using Pearson's Test (Tables 3 and 4). Differences in plasma distribution of AmpB and ABLC following incubation in plasmas obtained from different human subjects with varying lipid concentrations were determined by two-way analysis of variance without repeated measures (INSTAT; Human Systems Dynamics). Critical differences were assessed by Newman–Keuls and Tukey post hoc tests. A difference was considered significant if the probability of chance explaining the results was reduced to less than 5% ($p < 0.05$). All data were expressed as a mean ± standard deviation.

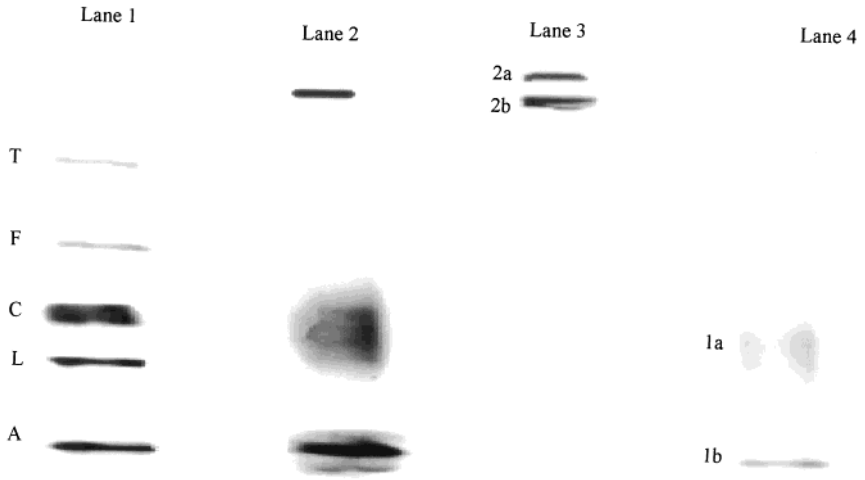

Figure 1—Human plasma samples run on the HDL gel formats. Lane 1 contains the standards: T, thyroglobulin; F, ferritin; C, catalase; L, lactate dehydrogenase; A, bovine serum albumin. Lane 2 is total HDL (including both HDL_3 and HDL_2) separated by ultracentrifugation. Lane 3 is HDL_2 separated by ultracentrifugation, 2a-HDL_{2b}, and 2b-HDL_{2a}. Lane 4 is HDL_3 separated by ultracentrifugation, 1a-HDL_{3a}, and 1b-HDL_{3b}. Lipoproteins and proteins in the standards were stained with Coomassie Brilliant Blue as described in the text (methods section).

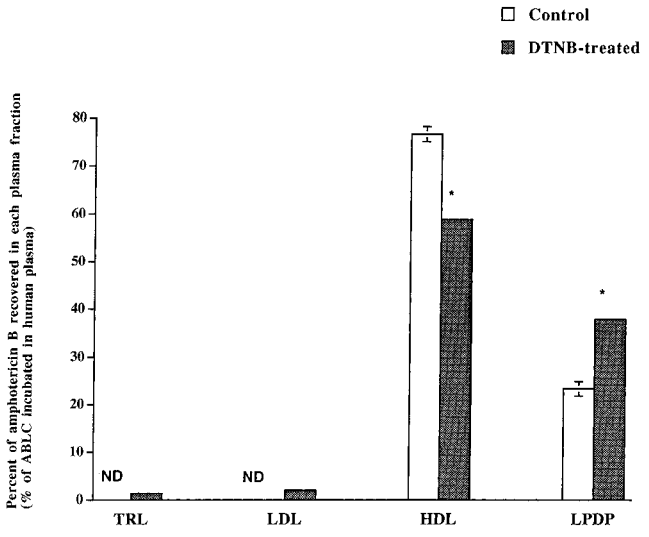

Figure 2—Distribution of amphotericin B lipid complex (ABLC) (20 μg of AmpB/mL of plasma) following incubation within human plasma that has been treated with DTNB (15 mM). ABLC was incubated in plasma pretreated with DTNB 18 h prior to the experiment or in untreated plasma (control) for 60 min at 37 °C. Following incubation the plasma was separated into its triglyceride-rich lipoprotein (TRL), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and lipoprotein-deficient plasma (LPDP) fractions by ultracentrifugation and assayed for AmpB by HPLC. $N = 3$; data are expressed as mean ± standard deviation. *$p < 0.05$ vs control.

Table 4—Correlational Analysis Comparing Amount of AmpB Recovered to Lipid and Protein Contents and Compositions of the Separated Lipoprotein and Lipoprotein-Deficient Plasma Fractions Following the Incubation of AmpB (20 μg/mL) for 60 min at 37 °C in Plasmas from Seven Different Human Subjects[a]

component or ratio	*r* value			
	TRL–AmpB	LDL–AmpB	HDL–AmpB	LPDP–AmpB
TC	–0.44	–0.32	–0.59	NA
CE				NA
fC	–0.10	–0.06	0.47	NA
TG	0.11	0.20	0.22	NA
PL	–0.34	–0.71	–0.20	NA
TP	–0.32	–0.63	0.58	
Core lipid content (CE + TG)	0.10	–0.72	–0.50	NA
Coat lipid content (fC + PL)	–0.30	–0.50	0.02	NA
TC:TP ratio	–0.49	0.39	–0.30	NA
TG:TP ratio	0.70	0.31	0.01	NA
PL:TP ratio	–0.56	0.47	–0.10	NA
TG:TC ratio	0.60	0.11	0.80[c]	NA

[a] Calculations are based on the Pearson correlation coefficient values with significance. Abbreviations: AmpB, amphotericin B; TRL, triglyceride-rich lipoproteins (which includes very low-density lipoproteins and chylomicrons); LDL, low-density lipoproteins; HDL, high-density lipoproteins; LPDP, lipoprotein-deficient plasma (which includes α-1-glycoprotein and albumin); TC, total cholesterol; CE, cholesteryl ester; fC, free cholesterol; TG, total triglycerides; PL, phospholipid; TP, total protein. [b] NA, not applicable; analysis was not performed due to a lack of sufficient data. [c] $P < 0.05$.

Results

Distribution of Amphotericin B (AmpB) and Amphotericin B Lipid Complex (ABLC) following Incubation in Plasma from Human Subjects with Varying Lipid Concentrations—Table 1 reports differences in total plasma and lipoprotein cholesterol (esterified and unesterified), triglyceride, and protein concentrations that were observed for the seven different patient samples measured.

Table 2 reports the human plasma lipoprotein and lipoprotein-deficient fraction distribution of AmpB and ABLC in seven different plasma samples following incubation at 37 °C for 60 min. For plasma incubated with AmpB, 0.5%−12.2% of the original AmpB concentration incubated was recovered in the TRL fraction, 1.3%−5.7% was recovered in the LDL fraction, 1.6%−12.3% was recovered in the HDL fraction, and 66.8%−89.8% was recovered in the LPDP fraction. For plasma incubated with ABLC, nondetectable levels to 0.9% of the original AmpB concentration incubated was recovered in the TRL fraction, nondetectable levels to 2.0% was recovered in the LDL fraction, 50.0%−89.7% was recovered in the HDL fraction, and 2.7%−42.6% was recovered in the LPDP fraction. Similar findings were observed following the incubation of AmpB and ABLC at 1, 5, and 10 μg AmpB/mL of plasma (data not shown).

When AmpB and ABLC were incubated in the LPDP fraction, the majority of the drug (>92%) was recovered in the density fraction from 1.21 to 1.25 g/mL, suggesting that the distribution of AmpB and ABLC is not a function of formulation density (data not shown).

When correlations between the amount of AmpB recovered within the TRL, LDL, HDL, and LPDP fractions following ABLC incubation and the amount of TC, CE, fC,

Table 5—Chemical Composition by Dry Weight of Plasma HDL_2 and HDL_3 and Amphotericin B Concentrations Recovered in HDL_2 and HDL_3 following the Incubation of Amphotericin B or Amphotericin B Lipid Complex (20 μg/mL) in Human Plasma for 60 min at 37 °C

component	units	HDL_2	HDL_3
ABLC	%[a]	ND	98.6 (0.2)*
AmpB	%[a]	ND	6.8 (0.2)* [c]
$M_r \times 10^{-6}$		0.36	0.18
density	g/cm^3	1.09	1.15
protein	%[b]	41	55
phospholipids	%[b]	30	23
triglycerides	%[b]	4.5	4.1
cholesteryl ester	%[b]	16	12
free cholesterol	%[b]	5.4	2.9

[a] Data are presented as mean ± standard deviation (*n = 3 replicates) for the drug distribution work. Abbreviations: ABLC, amphotericin B lipid complex; AmpB, amphotericin B. HDL, high-density lipoproteins; M_r; molecular weight; ND, non detectable concentration. [b] Percent of initial drug incubated. [c] Percent chemical composition by dry weight from ref 26. [d] $P < 0.05$ vs ABLC.

Table 6—Plasma Lipid, Protein and Amphotericin B (AmpB) Concentrations Recovered in HDL_2 and HDL_3 following the Incubation of Amphotericin B Lipid Complex (20 μG/mL) in Human Plasma (control) or Human Plasma Pretreated with Dithionitrobenzoate (DTNB) for 60 min at 37 °C

high-density lipoprotein fraction	coat lipid content (fC + PL) mg/dL	CE, mg/dL	AmpB, (%)[a]
	HDL_3		
control	101	26.0	60.8
	(13)	(1.7)	(6.6)
DTNB-treated	126*	25.2	15.1*
	(10)	(5.3)	(3.6)
	HDL_2		
control	51	30.4	11.6
	(13)	(9.4)	(0.6)
DTNB-treated	39	18.4	8.6*
	(6)	(5.3)	(0.6)

[a] Data are presented as mean ± standard deviation (n = 6 replicates). *$P < 0.05$ vs control. Abbreviations: ABLC, amphotericin B lipid complex; AmpB, amphotericin B. HDL, high-density lipoproteins; TC, total cholesterol; CE, esterified cholesterol; PL, phospholipid. [a]percent of the original ABLC concentration incubated in human plasma.

TG, PL, TP, core lipid content, and coat lipid content within these fractions were calculated for all seven patient plasma samples, only one statistically significant relationship was observed. As HDL coat lipid content (fC + PL) increased, the amount of AmpB recovered in this fraction proportionally decreased (Table 3).

When correlations between AmpB amount recovered in each lipoprotein and lipoprotein-deficient fraction and lipoprotein composition were determined following AmpB incubation, only one statistically significant relationship was observed. As the HDL TG:TC ratio increased, the amount of AmpB recovered in HDL proportionally increased (Table 4).

Amphotericin B (AmpB) and Amphotericin B Lipid Complex (ABLC) Distribution within Plasma HDL_2 and HDL_3 Subfractions—Table 5 shows the human plasma HDL_2 and HDL_3 distribution of AmpB and ABLC in plasma. The distribution of AmpB and ABLC in the HDL_2 and HDL_3 and LPDP fractions of plasma incubated at 37 °C for 60 min showed the following differences. For plasma incubated with AmpB and separated into HDL_2 and HDL_3 subfractions, only 6.8% of the total drug incubated was recovered from the HDL_3 fraction, with the remaining drug being found within the LPDP fraction (Table 5). For plasma incubated with ABLC and separated into HDL_2 and HDL_3 subfractions, >95% of the initial concentration of the drug incubated was recovered from the HDL_3 fraction, with the remaining drug located within the LPDP fraction (data not shown).

When ABLC was incubated in plasma pretreated with DTNB for 18 h, the percentage of AmpB recovered in the HDL fraction was significantly decreased and the percentage recovered in the LPDP fraction was significantly increased compared to controls (Figure 2). Furthermore, the percentage of AmpB recovered in the HDL_3 and HDL_2 fractions was significantly decreased compared to the HDL_3 and HDL_2 fractions in untreated control plasma (Table 6).

Discussion

We have previously observed that AmpB predominantly associates with HDL in human serum when AmpB is incorporated into a lipid complex containing DMPC and DMPG.[16] When annamycin (Ann), an anticancer anthracycline analogue, and Nys were incorporated into liposomes with the same phospholipid composition, the majority of Ann and Nys was recovered in the HDL fraction.[8,18,22] Since HDL and LDL are not found in an equimolar ratio in human plasma but at LDL cholesterol/HDL cholesterol ratios varying from 4:1 to 6:1,[7] these data suggest that a mechanism(s) besides random probability or mass lipoprotein cholesterol levels must drive these drug—liposome complexes toward HDL rather than LDL. One such mechanism appears to be related to liposome composition. We have observed that the DMPG component of ABLC predominantly distributes into HDL because of its interaction with the protein components (apolipoproteins AI and AII) of HDL.[7,19]

In this study, consistent with our previous findings,[7,19] differences in AmpB lipoprotein distribution were observed following the incubation of free AmpB or ABLC in human plasma (Table 2). In particular, independent of plasma lipoprotein lipid and protein concentration, the majority of AmpB was recovered in the LPDP fraction (which contains albumin and α-1-glycoprotein) following the incubation of free AmpB (Table 2). However, the majority of AmpB was recovered in the HDL fraction following the incubation of ABLC (Table 2).

Previous studies with AmpB have suggested that an alteration in plasma lipid concentrations modify this drug's pharmacological behavior. Chavanet and co-workers have demonstrated that an increase in plasma triglyceride concentration led to a reduction in AmpB toxicity in rats.[24] These findings suggested that triglycerides, or their main vehicle in serum, chylomicrons, and VLDL, were involved in the protective effect against AmpB toxicity. Souza and co-workers have further shown that a triglyceride-rich emulsion that behaves in vivo in rats as chylomicrons was able to reduce the in vivo and in vitro toxicity of AmpB.[25] Our laboratory has recently shown enhanced AmpB-induced kidney toxicity within patients who exhibited elevated serum LDL cholesterol concentrations.[14]

In the present study, we have observed differences in the plasma distribution of AmpB when ABLC was incubated in plasmas from seven different human subjects (Table 2). It appears that these differences can be attributed to differences in the lipoprotein lipid and protein concentration profile of the plasmas (Table 1). In particular, increases in HDL coat lipid content (which contains free cholesterol and phospholipid) resulted in less AmpB recovered in this fraction following the incubation of ABLC (Table 3). However, increases in the TG:TP ratio within HDL resulted in more AmpB recovered in this fraction following the incubation of free AmpB (Table 4). These findings suggest that the AmpB lipoprotein distribution following the incubation of free AmpB is regulated by

different plasma HDL components (triglycerides and cholesterol) than following the incubation of ABLC, which appears to be regulated by HDL coat lipid content (free cholesterol and phospholipids).

We further observed that the majority of the AmpB recovered in the HDL fraction following the incubation of ABLC was found in the HDL_3 fraction following experiments in two different human plasmas (Tables 5 and 6). In addition, when the HDL_3 coat lipid content (fC + PL) was artificially elevated by DTNB, the percentage of AmpB recovered in this fraction was significantly decreased compared to controls following the incubation of ABLC (Table 6 and Figure 2). These findings suggest that the compositional and structural differences of HDL_3 as compared to HDL_2 may play an important role in determining the plasma distribution of the drug. In particular, since HDL_3 has a lower percentage by weight coat lipid content (free cholesterol and phospholipids) than does HDL_2[26] (Table 5), the lower percentage of AmpB recovered in the HDL fraction as HDL coat lipid content increased (Table 3) may be a function of the percentage of HDL_3 particles within the HDL fraction. This is supported by our observation that increases in the HDL_3/HDL_2 particle ratio in plasmas from different subjects resulted in a greater percentage of AmpB recovered in the HDL fraction ($r > 0.70$; $P = 0.05$) following the incubation of ABLC (data not shown). Taken together, these findings suggest that HDL coat lipid content (specifically HDL_3) may be an important factor in determining which lipoprotein AmpB associates following the incubation of ABLC.

In conclusion, we have determined that the plasma distribution of AmpB is altered when incorporated into a lipid complex composed of DMPC and DMPG. Furthermore, not only does the relative levels of individual lipoproteins but also the HDL coat lipid content defines the distribution of AmpB among plasma lipoproteins of different human subjects and may be an important consideration when predicting and/or evaluating the pharmacokinetics and toxicity of these compounds following administration to patients with varying lipid profiles.

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