The Influence of Lipid Transfer Protein I on the Binding and Transfer of Cyclosporine A Between Lipoproteins

by

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ABSTRACT

Lipid transfer protein I (LTP I) is responsible for all facilitated transfer of the core lipoprotein neutral lipids, cholesteryl ester and triglycerides, and approximately one-third of the coat lipoprotein lipid, phosphatidylcholine (PC) between different plasma lipoproteins. LTP I has a binding site for neutral lipid and monoclonal antibodies against this site has been developed. It has been found LTP I can facilitate the transfer of lipophilic drugs between different lipoprotein subclasses. Our hypothesis is the human body may appear to recognize exogenous lipophilic drugs as lipid-like particles, resulting in these compounds interacting with lipoproteins just like endogenous plasma lipids. Thus their transfer between lipoproteins may be facilitated by plasma LTP I. Previous work has demonstrated LTP I facilitates the co-transfer of CSA with the neutral lipids, CE and TG, from low density lipoproteins (LDL) to high density lipoproteins (HDL) at the neutral lipid binding site. However the role of LTP I in the facilitated co-transfer of PC and CSA between lipoproteins has not been studied.

Purpose: The purpose of this work was to further understand the role of LTP I in the transfer of lipophilic drugs using CSA as a model lipophilic drug. There were two working hypotheses in this work: 1) LTP I is involved in the facilitated co-transfer of the CSA and PC between lipoproteins and 2) LTP I directly binds CSA to its neutral lipid binding site

Methods: To assess if LTP I facilitated PC transfer activity regulates the plasma lipoprotein distribution of CSA, $[^{14}C]$=PC- or $[^3H]$-CSA-enriched HDL or LDL were incubated in T150 buffer containing a radiolabeled free counterpart with purified LTP I or in delipidated human plasma (1.2μg/mL) in the absence and presence of a monoclonal antibody, TP1 (30μg protein/mL), directed against LTP I at the neutral lipid binding site at 37°C. The transfer was determined by radioactive scintillation counting. To assess if CSA binds to LTP I at the neutral lipid binding site, a series of samples (LTP I alone, $[^3H]$-CE liposomal vesicles alone, $[^3H]$-CSA liposomal vesicles alone, $[^3H]$-CE liposomal vesicles with LTP I, or $[^3H]$-CSA liposomal vesicles with LTP I) were injected onto a FPLC Sephacryl XK (18mm x 20cm) column set at a sample load of 0.25mL/min for 3

minutes followed by the collection of 1mL fractions from 3 to 35 at a flow rate of 0.5mL/min. CE binding to LTP I at the neutral lipid binding site was evaluated as a positive control. The LTP I, protein, and radioactivity concentrations of the eluted FPLC fractions were determined accordingly via an LTP I ELISA, protein enzymatic assay, and scintillation counting, respectively.

Results: From the transfer studies in this thesis, it appears the distribution of CSA is not influenced by LTP I-mediated PC transfer activity between the lipoproteins HDL and LDL but is partially dependent on the LTP I-mediated CE and TG transfer activities as demonstrated in previous experiments. The LTP I in fractions eluted from [^{3}H]CE-liposomal vesicles/LTP I injections and [^{3}H]CSA-liposomal vesicles/LTP I injections were similar but the radioactive elution profile was not similar. The FPLC studies suggest CSA may bind with LTP I at the neutral lipid binding site, at other regions, to albumin, or non-specifically to the FPLC column.

In conclusion, LTP I mediated transfer of CSA between lipoproteins may be a result of the direct binding of CSA to LTP I at the neutral lipid binding site.

The distribution/redistribution of drugs among plasma lipoproteins by the facilitated transfer of LTP I may serve as a possible mechanism for determining the ultimate fate of drug compounds. The work presented represents a prerequisite for further studies both, *in vitro* and *in vivo* testing, of the complex between various drugs and LTP I. A challenge in the future could be the development of LTP-mediated transport and controlled release of low molecular weight drugs.

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LIST OF ABBREVIATIONS

AmpB   amphotericin B

ABLC   amphotericin B lipid complex

CE   cholesteryl ester

CETP   cholesteryl ester transfer protein

CPM   counts per minute

CSA   cyclosporine A

EDTA   ethylenediaminetetraacetate

FPLC   fast protein liquid chromatography

HDL   high density lipoprotein

IDL   intermediate density lipoprotein

IL-2   interleukin-2

LCAT   lecithin cholesterol acyl transferase

LDL   low density lipoprotein

LPDF   lipoprotein deficient fraction

LTP I   lipid transfer protein I

LTP II   lipid transfer protein II

mab   monoclonal antibody

OD   optical density

PC   phosphatidylcholine

PL	phospholipid
PLTP	phospholipid transfer protein
RCT	reverse cholesterol transport
TCR	T cell receptor
TG	triglyceride
TP 1	monoclonal antibody directed against lipid transfer protein I
TP 2	monoclonal antibody directed against lipid transfer protein I
VLDL	very low density lipoprotein

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DEDICATION

This work is dedicated to my parents, Cindy *M*ee Sin and Simon *N*i Wah Kwong, and to my brothers, David, Vincent and Evan. Thank you for your faith.

Chapter 1

INTRODUCTION

1.1 Cyclosporine A

Transplantation success and the treatment of autoimmune diseases have benefited from the introduction of immunosuppressant agents. The main aim in transplantation is to increase graft survival by preventing the development of acute and chronic rejections, with the result being an increase in patient survival. In the 1960s, steroids provided part of the basis of immunosuppressive therapy, particularly for acute rejection. For the next two decades, steroids provided the backbone of maintenance therapy [1]. However, steroids are non-selective immunosuppressants and are associated with many long-term side effects. The most clinically useful immunosuppressive agents are ones that target specific parts of the immune system. One such example is Cyclosporine A (CSA), which acts by specifically inhibiting the T cell response.

Cyclosporine A is an immunosuppressant commonly used in organ transplantation and in the treatment of several autoimmune disorders [2]. Sandoz originally discovered it in the drug screening process for antifungal drugs. CSA was originally obtained from the fermentation products of the fungal species, *Tolypocladium inflatum Gams* [3]. The structure of CSA (Figure 1) was elucidated by X-ray crystallography as well as chemical degradation [4, 5]. It is a cyclic undecapeptide ($C_{62}H_{111}N_{11}O_{12}$) with a molecular weight of 1202.63. The structure consists of eleven amino acids cyclically linked with seven N-methylated and one unique 9-carbon amino acid, now named (4R)-4-[(E)-2-butenyl]-4-methyl-L-threonine (*MeBmt*) [6, 7]. CSA is a highly lipophilic with the aliphatic amino acids responsible for this property. It has a low solubility of 0.04 mg/mL in water [8]. It has an experimental log P_{oct} value of 2.92 and a calculated log P_{oct} value of 14 [9]. The partition co-efficient, log P_{oct}, is a physicochemical

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property describing a partitioning equilibrium of solute molecules between a water and an organic solvent, the organic solvent was octanol in this case. Several analogues exhibit *in vitro* biologic activities, but only CSA and some analogues cause selective inhibition of the immune response *in vivo* [2]. CSA was the most potent immunosuppressant and was purified from these analogues in 1973. The introduction of CSA in the 1980's revolutionized transplantation by not only increasing graft survival, but also by reducing the use of steroids and their associated side effects [2]. In addition to transplantation use, CSA is indicated for and has successfully treated autoimmune disorders including such conditions as psoriasis [8, 10], uveitis [11], and rheumatoid arthritis [2, 8, 12].

T cells contribute to immune response by the recognition of foreign antigens. The binding of foreign antigens results in the transcription of cytokines and creates an effective immune response by activating other components of the immune response such as T cells and B cells. T cell activation begins with the binding of an antigen to the T cell receptor (TCR) which is coupled to a cell surface protein [2].

CSA functions as an immunosuppressant by the selective inhibition of T lymphocyte signal transduction (Figure 2) [2]. It targets calcineurin, an enzyme that phosphorylates other proteins as part of the signal transduction pathways and is recognized as one of the rate limiting steps in lymphocyte activation [2]. CSA alters the activity of calcineurin to its substrates in T cells by binding to several proteins in the cell of which the cyclophilins are most important. The CSA/cyclophilin complex blocks the enzymatic function of the calcium-activated calcineurin, which in turn prevents dephosphorylation and activation of the interleukin-2 (IL-2) enhancer, the antigen-inducible transcription factors NFAT1 (nuclear factor of activated T cells), and the DNA binding proteins [2]. As a result, translocation to

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the nucleus is prevented, IL-2 synthesis is down regulated, and an effective immune response is not generated.

Due to its lipophilicity, CSA must be solubilized for administration. CSA can be administered intravenously as a 50 mg/mL solution made of an ethanol-polyoxyethylated castor oil mixture [7]. It can also be administered orally as a soft gelatin capsule (Sandimmune®) or as the newer oral microemulsion formulation (Sandimmune Neoral®). The original soft gelatin capsule formulation has a wide range in bioavailability ranging from 20% to 50% [7]. This was shown to be due more to a variability in absorption rather than a first pass effect [13]]. The new microemulsion formulation was developed to improve the absorption characteristics of CSA. It is a mixture of CSA with DL-α-tocopherol, castor oil, propylene glycol, corn oil and ethanol which forms a microemulsion upon contact with gastrointestinal fluids. The bioavailability of CSA in this microemulsion formulation is improved by 40-60% compared to the original oral formulation and there has been demonstrated reduction in the inter- and intrapatient variability of pharmacokinetic parameters [14, 15]. Food does not affect the absorption of CSA in the microemulsion formulation but a fatty meal does delay the absorption of CSA in the original oral formulation[15, 16]. Peak levels of CSA occur from 1.3 to 4 hours after oral administration [17]. CSA has a high volume of distribution with values of 3-5 L/kg reported in solid transplant patients [8].

CSA is extensively metabolized in the liver by the cytochrome P450 3A enzymes and to a lesser extent in the gastrointestinal system and the kidney [8]. More than 25 metabolites have been reported from patients taking CSA [6, 7, 18]. The majority of metabolites produced are N-demethylated and/or hydroxylated with the cyclic backbone remaining intact

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[6, 19]. Some of these metabolites exhibit immunosuppressive activity and may contribute to the varied CSA pharmacokinetics, therapeutic outcomes and severity of side effects found in patients taking this therapy [18]. Only 0.1% of CSA is excreted unchanged in urine and elimination is primarily via the biliary route [8].
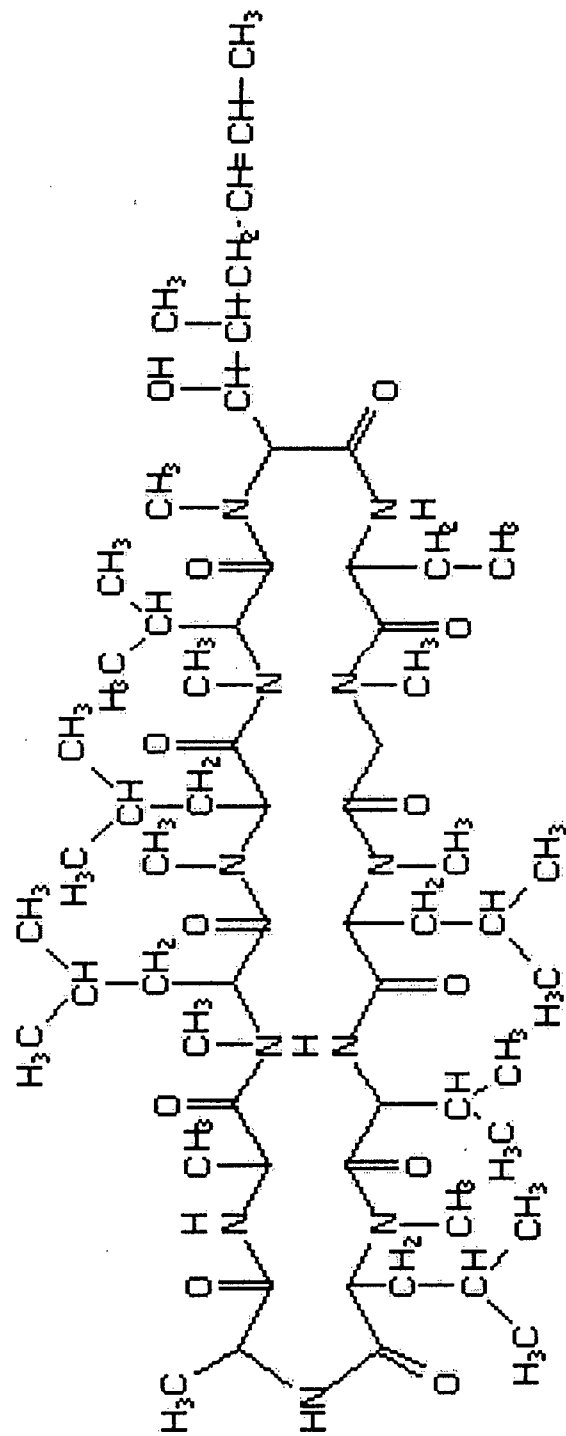
Figure 1 Molecular structure of cyclosporine
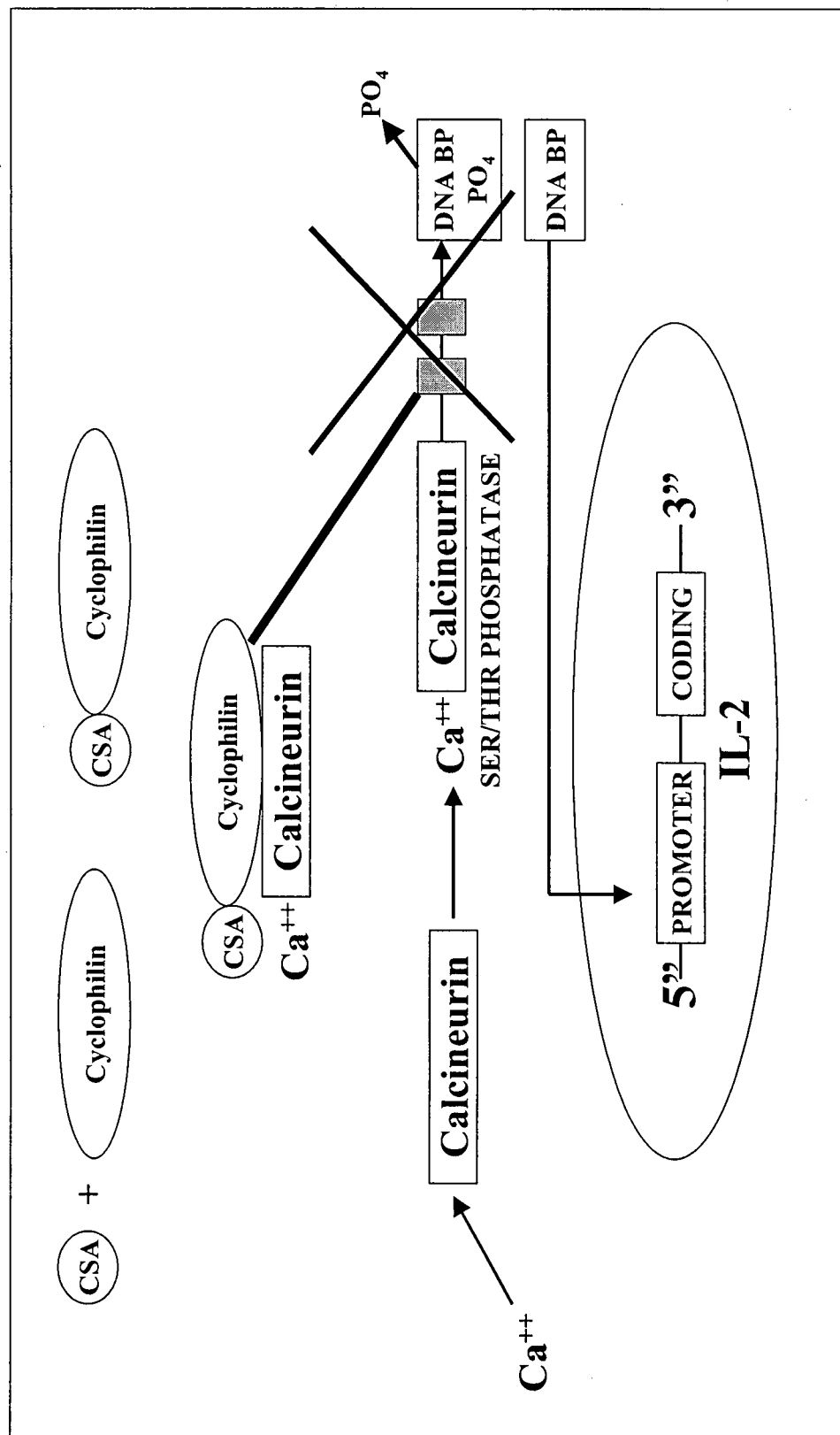
Figure 2 Mechanism of action of cyclosporine, modified from [2] [see text for details; CSA: cyclosporine, SER/THR PHOSPHATASE: serine/threonine phosphatase, Ca^{++}: calcium, IL-2: interleukin-2)

Despite its effectiveness, CSA is associated with a number of side effects. Its clinical use is limited by its nephrotoxicity which is indicated by increased serum creatinine levels and changes in creatine clearance [2]. A complicating factor accompanying CSA nephrotoxicity is distinguishing this adverse effect from chronic rejection. Nephrotoxicity can be minimized by drug combinations or by dose reduction during chronic maintenance therapy [20]. Other adverse effects of CSA include hypertension, hepatotoxicity, neurotoxicity, hirsutism, gingival hyperplasia, and gastrointestinal toxicity [8].

Many disease states have abnormal metabolic or physiological conditions that can affect drug characteristics. In the diseased state, lipid metabolism can be affected. This may result in dyslipidemic plasmas such as hypocholesterolemia and hypertriglyceridemia, which may alter the concentration of the different lipoprotein classes [21]. A number of lipophilic drugs bind to lipoproteins including halofantrine (Hf) [22, 23], amphotericin B [24, 25], probucol [26], nystatin [27], and cyclosporine [28]. Hence it is possible that changes in lipoprotein profiles may affect the association of a compound with lipoproteins as well as its distribution amongst the lipoprotein subclasses.

1.2 Plasma lipoproteins

Lipoproteins are complexes carrying lipids and proteins in plasma that primarily transport lipids throughout the vascular and extravascular body fluids to cells requiring energy [21]. Lipoproteins contain a hydrophobic core of cholesteryl ester (CE) and triglyceride (TG) surrounded by a monolayer of phospholipid (PL) embedded with both unesterified

cholesterol and apolipoproteins (Figure 3). The types of PL include phosphatidylcholine (PC), phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, and sphingomyelin with PC being the most abundant. The lipids found in lipoproteins are delivered from the liver and intestine to other tissues in the body for either storage or in the production of energy.
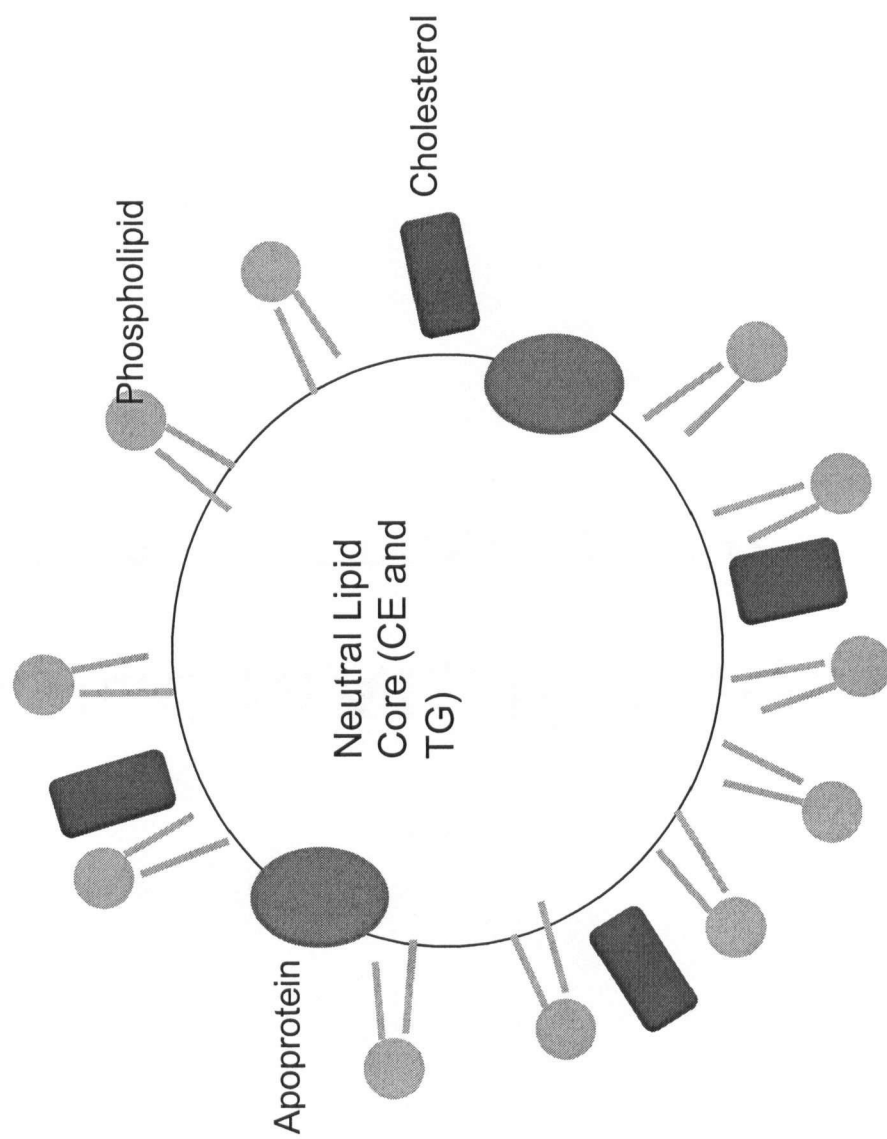
Figure 3 General structure of a lipoprotein [21, 29]

Lipoproteins are traditionally classified according to density, with those having a high protein to lipid ratio being of higher density [21]. There are four main categories of lipoproteins: triglyceride rich lipoproteins composed of chylomicrons and very low density lipoprotein (VLDL), low density lipoprotein (LDL), intermediate density lipoprotein (IDL), and high density lipoprotein (HDL).

Table 1 Physical chemical characteristics of major lipoprotein subclasses [30]

	Chylomicrons	VLDL	IDL	LDL	HDL
Diameter (nm)	75-1200	30-80	25-35	18-25	5-12
Density (g/mL)	0.95	0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.21
Composition (% dry weight)					
Cholesterol	5	19	38	50	19
Triglycerides	86	55	23	6	4
Phospholipids	7	18	20	22	30
Protein	1-2	8	19	22	47
Apoproteins	A1, A2				A1, A2
	B-48	B-100	B-100	B-100	
	C1, C2, C3	C1, C2, C3	C1, C2, C3		C1, C2, C3
	E	E	E		E

Chylomicrons are the largest of the lipoprotein particles and are most abundant after a meal. They range in diameter from 75 to 1200 nm with the largest component comprised of TG.

VLDL has a diameter range of 30 to 80 nm, and is the major transport vehicles for endogenous TG in the plasma. It is synthesized in the liver [31] and to a lesser degree in the intestine. Excess fatty acids and carbohydrates in the body are converted into TG and are stored in VLDL. The TG in both chylomicrons and VLDL are used as a cellular energy source or stored in adipose tissue after hydrolysis by lipoprotein lipase to release free fatty acids.

Intermediate-density lipoproteins (IDL) remain after the TG in the VLDL has been hydrolysed. IDL undergoes further hydrolysis of core TG and form LDL which is rich in CE. LDL has a diameter of 18 to 25 nm and is the major transport vehicle for cholesterol. LDL is removed from the circulation primarily via the LDL-receptor pathway. Once endocytosed, its contents are hydrolysed with free cholesterol being stored or incorporated into membranes.

HDL, the smallest lipoproteins with a diameter of 5 to 12 nm, has the highest protein content, and are involved in a process called reverse cholesterol transport (RCT) (Figure 4). RCT is the process of transporting cholesterol from peripheral tissues back to the liver [32-34]. Disc shaped nascent HDL particles are precursors to HDL. The esterification of the free cholesterol in the nascent HDL by lecithin cholesterol acyl transferase (LCAT) and its accumulation in the core result in a spherical shaped particle known as HDL_3. The further exchange of CE in HDL_3 for TG from triglyceride rich lipoproteins is facilitated by lipid transfer protein I (LTP I), also known as cholesteryl ester transfer protein (CETP). The end result of this process is the mature HDL_3 particle, called HDL_2. The liver then takes the triglyceride rich lipoproteins back and the cholesterol obtained from HDL_3 particles is removed, hence the term RCT. The HDL_2 particle regenerates into HDL_3 particles by the hepatic lipase mediated hydrolysis of TG and the cycle of cholesterol removal can begin again.
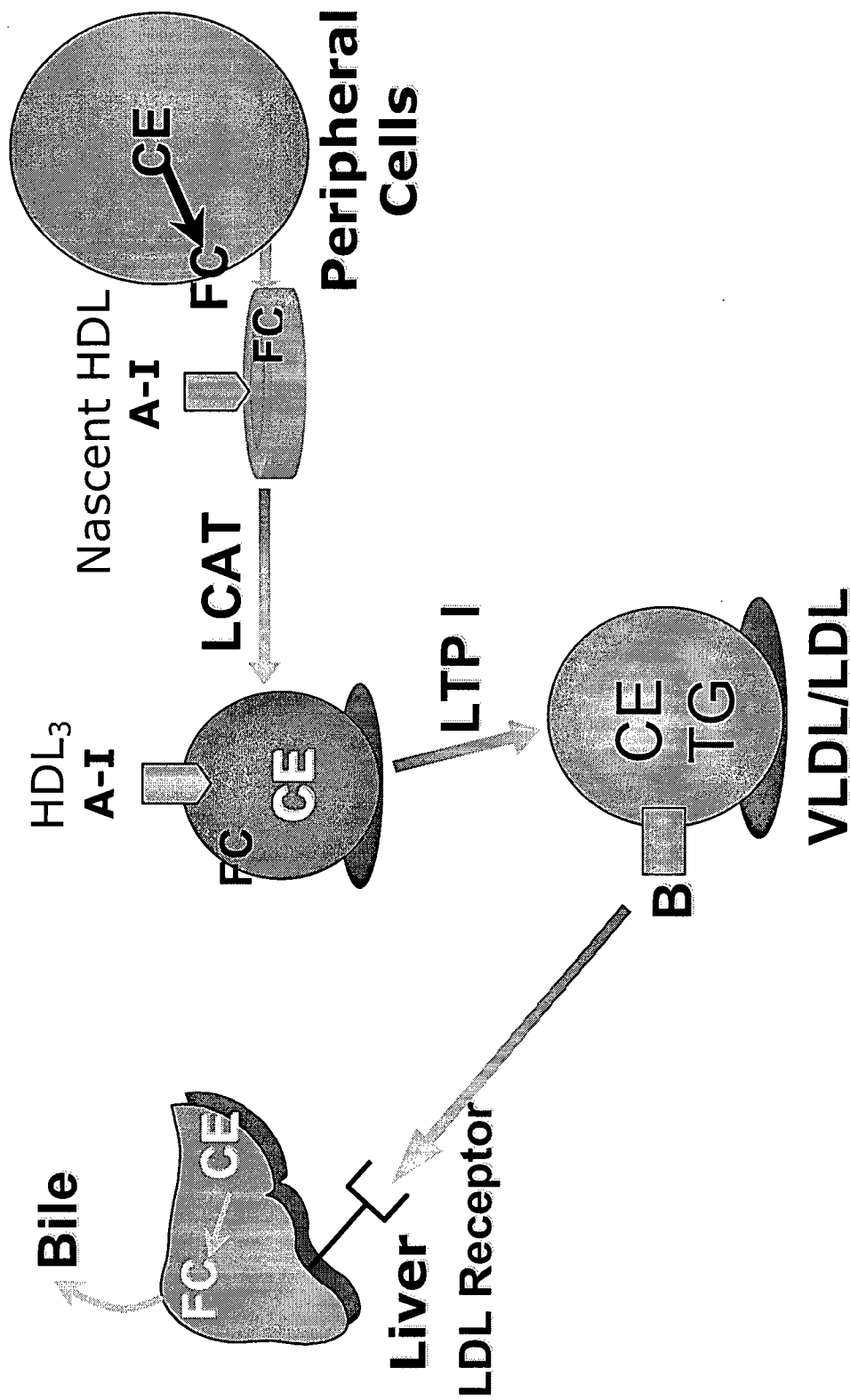
Figure 4 Reverse cholesterol transport (adapted from [35, 36])

Lipoproteins appear to have a wider biological role than simply the movement of water-soluble lipids from the systemic circulation to tissues. They are also involved in the binding and subsequent transport of a number of lipophilic compounds [22-28]. Lipophilic compounds associate with different lipoproteins with varying effects on their efficacy and toxicity and this will be discussed in the thesis.

1.3   CSA Association with plasma lipoproteins

The lipophilic properties of CSA facilitate its association with lipoproteins in blood, serum, and plasma [24, 37]. In blood, CSA is mainly found in erythrocytes (40-50%) and plasma (30-40%) with the remainder located in granulocytes and lymphocytes [28]. In plasma, CSA is mainly associated with lipoproteins of which approximately 33-46% is associated with HDL, 21%-31% with LDL, and 8%-19% with VLDL [37]. LDL and HDL appear to be the lipoproteins mostly associated with CSA.

The interaction of CSA with lipoproteins is poorly understood. Researchers have shown the activity of CSA may be dependent to which lipoprotein it is bound. One proposed consequence of CSA association with lipoproteins is the decrease in the pharmacological effect of the drug. It has been suggested that the pharmacological or toxic effects of CSA may depend to which lipoprotein CSA is bound [28]. We have further shown changes in the total and plasma lipoprotein lipid concentration and composition influence the lipoprotein binding of CSA [24]. Other studies have reported decreases in CSA activity in patients with elevated plasma triglyceride levels [38, 39], while an increase in CSA toxicity has been associated in patients with hypolipidemia [40], in heart transplant patients with high total

plasma cholesterol levels [41]. It appears CSA association with lipoproteins may result in alterations in its distribution and toxicity profile in different patient populations.

Transplantation patients who are administered CSA often exhibit or will exhibit plasma dyslipidemias [41-44], including hypocholesterolemia and hypertriglyceridemia. Plasma LTP I activity has been shown to be influenced by a number of plasma factors including neutral lipids and free cholesterol [45]. Dyslipidemias may also have an elevation in LTP I levels [46-51]. Other work has proposed CSA to be transferred by a facilitated process between lipoproteins rather than through simple diffusion [52].

Since the human body may recognize lipophilic compounds as lipid-like particles, LTP I is *hypothesized* to be involved in the facilitated transfer of the lipophilic drug, CSA, similar to how it facilitates the transfer of lipids.

1.4 Lipid transfer protein I

LTP I is a 476 amino acid glycoprotein with a molecular weight of 74,000 daltons [53]. LTP I and phospholipid transfer protein (PLTP or LTP II), are believed to have evolved from a common ancestor belonging to the family of lipopolysaccharide binding proteins. These two proteins share a 20% homology to each other and have homologous regions to lipopolysaccharide binding proteins [32, 54, 55]. LTP I expression between mammalian species is variable, with undetectable levels in rats and mice but moderate levels in humans and rabbits [45, 56]. In humans, LTP I is mainly synthesized in the liver [57], with lower levels synthesized in the adipose, kidney, heart and spleen [58]. It has been shown that LTP I is responsible for all facilitated transfer of the core lipoprotein lipids (CE and TG) and

approximately one-third of the coat lipoprotein lipid (PC) [53, 59-62]. It is involved in the removal of CE generated by the LCAT reaction through transferring from HDL_3 to triglyceride rich lipoproteins [21] with a reciprocal transfer of TG [53, 63, 64]. LTP I, along with LTP II, are involved in the metabolism and remodeling of plasma lipoproteins [32, 65]. LTP I may also play a role in certain disease processes, such as atherosclerosis, by redistributing cholesterol from the anti-atherogenic HDL particles to the pro-atherogenic LDL particles. Conversely, LTP I as mentioned previously, it is also involved in the process of RCT, which removes cholesterol from peripheral tissues and is thus viewed as anti-atherogenic (Figure 4). The exact role of LTP I in the development atherosclerosis remains uncertain [58].

LTP I is regulated by cholesterol levels, with an increase in activity and expression seen in response to cholesterol [66]. In studies using human-LTP I transgenic mice, the increased activity is attributed to an increase in transcription of the LTP I gene [67]. Conversely, LTP I activity has been reduced in response to corticosteroids and lipopolysaccharides [68, 69].

The LTP I binding domain for neutral lipids (CE and TG) is located in the 26 amino acids comprising the carboxyl-terminus of LTP I [53, 59-62]. A LTP I variant lacking the amino acid residues 470-475 cannot bind CE or TG [60, 70]. Binding to LTP I is also blocked by anti-LTP I monoclonal antibodies (mAb), such as TP1 and TP2, specific to the neutral lipid binding site of LTP I [59, 61, 62, 71]. TP2 has a higher affinity than TP1. This is illustrated by Table 3 [71]. Table 3 summarizes the differences in affinity to immobilized LTP I of different mAbs raised against LTP I [71].

Table 2 Dissociation Constants of antibody binding to immobilized LTP I [71]

MAb	K_d (M^{-1})
TP1	2.4×10^{-8}
TP2	1.9×10^{-8}
TP4	2.2×10^{-8}
TP5	5.4×10^{-8}
TP7	1.3×10^{-8}
TP8	2.3×10^{-7}
TP9	3.9×10^{-7}
TP10	5.5×10^{-8}
TP11	1.2×10^{-8}
TP12	5.3×10^{-7}
TP13	6.2×10^{-8}
TP14	4.8×10^{-9}
TP15	7.9×10^{-9}
TP16	4.8×10^{-7}
TP17	4.9×10^{-8}
TP18	9.7×10^{-9}
TP19	4.1×10^{-9}
TP20	1.6×10^{-9}

Another group [71] has prepared other anti-human LTP I mAbs with a proposed LTP I epitope map (Figure 5). The domain is comprised of an amphipathic helix, consisting of a charged/polar residue face and a hydrophobic residue face to which binding of neutral lipid occurs [72]. The binding of neutral lipids may induce a conformational change which in turn enhances binding to lipoproteins [62].

(A)

(B)
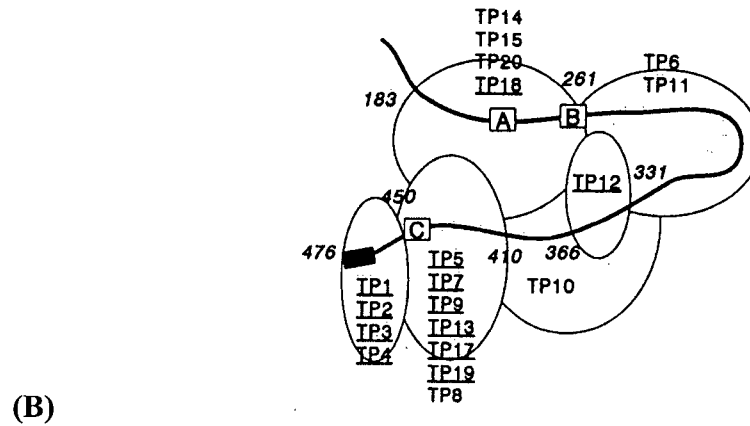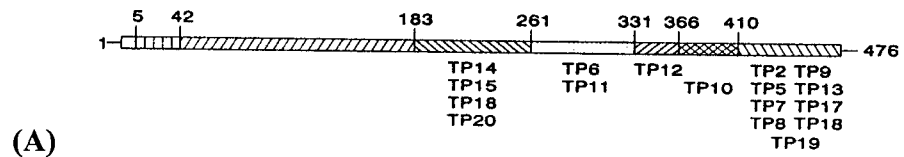
Figure 5 (A) An epitope map of LTP I based on the pattern of reactivities of mAbs with expressed LTP I fragments (B) Model of the carboxyl-terminal of LTP I based on results of epitope mapping and antibody competition experiments. Antibodies that are capable of greater than 20% inhibition of LTP I-mediated CE transfer are underlined (from [71])

LTP I facilitated lipid transfer has been proposed to occur by one of two methods: 1) a carrier mediated process [73], in which LTP I acts a shuttle between the donor and acceptor lipoprotein and 2) a ternary mechanism [74], in which a donor-LTP I complex collides with an acceptor forming a ternary complex.

In the carrier-mediated process LTP I binds to the donor lipoprotein (HDL), which could induce a conformational change in the protein, exposing a neutral lipid binding site. A molecule of CE is then relocated to the this binding site and LTP I dissociates from the HDL particle. This LTP I/CE complex then diffuses and will eventually bind to an acceptor lipoprotein (VLDL or LDL) where the CE is exchanged for a molecule of TG. The LTP I then dissociates from the acceptor lipoprotein and the process repeats.

In the ternary mechanism of lipid exchange, the LTP I/HDL complex would not dissociate from each other, rather this complex would collides and binds to the acceptor lipoprotein to form a ternary complex. The exchange of a CE molecule for a TG molecule is facilitated by LTP I. Once this exchange is completed, the three components dissociate into their separate entities and the process repeats again. LTP I is believed to transfer these lipids via a carrier mediated mechanism rather than by the formation of a ternary complex comprised of LTP I, the donor, and the acceptor lipoproteins [75].

Since the human body may recognize lipophilic compounds as lipid-like particles, LTP I was originally *hypothesized* to be involved in the facilitated transfer of lipophilic drugs amongst lipoproteins at the same neutral binding lipid site as where it facilitates the transfer of lipids.

Evidence to support this hypothesis is presented in the subsequent sections with the discussion of the role of LTP I in the lipoprotein distribution of Hf, AmpB, and CSA. The understanding of the mechanism by which these drugs distribute into lipoprotein fractions may be important in understanding their efficacy and toxicity. The aims presented in this thesis further explores the role of LTP I in the distribution of CSA between lipoproteins.

1.5   LTP I mediated transport of lipophilic drugs

1.5.1   LTP I and Halofantrine

Hf is a lipophilic agent indicated for the treatment of malaria, particularly against *Plasmodium falciparum* and other multi-drug resistant strains [76]. One study has suggested HDL may be a source of phospholipids for *Plasmodium falciparum* to support its growth *in vitro* [77]. There has been observed decreases in plasma HDL-cholesterol levels and increases in plasma VLDL-TG levels in patients infected with malaria compared to non-infected patients [78, 79].

Hf has been shown to bind to lipoproteins upon incubation in human blood [23] and in plasma [22]. It was observed that Hf interacts and binds to LDL and HDL upon incubation in human blood from both malaria infected individuals and noninfected individuals[23]. Another study has observed the relative pre-prandial and post-prandial lipoprotein profiles may influence the lipoprotein distribution of Hf within fasted and fed beagle plasma [80]. This group has also demonstrated the IC_{50} of Hf significantly increases when incubated in the presence of 10% post prandial serum with an elevation in triglyceride rich lipoproteins compared with normolipidemic serum in an *in vitro* culture of *Plasmodium falciparum* [81]. These differences may be

regulated by core neutral lipoprotein lipids [22]. As stated before, LTP I is involved in the facilitated transfer of neutral lipids between lipoproteins. Hence, from these studies, experiments were done to determine the role of lipoprotein lipid and protein concentration and the role of LTP I in the plasma distribution of Hf [82].

In these studies, Hf (1000 ng/mL) was incubated in plasma from three different patients (hypo-, normo-, or hyper-lipidemic human plasma) for 60 minutes at 37°C. Following incubation, the plasma was cooled to 4°C to prevent drug redistribution, then separated into its lipoprotein and lipoprotein deficient fractions by density gradient ultracentrifugation. Each fraction was subsequently quantified for Hf by high pressure liquid chromatography [82]. The activity of LTP I in these dyslipidemic plasma samples was determined in terms of its ability to transfer CE between LDL and HDL. The fraction of lipids and drug transferred (kt) was calculated as described by Pattnaik and Zilversmit [83].

$$kt = -ln\ (1-A_t/D_0)$$

where D_0 and A_t are the radioactivities of the donor at time 0 and the acceptor at time t, respectively. The constant k is the fraction of the radio-labeled compound transferred per unit time (t). Acceptor radioactivity in the absence of LTP I (usually < 2-3%) is subtracted before calculating kt values. Calculations assume steady-state conditions in which all lipid and drug transfer are in an exchange process.

From these experiments, it was observed, that as the total plasma and lipoprotein lipid concentrations increased from the hypolipidemic plasma to the hyperlipidemic plasma, LTP I

activity increased from 2.2 ± 0.8 %kt to 14.6 ± 2.5 %kt. As the LTP I mediated transfer of CE increased, the amount of Hf recovered in the HDL fraction decreased with an increased amount recovered in the triglyceride rich lipoprotein fractions. In this work [82], fasted normolipidemic plasma was further supplemented with an exogenous supply of LTP I (0.67µg protein/mL) to determine if these differences in Hf lipoprotein distribution are a function of LTP I. A significantly greater percentage of Hf was recovered in the TRL fraction with a significantly lower percentage of Hf recovered in the LDL, HDL, and lipoprotein deficient plasma fractions compared to normolipidemic plasma [82]. Hence, these studies demonstrated as LTP I activity increases, the proportion of Hf associated with HDL decreases and increases with the TRL fraction. Since LTP I catalyses the transfer exchange of neutral lipids between lipoproteins, the studies suggest Hf plasma distribution is related to its lipoprotein neutral lipid content or to LTP I.

1.5.2   LTP I and Amphotericin B & Amphotericin B Lipid Complex

Despite the development of a number of new lipid-based anti-fungal formulations [84], AmpB, formulated as a colloidal suspension, remains one of the most effective and affordable agents in the treatment of systemic fungal infections [84]. However, the clinical use of AmpB has been limited by dose-dependent renal toxicity.

AmpB's association with serum LDL is involved in the development of AmpB-induced kidney toxicity. This association is regulated by an increase in LTP I [63, 64, 85-87]. However, when AmpB is incorporated into non-toxic phospholipids to form AmpB-lipid complex (ABLC),

AmpB binding to serum LDL decreases and AmpB-induced kidney toxicity is significantly reduced [88, 89].

When AmpB was incubated in human serum for 1 hour at 37°C, over 70% of the drug was bound with serum HDL [90]. However, the preliminary findings of the research suggest that an increase in LTP I concentration increased the binding of AmpB with serum LDL [90]. This implies that changes in LTP I concentration may regulate the distribution of AmpB between the HDL and LDL fractions of human serum. This observation was recently supported by other research [91] which reported AmpB forms a complex with plant lipid transfer protein I, even though no binding affinity could be determined.

An increase in LTP I concentration, however, did not increase the binding of AmpB with serum LDL when ABLC was incubated in human serum [90]. Furthermore, the presence of ABLC decreased the ability of LTP I to transfer CE from HDL to LDL [90]. These observations suggest that the presence of lipid complexes in serum result in the reduction of LTP I-mediated transfer of CE from HDL to LDL. Since AmpB binds to unesterified and esterified cholesterol in serum [92], this finding may partially explain the decreased association of AmpB with serum LDL when formulated into these lipid complexes. Taken together these, studies suggest that AmpB is related to its lipoprotein neutral lipid content.

1.5.3 LTP I and Cyclosporine

As mentioned in previous sections, LTP I has a role in the distribution of the lipophilic drugs, Hf and AmpB. In the research of this thesis, CSA was used as a model of a lipophilic drug to further explore the relationship with LTP I.

Because CSA's activity and toxicity may be dependent upon which lipoprotein it is bound to and differences in activity and toxicity are noted in patients with different lipoprotein lipid concentration and composition [38-41], understanding the mechanism of how this drug is transferred from one lipoprotein to another may be important in understanding the efficacy and toxicity of this drug. Exploring the relationship of CSA with LTP I may also be important because of its relationship with dyslipidemic plasmas. Transplantation patients who are administered CSA often exhibit plasma dyslipidemias [41-44], and dyslipidemias may have an elevation in LTP I levels [46-51].

Previous data suggest LTP I has a direct role in lipoprotein distribution of CSA [93]. CSA incubated in plasma results in a predominant association with HDL and LDL [24]. When this plasma was supplemented with exogenous LTP I, CSA redistributes from LDL to HDL, as shown in Figure 6 [93].
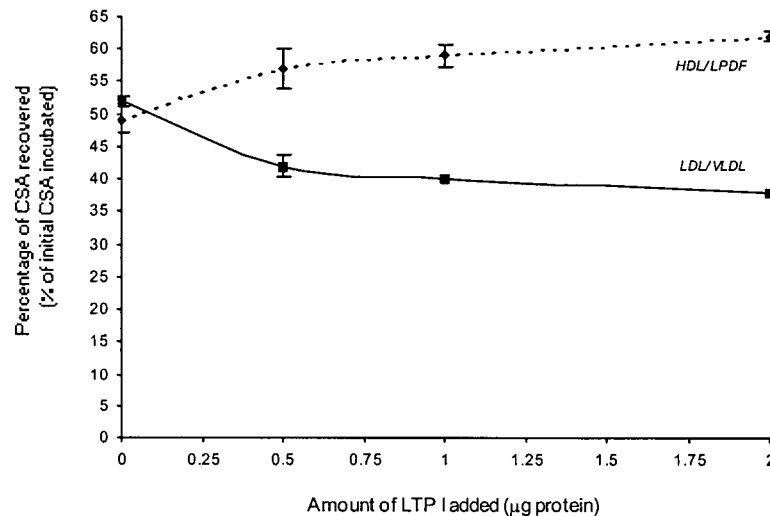
Figure 6 Percent recovery of cyclosporine in the HDL/LPDF and VLDL/LDL fractions in human plasma supplemented with increasing amounts of exogenous LTP I. Cyclosporine was incubated at a concentration of 1000ng/mL for 60 min at 37°C. After incubation, plasma was separated by affinity chromatography and the percentage of cyclosporine recovered in each fraction was determined by radioactivity. Data expressed as mean SD (n=6). (From [93]).

LTP I has been shown to facilitate the transfer of mainly the neutral lipids contained in the core

of lipoproteins at the neutral lipid binding site located in the 26 amino acids comprising the

carboxyl-terminus of LTP I. We had originally hypothesized LTP I may transfer lipophilic

compounds in the same way as lipids. Thus, we investigated the role of the neutral core lipids,

CE and TG, with the transfer of CSA at the neutral lipid binding site. We have demonstrated

the transfer of CSA between LDL and HDL is partially dependent on the LTP I facilitated

transfer of CE and TG [93, 94]. Briefly, in these experiments there was an incubation of radio-

labeled enriched CE or CSA HDL or LDL with a corresponding non-labeled counterpart in the

presence of a purified source of LTP I in T150 buffer or in the presence of a delipidated source

of plasma containing LTP I and other endogenous transfer proteins. In a further experiment, a

mAb against the neutral lipid binding site of LTP I was also added. Transfer of the radio-

labeled lipoprotein compound of interest to its non-radiolabeled counterpart was quantitated for each of these experiments. The experiments were repeated for radio-labeled enriched TG or CSA lipoproteins. To account for diffusional transfer, radio-labeled donor lipoproteins and its corresponding non-labeled counterpart were added in the volume equivalent to 10μg cholesterol or triglyceride. The fraction of lipids and drug transferred (kt) was calculated as described by Pattnaik and Zilversmit, as previously described [83].

These studies showed LTP I is partially involved in the transfer of CSA from LDL to HDL. Transfer rates were increased when the experiment was performed in delipidated plasma compared to the experiment in T150 buffer system with CE (Figure 7). The percent transfer of CSA from only LDL to HDL was greater in T150 buffer than in human plasma with TG [94]. When the transfer of CE and CSA (Figure 7) and TG and CSA (Figure 9) were determined in the presence of a mAb against the neutral lipid binding site, the percent transfer from LDL to HDL were significantly decreased compared to the controls. This suggests the neutral lipid binding site of LTP I has a role in the facilitated co-transfer of either CE or TG with CSA.

The transfer of CSA from HDL to LDL does not appear to be LTP I facilitated. When a mAb against the neutral lipid binding site of LTP I was added in amounts to significantly reduce the transfer of CE (Figure 8) from HDL to LDL there was no significant change in the transfer of CSA. An undetectable transfer of CSA from HDL to LDL was observed in T150 buffer containing purified LTP I when TG transfer was observed (Table 3). These findings suggest that along with LTP I, there may also be other factors involved in the transfer of CSA, such as other endogenous plasma transfer proteins. Our data also suggest LTP I may partially regulate

CSA distribution amongst different lipoprotein classes in human plasma. This transfer could be explained by CSA:

- binding to lipoprotein core neutral lipids, CE or TG, or to the coat phospholipid, PC, which then binds to LTP I at the neutral lipid binding site

- binding to the neutral lipid binding site of LTP I, independently of the lipids

- binding to an alternative site(s) on LTP I

Since LTP I is also involved in the transfer of one-third of the coat lipoprotein lipid, the role of PC in the transfer of CSA was further investigated in this thesis to determine if only the neutral lipids in the core of the lipoproteins play a role in the transfer of CSA at the neutral lipid binding site. The research in this thesis also investigated if CSA independently binds to LTP I at the neutral lipid binding site.
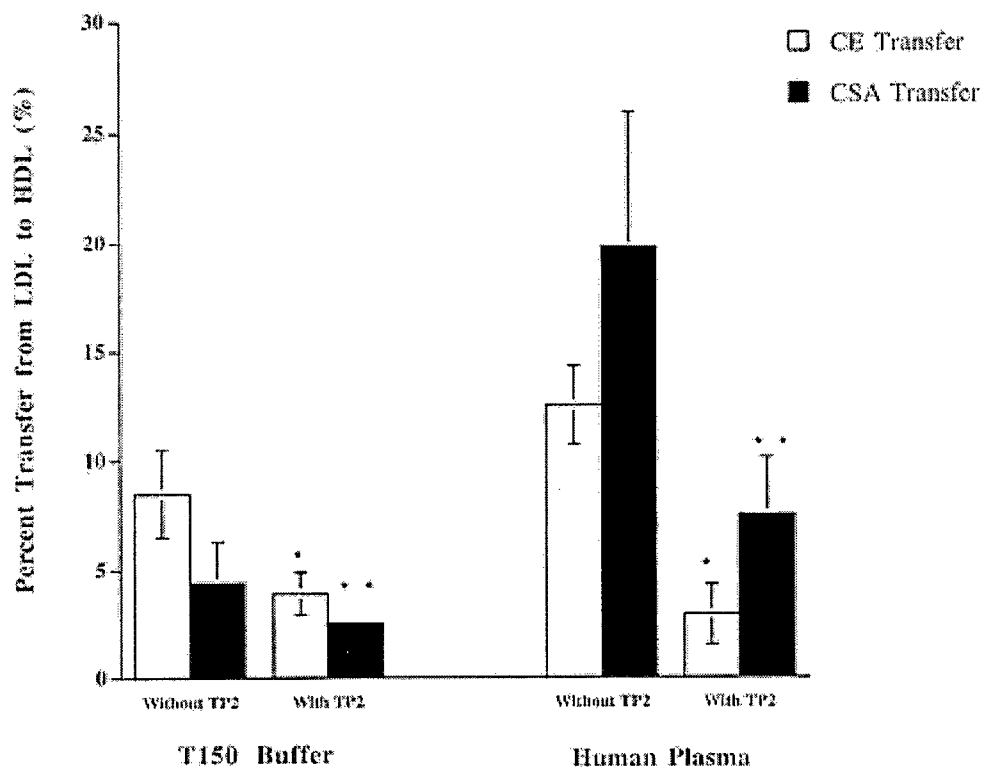
Figure 7 Cholesteryl ester (CE) and cyclosporine (CSA) percent transfer from LDL to HDL, in the presence or absence of a monoclonal antibody (TP2) directed against LTP I. Experiment involved incubation of radiolabeled CE- and CSA-enriched LDL with unlabeled HDL (at 10 µg lipoprotein cholesterol) for 60 min at 37°C in T150 buffer supplemented with LTP I (1.0 µg protein/ml) or delipidated human plasma which contained 1.0 µg protein/ml of LTP I. Data expressed as mean ± S.D. (n = 6). *P < 0.05 $vs.$ CE or CSA percent transfer without TP2. (From [93]).

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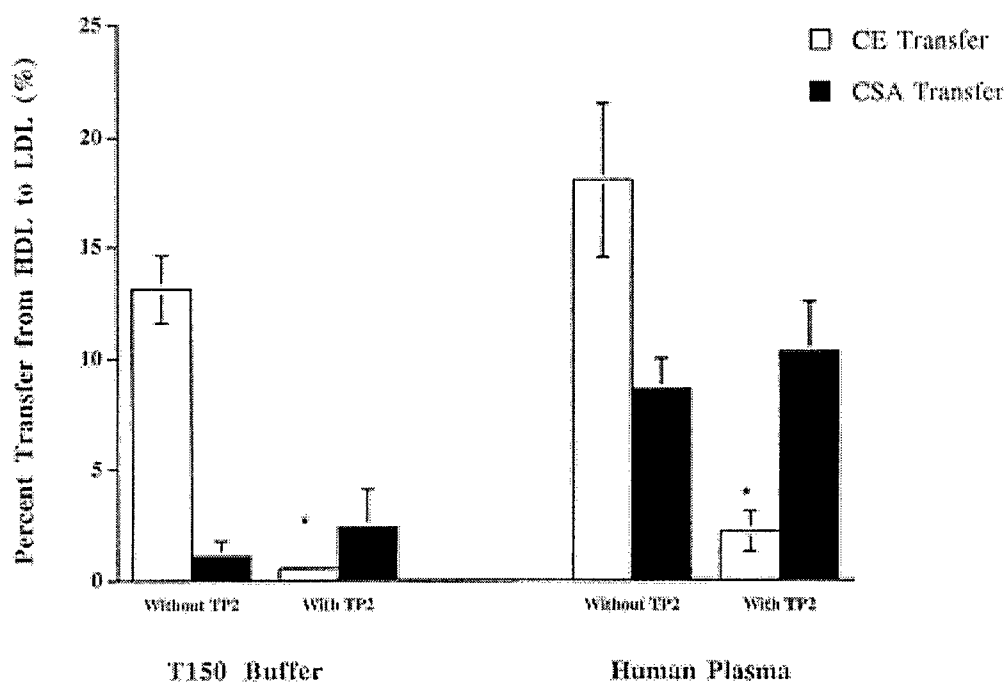
Figure 8 Cholesteryl ester (CE) and cyclosporine (CSA) percent transfer from HDL to LDL, in the presence or absence of a monoclonal antibody (TP2) directed against LTP I. Experiment involved incubation of radiolabeled CE- and CSA-enriched HDL with unlabeled LDL (at 10 μg lipoprotein cholesterol) for 60 min at 37°C in T150 buffer supplemented with LTP I (1.0 μg protein/ml) or delipidated human plasma which contained 1.0 μg protein/ml of LTP I. Data expressed as mean ± S.D. (n = 6). *P < 0.05 vs. CE or CSA percent transfer without TP2. (From [93])
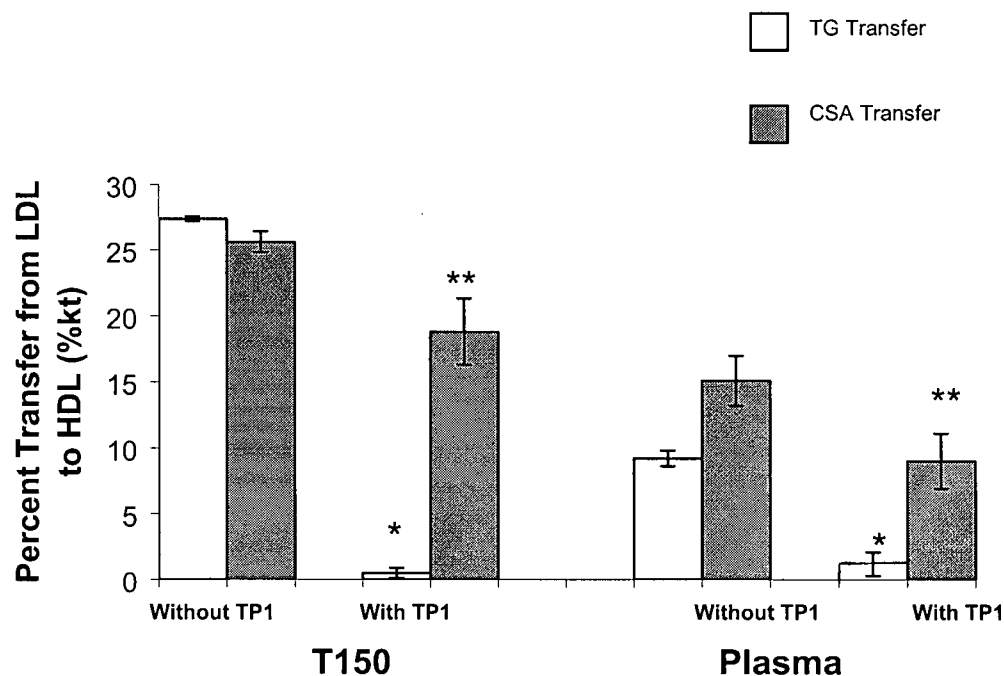
Figure 9 Triglyceride (TG) and cyclosporine (CSA) percent transfer from LDL to HDL, in the presence or absence of a monoclonal antibody (TP1) directed against LTP I. Experiment involved incubation of radiolabeled TG- and CSA-enriched LDL with unlabeled HDL (at 10 μg lipoprotein triglyceride) for 60 min at 37°C in T150 buffer supplemented with LTP I (1.0 μg protein/ml) or delipidated human plasma which contained 1.0 μg protein/ml of LTP I. Data expressed as mean ± S.D. (n = 6). *P < 0.05 $vs.$ TG or CSA percent transfer without TP1. (From [94])

Table 3 %kt [^{3}H]-CSA or [^{3}H]-TG transfer from HDL to LDL lipoproteins with plasma source of LTP I or purified source of LTP I corrected for TG

Treatment Groups	Net TG transfer (kt%)	Net CSA transfer (kt%)
Plasma LTP I	18.6 ± 2.1	16.8 ± 3.2
Purified LTP I	32.4 ± 1.7*	ND

Data expressed as mean ± standard deviation (n=4); *p < 0.05 vs. TG percent transfer with plasma LTP I ; ND = nondetectable
Experiment involved incubation of radiolabeled TG- and CSA-enriched LDL with unlabeled HDL (at 10 μg lipoprotein triglyceride) for 60 min at 37°C in T150 buffer supplemented with LTP I (1.0 μg protein/ml) or delipidated human plasma which contained 1.0 μg protein/ml of LTP I

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Objective

The overall aims of this proposal was to understand the role of LTP I in the facilitated co-transfer of CSA with PC between lipoproteins and to investigate if CSA binds to LTP I at its neutral lipid binding site.

1.6   Significance of research

Dyslipidemias may result in a different pharmacological or toxic profile of a lipophilic drug by differences seen in its lipoprotein distribution. Many transplantation patients exhibit lipid disturbances [41-44] and dyslipidemias may also have an elevation in LTP I levels [47-51, 95]. Since LTP I facilitates the transfer of mainly lipids between lipoproteins in the body then it may also facilitate the transfer of lipophilic drugs. Understanding the role of LTP I in the transfer of lipophilic drugs between lipoproteins may help provide a mechanism by which these drugs distribute into lipoprotein fractions. This may be important in understanding the efficacy and toxicity of lipophilic drugs when distributed into lipoproteins of patients with different lipid profiles.

1.7   Hypothesis

There are two hypotheses in this thesis:

1. LTP I is involved in the facilitated co-transfer of the CSA and PC between lipoproteins.

2. LTP I directly binds CSA to its neutral lipid binding site.

Chapter 2

AIMS

2.1   Specific Aims

Aim 1:

To determine if LTP I there is facilitated co-transfer of PC, the major coat lipid of lipoproteins, with CSA between lipoproteins.

Aim 2:

To determine if CSA directly binds to LTP I at the neutral lipid binding site by using a modified fast protein liquid chromatography (FPLC) method.

2.2   Rationale

Aim 1:

In a preliminary experiment, we have demonstrated LTP I can influence the lipoprotein distribution of CSA [93]. In this experiment, the CSA incubation in plasma resulted in a predominant association with HDL and LDL [93]. When the plasma was supplemented with exogenous LTP I, CSA redistributed from LDL to HDL [93]. This result suggested LTP I may have a role in facilitating the transfer of CSA.

Hence, we started to study the role of LTP I in the transfer of CSA in *in vitro* systems utilizing isolated [^{3}H]-CSA lipoprotein complexes. Because LTP I primarily is involved in the transfer of the neutral lipids, CE and TG, between lipoproteins at the neutral lipid binding site, we

started to investigate the role of LTP I in the co-transfer of CSA along with CE or TG at the neutral lipid binding site as previously discussed. We reported LTP I facilitated transfer of CSA between HDL and LDL is only partially dependent on LTP I's CE and TG transfer activities from LDL to HDL [93, 94]. This was shown by evaluating CE, TG, and CSA co-transfer between lipoproteins, using radioactivity, mAbs directed against the neutral lipid binding site of LTP I, and two different sources of LTP I; lipoprotein-deficient plasma or a purified source. The lipoprotein-deficient plasma source of LTP I also contained other endogenous transfer proteins.

Since LTP I is also involved in the transfer of one-third of the lipoprotein coat lipid, the role of the coat lipid, PC, in the transfer of CSA at the neutral lipid binding site was further investigated in this thesis. This was done to determine if only the neutral lipids in the core of the lipoproteins play a role in the transfer of CSA. To date, the role of the main coat lipoprotein lipid, PC, on the transfer of CSA between lipoproteins with LTP I has not been investigated.

The purpose of this aim was to establish if LTP I facilitated a co-transfer of PC with CSA between lipoproteins. An assay to measure LTP I facilitated transfer of PC and CSA, utilizing a mAb against the neutral lipid binding site of LTP I was developed to answer this aim.

Aim 2:

From the results of the experiments completed in Aim 1 and in previously published experiments [93, 94], it appears CSA is partially dependent on the LTP I facilitated transfer of the core neutral lipids, CE and TG, between lipoproteins but not of PC at the neutral lipid

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binding site. LTP I was only involved in the some of the facilitated co-transfer of CE and TG with CSA between lipoproteins from LDL to HDL. This suggests a number of possibilities:

- Spontaneous transfer of CSA and/or

- Facilitated transfer of CSA by other endogenous transfer proteins and/or

- Facilitated transfer of CSA by LTP I at the neutral lipid binding site and/or at other binding site(s) either independently or co-transferred along with the core neutral lipids, CE or TG

To start understanding if CSA is independently transferred at the neutral lipid binding site of LTP I, the purpose of the second aim was to investigate if CSA binds directly to the neutral lipid binding site of LTP I. This was accomplished by the determination of LTP I, protein, and radioactive concentrations of eluted FPLC fractions, when appropriate, following an injection onto the column (LTP I alone, [^{3}H]-CE liposomal vesicles alone, [^{3}H]-CSA liposomal vesicles alone, [^{3}H]-CE liposomal vesicles with LTP I, or [^{3}H]-CSA liposomal vesicles with LTP I).

Chapter 3

MATERIALS AND METHODS

3.1 Materials and Reagents

Radio-labeled CSA ([*mebmt*-β-^{3}H] Cyclosporine A dissolved in 100% ethanol solution; Specific Activity, 5.82 mCi/mg), radio-labeled PC (L-3-Phosphatidylcholine,1-palmitoyl-2-[1-^{14}C]oleoyl dissolved in a 50:50 toluene:ethanol solution; Specific Activity,72.2μCi/mg), and radio-labeled cholesteryl ester ([1α,2α(n)-^{3}H]-cholesteryl oleate dissolved in 100% ethanol solution; Specific Activity, 73.4 mCi/mg) were purchased from Amersham Pharmacia Biotech (Oakville, Ontario, Canada). Tris-HCl, sodium azide, ethylenediaminetetraacetate (EDTA), sodium bromide, sodium chloride, sodium carbonate, sodium bicarbonate, sodium phosphate (anhydrous), potassium phosphate monobasic, potassium chloride, bovine serum albumin, cholesteryl oleate, sodium hydroxide, p-nitrophenyl phosphate, triton x-100, and cyclosporine A were purchased from Sigma Chemical Company (St. Louis, MO, USA). LTP I (1690 μg protein/mL, 30μg LTP I/mL), stabilized with albumin in a buffer was donated by Dr. Richard E. Morton (Cleveland Clinic Lerner Research Institute, Cleveland, OH, USA) and was purified from human lipoprotein-deficient plasma as previously described [63]. Scintillation fluid was purchased from ICN Ltd (Montreal, QC, Canada). Phospholipids were bought from Avanti Lipids (Alabaster, AL, USA). Affinity purified goat anti-mouse phosphatase conjugate was purchased from Promega (Madison, WI, USA). Normolipidemic fasted pooled human plasmas (total plasma cholesterol concentrations ranging 135-150 mg/dL were obtained from Canadian Blood Services (Vancouver, BC, Canada) and Bioreclamation (Hicksville, NY, USA). TP 1 and TP 2 were obtained from the Ottawa Heart Institute (Ottawa, ON, Canada). Total plasma and lipoprotein triglycerides, cholesterol (free and cholesteryl ester), phospholipids and protein

concentrations were determined by enzymatic assays purchased from Sigma Diagnostics (St.

Louis, MO, USA), Hoffmann-La Roche Diagnostics (Laval, QC, Canada), and Biorad

Laboratories (Mississauga, ON, Canada). BSA protein standards were purchased from Sigma

Diagnostics (St. Louis, MO, USA). Sterile 0.2 μm syringe filters were purchased from

Millipore (Bedford, MA, USA). A XK column (18mm x 20cm) and Sephacryl HR 100 packing

were obtained from Amersham Pharmacia Biotech (Oakville, ON, Canada). Chloroform and

Nunc Maxisorp brand 96 well plates were obtained from Fisher Scientific (Nepean, ON,

Canada). A Lipex ® extruder and 100nm filters were obtained from Northern Lipids

(Vancouver, BC, Canada).

3.2   Lipoprotein Preparation

3.2.1   Radiolabeling of Plasma Lipoproteins

$[^{14}C]$-PC (2300 ng/ml) or $[^{3}H]$-CSA (1000 ng/ml) was incubated in plasma for 20-24 hours in

plasma at 37°C. The plasma was then separated into its HDL and LDL fractions by the

ultracentrifugation procedure described below.

3.2.2   Lipoprotein separation

The lipoprotein subfractions, HDL and LDL, were obtained by separation of plasma by gradient

ultracentrifugation [96, 97]. An additional sequential ultracentrifugation for concentrated HDL

was done. Labeling of the donor lipoproteins with $[^{14}C]$-PC or $[^{3}H]$-CSA was done as described

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above, prior to separation. All plasma and sodium bromide solutions were kept at 4°C prior to layering.

Human plasma samples (3.0 mL) were placed in centrifuge tubes and its solvent densities were adjusted to 1.25mg/mL by the addition of 1.02 g of sodium bromide. This was followed by layering with 2.8mL of the appropriate density solutions on top (1.21mg/mL, 1.063mg/mL, and 1.006mg/dL) to form a gradient.

Following gradient ultracentrifugation on a SW 41 Ti swinging bucket rotor (Beckman, Palo Alto, CA, USA) at 40,000 rpm for 18 hours at 15°C in a Beckman L8-80M Ultracentrifuge (Beckman, Palo Alto, CA, USA), only the LDL-rich and HDL-rich sub-fractions were recovered from each tube. Figure 10 shows a sample tube with four visibly distinct regions represented by the VLDL, LDL, HDL, and lipoprotein deficient plasma fractions. To further concentrate the HDL-rich fraction, 0.9g sodium bromide was added to each 10mL of HDL-rich fraction, and the resulting solution was spun at 40,000 rpm for an additional 7 hours at 15°C. The HDL-rich concentrated fraction was then recovered.

Fractions were further purified by dialyzing against sodium chloride 0.9% solution (500mL) for 16-20 hours at 4°C using dialysis tubing with a molecular weight cut-off of 500 or 1000. Following dialysis, lipoprotein fractions were filtered through a 0.2-micron filter. The dialysis and filtration steps were performed to remove PC or CSA not incorporated into either the coat or core of HDL and LDL.
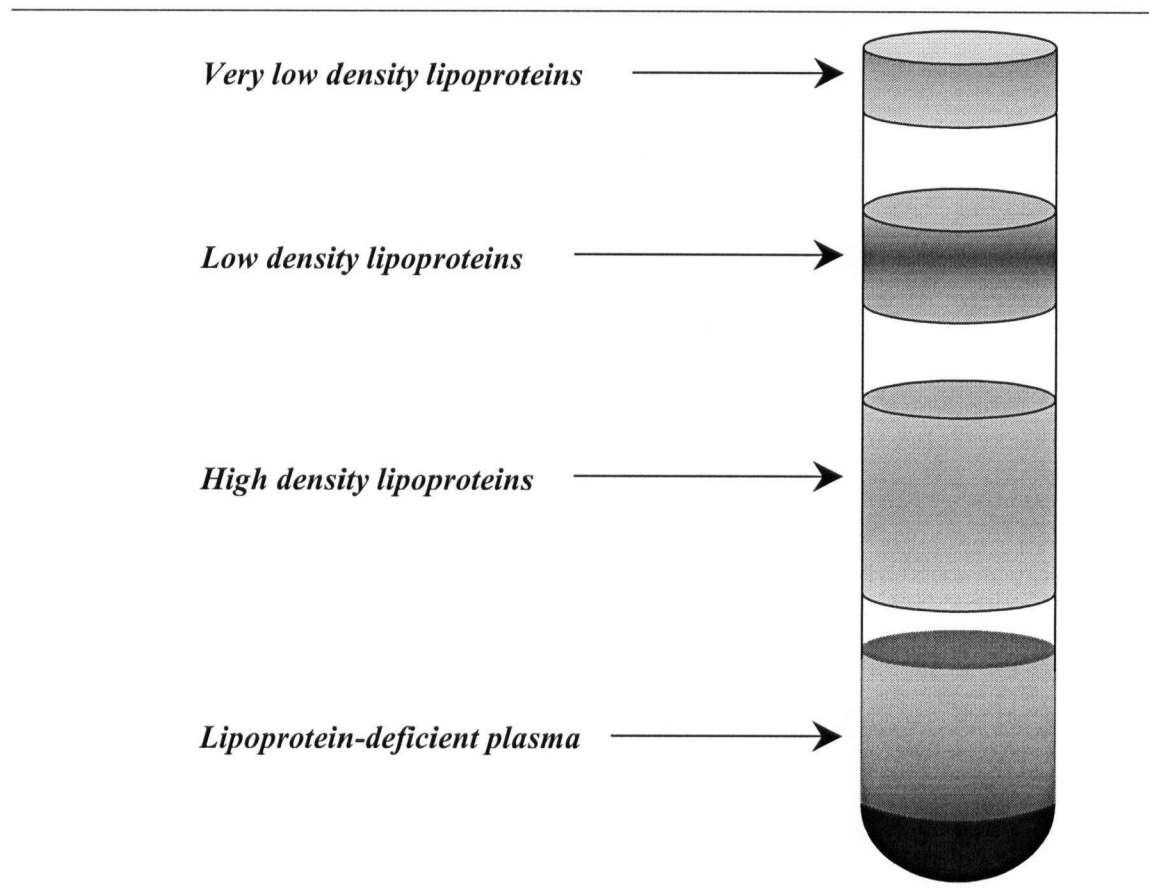
Figure 10 A representation of the separation of plasma lipoprotein fractions in an ultracentrifuge tube following ultracentrifugation in a Beckman SW41-Ti swinging bucket rotor at 40,000 rpm and 15°C for 18 hours in a Beckman L8-80M ultracentrifuge. Separation is based on the density of the plasma components; therefore the constituents of low density will rise to the top and those of heavier density will remain at the bottom. Only the LDL and HDL fractions were collected and used.

3.3 Lipid Transfer Protein I Transfer Activity

3.3.1 Determination of amount of acceptor and donor lipoproteins to add

For all transfer studies, radio-labeled HDL and LDL and their non-radio-labeled acceptor counterparts were added in equal amounts by using a volume equivalent to 20μg of PL, as determined by an enzymatic assay and calculated from a standard curve. This was done to account for diffusional transfer.

The following enzymatic procedure was used to determine phospholipids concentration. Phospholipase D is used to catalyse the conversion of PL to produce choline and phosphatidic acids. The choline is then oxidized by choline oxidase to yield betaine and hydrogen peroxide (H_2O_2) which then reacts with 4-aminophenazone and phenol in the presence of peroxidase to produce 4-(p-benzo-quinone-monoimino)-phenazone (the chromagen) and water [98]. The chromagen is a coloured dye with an absorbance maximum at 500nm. In summary, the series of reactions are as follows:

$$\text{phospholipids} + H_2O \xrightarrow{\text{phospholipase D}} \text{choline} + \text{phosphatidic acids}$$

$$\text{choline} + 2O_2 + H_2O \xrightarrow{\text{choline oxidase}} \text{betaine} + 2H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{4-}(p\text{-benzoquinone-monoimino) phenazone} + 4H_2O$$
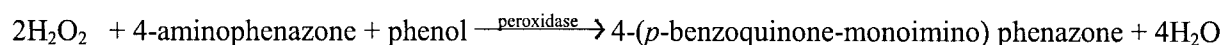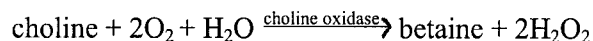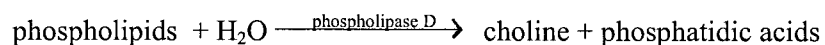
Phospholipid reagent was prepared by dissolving the contents of one enzyme reagent bottle in

40mL of the provided buffer solution. This working reagent solution contained phenol

20mmol/L, 4-aminophenazone 8 mmol/L, phospholipase D $\geq$ 1000 U/L, choline oxidase $\geq$ 1400

U/L, and peroxidase $\geq$ 800U/L. This solution was stable for up to two weeks when stored at 2

to 8°C. A standard curve was developed by serial dilution of the standardized stock solution of

300 mg phospholipids/dL equivalent to 54.1 mg/dL choline chloride rather than using a one

point standard as suggested by the kit. This standard curve was preformed each time new

batches of lipoproteins were made. 10 μL of the sample or standard was added to 1.5 mL of the

prepared phospholipid reagent solution, mixed, and incubated at 37°C for 10 minutes and read

against the reagent blank at 500nm using a Hewlett Packard 8452A Diode Array

Spectrophotometer interfaced with a Hewlett Packard Vectra N2 Personal computer with UV-

Vis software. The absorbance values of the standards were used to generate a standard curve

(Figure 11) and the volume equivalent to 20μg of PL of radio-labeled HDL and LDL and their

non-radio-labeled acceptor counterparts were calculated from the fitted linear equation.
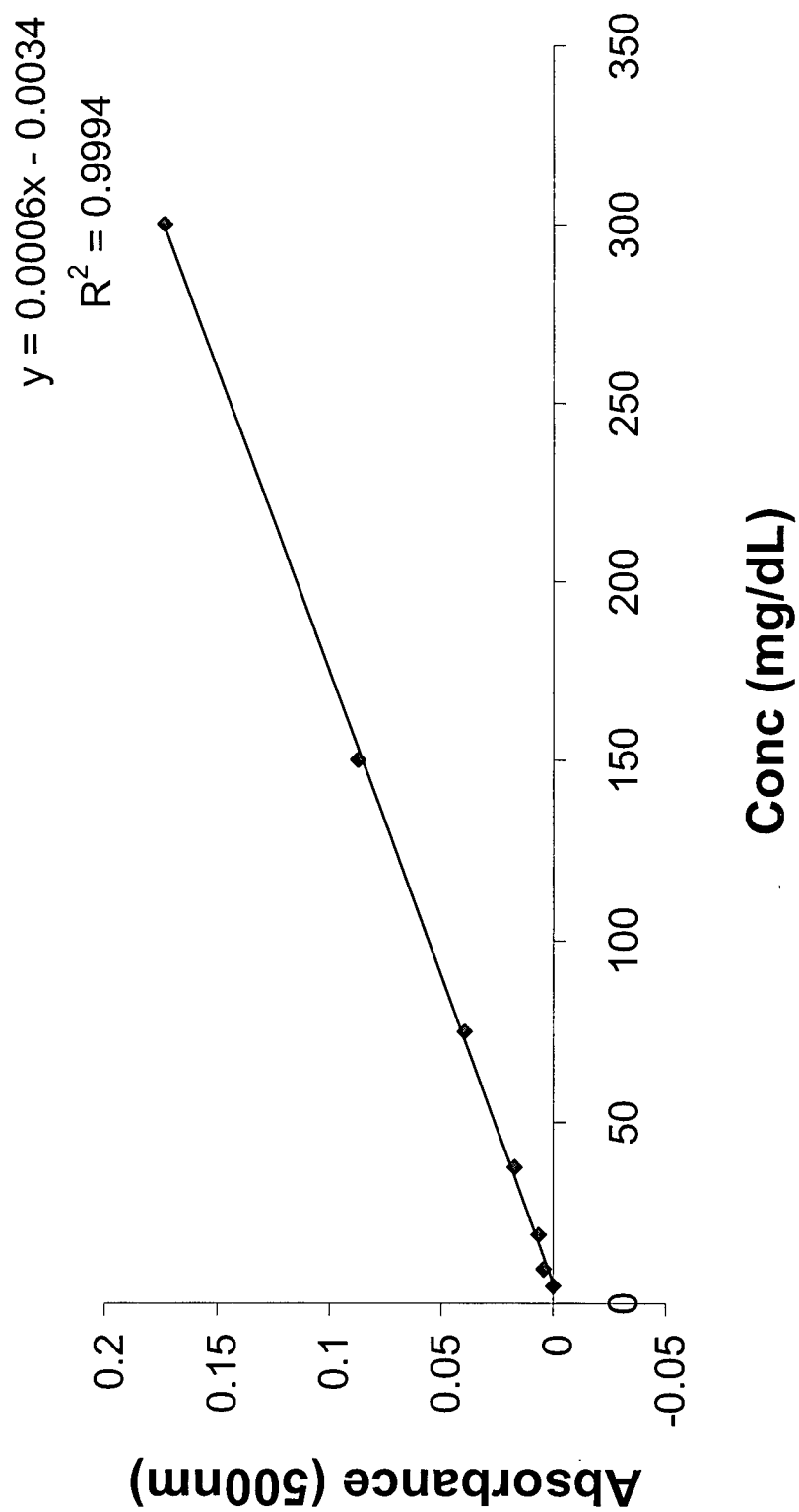
Figure 11 Representative standard curve for determination of PL. Lipoprotein volumes added in transfer assays were equivalent to 20 μg of PL as determined in triplicates from the standard curve.

3.4　LTP I transfer of cyclosporine and phospholipid

To investigate the hypothesis that the transfer of CSA between lipoproteins is regulated by LTP I facilitated PC transfer activity, two strategies involving the supplementation and inhibition of LTP I were used. LTP I transfer activity was measured following a modified protocol of Pattnaik and Morton [63, 64, 83, 99].

The first strategy involved incubation of [^{3}H]-CSA-enriched HDL or LDL with a corresponding drug-free lipoprotein counterpart in a T150 buffer (50 mM Tris-HCl, 150 mM NaCl, 0.02% sodium azide, 0.01% disodium EDTA, pH 7.4) in the presence of a purified source of LTP I (1.2 µg protein). Endogenous LTP I concentration within normolipidemic human plasma is usually 1-2 µg protein/ml [63]. In a further experiment LTP I was co-incubated with TP1 (30 µg protein per system), a monoclonal antibody (mAb) directed against LTP I at the neutral lipid binding site, before transfer studies were done [59].

The second strategy was to incubate delipidated human plasma as a source of LTP I with [^{3}H]-CSA-enriched HDL or LDL with a corresponding drug-free lipoprotein counterpart. This human plasma source was chosen from bags of pooled normolipidemic plasma to obtain a transfer activity of at least 15%. To confirm the transfer of CSA is due to LTP I and not other endogenous plasma factors, mAb TP1 (30 µg protein per system) was co-incubated with the delipidated plasma before transfer studies were started. The above 2 strategies were designed to determine if the movement of CSA between lipoprotein particles was partially facilitated by

LTP I and/or was a result of non-facilitated drug transfer rather than from the influence of other plasma components.

[^{14}C]-PC transfer between HDL and LDL in the presence and absence of TP1 was also determined for both sources of LTP I to observe if TP1 plays a role in inhibiting LTP I facilitated PC transfer activity. For all the aforementioned experiments, incubations were carried out for 90 minutes at 37°C.

For experiments with TP1, an additional 90-minute incubation time with the LTP I source was carried out at 37°C before transfer studies were started. Following the final incubation, samples were separated out into their individual lipoprotein constituents by a manganese and phosphate precipitation step (0.1M manganese chloride solution and 0.45M sodium phosphate, 0.02% sodium azide, pH 7.4 solution).

For all experiments, spontaneous transfer of the component of interest was accounted for by the performance of the transfer assay devoid of a LTP I source and this was subtracted from the total transferred.

Lipid and drug transfer between donor and acceptor lipoprotein was quantitated by scintillation counting using a Beckman LS6000TA scintillation counter. Figure 12 shows a diagram of the steps preformed in these transfer experiments.

As discussed earlier in the thesis, the fraction of lipids and drug transferred (kt) was calculated as described by Pattnaik and Zilversmit [83].

$$kt = -ln\ (1-A_t/D_0)$$

where D_0 and A_t are the radioactivities of the donor at time 0 and the acceptor at time t, respectively. The constant k is the fraction of the radiolabeled compound transferred per unit time (t). Acceptor radioactivity in the absence of LTP I (usually < 2-3%) is subtracted before calculating kt values. Calculations assume steady-state conditions in which all lipid and drug transfer are in an exchange process.
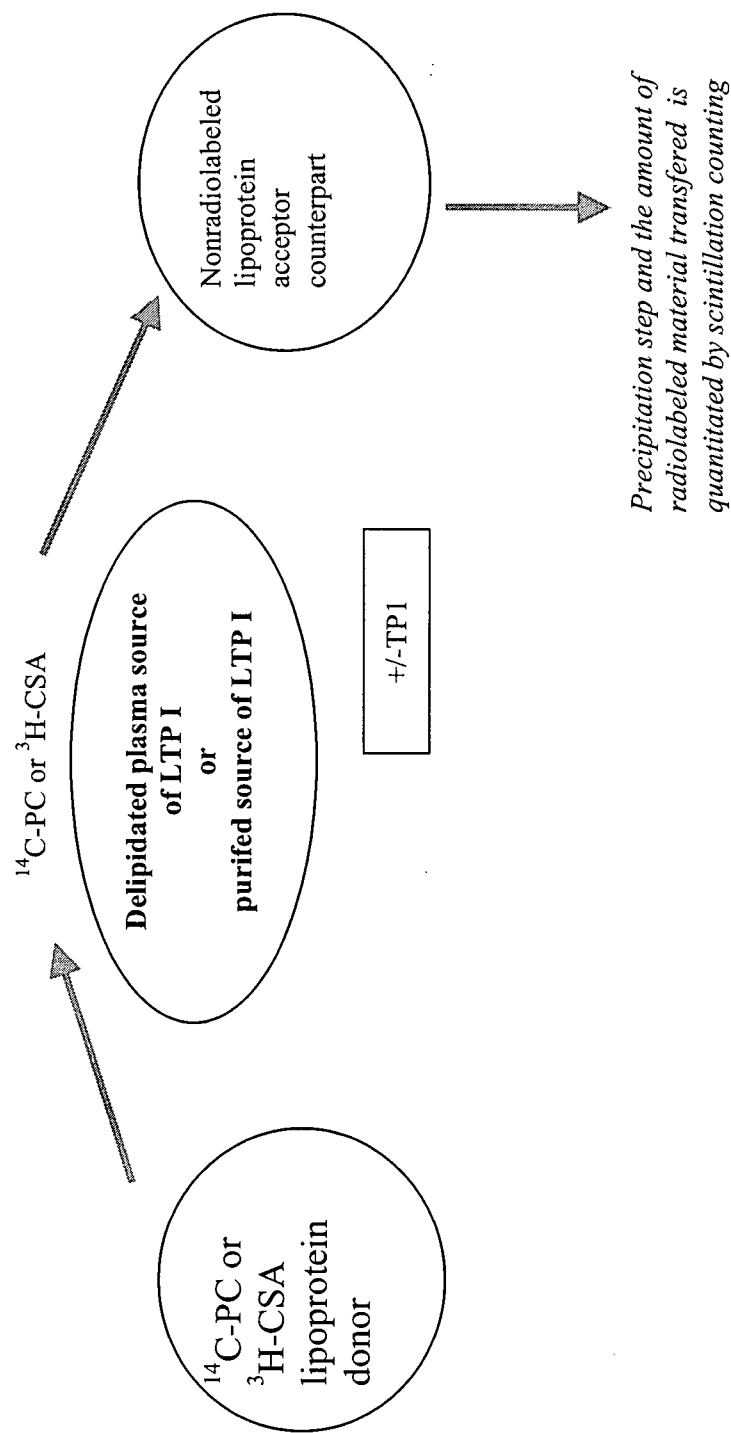
Figure 12 Diagram of LTP I lipoprotein transfer experiments with radiolabelled PC or CSA.
The transfer of radiolabelled [¹⁴C]-PC or [³H]-CSA lipoprotein (HDL or LDL donor) to its nonradiolabeled counterpart (LDL or HDL) was determined with and without a monoclonal antibody (TP1 30µg) against the neutral lipid binding site of LTP I. Two sources of LTP I was used (1.2µg protein/mL): a delipidated source of LTP I containing other transfer proteins and a purified LTP I source in a T150 buffer (50 mM Tris-HCl, 150 mM NaCl, 0.02% sodium azide, 0.01% disodium EDTA, pH 7.4). The amount of lipoproteins added was corrected to 20µg PL to account for diffusional transfer.

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3.5 Binding of CE, CSA, and Egg PC liposomal vesicles to LTP I

3.5.1 Binding of CE to LTP I

Binding of CE to LTP I was evaluated as a positive control since CE has been shown to bind to LTP I by other investigators [61, 70]. [^{3}H]-CE liposomal vesicles were firstly made. 21.6μmol of egg PC, 0.328μmol of cholesteryl oleate, 80μL of [^{3}H]-cholesteryl oleate (73.4mCi/mg, Amersham) were mixed in chloroform, dried to a thin layer in a glass tube under a stream of nitrogen over a hot water bath (> 25°C), and then placed in a vacuum for 2 hours. The lipids were then resuspended in 7mL of buffer (10mM Tris-HCl and 1mM EDTA, pH 7.4) and vortexed. The solution was aliquoted in 1mL aliquots into cyrotubes and subjected to 5 freeze thaw cycles using liquid nitrogen and a hot water bath. The samples were put away in a -70°C freezer until extrusion. Samples were extruded through a 100nm filter in a Lipex® extruder 10 times then sized by a Malvern Zetasizer and used for experiments. The total recovery after extrusion was 96.2%.

The FPLC column was equilibrated for 2 hours prior to any injections with a mobile phase buffer consisting of 10mM Tris Cl and 1mM EDTA buffer, pH 7.4. Egg PC (590 nmol) vesicles containing cholesteryl oleate and [^{3}H]-cholesteryl oleate (9.06nmol total) were incubated with LTP I (9μg) for 1 hour in a total volume of 0.5mL. As stated before, the LTP I solution used is stabilized with albumin. The mixture was loaded onto a Sephacryl HR 100 XK column (18mm x 20cm) controlled by a FPLC apparatus and mobile phase was run through to separate the components. The flow rate was set at 0.25mL/min for a 3-minute load and adjusted

to 0.5mL/min for collection. The sample loop used was 0.5mL. Fractions of 1 mL were collected and elution fractions were assayed for [3H]-CE profile, protein mass (Biorad protein DC assay), LTP I mass (ELISA), and PC profile (enzymatic assay as previously described).

LTP I injected alone on the column and [3H]-CE vesicles injected alone on the column served as controls. The column was washed with 300mL of buffer after each run. As an added step, a 0.5mL plasma sample was first injected onto the column to determine how dilute the eluted fractions were in order to minimize the usage of the pure LTP I source. A LTP I assay and a protein assay were conducted on the collected plasma fractions (Appendix G).

3.5.1.1   Protein mass analysis

All eluted fractions from the column were analyzed for protein content to determine the amount of LTP I and other proteins in the fractions. As stated before, the LTP I solution used is stabilized with albumin. This assay was done to further confirm the presence of this protein in the eluted fractions. The LTP I ELISA described later is more specific for LTP I.

The method for measuring protein concentration levels utilized a modification of the Lowry assay [100, 101]. Protein standard solution equivalent to BSA 400μg/mL was prepared by addition of distilled water to the vial as indicated by the labeled instructions. The appropriate amount of standard or 5μL of sample from each of the eluted fractions were added, either in duplicate or triplicate, in wells of a microtitre plate. 25μL of Reagent A (alkaline copper tartrate) were added to each well followed by 200μL of Reagent B (Folin reagent). Alkaline copper tartrate reacts with proteins and forms a blue colour when the Folin reagent is added. The resulting blue colour has a maximum absorbance of 750nm. The plate was gently mixed in

a plate shaker for 15 minutes after which the absorbance was read at 620nm with a plate reader (Labsystems Multiskan Ascent®). A standard curve (Figure 13) was constructed from the absorbance readings for the standards and the protein concentration of each sample was determined.
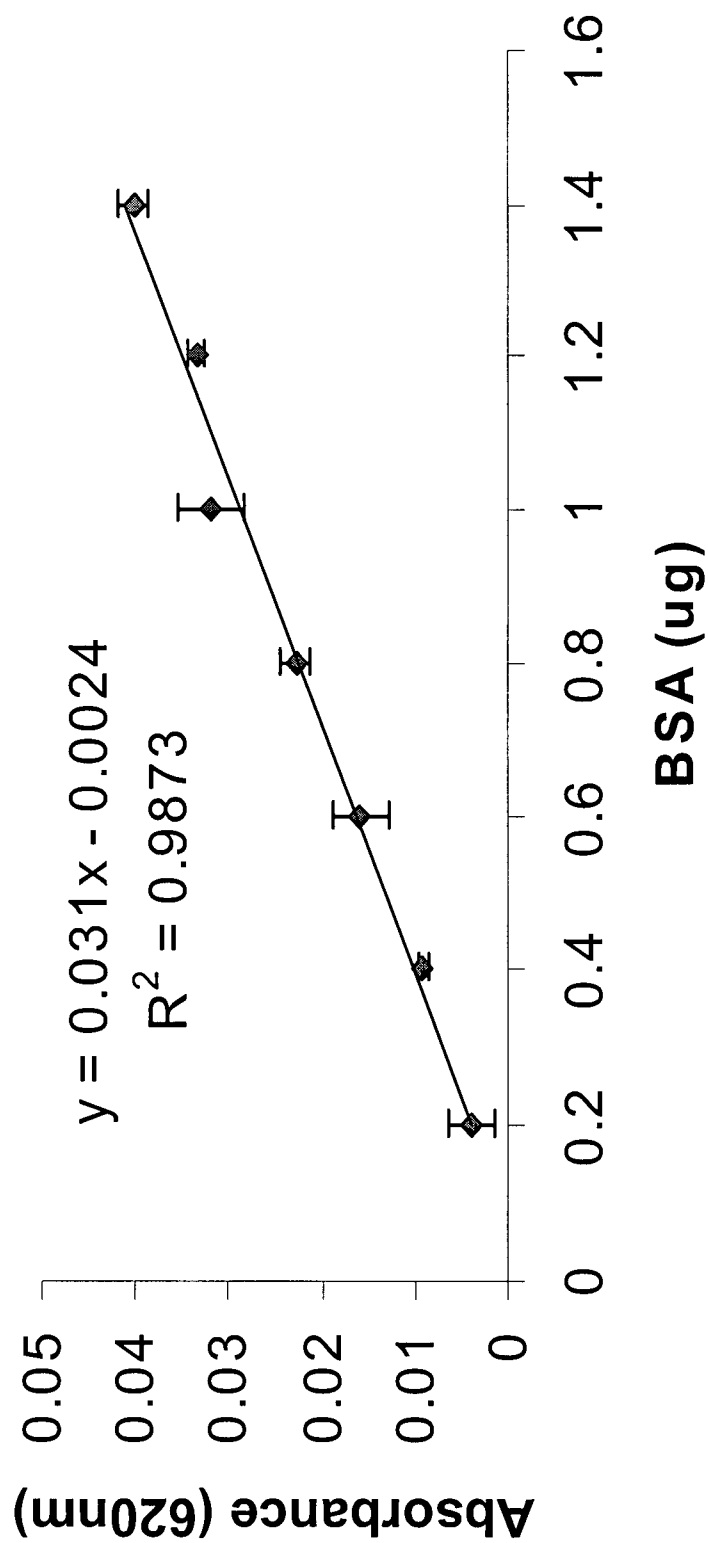
Figure 13 Sample protein concentration standard curve. n=3 for each standard point; data represented as average +/- std dev

3.5.1.2   Radioactive profile

20uL of the collected eluted [^{3}H]-CE 1mL fractions from the experiments incubated with and without LTP I was aliquoted into scintillation vials with 5.4mL scintillation fluid and read in a scintillation counter (Beckman LS 6000TA) to detect tritium for a time period of 3 min per sample. CPM per mL was reported.

3.5.1.3   LTP I ELISA

A widely used assay for measurement of LTP I protein in samples is a radioimmunoassay (RIA) that utilizes a monoclonal antibody to LTP I [102]. Glenn and Melton (1996) [103] have modified this type of method to eliminate the need for radiolabeled mAb. They have developed an ELISA involving competitive immunoreaction of unlabelled TP2, a mAb against the neutral lipid binding site of LTP I, between the LTP I absorbed to 96 well polystyrene plates and the samples of LTP I. The amount of the primary mAb, TP2, bound to the immobilized LTP I is detected with a secondary mAb consisting of an affinity-purified goat anti-mouse immunoglobulin G (IgG) linked to alkaline phosphatase. *P*-nitrophenyl phosphate is added as a substrate for the reaction to occur, and the end product, p-nitrophenol, is read at an absorbance of 405nm with a reference wavelength of 620nm. The measured level of TP2 bound to immobilized LTP I on the plates is indirectly proportional to the amount of soluble LTP I competing for immunoreaction.

Because TP2 binds to the neutral lipid binding site of LTP I and our results from previous experiments have shown a partial transfer of CSA by this site [93, 94], this procedure should have been modified using a mAb not directed at this neutral lipid binding site [71]. However, due to material constraints, only TP2 was used to duplicate the published procedure to ensure

replication [103]. If a mAb not against the neutral lipid binding site of LTP I was used, then the LTP I ELISA procedure would have to optimized which would require the use of more materials.

The procedure was followed from Glenn and Melton (1996) [103]:

3.5.1.3.1 Plate Coating

Partially purified LTPI was diluted in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, 0.02% (w/v) NaN_3, pH 9.8) to 0.4μg/mL and dispensed at 100uL/well, leaving 3 wells on the top left as controls in a 96-well Nunc Maxisorp plate. Following an overnight incubation at room temperature, the plates was rinsed twice with washing buffer (138mM NaCl, 8.1mM Na_2HPO_4, 1.2mM KH_2PO_4, 2.7mM KCl, pH 7.2). 250μl/well saturation buffer (washing buffer containing 1% (w/v) BSA, 1mM EDTA) was added per well prior to incubation at room temperature for 4 hours. Plates were stored at -20°C or used immediately.

3.5.1.3.2 ELISA Procedure

The saturation buffer treated plates were rinsed 4 times with washing buffer. Dilutions of LTP I standards were done first. Standards were dispensed as 100μL aliquots into triplicate wells of 10, 20, 40, 80, 160, and 320 ng/well. 100uL of triton buffer (saturation buffer containing 0.2% (w/v) Triton X-100) was added to the 3 uncoated LTP I wells and to 3 additional coated LTP I wells to serve as controls. Test samples were dispensed in 100uL aliquots/well in duplicates. 10mL at a concentration of 100ng/mL in TB, was prepared from an aliquot of stock TP2. 100μL/well of this solution was dispensed in all 96 wells of each plate and the plates were incubated at 37°C for 4 hours.

Each plate was rinsed four times with washing buffer prior to addition of 100μL/well of the goat anti-mouse alkaline phosphatase conjugate (a 1:7000 dilution of stock using saturation buffer). The plate was incubated at 37°C for one hour and rinsed four times with washing buffer. 100μL alkaline phosphatase substrate was added to each well and the plates were incubated at 37°C for 30 minutes. Non-LTP I coated wells were used as a blank. Plates were read at 405nm with a reference wavelength of 620nm. Figure 14 shows a simplified diagram of the LTP I ELISA procedure.

Each LTP I ELISA assay was done independently for each injection containing LTP I. A LTP I standard curve was run each time. The LTP I standard curve was plotted and a linear regression analysis of LTP I concentration relative to the mean optical density (OD) was used to calculate LTP I levels in test samples (Figure 15). The standard curve was best fitted linearly. However, validation of this assay in our laboratory should be done further with more standard curves to evaluate if a semi-log fit is more appropriate. Validation should also be done with different operators over a longer period of time.

1. Coat LTP I (antigen) on plate (overnight incubation)

2. Wash plate then add saturated buffer to uncoated parts of plate (incubation). Store plates away or use right away.

3. Wash plate then add samples containing LTP I and primary mAb (TP2) (incubation). The more LTP I in the sample, the less antibody remains to react with LTP I on plate.

4. Wash plate. The samples containing LTP I bound to the mAb will be washed away.

5. Add the secondary mAb conjugated to alkaline phosphatase (incubation). Substrate is then added (incubation) then the plate is read at 405nm with a reference wavelength of 620nm.

Figure 14 Basic description of the steps in the LTP I ELISA procedure
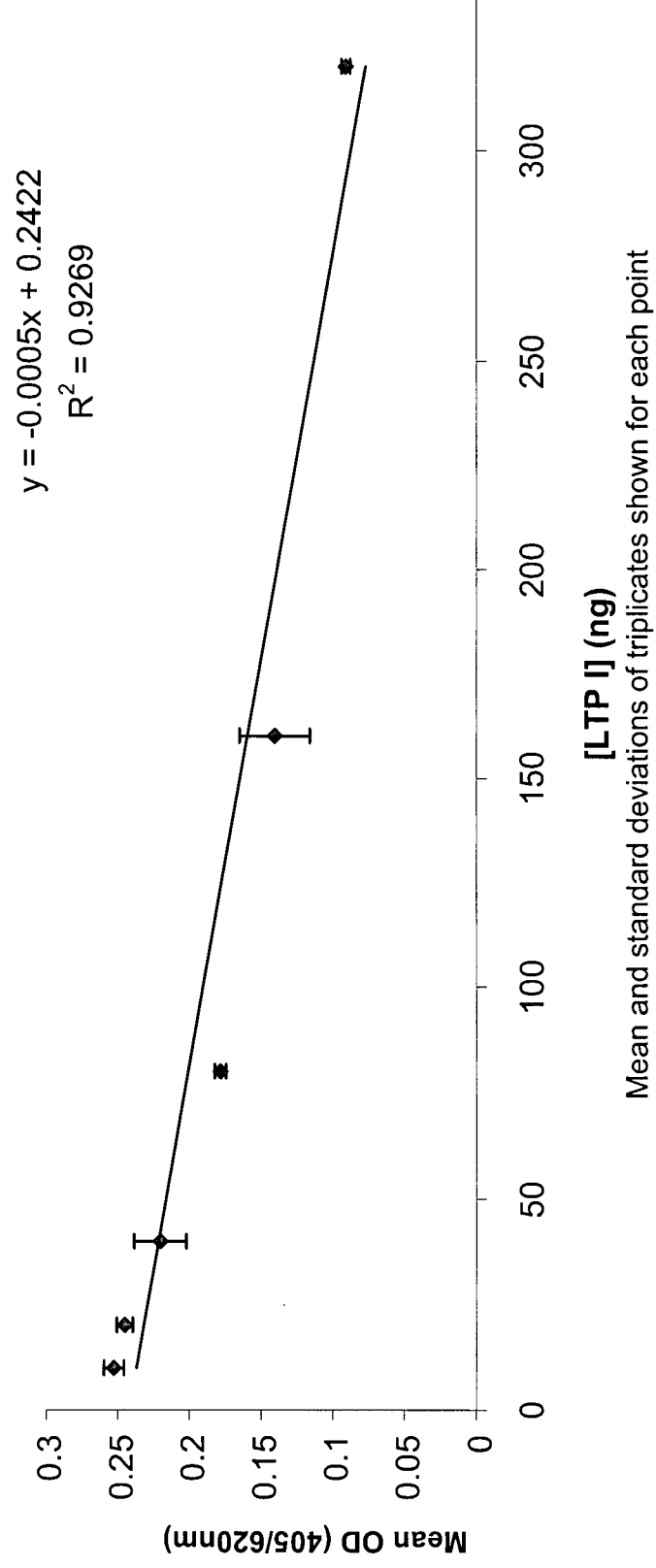
Figure 15 Effect of LTP I concentration on measured optical density in the LTP I ELISA using TP2 monoclonal antibody (sample standard curve) Mean and standard deviations of triplicates are shown for each point

3.5.2   Binding of CSA to LTP I

Egg PC liposomal vesicles containing CSA and [^{3}H]-CSA were prepared in a manner similar to the preparation of [^{3}H]-CE labeled liposomal vesicles. Cold and radiolabeled CSA were added at the beginning of the procedure in place of cold CE and radio-labeled CE to the egg PC in chloroform. Total recovery after extrusion was 82.74%.

The same procedure as that used for CE was used in evaluating the binding assay for CSA to LTP I. Purified LTP I (9µg) was incubated with egg PC liposomal vesicles (510 nmol) containing CSA and [^{3}H]-CSA (8.10nmol total) for 1 hour in a total volume of 0.5mL. The sample was analyzed the same way as described for [^{3}H]-CE binding to LTP I using a Sephacryl HR 100 XK column (18mm x 20cm), FPLC system, scintillation counting, LTP I ELISA, and PC analysis. [^{3}H]-CSA liposomal vesicles injected alone on the column served as a control.

3.6   Statistical Analysis

Differences in LTP I mediated CE and CSA transfer activity in the presence of different treatment groups were determined by paired sample t-test (JumpIn, SAS Institute Inc.) with critical differences set at $p<0.05$, data expressed as mean +/- standard deviation. Data in other experiments were expressed as mean +/- standard deviation.

Chapter 4

RESULTS

4.1 PC and CSA transfer between HDL and LDL

These experiments were done to assess the role of the lipoprotein coat lipid, PC on the transfer of CSA by LTP I. To date, only the role of the lipoprotein neutral core lipids, TG and CE, was assessed in the transfer of CSA between lipoproteins [93, 94].

To assess the ability of LTP I to promote the co-transfer of PC and CSA from HDL to LDL, radiolabeled PC or CSA enriched HDL was incubated in T150 buffer containing purified LTP I (1.2µg/mL) or in lipoprotein deficient plasma with endogenous LTP I (1.2µg/mL). Detectable PC transfer occurred from HDL to LDL with both sources of LTP I, with no statistical difference when TP1 was added (Figure 16). This percent transfer was greater with the delipidated human plasma as compared to the purified source (Figure 16). Undetectable [^{3}H]-CSA transfer occurred from HDL to LDL in T150 buffer containing purified LTP I and in the delipidated human plasma containing LTP I in the presence and absence of TP1 (Table 4). The percent transfer of PC and CSA from LDL to HDL was greater in delipidated human plasma containing LTP I than in T150 buffer containing purified LTP I (Figure 17). The percent transfer of PC determined in the presence of TP1 from LDL to HDL was significantly decreased in T150 buffer with purified LTP I as well as with the delipidated human plasma compared to controls (Figure 17). CSA transfer from LDL to HDL in T150 buffer containing purified LTP I was non-detectable (Figure 17).
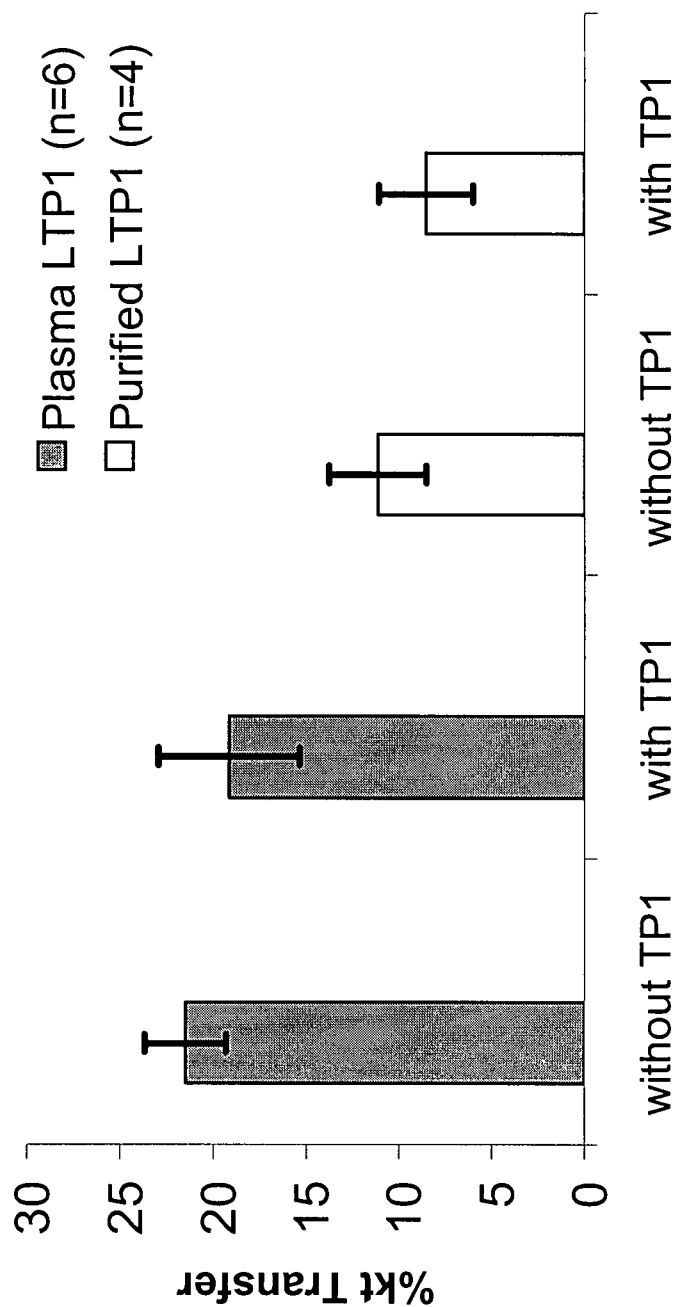
Figure 16 [^{14}C] -PC percent kt transfer from HDL to LDL in the presence and absence of a monoclonal antibody (TP1 30μg) directed against LTP I, following incubation of radiolabeled PC enriched HDL with unlabeled LDL (at 20μg lipoprotein PC) for 90 minutes at 37°C in T150 buffer supplemented with purified LTP I (1.2μg protein/mL) or in delipidated human plasma as a source of LTP I. Abbreviations: PC, phosphatidylcholine; HDL, high-density lipoproteins; LDL, low-density lipoproteins; TP1, mAb directed against LTP I; k – the fraction of label transferred per unit time; t, time. Data expressed as mean +/- std (n=4-6). No statistical significance; t-test within same LTP I source (from [104])

Table 4 Net %kt [^{3}H]-CSA transfer between HDL and LDL lipoproteins with plasma source of LTP I or purified LTP I in the absence and presence of TP1 (30 μg total protein) corrected for PC (from [104])

	% kt transfer with Plasma LTP I		% kt transfer with Purified LTP I	
	No TP I	TP I (30ug)	No TP I	TP I (30ug)
LDL to HDL	21.95 ± 2.4 (n=6)	22.85 ± 1.84 (n=6)	nd (n=6)	nd (n=6)
HDL to LDL	nd (n=6)	nd (n=6)	nd (n=4)	nd (n=4)

Data expressed as mean ± SD, LTP I – lipid transfer protein I, TP1 – monoclonal antibody directed against LTP I, LDL – low density lipoprotein, HDL - high density lipoprotein, nd – nondetectable
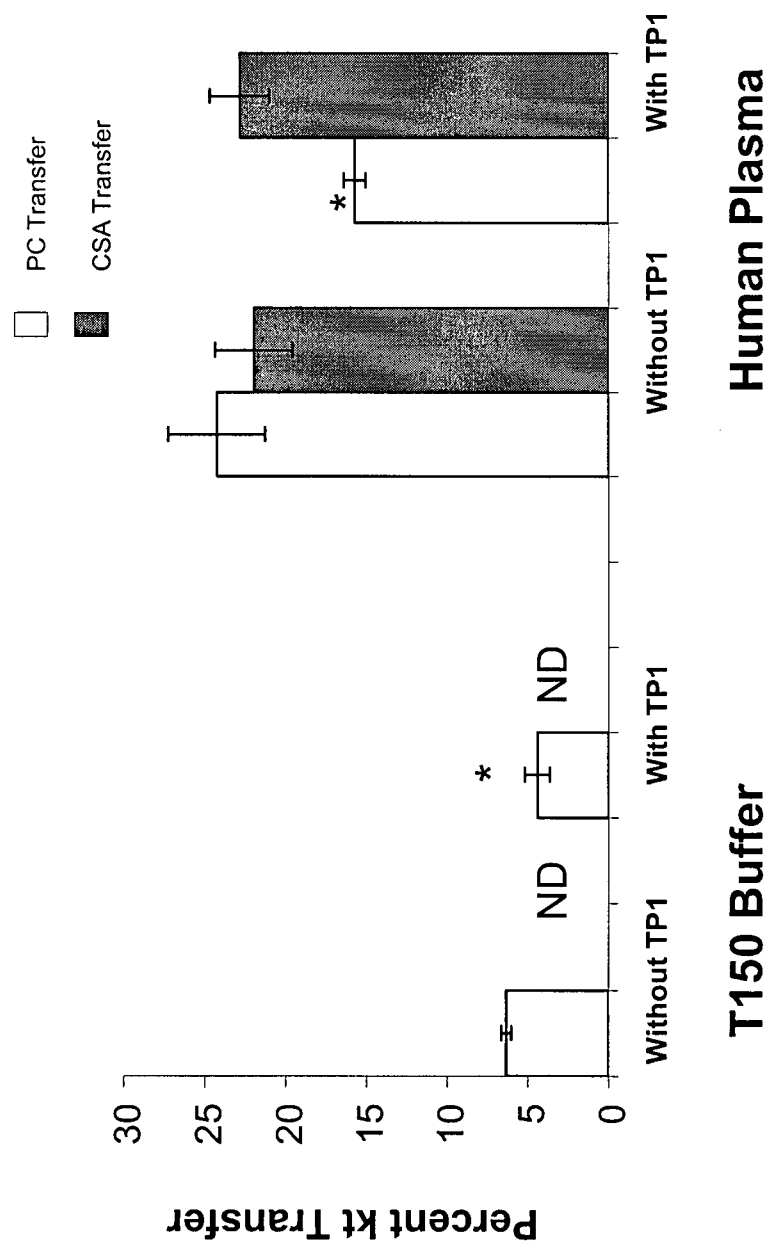
Figure 17 [^{14}C]-PC and [^{3}H]-CSA percent kt transfer from LDL to HDL in the presence and absence of a monoclonal antibody (TP1) directed against LTP I, following incubation of radiolabeled PC and CSA enriched LDL with unlabeled HDL (at 20ug lipoprotein PC) for 90 minutes at 37°C in T150 buffer supplemented with purified LTP I (1.2ug protein/mL) or in delipidated human plasma as a source of LTP I. Abbreviations: PC, phosphatidylcholine; CSA, cyclosporine; HDL, high-density lipoproteins; LDL, low-density lipoproteins; TP1, mAb directed against LTP I; k – the fraction of label transferred per unit time; t, time. Data expressed as mean +/- std (n=5-6). *p<0.05 vs. PC percent transfer without TP1; t-test within the same LTP I source (from [104])

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4.2   LTP I and protein elution profiles

LTP I interactions with the lipids CE, TG, and PC in defined assays have been evaluated

using FPLC by other groups [61, 70]. Our previous work [93, 94] has shown CSA to be

partially dependent on the facilitated co-transfer of the neutral lipids, CE and TG, from LDL

to HDL but not of PC. Since the neutral lipid binding site of LTP I is responsible for the

transfer of the neutral lipids between lipoproteins, there is a possibility that the neutral lipid

binding site also binds CSA. Previous experiments have demonstrated CE, TG, and PC

binding the neutral lipid binding site of LTP I using FPLC [61, 70]. As a positive control,

CE binding to LTP I at the neutral lipid binding site was assessed using a modification of this

FPLC method. PC binding to LTP I at this site was also assessed. This FPLC method was

also used to assess CSA independent binding to LTP I at the neutral lipid binding site [61,

70].

4.2.1   LTP I Alone

First, LTP I (9μg) was separated by FPLC (Figure 18a). The LTP I elution profile was

determined with a LTP I ELISA and the protein elution profile was determined by a protein

assay [103]. LTP I injected alone on the column eluted out into three main LTP I areas, at

fraction numbers 10, 14, and 30, and there were minor peaks at fractions 18 and 27. There

was a major protein peak at fraction 14 and also minor peaks at fractions 19, 27, and 30,

corresponding to the some of the LTP I peaks.

4.2.2   LTP I and CE

As a positive control, CE binding to LTP I was first assessed. Unilamellar egg PC liposomal vesicles containing cold CE and [^{3}H]-CE were incubated with purified LTP I (9µg) then separated by FPLC. The elution profile of LTP I and protein were determined by both a LTP I ELISA and a protein assay (Figure 18b). The LTP I peak at fraction 10 decreased and fraction 30 decreased towards baseline. The LTP I peaks obtained from the combination LTP I and [^{3}H]-CE liposomal vesicle injection at fractions 11, 18, and 26 increased by 2x, 1.6x, and 4.4x compared to the control LTP I fractions at 14, 18, and 26. There was an additional peak appearing at fraction 34. The protein assay resulted in only a peak at fraction 12 (Figure 18b) that was 1.6x the magnitude of the protein peak found in the control at fraction 14 (Figure 18a).

4.2.3   LTP I and CSA

Unilamellar egg PC liposomal vesicles containing cold CSA and [^{3}H]-CSA incubated with purified LTP I (9µg) was separated by FPLC. The elution profile of LTP I and protein were determined (Figure 18c) by a LTP I ELISA and a protein assay as previously described. A pattern similar to similar to the [^{3}H]-CE and LTP I injection was seen. There was a decrease in fraction 10 and fraction 30 decreased towards the baseline. The LTP I peak obtained from the combination LTP I and [^{3}]H-CSA liposomal vesicle injection at fractions 11, 18, and 25 increased by 2x, 1.8x, and 4.4x compared to the control LTP I fractions at peaks 14, 18, and 26. There was also an additional peak seen at fraction 34. The protein pattern seen with the [^{3}H]-CSA/LTP I injection was different than the [^{3}H]-CE/LTP I injection. There were more peaks seen with the [^{3}H]-CSA/LTP I injection (Figure 18c) which followed some of the

minor peaks seen with the control LTP I run (Figure 18a). The protein peaks at 12, 19, 28, and 33 were 2.1x, 1.3x, 1.9x, and 1.8x greater than the control LTP I fractions at peaks 14, 19, 27, and 32 respectively (Figures 18c and 18a).
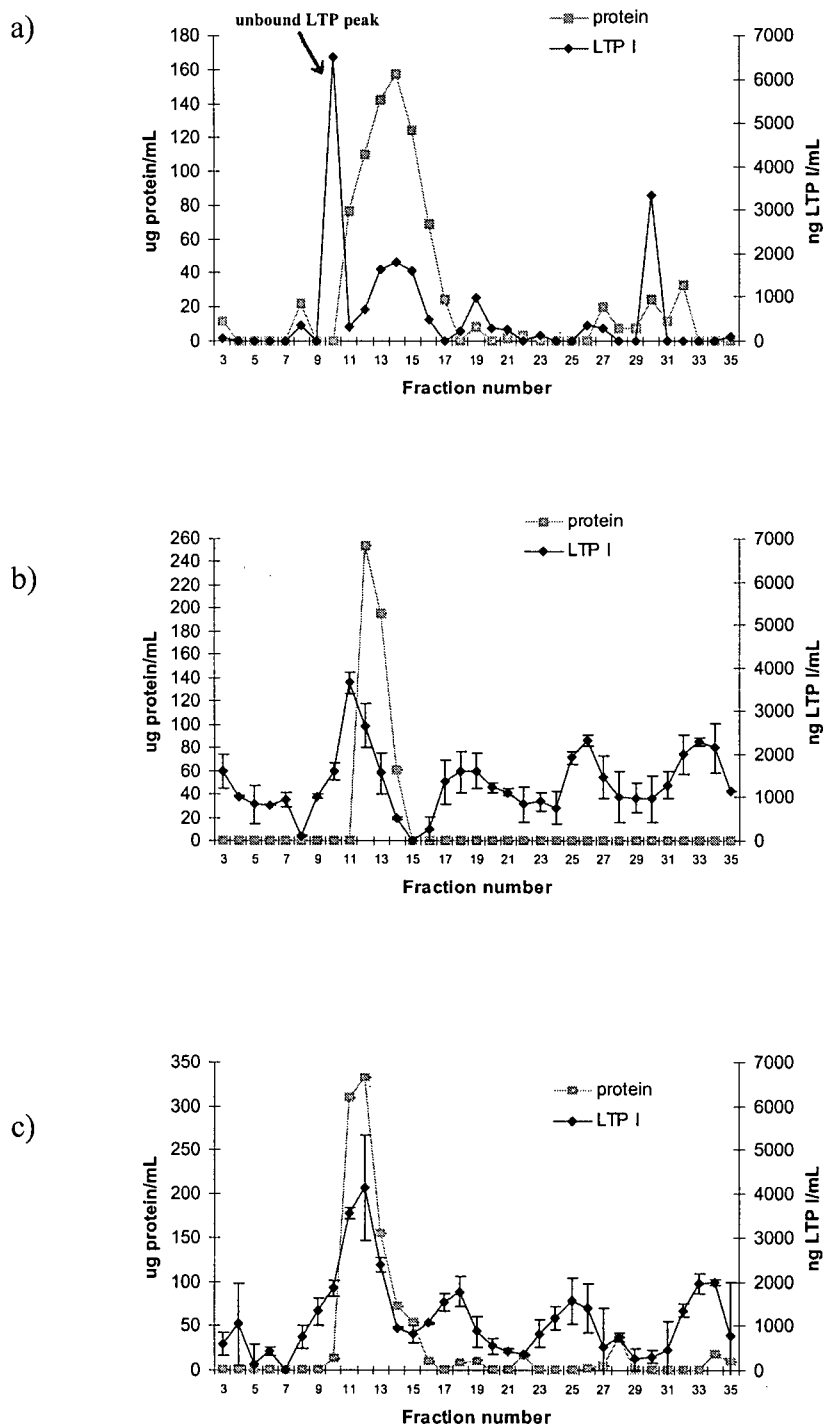
Figure 18 LTP I concentration (ng/mL) and protein concentration (μg/mL) in eluted fractions from injections onto a FPLC system with a Sephacryl HR 100 XK column (18mmx20cm) a) 9 μg LTP I alone b) LTP I 9μg + [^{3}H]-CE liposomal vesicles (egg PC 590nmol, CE 9.06nmol) b) LTP I + [^{3}H]-CSA liposomal vesicles (egg PC 510nmol, CSA 8.10nmol) Flow rate was set at 0.25mL/min for a 3 min load and adjusted to 0.5mL/min for collection. The sample loop used was 0.5mL. Fractions of 1mL were collected. n=1 for injections; eluted sample concentrations were determined n=1 to 2 for each assay

4.3   CE elution profiles

The elution profile [^{3}H]-CE is reported in Figure 19a following the injection of [^{3}H]-CE liposomal vesicles on the column. The radioactivity peak at fraction 12 was the [^{3}H]-CE eluting out in the void volume. When [^{3}H]-CE liposomal vesicles were preincubated with LTP I (9μg), the amount of [^{3}H]-CE eluting out at the void volume increased by 1.9x (Figure 19b) compared to injection of [^{3}H]-CE liposomal vesicles alone (Figure 19a). There was also [^{3}H]-CE eluting past fraction 18 but the magnitudes of these radioactive peaks closely match that of the control (Figures 19a and 19b insets). The total radioactivity recovery was 29% from the control [^{3}H]-CE liposomal vesicles as compared to 44% from the [^{3}H]-CE liposomal vesicles injected with LTP I.
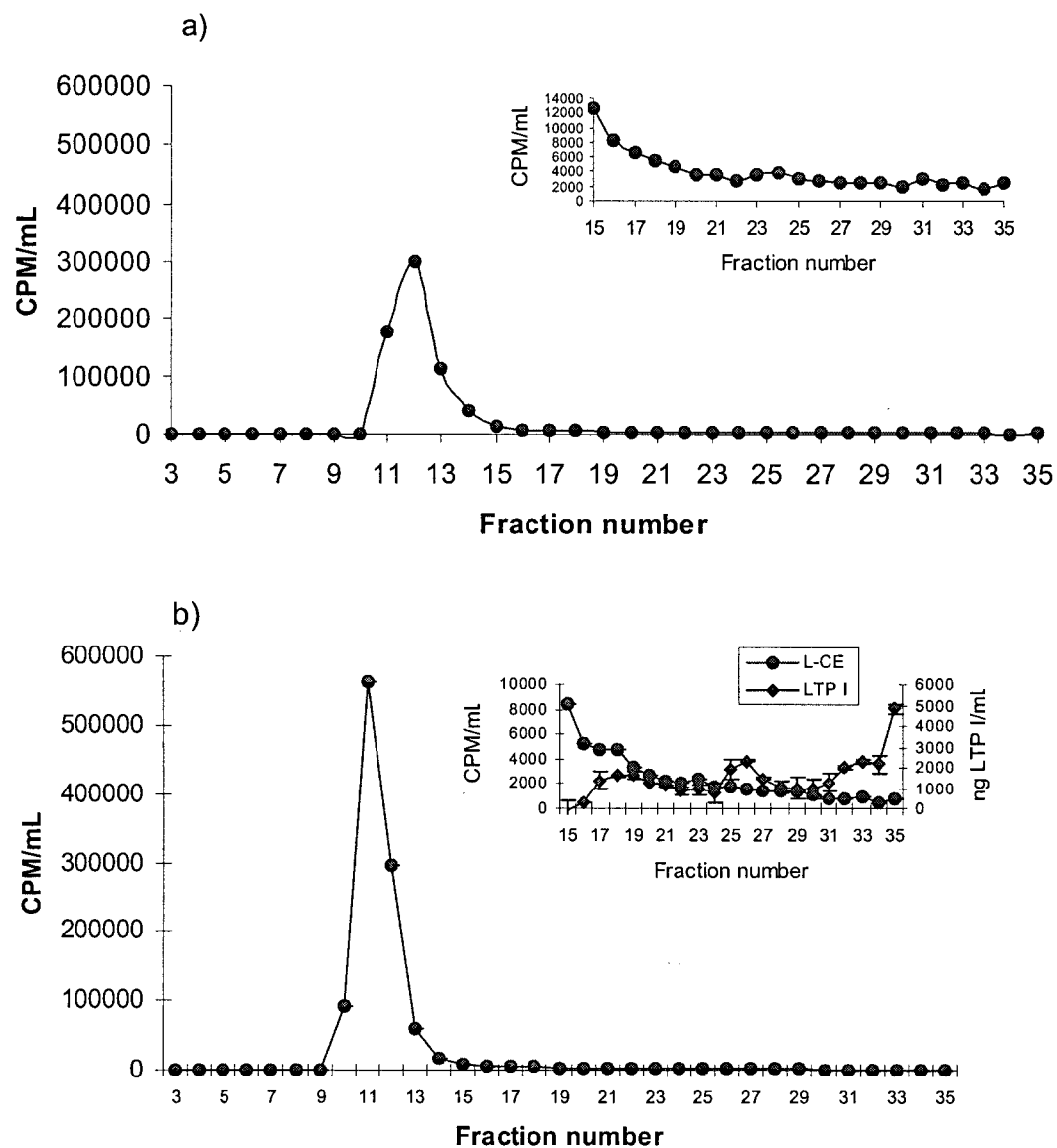
Figure 19 [^{3}H]-CE radioactivity (CPM/mL) in eluted fractions from injections onto a FPLC system with a Sephacryl HR 100 XK column (18mmx20cm) a) [^{3}H]-CE liposomal vesicles (egg PC 590nmol, CE 9.06nmol) b) LTP I 9μg + [^{3}H]-CE liposomal vesicles (egg PC 590nmol, CE 9.06nmol) Insets show fractions 15 to 35 with an additional y axis showing amount of LTP I in the eluted fractions. Flow rate was set at 0.25mL/min for a 3 min load and adjusted to 0.5mL/min for collection. The sample loop used was 0.5mL. Fractions of 1mL were collected. n=1 for injections; eluted sample concentrations were determined n=1 to 2 for each assay

4.4   CSA elution profiles

The distribution of [^{3}H]-CSA is shown in Figure 20a for [^{3}H]-CSA liposomal vesicles injected onto the column. The radioactivity peaking at fraction 12 is the CSA eluting out in the void volume. When [^{3}H]-CSA liposomal vesicles were preincubated with LTP I (9μg), the amount of [^{3}H]-CSA eluting out at the void volume decreased by 0.4x as compared to control counts. There was also [^{3}H]-CSA eluting out at later fractions (Figure 20b). The [^{3}H]-CSA eluting out at fractions 18, 26, and 36 corresponded to the LTP I peaks at these fractions (Figure 20b inset). The magnitude of the radioactive peak at 18 closely matches to that of the control but the magnitude was 3x and 1.4x higher at fractions 26 and 34 . The total radioactivity recovery was 14% from the control [^{3}H]-CSA liposomal vesicles compared to 8% from the [^{3}H]-CSA liposomal vesicles injected with LTP I.
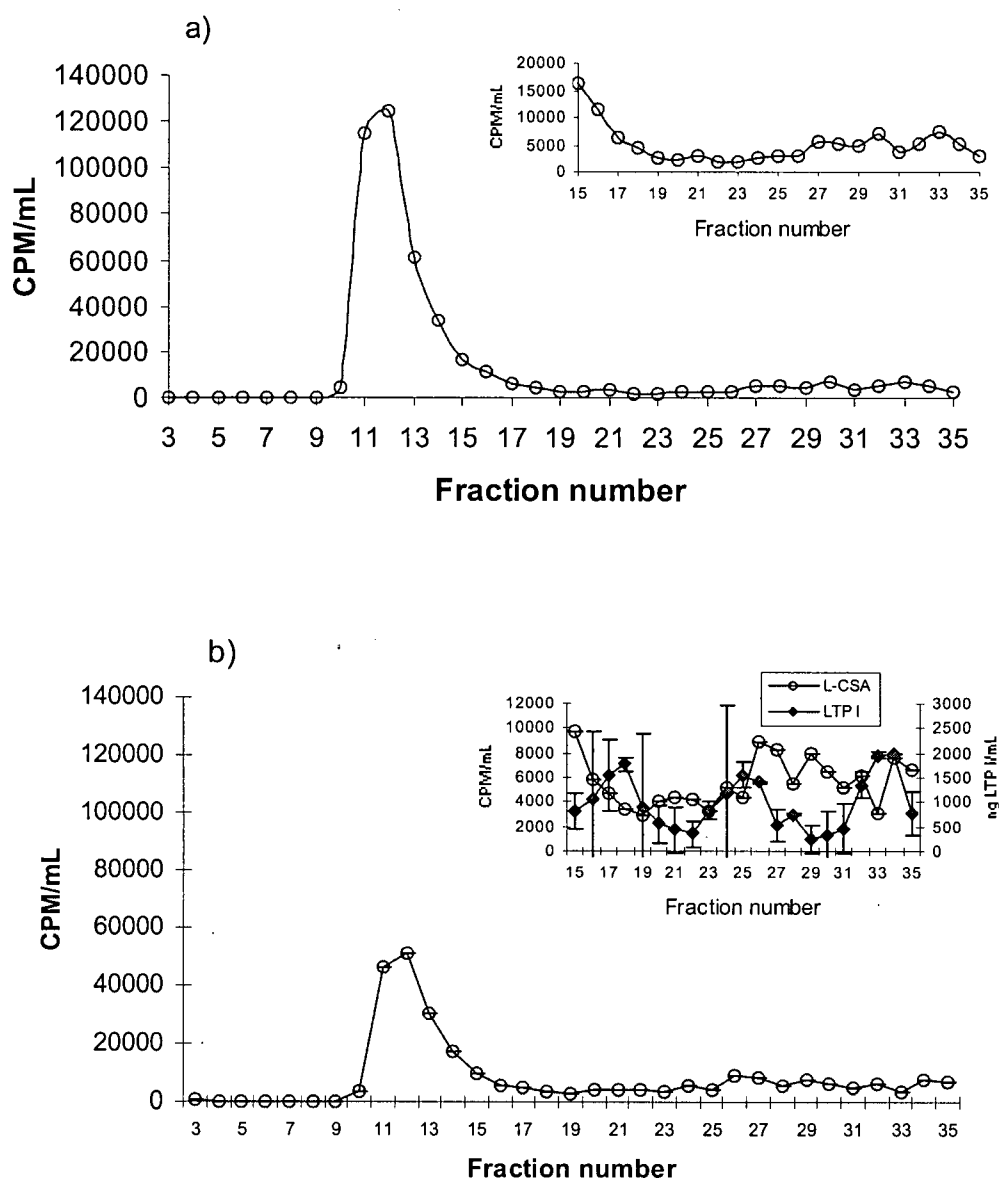
Figure 20 [³H]-CSA radioactivity (CPM/mL) in eluted fractions from injections onto a FPLC system with a Sephacryl HR 100 XK column (18mmx20cm) a) [³H]-CSA liposomal vesicles (egg PC 510nmol, CSA 8.10nmol) b) LTP I 9μg + [³H]-CSA liposomal vesicles (egg PC 510nmol, CSA 8.10nmol) Insets show fractions 15 to 35 with an additional y axis showing amount of LTP I in the eluted fractions. Flow rate was set at 0.25mL/min for a 3 min load and adjusted to 0.5mL/min for collection. The sample loop used was 0.5mL. Fractions of 1mL were collected. n=1 for injections; eluted sample concentrations were determined n=1 to 2 for each assay

Chapter 5

DISCUSSION

5.1 LTP Transfer of PC and CSA

LTP I is responsible for all facilitated transfer of the core lipoprotein neutral lipids, CE and TG, and approximately one-third of the coat lipoprotein lipid, PC [53, 59-62]. In previous studies, the LTP-I facilitated transfer of CSA was found to be partially dependent on its CE and TG transfer activity [93, 94] from LDL to HDL. In this thesis, since LTP I also facilitates the transfer of PC, we chose to assess if there was LTP I facilitated co-transfer of PC with CSA between lipoproteins.

In the experiments that were designed to directly measure the potential role of LTP I to facilitate CSA transfer based on HDL and LDL PC content, LTP I-mediated percent transfer of PC from LDL to HDL in delipidated human plasma containing other endogenous transfer proteins and in T150 buffer containing purified LTP I was different from that of CSA. The percent transfer of CSA from LDL to HDL is undetectable in T150 buffer containing purified LTP I, while PC transfer was observed and decreased in the presence of TP1 (Figure 17) [104]. In delipidated plasma, the percent transfer of CSA from LDL to HDL was not significantly different than with TP1, while PC transfer was observed and decreased in the presence of TP1 (Figure 17) [104]. The percent transfer of CSA from HDL to LDL is undetectable in T150 buffer containing purified LTP I and in delipidated plasma (Table 4). PC transfer is noted and not decreased in the presence of TP1 with both purified LTP I and delipidated plasma LTP I (Figure 16) [104]. Taken together, these findings found that the distribution of CSA is not influenced by LTP I-mediated PC transfer activity between HDL and LDL. These findings further support the notion that the PC transfer activity may be due

to facilitated transfer by other endogenous plasma factors (such as LTP II) and/or facilitated by LTP I at another site.

Sgoutas and coworkers have proposed the nature of CSA's association with HDL and LDL particles appears to be non-specific and with low affinity and high capacity, suggesting CSA is physically dissolved within the lipoprotein-lipid component [37]. From previous studies, assessing LTP I facilitated co-transfer of CE or TG with CSA, it appears CSA is only partially recognized by LTP I as an endogenous lipid compound [93, 94]. The distribution of CSA between lipoproteins does not seem to be influenced by LTP I-mediated PC transfer activity between HDL and LDL but is partially dependent on the LTP I-mediated CE and TG transfer activities [93, 94, 104]. Since the neutral lipids, CE and TG, are found in the lipid core of lipoproteins and are transferred between lipoproteins by binding to the neutral lipid binding site of LTP I this lead us to hypothesize that LTP I may facilitate CSA's transfer by directly binding CSA to its neutral lipid binding site. The next experiments in this thesis addressed whether there is independent binding of CSA to the neutral lipid binding site of LTP I.

5.2   LTP I Binding

The binding of LTP I with CE, TG, and PC in defined assays have been assessed by other groups [61, 70]. We had originally hypothesized that the human body may recognize lipophilic compounds as lipid-like particles, thus we modified these assays to assess the interaction of LTP I with CSA. CE binding to LTP I was evaluated as a positive control. PC binding to LTP I was also evaluated because the [^{3}H]-CE liposomal vesicles and [^{3}H]-CSA liposomal vesicles were made of PC. However, the PC in the diluted buffer fractions was

below the detection limit of the enzymatic assay employed. A further experiment should be done to evaluate LTP I binding with PC using radioactivity.

In experiments that were designed to evaluate CE and CSA binding to the neutral lipid binding site of LTP I, [^{3}H]-CE liposomal vesicles or [^{3}H]-CSA liposomal vesicles were co-incubated with LTP I (9μg) and injected onto a FPLC. The resulting LTP I elution profiles were found to be similar for both these experiments but the protein elution profiles (Figure 18) and radioactive elution profiles were different (Figure 19 and Figure 20).

First LTP I (9μg) was injected onto the column. The LTP I elution profile showed peaks coinciding with the protein elution profile (Figure 18) except there was one additional peak at fraction 10. The magnitude of the protein peaks were also much larger than the LTP I peaks. This suggests that there are unbound and protein bound LTP I available. Since the LTP I preparation contains both LTP I and albumin in a buffer, the higher magnitude of the protein peaks corresponding to the LTP I peaks suggest that albumin is binding to LTP I. Because the LTP I ELISA detects LTP I using a primary mAb against the neutral lipid binding site of LTP I, the LTP I detected in the elution fractions is not occupied at the neutral lipid binding site of LTP I. If this site was occupied then the LTP I would not be detected.

In the next experiments, [^{3}H]-CE liposomal vesicles or [^{3}H]-CSA liposomal vesicles were co-incubated with LTP I (9μg) and injected onto a FPLC. A similar LTP I elution pattern was seen with both these experiments. There was a disappearance of the unbound LTP I detected at fraction 10 in the control experiment, an increase of some elution peaks by the same magnitude (peaks located at fractions 11, 18, 25 or 26), a disappearance of the peak at fraction 30, and an appearance of a peak at fraction 34. The disappearance of the unbound

LTP I at fraction 10 suggest both CE and CSA bind to the neutral lipid binding site of LTP I. The similar pattern seen with the LTP I detected in the other eluted fractions could be explained by PC associated LTP I since PC is the common component to both the radioactive liposomal vesicles and it can be transferred by LTP I [32]. The two protein peaks at the void volume from the [^{3}H]-CE vesicles or [^{3}H]-CSA vesicles co-injection with LTP I were also higher than that of control (Figure 18). This could be due in part to the nonspecific interaction of the PC or CE or CSA with albumin which then could interact with LTP I at a non-neutral lipid binding site.

The disappearance of the unbound LTP I peak at fraction 10 does suggest CE and CSA binding to the neutral lipid binding site of LTP I as discussed before in Figure 18. However, further evaluation of the radioactive data suggest CSA binds differently than CE.

Co-administration of [^{3}H]-CE liposomal vesicles with LTP I resulted in an *increase* in [^{3}H]-CE radioactivity recovered at the fraction number where unbound LTP I eluted versus the [^{3}H]-CE radioactivity following [^{3}H]-CE liposomal vesicles injected alone onto the column (Figure 19). This confirms CE binding to the neutral lipid binding site of LTP I. Co-administration of [^{3}H]-CSA liposomal vesicles with LTP I resulted in the *decrease* of the [^{3}H]-CSA radioactivity recovered at the fraction number where unbound LTP I eluted compared to control [^{3}H]-CSA vesicles injected alone on the column, however there was still a significant amount of [^{3}H]-CSA recovered (Figure 20). [^{3}H]-CSA radioactivity was also recovered at other fraction numbers, some of which corresponded to where the protein–bound LTP I eluted (Figure 20 inset). Taken together, this data suggest CSA does bind to the neutral lipid binding site of LTP I, but it may also be binding to albumin, other regions on LTP I, or non-specifically to the FPLC column.

In these experiments, both CE and CSA were found to have non-specific interaction with the column by the determination of radioactive recovery rates. There was a low radioactive recovery from the column for both radioactive samples, demonstrating non-specific interactions: only 29% for the [³H]-CE liposomal vesicle injection, 44% for the [³H]-CE liposomal vesicle/LTP I injection, 14% for the [³H]-CSA liposomal vesicle injection, and 8% for the [³H]-CSA liposomal vesicle/LTP I injection were recovered calculated to fraction 35. [³H]CSA was also chaotically distributed passed fraction 26 at levels higher than control with the [³H]-CSA liposomal vesicle/LTP I injection (Figure 20) which could suggest that there are more non-specific interactions occurring with CSA compared to CE. CSA may have more non-specific interactions due to its size since it is almost double in molecular weight compared to CE. Some of this non-specific binding not solely be due to the column, but could be due to non-specific interactions with the tubing, or components used to transfer the sample. Analysis to fraction 82 showed 15% recovery in the [³H]-CSA liposomal vesicle injection and 11% in the [³H]-CSA liposomal vesicle/LTP I injection. Non-specific binding could be occurring with the resin of the column and the compound could be washing out at later fractions with the mobile phase as it flows through the column. In a previous experiment done by our laboratory, CSA was injected onto a Sepharose column without incorporation into a vesicle. This resulted in the CSA being stuck on the column indefinitely.

In this set of experiments, we have determined both CE and CSA bind to LTP I at the neutral lipid binding site. CE binding to LTP I was a positive control. These binding experiments demonstrate CSA may also be binding to albumin, other regions on LTP I, or non-specifically to the FPLC column.

5.3   Conclusion

In the current studies, LTP I has been studied to see if it plays a role in the transfer of CSA between lipoproteins by binding at the neutral lipid binding site. In the first set of experiments in this thesis, it was found LTP I does not facilitate the transfer of CSA with PC amongst lipoproteins while our previous studies have shown that LTP I does facilitate the transfer of CSA with the core neutral lipids, CE and TG [82, 93], suggesting CSA may be residing in the core of the lipoproteins. In the second set of experiments in this thesis, the results suggested LTP I binds to CE and CSA at the neutral lipid binding site. The data also suggest CSA may bind with LTP I at other regions, bind to albumin, or non-specifically to the FPLC column.

In conclusion, LTP I mediated transfer of CSA between lipoproteins may be a result of the direct binding of CSA to LTP I at the neutral lipid binding site. Figure 21 describes a model of the interaction of LTP I with the lipoprotein lipids and CSA.

5.4   Significance

The distribution/redistribution of drugs among plasma lipoproteins facilitated by LTP I may serve as a possible mechanism for determining the ultimate fate of drug compounds. LTP I may have potential application in the field of drug delivery since it can facilitate the transfer of lipophilic drugs such as Hf, AmpB, and CSA between different lipoprotein subclasses. The work presented represents a prerequisite for further studies both, *in vitro* and *in vivo* testing, of the complex between various drugs and LTP I. When drugs have to penetrate lipid membranes, the use of LTP I could be evaluated since it exhibits a transfer activity. A recent

paper [91] suggests a potential application of plant lipid transfer proteins for drug delivery. This group reports that skin lipids such as sphingosine, sphingomyelin and cerebroside (an azole derivative with antitumoral and/or antileishmania properties), and amphotericin B were shown to bind to plant lipid transfer protein I. These binding studies suggest a challenge for the future could be the development of LTP-mediated transport and controlled release of low molecular weight drugs.
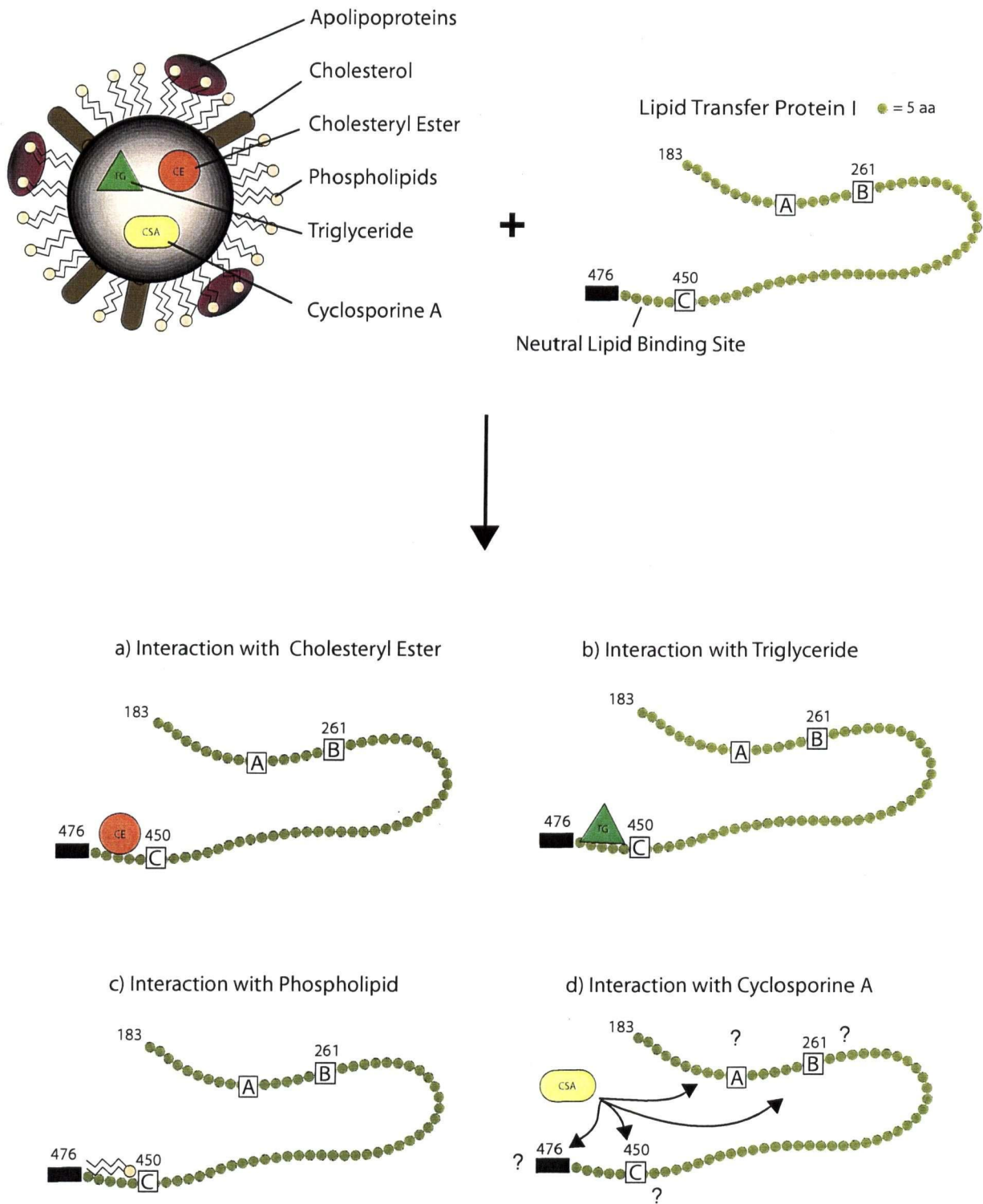
Figure 21 MODEL OF LTP I INTERACTIONS WITH CE, TG, PC, AND CSA

5.5   Problems encountered and limitations

The major limitation in all these studies was the lack of available purified LTP I, resulting in the performance of only one experiment each with the purified source. In the transfer studies, two transfer studies were done on different days with a delipidated source of LTP I before using a delipidated source of LTP I along with the purified source of LTP I in the third transfer study. In the binding studies, each experiment was only done once. Purified LTP I was used to coat enough ELISA plates to almost run duplicates of each fraction from each experiment for the determination of LTP I concentration. Considerable time was spent to determine if a commercial source of LTP I could be used in these experiments. However, this source was not purified enough for our studies. Transfer studies did not perform with this source of LTP I and ELISA plates could not be coated with this source, nor could it be quantitated with ELISA plates coated with the old source of LTP I.

Another limitation of the study was the LTP I ELISA used the primary mAb TP2 against the neutral lipid binding site of LTP I. We had expected if there was binding at the neutral lipid binding site of LTP I, then LTP I would not be detected with this particular ELISA. A more ideal experiment would be to use a primary mAb that is not against this site. This way, the LTP I would still be detected if the component of interest was bound to the neutral lipid binding site of LTP I. This particular LTP I ELISA was not developed first due to the lack of purified LTP I.

Control radio-labeled liposomal vesicles eluted out at the same location as some of the control LTP I. A longer column may have allowed enough column interactions to occur, enabling the control radio-labeled liposomal vesicles and the control LTP I to elute at

different fractions. If binding of CE and CSA with LTP I were to occur, then there may be a more distinctive visual decrease in the location of the control peaks. If the compound of interest was to bind with LTP I then there would also be an additional peak appearing.

Since the lipoprotein coat lipid, PC, is known to interact with LTP I with one-third of its transfer between lipoproteins being mediated with LTP I [32], it should be further evaluated with regards to its binding with LTP I. PC and LTP I binding was evaluated as part of this thesis, however an enzymatic reaction was used to detect the PC content in each fraction. The samples fell below the limit of detection to be of use. A more ideal experiment would be to use a radioactive isotope of PC to increase the sensitivity of detection. Dual-labeled liposomal vesicles of radioactive PC and radioactive lipid or CSA could also be incubated with LTP I and eluted out on the FPLC.

5.6   Future studies

The data suggest CSA does bind to the neutral lipid binding site of LTP I but the role of the neutral core lipids of lipoproteins in this interaction has not been evaluated. CSA could be binding to this neutral lipid binding site independently or with the neutral lipids as well. The relationship between the neutral core lipids, CE and TG, and CSA could be explored further. A simple experiment to do would be to repeat the transfer experiments from one lipoprotein component to another, utilizing purified LTP I and an increasing amount of CSA with the same concentration of a neutral lipid (CE or TG) and vice versa. This would determine if neutral lipid influences the transfer of CSA. Further experiments could utilize dual labeling of CSA and the neutral lipid (CE or TG) to follow both components in both transfer studies and binding studies.

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The data also suggest the control LTP I injected onto the FPLC column is free at the neutral lipid binding site. A transfer study of neutral lipids (CE or TG) between lipoproteins using the control LTP I elution fractions from the FPLC with and without a mAb against the neutral binding site could be done to confirm this. This experiment can also confirm whether the LTP I is still active.

As mentioned, in our experiments, role of PC binding to LTP I could not be determined due the low sensitivity of the PC assay. This experiment should be repeated with radioactive PC liposomal vesicles.

To determine if other binding sites play a role in CSA transfer between lipoproteins, transfer experiments from one lipoprotein component to another, utilizing LTP I and other mAb against LTP I at other sites or utilizing mutant LTP I could be done. A FPLC procedure could then be developed further to determine the non-neutral lipid binding site if warranted.

Since the human body may recognize lipophilic compounds as lipid-like particles, we had originally hypothesized LTP I may also be involved in the facilitated transfer of the lipophilic drug, CSA. CSA was used as a model lipophilic compound. Other lipophilic drugs should also be tested. One limitation of this would be the lack of commercial readily available radio-labeled lipophilic drug compounds. Radioactivity provides for more sensitivity in detection especially since the samples are diluted in FPLC. While this would be a logical study, the synthesis of radio-labeled compounds may need to be contracted out. Cost would be an important consideration.

In order to accomplish these experiments, the purification of LTP I should be brought in-house to get a consistent supply and ongoing volume of pure LTP I for replication of the experiments. LTP I could be purified from human plasma [63] or from a cell line [105].

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APPENDIX A   PREPARATION AND COMPOSITION OF DENSITY SOLUTIONS EMPLOYED FOR SEPARATION OF PLASMA COMPONENTS INTO LIPOPROTEIN AND LIPOPROTEIN-DEFICIENT FRACTIONS BY GRADIENT ULTRACENTRIFUGATION

Solution Density	Composition	Amount
1.006 g/mL	Sodium chloride	11.4g
	1M Sodium hydroxide	1 mL
	Water	qs 1000 mL
1.478 g/mL	Sodium bromide	195.8 g
	1.006 g/mL density solution	250 mL
1.063 g/mL	1.006 density solution	400 mL
	1.478 density solution	55 mL
1.210 g/mL	1.006 density solution	200 mL
	1.478 density solution	152.2 mL

APPENDIX B   SUMMARY SHEET OF THE LIPID TRANSFER PROTEIN ASSAY

Materials

- 3.5% bovine serum albumin in 50mM Tris-HCl, 0.02% NaN_3, pH 7.4 (37°C)
- 50mM Tris-HCl, 150 mM NaCl, 0.02% NaN_3, 0.01% disodium EDTA, pH 7.4 (T150)
- unlabeled HDL or LDL
- radiolabeled LDL or HDL
- 0.1 M $MnCl_2$ in H2O
- 0.45 M sodium PO_4, 0.02% NaN_3, pH 7.4, in H_2O
- LTP source (Purified LTP I or Delipidated Plasma)

Procedure

1. In 1.5 ml microfuge tubes, combine the following amounts of reagents for assay blanks, assay samples, and assay totals: (For LTP I human source, in a separate microfuge tube, add 200µL $BaCl_2$ and 250µL 0.4% PTA in NaOH solution, leave it for 15 minutes, spin at 6740 x g (9000 rpm) for 3 minutes in a Beckman Microfuge 12, or equivalent and remove the supernatant).

Reagent		3 Assay Blanks	(6x*) Assay Samples***	(2x*) Assay Totals
3.5% BSA		200 µl	200 µl	200 µl
LDL	Note:	L$^+$ µl	L µl	L µl
HDL	These three should total 200 µl.	H$^\psi$ µl	H µl	H µl
H_2O		(200-H-L) µl	(200-H-L) µl	(200-H-L) µl
LTP source**			200 µl	200 µl
T150		300 µl	100 µl	100 µl
Mix well – Incubate all tubes at 37°C for 90 minutes (typical)				
PO_4 reagent		100 µl	100 µl	H_2O 250µl
Mix well				
$MnCl_2$ reagent		150µl	150 µl	
Mix well – Allow to stand at room temperature for 15 minutes				

* where x = number of samples
$^+$ where L = volume equivalent to give 20 µg of phospholipid, as determined by BM Phospholipid Assay or
$^\psi$ where H = volume equivalent to give 20 µg of phospholipid, as determined by BM Phospholipid Assay or
** for pure source of LTP I, this may be less; the volume will be added to the T150 buffer in this case
***6x assay samples with a mAb can be added; a preincubation of 90 min at 37°C with LTP I is done first; the volume will be deducted from the T150 buffer in this case

2. Centrifuge blank and sample tubes at 6740 x g (9000 rpm) for 3 minutes in a Beckman Microfuge 12, or equivalent, to pellet the LDL.

3. For blanks and samples, remove all supernatant into scintillation vials. For totals, remove entire volume into scintillation vials. Determine radioactivity by scintillation counting. (Use 5.4 mL of scintillation fluid.)

(For HDL to LDL transfer blanks and samples, discard all supernatants. Pipette 1mL of normal saline to each sample to suspend the precipitated LDL, and pipette into scintillation vials.)

4. After appropriate corrections of raw counting data for background radioactivity, calculate the extent of lipid transfer as follows. For LDL to HDL Transfer:

$$kt = -\ln (1 - ((\text{sample } HDL_t \text{ cpm} - \text{blank } HDL_t \text{ cpm}) / \text{total } LDL_0 \text{ cpm}))$$

where HDL_t is the supernatant radioactivity at time t for the assay sample and assay blank, and LDL_0 is the total LDL radioactivity added at time 0. Transfer values are reported as percent lipid transfer; i.e., 100 x kt or %kt.

For HDL to LDL Transfer:

$$kt = -\ln (1 - ((\text{sample } LDL_t \text{ cpm} - \text{blank } LDL_t \text{ cpm}) / \text{total } HDL_0 \text{ cpm}))$$

APPENDIX C   PACKING A SEPHACRYL HR 100 XK COLUMN

1. Slurry the gel in a total volume of 1.5x that of the final packed column (use buffer).
2. Dilute to 2x volume of the final packed column volume for the slurry to be poured into the column.
3. Level the column with a 'leveler' before clamping it.
4. Prepare the adapter by removing the air with 20% EtOH so no bubbles are in the net. Close the tube once all air is removed and leave in a beaker until needed. Repeat this process with the bottom piece and insert this piece on the column.
5. Initially pour about 5cm of buffer in the column.
6. Attach the reservoir. Pour in the homogeneous slurry. Make sure the reservoir is filled to the top with buffer and open the tubing on the bottom of the column to allow outflow of buffer.
7. Once the gel is poured into the column, pump the buffer at the specified rate (1.0mL/min for 2 hours then 1.8mL/min for 1 hour for Sephacryl HR 100). Afterwards, stopper the tubing that is coming out of the bottom of the column.
8. Stop the pump, detach tubing from the top of the reservoir then detach the column from the post.
9. Remove the reservoir over a beaker or the sink and pour out the remaining buffer.
10. Remove excess slurry so the gel bed is visible underneath the black top piece (mark level), and fill to top with buffer. Insert the 'wetted' adapter top piece on top of gel bed at 45 degrees, making sure not to trap air.
11. Tighten nut and remove excess liquid around the top of the adaptor.
12. Remove the cap from the adapter tubing. Screw on red top of the adapter and push gently down to remove the excess buffer from the top of the column. "2 clicks on the video"
13. Start pump at low flow and flush all bubbles from the VALVE before connecting the tubing.
14. Connect the adapter tubing to the valve ad open the bottom tubing.
15. Once the bed is stabilized, mark level, sop the pump and close the outlet from the bottom of the column.
16. Switch the valve to connect the column and the sample port.
17. Adjust the adapter to the new bed height slightly below the bed height (5mm), allowing excess buffer to run out through the open tube leading out from the adapter.
18. Start pump and connect the column and pump.
19. Equilibrate column with 2 volumes of buffer.
20. Test column with a small volume of acetone to measure peak symmetry:
    Leading – too high a packing rate will cause small cracks in the gel bed or poor contact between the gel bed and column wall.
    Tailing – caused by too low a packing rate or large dead volume or air under the top net (filter). The valve may be the source of bubbles, so make sure the same channel for pump-to-column and port-to-column is used.
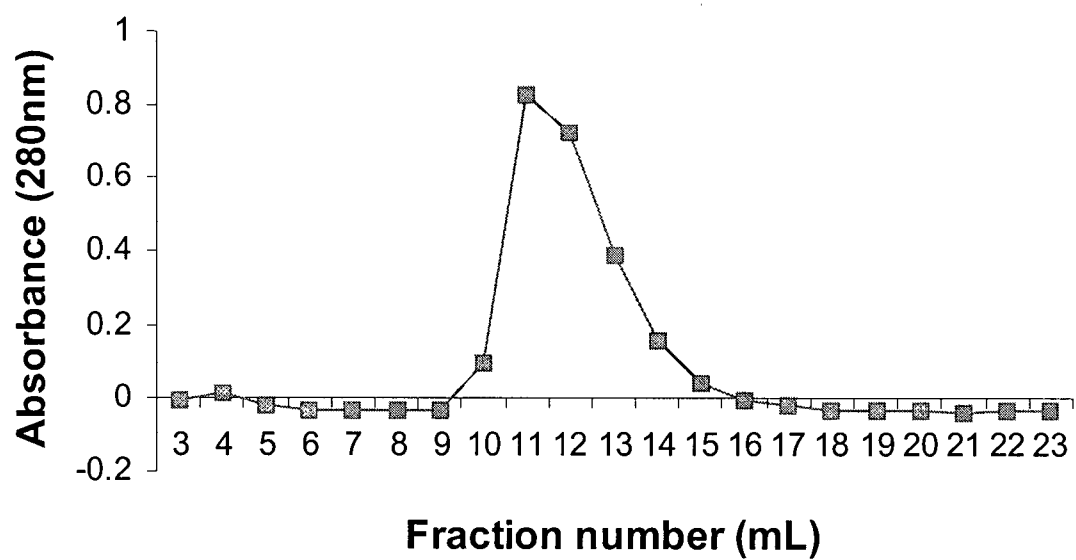
APPENDIX E   MAKING PC ENCAPSULATED LIPOSOMALVESICLES

1 Litre Buffer – adjust to pH 7.4 and filter

Tris-HCl   157.6 MW
EDTA   372.2 MW

10mM = 0.01 M   Tris-HCl   → need 1.576g
 1mM = 0.001 M   EDTA   → need 0.3722g

Add Tris-HCl and EDTA to distilled water to make up to 1L, mix, then filter, and store in the fridge.

Procedure

1. Take out the PC and cholesterol oleate (cold) from the freezer. In a capped test tube, quickly weigh out enough lipid to make 2mL of a 20mg/mL in chloroform for each. Back calculate to add the appropriate amount of chloroform. Vortex well. You now know that you have x mg lipid in x mL of chloroform. Take 2 x 20uL samples of this for scintillation counting. Dry the chloroform off the samples (chloroform quenches counts).
2. Set up a vacuum system in the fume hood for step 4.
3. Dry down in the fume hood with N2 gas. Put the adapter on the gas cylinder and put a glass pipette on the end of the rubber hose. Get the N2 gas to blow very gently – check the gas flow on your hand – it should be very light. Get a beaker ¾ full of hot tap water. Dip test tube inside and blow down the liquid with the glass pipette – GO SLOW and blow down until you get a thin layer with lots of bubbles.
4. Once you've dried your lipid to a thin layer, keep it WARM. The warmer the sample is the easier it is to boil away the excess chloroform. Cover the top of the test tube with kimwipe taped or rubber band bound it to the top of the test tube. This will absorb some of the liposome if it shoots up into the vacuum. Put the test tube into the erlenmyer vacuum flask and start the vacuum slowly and watch as the chloroform boils off. You may get a thick film building up. Just swirl the test tube until the film breaks. Be careful – if you have the vacuum on too high and the film breaks, the sample will shoot right out of the test tube. The lipid should foam and bubble as the chloroform boils off and you should be left with a fluffy lipid film (depending on the lipid). Keep the vacuum on for 2 hours.
5. At this point you may wish to stop. You can use a cap and parafilm seal the test tube and store it in the freezer. This is handy so that if you want fresh liposomes, you just have to reconstitute from this step rather than starting at step 1.
6. Set up a hot water bath. Heat your buffer and the lipid. Add 7mL of buffer to the film of lipid. Vortex very thoroughly.

7. Fill a dewar with liquid N2. Transfer the lipid mixture into plastic cyro tubes (I put 1mL in the 1.8mL cyro tubes). (glass test tubes will shatter when you freeze thaw and the cyro tubes cannot be used with chloroform at the beginning step because chloroform dissolves plastic). Attach cyro tubes to the lower portions of the metal crane. Freeze the sample in liquid N2 and then thaw in the hot water bath at the transition state temperature. ***Repeat this 5 times.***

8. You can stop here if you run out of time at the freezing part and put away in a -70 C freezer.

9. Assemble the extruder and put sample through the extruder 10 times.

10. Size the sample with a particle sizer. Take about a drop or two and dilute with your filtered buffer. You should run the sizer again using a 2x dilution of the sample you just ran. If the two samples agree with +/- 5nm then it is fine. If not, keep on diluting until you have this error. (The particle sizer works by measuring the movement of the molecules. If the solution is too concentrated then the molecules will be bigger than they actually are).

11. Take 2 x 20uL samples of the final solution to determine the final concentration. For interest sake the filters can be counted to see how much lipid is stuck to the filters. Cap the sample and store in the fridge.

12. Wash the extruder with detergent then rinse very well (detergent will lyse any liposomes that are extruded next time if you don't rinse well). Wash well with radiowash as well if radioactivity is used. Rinse with 70% isopropranol then with distilled water and leave to air dry.

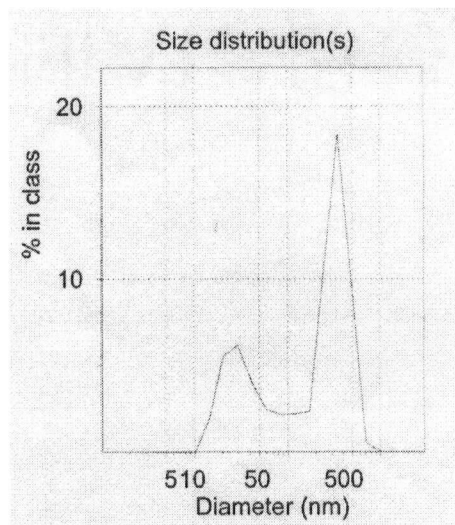**Appendix F   EXTRUDED VS SONICATED [³H]-CE LIPOSOMALVESICLES**

Figure F 1 Particle size distribution of sonicated [³H]-CE liposomal vesicles (mean 318nm after 24min)
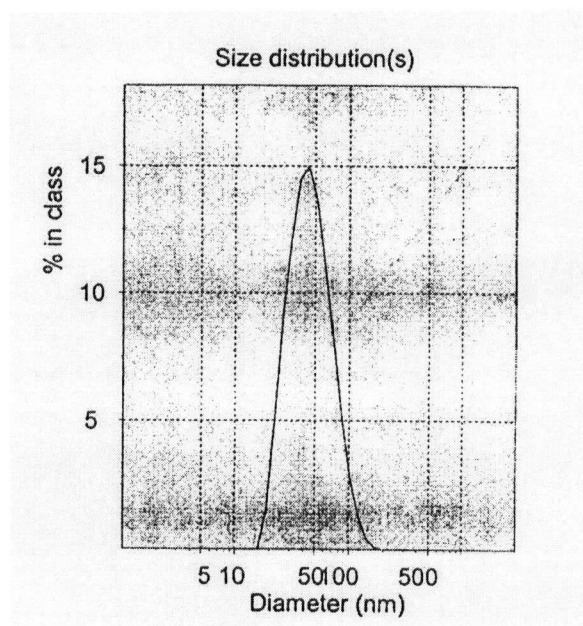

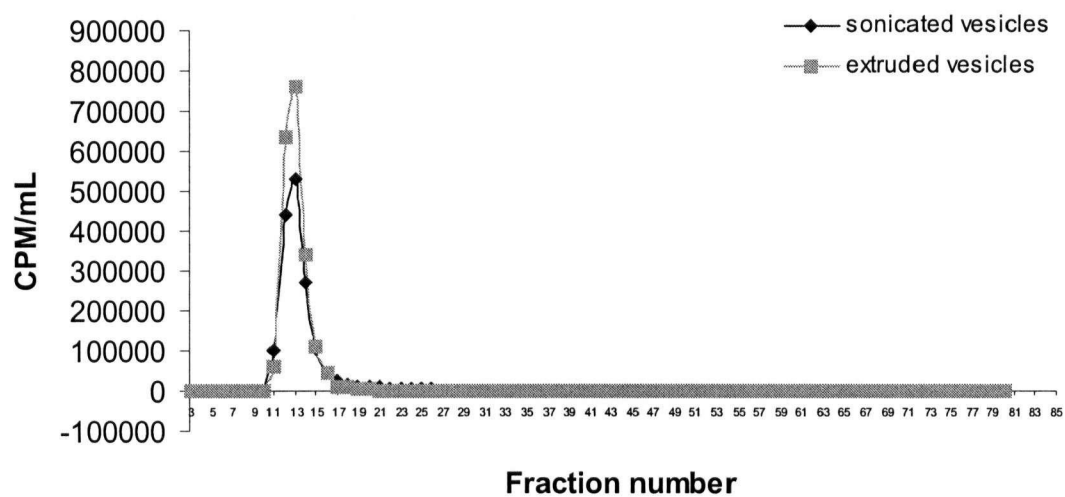Figure F 2 Particle size distribution of extruded [³H]-CE liposomal vesicles (mean 48.7nm)

Figure F 3 [^{3}H]-CE Elution of sonicated versus extruded [^{3}H]-CE liposomal vesicles injected through a Sephacryl HR 100 XK column

APPENDIX G   ELISA SOLUTION CALCULATIONS

		MW	Amt needed for 2L	Amt needed for 1L	Amt needed for 500mL	Amt needed for 100mL
Coating Buffer						
15mM Na2Co3	0.015	106.004	3.18012	1.59006	0.79503	0.159006
35mM NaHCo3	0.035	84.015	5.88105	2.940525	1.4702625	0.2940525
0.02%(w/v) NaN3		n/a	0.4	0.2	0.1	0.02
pH 9.8						
qs with distilled water						
Washing Buffer						
138mM NaCl	0.138	68.454	18.893304	9.446652	4.723326	0.9446652
8.1mM Na2HPO4	0.00081	141.969	2.2998978	1.1499489	0.57497445	0.11499489
1.2mM KH2PO4	0.0012	136.075	0.32658	0.16329	0.081645	0.016329
2.7mM KCl	0.0027	74.557	0.4026078	0.2013039	0.10065195	0.02013039
pH 7.2						
qs with distilled water						
Saturation Buffer						
1mM EDTA	0.001	372.2	0.7444	0.3722	0.1861	0.03722
1% (w/v) BSA		n/a	20	10	5	1
qs with WB						
Triton Buffer	0.2% (w/v) Triton X-100	n/a	4	2	1	0.2
qs with SB						

APPENDIX H   LTP I AND PROTEIN DETERMINATION OF ELUTION FRACTIONS FROM A PLASMA INJECTION (0.5ML) THROUGH A SEPHACRYL HR 100 XK COLUMN