THE ROLE OF THE LOW DENSITY LIPOPROTEIN RECEPTOR IN TARGETED DRUG DELIVERY MEDIATED BY ANIONIC LIPOSOMES

by
Ketan Amin

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THE ROLE OF THE LOW DENSITY LIPOPROTEIN RECEPTOR IN TARGETED DRUG DELIVERY MEDIATED BY ANIONIC LIPOSOMES

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Under the supervision of Associate Professor Timothy D. Heath

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Anionic liposomes have long been proposed as ideal carriers for the delivery of drugs and other macromolecules. Fundamental to the practical development of anionic liposomal formulations for use in drug delivery is the characterization of the cellular mechanisms involved in their uptake. The present work examines how the characteristics of anionic liposomal formulations impact the cellular mechanisms involved in their uptake.

Association of liposomal aqueous contents and liposomal lipid with CV1-P and CHO wild type cells, which both express the low density lipoprotein receptor (LDLr), and CHOldlA7, which lacks the LDLr, was measured. Additionally, CHOldlA7 was transfected with the human LDLr and further association studies were performed using the anti-LDLr and anti-apolipoprotein B100 (apoB100) monoclonal antibodies, IgG C7 and IgG 5E11, respectively.

Association of liposomes composed of 75-100 mole percent egg phosphatidylglycerol (ePG), a fluid anionic phospholipid, with cells was found to be mediated by LDL and the classical LDLr. Binding of liposomes composed of 75-100 mole percent ePG was blocked by the anti-LDLr antibody IgG C7 and the anti-apoB100 antibody IgG 5E11.

Cellular association of 75-100 mole percent ePG vesicles in a serum free growth medium allowing for a maximal LDLr expression led to the functional delivery of a series of model liposome dependent drugs. Findings strongly indicate that 75-100 mole percent ePG liposomes interact with the LDLr in an apoB100-dependent fashion, and the interaction results in the delivery of contents to cells. An attractive strategy would be to utilize these ePG liposomes for targeted drug delivery to highly proliferative cancer cells known to over express the LDLr.

Serum, but not LDL or HDL, induced association of 25-50 mole percent ePG liposomes with both CV1-P and CHO wild type, but not CHOldlA7. This form of liposome binding appears not to involve LDL or LDLr, but requires a receptor, currently unknown, and a serum component other than LDL or HDL. Findings indicate that LDLr is not involved in the interaction of 25-50 mole percent ePG vesicles with cells, and that CHOldlA7 lacks at least one other surface protein in addition to LDLr.

APPROVED:

Timothy D. Heath

Timothy D. Heath, Associate Professor

Date 2/5/01

To Kinnari,
the love of my life.

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ABBREVIATIONS

apoB48, apolipoprotein B-48

apoB100, apolipoprotein B-100

CHOwt, CHO wild type

DPPG, dipalmitoylphosphatidylglycerol

DSPG, distearoylphosphatidylglycerol

ePC, egg phosphatidylcholine

ePG, egg phosphatidylglycerol

FO, fluoroorotic acid

HDL, high density lipoprotein

LDL, low density lipoprotein

LDLr, low density lipoprotein receptor

Mtx, methotrexate

Mtx-γ-asp, methotrexate-γ-aspartate

PALA, N-phosphonacetyl-L-aspartic acid

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CHAPTER ONE: INTRODUCTION

1. Liposomes

It was in the 1960's that Bangham and Mueller introduced the concept of lipid bilayers used as models for biological membranes.[1,2] These lipid bilayers, now called liposomes, have taken on applications in the basic sciences, cosmetic industry and pharmaceutical industry. A liposome may be defined as any structure composed of lipid bilayers that encloses a volume.[3] The most commonly used lipids are phospholipids, which consist of a hydrophobic tail group attached to a hydrophilic head group (Figure 1). When placed in excess water these amphipathic molecules arrange in a manner such that the hydrophilic head groups tend to be in contact with water while the hydrophobic hydrocarbon chains prefer to be hidden from water resulting in the formation of a liposome.[4]

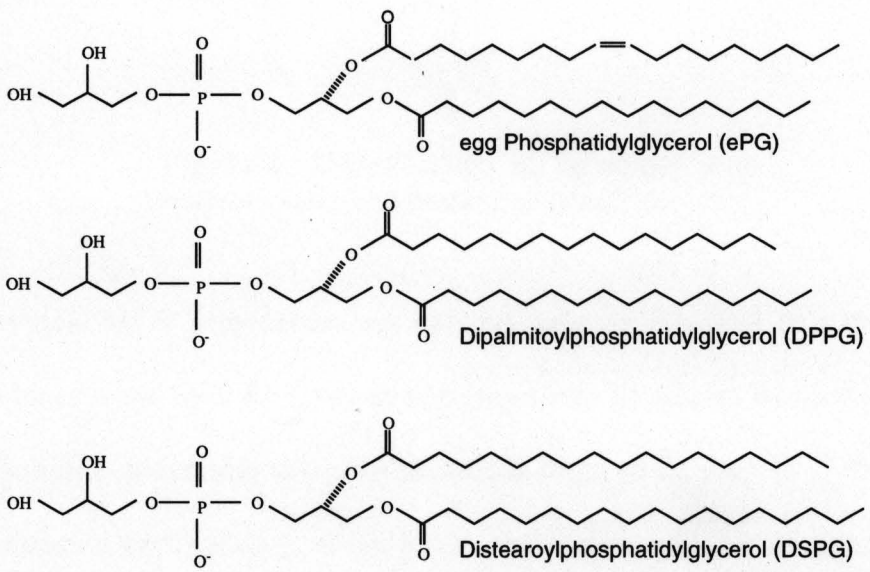


Figure 1. Anionic Phospholipid Structures. Fatty acid content of ePG is given in the legend of Table II. The designated structure for ePG represents the predominant fatty acid composition.

Liposomes can be large or small and may consist of numerous concentric bilayers. With respect to size and number of lamellae, they can be classified as small unilamellar vesicles, SUV (0.02-0.08 μm), large unilamellar vesicles, LUV (0.1-1.0 μm) or multilamellar vesicles, MLV (1-5 μm) (Figure 2). Selection of a method for liposome preparation for drug delivery involves a multitude of variables such as encapsulation efficiency, drug to lipid ratio, captured volume, stability and pharmaceutical acceptability. Although no single method will encompass all of these variables, there are numerous methods of liposome preparation detailed elsewhere, one of which will result in a final product with acceptable properties.[5,6,7]

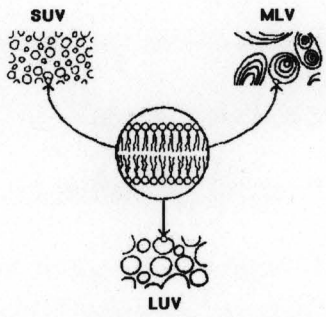


Figure 2. Classification of liposomes with respect to size and number of lamellae.

In a typical MLV preparation, an aqueous solution is added to a dry lipid film. Agitation loosens the lipid film, which aggregates into liposomes while surrounding the aqueous solution containing the pharmaceutical cargo to be carried by the liposome.[8] Further extrusion or sonication of the MLV preparation will lead to a decrease in the number of lamellae and formation of LUV or SUV. Though relatively easy to prepare, MLV and SUV typically have a lower captured volume and encapsulation efficiency.

When working with expensive lipids and drugs it is desirable to optimize captured volume and encapsulation efficiency, thus leading to the development of various methods to prepare LUV. The reverse-phase evaporation (REV) technique allows the formation of LUV with characteristics highly favorable for drug delivery. Vesicles are formed when an aqueous buffer is introduced into a mixture of phospholipid and organic solvent and the organic solvent is subsequently removed by evaporation under reduced pressure.[9]

2. Physicochemical Properties of Phospholipid Vesicles

Since phospholipids are the major component of liposomal drug delivery systems it is necessary to discuss their physical chemistry, in particular the gel-liquid crystalline phase behavior. Depending on temperature, membrane lipids can exist in a frozen gel state (L_β) or fluid liquid-crystalline state (L_α). There is a distinct phase, called the ripple phase (P_β), between the gel (L_β) and liquid crystalline (L_α) forms. The $P_\beta \rightarrow L_\alpha$ transition is known as the main transition (T_m) whereas the $L_\beta \rightarrow P_\beta$ transition is called the pretransition (T_p). The L_α phase is characterized by considerable disorder in the acyl chains with a resultant increase in molecular mobility. Conversely, the L_β phase features tightly packed molecules whose acyl chains are much more highly ordered. Because the chains are maximally extended in the gel phase, the bilayer thickness is greater than in the liquid crystalline phase. Transitions between the gel and liquid-crystalline phases can be monitored by a variety of techniques, such as nuclear magnetic resonance. However, differential scanning calorimetry (DSC), which measures the heat absorbed (or released)

by a sample as it undergoes an endothermic (or exothermic) phase transition, is particularly useful.

The T_m is highly dependent on the fatty acyl chain because of the importance of van der Waals forces in determining the relative stability of the gel and liquid crystalline phases. Additionally, longer chain lengths result in higher T_m values, because of the increased van der Waals interaction of the longer chains. A trans double bond reduces the T_m as this will disrupt the ability of the chains to interact optimally in the gel state. Finally, a cis double bond has a greater affect on T_m than would a trans double bond.[10]

The role of cholesterol as a membrane component has been studied rather extensively. This lipid has the ability to inhibit the crystallization of lipids to form gel-state systems. The enthalpy of the transition is progressively reduced as the cholesterol content is increased. For phosphatidylcholine: cholesterol molar ratios of 2:1, no transition is observable.[11] Cholesterol incorporation into phospholipid membranes produces considerable restriction of molecular motion and a decrease in membrane permeability. Studies have found cholesterol to decrease permeability of phospholipid vesicles to glucose and other non-electrolytes.[12] Additionally, a number of purified proteins greatly increase the permeability of phospholipid membranes.[13] Papahadjopoulos *et al.* reported cholesterol incorporation into phospholipid membranes to greatly inhibit the ability of proteins to increase the diffusion rates for Na^+.[14] Carboxyfluorescein loaded liposomes were found to retain their contents in the presence of mouse whole blood, plasma or serum in a cholesterol dependent manner.[15]

In addition to an ability to adopt a gel or liquid-crystalline bilayer organization, lipids can also adopt entirely different liquid-crystalline structures on hydration.[16] A qualitative understanding of lipid polymorphism is possible by considering the packing requirements of different lipids and the constraints imposed by simple geometric considerations. Lipids can be simply classed as cones, cylinders, or inverted cones depending on the relative packing requirements of the polar headgroup and hydrocarbon portion of the lipid.[10] Considering the molecular shape of lipids, hexagonal phase lipids have a cone shape, detergent-type lipids which form micellar structures are suggested to have reversed geometry corresponding to an inverted cone shape, and bilayer phase lipids are proposed to exhibit cylindrical geometry compatible with that organization.[11]

3. Liposome-Dependent Drugs

Liposomes are usually made from naturally occurring substances and are therefore regarded as being biocompatible and biodegradable. These favorable characteristics have led to the encapsulation of a wide variety of pharmaceutical agents for the purposes of targeting pharmaceuticals to specific cells or to reduce toxicities associated with drugs with narrow therapeutic indices. Antineoplastic agents, anitmicrobials and anti-inflammatory agents have all been encapsulated in liposomes.[17,18,19]

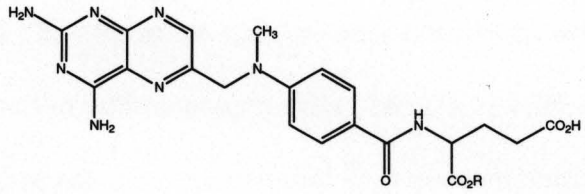
Drug delivery using liposomes is based upon several concepts such as controlled release and localization. Controlled release is a mechanism for increasing the efficacy of a drug by maintaining therapeutic drug levels for extended periods. Localization increases the efficacy of a drug by delivering it to its site of action. Based on this

concept, Heath defined liposome dependent delivery as the process by which a liposome delivers its contents to intracellular compartments. Conversely, if a drug enters a cell after release from the liposome, then its delivery to the cell is independent of the liposome.[20] Taking advantage of increased potency of macromolecules by liposomal encapsulation was shown by several investigators working with polio RNA, SV40 DNA, gelonin and diphtheria toxin fragment A.[21,22,23,24] These molecules exert a significant effect once inside cells, yet are unable to enter cells without the assistance of liposome encapsulation. Keeping in line with this approach, I have utilized the model liposome dependent drugs, methotrexate-γ-aspartate (Mtx-γ-asp), methotrexate (Mtx), fluoroorotic acid (FO), and N-phosphonacetyl-L-aspartic acid (PALA), to probe fundamental issues pertaining to the uptake of anionic liposomes via the LDLr (Figure 3).

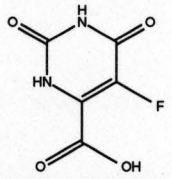
Mtx-γ-asp and Mtx are members of the pteridine antifolates, inhibitors of dihydrofolate reductase (DHFR), and are transported into cells by the folate transport system.[25] The γ-substituents of Mtx are unable to enter cells, but are fully effective inhibitors of DHFR.[26] Utilizing a growth inhibition assay, in which the concentration of drug required to inhibit 50% of cell growth (IC_{50}) is determined, Heath *et al.* were able to demonstrate increased potency of Mtx-γ-asp encapsulated in negatively charged liposomes.[27] Mtx-γ-asp is at least 100 times less able to enter cells than Mtx, and is a 150 times less potent cytotoxic agent than Mtx.[20] By using a cell line susceptible to drug delivery by liposomes or by using short exposure times, Mtx can also be studied as a liposome dependent drug. CV1-P cells proved to be ten-times more sensitive to

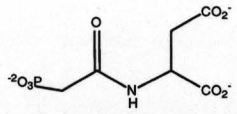
encapsulated Mtx, whereas all other cell lines tested were either less sensitive or no more sensitive to the encapsulated drug.[28]

FO, a fluorouracil derivative, unable to penetrate cells is used as another liposome dependent drug. FO is metabolized to fluorouridine monophosphate and incorporated into newly transcribed RNA, altering the function of various ribosomal species.[29] When incorporated into negatively charged liposomes, FO, was up to 30 times more potent for growth inhibition of cells.[30]



Methotrexate (Mtx): R = H
Methotrexate-γ-aspartate (Mtx-γ-asp): R = L-Asp





Fluoroorotic acid (FO) N-phosphonacetyl-L-aspartic acid (PALA)

Figure 3. Liposome-Dependent Drugs.

PALA, an inhibitor of aspartate transcarbamylase, exhibits a considerable increase in its *in vitro* growth inhibitory potency when it is encapsulated into liposomes.

Encapsulation in negatively charged liposomes increases the growth inhibitory potency of PALA by up to 360 times.[31] Encapsulated PALA in negatively charged liposomes was also shown to be up to 570 fold more potent for growth inhibition on human ovarian tumor cells *in vitro*.[32]

4. Targeting of Liposomes

Targeting liposomes to a specific cell type *in vivo* requires the successful completion of several steps. First, the liposomes must have access to and eventual recognition by the appropriate target cell. The liposome must interact selectively with the target cell while having minimal interaction with nontarget cells. Next, it is critical that the drug remain associated with the liposome until the required drug concentration is delivered. Finally, drug targeting should not produce unacceptable levels of toxicity.[33,34]

The most common route of administration of liposome preparations is by intravenous (IV) injection. After, IV dosing, liposomes are quickly cleared from the circulation by the mononuclear phagocytes of the reticuloendothelial system (RES) in the liver and spleen and circulating blood macrophages.[35] The RES is a major host defense system in the body consisting of circulating blood macrophages and the fixed macrophages of the liver (Kupffer cells), spleen and bone marrow.[36,37] In addition to removing foreign particles such as particulate matter, microorganisms, effete cells, dead cells and cellular debris from the circulation, the functional state of the RES helps determine the host response to events such as hemorrhage, circulatory and septic shock, and trauma.[38]

Though the function of the RES makes it difficult to target other cells, it is possible to passively target liposomes to these cells in disease states that affect the RES. Liposomes have been used to passively target macrophage activating factors, thus, allowing activated macrophages to distinguish between tumorigenic and nontumorigenic cells.[38] Liposomes have also been used to passively target therapeutic compounds to the cells of the RES infected with *Leishmania donovani* and human immunodeficiency virus (HIV).[39,40]

Liposomes can also be actively targeted to cells by the attachment of various ligands to the surface of the liposome. The most widely used molecules for targeting liposomes have been monoclonal antibodies. Monoclonal antibodies, selected for their ability to bind to cancer-associated epitopes on the surface of cancer cells, have been coupled to liposomes to produce targeted drug carriers, often referred to as immunoliposomes.[41] Malignant cells often overexpress receptors for growth factors or other molecules which help to maintain their highly proliferative growth patterns. Subsequently, ligands for growth factor receptors, such as the transferrin receptor and the LDLr, have been complexed to liposomes for targeting to cancer cells.[42,43]

5. Liposome-Cell Interactions *In Vitro*

Liposomes can interact with cells by adsorption, lipid exchange, fusion or endocytosis (Figure 4). The attachment of liposomes onto the cell surface is referred to as adsorption. Upon binding to the cell surface, liposomal contents are released into the extracellular fluid, allowing some of this released fluid to cross the cell membrane. Lipid exchange involves the transfer of liposomal lipids to the cell membrane with concomitant

transfer of cellular lipids to the liposome membrane.[44] Transfer of lipophilic drugs contained within the liposome membrane may occur during the lipid exchange process.[45] The unification of the liposomal membrane with the cell membrane is known as fusion. This process involves the dumping of liposomal contents into the cytoplasm of the cell. The early notion was that fusion was a major interaction, however, later studies showed this to be a rare event.[46,47]

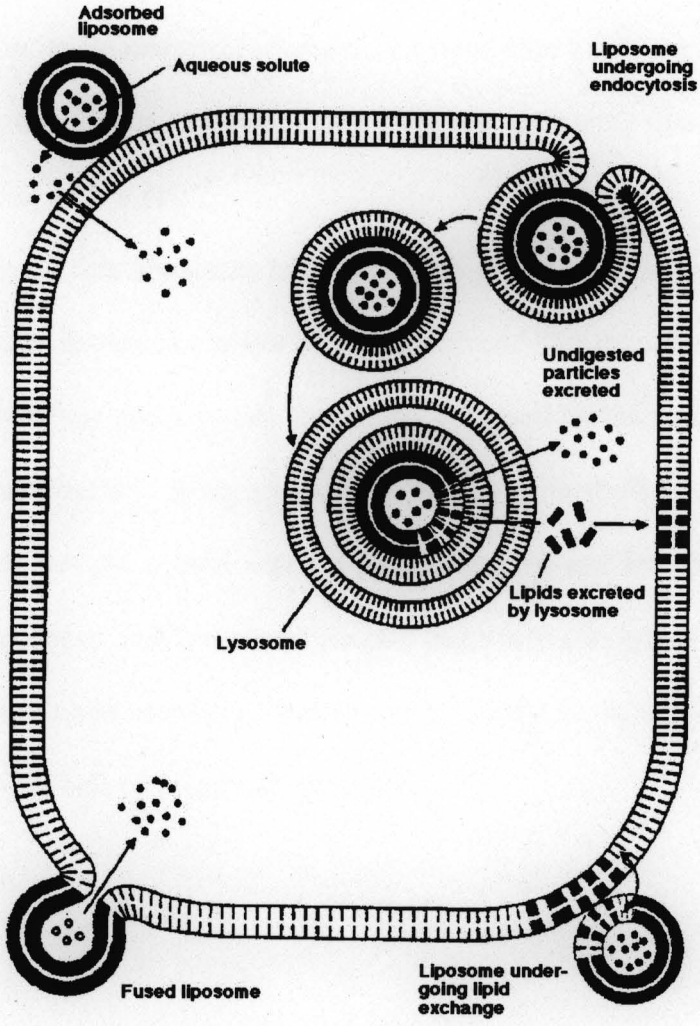
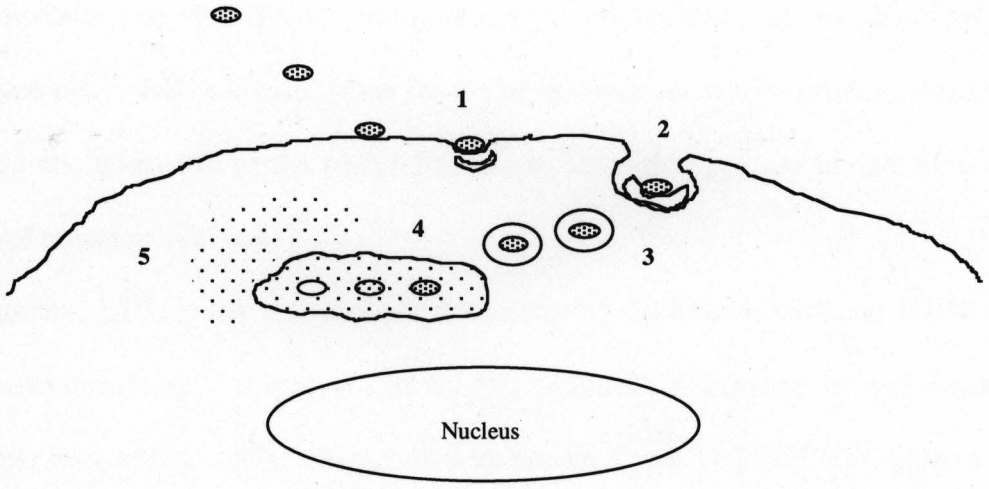


Figure 4. Liposome-Cell Interactions *In Vitro*.[45]

Though liposomes may enter cells through a variety of postulated mechanisms, the predominant route is endocytosis via coated pits.[34] Utilizing fluorescence and electron microscopy, Straubinger *et al.*were able to demonstrate that anionic liposomes are endocytosed by coated pits in a manner similar to that of a number of macromolecules with specific surface-bound receptors, such as LDL and transferrin.[36] Lipid vesicles containing the pH sensitive fluorophore 8-hydroxy-1,3,6-pyrenetrisulfonate (HPTS) were used as probes for the study of liposome entry and intracellular fate.[48] HPTS underwent a pH dependent shift in its fluorescence intensity one hour after exposure to CV1-P cells. The pH dependence was effected by NH_4Cl and monensin indicating that the dye resided in intracellular vesicles of low pH.[49]

These studies reveal that liposomes bind to regions on the plasma membrane known as coated pits of which clathrin is a major protein involved. The involvement of clathrin coated vesicles in liposome endocyctosis has been confirmed by microinjection of anti-clathrin antibodies into cells.[50] Invagination occurs leading to the formation of coated vesicles. Within seconds the coated vesicles shed their coat and are able to fuse with endosomes and lysosomes. The lysosomes contain degradative enzymes which are able to break down the liposomal membrane leading to the escape of liposomal contents and eventual release into the cell cytoplasm (Figure 5).[51]



1. Coated Pit
2. Coated Vesicle
3. Endosome
4. Lysosome
5. Lysosomal Degradation and Drug Release

Figure 5. Endocytic Cycle for Liposomal Drug Delivery.[51]

6. Low Density Lipoprotein and the LDL Receptor

Plasma lipoproteins are macomolecular aggregates of lipids and proteins classified in terms of their density, as determined by centrifugation. The standard lipoprotein classification includes chylomicrons (d<1.00 g/ml), very low-density lipoprotein (VLDL) (d<1.006 g/ml), intermediate density lipoprotein (IDL) (d=1.006-1.02 g/ml), low density lipoprotein (LDL) (d=1.02-1.063 g/ml) and high density lipoprotein (HDL) (d=1.063-1.21 g/ml). Triacylglycerol-rich chylomicron particles are the major lipid secretory products of the intestine. These chylomicrons are packaged with apoB48, an apolipoprotein consisting of 48% of the amino terminal portion of apoB100, and are rapidly metabolized.[52] VLDL is the major lipoprotein particle produced by the liver and

is responsible for delivering endogenously synthesized fat in the form of triacylglycerols.[53] HDLs are a diverse family of lipoproteins whose primary function is to remove cholesterol from the peripheral tissues through a process known as reverse cholesterol transport (Table I).[54]

In humans, LDL is the major cholesterol-carrying lipoprotein with apoB100 as its only apolipoprotein.[52] Lipolysis of VLDL produces a cascade of intermediates collectively termed IDL whose interaction with hepatic lipase yields LDL.[53] Plasma LDL particles contain a cholesterol ester core surrounded by a shell of esterified cholesterol and phospholipid all of which is surrounded by a single apoB100 (Figure 6).[55]

Table I. Density, Size, and Physical Composition of Human Plasma Lipoproteins.[52,53]

characteristic	Chylomicrons	VLDL	IDL	LDL	HDL
Density (g/ml)	< 1.00	< 1.006	1.006-1.02	1.02-1.063	1.063-1.21
Diameter (nm)	75-1200	30-80	25-35	18-25	5-12
Composition (% dry wt)					
Protein	1-2	8	19	22	47
Triglycerides	86	55	23	6	4
Cholesterol	5	19	38	50	19
Phospholipids	7	18	20	22	30
apolipoproteins	A1, A2				A1, A2
	B48	B100	B100	B100	
	C1, C2, C3	C1, C2, C3	C1, C2, C3		C1, C2, C3
	E		E		E

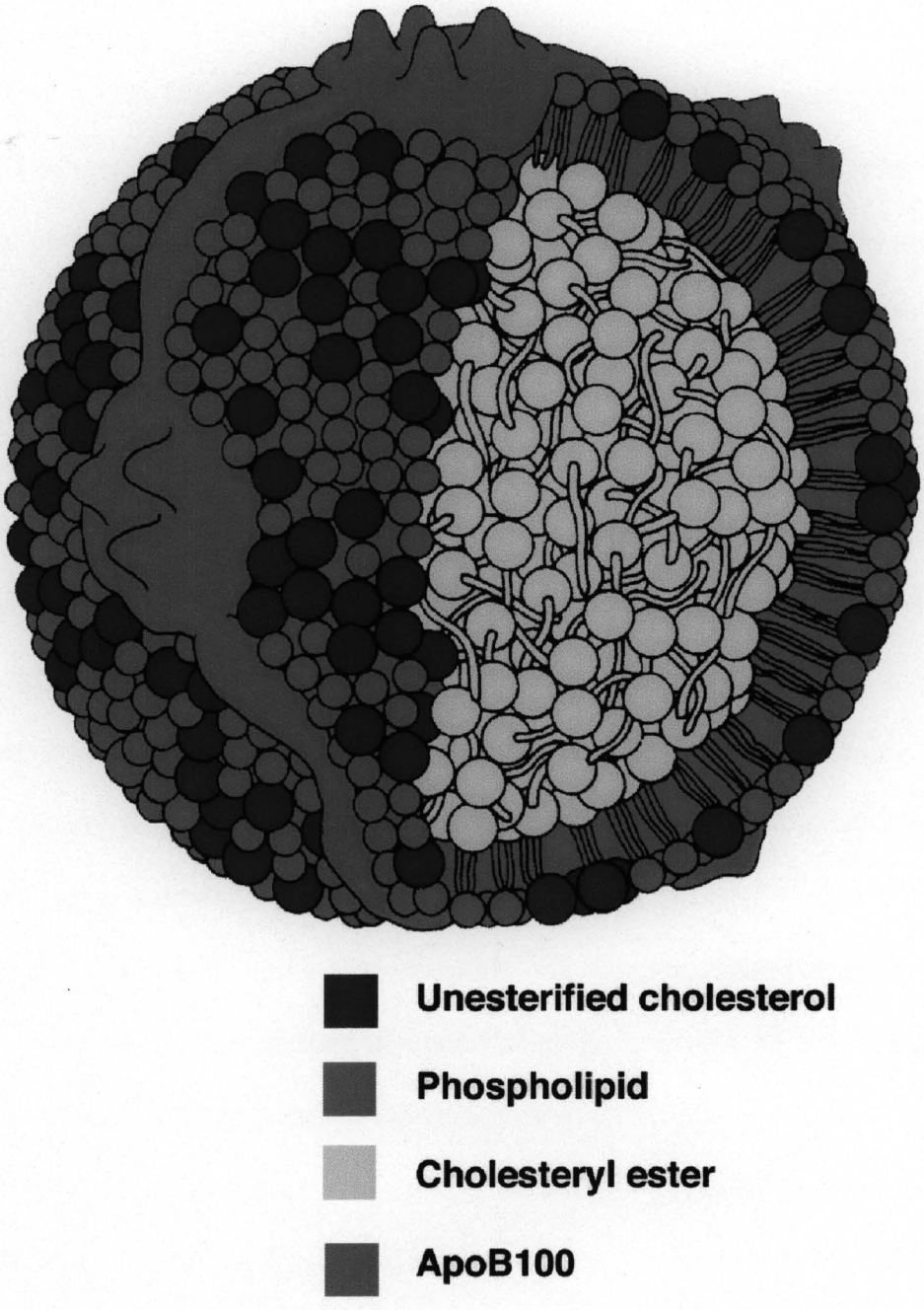


Figure 6. Schematic model of low density lipoprotein.[55]

ApoB100 is a 514 kD protein and one of the largest single chain proteins ever to be sequenced. The structural motifs responsible for the association of apoB100 with lipids are complex and are likely to involve several domains having different secondary structures.[53] The presence of α helices and β sheets consisting of numerous hydrophilic and hydrophobic domains are responsible for binding the protein to the surface coat lipids. The hydrophilic face of the protein is surrounded by the aqueous compartment (i.e. the plasma) whereas the hydrophobic face is found embedded with the lipid coat.[52,56] Additionally, apoB100 contains several cationic domains which are able to bind with glycosaminoglycans, such as heparin, with high affinity.[57] The positively charged clusters of arginine and lysine on the carboxy terminus are responsible for binding to the LDLr. Interestingly, heparin is able to tightly bind to apoB100 as well as apoB48, which lacks a LDLr binding domain.[58]

Cellular uptake of LDL occurs predominantly via receptor mediated endocytosis whereby the receptor binding domain of apoB100 binds to a specific ligand binding domain on the LDLr. After synthesis in the endoplasmic reticulum, the LDLr matures in the Golgi before ultimately appearing on the cell surface in areas associated with coated pits. After binding LDL, the LDL-LDLr complex appears in the endosome where the acidic pH causes dissociation of the ligand from the receptor. The receptor can return to the cell surface while LDL proceeds to the lysosome where cholesterol released can then act to down regulate synthesis of the LDLr (Figure 7).[59]

The LDLr is highly conserved consisting of five domains which are: 1) the ligand binding domain; 2) a domain that has a high degree of homology with the precursor to the epidermal growth factor; 3) a domain that contains a cluster of 0-linked carbohydrate chains; 4) a transmembrane domain; and 5) a short cytoplasmic region.[60] It is the ligand binding domain which mediates the interaction between the LDLr and apoB100 and/or apoE.[61] The presence of seven repeats containing negatively charged clusters of serine, aspartic acid and glutamic acid are thought to bind to apolipoproteins by means of an electrostatic interaction (Figure 8).

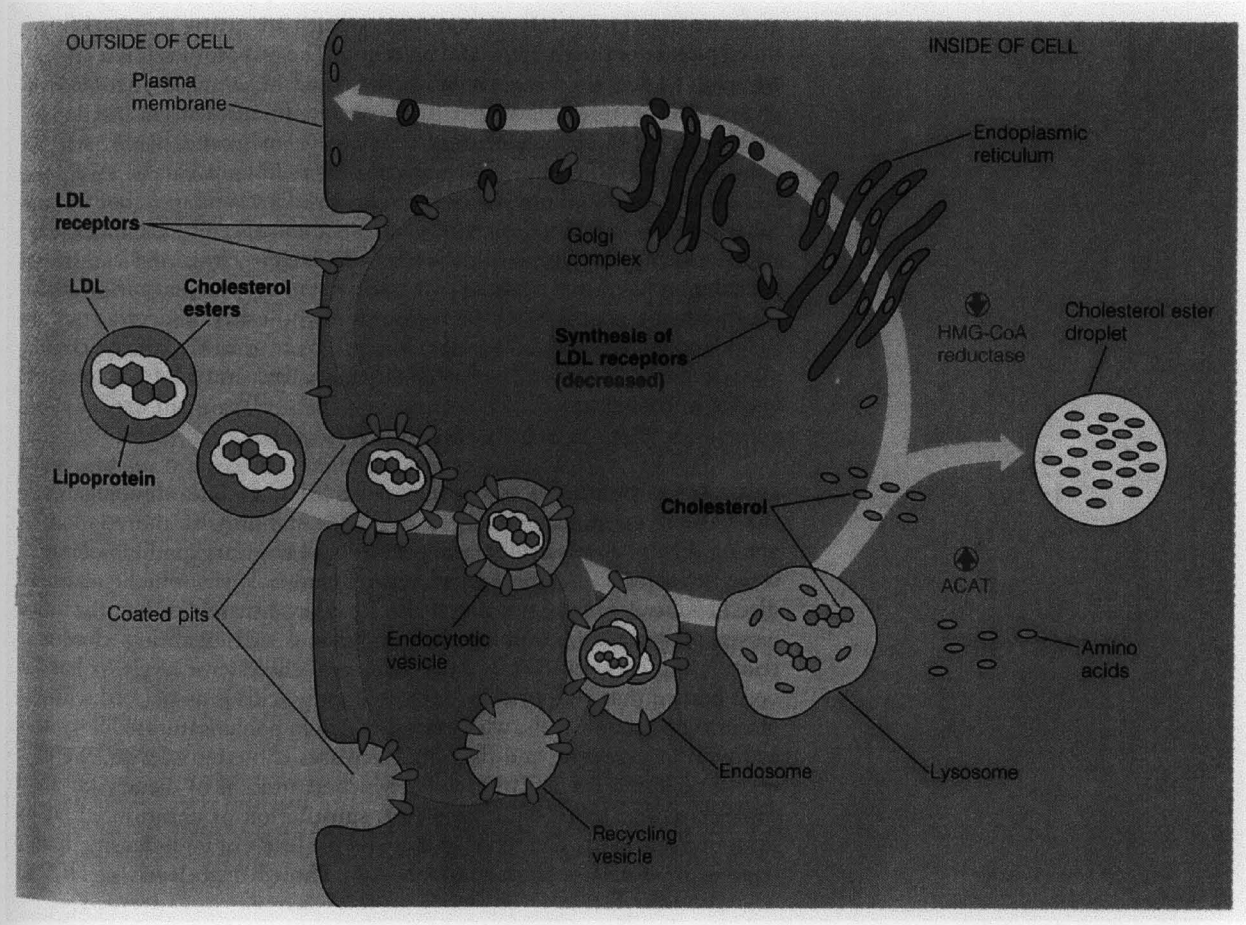


Figure 7. Receptor Mediated Endocytosis. LDL-LDLr complex is internalized in a coated vesicle which becomes uncoated and acidified, resulting in an endosome. From there LDL is delivered to the lysosome, whereas the LDLr is recycled back to the cell surface. The LDL apoB100 is degraded and cholesterol has various fates. Regulatory actions of cholesterol are shown in red. HMG CoA reductase denotes 3-hydroxy-3-methylglutaryl CoA reductase and ACAT denotes acyl CoA:cholesterol acyltransferase.[59]

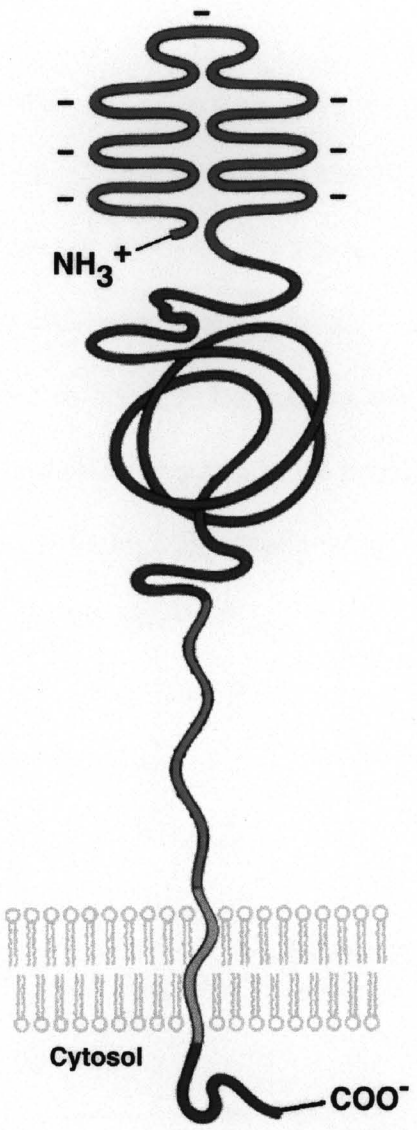


Figure 8. Low Density Lipoprotein Receptor. The LDLr is highly conserved consisting of five domains which are: (green) the ligand binding domain; (purple) a domain that has a high degree of homology with the precursor to the epidermal growth factor; (blue) a domain that contains a cluster of 0-linked carbohydrate chains; (yellow) a transmembrane domain; and (red) a short cytoplasmic region.[55]

7. Liposome Stability

Upon IV injection, liposomes immediately come into contact with various plasma proteins which have a destabilizing effect upon lipid vesicles. Scherphof *et. al.* working with radiolabeled PC liposomes, encapsulating ^{125}I-albumin, noticed a massive release of entrapped ^{125}I-albumin during incubation with plasma. Additionally, they noticed the transfer of PC from liposomes to HDL.[62] Liposomes made of high phase transition temperature lipids were shown to be more stable in the presence of HDL presumably due to less membrane fluidity at physiological temperatures.[63] PC liposomes containing cholesterol exposed to rat HDL were shown to exhibit no appreciable loss of encapsulated carboxyfluorescein.[64] The addition of cholesterol to the phospholipid bilayer membrane results in relatively loose bilayers packing more tightly, thus, reducing the removal of phospholipid by HDL.[15]

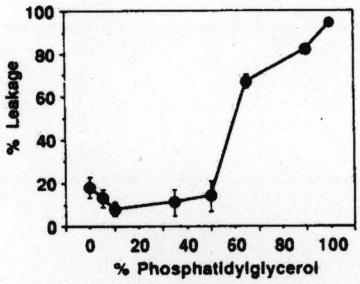


Figure 9. Effect of negative charge on liposome leakage in 10% serum.[66]

Table II. Leakage of Mtx-γ-asp from Liposomes (phospholipid: cholesterol ratio 2:1) in the presence of 10% Newborn Calf Serum.[66]

Phospholipid	Chain	Mean % Leakage	T_m (°C)[65]
DOPG	18:1	70	-18
DLPG	12:0	84	-3
ePG	16:0 – 18:1*	94	-2
DMPG	14:0	53	23
DPPG	16:0	17	41
DSPG	18:0	17	55
ePC	16:0 – 18:1*	18	-2

*Fatty acid content: 16:0, 34.0%; 18:1 31.1%; 18:2 17.7%; 18:0, 10.5%.[65]

These earlier liposome stability studies utilized lipid concentrations considerably higher than those used for drug delivery studies. Earlier work from this lab, using cholesterol containing liposomes, has shown that when performing leakage studies at concentrations relevant to drug delivery, liposomes prepared from low phase transition temperature lipids leak their contents extensively in the presence of serum. High phase transition temperature lipids, such as DSPG and DPPG, were far more stable than the low phase transition temperature lipid ePG (Table II). Additionally, ePG has no effect on serum induced leakage when 50 mol/ 100mol of phospholipid or less is present in the liposome bilayer (Figure 9). When specific lipoproteins were studied, leakage of Mtx-γ-asp in ePG liposomes was greatest in the presence of LDL (Table III) at concentrations similar to those found in serum.[66] These results using lipid concentrations seen in drug delivery studies implicated LDL as a factor in liposome stability. The fortuitous behavior of ePG liposomes to exhibit an interaction with LDL, though resulting in leakage of liposomal aqueous contents, raises the possibility that this ePG liposome-LDL interaction can be utilized to deliver a pharmaceutical cargo to cells via the LDLr. Lundberg *et al.* have covalently attached

Table III. Leakage of Mtx-γ-asp from Liposomes (phospholipid: cholesterol ratio 2:1) in the presence of Lipoproteins and Apolipoprotein A-I.[66]

	Mean % Leakage		
Phospholipid	HDL	LDL	Apo A-I
ePG	29	73	25
DOPG		57	
DPPG	10	16	
ePC	13	20	19

apoB to liposomes for this purpose, but the spontaneous interaction with anionic vesicles suggested by the leakage data (Table III) is a simpler means to achieve this end.[43]

8. Lipoprotein Interactions with Liposome Based Formulations

Upon entering the bloodstream, parenterally administered drugs associate with various plasma proteins. Typically, most studies limit this association to proteins such as albumin or glycoproteins neglecting the role of lipoproteins. Since lipoproteins are involved in liposome stability and the composition and content of lipoproteins is frequently altered in disease states it becomes imperative to examine the association of lipoproteins with liposome based drug formulations. Resulting lipoprotein interactions can have a profound impact upon the pharmacological action of the drug.

Amphotericin B (AmpB) is an antifungal agent frequently encapsulated in liposomes in an attempt to reduce dose dependent renal toxicity associated with the free drug. Wasan *et al.* showed that cholesterol free anionic liposomes (DMPC:DMPG, 7:3 [wt/wt]) encapsulating AmpB associated with the HDL fraction of human serum.[67] It has also been observed that cellular uptake of free AmpB is via the LDLr.[68,69] Reduced nephrotoxicity of liposomal AmpB may be due to the lack of high affinity HDL receptors in kidney tissue. Conversely, this tissue is rich in LDL receptors leading to the toxicity of the free drug.[70] By properly selecting your liposome formulation you may achieve favorable alterations in pharmacological response hence yielding a more desirable product.

As previously mentioned, the incorporation of cholesterol into liposomes impacts the stability of the drug formulation. The addition of cholesterol also leads to the enhancement of liposomal interactions with LDL. Thus, by changing the physico-chemical properties of a liposomal preparation through cholesterol addition it is possible to regulate its interaction with serum lipoproteins.[71] Additionally, cholesterol containing liposomes made from high phase transition temperature lipids interacted with fewer serum proteins than those liposomes made from low phase transition temperature lipids.[72] The association of cholesterol containing liposomes, made from low phase transition temperature lipids, with LDL possibly allows for a natural targeting of such drug formulations to various cancer cells rich in LDL receptors.

9. Statement of the Purpose

Anionic liposomes have long been proposed as ideal carriers for the delivery of drugs and other macromolecules. Fundamental to the practical development of anionic liposomal formulations for use in drug delivery is the characterization of the cellular mechanisms involved in their uptake. The research presented in this thesis examines how the characteristics of anionic liposomal formulations impact the cellular processes involved in their uptake.

Association of liposomal aqueous contents and liposomal lipid was measured using CV1-P and CHO wild type cells, which both express the LDLr, and CHOldlA7, which lacks the LDLr. Additionally, CHOldlA7 was transfected with the human LDLr and further association studies were performed using the anti-LDLr and anti-apoB100 monoclonal antibodies, IgG C7 and IgG 5E11 respectively. Liposomes containing 75-100 mole percent ePG were found to utilize the LDLr for cellular association and for the functional delivery of a series of model liposome dependent drugs. Findings indicate that LDL and LDLr are not involved in the interaction of 25-50 mole percent ePG vesicles with cells. An attractive strategy would be to utilize the 75-100 mole percent ePG vesicles for targeted drug delivery to highly proliferative cancer cells known to over express the LDLr. .

CHAPTER TWO: MATERIALS AND METHODS

1. Reagents

Egg phosphatidylglycerol (ePG), egg phosphatidylcholine (ePC), dipalmitoylphosphatidylglycerol (DPPG) and distearoylphosphatidylglycerol (DSPG) were obtained from Avanti Polar Lipid (Birmingham, AL) and stored as a chloroform solution at -20°C under argon gas in sealed ampules. Cholesterol, methotrexate (Mtx), fluoroorotic acid (FO), folic acid, reduced nicotinamide adenine dinucleotide phosphate (NADPH) and dithiothreitol (DTT) were obtained from Sigma Chemical Co. (St. Louis, MO).[73] N-phosphonacetyl-L-aspartic acid (PALA) was provided by Dr. V.L. Narayanan of the Division of Cancer Treatment, National Cancer Institute.[74] Methotrexate-γ-aspartate (Mtx-γ-asp) was a generous gift from Dr. J. R. Piper, Southern Research Institute.[75] All other reagents for cell culture were obtained from Sigma and all solutions for cell culture were prepared in endotoxin free water.

2. Preparation of Dihydrofolic Acid

Dihydrofolic acid (DHF) was prepared from folic acid by the method of Blakley.[76] Ultrapure water was degassed and purged with argon gas. This water was subsequently used to prepare 5 mM HCl and 1 mM HCl, both of which were kept cold. Additionally, 12 ml of this degassed, argon purged water was used to dissolve 9 g of ascorbic acid followed by slow pH adjustment to 6.0 with NaOH. A folic acid solution was prepared by dissolving 170 mg folic acid into approximately 7 ml of 0.1N NaOH. The pH of this solution was increased by addition of 1N NaOH, until all folic acid had dissolved, but not

allowing the pH to exceed 7.6. Folic acid solution was mixed with almost half of the ascorbic acid solution and to this solution 1.8 g of sodium dithionite was added. An ice bath was placed around the beaker until the temperature fell below 5°C. Using a burette, 1N HCl was added at 0.1 ml/min with stirring at 0-5°C until the pH fell below 2.8. The solution was stirred for an additional 5 min to allow complete precipitation. The precipitate was recovered by centrifugation for 5 min, 4°C, 2500 rpm in a Beckman GPR centrifuge. The heavy white precipitate was dissolved in the remaining cold ascorbic acid solution, placed in an ice bath, adjusted to pH 6.0 with 1N NaOH, and stirred at 0°C for 5 min. The dissolved DHF was crystallized from the solution by slowly adding 1N HCl at a rate of 4 drops/min. The crystallization was complete 5 min after reaching pH 2.8 and the white suspension was centrifuged to isolate the crystals. The precipitate was resuspended in 100 ml cold 1 mM HCl centrifuged, resuspended, and centrifuged one last time. The final pellet was resuspended in 70 ml degassed cold 5 mM HCl, stored in 1 ml aliquots, purged with argon gas, and stored at –70°C until used.[77]

To check the purity of DHF, one frozen microtube containing DHF in 5 mM HCl was thawed in cold water. DHF was pelleted by spinning for 60 sec in a Tomy HF-120 microcentrifuge. The supernatant was discarded and 1 ml of cold 0.1 M phosphate buffered solution, pH 7.4, 10 mM DTT, 290 mmol/kg was added to dissolve the pellet. The dissolved DHF was then transferred to a 15 ml centrifuge tube. The microtube was then rinsed with an additional 1 ml of the cold 0.1 M phosphate buffered solution and this solution was also placed in the 15 ml centrifuge tube. The absorbance between 220 nm and 400 nm of a 1:71 dilution of DHF was taken with a Hitachi U3000

Spectrophotometer (Figure 10). A peak which appears at 280 nm, then followed by a shoulder around 300 nm is indicative of pure DHF. Contamination with folic acid will be detected if a peak is observed at 370 nm.[76] For liposome-cell association studies the concentration of DHF was adjusted to 200 μM (ε^{282nm} = 22400 $cm^{-1} M^{-1}$) by addition of the previously mentioned 0.1 M phosphate buffered solution and used on the day of preparation.

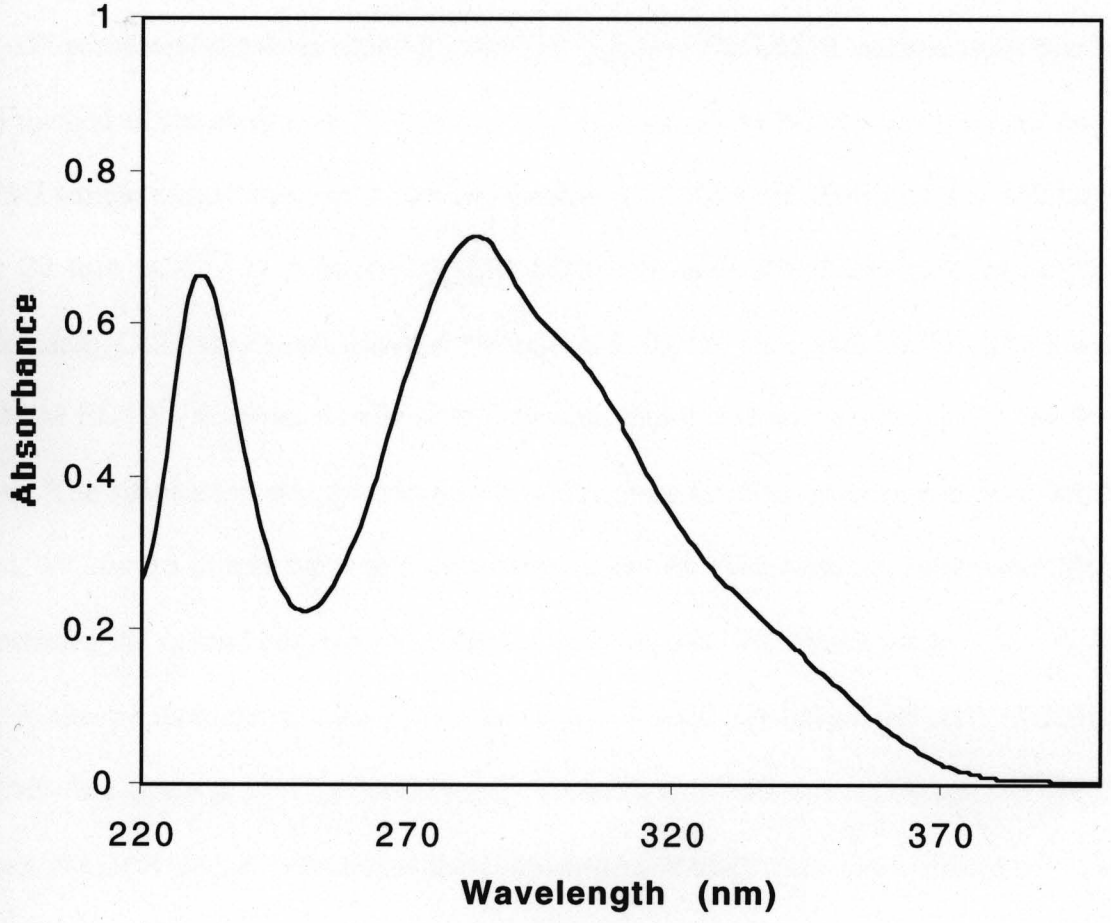


Figure 10. Absorption spectra of 32 μM solution of DHF in 0.1 M phosphate buffered solution, pH 7.4, 10 mM DTT, 290 mmol/kg.

3. Isolation of DHFR from lymphoma cell line L1210

Dihydrofolate reductase (DHFR) was isolated from the L1210 leukemia cell line by the method of Sirotnak *et al.*[78] Briefly, cells were grown to 2-3 million cells per ml in RPMI supplemented with 10% fetal bovine serum. Cells were centrifuged at 1500 rpm for 20 min at 4°C in a Beckman GPR centrifuge after which the supernatant was discarded. Cells were resuspended at 10^8 cell/ml in 0.01M phosphate buffer, pH 6.8 with 0.001M EDTA. Suspended cells were then sonicated in 1-2 ml aliquots at 4°C for 5-10 min. The suspension was centrifuged in a Beckman L5-50B ultracentrifuge at 25000 rpm, 4°C for 15 min in order to remove cellular debris. The resultant supernatant fluid containing the desired enzyme was collected and stored at –70°C until used.

A spectrophotometric determination (Hitachi U3000 Spectrophotometer) of DHFR activity was made at 37°C by following the decrease in absorbance at 340 nm that occurs when NADPH and dihydrofolate are converted to $NADP^+$ and tetrahydrofolate. The overall molar absorption coefficient for the reaction was taken as 12000 cm^{-1} M^{-1} as reported by Hillcoat *et al.*[79] The assay mixture contained 0.1M pH 7.4, 290 mmol/kg phosphate buffered saline, 150 μM NADPH, and 100 μM DHF. Approximately 5-10 μL of the L1210 enzyme solution was used to initiate the reaction. One unit of enzyme activity is defined as the amount which catalyzes the reduction of 1 μmole of dihydrofolate per min.[77]

4. Lipoprotein Isolation

LDL and HDL were isolated from fresh bovine blood, obtained from the Meat Science Laboratories (UW-Madison), by the method of Burstein *et al.* utilizing 4% citric acid (tripotassium salt, Sigma) as anticoagulant.[80] In some instances to prevent degradation of apolipoproteins, thimerosal (0.1 mg/ml), sodium azide (0.2 mg/ml), EDTA (1.0 mg/ml), and phenylmethanesulfonyl fluoride (0.17 mg/ml) was added as a proteolysis inhibitory cocktail.[81,82] Blood was then centrifuged at 3000 rpm for 30 min at 20°C in a Sorvall RC-5B centrifuge. The plasma was decanted, brought to 37°C, filter sterilized, and stored for no longer than 4 days before further treatment. To 500 ml of plasma, 50 ml of a 4% sodium phosphotungstate, pH 7.6, solution and 12.5 ml 2M $MgCl_2$ (Mallinckrodt) was added, precipitating LDL and VLDL. The supernatant, labeled Supernatant I, containing the solubilized HDL was stored at 4°C and HDL was extracted within 4 days. The precipitate was centrifuged at 6000g for 10 min at 20°C and subsequently redissolved in 500 ml of 0.2% sodium citrate (Sigma). LDL and VLDL were reprecipitated upon addition of 5 g NaCl and 15 ml of 2M $MgCl_2$ and centrifuged at 6000g for 10 min in order to eliminate protein impurities. The precipitate was dissolved in approximately 15 ml 10% sodium citrate at room temperature followed by dialysis for 5 hr at 4°C against a solution containing 5% sodium citrate and 10% ammonium chloride in order to remove phosphotungstate. The isolated LDL was further dialyzed for 24 hrs at 4°C against 2000 ml of 0.02M Tris HCl/ 1% NaCl/ pH 7.7. Final LDL samples were stored at –20°C and used within 6 months.

To Supernatant I, containing HDL, 450 ml 4% sodium phosphotungstate was added followed by centrifugation at 6000g for 10 min. The resultant precipitate was discarded and 87.5 ml of 2M $MgCl_2$ was added to the supernatant followed by centrifugation at 20000g for 30 min. The supernatant was decanted and the precipitate was washed with 250 ml of a solution containing 1% NaCl, 0.4% sodium phosphotungstate and 0.1M $MgCl_2$. The precipitate was recovered by centrifugation at 6000g for 10 min and resuspended in 40 ml of 1% NaCl. Approximately 10 ml of 10% sodium carbonate was added to dissolve the precipitate, yielding a concentrated HDL solution. Further purification was done by adjusting the density to 1.22 g/ml, by adding 10g NaCl and 23g KBr (Sigma) and bringing the volume to 100 ml. Ultracentrifugation of the concentrated HDL solution was performed at 105000g (35000 rpm) for 24 hrs at 4°C. HDL was dialyzed against 0.02M Tris HCL/1% NaCl/pH 7.7 at 4°C for 24 hrs. Final LDL samples were stored at –20°C and used within 6 months. Protein concentration of LDL and HDL was determined using a Bradford reagent (BioRad, Melville, NY).[83] Defined medium was supplemented with lipoprotein at concentrations relevant to those normally found in serum.

LDL characterization was performed utilizing nondenaturing polyacrylamide gel electrophoresis (PAGE) on a 5% gel based on the method of Nichols *et al.*[84] Plasma lipoproteins encompass a wide range of densities, molecular weights and particle sizes. Separation by nondenaturing PAGE is based upon the difference in migration in a polyacrylamide gel for particles with different sizes, such as HDL and LDL (Table I). For particle size calibration, standards were run which included apoferritin (Sigma) and

thyroglobulin (Sigma) (diameters 12 nm and 17 nm respectively). Approximately 2.5 μg of each of the above standard proteins was applied to the gel. Samples were mixed with loading buffer (0.5M Tris HCl, pH 6.8, 25% glycerol, 0.02% bromophenol blue) and aliquots (10-15 μl) were applied to lanes. Gels were electrophoresed in Tris HCl (6 g/L) and glycine 28.8 g/L for up to 3 hrs at 100V using equipment supplied by BioRad. Gels were stained overnight in a solution of Coomassie blue stain (Coomassie blue stain: 600 mg Coomassie brilliant blue into a mixture containing 260 ml water, 240 ml ethanol, and 100 ml acetic acid). Destaining was accomplished by exposure of the stained gel to a destaining solution (destaining solution: 260 ml water, 240 ml ethanol, and 100 ml acetic acid). Scanning of the stained gels shows LDL to have a diameter of approximately 18 nm and greater. As expected, the particle size of the isolated LDL was greater than that of thyroglobulin and apoferritin. (Figure 11)

Characterization of LDL and HDL was also done by separation of apolipoproteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) on a 5% or 10% gel, respectively, based on the method of Ordovas.[85] Apolipoproteins display a wide range of molecular weights from 514 kD for apoB100 to 28 kD for apoA1. Approximately 3 μg of lipoprotein was mixed with sample buffer (125 mM Tris HCl, pH 6.8, 20% glycerol, 4% SDS, 0.05% bromophenol, 40 μl/ml β-Mercaptoethanol) and placed on to the gel along with a molecular weight standard (BioRad). Gels were electrophoresed in 0.25 M Tris HCl, 1.92 M glycine, 1% SDS, pH 8.3 for up to 3 hrs at 100V using equipment supplied by BioRad. Gels were stained overnight in a solution of Coomassie blue stain (Coomassie blue stain: 600 mg Coomassie brilliant blue into a

mixture containing 260 ml water, 240 ml ethanol, and 100 ml acetic acid). Destaining was accomplished by exposure of the stained gel to a destaining solution (destaining solution: 260 ml water, 240 ml ethanol, and 100 ml acetic acid). Scanning of the stained gels shows isolated LDL to be a lipoprotein containing a single protein, whose molecular weight is greater than 208 kD and is unable to enter a 5% gel. ApoB100, which is the apolipoprotein associated with LDL, has a molecular weight of 514 kD and would be unable to enter a 5% gel. (Figure 12) Additionally, scanning of the stained gels shows isolated HDL to be a lipoprotein containing a single, approximately 28 kD, protein which corresponds to the molecular weight of apoA1 the apolipoprotein associated with all classes of HDL. (Figure 13)

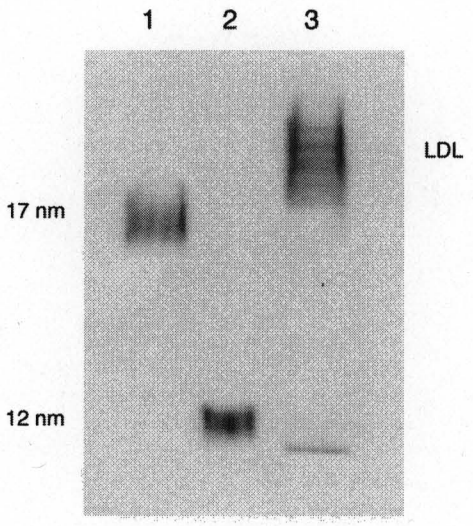


Figure 11. Nondenaturing PAGE showing the difference in migration of particle size standards thyroglobulin (lane 1) and apoferritin (lane 2) versus isolated LDL (lane 3) on a 5% gel.

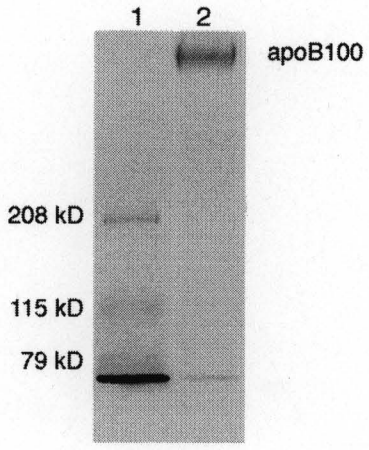


Figure 12. 5% SDS PAGE showing the major apolipoprotein in isolated LDL (lane 2). Lane 1, molecular weight markers.

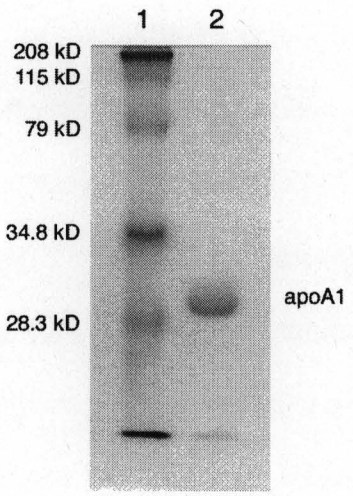


Figure 13. 10% SDS PAGE showing the major apolipoprotein in isolated HDL (lane 2). Lane 1, molecular weight markers.

5. Cell Culture

CV1-P, an African green monkey kidney cell line, was obtained from P. Berg, Stanford University, and was grown in Dulbecco's modified Eagles medium (Cellgro) containing 1g/liter glucose, supplemented with 100 units/ml penicillin/streptomycin and 10% fetal bovine serum (Cellgro).[86] Defined medium consisted of Dulbecco's modified Eagles medium supplemented with 0.456 g/l glutamine, 0.288 g/l pyruvic acid, 100 units/ml penicillin and streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, 5ng/ml sodium selenite, 1 mg/ml salt fractionated bovine serum albumin, 7.3 μg/l biotin, 0.68 mg/l vitamin B12 and 88 μg/l linoleic acid.

CHO wild type (CHOwt) and CHOldlA7, a mutant which specifically lacks the LDLr, were obtained from Dr. M. Krieger, Massachusetts Institute of Technology.[87] Both CHO cell lines were grown in RPMI 1640 (Cellgro), supplemented with 100 units/ml penicillin/streptomycin and 5% fetal bovine serum (Cellgro). Defined medium consisted of RPMI 1640 supplemented with 0.456 g/l glutamine, 0.288 g/l pyruvic acid, 100 units/ml penicillin and streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, 5ng/ml sodium selenite, 1 mg/ml salt fractionated bovine serum albumin, 7.3 μg/l biotin, 0.68 mg/l vitamin B12, 8.9 mg/l l-alanine, 14.5 mg/l proline, 0.27 mg/l thioctic acid, 0.161 mg/l putrescine hydrochloride and 88 μg/l linoleic acid.

6. Plasmids and Transfected Cell-Lines

pLDLr2, a recombinant plasmid containing a 5.3 kilobase cDNA for the human LDLr, was generously provided by Dr. Alan Attie (UW-Madison). pLDLr2 and pCDNA3 were cotransfected into CHOldlA7, a mutant CHO cell that lacks the LDLr, by the calcium phosphate precipitation technique.[88] On day one, 1.5×10^5 cells were plated onto duplicate wells of a 6 well plate containing 2 ml of RPMI 1640, supplemented with 100 units/ml penicillin/streptomycin and 5% fetal bovine serum. On day two, the medium was removed and a calcium phosphate precipitate of pLDLr-2 and pCDNA3 (5 μg) in 600 μl of Hepes buffered saline (HBS) was added to the duplicate wells. After incubation for 20 min at room temperature, 2 ml of RPMI 1640 was added to each well and the cells were incubated for a further 4 hrs at 37°C. The cells were then washed once with phosphate buffered saline (PBS), followed by a 2 min incubation at room temperature with 15% glycerol in HBS. The cells were than washed twice with PBS, placed in 2 ml of RPMI 1640 containing 5% fetal bovine serum and antibiotics. On day 4, medium was aspirated, cells were trypsinized, and replated at several dilutions ranging from 1:10 to 1:400 in selective medium containing 700 μg/ml G418 (Calbiochem). Selective medium was replaced every 2-4 days for approximately 14 days after which individual colonies were isolated. Colonies expressing functional LDL receptors, as identified by means of the liposome cell association assay, were used for further study.

7. Liposome Preparation

Large unilamellar vesicles were prepared under sterile conditions using the reverse phase evaporation (REV) technique.[9] The phospholipid:cholesterol ratio for all liposomes was 2:1, and all preparations will be referred to subsequently by the phospholipid content followed by the drug encapsulated. For ePG, the solvent was diethyl ether and sonication and ether evaporation were performed at 36°C. For DPPG and DSPG, the solvent was isopropyl ether and sonication and evaporation were performed at 55°C. The lipid content of liposomes was determined by the method of Bartlett.[89]

Mtx solution was prepared for encapsulation at a concentration of 20 mM in buffer containing 50 mM morpholinoethanesulfonic acid (MES), 50 mM N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1 mM ethylenediaminetetraacetate (EDTA), adjusted to pH 7.2 with NaOH and adjusted to 290 mmol/kg with NaCl. The tonicity of all drug solutions was measured with an osmometer (Wescor, Logan, UT). Unencapsulated Mtx and residual ether were removed by gel chromatography on a 1 X 10 cm sterile Sephadex G-50 column eluted with buffer of identical composition. After solubilizing a small portion of the liposomes in 1:3:1 chloroform:methanol:water a spectrophotometric determination of Mtx concentration was made using an extinction coefficient of 7943 M^{-1} cm^{-1} at 370 nm.

FO solution was prepared at a concentration of 50 mM in buffer containing 50 mM MES, 50 mM morpholinopropanesulfonic acid (MOPS), 2 mM EDTA, adjusted to pH 7.4 with LiOH and adjusted to 290 mmol/kg with LiCl. Unencapsulated FO was

removed by passage through a 1 X 10 cm sterile Sephadex G-75 column eluted with buffer of identical composition. After solubilizing a small portion of the liposomes in 1:3:1 chloroform:methanol:water a spectrophotometric determination of FO concentration was made using an extinction coefficient of 7100 M^{-1} cm^{-1} at 284 nm.

PALA solution was prepared at a concentration of 60 mM in buffer containing 50 mM MES, 50 mM HEPES, 1 mM EDTA, adjusted to pH 6.7 with NaOH and adjusted to 290 mmol/kg with NaCl. Calcein was also included in the PALA solution at a concentration of 2 mM and was used as an indicator of PALA encapsulation. Liposomes were separated from unencapsulated PALA by passage through a 1 X 10 cm sterile Sephadex G-75 column eluted with buffer of identical composition. After solubilizing a small portion of liposomes in 0.1% Triton X-100 the concentration of PALA was indirectly determined from the concentration of co-encapsulated calcein. Spectrophotometric determination of calcein concentration was made using an extinction coefficient of 55053 M^{-1} cm^{-1} at 493 nm.

Liposomes with gold particles were prepared by generating the colloid within preformed liposomes which had encapsulated soluble gold chloride.[36,90] A gold chloride solution consisting of 3.18 mM $HAuCl_4$, 2.5 mM K_2CO_3, 10.2 mM trisodium citrate, 55 mM NaCl, pH 6.2 was prepared. The gold chloride solution was added to 10 μmol of ePG dissolved in 1 ml of diethyl ether followed by sonication and evaporation at 36°C. Colloidal gold was formed by incubating the liposome suspension for one hour at 37°C. The unentrapped gold granules, either free or associated with the external liposome surface, were removed by passing the liposome suspension through a sterile 1 X 4 cm

DEAE-cellulose column eluted with a 5 mM HEPES, 100 mM NaCl, pH 7.0 buffer. Colloidal gold is negatively charged and will bind tightly to DEAE-cellulose. To minimize binding of negatively charged ePG, the DEAE-cellulose column was pretreated with empty ePG liposomes. Gold-liposomes were stored at 4°C under argon and used within one month. Approximately 80-90% of the liposomes prepared by this method contained gold particles.

The preparation of fluorescein labeled liposomes was done by incorporating 10 mole percent L-α-phosphatidylethanolamine-N-fluorescein (PE-fluorescein, Avanti) in the ePG lipid mixture. The solvent was diethyl ether and sonication and ether evaporation were performed at 36°C. Residual ether was removed by gel chromatography on a 1 X 10 cm sterile Sephadex G-75 column eluted with a 50 mM MES, 50 mM HEPES, 1 mM EDTA, pH 7.2 and 290 mmol/kg buffer.

8. Liposome Aqueous Content Association

Association studies were performed based on the method previously developed by Ng and Heath.[91] Cells were plated in 24 well culture plates (Corning, NY) in incubation medium at a concentration of 3 X 10^4 cells per ml, 1 ml per well, followed by overnight incubation at 37°C prior to the experiment. Triplicate wells were then treated for the indicated time with Mtx in either ePG or DSPG liposomes at a final Mtx concentration of 20 nM and final lipid concentration of approximately 200 nM. For samples tested in the defined medium supplemented with lipoprotein, liposomes were added to wells after a 30 min preincubation with lipoprotein. After incubation was complete, growth medium was

gently aspirated, and the cells were washed twice with 0.5 ml of phosphate buffered saline containing 0.36 mM $CaCl_2$ and 0.42 mM $MgCl_2$ (PBS C/M) to remove unbound liposomes and Mtx. The cells were resuspended by treatment with 0.3 ml of 0.1% trypsin and 1 mM EDTA in PBS at 37°C for 20 min, harvested and transferred to a 1.5 ml eppendorf microtube. The tubes were placed in a boiling water bath for 15 min to release the cell associated Mtx.[92]

The concentration of cell associated Mtx is determined by an enzyme inhibition assay involving the decrease in activity of L1210 DHFR in the presence of Mtx. DHFR activity is determined from the dihydrofolate dependent oxidation of NADPH at 340 nm. All reagents were dissolved in 0.1 M PBS, pH 7.4, 290 mmol/kg, 10% serum and 10 mM DTT. In all cases, 0.2 ml samples were analyzed in triplicate at the same time as a series of standards containing 0 to 0.3 pmole of Mtx. Incubation times were controlled by adding reagents to successive tubes in 4 second intervals. Freshly prepared DHFR (0.25 mU) and NADPH (60 nmol in 0.1 ml of buffer) was added followed by vortexing. Freshly prepared DHF (20 nmol in 0.1 ml of buffer) was added, the mixture was vortexed and placed in a 37°C water bath for 12 min. The reaction was stopped by adding 0.1 ml of 200 nM Mtx in buffer without serum. The absorbance of the samples was measured at 340 nm in a Beckman DU-64 spectrophotometer. The concentration of Mtx was determined by a plot of A_{340} versus Mtx concentration, the plot being linear up to 0.3 nM Mtx (Figure 14). Percent Mtx associated is expressed as the percent of Mtx originally added to the cell culture.

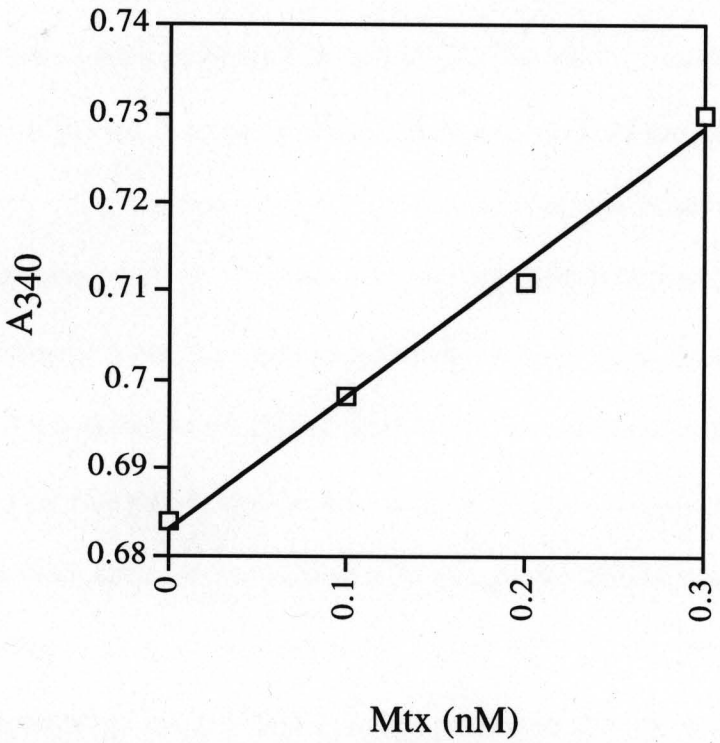


Figure 14. Standard curve for the enzyme inhibition assay involving the decrease in activity of L1210 DHFR in the presence of Mtx. The plot of absorbance at 340 nm versus concentration Mtx was linear up to 0.3 nM.

9. Liposome Lipid Association

Cells were plated in 24 well culture plates in incubation medium at a concentration of 3 X 10^4 cells per ml, 1 ml per well, followed by overnight incubation at 37°C prior to the experiment. Triplicate wells were then treated for one hour with fluorescein labeled liposomes incorporating 10 mole percent L-α-phosphatidylethanolamine-N-fluorescein (PE-fluorescein, Avanti) in the lipid mixture. A final lipid concentration of 200 nM was utilized which corresponds to the final lipid concentration in the liposome association studies. For samples tested in the defined medium supplemented with lipoprotein, liposomes were added to wells after a 30 min preincubation with lipoprotein. The cells were resuspended by treatment with 1.0 ml of 0.1% trypsin and 1 mM EDTA in phosphate buffered saline (PBS) at 37°C for 20 min, harvested and lysed with 100 µl of 10% (w/v) deoxycholate. Fluorescein fluorescence was determined (excitation/emission maxima 497/521 nm) using a Hitachi F-3010 fluorescence spectrophotometer. The concentration of lipid was determined by a plot of fluorescence intensity versus lipid concentration (Figure 15). Percent lipid associated is expressed as the percent of lipid originally added to the cell culture.

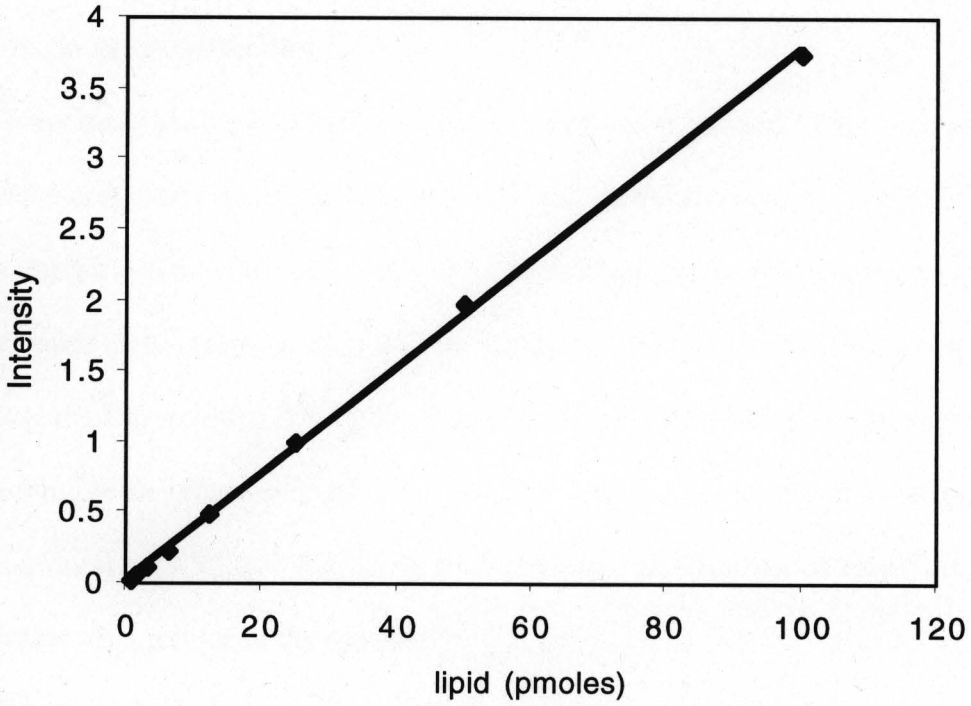


Figure 15. Lipid Association Standard Curve for ePG Liposomes. The linear plot of intensity as a function of lipid concentration allows the determination of liposomal lipid associated at the pmole level.

10. Monoclonal Antibodies

Monoclonal antibodies (Mab) that interact with apoB100 and LDLr were selected for use in the previously described liposome and lipid association assays. Mab 5E11 (Ottawa Heart Institute Research Corporation, Ottawa, Canada) binding has been mapped to amino acids 3441-3569 on apoB100, and binding of 5E11 to LDL is known to inhibit binding of LDL to LDLr.[93,94] IgG C7, provided by Dr. K. Wasan (University of British Columbia), is an extensively studied anti-LDLr Mab. C7 is known to bind to the ligand binding domain of LDLr (Figure 8), thus inhibiting the binding of apoE and apoB100 containing lipoproteins to the receptor.[95]

For association studies utilizing 5E11, liposomes were first preincubated with LDL allowing for the formation of the putative liposome-LDL complex. 5E11 was subsequently added to liposomes preincubated with LDL, at a ratio of 20 μg 5E11:100 μg LDL, for 2 hrs at 4°C. All association studies with the preformed liposome-LDL-5E11 cocktail were performed as previously described at 37°C.

Association studies utilizing C7, were conducted at 4°C based on methods previously described by Goldstein *et al.*[96] Briefly, on the day of the experiment cells were exposed to 0.5 μg/ml C7 for 1 hr at 4°C followed by 1 hr, 4°C exposure to liposomal Mtx or fluorescein labeled liposomes. After liposomal exposure, samples were assayed for liposome and lipid association assays as previously described.

11. Scanning Electron Microscopy Studies

Cell monolayers for electron microscopy studies were grown on glass cover slips in 24 well culture dishes. CHOwt and CHOldlA7 cells were plated at 3 X 10^4 cells per ml, 1 ml per well, and allowed to incubate overnight. Following exposure to ePG-Au liposomes for varying amounts of time, cells were fixed with 2.5% glutaraldehyde in PBS for 30 min then washed with PBS. Cells were then postfixed sequentially with 1% osmium tetroxide in PBS for 30 min and 1% tannic acid in PBS for 30 min. Cells were then dehydrated in increasing concentrations of ethanol. Once in 100% ethanol, samples were dried by the critical-point procedure. Dried samples were coated via ion beam sputtering with a 1-2 nm ultrathin layer of platinum and examined at 10Kv accelerating voltage in a field emission, in-lens, high-resolution scanning electron microscope, Hitachi S-900, at the University of Wisconsin Microscopy Resource, UWMR, UW-Madison.

12. Fluorescence Microscopy Studies

Cell monolayers for fluorescence microscopy studies were grown on glass cover slips in 24 well plates. CHOwt and CHOldlA7 cells were plated at 3 X 10^5 cells per ml, 1 ml per well, and allowed to incubate overnight. Following exposure to ePG-fluorescein liposomes for varying amounts of time, cells were washed twice and viewed in PBS C/M using a Nikon Diaphot inverted light microscope. Epifluorescence and transmitted light images were obtained using a Photometrix PXL cooled CCD camera. Digital images were collected and archived using Metamorph/Metaflour imaging software.

Fluorescence images and transmitted light wide field images were overlaid using Adobe Photoshop 5.0.

13. Growth Inhibition Studies

CHOwt and CHOldlA7 cells were plated at 3 X 10^4 cells per ml, 1 ml per well, in 24 well plates. Following overnight incubation at 37°C, the cells were treated in triplicate with 10 µl of drug from a half logarithmic dilution series. Three wells were used to obtain the original cell count and three wells were treated with 10 µl of buffer. The cells were allowed to grow for 48-72 hours, the medium was aspirated, the cells were trypsinized, harvested and counted in a Coulter model ZM counter. In wash studies, cells were freed of drug after 1-24 hours of incubation and then returned to culture in medium without drug for the remainder of the 48 hour growth period. The concentration of drug that inhibits the cell count by 50% (IC_{50}) was determined from a plot of percent growth versus drug concentration.

CHAPTER THREE: STUDIES IN DEFINED MEDIUM

1. Introduction

Comiskey *et al.* have demonstrated that cholesterol containing liposomes, at concentrations relevant to drug delivery, leak their contents in the presence of serum. Leakage occurs for liposomes prepared from low phase transition temperature anionic lipids, such as ePG, but not for high phase transition temperature lipids, such as DSPG and DPPG, or for neutral phospholipids. Additionally, serum caused liposome leakage only when 75-100 mole percent of the phospholipid was anionic. When specific lipoproteins were studied at their normal serum concentrations, leakage from ePG liposomes was greatest with LDL suggesting that LDL is the major cause of serum-induced leakage.[66] The fortuitous behavior of ePG liposomes to exhibit an interaction with LDL raises the possibility that this ePG liposome-LDL interaction can be utilized to deliver a pharmaceutical cargo to cells via the LDLr. Therefore, in the study presented in this chapter I have chosen to investigate the association and potency of various anionic liposomal formulations in a chemically defined, lipoprotein and apolipoprotein free growth medium in an attempt to understand the role of the LDLr in targeted drug delivery mediated by anionic liposomes.

2. Results

The results of liposome-cell association studies in defined medium utilizing (100 mole percent) ePG encapsulated Mtx are summarized in Table IV. The amount of association observed for incubation times between 10 and 60 minutes was quite constant with no specific trend during the 60 min period studied. Consequently, Table IV shows

the average amount associated for incubation periods up to 60 minutes. The addition of 0.1 mg/ml and 1.0 mg/ml LDL yielded approximately a 4 and 5 fold increase, respectively, in Mtx association in the CV1-P cell line. Similarly, the addition of 0.1 mg/ml and 1.0 mg/ml LDL yielded approximately a 6 and 7 fold increase, respectively, in association of Mtx with the CHOwt cell line. Conversely, the addition of LDL at either concentration had a negligible effect upon Mtx association with the CHOldlA7 cell line. The addition of HDL at either concentration did not have any substantial impact upon cellular association of Mtx with the CHOwt and CHOldlA7 cells. However, HDL caused nearly a two fold elevation of ePG liposome association by CV1-P cells. The heightened increase in ePG-Mtx association in the presence of LDL supports the theory that ePG liposomes utilize the LDLr and this route is directly responsible for the increased uptake seen in the wild type and CV1-P cell lines.

The results of liposome-cell association studies in defined medium utilizing DSPG encapsulated Mtx are also summarized in Table IV. Association was rapid and fairly constant over the incubation time with no specific trend during the 60 min period studied. Association of DSPG-Mtx to cell surface components is more extensive than the association observed with ePG-Mtx. However, the addition of LDL at either concentration tested did not enhance cellular association of DSPG-Mtx in any of the cell lines tested. Association was comparable in both CHO cell lines indicating that these liposomes do not utilize the LDLr for uptake. Addition of HDL did not significantly alter uptake in the CHOwt and CV1-P cells but the presence of HDL at either concentration eliminated all detectable uptake in the mutant cell line. Taken as a whole the DSPG

association studies imply that these liposomes do not extensively employ the LDLr for uptake but that uptake in the mutant cell line exists via an alternative pathway that is inhibited by HDL.

Liposome-cell association studies correlating the uptake of liposomal aqueous contents as a function of anionic phospholipid content are shown in Figures 16-18. In defined medium, for all cell lines tested, there was a low level of uptake for all formulations with at least 25 mol/100 mol ePG (Figure 16). Preparations with 0 mol/100 mol anionic phospholipid content displayed negligible amounts of Mtx uptake for all cell lines tested. The addition of HDL 1.0 mg/ml did not significantly alter the uptake of ePG-Mtx, regardless of anionic phospholipid content, for either of the CHO cell lines (Figure 17B). In contrast, CV1-P displayed increasing levels of uptake, upon addition of 1.0 mg/ml HDL, as anionic content was increased. Similar results to those obtained with 1.0 mg/ml HDL were seen for all cell lines with the addition of 0.1 mg/ml HDL (Figure 17A). The enhanced uptake of 100 mole percent ePG-Mtx upon addition of LDL 1.0 mg/ml seen in CHOwt and CV1-P also existed for formulations with at least 75 mole percent ePG, but did not occur for liposomes with lower ePG content (Figure 18B). Similar observations were made for these two cell lines with LDL 0.1 mg/ml (Figure 18A). CHOldlA7 did not show an LDL mediated enhancement of uptake for any of the charge ratios tested.

As the previous studies monitored the fate of liposomal aqueous contents, equivalent lipid association studies were conducted to monitor the fate of liposomal lipid. In defined medium, for all cell lines tested, there were low levels of uptake for all preparations

(Figure 19). The addition of HDL 0.1 mg/ml (Figure 20A) or 1.0 mg/ml (Figure 20B) did not alter the lipid association properties in any of the cell lines. However, the addition of LDL 1.0 mg/ml resulted in a substantial increase in lipid association for 100 mole percent (25 fold increase) and 75 mole percent (23 fold increase) ePG vesicles in CV1-P (Figure 21B). Comparable increases in lipid association were seen in CHOwt but not in CHOldlA7. Parallel studies utilizing LDL 0.1 mg/ml yielded similar findings (Figure 21A). Consistent with cell association studies (Table IV), the addition of LDL at either concentration tested did not enhance lipid association of DSPG vesicles in any of the cell lines tested (Table V). Liposomal lipid association for 75-100 mole percent ePG in the presence of LDL is much higher than liposome content association. This is quite consistent with prior observations showing that LDL induces leakage of contents for these two compositions. Based on the difference between contents and lipid association, I estimate the leakage to be 89% and 94% for 75 and 100 mole percent ePG respectively. These numbers closely agree with prior measurements of leakage.[66]

The growth inhibitory effects of ePG encapsulated PALA were determined in the presence of lipoproteins (Table VI). The addition of LDL 0.1 mg/ml and 1.0 mg/ml yielded approximately a 7 and 5 fold increase, respectively, in potency in the CV1-P cell line. Similarly, the addition of LDL 0.1 mg/ml and 1.0 mg/ml enhanced potency approximately 3 and 6 fold, respectively, in the CHOwt cell line. The addition of LDL at either concentration led to approximately a two fold decrease in potency on the CHOldlA7 cell line. The addition of HDL did not augment the potency of ePG-PALA for any of the cell lines tested. In the CHOldlA7 and CV1-P cell lines the addition of

HDL actually led to a decrease in potency of the ePG-PALA formulation. Growth inhibition studies utilizing ePG encapsulated FO or Mtx-γ-asp produced similar findings, whereby LDL addition led to enhanced potency of ePG formulations on CHOwt and CV1-P but not on CHOldlA7 and HDL addition caused either minimal effect or a decrease in growth inhibition.

The growth inhibitory properties of DSPG encapsulated PALA were also determined in the presence of lipoproteins (Table VII). The addition of LDL at either concentration did not result in the potency enhancement seen for the ePG formulations. In fact the DSPG formulation exhibited a decrease in potency (CHOwt) or negligible effect (CV1-P) upon the addition of LDL to the growth medium. Similar experiments with DPPG encapsulated FO resulted in analogous findings. As with the ePG formulations, the DSPG preparation showed decreased potency upon lipoprotein addition in the CHOldlA7 cell line. Upon HDL addition at either concentration, DSPG encapsulated PALA exhibited no change in CHOwt or a decrease in potency as observed with CV1-P. Similar findings were observed in experiments with DPPG encapsulated FO.

Figures 22-24 display results from growth inhibition studies comparing potency with the anionic phospholipid content of ePG vesicles. The LDL mediated enhancement in potency of the ePG-PALA preparation also occurred with vesicles containing 75 mole percent ePG for CV1-P and CHOwt. There was no LDL enhanced potency for any of the formulations on CHOldlA7. The increased liposomal uptake seen in LDL supplemented defined medium for preparations containing at least 75 mole percent ePG is consistent with the findings of these growth inhibition studies. Contrary to the LDL effect, addition

of HDL did not yield an increase in potency for any of the charge ratios tested. Parallel growth inhibition studies with the liposome dependent drugs FO and Mtx-γ-asp gave analogous findings (Figures 23 & 24).

		% Mtx Associated ± standard deviation				
Lipid	Cell Line	Control	LDL (0.1 mg/ml)	LDL (1.0 mg/ml)	HDL (0.1 mg/ml)	HDL (1.0 mg/ml)
ePG:	CV1-P	0.38 ± 0.08	1.35 ± 0.13*	2.01 ± 0.14*	0.59 ± 0.15	0.72 ± 0.13
	CHOwt	0.26 ± 0.20	1.43 ± 0.10*	1.84 ± 0.14*	0.26 ± 0.14	0.16 ± 0.16
	CHOldlA7	0.14 ± 0.19	0.12 ± 0.15	0.05 ± 0.08	0.04 ± 0.09	0.11 ± 0.11
DSPG:	CV1-P	0.61 ± 0.09	0.60 ± 0.08	0.67 ± 0.16	0.63 ± 0.18	0.63 ± 0.18
	CHOwt	0.46 ± 0.16	0.27 ± 0.14	0.32 ± 0.12	0.39 ± 0.15	0.46 ± 0.18
	CHOldlA7	0.37 ± 0.19	0.20 ± 0.15	0.20 ± 0.15	0	0

Table IV. Cellular association of ePG and DSPG encapsulated Mtx. The association of liposomal aqueous contents was determined in the presence of defined medium either alone (control), with 0.1 mg/ml LDL, with 1.0 mg/ml LDL, with 0.1 mg/ml HDL, or with 1.0 mg/ml HDL. Mtx was encapsulated in liposomes prepared from ePG or DSPG together with cholesterol at a phospholipid:cholesterol ratio 2:1. Cells were exposed to encapsulated Mtx at a final Mtx concentration of 20 nM, which corresponds to final lipid concentration of approximately 200 nM. *, $P < 0.01$ compared to control.

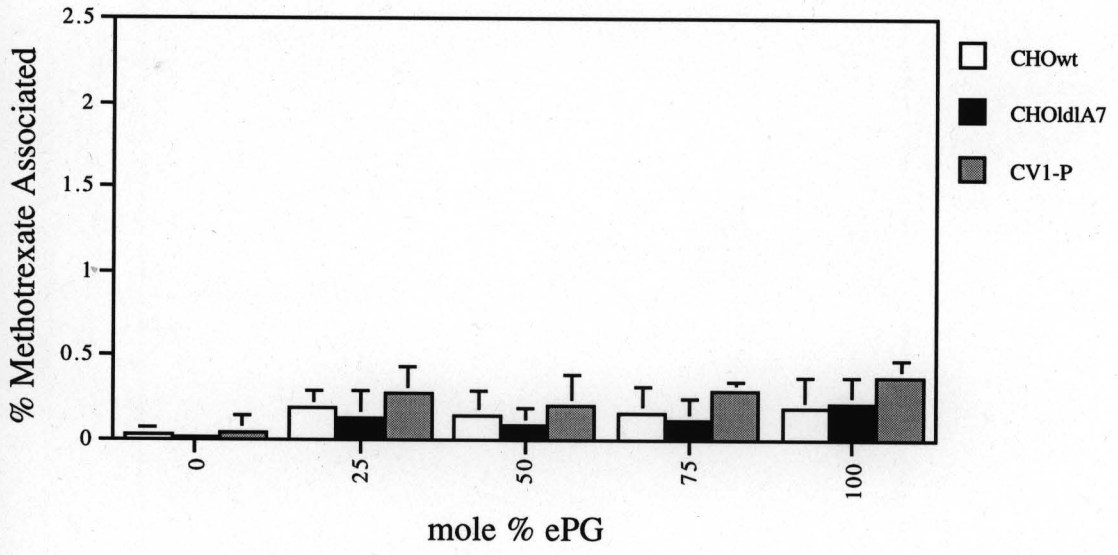


Figure 16. Cellular association of liposome contents for CHOwt (white bars), CHOldlA7 (black bars), and CV1-P (gray bars) cells as a function of anionic phospholipid content. The association of liposomal aqueous contents was determined in the presence of defined medium. Mtx was encapsulated in liposomes prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid: cholesterol ratio 2:1. Cells were exposed for 60 min to encapsulated Mtx at a final Mtx concentration of 20 nM, which corresponds to a final lipid concentration of approximately 200 nM. Each bar is the mean of three determinations ± the standard deviation.

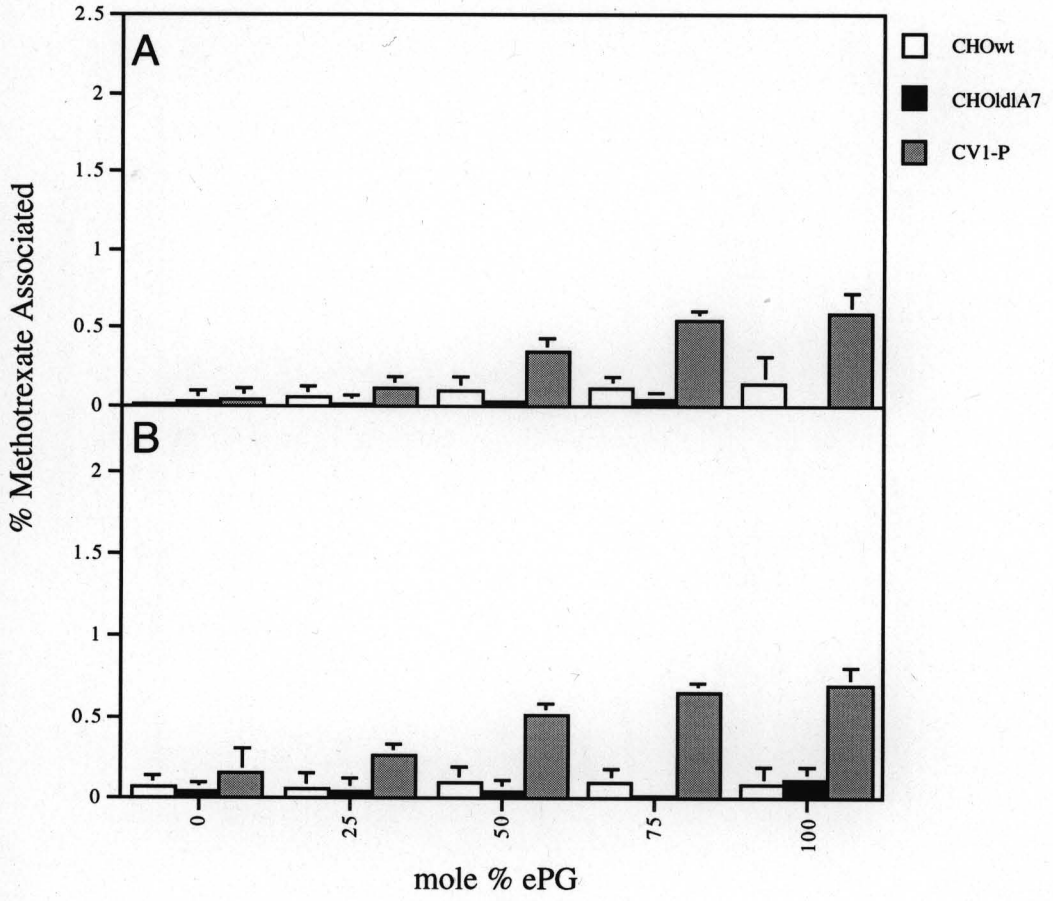


Figure 17. Cellular association of liposome contents for CHOwt (white bars), CHOldlA7 (black bars), and CV1-P (gray bars) cells as a function of anionic phospholipid content. The association of liposomal aqueous contents was determined in the presence of defined medium supplemented with either 0.1 mg/ml HDL (A) or with 1.0 mg/ml HDL (B). Mtx was encapsulated in liposomes prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid: cholesterol ratio 2:1. Cells were exposed for 60 min to encapsulated Mtx at a final Mtx concentration of 20 nM, which corresponds to a final lipid concentration of approximately 200 nM. Each bar is the mean of three determinations ± the standard deviation.

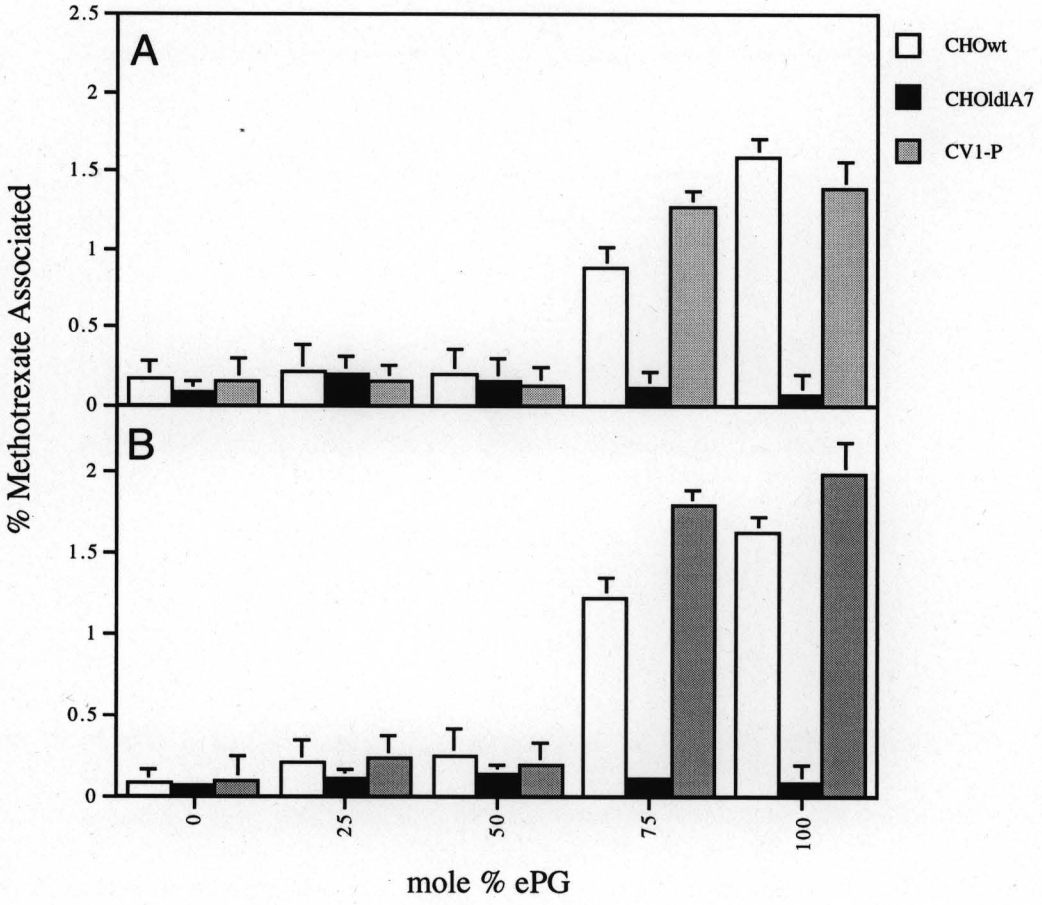


Figure 18. Cellular association of liposome contents for CHOwt (white bars), CHOldlA7 (black bars), and CV1-P (gray bars) cells as a function of anionic phospholipid content. The association of liposomal aqueous contents was determined in the presence of defined medium supplemented with either 0.1 mg/ml LDL (A) or with 1.0 mg/ml LDL (B). Mtx was encapsulated in liposomes prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid: cholesterol ratio 2:1. Cells were exposed for 60 min to encapsulated Mtx at a final Mtx concentration of 20 nM, which corresponds to a final lipid concentration of approximately 200 nM. Each bar is the mean of three determinations ± the standard deviation.

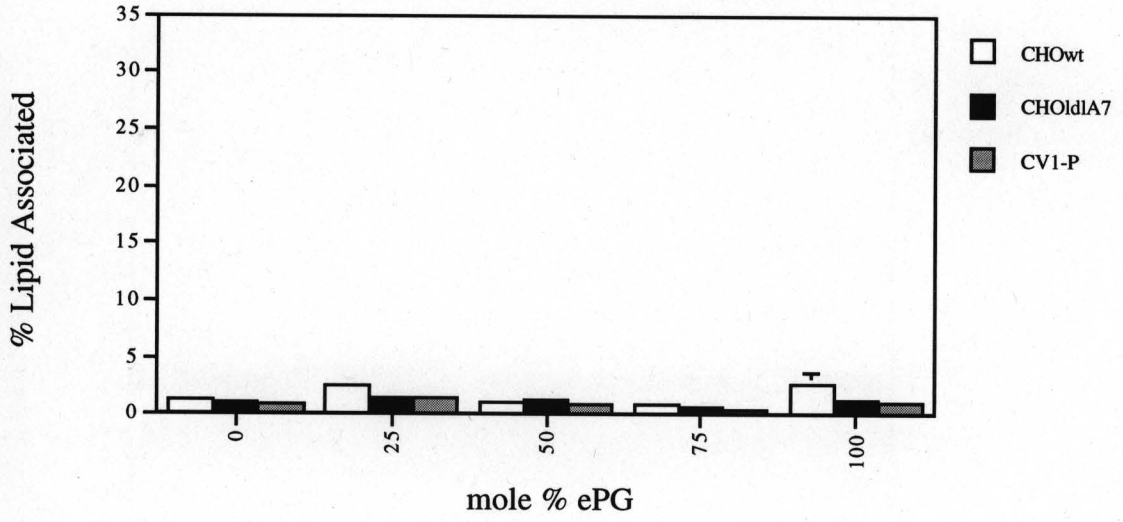


Figure 19. Cellular association of liposomal lipid for CHOwt (white bars), CHOldlA7 (black bars), and CV1-P (gray bars) cells as a function of anionic phospholipid content. The association of liposomal lipid was determined in the presence of defined medium. Fluorescein labeled liposomes were prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid: cholesterol ratio 2:1. Cells were exposed for 60 min to labeled vesicles at a final lipid concentration of 200 nM. Each bar is the mean of three determinations ± the standard deviation.

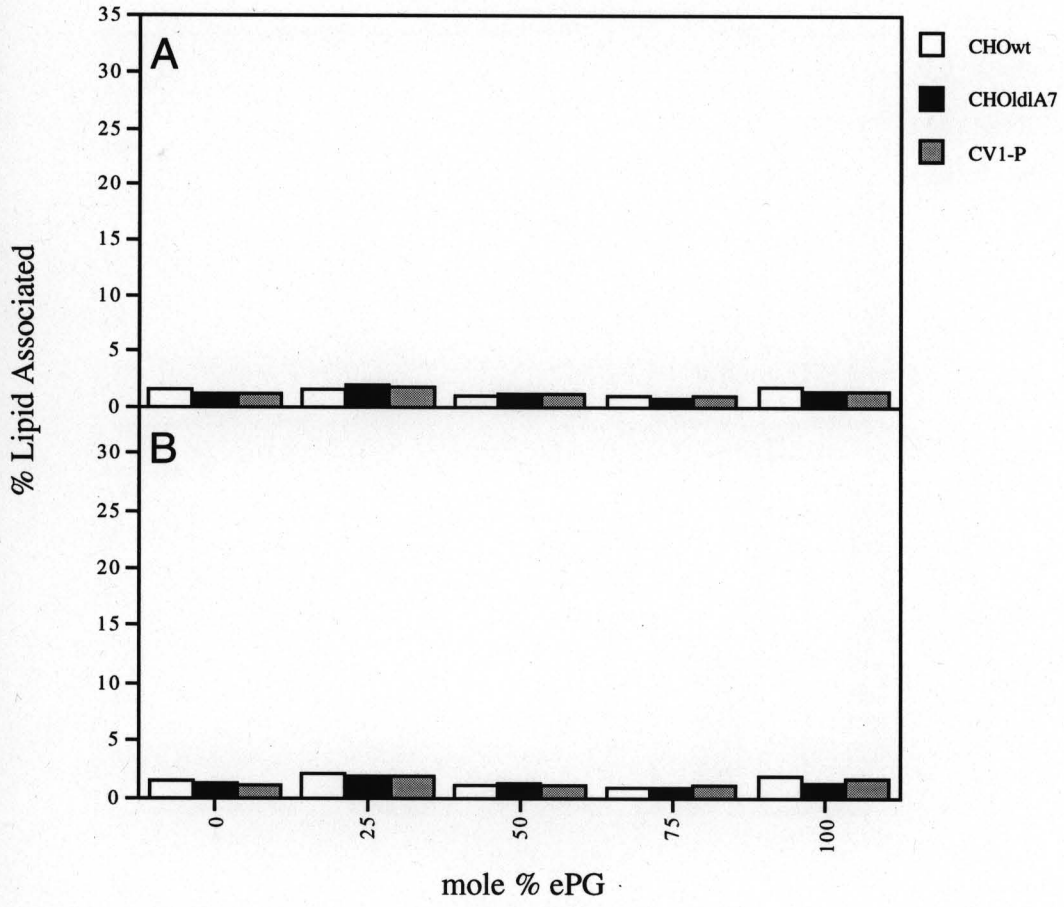


Figure 20. Cellular association of liposomal lipid for CHOwt (white bars), CHOldlA7 (black bars), and CV1-P (gray bars) cells as a function of anionic phospholipid content. The association of liposomal lipid was determined in the presence of defined medium supplemented with either 0.1 mg/ml HDL (A) or with 1.0 mg/ml HDL (B). Fluorescein labeled liposomes were prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid: cholesterol ratio 2:1. Cells were exposed for 60 min to labeled vesicles at a final lipid concentration of 200 nM. Each bar is the mean of three determinations ± the standard deviation.

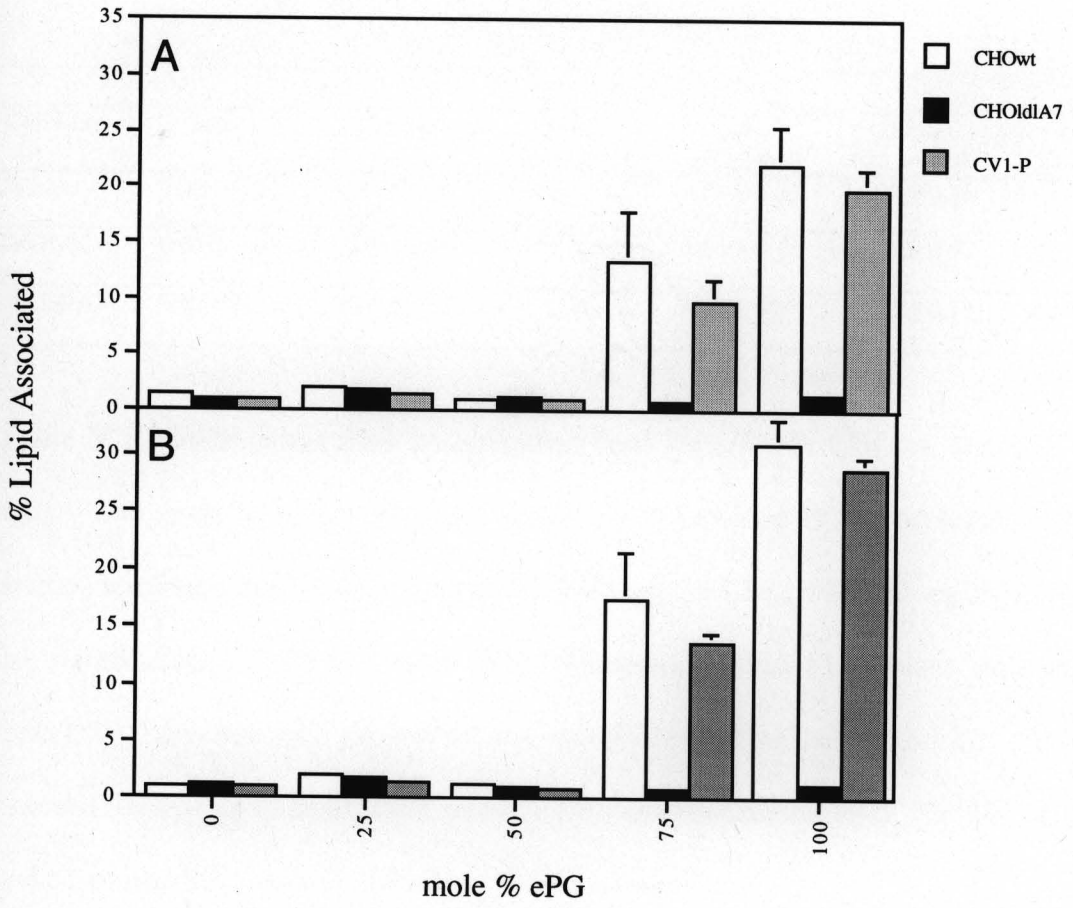


Figure 21. Cellular association of liposomal lipid for CHOwt (white bars), CHOldlA7 (black bars), and CV1-P (gray bars) cells as a function of anionic phospholipid content. The association of liposomal lipid was determined in the presence of defined medium supplemented with either 0.1 mg/ml LDL (A) or with 1.0 mg/ml LDL (B). Fluorescein labeled liposomes were prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid: cholesterol ratio 2:1. Cells were exposed for 60 min to labeled vesicles at a final lipid concentration of 200 nM. Each bar is the mean of three determinations ± the standard deviation.

	% Lipid Associated					
Cell Line	Serum	Control	LDL (0.1 mg/ml)	LDL (1.0 mg/ml)	HDL (0.1 mg/ml)	HDL (1.0 mg/ml)
CV1-P:	1.40 ± 0.10	2.35 ± 0.24	1.97 ± 0.46	1.26 ± 0.09	1.88 ± 0.26	1.64 ± 0.23
CHOwt:	0.62 ± 0.08	0.61 ± 0.08	0.64 ± 0.10	0.60 ± 0.13	0.54 ± 0.07	0.33 ± 0.04
CHOldlA7:	0.72 ± 0.15	0.86 ± 0.14	0.63 ± 0.10	0.75 ± 0.07	0.62 ± 0.10	0.38 ± 0.10

Table V. Cellular association of liposomal lipid for CV1-P, CHOwt, and CHOldlA7 cells. The association of liposomal lipid was determined in the presence of serum, defined medium either alone (control), with 0.1 mg/ml LDL, with 1.0 mg/ml LDL, with 0.1 mg/ml HDL, or with 1.0 mg/ml HDL. Fluorescein labeled liposomes were prepared from DSPG together with cholesterol at a phospholipid: cholesterol ratio 2:1. Cells were exposed for 60 min to labeled vesicles at a final lipid concentration of 200 nM. Each bar is the mean of three determinations ± the standard deviation.

		IC_{50} (μM)				
Cell Line	Drug	Control	LDL (0.1 mg/ml)	LDL (1.0 mg/ml)	HDL (0.1 mg/ml)	HDL (1.0 mg/ml)
CV1-P:	PALA	0.26 ± 0.03	0.04 ± 0.01*	0.05 ± 0.01*	0.21 ± 0.02	0.47 ± 0.13
	FO	0.86 ± 0.31	0.21 ± 0.03*	0.19 ± 0.02*	0.81 ± 0.04	0.71 ± 0.16
	Mtx-γ-asp	0.15 ± 0.04	0.06 ± 0.01*	0.04 ± 0.01*	0.15 ± 0.03	0.16 ± 0.02
CHOwt:	PALA	2.42 ± 0.22	0.88 ± 0.08*	0.38 ± 0.07*	2.55 ± 0.29	2.64 ± 0.28
	FO	4.82 ± 0.75	2.51 ± 0.64*	0.94 ± 0.45*	5.32 ± 0.61	5.30 ± 1.02
	Mtx-γ-asp	0.72 ± 0.11	0.30 ± 0.01*	0.13 ± 0.02*	0.71 ± 0.09	0.83 ± 0.06
CHOldlA7:	PALA	2.72 ± 0.23	5.50 ± 0.39	6.06 ± 0.10	3.37 ± 0.07	3.53 ± 0.14
	FO	6.00 ± 0.32	7.08 ± 0.32	6.41 ± 0.62	6.58 ± 0.57	5.44 ± 0.61
	Mtx-γ-asp	0.49 ± 0.06	0.52 ± 0.01	0.52 ± 0.01	0.55 ± 0.09	0.56 ± 0.02

Table VI. The potency of ePG encapsulated liposome dependent drugs. Growth inhibition was determined in defined medium either alone (control), with 0.1 mg/ml LDL, with 1.0 mg/ml LDL, with 0.1 mg/ml HDL, or with 1.0 mg/ml HDL. PALA/ePG lipid ratio = 0.440 mol/mol, FO/ePG lipid ratio = 0.437 mol/mol, and Mtx-γ-asp/ePG lipid ratio = 0.120 mol/mol. *, $P < 0.01$ compared to control.

		IC_{50} (μM)				
Cell Line	Drug	Control	LDL (0.1 mg/ml)	LDL (1.0 mg/ml)	HDL (0.1 mg/ml)	HDL (1.0 mg/ml)
CV1-P:	PALA	0.15 ± 0.03	0.23 ± 0.03	0.23 ± 0.05	025 ± 0.01	0.19 ± 0.06
	FO	0.74 ± 0.20	0.73 ± 0.14	1.85 ± 0.18	2.01 ± 0.53	6.50 ± 0.30
CHOwt:	PALA	1.90 ± 0.34	3.77 ± 0.41	4.49 ± 0.94	1.93 ± 0.06	1.86 ± 0.25
	FO	6.28 ± 1.13	7.25 ± 0.71	6.92 ± 0.75	5.38 ± 1.03	5.49 ± 0.54
CHOldlA7:	PALA	2.31 ± 0.18	4.72 ± 0.27	5.72 ± 0.29	4.54 ± 0.52	6.26 ± 0.18
	FO	6.57 ± 0.54	7.23 ± 0.38	7.13 ± 0.25	6.87 ± 0.66	6.43 ± 0.54

Table VII. Potency of DSPG encapsulated PALA and DPPG encapsulated FO. Growth inhibition was determined in defined medium either alone (control), with 0.1 mg/ml LDL, with 1.0 mg/ml LDL, with 0.1 mg/ml HDL, or with 1.0 mg/ml HDL. PALA/DSPG lipid ratio = 0.562 mol/mol and FO/DPPG lipid ratio = 0.451 mol/mol.

Figure 22. The potency of liposome-encapsulated PALA for CV1-P (A), CHOwt (B), and CHOldlA7 (C) as a function of anionic phospholipid content. PALA was encapsulated in liposomes prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid:cholesterol ratio 2:1. Potency was determined either in defined medium alone (□), with 0.1 mg/ml HDL (Δ), with 1.0 mg/ml HDL (▲), with 0.1 mg/ml LDL (○), or with 1.0 mg/ml LDL (●). The dotted line denotes the IC_{50} of free drug in the defined medium. Each point is the mean of three determinations ± the standard deviation. The drug lipid ratios were: 0 mol ePG/100 mol lipid, 0.434 mol/mol; 25 mol ePG/100 mol lipid, 0.735 mol/mol; 50 mol ePG/100 mol lipid, 0.452 mol/mol; 75 mol ePG/100 mol lipid, 0.412 mol/mol; 100 mol ePG/100 mol lipid, 0.440 mol/mol.

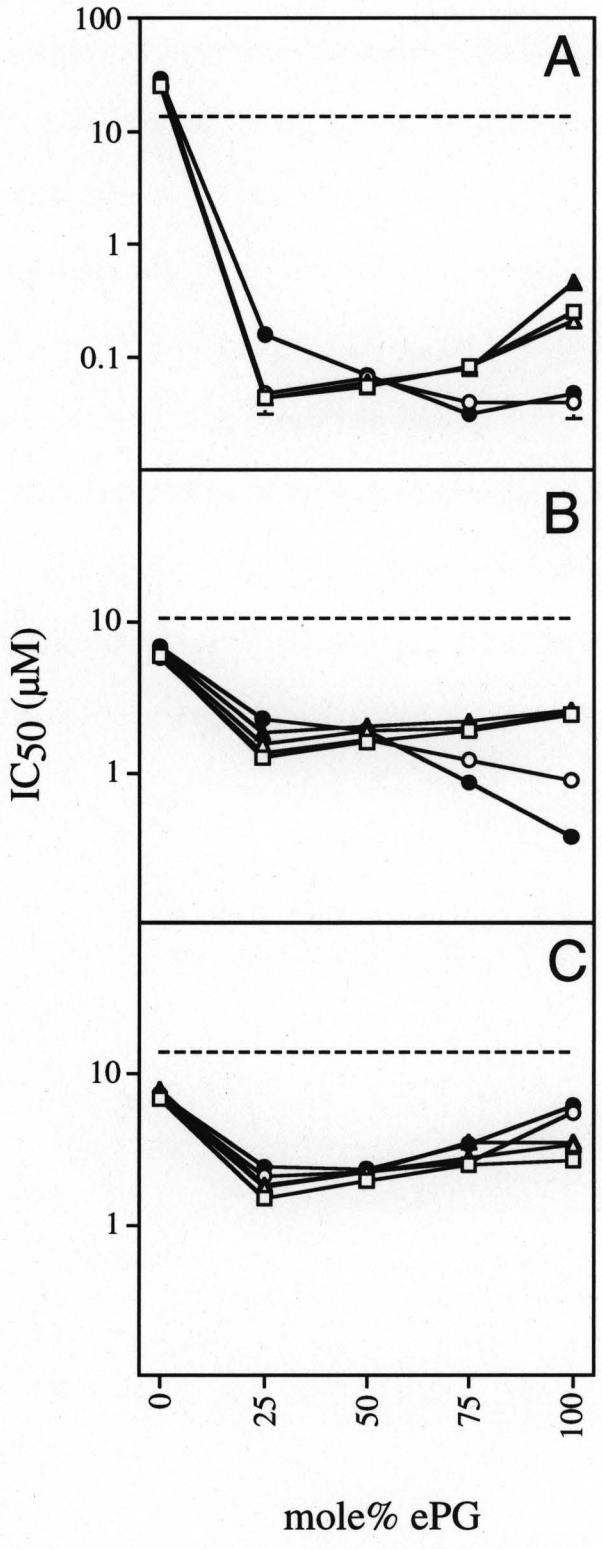

100
10
1
0.1
A
B
C
IC50 (μM)
0
25
50
75
100
mole% ePG

Figure 23. The potency of liposome-encapsulated FO for CV1-P (A), CHOwt (B), and CHOldlA7 (C) as a function of anionic phospholipid content. FO was encapsulated in liposomes prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid:cholesterol ratio 2:1. Potency was determined either in defined medium alone (□), with 0.1 mg/ml HDL (Δ), with 1.0 mg/ml HDL (▲), with 0.1 mg/ml LDL (○), or with 1.0 mg/ml LDL (●). The dotted line denotes the IC_{50} of free drug in the defined medium. Each point is the mean of three determinations ± the standard deviation. The drug lipid ratios were: 0 mol ePG/100 mol lipid, 0.607 mol/mol; 25 mol ePG/100 mol lipid, 0.673 mol/mol; 50 mol ePG/100 mol lipid, 0.547 mol/mol; 75 mol ePG/100 mol lipid, 0.689 mol/mol; 100 mol ePG/100 mol lipid, 0.437 mol/mol.

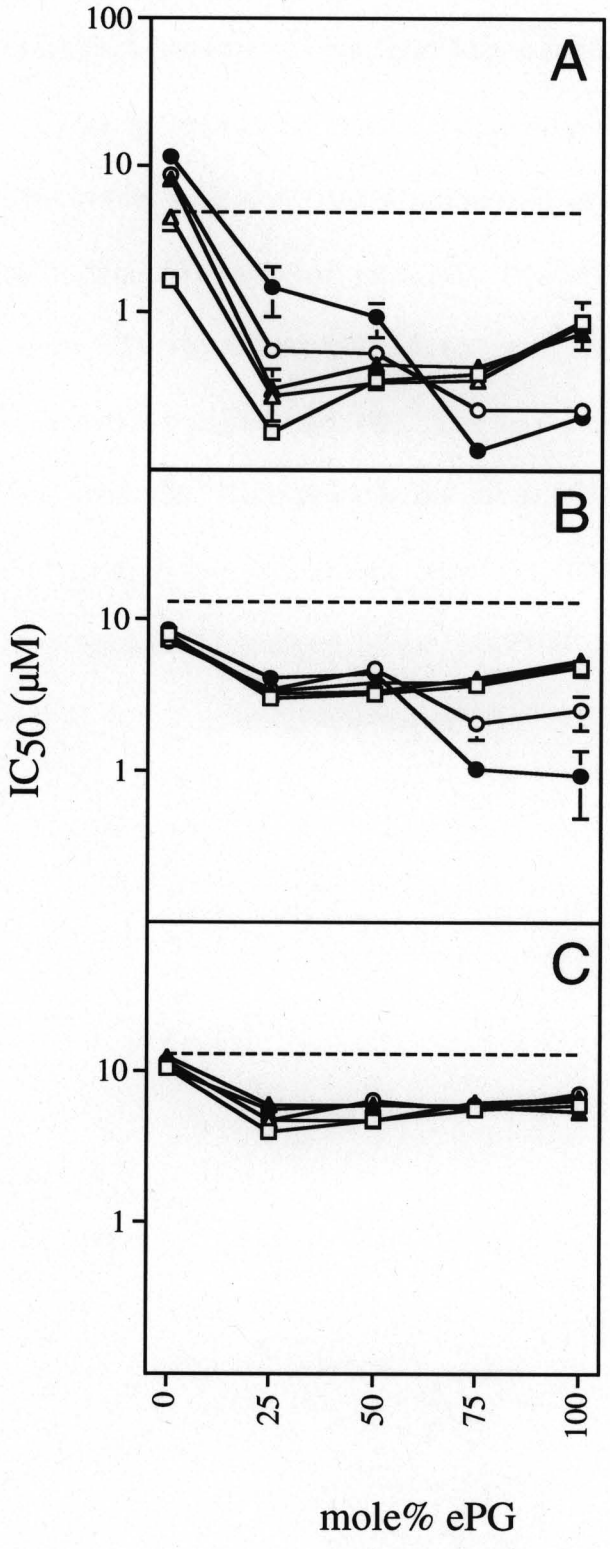
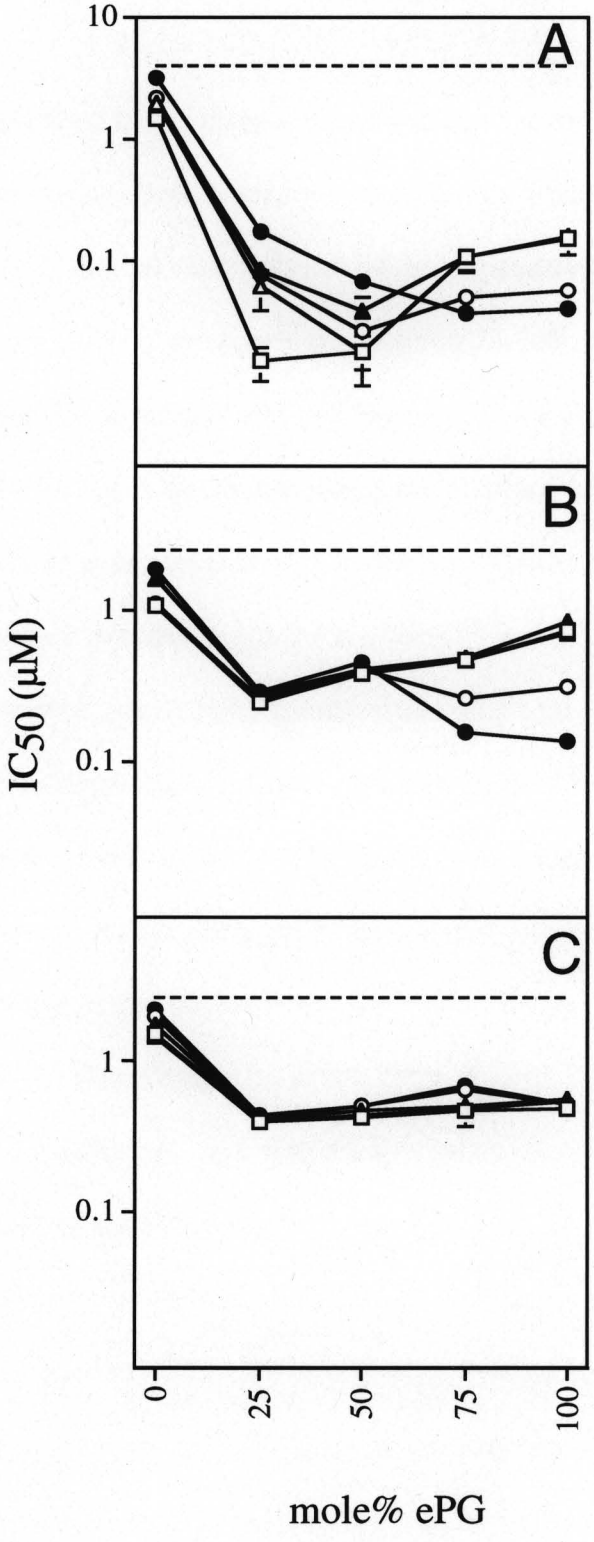

100
10
1
A
B
C
IC50 (μM)
0
25
50
75
100
mole% ePG

Figure 24. The potency of liposome-encapsulated Mtx-γ-asp for CV1-P (A), CHOwt (B), and CHOldlA7 (C) as a function of anionic phospholipid content. Mtx-γ-asp was encapsulated in liposomes prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid:cholesterol ratio 2:1. Potency was determined either in defined medium alone (□), with 0.1 mg/ml HDL (Δ), with 1.0 mg/ml HDL (▲), with 0.1 mg/ml LDL (○), or with 1.0 mg/ml LDL (●). The dotted line denotes the IC_{50} of free drug in the defined medium. Each point is the mean of three determinations ± the standard deviation. The drug lipid ratios were: 0 mol ePG/100 mol lipid, 0.241 mol/mol; 25 mol ePG/100 mol lipid, 0.151 mol/mol; 50 mol ePG/100 mol lipid, 0.145 mol/mol; 75 mol ePG/100 mol lipid, 0.132 mol/mol; 100 mol ePG/100 mol lipid, 0.120 mol/mol.


A
B
C
10
1
0.1
IC50 (μM)
0
25
50
75
100
mole% ePG

3. Discussion

The study described in this chapter documents one means by which anionic liposomes can interact with cells. Both liposome contents and lipid associate with CV1-P and CHOwt cells in a manner that appears to be dependent on the presence of LDL. Association occurs only for liposomes that contain 75-100 mole percent ePG, and is not seen with CHOldlA7, a mutant cell line known to lack the LDLr. Drug delivery to CHOwt and CV1-P cells is also enhanced by the presence of LDL, but under only those circumstances where association is also increased. These results correlate closely with earlier studies, which documented that the effects of serum on the leakage of drug from liposomes were caused primarily by an interaction with LDL. In those studies, leakage of liposome contents occurred only for liposomes prepared from low phase transition temperature anionic lipids such as ePG, and the ePG content had to be 75-100 mole percent in order to induce leakage.[66] In the present study, enhancement of both association and drug delivery also occurs only for ePG liposomes containing more than 50 mol ePG/100 mol phospholipid.

From these observations, the following hypothesis for how LDL interacts with anionic liposomes and the consequent cell interaction can be proposed:

Step 1: LDL binds to the liposomes via an electrostatic interaction that requires the presence of more than 50 mol ePG/100 mol phospholipid in the liposome bilayer. The binding of LDL to a highly anionic liposome may not involve the portion of LDL that binds to the receptor because polyanions such as heparin are known not to bind to the region of apoB100 responsible for binding to the LDLr.[97] Similarly, it is plausible that

LDL binds to these anionic vesicles in a manner such that the LDLr binding region remains accessible to the LDL receptor.

Step 2: The LDL particle coalesces with the liposome membrane, causing a substantial loss of liposome aqueous contents. This coalescence requires that the bilayer be in the liquid crystalline (fluid) state. The final product is a particle that retains an internal aqueous space and thereby retains some of the original liposome contents. Damen *et al.*, though working with neutral phospholipids, found that cholesterol containing liposomes were able to maintain their integrity in the presence of rat plasma or plasma fractions.[98]

Step 3: The resultant hybrid particle binds to the cell membrane via an interaction of apoB100 with the LDLr. The delivery of the liposome-LDL complex to the LDLr seen in our system is analogous to the findings of Greenspan *et al.* in which they demonstrate that ePG vesicles can form a complex with LDL facilitating its phagocytosis by macrophages via scavenger receptors.[99]

The observations described in this chapter are consistent with prior studies, both from this lab and from others. In their studies on the aggregation of DMPG liposomes by LDL, Lauraeus *et al.* suggested that an electrostatic interaction between the acidic phosphate of DMPG and cationic residues in apoB100 were responsible for the LDL induced aggregation of DMPG vesicles.[100] Involvement of apoB100 was implicated by the finding that tryptic digestion of LDL abrogated its ability to cause aggregation. Interestingly, aggregation of DMPG liposomes by LDL was observed in the liquid crystalline state but not in the gel state. Additionally, increasing the content of DMPC in

DMPG liposomes reduced aggregation and at 50 mole percent anionic phospholipid content no aggregation was evident.

Despite the effect of LDL upon drug delivery, it should be mentioned that substantial drug delivery effects occur that are not induced by LDL. This effect is produced by a lower level of drug binding that occurs with liposomes prepared from DSPG, and with ePG liposomes containing 25-50 mol ePG/100 mol phospholipid. The nature of this interaction or interactions remains to be characterized. However, the identification of the scavenger receptor SR-BI as an HDL receptor on the CHO cell lines provides one possible alternative pathway for the uptake seen in these studies.[101] Additionally, gp330, a major kidney membrane protein and a member of the LDL receptor gene family, may be involved in liposomal association in CV1-P cells.[102] Other members of the LDLr gene family, such as LDL receptor related protein or cubulin, may be involved with liposome association but neither of these receptors has been identified on the cell lines used here.

It should be noted that for the LDL-mediated effect, the extent of lipid binding is much greater than the extent of drug binding. This is entirely in keeping with prior leakage studies, which showed that LDL induced an instantaneous loss of 70% of the contents of ePG liposomes.[66] Hence, the leakage of contents would result in a lower fraction of liposome content association. As shown above, leakage values based on the disparity between lipid and contents association have been calculated, and these that are very similar to those previously measured.[66] It should also be pointed out that DSPG liposomes leak very little in contact with serum or LDL. Consequently, the DSPG lipid and contents association will be similar and very low.

The nature of the effect of HDL on binding to CV1-P cells is not presently clear, and does not lead to an increase in drug delivery. While the percentage of drug binding to CV1-P can be as much as half that induced by LDL, the level of lipid binding is much lower, and is comparable to the extent of drug binding. This also is in keeping with the limited extent to which HDL induces leakage from these liposomes.[66] The inhibition of association of liposomes with CHOldlA7 by HDL and LDL may well indicate that the form of association is mediated by the scavenger receptor SR-BI, discussed above.

The interaction of anionic liposomes with cells has been explored by a number of investigators.[103,104] Unfortunately, it is difficult to compare the current results to prior studies, because the present work has utilized a concentration of lipid relevant to the drug delivery studies described here, and this concentration is much lower than that used by other investigators. Consequently, it is quite possible that the LDL-mediated processes seen in the current work might not have been a major part of association in other studies, particularly if the LDL receptors were to become saturated as lipid concentration is increased.

In conclusion, the results described in this chapter demonstrate that the interaction of LDL with fluid liposomes bearing at least 75 mol anionic phospholipid/100 mol phospholipid leads to cell association of the liposomes via an interaction with the LDLr. The association of the liposomes and their contents with cells subsequently produces drug delivery effects, manifest by an increase in the potency of encapsulated drug.

CHAPTER FOUR: STUDIES IN SERUM-CONTAINING MEDIUM

1. Introduction

Although interaction of LDL with anionic liposomes causes extensive leakage, it also provides a possible means, by which these liposomes may interact with cells. In Chapter III I described *in vitro* experiments in a chemically defined, lipoprotein and apolipoprotein free, growth medium establishing that anionic liposomes interact with LDLr, in a LDL mediated event, in which cellular association led to functional delivery of a series of liposome dependent drugs.[105] Association of liposomes containing at least 75 mol ePG/100 mol phospholipid with cells grown in defined medium supplemented with 1.0 mg/ml LDL was up to 30 fold higher with CV1-P or CHOwt cells than with CHOldlA7, which lack the LDLr. The addition of LDL did not yield any elevation of cellular association of DSPG liposomes. Increased association was paralleled by a corresponding increase in potency of three liposome dependent drugs, N-phosphonacetyl-L-aspartic acid, fluoroorotic acid, and methotrexate-γ-aspartate.

Targeting of delivery systems such as liposomes directly to the LDLr is a potentially attractive therapeutic strategy, because a localized tumor mass may exhibit increased LDLr expression not seen on normal healthy tissue. This arises, because rapidly dividing tumor cells often have a much greater requirement for cholesterol than normal cells.[106] The previous studies (Chapter III) were performed utilizing a defined medium, which deprived cells of exogenous sources of cholesterol thereby allowing for maximal LDLr activity. Therefore, in the present study I have investigated cell binding and drug delivery potency of various anionic liposomal formulations in a serum supplemented growth medium, in order to understand the role of the LDLr in targeted drug delivery

mediated by anionic liposomes under conditions where cells do not exhibit maximal receptor expression.

2. Results

The results of liposome-cell association studies utilizing ePG and DSPG encapsulated Mtx are summarized in Figure 25. There is a nearly 30 fold difference in the level of ePG-Mtx association between cells expressing (CV1-P, CHOwt) and cells lacking (CHOldlA7) the LDLr. Additionally, a moderate level of DSPG-Mtx association is observed for CV1-P, but not for CHOwt and CHOldlA7. Therefore, in serum-containing medium, the aqueous contents of ePG vesicles associate with cells mostly via the LDLr, while DSPG vesicles did not rely upon the LDLr for uptake. The moderate level of DSPG-Mtx association seen for CV1-P suggests the presence of additional mechanisms of uptake in this cell line.

Liposome-cell association studies correlating the uptake of liposomal aqueous contents as a function of anionic phospholipid content are shown in Figure 26. Preparations with 0 mol/100 mol anionic phospholipid content displayed negligible amounts of Mtx uptake for all cell lines tested. Additionally, CHOldlA7 displayed minimal association of liposomal aqueous contents for all charge ratios tested. In contrast, CHOwt and CV1-P displayed significant levels of liposomal cellular association with as little as 25 mole percent ePG.

Figure 27 shows the association of liposomal lipid as a function of anionic phospholipid content. As with the association of liposomal aqueous contents, liposomal

lipid associated to a minimal extent with CHOldlA7 for all charge ratios tested. Lipid association with both CV1-P and CHOwt was much more extensive than Mtx association, with approximately 20% and 4% lipid associated, respectively, for 100 mole percent and 75 mole percent ePG vesicles. In view of the extensive serum mediated leakage of contents from liposomes prepared from greater than 50 mole percent ePG, CV1-P and CHOwt should associate with a much larger fraction of lipid at high charge ratios in order to produce the levels of Mtx association described above (Figure 26).[66]

Table VIII shows the growth inhibitory effects in serum containing medium of the liposome dependent drugs, FO and PALA, on the two CHO cell lines. The free drugs have similar effects on both cell lines during a 48 hour exposure period. Both PALA and FO show significantly increased potency, whether used in ePG, DSPG or DPPG liposomes. The potency of DPPG or DSPG formulations was similar to that of ePG formulations despite negligible cellular association of DSPG-Mtx observed in serum containing medium (Figure 25). Additionally, the efficacy of the ePG formulations was also identical on the two cell lines despite the almost 30 fold difference in cellular association of ePG-Mtx in serum-containing medium (Figure 25).

Previously, Heath *et al.* have shown by plotting IC_{50} x exposure length (Cxt) versus exposure length that both FO and PALA may be more effective if delivery occurs rapidly.[107] In light of these findings, growth inhibition wash studies were conducted to determine if LDL dependent differences in potency could be detected with shorter exposure lengths. Figure 28 shows the effect of exposure length on the potency of ePG encapsulated FO and PALA for the growth inhibition of CHOwt and CHOldlA7 cells in

serum-containing medium. All ePG preparations exhibit growth-inhibitory effects that are dependent on the exposure length, as shown by the decrease in the IC_{50} as the exposure length is increased. No differences between the two cell lines were evident at any exposure length, despite the nearly 30 fold difference in cellular association of ePG-Mtx in serum containing medium (Figure 25). These results were surprising, particularly for the shorter exposure lengths.

The studies described above show that in serum-containing medium there is no LDL-dependent increase in drug potency despite a considerable effect upon the extent of liposome association with cells. However, in the previous chapter a correlation between increased uptake of ePG vesicles and enhanced potency of ePG liposomal formulations in CV1-P and CHOwt in defined medium supplemented with 1.0 mg/ml LDL was reported.[105] In order to resolve this difference, I have measured the potency of FO and PALA in ePG liposomes on all three cell lines in defined medium supplemented with LDL, and I have added the LDL either 30 minutes prior to the addition of the liposomes as was done in the earlier defined medium studies (Chapter III), or 12 hours prior to liposome addition. Table IX shows that the prolonged LDL pre-exposure eliminates the LDL-dependent increase in potency of ePG-PALA and ePG-FO seen for CV1-P and CHOwt, but not CHOldlA7. Prolonged exposure to LDL prior to liposome addition may induce down regulation of LDL receptors, resulting in a situation analogous to LDLr expression in serum supplemented growth medium.

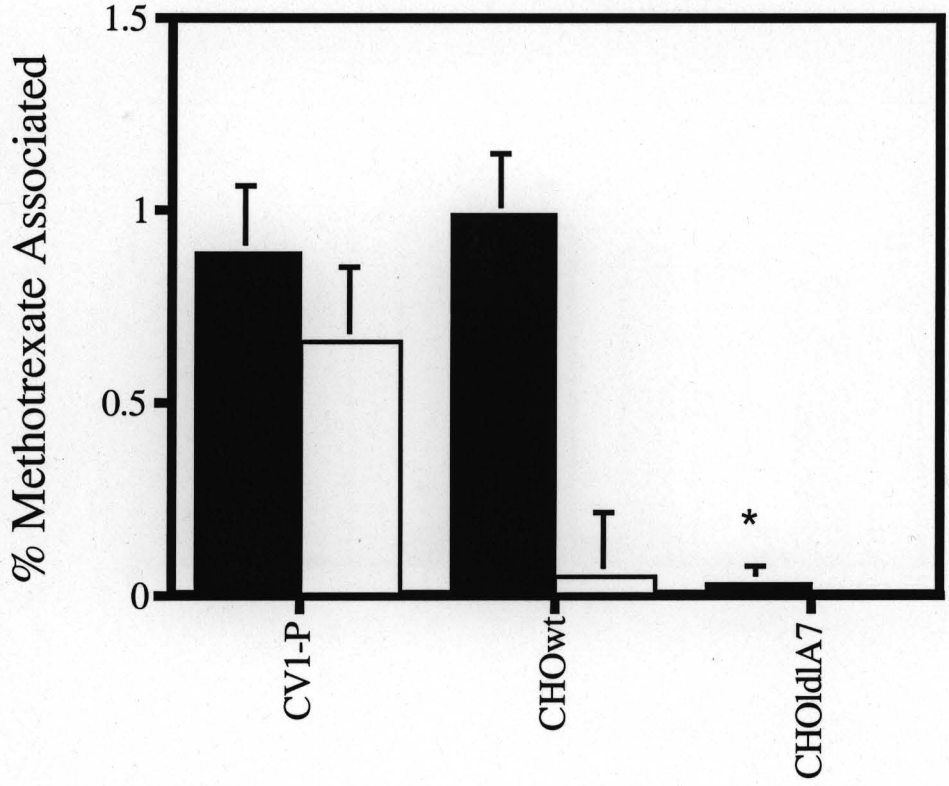


Figure 25. Cellular association of ePG (black bars) and DSPG (white bars) encapsulated Mtx for CV1-P, CHOwt, and CHOdlA7 cells in serum supplemented growth medium. Mtx was encapsulated in liposomes prepared from a mixture of phospholipid and cholesterol at a phospholipid: cholesterol ratio 2:1. Cells were exposed for 60 min to encapsulated Mtx at a final concentration of 20 nM which corresponds to a final lipid concentration of approximately 200 nM. Each bar is the mean of nine determinations ± the standard deviation. *, $P < 0.01$ compared to CHOwt.

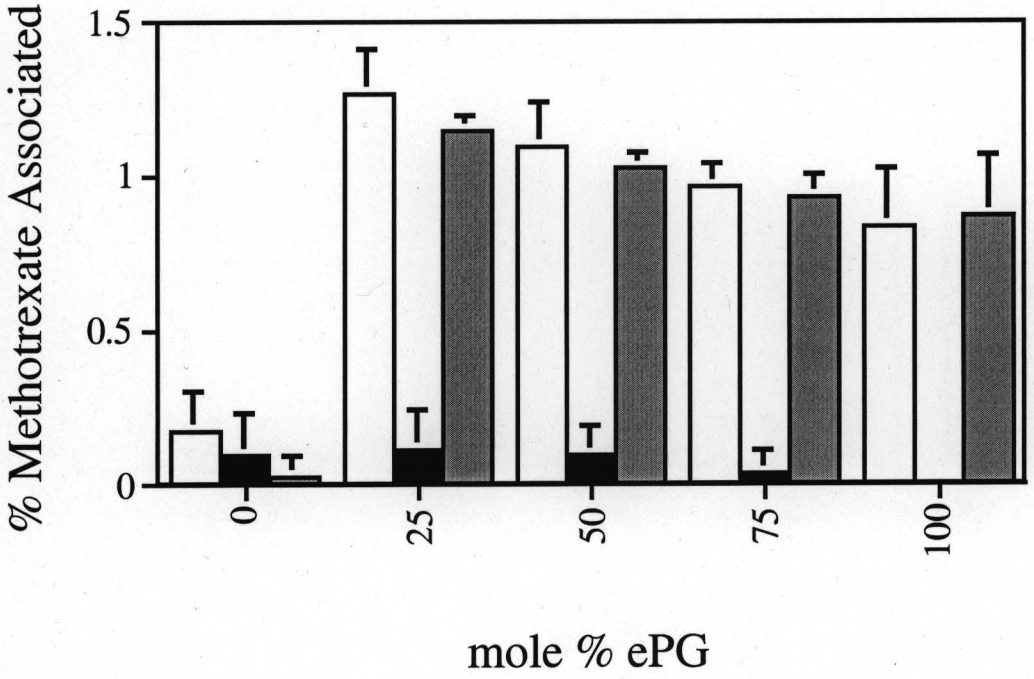


Figure 26. Cellular association of liposome contents for CHOwt (white bars), CHOldlA7 (black bars), and CV1-P (gray bars) cells as a function of anionic phospholipid content. Mtx was encapsulated in liposomes prepared from a mixture of phospholipid and cholesterol at a phospholipid: cholesterol ratio 2:1. Cells were exposed for 60 min to encapsulated Mtx at a final concentration of 20 nM which corresponds to a final lipid concentration of approximately 200 nM. Each bar is the mean of nine determinations ± the standard deviation.

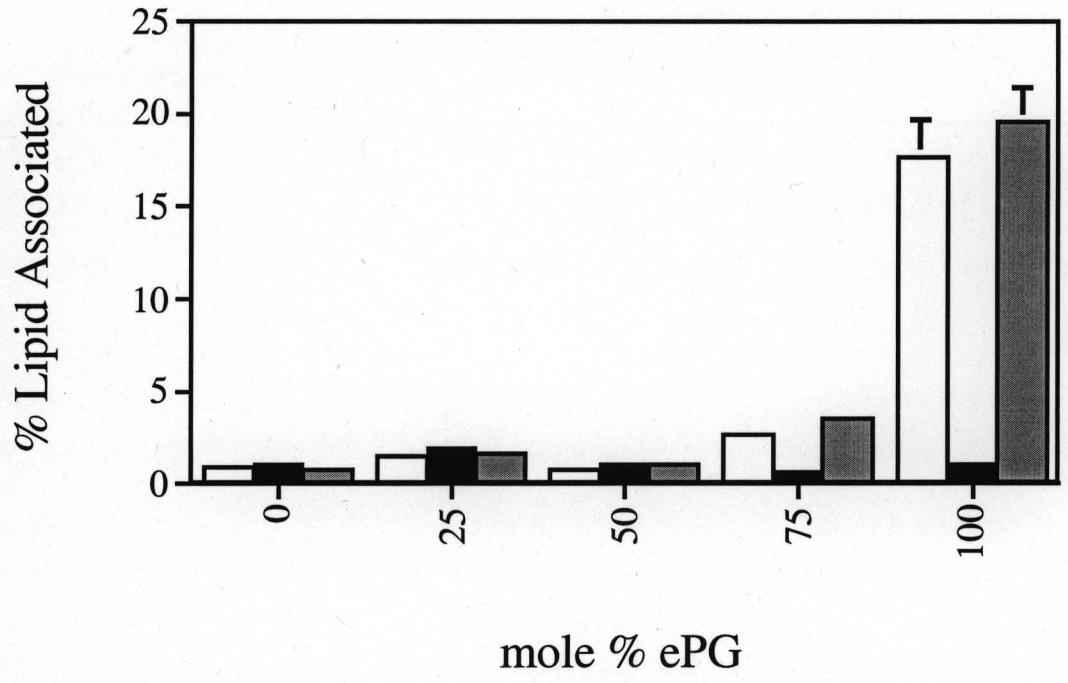


Figure 27. Cellular association of liposomal lipid for CHOwt (white bars), CHOldlA7 (black bars), and CV1-P (gray bars) cells as a function of anionic phospholipid content. Fluorescein labeled liposomes were prepared from a mixture of phospholipid and cholesterol at a phospholipid: cholesterol ratio 2:1. Cells were exposed for 60 min to labeled vesicles at a final lipid concentration of 200 nM. Each bar is the mean of three determinations ± the standard deviation.

Drug Preparation	IC_{50} (μM) ± standard deviation	
	CHOwt	CHOldlA7
FO	4.86 ± 1.82 (11)	3.91 ± 1.02 (11)
ePG-FO	0.62 ± 0.11 (4)	0.56 ± 0.05 (4)
DPPG-FO	0.95 ± 0.14 (6)	1.35 ± 0.78 (8)
PALA	22.91 ± 3.41 (7)	23.34 ± 3.64 (8)
ePG-PALA	0.54 ± 0.08 (4)	0.53 ± 0.11 (4)
DSPG-PALA	0.37 ± 0.18 (4)	0.31 ± 0.05 (4)

Table VIII. Potency of free and encapsulated FO and PALA in serum supplemented medium following drug exposure for 48 hours. The values are expressed as the mean ± the standard deviation, and the number in parentheses is the number of determinations. ePG-FO and ePG-PALA liposomes were prepared from ePG/cholesterol (67:33) and drug/lipid ratio was 0.423 mol/mol and 0.820 mol/mol respectively. Values for DPPG liposomes were obtained using liposomes prepared from DPPG/cholesterol (67:33) derived from two preparations with the following drug/lipid ratios: 0.352 and 0.392 mol/mol. DSPG liposomes were prepared from DSPG/cholesterol (67:33) and the drug/lipid ratio was 0.474 mol/mol.

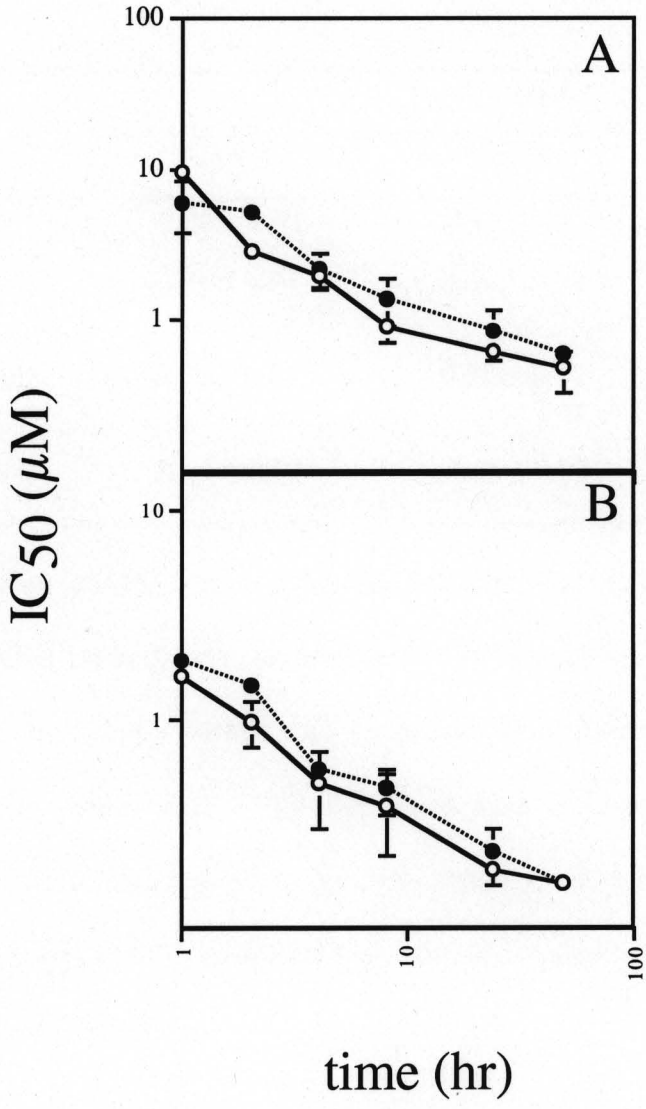


Figure 28. The effect of exposure length on the potency of ePG-FO (A) and ePG-PALA (B) in serum supplemented growth medium. CHOwt (O) and CHOldlA7 (●) cells were exposed to the drug for the specified time, washed free of drug and returned to culture in fresh serum containing medium for the remainder of the 48 hour incubation. FO:ePG lipid ratio = 0.423 mol/mol and PALA:ePG lipid ratio = 0.440 mol/mol.

	IC_{50} (μM)		
	CV1-P	CHOwt	CHOldlA7
Control	0.26 ± 0.03	2.42 ± 0.22	2.72 ± 0.23
LDL (1.0 mg/ml) ~0 minutes	0.06 ± 0.02*	0.40 ± 0.06*	5.98 ± 0.10
LDL (1.0 mg/ml) ~30 minutes	0.05 ± 0.01*	0.38 ± 0.07*	6.06 ± 0.10
LDL (1.0 mg/ml) ~12 hours	0.25 ± 0.03	2.35 ± 0.23	4.74 ± 0.13

Table IX. Potency of ePG-PALA in defined medium alone (control) or with LDL 1.0 mg/ml added either immediately, 30 minutes, or 12 hours prior to liposome exposure. Defined medium was supplemented with lipoprotein at concentrations relevant to those normally found in serum. ePG-PALA liposomes were prepared from ePG/cholesterol (67:33) and drug/lipid ratio was 0.440 mol/mol. Potency was determined following drug exposure for 72 hours and the values are expressed as the mean ± the standard deviation. *, $P < 0.01$ compared to control.

3. Discussion

In chapter III, it was shown that anionic liposomes composed of at least 75 mol ePG/100 mol phospholipid interact with cells bearing LDL receptors, provided purified LDL is added to the defined (lipoprotein free) growth medium.[105] In the present chapter, this interaction is also shown to occur with LDLr-bearing cells grown in serum-containing medium, whose components can be expected to include LDL at a concentration comparable to that added into defined medium in chapter III. I have previously discussed the possible mechanism of this interaction, and postulated that fluid liposomes containing at least 75 mol ePG/100 mol phospholipid coalesce with LDL particles, thereby causing partial but not total loss of contents. [105] The resultant liposomes, which would have apoB100, and possibly also apoE, on their surface, would bind to the LDLr through an interaction of the receptor and apolipoprotein domains normally involved in the interaction of native LDL with the receptor.[61]

Prior studies (Chapter III) showed that this interaction also resulted in an increase in potency of encapsulated liposome-dependent drugs, as one might expect for any process that increases cellular uptake of liposomes. LDL is known to be taken up by the endocytic pathway, and the predominant route by which liposomes gain entry into cells is endocytosis via coated pits.[36,108] However, in the present study described in this chapter, there is no observed increase in drug potency for cells grown continuously in serum. Specifically, association of liposomal aqueous contents was up to 30 fold higher with CV1-P or CHOwt cells than with CHOldlA7, although neither of the ePG encapsulated

liposome dependent drugs tested is any more effective for growth inhibition of CHOwt than it is for CHOldlA7.

The discrepancy between association and potency in serum supplemented growth medium appears to involve differences in LDLr expression between cells grown in serum supplemented medium and cells grown in a defined medium lacking lipoproteins. CHOwt grown in the lipoprotein and apolipoprotein-deficient defined medium was 6 times more sensitive to encapsulated drugs than CHOldlA7 when defined medium was supplemented with LDL either immediately prior or 30 minutes prior to liposome exposure, but not if the medium was supplemented with LDL 12 hours prior to liposome exposure. This result indicates that CHOwt cells continuously exposed to LDL associate with liposomes, but are not sensitive to drug delivery. Goldstein *et al.* have reported that cells grown in medium containing 10% serum express a level of LDL receptors that is 10-20% of that found when the cells are cultured for 36-48 hours in medium containing 10% lipoprotein-deficient serum.[96] Moreover, cells grown in lipoprotein deficient medium rapidly internalize LDL receptors once exposed to LDL.[109] This rapid transient uptake of the receptors together with the liposomes associated with them may stimulate drug uptake leading to the observed increase in sensitivity.

It is clear from the present study that the LDLr is not the only potential mechanism of uptake for negatively charged liposomes by cells. Previously, it was observed in LDL-supplemented defined medium that Mtx in ePG liposomes was taken up significantly only if the ePG content was at least 75 mol/100 mol phospholipid (Figure 18). This observation is in perfect accord with both lipid uptake and LDL induced leakage, both of

which occur only if the ePG content is at least 75 mol/100 mol phospholipid. In the present study, we observe significant Mtx uptake when the liposomes contain as little as 25 mol ePG/100 mol phospholipid, indicating that serum promotes uptake of liposomes containing 25-50 mol ePG. The amount of lipid taken up is small, but the amount of Mtx associated is comparable to that achieved with 75-100 mol percent ePG, because leakage of contents is minimal below 75 mol percent ePG. These results suggest that a ligand which is present in serum and is not present in purified LDL is promoting liposome association with the cells. The receptor for this interaction is present on CV1-P cells and CHOwt cells, but is absent on CHOldlA7.

The association of ePG and DSPG liposomes with CHO cells in defined medium lacking lipoproteins suggests that a receptor or receptors other than the LDLr can be involved in liposome cell association without prior interaction of a ligand with the liposomes.[105] Moreover, drug delivery results similarly suggest such uptake mechanisms. The scavenger receptor SR-BI is an HDL receptor on CHO cells, and may be involved in this uptake mechanism.[101] Additionally, Rigotti *et al.*, despite working with higher lipid concentrations than those used here, found that SR-BI can tightly bind negatively charged phosphatidylserine (PS) and phosphatidylinositol (PI) liposomes but not neutral phosphatidylcholine (PC) liposomes.[110]

In conclusion, the results described here characterize the propensity for fluid ePG liposomes to associate to a greater extent with cells expressing the LDLr. Additionally, the existence of an ePG liposome-LDL interaction not present with DSPG liposomes is the likely source of the increased uptake of our ePG liposome formulation. Liposome-

cell association studies, using liposomes of varying ePG content determined significant levels of cellular association with as little as 25 mole percent ePG. These results suggest that a ligand which is present in serum and is not present in purified LDL is promoting liposome association with CV1-P and CHOwt for formulations containing 25-50 mole percent ePG. The failure of this association to increase drug delivery seems to be caused by the down regulation of LDLr expression when cells are continuously exposed to LDL.

CHAPTER FIVE: MONOCLONAL ANTIBODIES AND TRANSFECTED CELL LINE STUDIES

1. Introduction

I have previously conducted *in vitro* experiments in a chemically defined, lipoprotein and apolipoprotein free, growth medium (Chapter III) and in serum supplemented growth medium (Chapter IV) suggesting that anionic liposomes containing 75-100 mole percent ePG interact with the LDLr, in a LDL dependent manner. In defined medium supplemented with LDL, cellular association led to functional delivery of a series of liposome dependent drugs, manifest by their lowered IC_{50} values. Association of liposomes containing 75-100 mol ePG/100 mol phospholipid with cells grown in defined medium supplemented with 1.0 mg/ml LDL was up to 30 fold higher with CV1-P or CHO wild type cells than with CHOldlA7.[105,111] These results confirmed that 75-100 mole percent ePG liposomes interact with cells in an LDL-dependent manner, and suggested, based on the reported properties of CHOldlA7, that the receptor involved is LDLr.

In addition to the LDL-dependent cell interaction of 75-100 mole percent ePG liposomes, I also observed a serum-dependent interaction of 25-50 mole percent ePG liposomes with CV1-P and CHOwt cells, but not with CHOldlA7. This result points to an interaction that requires a serum component other than LDL or HDL, but which may involve the LDLr, since this is the only receptor known to be missing from CHOldlA7.

In the present chapter, I confirm the role of both LDL and LDLr in the interaction of 75-100 mole percent ePG liposomes by carrying out blocking studies with C7, a monoclonal specific for LDLr, and 5E11, a monoclonal specific for apoB100. I also confirm the role of LDLr by transfecting CHOldlA7 with a plasmid expressing the

human LDLr. These experiments have also shown that LDLr is not involved in the interaction of 25-50 mole percent ePG liposomes with cells, and indicate that CHOldlA7 lacks at least one other surface protein in addition to LDLr.

2. Results

Figure 29 shows the binding of liposomes to CV1-P cells, grown in serum, as a function of liposomal ePG content. Association is monitored both by encapsulated Mtx (Panel 29A) and lipid label (Panel 29B). This study was carried out at 4°C in order to facilitate blocking by C7, while work described in chapters III and IV was done at 37°C. Association at 4°C is at least two fold more extensive than was previously seen at 37°C.[111] Increased cellular association at 4°C was expected based on the findings of Brown and Goldstein who have shown that the affinity of the fibroblast LDLr for LDL is ~10 fold lower at 37°C, as compared to 4°C, due, at least in part, to a more rapid dissociation at the higher temperature.[109] Additionally, Beisiegel *et al.* reported C7 to exhibit an affinity for the LDLr that is ~75 fold lower at 37°C, as opposed to 4°C.[112] Association of liposomal aqueous contents (Figure 29A) and liposomal lipid (Figure 29B) was inhibited by at least 70% upon addition of C7 at a final concentration of 3 nM. Inhibition was only observed for vesicles containing 75-100 mole percent ePG, suggesting that association of 25-50 mole percent ePG liposomes is not mediated through LDLr. The extent of C7 induced inhibition is consistent with the findings of Beisiegel *et al.* who found binding of ^{125}I-LDL to human fibroblasts to be reduced by 70% at a C7

concentration of 2 nM.[112] Parallel studies using CV1-P cells in defined medium supplemented with 0.1 or 1.0 mg/ml LDL are shown in Figure 30. Once again, the use of a lower incubation temperature increases the extent of association. C7 also blocks association by up to 70%.

The association of encapsulated Mtx with both CHOldlA7 and a clone of this line transfected with the cDNA for the human LDLr (A7(pLDLr2)) is shown in Figure 31. Association is shown in both serum-supplemented medium (Panel 31A) and defined medium supplemented with 1.0 mg/ml LDL (Panel 31B), and is determined for liposomes containing 0-100 mole percent ePG. In both conditions, association with the transfected line is significantly elevated only for liposomes containing 75-100 mole percent ePG. This further confirms that association of 75-100 mole percent ePG liposomes is mediated by LDLr. The failure of 25-50 mole percent ePG liposomes to show elevated association with A7(pLDLr2) in serum supplemented medium confirms that LDLr is not responsible for the association of 25-50 mole percent ePG liposomes with CV1-P and CHOwt cells in the presence of serum.

The effect of C7 upon the association of liposomal Mtx with A7(pLDLr2) for vesicles containing 75-100 mole percent ePG is shown in Figure 32. As was noted earlier, association was two fold higher at 4°C than was observed at 37°C. Association of liposomal aqueous contents was inhibited by at least 70% upon addition of C7 at a final concentration of 3 nM. Inhibition was observed in serum supplemented growth medium (Figure 32A) and in defined medium supplemented with 1.0 mg/ml LDL (Figure 32B) for

vesicles containing 75-100 mole percent ePG. This confirms that association with A7(pLDLr2) occurs through the presence of LDLr on the cell surface.

The inhibition of liposome cell association by the anti-apoB100 Mab 5E11 is shown in Figure 33 for CV1-P and CHOwt. Both cell lines exhibited an approximately 80% decrease in association of liposomal aqueous contents for vesicles containing 75-100 mole percent ePG. The extent to which 5E11 inhibited LDLr binding is comparable to inhibition observed by Pease *et al.* for other anti-apoB100 Mabs that have almost identical epitopes as 5E11.[94] This confirms the LDL dependent interaction of 75-100 mole percent ePG liposomes and further shows that this interaction is mediated through apoB100.

In order to further investigate the nature of the LDL-mediated binding of 100 mole percent ePG liposomes to LDLr, morphological studies were conducted using two different labels. In the first, CHOwt and CHOldlA7 cells in serum containing medium were exposed to ePG-Au liposomes at time intervals similar to those used in the cell-association studies described above. CHOwt cells exposed to ePG-Au for 10 min (Figure 34A) and 60 min (Figure 34C) were compared to CHOldlA7 cells exposed to liposomal gold for identical time intervals (Figures 34B and 34D). The wild type displayed greater uptake of ePG-Au compared to the mutant cell line for both time points studied (see white arrows in Figure 34). Gold particles on the wild type are clearly visible in backscatter detection mode as clusters just below the cell surface. Hence, the liposomes appear to have been internalized and sequestered within organelles close to the surface of the cell.

The increased uptake of ePG liposomes by way of the LDLr seen in previous cell-association studies and the SEM studies (Figure 34) was further established by means of fluorescence microscopy. CHOwt (Figure 35A) and CHOldlA7 (Figure 35B) cells were exposed to ePG-fluorescein for 60 min and washed of unbound liposomes with PBS C/M then compared. Greater amounts of fluorescent material was shown to be associated with the surface of wild type cells while bare traces of fluorescence were seen in the mutant cell line. These results confirm the more extensive surface interaction of 100 mole percent ePG liposomes with cells bearing the LDLr.

Figure 29. Cellular association of liposomal aqueous contents (Panel A) and liposomal lipid (Panel B) for CV1-P cells in serum supplemented growth medium. Liposomes were prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid: cholesterol ratio 2:1, and cells were exposed for 60 min either in the absence (black bars) or presence of 3 nM IgG C7 (dashed bars) at 4°C. Mtx was encapsulated in liposomes for liposomal aqueous content studies and fluorescein labeled liposomes were utilized for liposomal lipid association studies. Each bar is the mean of three determinations ± the standard deviation. *, $P < 0.01$ compared to CV1-P in the absence of IgG C7.

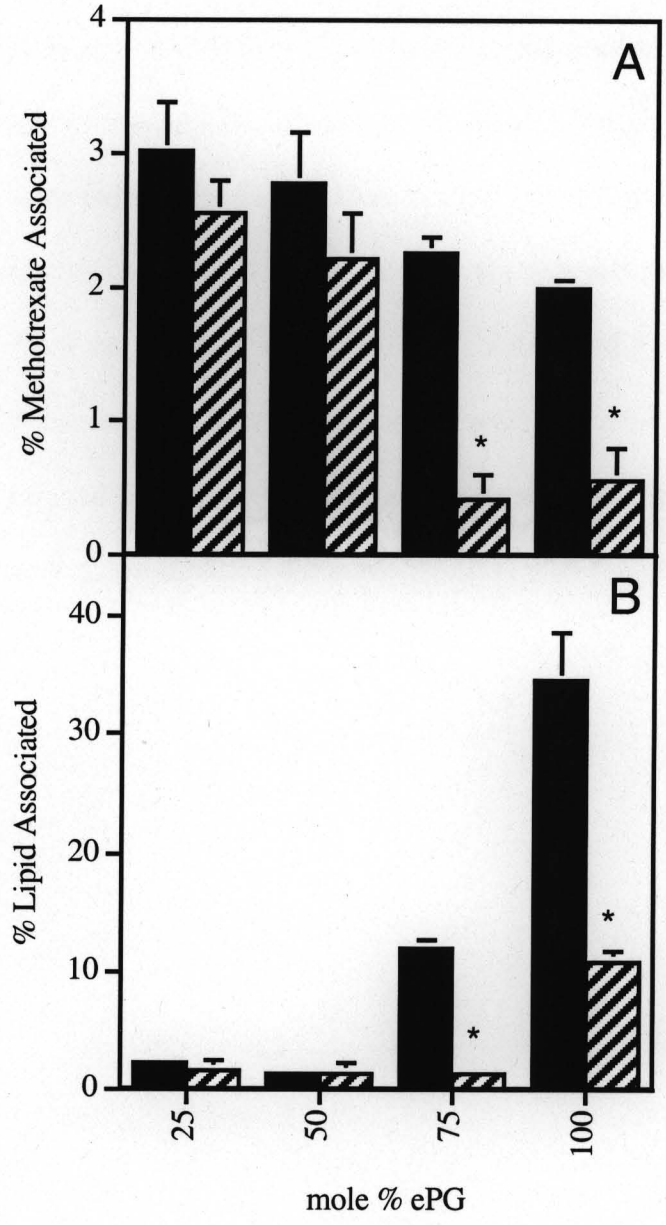

A
B
% Methotrexate Associated
% Lipid Associated
4
3
2
1
0
40
30
20
10
0
*
*
*
*
25
50
75
100
mole % ePG

Figure 30. Cellular association of liposomal aqueous contents (Panels A and B) and liposomal lipid (Panels C and D) for CV1-P cells in the presence of defined medium supplemented with 0.1 mg/ml LDL (Panels A and C) or 1.0 mg/ml LDL (Panels B and D). Liposomes were prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid: cholesterol ratio 2:1, and cells were exposed for 60 min either in the absence (black bars) or presence of 3 nM IgG C7 (dashed bars) at 4°C. Mtx was encapsulated in liposomes for liposomal aqueous content studies and fluorescein labeled liposomes were utilized for liposomal lipid association studies. Each bar is the mean of three determinations ± the standard deviation. *, $P < 0.01$ compared to CV1-P in the absence of IgG C7.

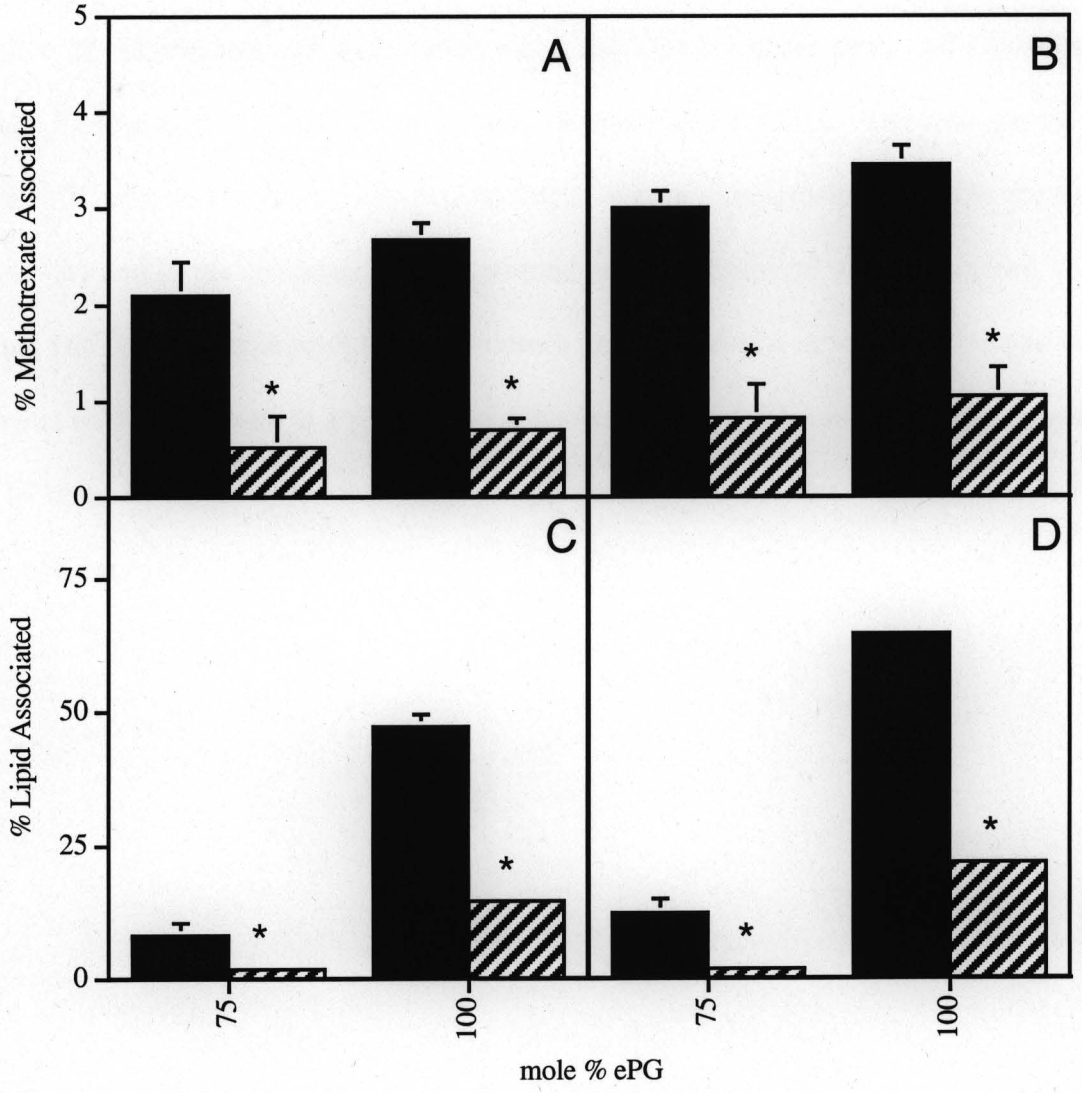

A
B
C
D
% Methotrexate Associated
% Lipid Associated
0
1
2
3
4
5
0
25
50
75
*
75
100
75
100
mole % ePG

Figure 31. Liposome-cell association for A7(pLDLr2) (white bars) and CHOldlA7 (black bars) cells as a function of anionic phospholipid content. The association of liposomal aqueous contents was determined in serum supplemented growth medium (Panel A) and in the presence of defined medium supplemented with 1.0 mg/ml LDL (Panel B). Mtx was encapsulated in liposomes prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid: cholesterol ratio 2:1, and cells were exposed for 60 min. Each bar is the mean of three determinations ± the standard deviation. *, $P < 0.01$ compared to A7(pLDLr2).

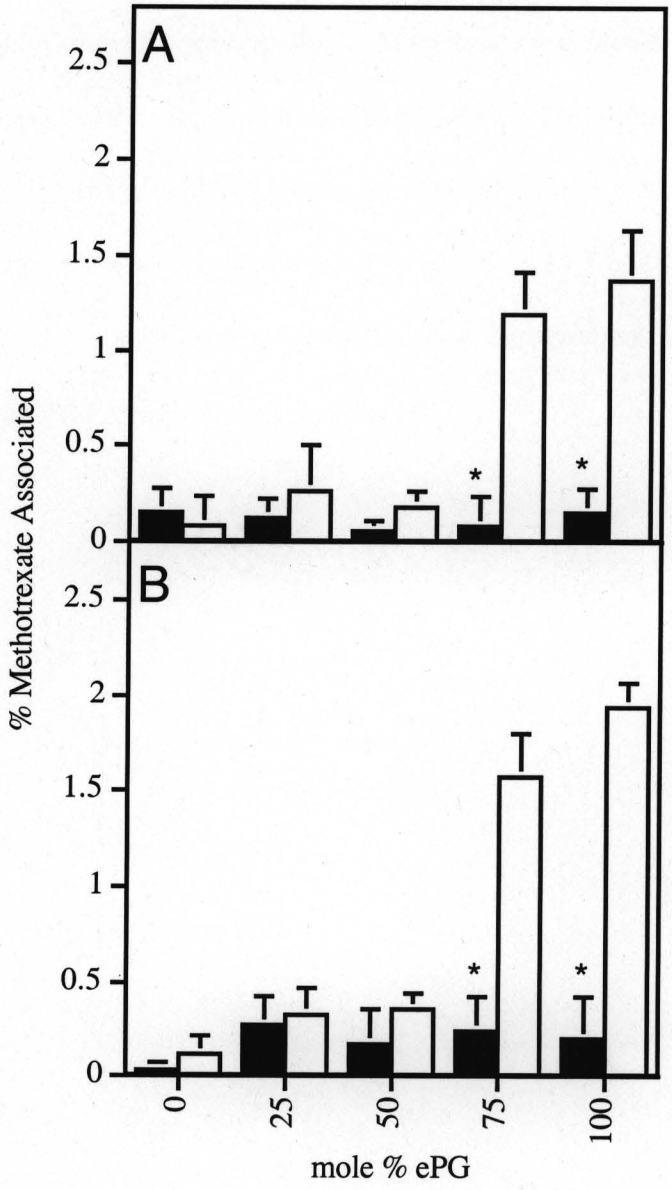

A
B
2.5
2
1.5
1
0.5
0
*
*
% Methotrexate Associated
0
25
50
75
100
mole % ePG

Figure 32. Cellular association of liposomal aqueous contents for A7(pLDLr2) cells in serum supplemented growth medium (Panel A) or in defined medium supplemented with 1.0 mg/ml LDL (Panel B). Liposomes were prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid: cholesterol ratio 2:1, and cells were exposed for 60 min either in the absence (white bars) or presence of 3 nM IgG C7 (gray bars) at 4°C. Each bar is the mean of three determinations ± the standard deviation. *, $P < 0.01$ compared to A7(pLDLr2) in the absence of IgG C7.

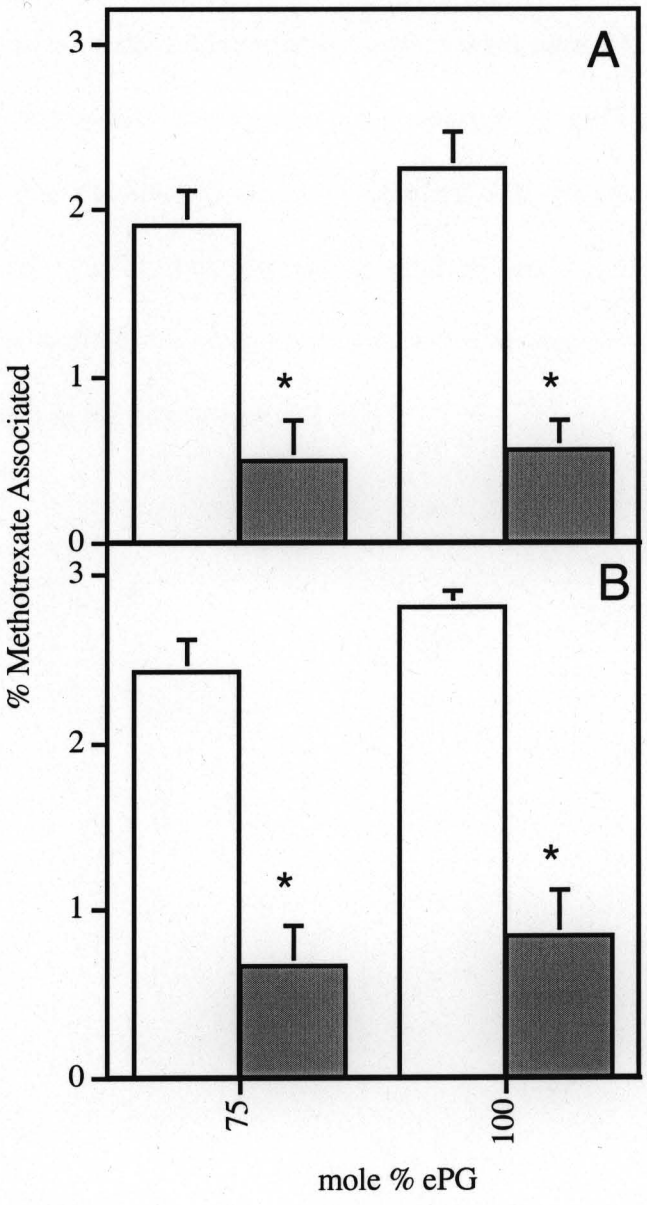

A
B
3
2
1
0
*
*
*
*
% Methotrexate Associated
75
100
mole % ePG

Figure 33. Liposome-cell association for CV1-P (black bars) and CHOwt (gray bars) cells in the presence of defined medium supplemented with 1.0 mg/ml LDL. Mtx was encapsulated in liposomes prepared from a mixture of ePG and ePC together with cholesterol at a phospholipid: cholesterol ratio 2:1, and cells were exposed for 60 min either in the absence (solid bars) or presence (dashed bars) of 20 μg/ml IgG 5E11. Each bar is the mean of three determinations ± the standard deviation. *, $P < 0.01$ compared to cellular association in the absence of IgG 5E11.

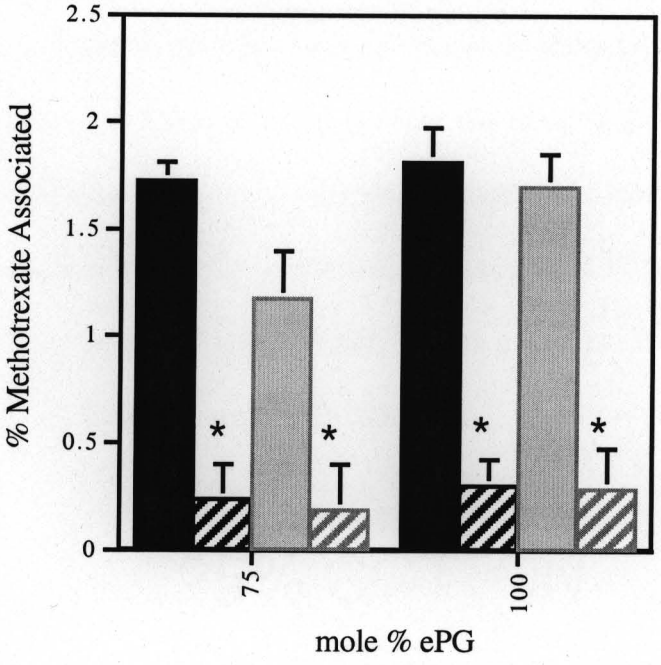

2.5
2
1.5
1
0.5
0
% Methotrexate Associated
*
*
*
*
75
100
mole % ePG

Figure 34. SEM micrographs of CHOwt and CHOldlA7 cells, in serum supplemented growth medium, exposed to ePG liposomes containing colloidal gold. Samples were viewed in the backscatter detection mode because the ensuing Z contrast enhancement allowed for gold particles to appear significantly brighter and easier to detect. Arrows indicate cell associated clusters of gold beads. Micrographs shown are representative figures from triplicate samples. (A: CHOwt, 10 min exposure; B: CHOldlA7, 10 min exposure; C: CHOwt, 60 min exposure; D: CHOldlA7, 60 min exposure).

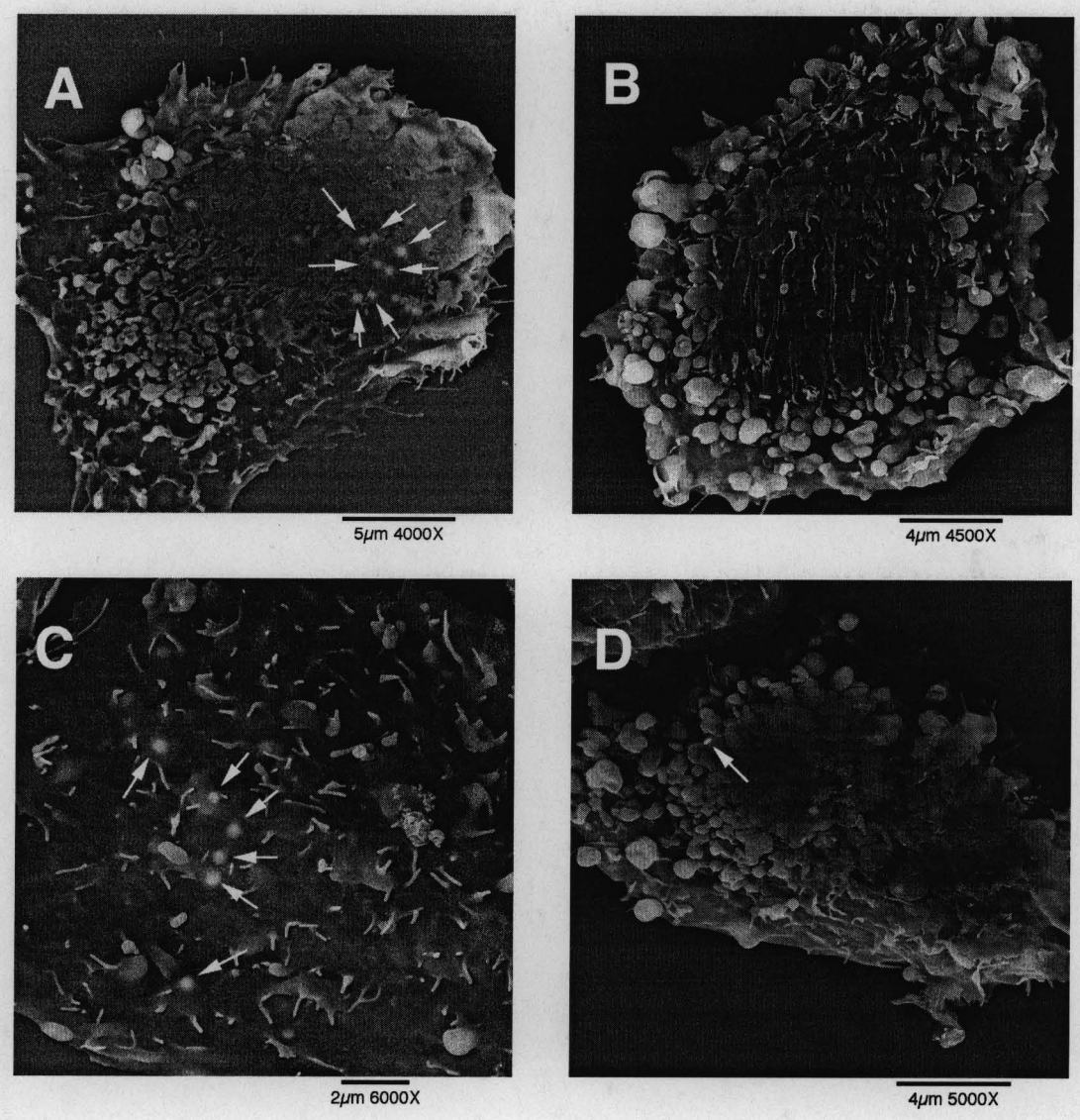
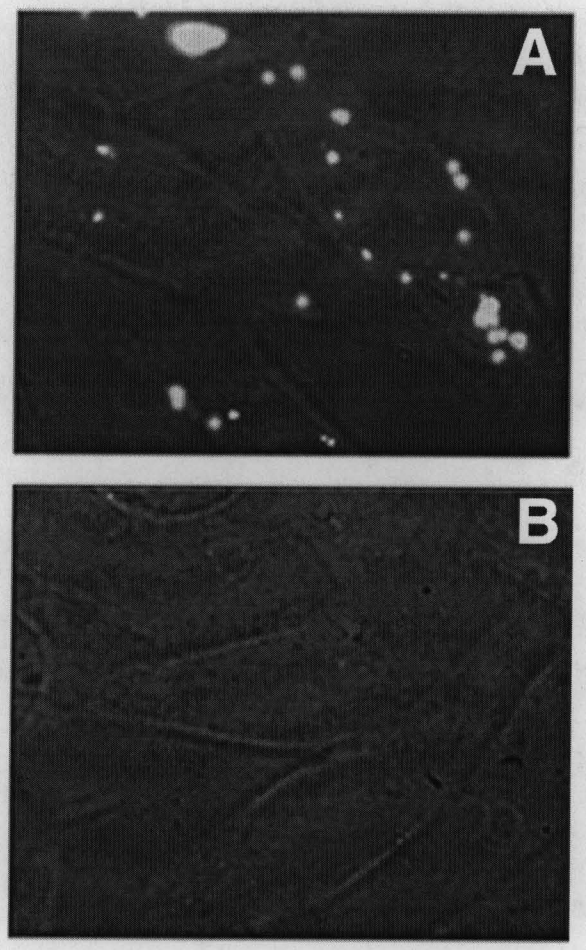

A
5μm 4000X
B
4μm 4500X
C
2μm 6000X
D
4μm 5000X

Figure 35. Fluorescence images of CHOwt and CHOldlA7 cells, in serum supplemented growth medium, exposed to fluorescein labeled ePG liposomes. Images shown are representative figures from triplicate samples. (A: CHOwt, 60 min exposure; B: CHOldlA7, 60 min exposure).


A
B

3. Discussion

The findings described in this chapter confirm that 75-100 mole percent ePG liposomes interact with cells through the LDLr, and that this interaction also involves LDL. It was suggested in chapter III that this interaction occurs, because LDL coalesces with fluid anionic liposomes of this composition, and that the particles so produced bind to LDLr through their apolipoprotein components. The concept of an ePG-LDL complex has been established by Greenspan *et al.* who have demonstrated that such complexes, upon formation, are phagocytosed by macrophages.[99] Lauraeus *et al.* have also established the involvement of electrostatic attractions between the acidic phosphate of DMPG and cationic residues in apoB100 which resulted in aggregation of DMPG liposomes by human plasma LDL for liposomes containing greater than 50 mole percent anionic phospholipid.[100] Comiskey *et al.* previously demonstrated that serum induces leakage of contents from 75-100 mole percent ePG liposomes, and that this leakage is caused by LDL.[66]

The study described in this chapter confirms the role of LDLr in the cellular association of 75-100 mole percent ePG liposomes by showing that it occurs for A7(pLDLr2), a clone of CHOldlA7 specifically transfected with the human LDLr. The role of LDLr is also confirmed by blocking of association of 75-100 mole percent ePG liposomes by the C7 monoclonal antibody. Blocking was observed for CV1-P cells, both in serum and in LDL-supplemented defined medium, and for A7(pLDLr2). C7 binds to the first of seven cysteine-rich repeats in the ligand-binding domain of the LDLr.[95] This first repeat is not involved in binding to lipoprotein and so inhibition of liposome cell

association by C7 may be due to a steric effect. The role of LDL in the interaction of 75-100 mole percent ePG liposomes was also confirmed with its inhibition by the anti-apoB100 Mab, 5E11, which suggests specifically that apoB100 and not apoE is responsible for the association.

In chapter IV, an interaction of 25-50 mole percent ePG vesicles with CV1-P and CHOwt in serum was demonstrated, and it was concluded that this interaction did not involve either LDL or HDL, but required an unknown serum component. The failure of CHOldlA7 to interact with vesicles under these circumstances suggested that LDLr might be the receptor involved, because LDLr is the only receptor known to be missing from this cell, which is a deletion mutant.[87] However, the data shown in this chapter shows clearly that LDLr is not involved in this interaction, both through the failure of C7 to block the association, and through the failure of A7(pLDLr2) to exhibit the association. A possible alternative candidate might be the LDL receptor related protein (LRP). LRP, also known as the α_2-macroglobulin receptor, is a member of the LDLr gene family whose ligand, α_2-macroglobulin, is known to associate with liposomes; thus, making this a likely candidate for the receptor involved in liposome cell association at 25-50 mole percent ePG. However, Black *et al.* demonstrated that α_2-macroglobulin associates with liposomes regardless of charge.[113] As a result, liposome association should have been observed at 0 mole percent ePG if this protein were involved. Though this may eliminate a role for α_2-macroglobulin, LRP is known to bind several ligands present in serum, such as lipoprotein lipase, apoE-enriched remnant lipoproteins, and plasminogen activator, and

may indeed be involved in liposome cell association for vesicles containing 25-50 mole percent ePG.[102]

In conclusion, the binding of 75-100 mole percent ePG liposomes to cells occurs through the LDLr and the apoB100 component of LDL has been confirmed. The LDLr has been ruled out as the site for binding of 25-50 mole percent ePG liposomes, whose interaction with cells occurs through the presence of a protein other than LDL or HDL. Both the receptor for 25-50 mole percent ePG liposomes and LDLr are missing from CHOldlA7 cells.

CHAPTER SIX: CONCLUSIONS

The results described demonstrate the interaction of LDL with fluid liposomes bearing at least 75 mol anionic phospholipid/100 mol phospholipid which leads to cell association of the liposomes via an interaction with LDLr. Hence, LDLr plays a major role in the cellular association of ePG liposomes but not DSPG liposomes. However, ePG and DSPG liposomes may bind to other receptors, such as the HDL receptor, as indicated by liposome cell association in the LDLr-deficient hamster cell line. The binding of 75-100 mole percent ePG liposomes to cells occurs through the LDLr and the apoB100 component of LDL. Use of the anti-LDLr monoclonal antibody, IgG C7, and a LDLr-deficient hamster cell line transfected with the human LDLr establish that liposomal formulations with 75-100 mole percent ePG display targeted cellular uptake via the LDLr. Additionally, use of the anti-apoB100 monoclonal antibody, IgG 5E11, establish that liposomal formulations with 75-100 mole percent ePG display targeted cellular uptake via an interaction between liposome associated apoB100 and the LDLr.[114]

Cellular association, in serum supplemented growth medium, of formulations with 25-50 mol ePG/100 mol phospholipid occurs through the presence of a ligand in serum that is not found in purified LDL or HDL, and through the action of a currently unknown receptor that is present on CHOwt and CV1-P cells, but is absent from CHOldlA7. The LDLr has been ruled out as the site for binding of 25-50 mole percent ePG liposomes, whose interactions with cells occurs through the presence of a protein other than LDL or HDL. Additionally, IgG C7 does not block this interaction, and transfected CHOldlA7 cells do not show this interaction. Both the receptor for 25-50 mole percent ePG liposomes and LDLr are missing from CHOldlA7.[114]

The association of liposomes and their contents with cells grown in a serum free growth medium supplemented with LDL subsequently produces drug delivery effects, manifest by an increase in the potency of encapsulated drug. In this lipoprotein and apolipoprotein free defined growth medium, which allows for maximal LDLr expression, ePG liposomes interacted with LDLr in an LDL-dependent fashion, and the interaction resulted in the functional delivery of a series of model liposome dependent drugs. Additionally, in the defined medium, formulations with at least 75 mole percent ePG displayed targeted cellular uptake via LDLr with an LDL mediated enhancement in delivery of liposomal contents.[105]

The possible mechanism of ePG vesicles interacting with LDL involves these fluid liposomes containing at least 75 mol ePG/100 mol phospholipid coalescing with LDL particles, thereby causing partial but not total loss of contents. The resultant liposomes, which would have apoB100 on their surface, would bind to the LDLr through an interaction of the receptor and apolipoprotein domains normally involved in the interaction of native LDL with the receptor. Therefore, the serum dependent association of fluid, anionic (ePG) liposomes with cells expressing the LDLr is caused by an interaction of ePG liposomes with LDL. The failure of this association to increase drug delivery in serum supplemented growth medium seems to be associated with the down regulation of LDLr expression when cells are continuously exposed to LDL.[111]

The pharmacological utility of the liposome-cell interactions described here depends on the ability of the liposomal formulation to effectively delivery pharmaceutical cargo to those cells over expressing the LDLr. The rationale for targeting to the LDLr is that

numerous cancer cell lines exhibit increased LDLr activity compared to normal cells.[115] In fact, several authors have proposed use of this receptor for the delivery of cytotoxic agents.[43,116] In the present study, a highly anionic fluid vesicle displayed targeted drug delivery via the LDLr with a functional delivery of liposomal aqueous contents by means of a spontaneous interaction with LDL. Additionally, the liposome-LDL complex proposed here would be an ideal drug delivery system such that all components are biocompatible and biodegradable. These liposome-LDL complexes would be quite analogous to the more extensively studied LDL-drug complexes, which have been shown to be effective *in vitro* and *in vivo*.[116,117] De Smidt and Van Berkel found an increased therapeutic effect of their LDL-drug complexes *in vivo* lending support to the view that liposome-LDL complexes may also prove useful in drug targeting.

An understanding of the cellular processes involved in the binding and internalization of liposomes is critical to the development of liposomal formulations that possess ideal characteristics for the delivery of various pharmaceuticals. The interaction of liposomes with cultured cells *in vitro* is complex and the cellular components involved in the entire process are not fully known. Lee *et al.* found that the differences observed in the uptake rate of liposomes with different lipid compositions seemed to be primarily due to the differences in the binding between liposomes and cell membrane components.[103] Here, I have attempted to identify the cellular components involved in the delivery of anionic liposomes to the LDLr. Additionally, I have sought to control the cellular processes involved by altering the components of the liposome. Vesicles containing 75-100 mole percent ePG associated with cells bearing the LDLr in a LDL mediated event. It is

obvious that there are other factors including a number of differing cell types as well as opsonization of liposomes that will contribute to liposome-cell interactions *in vivo*. Nevertheless, a better understanding of liposome-cell interactions *in vitro* will provide clues as to the behavior of liposomes *in vivo*.

CHAPTER SEVEN: REFERENCES

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