Differences in Lipoprotein Concentration and Composition Modify the Plasma Distribution of Free and Liposomal Annamycin

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Purpose. The purpose of these studies were to determine the distribution of a lipophilic antineoplastic agent, annamycin (Ann), and its liposomal counterpart (LAnn) in plasma which had been altered in its lipoprotein concentration and lipid composition.
Methods. Ann, LAnn, and doxorubicin (a hydrophilic control) were incubated in human plasma for 1 hour at 37°C. Following incubation plasma samples were assayed by fluorimetry for drug in each of the lipoprotein and lipoprotein-deficient plasma (LPDP) fractions. To assess the influence of modified lipoprotein concentrations and lipid composition on plasma distribution of Ann and LAnn, either Ann or LAnn were incubated in human plasma which had been supplemented with very low density lipoproteins (VLDL) or low density lipoproteins (LDL).
Results. When unbound Ann or doxorubicin was incubated in plasma for 1 hour at 37°C, the majority of drug was found in the LPDP fraction. However, when Ann was incorporated into liposomes composed of dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol (LAnn) the majority of Ann was recovered in the high-density lipoprotein (HDL) fraction. Elevation of plasma LDL-cholesterol or VLDL-triglyceride concentrations increased the amounts of Ann and LAnn associated with these lipoprotein classes. Alterations in HDL composition decreased the amount of Ann, but increased the amount of L-Ann within the HDL fraction. Lipid transfer protein (LTP) activity did not significantly modify the plasma distribution of Ann and LAnn in short-term experiments, but the modified lipoprotein composition that LTP facilitates in long-term incubations reduced the capacity of VLDL and LDL to accept drug.
Conclusions. These findings suggest that lipoprotein concentration and composition alter the plasma distribution of Ann and LAnn and may help to explain the discrepancies observed in the pharmacokinetics of Ann and LAnn when they are administered to healthy versus cancer patients.

KEY WORDS: annamycin; liposomes; lipoproteins; dyslipidemia.

INTRODUCTION

Anthracycline compounds are effective agents in the treatment of human leukemia, lymphoma, breast carcinoma, osteosarcoma, and soft tissue sarcoma (1). However, their use is limited by acute myelosuppression, chronic cardiotoxicity (2), and/or multidrug resistance (MDR) (3). The emergence of MDR has been linked to the over-abundance of the cell membrane P-glycoprotein, which acts as an energy-dependent drug efflux pump (4).

Liposomes have been shown to improve the therapeutic index of one such anthracycline compound, doxorubicin (Dox), by modifying its pharmacokinetics and organ distribution (5). The incorporation of Dox into liposomes has resulted in decreased cardiotoxicity without decreasing its effectiveness as compared to its unbound counterpart (5). A number of liposomal-Dox formulations that contain cardiolipin and phosphatidylinositol have been reported to overcome MDR (6). However, an alternative approach in overcoming MDR has been through the synthesis of anthracycline analogs that are not substrates for P-glycoprotein. Annamycin (Ann) is one such compound (7). Currently, Ann is being developed into a liposomal formulation (LAnn) because of its high affinity for lipid membranes and insolubility in water (7).

LAnn has shown to have markedly enhanced antitumor activity compared with Dox in several *in vivo* tumor models, including human xenografts which express the MDR 1 phenotype (8). Preliminary studies have demonstrated that L-Ann or Ann have a significantly higher cellular uptake compared to Dox (8), and the incorporation of Ann into liposomes prolongs the serum half-life of Ann compared to unbound Ann and Dox (9). Furthermore, the incorporation of Ann into these liposomes resulted in a markedly increased tumor uptake of Ann as compared to unbound Ann or Dox (9). However, an explanation for the altered pharmacokinetics, organ distribution, and pharmacodynamics of Dox and Ann when entrapped into these multilamellar lipid vesicles remains unknown.

Lipoproteins are macromolecules of lipid and protein that transport lipids through the vascular and extravascular bodily fluids. Great diversity in the composition and physical properties of lipoproteins is possible, particularly in diseased states. However, it is becoming apparent that lipoproteins have a wider biological significance than simply in lipid transport. It has been demonstrated that the interaction of several compounds, including amphotericin B (AmpB) (10,11) with plasma lipoproteins modifies their pharmacokinetics, tissue distribution, and pharmacological activity.

We have previously demonstrated that AmpB initially associates with the high-density lipoprotein (HDL) fraction upon incubation in plasma (12). Over time, AmpB redistributes from HDL to low-density lipoprotein (LDL). This redistribution appears to be regulated, in part, by lipid transfer protein (LTP) (13), a protein which catalyzes the transfer of cholesteryl esters (CE), triglycerides (TG), and phospholipids between plasma lipoproteins. However, when AmpB is incorporated into liposomes (L-AmpB) composed of negatively or positively charged phospholipids, LTP's capability to affect the transfer of AmpB from HDL to LDL diminished and AmpB remains retained with only the HDL fraction (13). Furthermore, we demonstrated that AmpB was less toxic to LLC PK1 (pig kidney endothelial cells) cells *in vitro* when associated with HDL than when associated with low-density lipoproteins (LDL) or when presented to cells in the unbound state (13).

Since cancer patients often display dyslipidemias (e.g., hypocholesterolemia and hypertriglyceridemia) resulting in modified lipoprotein-lipid composition, we conducted studies to determine whether the distribution of Ann and L-Ann within

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human plasma is affected by changes in lipoprotein concentration and lipid composition. Since unbound Ann is lipophilic, our working hypothesis was that its association with different lipoprotein classes within the plasma component of the bloodstream would be influenced by the composition and relative abundance of these lipid-protein complexes.

MATERIALS AND METHODS

Chemicals, Lipids, and Plasma

Free (Ann) Annamycin was provided by Argus Pharmaceuticals (Woodlands, TX). Chromatographically pure DMPC and DMPG were obtained from Nippon Fine Chemical (Tokyo, Japan). Sodium bromide and dithionitrobenzoate (DTNB) were purchased from Sigma Chemical Company (St. Louis, MO). Methanol and chloroform were obtained from Fisher. Human plasma was obtained from the American Red Cross (Cleveland, OH). Ten μl of 0.4M ethylenediaminetetraacetic acid pH 7.1 (EDTA, Sigma Chemical Company) was added to 1.0 ml of whole blood. The blood was centrifuged (10,000g) for 20 minutes at 4°C and the plasma was removed and stored at 4°C. Plasma from chronic ambulatory peritoneal dialysis (CAPD) patients was obtained from residual clinical samples. For all Ann plasma distribution studies, Ann was dissolved in a 10% dimethyl sulfoxide (DMSO) solution containing normal saline. This drug-free solution did not modify lipoprotein composition or LTP activity.

Liposomal Annamycin (LAnn) Formulation

The method of preparing LAnn and drug-free liposomes has been described previously (8,9). Briefly, DMPC and DMPG, at a molar ratio of 7:3, and Ann were dissolved in chloroform and methanol (5:1 v/v) solvent and the organic phase was evaporated in a rotary evaporator under reduced pressure at 42°C. A thin lipid film was obtained. Normal saline was added (2 mg Ann/ml) and the liposome suspension was obtained by rotation of the flask at 30–40 rpm for 1.5 h at 30–35°C and subsequently shaking it for 30 minutes at 30°C in a mechanical shaker (Versa-Bath S Model 224, Fisher Scientific, Pittsburgh, PA). The liposome suspension was sonicated in a bath sonicator (Model G1125PIT, Lab. Supplies, Hicksville, NY) for 30 seconds and passed through a 5 μm syringe filter. The final Ann concentration was determined by fluorimetry and adjusted to 1.0 mg/ml. The final lipid-to-drug ratio was 15:1 (wt/wt).

The mean vesicle size and size distribution of LAnn were determined by disc centrifugation as previously described (8,9). LAnn samples were diluted 100 times in normal saline and injected into a BI-DCP particle sizer (Brookhaven Instrument, Long Island, NY). The disc speed was 1500 rpm and the centrifugation time was 2 hours at 25°C. Liposome morphology was assessed by scanning electron microscopy as previously described (8,9). Liposomes used in this study ranged in size from 1 to 1.5 microns in diameter.

Lipoprotein Separation

The plasma was separated into its HDL, LDL, VLDL, and lipoprotein deficient plasma (LPDP) fractions by ultracentrifugation (13). Briefly, human plasma (1.0 ml) samples were placed in centrifuge tubes and their solvent densities adjusted to 1.006 g/ml by sodium bromide. Following centrifugation (L7-65 Beckman Instruments) at 50,000 rpm for 17.5 hours at 4°C the VLDL-rich and VLDL-deficient fractions were recovered. Following this initial spin the VLDL-deficient fraction was adjusted to a solvent density of 1.063 g/ml with sodium bromide and respun at 50,000 rpm for 17.5 hours at 4°C to separate the LDL-rich and HDL/LPDP fractions. The HDL/LPDP fraction was adjusted to a solvent density of 1.21 g/ml with sodium bromide and respun at 50,000 rpm for 21.4 hours at 4°C to separate the HDL and LPDP fractions. All isolated lipoprotein and lipoprotein deficient fractions were dialyzed against a PBS solution (4000 ml) for 18 hours before Ann quantification. The molecular weight cutoff of the dialysis tubing used was 10,000–14,000.

Isolation and Purification of Lipid Transfer Protein (LTP)

LTP was purified from human lipoprotein-deficient plasma as has been previously described (13). Briefly, citrated human plasma was made lipoprotein-deficient by the dextran-$MnCl_2$ procedure of Burstein and co-workers (14). LTP was then partially purified by sequential chromatography on phenyl-Sepharose and carboxy-methylcellulose gel (CM-52, Whatman Inc., Chifton, NJ). Partially purified LTP (1.05 mg protein/ml), enriched 800-fold relative to lipoprotein-deficient plasma, was stored at 4°C in 0.01% disodium EDTA pH 7.4. The CM-cellulose fraction of LTP was used in all experiments.

Quantification of Annamycin (Ann), Doxorubicin (Dox), Plasma Triglyceride (TG), Plasma Cholesterol, and VLDL-Protein

HDL, LDL, VLDL, and LPDP fractions were analyzed for Ann and Dox against external standard calibration curves by fluorimetry (8,9). Dialyzed fractions (1.0 ml) were mixed with 3 ml of a chloroform and methanol solution (1/2 v/v), followed by 2 ml of chloroform, and then 1 ml of distilled water. The sample was vortexed for 10 s between each addition of each, and then centrifuged at 2,000 rpm for 15 minutes at 4°C (13). Drug concentration was determined by fluorescence spectroscopy (LS-3 Perkin-Elmer, Norwalk, Conn). For Ann, the excitation wavelength was 480 nm and the emission wavelength was 565 nm; for Dox the excitation wavelength was 480 nm and the emission wavelength was 585 nm. The minimum sensitivity of this assay for both Ann and Dox was 156 ng/ml, with an intraday coefficient of variation of 5% (linear range 0.156–50 μg/ml; $R^2 = 0.98$). Total triglyceride and cholesterol concentrations were determined by enzymatic assay kits from Sigma Diagnostics (St. Louis Mo.). VLDL-protein concentrations were determined by a modified Lowry method (15).

Plasma Distribution Studies of Free and Liposomal Annamycin

Studies were designed to assess if changes in LTP activity, lipoprotein concentration, or lipid composition altered the plasma distribution of Ann and LAnn. Initially, the distribution of Dox, Ann, and L-Ann within human plasma was determined. In addition, studies that incubated drug-free liposomes composed of DMPC/DMPG (7:3 molar ratio), DMPC alone, or DMPG alone concurrently with Ann were done. Furthermore,

Table 1. Distribution of Doxorubicin, 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 Fractions

Drug Compound	Drug Concentration (μg/ml)	LPDP Fraction[a] %[b]	HDL Plasma Fraction %	LDL Plasma Fraction %	VLDL Plasma Fraction %	Percent Recovery %[c]
Doxorubicin	5	70.6 ± 6.2	2.8 ± 2.3	0.6 ± 0.3	11.0 ± 2.3	85.0 ± 2.8
	10	68.8 ± 18.3	4.0 ± 2.1	0.8 ± 0.4	12.7 ± 2.8	86.3 ± 5.9
	20	78.7 ± 5.6	5.2 ± 1.3	1.1 ± 0.4	16.8 ± 1.0	101.7 ± 2.1
Annamycin	5	55.9 ± 8.1[d]	9.1 ± 3.1[d]	8.7 ± 4.4[d]	17.8 ± 5.1	91.5 ± 5.2
	10	48.7 ± 2.9	14.1 ± 4.0[d]	11.5 ± 1.1[d]	29.0 ± 7.0[d]	103.3 ± 3.8[d]
	20	50.5 ± 3.6[d]	11.2 ± 5.0	11.5 ± 1.7[d]	22.1 ± 1.0[d]	95.3 ± 1.8
Liposomal Annamycin	5	10.8 ± 5.5[d,e]	89.8 ± 11.0[d,e]	1.1 ± 1.0[e]	1.8 ± 1.4[d,e]	103.8 ± 4.7[d]
	10	10.9 ± 4.4[d,e]	79.8 ± 7.1[d,e]	5.4 ± 2.0[d,e]	5.4 ± 2.2[d,e]	101.5 ± 3.9[d]
	20	15.4 ± 4.0[d,e]	68.7 ± 4.1[d,e]	9.0 ± 4.1[d]	6.7 ± 5.2[d,e]	99.7 ± 4.3

Note: Data expressed as mean ± standard deviation (n = 5).
*Composed of phospholipids DMPC and DMPG in a lipid:drug ratio of 15:1 wt/wt.
[a]Lipoprotein deficient plasma fraction.
[b]Percent of initial doxorubicin or annamycin concentration.
[c]Percent of initial drug incubated.
[d]$p < 0.05$ vs. doxorubicin of corresponding concentration.
[e]$p < 0.05$ vs. annamycin of corresponding concentration.

studies were designed to determine the fate of the liposome following incubation in plasma.

To assess the influence of LTP on the distribution of Ann and LAnn within human plasma, Ann or LAnn was incubated in human plasma devoid of, with native, with restored, and with enhanced LTP activity.

To assess the influence of modified lipoprotein concentrations on plasma distribution of Ann and LAnn three different approaches were used. Ann or LAnn were incubated in human plasma which had been supplemented with VLDL or LDL prior to, or concurrent with, drug addition. A second approach was to determine the plasma distribution of Ann and LAnn within plasma of continuous ambulatory peritoneal dialysis (CAPD) patients. CAPD patients exhibit hyperlipidemia, in particular hypertriglyceridemia. A third approach was to determine if Ann or LAnn added to plasma could be transferred from the lipoprotein classes they associated with, to VLDL by adding exogenous VLDL to the plasma.

Statistical Analysis

Differences in drug distribution within plasma lipoproteins and differences in LTP-mediated CE transfer activity in the presence of different treatment groups were determined by a two-way analysis of variance (PCANOVA; Human Systems Dynamics). Critical differences were assessed by Neuman-Keuls posthoc tests. Differences were considered significant if p was < 0.05. All data are expressed as mean ± standard deviation.

RESULTS

Plasma Distribution of Liposomal Annamycin (LAnn), Annamycin (Ann), and Doxorubicin (Dox)

Incubation of Dox (control) at 5, 10, and 20 μg/ml with human plasma for 60 minutes at 37°C resulted in 11–16.8% of the initial Dox concentration in the VLDL fraction and 68.8–78.7% in the lipoprotein deficient plasma (LPDP) fraction (Table 1). In contrast, 9.1–14.1% of Ann was recovered in the HDL fraction, 8.7–11.5% in the LDL fraction, 17.8–29% in the VLDL fraction, and 48.7–55.9% in the LPDP fraction (Table 1). The distribution of Ann following incubation of LAnn in human plasma for 60 minutes was 68.7–89.8% of the HDL fraction and 10.8–15.4% in the LPDP fraction (Table 1). Similar results were observed when these compounds were incubated in human plasma for 5,15, or 30 minutes (data not shown). The incubation of drug-free DMPC/DMPG, DMPC alone, or DMPG alone liposomes concurrently with free Ann did not alter Ann plasma distribution (data not shown).

The liposomal structure of LAnn was quickly lost when mixed with plasma (Table 2). Most of LAnn (88.7%) was

Table 2. Estimation of the Liposome Structure of Liposomal Annamycin[a] (20 μg/ml) by Filtering through a Microfilter[b] Before and After the Addition of Human Plasma

	Retained by Filter %[c]	Pass thru Filter %	Percent Recovery %
Liposomal Annamycin before plasma addition[d]	88.7 ± 4.3	7.1 ± 3.5	95.8 ± 3.9
Liposomal Annamycin after plasma addition[e]	2.1 ± 1.9*	86.4 ± 8.4	88.5 ± 5.2

Note: Data expressed as mean ± standard deviation (n = 3).
*$p < 0.05$ vs. before plasma addition.
[a]Composed of phospholipids DMPC and DMPG in a lipid:drug ratio of 15:1 wt/wt.
[b]Filter retains molecules that are 0.45 μm in diameter and greater.
[c]Percent of initial annamycin concentration added to filter.
[d]Dissolved in normal saline at 23°C.
[e]Incubation in human plasma at 37°C for 5 minutes.

Table 3. Distribution of Annamycin (20 μ/ml) after 60 Minutes of Incubation at 37°C within Human Plasma that Has Been Enriched or Pre-enriched with Low-density Lipoproteins (LDL) or very Low-density Lipoproteins (VLDL). Ann Was Incubated in Plasma Either Pre-treated with LDL or VLDL 24 Hours Prior to the Experiment or Incubated at the Same Time as LDL or VLDL for 60 Minutes

Plasma Treatment	HDL Fraction + Plasma Component Fraction %[a]	LDL Fraction %	VLDL Fraction %	Percent Recovery %**
Non-Treated Plasma	62.9 ± 10.8	6.6 ± 4.5	14.1 ± 9.5	83.6 ± 8.3
LDL-enriched Plasma[b]	30.3 ± 5.3*	64.5 ± 4.3*	14.5 ± 4.5	109.3 ± 4.7
VLDL-enriched Plasma[c]	18.2 ± 6.9*	12.8 ± 2.5	59.9 ± 15.9*	90.9 ± 8.4
Plasma pre-enriched with LDL[d]	50.9 ± 3.5	37.9 ± 8.7*	15.0 ± 5.0	103.8 ± 5.7
Plasma pre-enriched with VLDL[e]	48.9 ± 4.1	9.9 ± 0.7	36.4 ± 3.4*	95.2 ± 2.7

Note: mean ± standard deviation, n = 5; *$p < 0.05$ vs. non-treated plasma group.
**Percent of initial annamycin incubated.
[a]Percent of initial Annamycin concentration.
[b]Drug and LDL (1288 μg LDL-cholesterol/ml plasma) were added to plasma at the same time.
[c]Drug and VLDL (300 μg VLDL-triglyceride/ml plasma) were added to plasma at the same time.
[d]LDL (1288 μg LDL-cholesterol/ml plasma) was added to plasma and incubated 24 hr at 37°C prior to the experiment.
[e]VLDL (300 μg VLDL-triglyceride/ml plasma) was added to plasma and incubated 24 h at 37°C prior to the experiment.

retained by a 0.45 micron filter before addition of plasma, but only 2.1% after 5 min incubation with plasma. The rapid disruption of the LAnn structure was consistent with its rapid distribution in plasma noted above.

Effect of Lipid Transfer Protein (LTP) on Plasma Distribution of Annamycin (Ann) and Liposomal Annamycin (LAnn)

To assess the influence of LTP on the distribution of Ann and LAnn within human plasma, Ann or LAnn (20 μg Ann/ml) was incubated for 1 hour in human plasma with minimal (plasma at 4°C), with native (plasma at 37°C), with restored (plasma at 4°C + exogenous LTP), and with enhanced (plasma at 37°C + exogenous LTP) LTP activity. For both Ann and LAnn, no positive relationship between LTP activity and plasma lipoprotein distribution existed (data not shown).

Influence of Lipoprotein Concentration on the Plasma Distribution of Annamycin (Ann) and Liposomal Annamycin (LAnn)

To assess the influence of lipoprotein concentration on the plasma distribution of Ann and LAnn four different approaches were used. When Ann was incubated in human plasma containing additional LDL for 60 minutes at 37°C, the amount of Ann recovered in the LDL fraction significantly increased while significantly decreasing in the HDL-LPDS fraction (Table 3). When Ann was incubated in human plasma pre-treated with LDL 24 hours prior to the experiment, the amount of Ann recovered in the LDL fraction also significantly increased but to a lesser extent than that seen when drug and excess LDL were added concurrently (Table 3). A similar pattern was observed when Ann was incubated in human plasma treated or pre-treated with VLDL. Likewise, when LAnn was incubated in human plasma treated or pre-treated with LDL, the amount of Ann recovered in the LDL fraction significantly increased while significantly decreasing in the HDL fraction (Table 4). When LAnn was incubated in human plasma treated or pre-treated with VLDL, the amount of Ann recovered in the VLDL fraction significantly increased, but the amount of Ann in the HDL-LPDS fraction significantly decreased only when VLDL and LAnn were incubated concurrently (Table 4).

To further confirm the effect of altered total plasma TG and cholesterol concentrations on the distribution of Ann and LAnn among plasma lipoproteins, Ann and LAnn were incubated with CAPD patient plasma samples which differed in total plasma TG and cholesterol concentrations. An increase in the total plasma TG concentration resulted in an increase in Ann and LAnn distribution within the plasma TG-rich VLDL fraction (Figure 1). However, among these plasma samples an increase in total plasma cholesterol did not systematically alter the distribution of Ann or LAnn within either LDL or HDL (data not shown). These findings suggest that although total cholesterol varied 3-fold, TG concentrations appear to be the dominant factor affecting drug distribution.

In an attempt to demonstrate whether Ann and LAnn already associated with lipoproteins could be transferred to other lipoprotein classes by the addition of exogenous lipoproteins, exogenous VLDL (862, 1724, or 2586 μg VLDL-TG/ml plasma) was added to Ann- or LAnn-containing plasma and allowed to incubate for 2 hours at 37°C. Following incubation, the VLDL fraction was reisolated by ultracentrifugation and the amount of Ann determined. Fresh VLDL was added to the plasma and re-incubated. This addition/incubation/centrifugation protocol was repeated twice. Repetitive additions of VLDL to Ann-containing plasma progressively transferred more Ann into the VLDL fraction, however this process was independent of the concentration of VLDL added (Figure 2A). This contrasts with LAnn-containing plasma where different quantities of VLDL resulted in more Ann transfer to this lipoprotein fraction (Figure 2B). These findings suggest that Ann, regardless of its initial origin (as free Ann or LAnn) can be repartitioned within plasma.

DISCUSSION

The objective of these studies was to determine the distribution of Ann and L-Ann within normal and modified human plasma. Unlike Ann and Dox, the incorporation of Ann into liposomes composed of DMPC and DMPG resulted in the

Table 4. Distribution of Liposomal Annamycin[f] (20 μg/ml) after 60 Minutes of Incubation at 37°C Within Human Plasma that Has Been Enriched or Pre-enriched with Low-density Lipoproteins (LDL) or Very Low-density Lipoproteins (VLDL). Ann Was Incubated in Plasma Either Pre-treated with LDL or VLDL 24 Hours Prior to the Experiment or Incubated at the Same Time as LDL or VLDL for 60 Minutes

Plasma Treatment	HDL Fraction + Plasma Component Fraction %[a]	LDL Fraction %	VLDL Fraction %	Percent Recovery %**
Non-Treated Plasma	78.0 ± 3.3	12.9 ± 3.6	2.9 ± 0.5	93.8 ± 2.5
LDL-enriched Plasma[b]	30.3 ± 5.3*	74.0 ± 4.3*	5.1 ± 2.4	109.4 ± 4.0*
VLDL-enriched Plasma[c]	32.8 ± 1.4*	10.5 ± 4.5	60.1 ± 6.1*	103.4 ± 4.0*
Plasma pre-enriched with LDL[d]	31.0 ± 5.3*	52.9 ± 8.7*	5.1 ± 2.5	89.0 ± 5.5
Plasma pre-enriched with VLDL[e]	79.5 ± 12.8	12.5 ± 2.0	14.9 ± 7.2*	106.9 ± 7.3*

Note: mean ± standard deviation, n = 5; *$p < 0.05$ vs. non-treated plasma group.
**Percent of initial annamycin incubated.
[a]Percent of initial Annamycin concentration.
[b]Drug and LDL (1288 μg LDL-cholesterol/ml plasma) were added to plasma at the same time.
[c]Drug and VLDL (300 μg VLDL-triglyceride/ml plasma) were added to plasma at the same time.
[d]LDL (1288 μg LDL-cholesterol/ml plasma) was added to plasma and incubated 24 hr at 37°C prior to the experiment.
[e]VLDL (300 μg VLDL-triglyceride/ml plasma) was added to plasma and incubated 24 hr at 37°C prior to the experiment.
[f]Composed of phospholipids DMPC and DMPG in a lipid:drug ratio of 15:1 wt/wt.

majority of the drug distributing into the HDL fraction (Table 1). Our data suggest that, unlike AmpB (13), LTP has no direct role in the distribution of Ann, L-Ann, or doxorubicin (data not shown) among plasma lipoproteins. However, changes in lipoprotein-lipid composition, which LTP facilitates, and lipoprotein concentration alter the distribution of Ann and L-Ann within human plasma.

We previously observed that AmpB predominantly associates with HDL in human serum and that the amount of AmpB associated with HDL increases when AmpB is incorporated into negatively charged liposomes (12). We have further shown that the DMPG component of liposomal AmpB (L-AmpB) predominantly distributes into HDL because of its interaction with the protein components (apolipoprotein A-I and A-II) of HDL (12). Since L-Ann is composed of the same phospholipids as L-AmpB, the increased distribution of Ann into the HDL fraction when incorporated into these liposomes may also be a result of DMPG's attraction for apolipoprotein AI and AII.

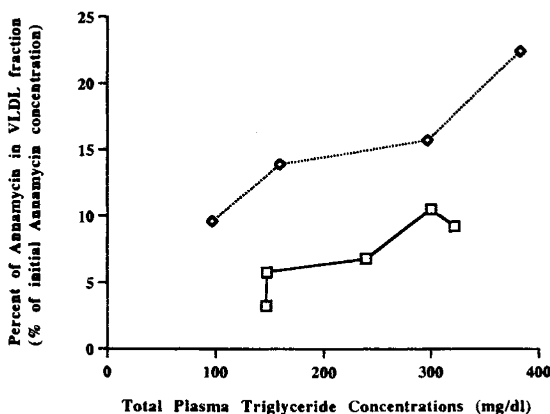

Fig. 1. Distribution of Annamycin (-□-) versus Liposomal Annamycin (-◇-) at 20 μg/ml incubated for 60 minutes at 37°C in plasma of chronic ambulatory peritoneal dialysis patients. Total Plasma Triglyceride Concentrations versus Annamycin distribution within the very-low density lipoprotein fraction.

We have further shown that the liposomal structure of Ann is not intact within 5 minutes incubation in plasma at 37°C (Table 2). This suggests that the liposomes may fuse with lipoproteins, especially HDL, once in contact with human plasma. Since circulating plasma HDL has a half-life of several days, the increased plasma circulation time of L-Ann may be attributed to the increased association of Ann with HDL when incorporated into these liposomes versus free Ann (69% vs. 11%; Table 1). Furthermore, the addition of exogenous VLDL to Ann- and LAnn-containing plasma shifts both Ann and LAnn into the VLDL fraction (Figure 2A, 2B); a shift of Ann or LAnn to VLDL should result in an shorter plasma half-life and an increased hepatic clearance of Ann and LAnn. Plasma VLDL has a half-life of several hours, and is rapidly cleared from the plasma by the liver. The pharmacological importance of this association remains to be explored.

We have previously demonstrated that the distribution of AmpB into the HDL and LDL fractions of human plasma is regulated by LTP. However, once AmpB is incorporated into liposomes composed of DMPC and DMPG, the ability of LTP to transfer AmpB and ^{3}H-CE from HDL to LDL is diminished (13). We concluded from these studies that since AmpB interacts with cholesterol and CE upon administration into the plasma component of the bloodstream, LTP's ability to transfer AmpB between HDL and LDL was due to its ability to transfer CE between HDL and LDL and not due to the direct transfer of AmpB between lipoprotein fractions. In the case of Ann, plasma distribution appears to be independent of LTP activity during short-term incubations.

Since Ann appears not to be recognized by LTP as an endogenous lipid compound, LTP's ability to transfer Ann between lipoprotein fractions is minimal. This suggests that other mechanism(s) exist which determine the distribution of Ann upon entrance into the plasma component of the bloodstream.

One such mechanism appears to be related to lipoprotein concentrations. We observed that an increase in plasma LDL-cholesterol or VLDL-TG concentrations increased the distribu-

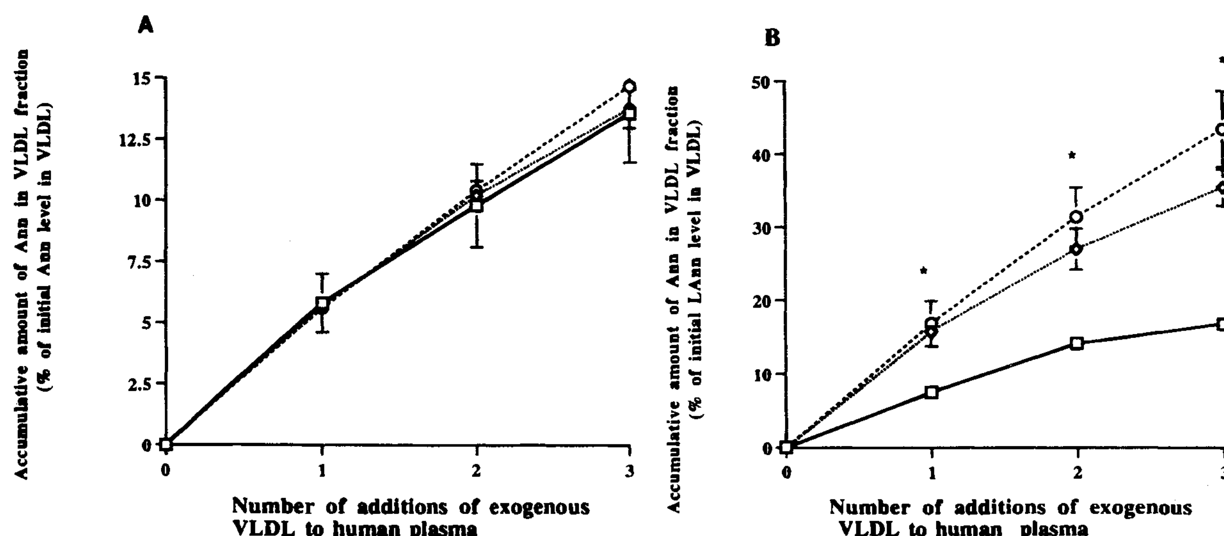

Fig. 2. Recovery of (A) annamycin and (B) liposomal annamycin (B) at 20 μg/ml in the VLDL fraction after adding exogenous VLDL to the plasma. Annamycin and liposomal annamycin were incubated for 18 hours at 37°C in human plasma. Following the first incubation, the plasma was ultracentrifuged, the VLDL fraction was removed, and assayed for annamycin or liposomal annamycin as described in the methods. Exogenous VLDL, 862 μg (-□-); 1724 μg (-◇-); or 2586 μg (-○-) VLDL-TG/ ml plasma was then added to sample and allowed to incubate for 2 hours at 37°C. Following the second incubation, the plasma was ultracentrifuged, the VLDL fraction removed, and assayed for annamycin or liposomal annamycin. This was repeated once more and the total amount of annamycin or liposomal annamycin that had moved into the VLDL fraction from the three incubations was determined. Endogenous TG level was 1063 μg/ml. Data expressed as mean ± standard deviation; n = 3. *$p < 0.05$ vs VLDL 862 μg VLDL-TG/ml plasma.

tion of Ann (Table 3) or L-Ann (Table 4) into those fractions respectively. However, what is of particular interest is that VLDL (300 μg TG) was more effective than LDL (1233 μg cholesterol) in altering drug distribution. At these concentrations, the total lipid surface area and lipid core volume for LDL were 7.3 and 5.6 greater, respectively, than that in VLDL. These findings suggest that VLDL is much more effective at binding these hydrophobic drugs than LDL. We further observed that the addition of different amounts of exogenous VLDL to Ann- and LAnn-containing plasma, shifts Ann and LAnn away from other lipoprotein classes and LPDP fraction and into VLDL (Figure 2A and 2B). These findings illustrate that the drug moved from one lipoprotein class to another and its movement could be influenced by different disease states (e.g. cancer) and adjunct therapies such as Intralipid infusion (11), where lipoprotein concentrations are altered. Furthermore, data from dyslipidemic plasma suggested that a direct correlation existed between total plasma TG concentrations and VLDL-Ann concentrations (Figure 1). A positive correlation between total cholesterol and LDL-Ann and HDL-Ann concentrations was not observed (data not shown).

In addition, tables 3 and 4 further illustrate that plasma plus LDL-cholesterol or VLDL-TG behaves differently if the lipoproteins are added with the drug versus when they are added 24 hours before the drug. When either LDL or VLDL are added concomitantly with the drug, both Ann and LAnn are mostly recovered in those fractions respectively. These findings suggest that drug lipoprotein distribution are regulated by mass plasma lipoprotein levels. However, adding the LDL-cholesterol or VLDL-TG 24 hours before the drug decreased the capacity of these lipoproteins to accept the drug. This suggests that the TG/CE ratio and the free cholesterol content of lipoproteins, which are altered by LTP and lecithin cholesteryl acyltransferase during the 24 hr pre-incubation, may also be important in defining the capacity of a lipoprotein to sequester drug.

In conclusion we have determined that Ann predominantly associates with HDL upon entrance into the plasma component of the bloodstream when incorporated into liposomes composed of DMPC and DMPG. However, the distribution of Ann among lipoproteins is defined by the relative levels of individual lipoproteins. Since many cancer patients exhibit lipid disturbances, including hypocholesterolemia and hypertriglyceridemia, these results may provide an explanation for the altered pharmacokinetics and pharmacodynamics of L-Ann when administered into these patients compared to non-diseased patients. Future studies will investigate the pharmacological implications of Ann's predominant association with HDL when incorporated into these liposomes and the increased association of Ann with VLDL when total plasma TG concentrations increase.

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