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Physical Characteristics and Lipoprotein Distribution of Liposomal Nystatin in Human Plasma

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The physical characteristics and lipoprotein distribution of free nystatin (NYS) and liposomal NYS (L-NYS) in human plasma were investigated. To determine the percentage of NYS that was lipid associated following incubation in human plasma, C_{18} reverse-phase extraction columns were used. To assess plasma drug distribution, NYS and L-NYS (20 μg/ml) were incubated in human plasma for 5, 60, and 120 min at 37°C. After each interval, plasma was removed and separated into its lipoprotein and lipoprotein-deficient plasma (LPDP) fractions by ultracentrifugation and assayed for NYS by high-pressure liquid chromatography. Further studies evaluated the liposome structure of L-NYS by filtering through a 0.14-μm-pore-size microfilter before and after the addition of human plasma. When reconstituted L-NYS (mean particle diameter ± standard deviation, 321 ± 192 nm) was applied to a C_{18} column, 67% ± 4% of the initial NYS concentration was associated with the lipid. When plasma samples containing L-NYS that had been incubated for 5 to 120 min at 37°C were applied to C_{18} columns, 66 to 76% of the NYS was lipid associated. Incubation of NYS in human plasma for 5 min at 37°C resulted in 3% ± 1% of the initial NYS concentration incubated in the low-density lipoprotein (LDL) fraction, 23% ± 4% of that in the high-density lipoprotein (HDL) fraction, and 66% ± 10% of that in the LPDP fraction. In contrast, the distribution of NYS following incubation of L-NYS in human plasma for 5 min was 13% ± 2% in the LDL fraction, 44% ± 5% in the HDL fraction, and 42% ± 5% in the LPDP fraction. Similar results were observed following 60 and 120 min of incubation. In addition, the liposome structure of L-NYS was quickly lost when mixed with plasma. These findings suggest that rapid disruption of the L-NYS structure upon incubation in human plasma is consistent with its rapid distribution in plasma. The preferential distribution of NYS into the HDL fraction upon incubation of L-NYS may be a function of its phospholipid composition.

Nystatin (NYS), a polyene macrolide antibiotic administered orally and topically, is currently being used in the treatment of superficial fungal infections of the skin and mucous membranes (8). The fungal cytotoxicity of NYS is related to its amphiphilic structure, which facilitates binding to cell membrane sterols that disrupt membrane integrity, and to its preferential binding to ergosterol in fungal membranes versus cholesterol in mammalian membranes (9). However, its clinical use is limited due to its poor absorption from the gastrointestinal tract (1). The incorporation of NYS into liposomes has allowed the preparation of a new parenteral NYS formulation that in animals can be safely administered at doses much higher than free NYS (28).

Previous studies have shown that polyene macrolide antibiotics such as amphotericin B (AMPB) and NYS are active against a variety of lipid-enveloped RNA and DNA viruses (11, 13) and that NYS in vitro inhibits human immunodeficiency virus type 1 replication in H9 cells (12). Furthermore, a phase I clinical trial has recently been completed which investigated the safety, tolerance, and pharmacokinetics of a single dose of liposomal NYS (L-NYS) in patients with human immunodeficiency virus infection (10). Pharmacokinetic analysis from this study has shown that following L-NYS administration, the drug appears to distribute initially into the blood circulation and is cleared with a terminal half-life of 5 h (10, 22). The kinetics of L-NYS appear to be dose independent in the range of 0.5 to 1 mg/kg of body weight; however, the area under the concentration-time curve appears to increase in proportion to the dose administered for doses ranging from 0.25 to 0.75 mg/kg (10, 22). No rationale to explain these results has been found.

Plasma lipoproteins are macromolecules of lipid and protein that transport polar and nonpolar lipids through the vascular and extravascular body fluids (3, 18). However, lipoproteins appear to have a wider biological significance than simply that of lipid transport. It has been demonstrated that interactions with plasma lipoproteins modify the pharmacokinetics, tissue distribution, and pharmacological activity of several lipophilic compounds, including AMPB, which is another antifungal agent that is chemically similar to NYS (17, 23, 25–27).

There is growing evidence that by increasing the association of AMPB with plasma low-density lipoproteins (LDL) an increase in AMPB-induced renal toxicity is observed. Our preliminary findings suggest that patients with higher plasma LDL cholesterol levels are more susceptible to AMPB-induced renal toxicity (16). Koldin et al. demonstrated the occurrence of elevated AMPB-induced nephrotoxicity when LDL-associated AMPB was administered to hypercholesterolemic rabbits (5). Kreiger has observed that the cellular uptake of AMPB is mediated through the LDL receptor (6). Furthermore, we have demonstrated that AMPB was toxic to kidney cells when associated with LDL; however, when the number of LDL re-

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ceptors expressed on these cells was reduced, LDL-associated AMPB was less toxic to these cells than unbound AMPB (24).

The studies presented here describe the physical characteristics of L-NYS and address the role of liposome incorporation in the distribution of NYS to plasma lipoproteins.

MATERIALS AND METHODS

Chemicals, lipids, and plasma. NYS and L-NYS were provided by Aronex Pharmaceuticals Inc. Methanol, tetrahydrofuran, and other organic solvents were purchased from Fisher Scientific Canada (Toronto, Ontario, Canada). Pooled human whole blood was obtained from the Vancouver Red Cross (Vancouver, British Columbia, Canada). Ten microliters of 0.4 M EDTA (pH 7.1; Sigma Chemical Company, St. Louis, Mo.) was added to 1.0 ml of whole blood. The blood was centrifuged (10,000 × *g*) for 20 min at 4°C, and the plasma was removed and stored at 4°C. Sodium bromide was purchased from Sigma Chemical Company. Free NYS was dissolved in 10% methanol, while L-NYS was reconstituted in normal saline for all experiments. The 10% methanol solution does not effect lipoprotein-lipid composition (data not shown).

Liposome preparation. The method of preparing multilamellar liposomes containing NYS is similar to how liposomal AMPB was prepared and has been described previously (7, 9). Briefly, NYS dissolved in methanol was mixed with chloroform solutions of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG). Organic solvents were removed by evaporation under a vacuum, and the resultant lipid film was dispersed by hand shaking in 0.9% sodium chloride to form liposomes. The final preparation contained a 7:3 (wt/wt) ratio of DMPC to DMPG and a total lipid/drug ratio of 10:1 (wt/wt), and the mean particle diameter as determined by quasi-electric light scattering (QELS) using a Nicomp submicron particle sizer (model 270; Pacific Scientific, Santa Barbara, Calif.) was 321 ± 192 nm (15). A number of L-NYS preparations with similar physical characteristics and the same phospholipids were prepared.

Lipoprotein separation. The plasma was separated into its high-density lipoprotein (HDL), LDL, very-low-density lipoprotein (VLDL), and lipoprotein-deficient plasma (LPDP) fractions by ultracentrifugation (4). Briefly, human plasma samples (3.0 ml) were placed in centrifuge tubes and their solvent densities were adjusted to 1.006 g/ml by sodium bromide. Following centrifugation (in a model L8-80M centrifuge; Beckman Canada) at 50,000 rpm for 18 h at 4°C, the VLDL-rich and VLDL-deficient plasma fractions were recovered. The VLDL-deficient plasma fraction was readjusted to a density of 1.063 g/ml and respun at 50,000 rpm for 18 h at 4°C to separate the LDL-rich and VLDL/LDL-deficient plasma fractions. This fraction was readjusted to a density of 1.21 g/ml and respun at 50,000 rpm for 18 h at 4°C to separate the HDL-rich and LPDP fractions.

To assure that the distribution of free NYS and L-NYS found in each of these fractions was a result of its association with each lipoprotein or lipoprotein-deficient fraction and not a result of the density of the formulation, the density of the free NYS and L-NYS formulation incubated in the LPDP fraction for 60 min at 37°C was determined by ultracentrifugation. The majority of free NYS and L-NYS (>90%) was found in the density range of 1.21 to 1.25 g/ml (data not shown), suggesting that the NYS distribution within the ultracentrifuge tubes following incubation in human plasma was not a function of formulation density.

NYS quantification. NYS was extracted from each lipoprotein and lipoprotein-deficient fraction by using methanol (3 parts methanol for 1 part of sample; three washes), and the drug level was analyzed against external calibration curves by high-pressure liquid chromatography (HPLC). This HPLC system consisted of a Waters 600 fluid handling unit and controller, a Waters 717 autosampler, and a Waters 486 UV-visible spectrum detector. The detector was set at a wavelength of 306 nm with absorbency sensitivity at 0.005 absorbance units, full scale. Results were recorded on a Waters 746 integrator. For chromatographic separation, a Zorbax C_8 reverse-phase column (150 by 4.6 mm) packed with trimethylsilyl phases (particle diameter, 5 μm) was used at ambient temperatures. The mobile phase consisted of a gradient system of acetonitrile and tetrahydrofuran with a flow rate of 1.5 ml/min. The sensitivity of this assay was at least 1.5 μg/ml, with an intraday coefficient of variation of 5% for each of the different components measured (Table 1).

Determination of plasma cholesterol (esterified and unesterified) and TG concentrations. Total plasma triglycerides (TG) and cholesterol concentrations were determined by enzymatic assays purchased from Sigma Chemical Co. Briefly, in this assay TG are first hydrolyzed by lipoprotein lipase to glycerol and free fatty acids. Glycerol is then phosphorylated by ATP, forming glycerol-1-phosphate and ADP in the reaction catalyzed by glycerol kinase. Glycerol-1-phosphate is then oxidized by glycerol phosphate oxidase to dihydrooxyacetone phosphate and hydrogen peroxide. A quinoneimine dye is produced by the peroxidase-catalyzed coupling of 4-aminoantipyrine and sodium *N*-ethyl-*N*-(3-sulfopropyl)*m*-anisidine with hydrogen peroxide. This dye shows a maximum absorbency of 500 nm and is directly proportional to the TG concentration of the sample. Absorbencies of plasma and lipoprotein samples were determined and compared to an external calibration curve for TG (linear range of 10 to 300 mg/dl; $R^2 = 0.95$).

In the determination of cholesterol concentrations, cholesterol esters are first hydrolyzed to cholesterol by cholesterol esterase. The cholesterol is then oxidized by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. A quinoneimine dye is produced by the peroxidase catalyzed coupling of 4-aminoantipyrine and *p*-hydroxybenzenesulfonate with hydrogen peroxide. This dye shows maximum absorbency at 500 nm and is directly proportional to the cholesterol concentration of the sample. Absorbencies of plasma and lipoprotein samples were determined and compared to an external calibration curve for cholesterol (linear range of 10 to 450 mg/dl; $R^2 = 0.96$). The total and lipoprotein lipid profiles of the human plasma used in these studies were determined.

TABLE 1. Linear calibration curves for NYS as determined in various fractions

Fraction in which calibration curve was determined	Equation[a]	Correlation coefficient	Range (μg/ml)	Recovery (%)[b]
Methanol	$y = 120{,}000x - 710{,}000$	0.975	0.3–50.0	
Human plasma	$y = 525{,}000x + 3{,}000{,}000$	0.982	1.5–100.0	>95
VLDL	$y = 356{,}000x - 640{,}000$	0.985	1.3–40.0	>92
LDL	$y = 329{,}000x - 330{,}000$	0.999	1.3–40.0	>92
HDL	$y = 324{,}000x - 33{,}000$	0.998	1.3–40.0	>95
LPDP	$y = 293{,}000x - 191{,}000$	0.998	1.3–40.0	>95

[a] y = peak area (in microvolts per second); x = concentration of NYS (in micrograms per milliliter).
[b] Percent recovery compared to that of NYS dissolved in methanol.

Efficiency of L-NYS incorporation. To determine the percentage of original Nys used in the liposomal preparation that is associated with lipid and that which is free, C_{18} reverse-phase extraction columns attached to a vacuum manifold apparatus were utilized (14). Each column was preconditioned by washing with methanol (1 ml; two washes) followed by phosphated saline buffer (1 ml; two washes). When reconstituted L-NYS was applied to a C_{18} column (pretreated with 1% bovine serum albumin), lipid-associated NYS was found to elute through the column in the aqueous wash while free NYS remained bound to the column (data not shown). When the column was then washed with methanol-free wash, NYS would elute off (data not shown). To confirm this technique, sham DMPC-DMPG liposomes containing a radiolabeled liposome marker, [1,2-^{3}H(N)]cholesteryl hexadecyl ether, and free NYS (dissolved in normal saline or methanol) were applied to the column.

Sizing of L-NYS. The mean diameter of L-NYS particles was determined by QELS, with a Nicomp submicron particle sizer (model 270; Pacific Scientific) operating at a wavelength of 632.8 nm. As described previously, QELS analysis using the Nicomp submicron particle sizer reports, for a given sample, the mean particle diameter, standard deviation (SD), and goodness of fit for a normal Gaussian distribution (15). The SD gives an indication of the degree of heterogeneity within a sample.

Influence of liposomal incorporation on NYS plasma lipoprotein distribution. To assess the distribution of NYS and L-NYS within human plasma, NYS or L-NYS (20 μg of drug/ml of plasma; concentration close to peak levels in plasma observed in mice after intravenous bolus) was incubated in human plasma for 5, 60, and 120 min at 37°C. After each time point, plasma samples were removed and each of the lipoprotein and LPDP fractions was assayed for drug levels as described above. Furthermore, studies to determine the fate of the liposome following incubation in plasma were designed. In these studies, the estimation of the liposome structure of L-NYS was determined by filtering through a microfilter (that retains molecules with diameters of 0.14 μm and more) before and after the addition of human plasma. L-NYS was either dissolved in normal saline at 23°C or reconstituted L-NYS (20 μg of NYS/ml) was incubated in human plasma at 37°C for 5 min and filtered through a microfilter.

Statistical analysis. Differences in NYS distribution within plasma lipoproteins and in lipid-associated versus lipid-free NYS were determined by a two-way analysis of variance (PCANOVA; Human Systems Dynamics). Critical differences were assessed by Neuman-Keuls post hoc tests. Differences were considered significant if P was <0.05. All data is expressed as means ± SDs.

RESULTS

HPLC determination of NYS and human plasma lipid analysis. The standard curves in methanol, plasma, and each lipoprotein plasma and LPDP fraction were linear for NYS concentrations over a range of at least 1.5 to 40 μg/ml, and the correlation coefficient was greater than 0.975 for each regression line (Table 1). Similar results were observed when L-NYS was incubated in the different lipoprotein plasma and LPDP fractions. The retention times of NYS following liquid-liquid extraction from human plasma, plasma lipoprotein, and plasma lipoprotein-deficient fractions were 8.96 and 9.14 min (data

TABLE 2. Effects of reconstitution and incubation association of NYS with lipid components[a]

L-NYS	L-NYS (%)[b]	Unassociated NYS (%)[b]	Recovery (%)	*n*
Reconstituted prior to incubation	67 ± 4	33 ± 1	100 ± 5	4
Incubated in plasma for:				
5 min	66 ± 10	30 ± 1	96 ± 9	3
60 min	76 ± 7	28 ± 9	104 ± 8	3
120 min	70 ± 7	29 ± 3	99 ± 6	3

[a] Reconstitution of L-NYS (20 μg/ml) was achieved with normal saline at 23°C, and incubation of L-NYS was carried out in human plasma at 37°C. Percentages are expressed as means ± SDs.

[b] Percent of original NYS incorporated into liposome and/or incubated in human plasma when using the C_{18} reverse-phase or fraction column method.

not shown). These two nonoverlapping peaks represent different isomers of NYS. The area of each peak was determined, and the sum for all peaks was used to generate the linear regression curves. The interday coefficient of variation for all the curves generated was between 5 and 8%.

Furthermore, the total and lipoprotein lipid profiles of the human plasma used in these studies were determined. Total cholesterol and TG concentrations in plasma were 213 ± 18 and 146 ± 6 mg/dl, respectively. Plasma HDL, LDL, and VLDL cholesterol concentrations were 26 ± 7, 125 ± 10, and 57 ± 9 mg/dl, respectively. Plasma HDL, LDL, and VLDL TG concentrations were 22 ± 7, 38 ± 5, and 69 ± 13 mg/dl, respectively. These values fall within the range of plasma lipid levels observed in nondiseased fasting patients (2).

Measurement of lipid-associated and lipid-free NYS. To characterize the liposome interaction with the C_{18} column, liposomes composed of DMPC and DMPG containing [1,2-^{3}H(N)]cholesteryl hexadecyl ether as a liposome marker were used. When these liposomes reconstituted with normal saline or plasma containing liposomes (total lipid concentration of 200 μg/ml) were applied to the solid-phase C_{18} column, more than 98% of the liposome-associated radioactivity passed through the column and was collected in the aqueous fraction, while less than 1% of the radioactivity remained bound to the column and was subsequently removed by methanol washes. Preliminary studies have demonstrated that NYS solubilized in methanol or normal saline and insoluble particulate NYS in normal saline bind to the C_{18} column (data not shown).

When reconstituted L-NYS with normal saline at 23°C was applied to the column, 67% ± 4% of the L-NYS passed through the column and was collected in the aqueous fraction (L-NYS), while 33% ± 1% of the NYS remained bound to the column (free NYS) (Table 2). Furthermore, when plasma samples containing L-NYS (20 μg of NYS/ml) that had been incubated for 5, 60, and 120 min at 37°C, were applied to the column, 66 to 76% of the NYS was lipid-associated while 28 to 30% of the NYS was free (Table 2). In addition, when plasma samples containing NYS, that had been incubated for 5, 60, and 120 min at 37°C were applied to the column, the majority of the drug was not lipid associated as indicated by the observation that more than 66% of the drug remained bound to the column (data not shown).

Effect of liposomal incorporation on NYS distribution with lipoproteins. Incubation of NYS (20 μg/ml) with human plasma for 5 min at 37°C resulted in 3% ± 1% of the initial NYS concentration in the LDL fraction, 23% ± 4% in the HDL fraction and 66% ± 10% in the LPDP fraction (Fig. 1A). The distribution of NYS following incubation of L-NYS in human plasma for 5 min was 13% ± 2% in the LDL fraction, 44% ± 5% in the HDL fraction, and 42% ± 5% in the LPDP fraction (Fig. 1A). Nondetectable levels of NYS in the VLDL fraction following the incubation of free NYS and L-NYS were observed. When the amount of NYS associated with each lipoprotein fraction was corrected for the amount of core lipoprotein-lipid (cholesterol and TG), the majority of drug was found in the HDL fraction (Fig. 1B). Similar results were observed when NYS and L-NYS were incubated in human plasma for 60 and 120 min (data not shown). The incubation of drug-free DMPC-DMPG liposomes or DMPC or DMPG liposomes concurrently with free NYS did not alter NYS distribution in plasma (data not shown).

The liposomal structure of L-NYS was quickly lost when mixed with plasma (Table 3). Most of L-NYS (65% ± 4%) was retained by a 0.14 μm-pore-size filter before addition of plasma, but only 4% ± 1% was retained after a 5-min incubation with plasma. The rapid disruption of the L-NYS structure was consistent with its rapid distribution in plasma noted above.

DISCUSSION

The objectives of these studies were to determine the physical characteristics and lipoprotein distribution of L-NYS upon incubation in human plasma. The incorporation of NYS into liposomes composed of DMPC and DMPG results in the majority of the drug distributing into the HDL fraction (Fig. 1). Our data further suggests that upon incubation in plasma, the liposome structure of L-NYS was quickly lost, although the drug remains lipid associated.

We have 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 liposomes containing DMPC and DMPG (23, 25). When annamycin (ANN), an anticancer anthracycline analog, is incorporated into liposomes with the same phospholipid composition, the majority of ANN is also found in the HDL fraction (19, 20). Since HDL and LDL are found not in an equimolar ratio in human plasma but at LDL/HDL ratios varying from 4:1 to 6:1 (21) (the LDL cholesterol/HDL cholesterol ratio is 4.8:1 in the human plasma used in our studies), the data suggests that a mechanism(s) besides random probability or mass lipoprotein lipid levels must drive these drug-liposome complexes towards HDL rather than LDL. This is further evident when the NYS lipoprotein distribution, expressed as the amount of NYS associated with each lipoprotein fraction, is corrected for the amount of core lipoprotein lipid (esterified and unesterified cholesterol and TG) (Fig. 1B).

TABLE 3. Estimation of the liposome structure of L-NYS by filtering[a]

L-NYS	% L-NYS		Recovery (%)
	Retained by filter[b]	Passed through filter	
Before plasma addition[c]	65 ± 4	35 ± 4	100 ± 5
After plasma addition[d]	4 ± 1*	90 ± 7*	94 ± 6

[a] L-NYS (20 μg/ml) was composed of DMPC and DMPG in a lipid/drug ratio of 10:1 (wt/wt). Before and after the addition of human plasma, L-NYS was filtered through a microfilter that retains molecules with diameters of 0.14 μm or more. Data is expressed as means ± SDs ($n = 5$). *, $P < 0.05$ compared to that before plasma addition.

[b] Percent of initial concentration of NYS added to filter.

[c] Dissolved in normal saline at 23°C.

[d] Incubation in human plasma at 37°C for 5 min.

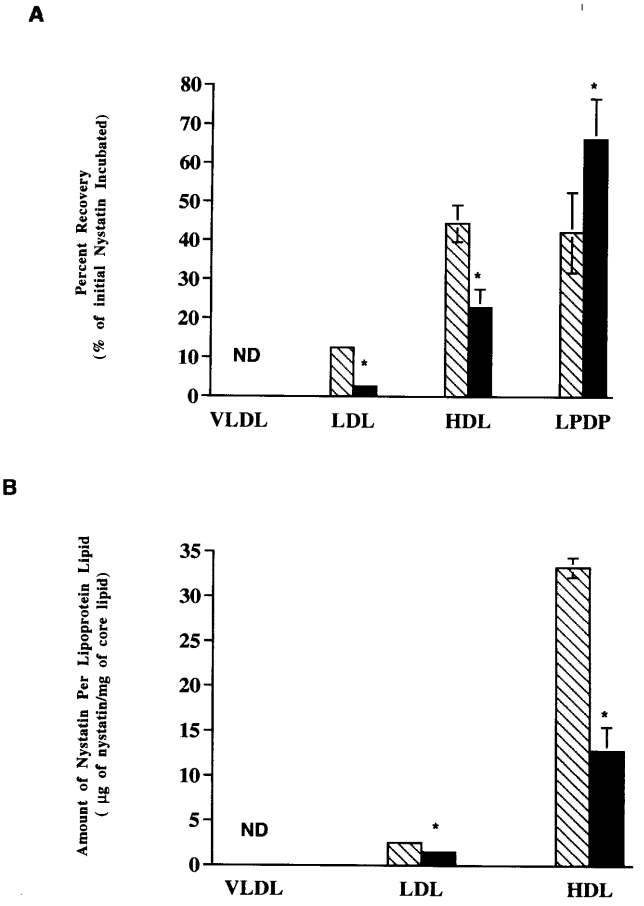

FIG. 1. Distribution of free NYS (■) and L-NYS (▨) (20 μg/ml) in human plasma following incubation for 5 min at 37°C. (A) Data expressed as percent of total NYS distributed into the LPDP, HDL, LDL, and VLDL fractions. (B) Data expressed as micrograms of NYS per milligram of lipoprotein lipid (esterified and unesterified cholesterol and TG) distributed into the HDL, LDL, and VLDL fractions. Data is reported as means ± SDs (error bars) ($n = 6$). *, $P < 0.05$ compared to L-NYS; ND, not detectable.

One such mechanism appears to be related to liposome composition. We have observed that the DMPG component of L-AMPB and L-ANN predominantly distributes into HDL because of its interaction with the protein components (apolipoproteins AI and AII) of HDL (25). Since L-NYS is composed of the same phospholipids as L-AMPB and L-ANN, the increased distribution of NYS into the HDL fraction when incorporated into these liposomes may also be a result of DMPG's attraction for apolipoproteins AI and AII.

We have further shown that the liposomal structure of NYS is not intact within 5 min of incubation in plasma at 37°C (Table 3). However, it appears that the majority of NYS remains lipid associated upon incubation in plasma (Table 2). These findings suggest that these liposomes quickly dissolve into lipid-drug complexes which may rapidly fuse with lipoproteins, especially HDL, once in contact with human plasma. Although we cannot clearly delineate the individual lipid-drug complexes in plasma from these studies, our previous and present observations that the majority of the DMPG (25) and NYS content (Fig. 1A) from these liposomes was recovered in the HDL fraction and the majority of NYS remains lipid associated upon incubation in plasma (Table 2) suggest that a lipid-NYS-HDL complex appears most likely to form. However, further studies are required to substantiate this conclusion.

In summary, we have determined that NYS predominantly associates with HDL upon entrance into the plasma component of the bloodstream when incorporated into liposomes composed of DMPC and DMPG. Furthermore, although a quick disruption of the L-NYS structure was observed, most of the drug remains lipid associated upon incubation in human plasma, suggesting that lipid-drug complexes may form which are then rapidly transferred to lipoproteins. Further studies are required to investigate the pharmacological implications of these findings.

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