DELIVERY OF CYTOTOXIC AGENTS USING LOW DENSITY LIPOPROTEIN LDL : PHYSICO-CHEMICAL AND BIOLOGICAL EVALUATION OF LDL-DRUG CONJUGATES

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DELIVERY OF CYTOTOXIC AGENTS USING LOW DENSITY LIPOPROTEIN [LDL] : PHYSICO-CHEMICAL AND BIOLOGICAL EVALUATION OF LDL-DRUG CONJUGATES

by

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A thesis submitted to the school of graduate studies in partial fulfilment of the requirements for the degree of Master of Science in Pharmacy

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"..Yet all experience is an arch wherethrough,
Gleams that untravelled world Whose margin fades
Forever and forever when I move."

From Ulysses, Alfred Lord Tennyson (1809-1892).

To my nephews and nieces with love.

ABSTRACT

Low density lipoprotein (LDL) particles appear very promising for delivering anticancer drugs specifically to tumor or macrophage cells by exploiting the LDL or the scavenger receptor pathway. I have chosen doxorubicin [Dox] to investigate the feasibility of this approach in cancer chemotherapy. Dox could be directly incorporated into LDL particles. A lipophilic cholesteryl ester [CE] analogue, cholesteryl iopanoate [CI] was proposed as a radiotracer to study the *in vivo* fate of LDL. CI was radioiodinated with ^{125}I by a pivalic acid exchange reaction and the radiochemical purity was determined by HPLC in conjunction with γ-counting and was found to be more than 95% pure.

A new reverse phase HPLC procedure with UV detection was developed for the quantitation of CI. The regression line, and intra- and interday variations for a set of standards were determined and were found to be statistically valid. The minimum detection range was less than 10 ng for the compound. The % recovery from LDL was found to be more than 95%. Plasma protein binding of Dox was studied ex-*vivo*. Dox was found to be more than 30% lipoprotein bound. The plasma distribution of Dox was refashioned by preincubating plasma with oleic acid. With oleic acid, Dox association with lipoproteins increased from less than 30% to approximately 70%.

Mainly the contact method or the direct addition method was adapted to incorporate drugs into LDL particles. The loading techniques were optimized in terms of incubation time, temperature, and stoichiometry of LDL-drug conjugates. A four to six hour protocol and 37° incubation temperature were chosen with a drug to protein ratios more than 1. The

effect of various wetting agents, such as Tween 20, 40, 60 and 80, Span 60, Triton-X, and Celite 545, and ethanol was investigated for LDL-Dox conjugates in the contact method. Tween 20 was chosen for its favorable loading efficiency (more than 5-fold compared to the dry film method). Liposomal Dox was prepared by an extrusion technique with 24% loading efficiency. The liposomal Dox preparation was found to be the most suitable (45 molecules of Dox/LDL particle) compared to other methods such as the dry film method, the contact method with Tween 20, and the direct addition method.

All these incorporation methods were found to be suitable for generating LDL-drug conjugates without disrupting the native integrity of LDL particles when characterized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis [SDS-PAGE], electron microscopy [EM], and differential scanning calorimetry [DSC].

An insect lipid transfer catalyst [LTP] was studied and found to enhance drug loading into LDL particles by at least 2 to 5-fold, depending on the drug molecules and the incubation conditions. The LDL-drug conjugates generated by this transfer particle were characterized by SDS-PAGE and EM and found to be similar to native LDL. The site of drug location in LDL was studied by DSC and UV-visible scanning. The drug was found to be located both in the core and the outer monolayer of LDL for Dox. This kind of enhancement was not observed with human cholesterol ester transfer protein [CETP].

Dox interference in bicinchoninic acid [BCA] protein assay method was examined and it was found that Dox interfered [33-fold more sensitive compared to protein] with the protein assay method. A solution to overcome this interference was also suggested using the

Bradford method.

To target macrophages [Mϕ], native LDL was modified by acetylation [acetylated LDL, AcLDL] and Dox was loaded in AcLDL and physico-chemically characterized by SDS-PAGE and EM. The loading efficiency of AcLDL-Dox conjugates was comparable to that of LDL-Dox conjugates.

Different formulations of Dox were evaluated in cell culture studies using a human tumor cervical cell line, HeLa, and a mouse Mϕ cell line, J774.A1. LDL-Dox conjugates were found to be greater than 18-fold more cytotoxic than the corresponding free drug Dox using the [3-[4,5-dimethylthiazoyl-2-yl]-2,5-diphenyl-tetrazolium bromide], [MTT] assay in Hela cells. When the cytotoxicity of AcLDL-Dox conjugates was examined in J774.A1 cells, a more than 7-fold increase in cytotoxic effects was observed in comparison to its free drug counterpart, Dox.

Key Words: drug targeting; low density lipoprotein; cytotoxic agents; doxorubicin; insect lipid transfer protein; drug loading; liposome; electrophoresis; electron microscopy; differential scanning calorimetry; UV-visible sanning; acetylated LDL; macrophages; cytotoxicity; HeLa cells; J774.A1 cells; MTT assay; drug interference; HPLC; cholesteryl iopanoate; cholestryl ester transfer protein. protein assay

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LIST OF ABBREVIATIONS AND SYMBOLS

λ	Wave length
ACAT	Acyl CoA cholesterol acyltransferase
ACEI	Angiotensin converting enzyme inhibitor
AcLDL	Acetylated LDL
AD 32	N-(Trifluoroacetyl)adriamycin 14-valerate
AIDS	Acquired immune deficiency syndrome
AML	Acute myeloid leukemia
Amp B	Amphotericin B
ANOVA	Analysis of variance
Apo B	Apoprotein B 100 (in LDL)
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CD	Circular dichroism spectroscopy
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CI	Cholesteryl iopanoate
CLL	Chronic lymphocytic leukemia
CM	Chylomicron
CPM	Counts per minute
CT	Computer tomography
CV	Coefficient of variation
Da	Dalton
DAG	Diacylglycerol
DMAC	Dimethylacetamide
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNM	Daunomycin
Dox	Doxorubicin
DP	Diphosphate
DSC	Differential scanning calorimetry
EGF	Epidermal growth factor
ELISA	Enzyme linked immuno-sorbent assay
EM	Electron microscopy
EYPC	Egg yolk phosphotidylcholine
FCS	Fetal calf serum
FGF	Fibroblast growth factor
ΔH_{cal}	Change in enthalpy in endotherm
HDL	High density lipoprotein

HDLp	High density lipophorin
HMG CoA	3-Hydroxy-3-methylglutaryl Co-A
HPLC	High performance liquid chromatography
IC_{50}	Concentration required to reduce cell survival to 50%.
IV	Intravenous
kDa	Kilodalton
LCAT	Lecithin cholesterol acyltransferase
LDL	Low density lipoprotein
LPDP	Lipoprotein deficient plasma
LTP	Lipid transfer particle
Mϕ	Macrophages
MDR	Multiple drug reistance
MeLDL	Methylated LDL
MLV	Multilamellar vesicle
MPS	Mononuclear phagocytic system
MTP	Microsomal triacylglycerol transfer protein
MTT	[(3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyl-tetrazolium bromide]
MW	Molecular weight
MWCO	Molecular weight cut off
OxLDL	Oxidized LDL
P	Partition coefficient
PBS	Phosphate buffered saline
PDI	Protein disulfide isomerase
P-gp	P-glycoprotein
PL	Phospholipid
PLTP	Phospholipid transfer protein
R_f	Rate of flow in TLC
RES	Reticulo-endothelial system
Rpm	Revolutions per minute
RNA	Ribonucleic acid
R_S	Peak resolution in HPLC
RT	Room temperature
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TAG	Triacylglyceride
T_m	Melting temperature of lipids
TGF	Tissue growth factor
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TNF	Tissue necrosis factor

T_R	Retention time
UV	Ultaviolet spectroscopy
VCR	Vincristine
VLDL	Very low density lipoprotein

CHAPTER 1:

GENERAL INTRODUCTION

1.1. Current status of cancer chemotherapy

The development of techniques to selectively deliver cytotoxic agents to cancer cells, without concurrent adverse effects in healthy cells, is one of the fascinating areas of research in cancer chemotherapy. This interest is specifically centered towards optimizing the delivery of cytotoxic agents, which possess an intrinsic ability to discriminate cancer cells from normal cells [Duncan, 1992]. Unfortunately, the administration of current cytotoxic agents almost invariably causes dose-dependent systemic toxicities due to their non-selective biodistribution, often warranting discontinuation of treatment, and thus failure to allow successful eradication of cancer cells [Keizer *et al.*, 1985]. Moreover, maintaining low level exposure of tumor cells to a cytotoxic drug may also induce resistance, so continued exposure must be viewed with caution [Duncan, 1992]. Theoretically, total eradication of cancer cells could be accomplished by total surgical removal of the tumor or complete destruction of all cancer cells by chemo- and/or radiotherapy. Current treatment regimens do not meet such a challenge and new approaches to solve this predicament are warranted.

The therapeutic efficacy of current cancer treatment is often complicated by several factors [e.g., existence of subpopulations of neoplastic cells within a tumor which considerably differ in their morphology, immunogenecity, rate of growth, capacity to metastasize, and response to chemotherapeutic agents] [Calabrashi *et al.*, 1980]. In addition,

the total blood flow, rate of perfusion, and vessel penetrability may vary within different regions of the same tumor tissue [Woodruff, 1983, Poste and Kirsh, 1983; Fidler and Hart, 1982; Dexter *et al.*, 1978]. Because of these differences, an increase in dose may be needed to reach the cytotoxic concentration within cancerous tissues. In solid tumors, the permeability of the cytotoxic agents is often too low to be effective when administered at usual therapeutic concentrations [Brouwers, 1996; Hori *et al.*, 1993]. This necessitates administration of maximum tolerable dose which in turn increases the dose dependent toxicity. Different parenteral routes have been attempted to increase the concentration of cytotoxic agents into the target areas e.g., intra-arterial to cancer tissues [Bufill *et al.*, 1996], intrathecal in brain metastases, and intraperitonium in peritoneum carcinoma. Limited success was achieved in these parenteral approaches [Buchwald *et al.*, 1980; Chen and Gross, 1980; Eckman *et al.*, 1974].

Despite extensive efforts in experimental pharmacology and theoretical considerations, the overall success in routine cancer therapy has been nominal [Daemen *et al.*, 1988; Blacklock *et al.*, 1986; Levin, 1986] . Chemotherapy, as well as radiotherapy, has been disappointing because of acute side effects and complications [Chiuten *et al.*, 1986; Collin *et al.*, 1985] . These difficulties have urged the need for developing "specialized systems" which may allow selective delivery of one or more chemotherapeutic agents to cancer cells as this would prevent effects on the physiology of the normal cells. To meet these requirements, numerous targeted drug delivery systems have been proposed over recent decades [Naeff, 1996; Torchilin and Trubetskoy, 1995; Allen *et al.*, 1995, Gabizon, 1995;

Fidler and Kleinerman, 1994; Allen, 1994; Jones, 1994; Jalil, 1990; Pozansky and Juliano, 1984; Tomlinson, 1986 to 1991].

1.2. Targeted drug delivery

Theoretically, targeted drug delivery systems can improve the outcome of chemotherapy due to the following processes [Table 1.1]: [1] by allowing a maximum

Table 1.1. Rationale for drug targeting*

Exclusive delivery to specific compartments.
Access to previously inaccessible sites [*e.g.,* intracellular infections].
Protection of body from unwanted deposition which would lead to untoward reactions, metabolism, etc.
Controlled rate and modality of delivery to pharmacological receptor.
Reduction in the amount of active principle employed

*[After Tomlinson, 1987]

fraction of the delivered drug molecules to react exclusively with cancer cells, without having any harmful effect on normal cells; and [2] by allowing preferential distribution of drug to cancer cells. The first process can be classified as absolute drug targeting. In the

second process, complete eradication of cancer cells is not possible without some degree of destruction to normal cells; this process therefore falls in the category of partial drug targeting.

Drug may be delivered by [1] carrier-dependent, and/or [2] carrier-independent routes. In the former case, after localization in the target tissue, the drug carrier is taken up by the target cells and the drug is released intracellularly in a controlled manner. In the latter case, the drug is released from the carrier extracellularly and hence the drug action inside the target cells is not influenced by the ability [or inability] of the carrier to be taken up by these cells. From this discussion, it is clear that carrier-dependent targeted delivery may allow utilization of drugs which are active intracellularly, but are normally discarded due to their poor intracellular uptake. In such situations, appropriate selection of a drug carrier may allow greater influx of drug to the intracellular components and hence increase the overall efficacy of drug delivery. Although the concept of a magic bullet pioneered by Ehrlich [1956] may still be an unrealized dream in the context of specific delivery of cytotoxic agents to tumor cells, a number of carrier systems have been proposed over the years to achieve partial or complete drug targeting. For example, first order targeting whereby the carrier takes the drug to a particular organ; second order targeting whereby the carrier is directed towards a particular diseased part of an organ; and third order targeting where the carrier takes the drug molecule into the cell by whatever mechanism that may be hypothesized.

Significant advances have been made with the identification of many delivery systems that achieve very effective organ/compartmental [first order] drug targeting.

Liposomes, particulate systems, and macromolecular carriers have been developed which can deposit a large percentage of an intravenous [IV] dose into the liver or lung [Gabizon, 1995]. One potential advantage of liposomes as drug carriers is that they are easy to load with drugs, both lipid- and water-soluble [Crommelin *et al.*, 1995]. Early *in vitro* experiments with liposomes were encouraging, but when drug-containing liposomes were administered to animals, the liposomes were recognized as non-self and hence rapidly removed from the circulation by the reticuloendothelial system [RES] [Poste, 1983; Woodle, 1995; Allen *et al.*, 1995]. A major problem with liposomes as drug carriers for treatment is the lack of a homing device to direct liposomes to tumor cells [Crommelin *et al.*, 1995]. Immunoliposomes were proposed to increase the targeting potential of liposomes. So far, very little success has been achieved *in vivo* [Mori and Huang, 1995]. A more frequently encountered limitation of liposomal delivery systems is their relatively low stability, both *in vitro* and *in vivo* [Naeff, 1996; Poste, 1985 and 1983; Allen and Cleland, 1980]. Stability problems and variable delivery to the tumor have been identified as major limitations of liposomal formulations of cytotoxic drugs [Janknegt, 1996; Codde *et al.*, 1993]. In short, this approach holds promise of regional delivery of cytotoxic drugs for treatment of primary or secondary disease, but clinical success has not been achieved.

To achieve tumor-specific [second order] targeting it is necessary to identify unique features of tumor cell biology that will concentrate drug[s] within the tumor. From the standpoint of selectivity, antibodies are very attractive as drug carriers. Most approaches have sought to produce monoclonal antibodies that will interact preferentially with tumor cell

surface antigens [Magerstadt, 1990]. Although tumor cells do express tumor enhanced, or specific-antigens, true tumor-specific antigens probably do not exist [Daemen *et al.*, 1995]. Another problem is the existing circulating antigens that could bind and neutralize the antibodies before they reach the tumor site. Also, the coupling of drug molecules to antibodies may interfere with antigen recognition and/or with the activity of the drug. Other potential limitations are: limited tumor access of these relatively large macromolecules; tumor cell heterogeneity; and the human antimouse antibody response experienced in patients [Duncan, 1992]. Even when using antibodies of the highest affinity and specificity a relatively small fraction of administered dose is delivered to the tumor *in vivo* [possibly less than 0.1% dose administered in man] [Gupta, 1990], but it is encouraging that this relatively small localization can theoretically be put to good use, exemplified by the antibody-directed enzyme prodrug approach [Springer *et al.*, 1991]. Immunoliposomes, the artificial combination of liposomes and antibodies, have drawn great attention recently [Mori and Huang, 1995]. Most of these immunoliposomes deliver drugs to the vicinity of the cells but fail to internalize the agents into cells. This reduces the efficacy of anticancer drugs that act intracellularly at the DNA level and must enter the cells by endocytic mechanisms. Repeated injections of immunoliposomes were ineffective in prolonging the survival of tumor bearing animals [Mori and Huang, 1995]. A spectrum of other cell surface receptors have been proposed as candidates for tumor selective targeting and examples of those explored experimentally are listed in Table 1.2.

There are many factors to consider when designing systems to target cell surface

receptors: the homogeneity of receptor expression within a tumor, the number of receptors available per cell and their ligand affinity; the possibility of up- and downregulation following exposure to the targeting ligand; and not least the cellular fate of the receptor ligand complex. In particular knowledge of the number of receptors expressed at any time is crucial as receptor saturation would obviously decrease efficiency of targeting [expressed as a percentage of the dose administered] if the dose given per bolus was increased without prior consideration of this point. Considering all these parameters, low-density lipoprotein [LDL] receptors seem to be the most realistic approach which will be discussed in detail elsewhere in this text.

1.3. Criteria of a drug carrier

My primary goal is to propose a targeting carrier for cytotoxic drugs. Theoretically, a good drug carrier for *in vivo* use should meet the following criteria:

1. the drug carrier conjugate must be stable, both during storage and *in vivo*.
2. the carrier should be biocompatible and biodegradable, and the drug carrier conjugate must not produce unacceptable levels of toxicity or immunological reactions.
3. the carrier should be suitable for targeting.
4. the drug carrier conjugate must allow release of the drug at the target site.
5. the carrier must not cause unspecific uptake by nontarget cells.
6. for large scale clinical use, the carrier system must be pharmaceutically acceptable in regard to formulation homogeneity, cost of manufacture, ease of handling and

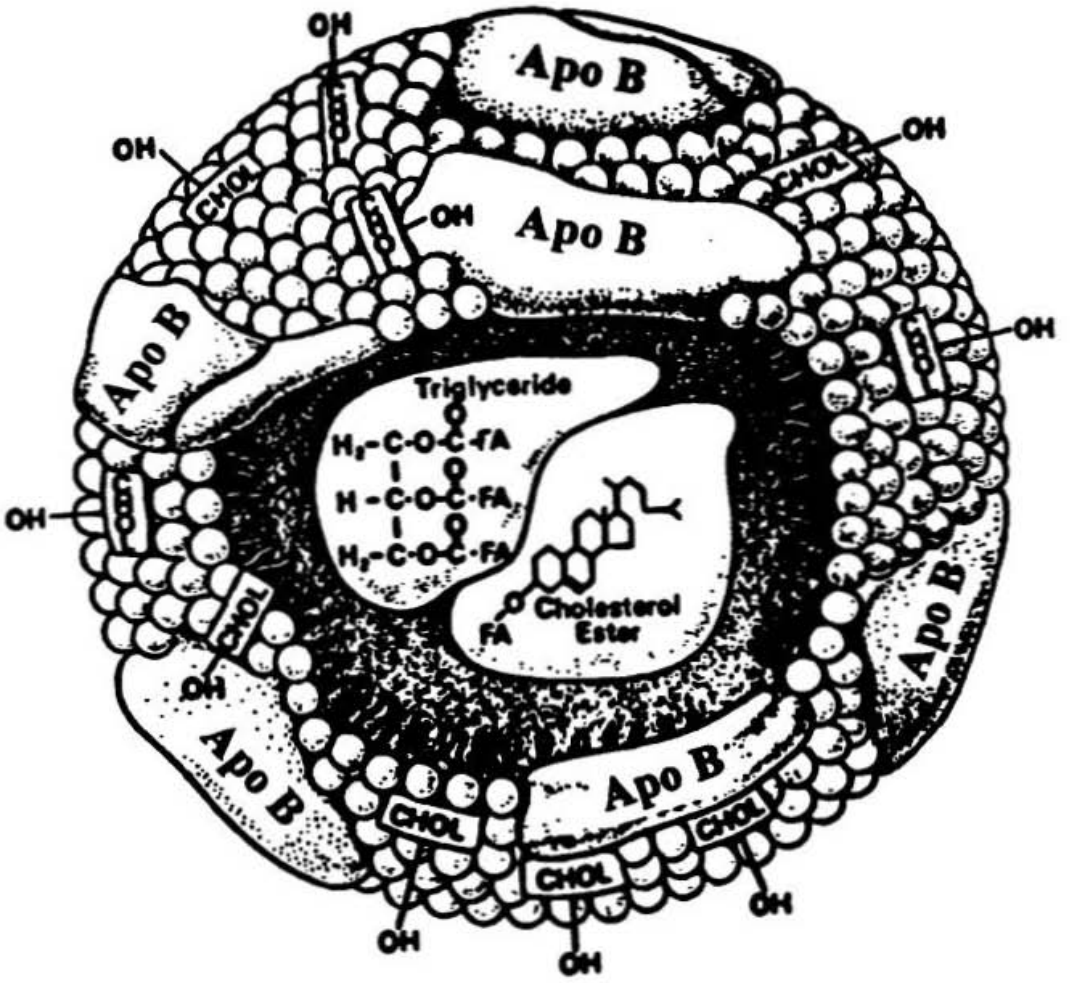

Figure 1.1. **Schematic model of low density lipoprotein [LDL].** The surface of the LDL particle contains the polar-head groups of the phospholipids. The apolipoproteins as well as cholesterol are intercalated between the polar-head groups of the phospholipids. The neutral lipids, cholesteryl esters and triglycerides, are localized in the core of the LDL particle. CHOL, cholesterol, FA, fatty acid. [After Brewer, 1994]

Table 1.2. Tumor markers as targets for second order drug delivery*

Type	Comments
Receptor involved in constitutive biochemical pathways	
LDL receptors	Selective delivery of cytotoxic agents to tumor cells is possible after appropriate up- and downregulation schemes discussed throughout the text.
Transferrin receptors	The surface density of transferrin receptors has been correlated with the degree of malignancy and proposed as a tumor selective target [Trowbridge and Domingo, 1981]. However, the broad cellular distribution of this receptor has prevented fruitful use for drug delivery.
Growth-factors receptors	Many tumors have been reported to overexpress receptors for growth factors such as epidermal growth factor [EGF] [King *et al.*, 1990] and fibroblast growth factor [FGF] [Robinson, 1991]. The EGF receptor is overexpressed in 20-30 % of breast cancers.
Melanocyte stimulating hormone [MSH]	Melanocytes and malignant melanoma have a receptor which recognizes the peptide hormone MSH. Binding of MSH increases the levels of intracellular cAMP and stimulates tyrosinase activity, in the [MSH] course of melanin production. Because of the relative selectivity this receptor has been used as a target for MSH-toxin constructs, antibody conjugates and other ligands [Ghanem *et al.*, 1988].
Cellular adhesion/recognition systems	
	Several aspects of cellular recognition and adhesion have been proposed as targets for chemotherapy, including laminin and fibronectin receptors.

*[Modified after Duncan, 1992]

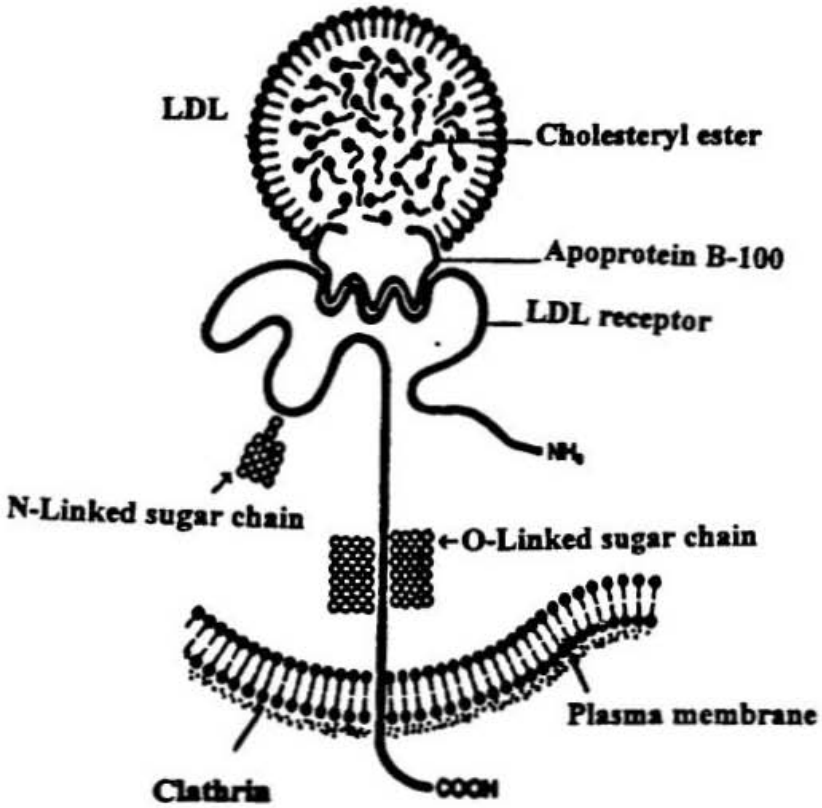

Figure 1.2. **Schematic representation of the LDL receptor,** a glycoprotein embeded in the plasma membrane of most body cells. The DNA's nucleotide sequence was determined and sites of attachment of sugar chains to nitrogen and oxygen atoms were identified. Additionally, [1] an eleven amino acid region enriched in arginine and lysine residues as an LDL receptor binding site and [2] potential lipid binding regions which contain proline-enriched beta-sheets [Knott *et al.*, 1985] were reported. (After Brown and Goldstein, 1994).

administration. Lipoproteins meet most of the above requirements for a satisfactory drug carrier. This issue will be discussed later.

1.4. Cholesterol and cancer

Before justifying LDL as a carrier in cancer chemotherapy brief background studies regarding cholesterol metabolism and cancer are presented. In the 1930s, Muller noted that a low plasma cholesterol level was frequently associated with leukemia [Muller, 1939]. Epidemiological studies done in recent decades have demonstrated a correlation between plasma cholesterol levels and risk of cardiovascular disease. In some of these studies, concomitant analysis of cancer incidence has shown an unexpected correlation between low plasma cholesterol levels and cancer [Sherwin *et al.*, 1987; Feinleib, 1983]. Two hypotheses have been proposed to explain this observation: [1] hypocholesterolemia is envisaged as a risk factor for the development of malignancy, and [2] hypocholesterolemia is conceived as a metabolic consequence of an existing cancer. With regard to the first hypothesis, it is possible that individuals maintaining a low blood cholesterol level excrete increased amounts of biliary sterols and that bacterial metabolism of these sterols in the gut could result in an increased production of carcinogenic sterols [Reddy, 1981]. The second hypothesis is supported by studies demonstrating that the lowest cholesterol levels were found in subjects who presented clinically overt cancer the soonest [within 2 years] after blood sampling [Rose and Shipley, 1980; Cambien *et al.*, 1980]. The clinical studies discussed above show that plasma cholesterol levels in newly diagnosed cancer patients are

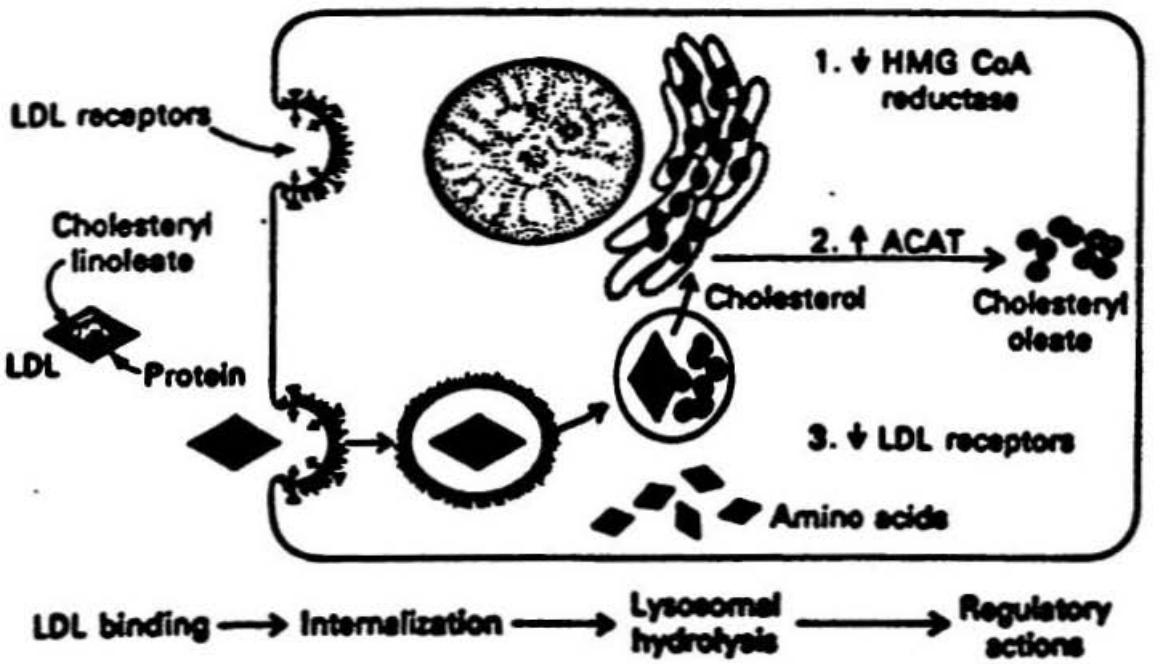

Figure 1.3. **Steps in the LDL pathway *in vivo*.** HMG CoA reductase denotes 3-hydroxy-3-methylglutaryl CoA reductase, and ACAT denotes acyl CoA: cholesterol acyltransferase. [After Brown and Goldstein, 1986].

related to tumor burden. It is now abundantly clear that it is cancer that brings about a reduction in cholesterol, and not low cholesterol that causes cancer [Kritz *et al.*, 1996]. The reduced cholesterol often observed in cancer patients is the result of uptake of LDL by the tumor and not the cause of the cancer [Kritz *et al.*, 1996; Lackner *et al.*, 1989].

1.5. Fate of LDL in the body

Structurally, LDL consists of an apolar core, composed mainly of cholesteryl esters [CE] and to a lesser extent, triacylglyerol [TAG], surrounded by a monolayer of phospholipid[s] [PL] in which cholesterol and apoprotein B-100 [apo B] are embeded [Figure 1.1 and Table 1.3]. LDLs are formed during the metabolism of very low density lipoproteins [VLDL]. LDL is slowly cleared from the circulation via specific LDL [apo B, and/or E] receptors [Figure 1.2] that interact with the apoprotein [Brown *et al.*, 1986]. After binding to the receptor, LDL is internalized and degraded in the lysosomal compartment [Figure 1.3]. The released unesterified cholesterol can be used for membrane or steroid synthesis. Alternately, it can be re-esterified for storage inside the cell by acyl Co-A:cholesterol acyl transferase [ACAT], an enzyme stimulated by available cholesterol inside the cell. High cholesterol content can suppress the transcription of the gene for 3-hydroxy-3-methylglutaryl Co-A [HMG-Co A] reductase, a key enzyme for *de novo* cholesterol biosynthesis. High cholesterol content inside the cell downregulates LDL receptor biosynthesis, and thus the additional uptake of LDL is inhibited [Sudhof *et al.*, 1985] [Figure 1.4]. The expression of LDL receptors on the surface of a cell is carefully regulated by the

cholesterol status of the cell [Goldstein and Brown, 1990].

Table 1.3. Physiochemical properties and composition of human LDL*

Density	1.019-1.063 g/mL	
Size	20-25 nm	
Phospholipids [PL]	18-24%	800 molecules
Cholesterol	6-8%	500 molecules
Cholesteryl esters [CE]	45-50%	1500 molecules
Triacylglycerols [TAG]	4-8%	500 molecules
Protein, apo B	18-22%	1 molecule

*[After De Smidt and Van Berkel, 1990]

1.5.1. Catabolism of LDL

In normal humans, the plasma half life of LDL is 3-4 days [Spady, 1991]. The rate of LDL uptake in hamster, rat, and rabbit, when expressed per gram of tissue, is the highest in the endocrine organs, liver, small intestine, and spleen. Somewhat lower rates of uptake are observed in the kidney, lung, colon, heart, and stomach. Importantly, extremely low rates of LDL uptake are found in the major tissue compartments of the body such as skeletal muscle, adipose tissue, skin and brain. Information concerning rates of LDL uptake in the various organs in humans is not available. However, circulating LDL levels were found to fall 50% in a patient with a genetic defect in the LDL receptor pathway who received a

normal liver transplant, suggesting that the liver also accounts for the majority of LDL turnover in humans [Bilheimer *et al.*, 1984]. In fact, this organ accounts for the clearance of at least 40% of the circulating LDL. Both the parenchymal liver cells and the Kupffer cells, are involved in the uptake of LDL [Van Berkel *et al.*, 1990].

1.5.2. Regulation of LDL receptor activity in malignant cells

Cancer cells show an increased uptake of LDL. This increased uptake is caused by an elevated LDL receptor expressed on the cellular surface. The evidence is 2-fold: measurements of LDL uptake by tumor cells and depletion of LDL in the blood of cancer patients resulting from high uptake by the tumor [*vide infra*] [Firestone, 1994]. However, it is not clear why LDL receptor activity is elevated in cancer cells. One possibility is that proliferating cells have an increased cholesterol demand for membrane synthesis [Gal *et al*,. 1981; Kruth *et al.*, 1979]. Alternatively cancer cells may lose more cholesterol from cell membranes than do normal cells because of an accelerated membrane turnover. Finally a defect in the regulation of the LDL receptor in cancer cells could be another explanation for the elevated receptor activity [Vitols, 1991; Ho *et al.*, 1978].

The first report regarding cancer and LDL demonstrated that human acute myeloid leukemia [AML] cells take up 3- to 100-fold more LDL than normal cells [Ho *et al.*, 1978]. Human AML cells take up 4- to 25-fold more LDL than normal white blood cells [Vitols *et al.*, 1984]. It was observed later that the high *in vitro* uptake of AML correlates with high *in vivo* uptake [Vitols *et al.*, 1990]. Human monocytic [FAB-M5] and myelomonocytic

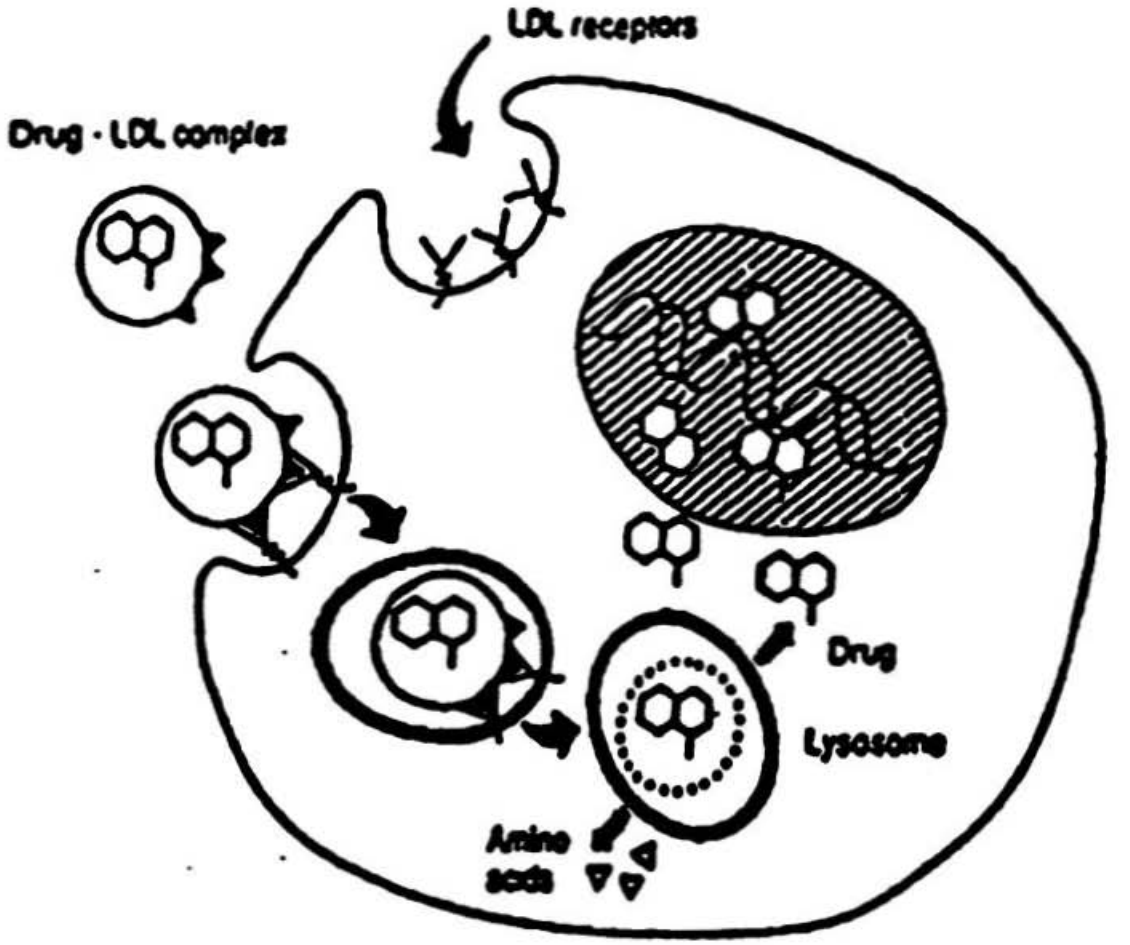

Figure 1.4. Receptor mediated endocytosis of LDL-drug conjugates. Circulating cytotoxic drug loaded LDL is taken into a cell by a receptor mediated endocytosis. Once internalized, LDL release toxic drug inside the cell where the drug exerts its cytotoxic action such as intercalation with DNA. [After Vitols, 1990].

[FAB-M4] leukemias and chronic myeloid leukemia in blast crisis [but not acute lymphoblastic leukemia] take up much more LDL than normal mononuclear cells, peripheral granulocytes, or nucleated bone marrow cells [Vitols *et al.*, 1984].

Some solid tumors are also avid for LDL. Epidermoid cervical cancer EC-50 absorbs 15-fold more LDL than fetal adrenal tissue [which has exceptionally high uptake] and 50-fold more than normal gynecologic tissue. Endometrial adenocarcinoma AC-258 absorbs 10-fold more than normal cells [Gal *et al.*, 1981a]. EC-50 and four other gynecologic cancers have greater LDL uptake than normal cervical tissues [Gal *et al.*, 1981b]. Gastric carcinoma and parotid adenoma exceed every normal cell type in term of LDL receptor numbers [Rudling *et al.*, 1990a]. Many brain tumors bind 2- to 3-fold more LDL than normal brain, especially medulloblastoma, oligodendroglioma, and malignant meningioma [Rudling *et al.*, 1990b]. In most of a group of nine patients, lung tumor tissues' uptake exceeded that of the neighboring normal lung by 1.5- to 43-fold [Vitols *et al.*, 1992]. Other tumors have been reported to have high LDL uptake; such as, glioma V-251MG [Vitols *et al.*, 1985], G2 hepatoma [Hep G2] [Dashti *et al.*, 1984; Havekes *et al.*, 1983], squamous lung tumor [Kerr *et al.*, 1988], and choriocarcinoma [Simpsons *et al.*, 1979]. Most human tumors have not yet been surveyed , so it is reasonable to suppose that many more will be found to have exceptionally high LDL requirements.

The most sinister aspect of cancer is its tendency to spread, or metastasize, throughout the body. This is most often the cause of death, even after resection of the primary tumor, because metastasized tumor cells are not only difficult to find but also

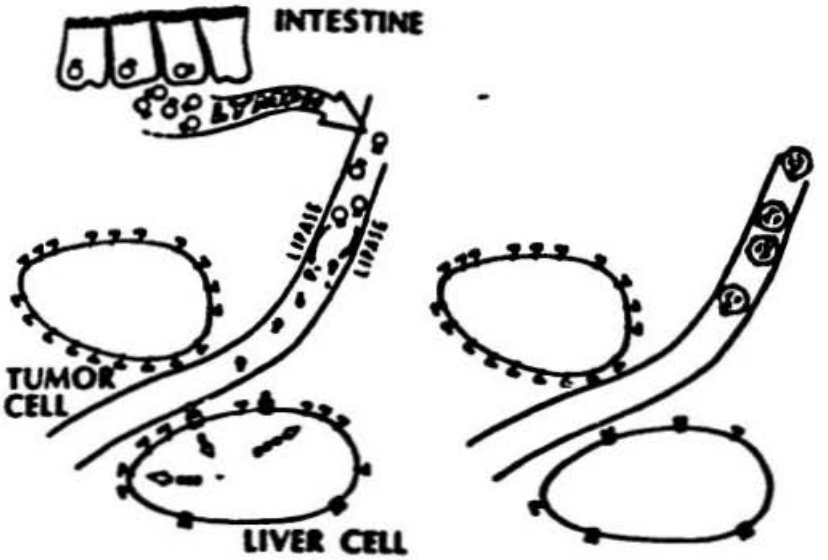

Figure 1.5. **Influence of dietary fat on LDL receptor regulation in liver and tumor cells.** When taking special diets, cholesterol and fatty acids are absorbed and processed in the intestine to become chylomicrons (CM). After hydrolysis of TAG on the capillary endothelial cells, CM are converted to remnants which are selectively internalized by liver cells. The liberated cholesterol and fatty acids will downregulate LDL receptor synthesis in these liver cells. Tumor cells will not be influenced by dietary fat [left]. After this pretreatment, a high degree of tumor specificity can be expected when cytotoxic LDL is injected in the bloodstream [right]: LDL receptors; chylomicrons; chylomicron remnant receptors; chylomicron remnants; LDL. [After Van Berkel *et al.*, 1990].

difficult to kill with standard chemotherapy. Therefore, particularly noteworthy is the small but growing body of evidence that tumor cells that are either exceptionally metastatic [Schroeder *et al.*, 1984; Cambien *et al.*, 1980], aggressive [Muller *et al.*, 1989; Rudling *et al.*, 1986; Peterson *et al.*, 1985], or undifferentiated [Zyada *et al.*, 1990; Ponec *et al.*, 1985; 1984] are also exceptionally high in their LDL requirements. If this is borne out in future studies, LDL based therapy will be even more valuable than it presently appears.

Some tumors, however, do not internalize great amounts of LDL, e.g., AML [Vitols *et al.*, 1984], chronic lymphocytic leukemia [CLL] [Juliusson and Vitols, 1988], several colon adenocarcinomas [Fabricant and Broitonan, 1990], Lewis rat renal carcinoma [Clayman *et al.*, 1986], cervical cancer EC-168 [Gal *et al.*, 1982], epithelioid carcinoma A-431 [Anderson *et al.*, 1981], and guinea pig leukemic lymphocytes [Saint-Marie *et al.*, 1986]. The latter three have ample LDL receptors, but internalization is deficient. Thus, it is important to show not only binding but also internalization of LDL before concluding that a given cell type is ripe for LDL targeting.

It is noteworthy to mention that some noncancerous pathogens have also been found to have high LDL requirements and therefore are candidates for LDL based therapy [Bakker, 1995; Coppens *et al.*, 1993, 1992, 1988; Bennet and Caulfield, 1991; Chaudhuri, 1989 Peterson and Alderete, 1984]. This non-neoplastic potential of LDL therapy will not be discussed here.

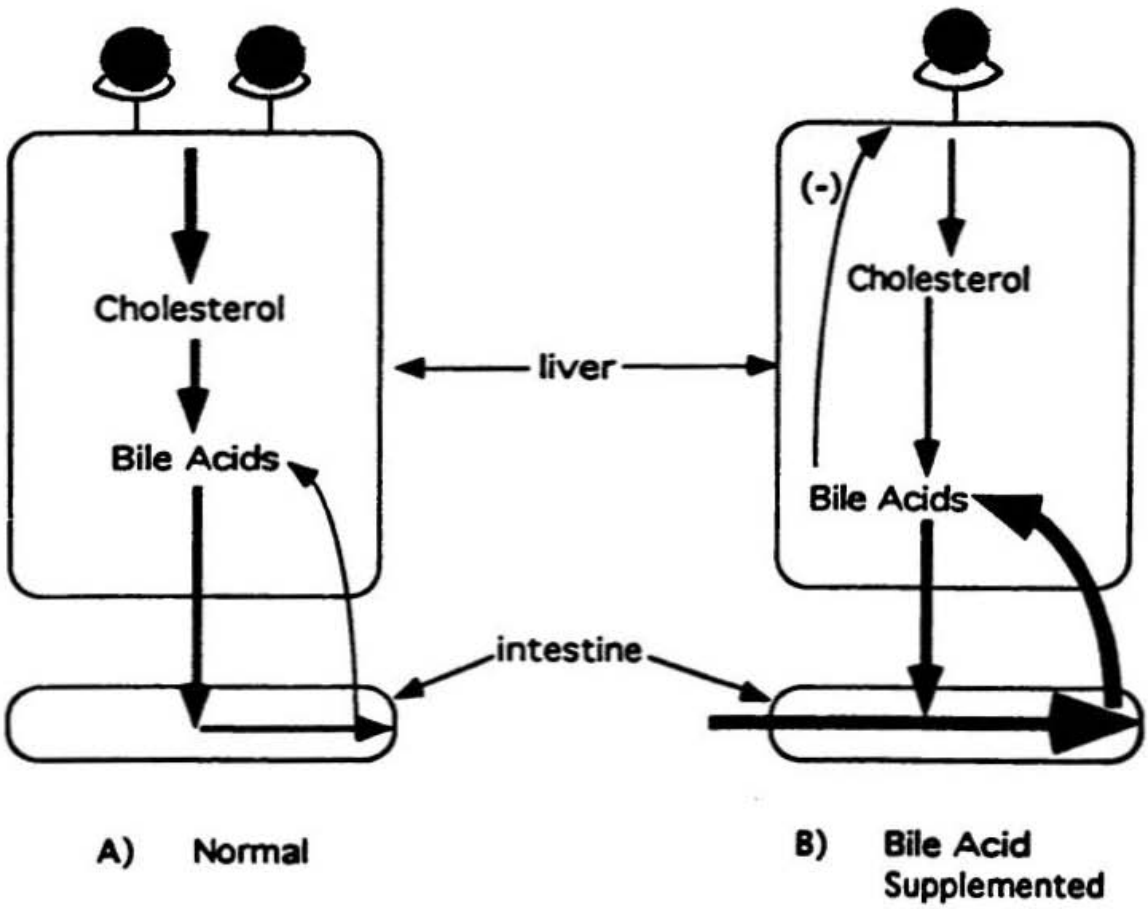

Figure 1.6. Rationale for the use of a bile acid supplements in the downregulation of LDL receptor uptake in liver. [After Brown and Goldstein, 1986].

1.5.3. Up- and downregulation of LDL receptors in cancer patients

It is not possible to obtain an absolute tumor specificity since normal cells also express LDL receptors. The organs most likely to suffer from the cytotoxic effects of LDL-drug conjugates are those with a high LDL uptake, such as the liver because of its size and LDL receptor content, and the adrenals whose uptake per gram is particularly high. The protection of the liver and adrenals is nevertheless desirable. The extent of liver toxicity is hard to predict since many drugs are degraded to less toxic metabolites in the liver. Although a LDL-drug conjugate may accumulate to large amounts in the liver, it is possible that the organ may metabolize and excrete the drug without suffering heavy damage [Vitols, 1991].

As indicated by animal studies, it may be possible to circumvent adrenal and liver toxicity by pretreatment with steroids [Hynds *et al.*, 1984] and bile acids [Dolecek *et al.*, 1996; Angelin *et al.*, 1983] [Figure 1.5]. Down regulation in the uptake of LDL by liver can be achieved by feeding saturated fats [Spady and Dietschy, 1985], cholesterol with hydrogenated coconut oil [Spady and Dietschy, 1985], and fasting [Shimano *et al.*, 1988], which is said to downregulate LDL receptors on healthy but not tumor cells [de Smidt and Van Berkel, 1990] [Figures 1.5 and 1.6]. Two new strategies were proposed by Firestone [1994]. The first strategy would be delivering cholesterol as acetylated LDL [AcLDL], methylated LDL [MeLDL], or oxidized LDL [OxLDL] to the liver through the scavenger receptor pathway. Cholesterol liberated from this pathway enters the metabolically active pool [Fox and Dicorleto, 1986] and thus presumably functions normally in downregulating

LDL receptor expression. Therefore, the liver which is rich in scavenger [Pitas *et al.*, 1985] and normal LDL receptors, might be down-regulated by cholesterol loaded AcLDL, MeLDL, or OxLDL which would not downregulate LDL receptor expression on tumor cells [Firestone, 1994]. Sinusoidal epithelial cells of the spleen, bone marrow, and ovaries also take up AcLDL [Pitas *et al.*, 1985] and might be down-regulated at the same time. The second strategy is related to using angiotensin II. Angiotensin II increases the uptake and receptor number of LDL in the adrenal gland [Leitersdorf *et al.*, 1985]. Therefore, angiotensin converting enzyme inhibitors (ACEI), which prevent angiotensin II formation, might then reduce LDL uptake by the adrenals.

Increasing the activity of LDL-mediated endocytosis in tumor cells would also be beneficial, provided normal cells were not concomitantly upregulated. LDL receptor activity is indeed stimulated in normal cells by 3-hydroxy-3-methylglutaryl Co-A [HMG CoA]-reductase inhibitors [Shaw *et al*,. 1990; Bilheimer *et al.*, 1983; Kovanen *et al.*, 1981], or bile acid sequestrants [Reihner *et al.*, 1990; Bilheimer *et al.*, 1983; Kovanen *et al.*, 1981], verapamil [Filipavic *et al.*, 1986], cachectin, and some growth factors [Horada *et al.*, 1990], tissue growth factor [TGF]-ß [Nicholson and Hajjar, 1992], tissue necrosis factor [α-TNF], and IL-1 [Hamanaka, 1992]. Compactin is reported to upregulate LDL receptors on Hep-G2 cells with little effect on normal human fibroblasts [Cohen *et al.*, 1984]. Oncostatin M potently upregulates LDL uptake by Hep G2 cells, more than normal cells [Grove *et al.*, 1991]. It is difficult to verify without clinical studies whether upregulation of tumor LDL receptor activity by the above methods would or would not be therapeutically beneficial.

1.6. Evaluation of LDL as drug carriers

Effective anticancer therapy requires a tumor specific carrier. LDL was proposed as a targeting vehicle in 1981-82 [Counsell and Pohland, 1982; Gal *et al.*, 1981] and has been reviewed several times since then [Van Berkel, 1993; Vitols, 1991; Peterson *et al.*, 1991; de Smidt and Van Berkel, 1990; Catapano, 1987]. The advantages of using LDL particles as drug carriers are:

1. they are endogenous and can avoid such typical carrier problems as immunological reaction and rapid plasma clearance due to uptake by RES; they have relatively a long half life in plasma and tissue fluids [in human $t_{1/2}$ is 2-4 days];
2. their intracellular uptake [>70%] via receptor mediated endocytosis enables the release of the incorporated drug in specific tissues;
3. the lipoprotein particle is totally biodegradable;
4. their small particle size allows penetration from the vascular to the extravascular compartment; and
5. they provide a biocompatible vehicle for lipophilic drugs, which is of special importance if the drug is prone to decomposition.

Although the LDL possesses favorable properties concerning loading factors, immunogenicity, toxicity, and applicability to disease processes, there are certain disadvantages associated in using lipoproteins or LDL as drug carriers, for example,

1. their complex nature and
2. their potential to produce cytotoxicity to normal cells through defective targeting.

1.6.1. LDL particles as carriers of cytotoxic agents

The potential of LDL as carriers for cytotoxic agents compared to other available carriers currently in development will be evaluated in this section. Growing interest in the use of drug delivery in cancer chemotherapy has been in part due to the limited progress in the successful development of effective new drug entities, but is also due to the realization that new approaches must be adopted if we are to achieve an improvement in therapeutic activity [Connors, 1989]. As pointed out earlier many different systems have been explored : low molecular weight prodrugs [Walker *et al.*, 1989], macromolecular carriers, immunoconjugates [Baldwin *et al.*, 1990], natural [Sezaki *et al.*, 1989] and synthetic polymers [Krinick *et al.*, 1991], vesicular or particulate systems, liposomes, [Rahman *et al.*, 1980], nanoparticles [Couvier *et al.* 1990], microparticles for regional therapy [Kerr and Keye, 1991], polymeric implants [Word *et al.*, 1989], and use of devices such as infusion pumps [Blackshear, 1989].

Several of these technologies have been tested clinically [infusion pumps are in routine use], but it is certainly true that many [including immunoconjugates and liposomes] have, as yet, failed to realize their promise clinically. Liposomes are attractive drug carriers because they are easy to load with drugs of different physicochemical properties. Antibodies have another attractive property since they specifically interact with the antigen. Ideally one should try to combine the attractive features of these two carrier systems. At least some of the problems encountered with liposomes and antibodies as drug carriers could be avoided using LDL as a natural liposome that is taken up into cells by a specific receptor-mediated

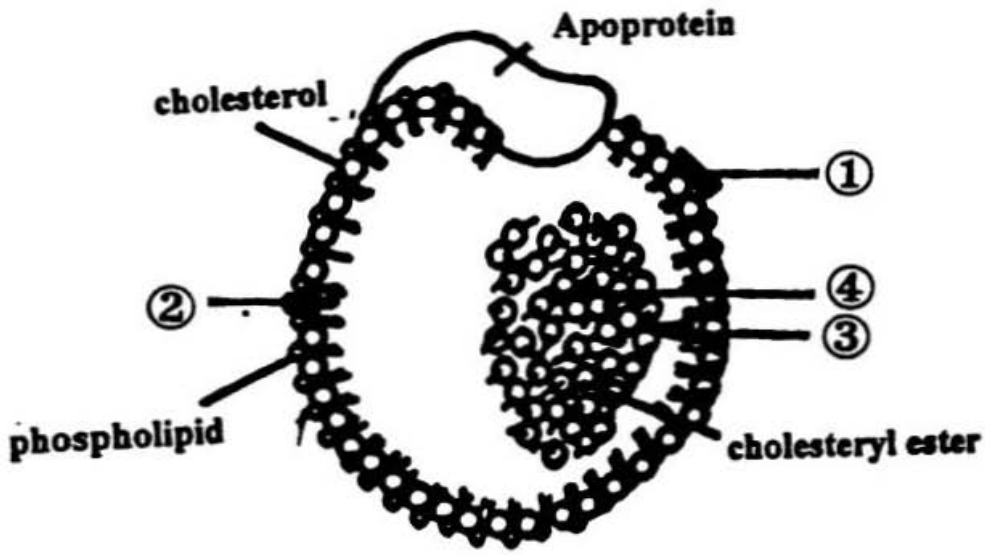

Figure 1.7. **Sites of incorporation of drugs and other active [and inactive] moieties onto or into LDLs:** [1] surface attachment; [2] penetration into the PL-cholesterol monolayer; [3] solubilization at the CE-monolayer interface; or [4] solubilization in the lipid interior of the particle. [After Florence and Halbert, 1991].

endocytosis. Since several malignant cell types express more LDL receptors than normal cells, selective uptake and cytotoxicity is possible as discussed earlier. In fact, one human trial of LDL-vincristine conjugates in Europe warrants further investigation of this approach in cancer chemotherapy [Breeze *et al.*, 1994; Filipowska *et al.*, 1992].

1.6.2. Sources of LDL for large scale use as targeted carriers in cancer chemotherapy

An advantage associated with LDL mediated cancer chemotherapy is the easy availability of lipoproteins clinically in addition to commercial source[s]. There are mainly two sources of human LDLs, [a] LDL isolated directly from the patient [Yamamoto *et al.*, 1992; Franceschini *et al.*, 1991, Schultis *et al.*, 1990; Behm *et al.*, 1989; Saal *et al.*, 1986; Parker *et al.*, 1986; Wieland and Seidel, 1983; Goldstein and Brown, 1977] and [b] LDL isolated from plasma of other individuals [Edelstein and Scanu, 1986; Schumaker and Puppione, 1986]. The use of plasmapheresis for the isolation of LDL and the preparation of LDL-drug conjugates from the patients' own LDL has also been proposed [Lundberg and Suominen, 1984].

1.7. Incorporation of biologically active molecules into lipoproteins

The nature of cytotoxic drug association with lipoproteins is a complex phenomenon. This topic is the main focus of this section. There are at least four potential sites for interaction, excluding covalent interactions [Figure 1.7], which have analogs in the sites of uptake into or onto lipoprotein system[s]:

1. Adsorption onto the surface either to the protein moiety or to PL head groups.
2. Incorporation into the PL monolayer.
3. Incorporation into the interface between the lipid core and the PL monolayer.
4. Incorporation into the lipid core.

Adsorption may be mediated by hydrophobic or electrostatic interactions. The principle hydrophobic interaction will be with the apoprotein, whereas electrostatic interactions may occur between the PL charged groups and oppositely charged solutes [Chen *et al.*, 1987; Davis and Illum, 1986; Willmott *et al.*, 1985] as the result of partitioning between the largely aqueous external phase and the particle, the ultimate destination [i.e., depth of penetration] of the solute will be determined by its polarity. Amphiphilic solutes are likely to be sited in the PL monolayer or in the lipid interior, provided they are miscible with the CE-TAG core. Lipophilic molecules will partition into the lipid core, while lipophilic, polar molecules will straddle the core and the PL region.

The surface attachment of drug molecules has certain disadvantages in the case of LDL, the LDL particles generally show a greater tendency to aggregate, possibly because of the loss of surface charge. Any ionic drug neutralizes the surface charge if bonded covalently with apo B protein. The most serious problem is the potential interference with the receptor specificity of the system if drugs are bound to the apoprotein [Mahley *et al.*, 1977].

1.7.1. Effect of drug location in LDL

The apolar core of the lipoprotein provides an ideal domain for highly lipophilic

drugs. Drugs that are stored in the core of a lipoprotein carrier are protected from the environment during transport. This will prolong their plasma half life and stability if the drug is readily degraded in plasma. Furthermore, a drug that is located inside the lipid moiety of a lipoprotein carrier will not disturb the recognition of the apoprotein present on the surface of the particle.

Drugs, for example, amphiphilic compounds, may not be incorporated into the apolar core, but into the more amphiphilic PL shell. In this case, the compound is not shielded from the environment during transport and it may also affect the specific uptake of the carrier by the receptor dependent pathway.

1.7.2. Designing of drugs to incorporate into LDL particles

An improvement of the lipophilicity of a drug increases the possibility of enhancing its ability to penetrate membranes and reach its site of action. This can be done by choosing molecules with high octanol/water partition coefficients [P] or those molecules with distinct lipophilic and hydrophilic regions [i.e. amphipathic]. Some highly lipophilic drugs [e.g., porphyrin containing compounds, diphenylhydantoin, reserpine, and estradiol] incorporate spontaneously into lipoproteins [Yoo *et al.*, 1986; Iwanik *et al.*, 1984; Yanovich *et al.*, 1984; Ramsen and Shireman, 1981]. However, most of the drugs that are currently used for the treatment of diseases are too hydrophilic for spontaneous incorporation. For incorporation, these drugs have to be rendered more lipophilic by the coupling of lipophilic groups. A water soluble drug can be rendered amphipathic by covalent linkage via acyl ester, amide,

or phosphate ester to biologically compatible lipophilic functional groups such as fatty acid, cholesterol, diacylglyceride (DAG), or PL.

Several studies suggest that if a compound is provided with certain groups that render it compatible with LDL's PL coat or lipophilic core, this will facilitate more drug incorporation in LDL. Oleyl, retinyl, and cholesteryl were proposed as suitable groups for this purpose [Marsh, 1974]. However, this suggestion was proven null and void as compounds with these groups did not show higher incorporation efficiency. Later, Shaw *et al.,* [1989] incorporated a variety of compounds with dissimilar structures that had lipophilic characters. Any lipophilic drug is considered suitable to load into LDL and there is no strict structural requirement considered to optimize drug loading efficiency [Barel *et al.*, 1986; Candide *et al.*, 1986; Reyftmann *et al.,* 1984; Counsell *et al.*, 1982].

To deliver a drug that will be pharmacologically active by the LDL mediated pathway, the following conditions have to be met.

[1] The drug needs to possess a high affinity for the lipid moiety of the lipoprotein carrier, either of its own, or after the attachment of hydrophobic residues.

[2] The [derivatized] drug should remain firmly associated with the carrier during transport in the circulation.

[3] If present, the hydrophobic anchor attached to the drug needs to be removed in the lysosomes.

[4] The parent drug should be released from the LDL carrier in the lysosomes but

should not be deactivated.

It is equally important that the released drug is able to pass the lysosomal membrane and become available in the cytoplasm. Also, the MW of drugs should not be too high, since lysosomal membrane permeability may then become a problem..

1.7.3. Limitations in loading drugs into LDL particles

1.7.3.1. Restoration of apo B protein integrity: When using synthetic or semisynthetic colloidal drug carriers, modifications can be made to the system to optimize carrying capacity, biodegradability, and perhaps targeting potential. It is unlikely that such approaches can be made with LDL particles, since the equilibrium state of the particle will be perturbed.

In the native particle the apo B is so arranged that approximately half of the molecule is exposed to the aqueous environment and the remainder resides in the lipid coat. Any disruption to this "coat", particularly through interaction with amphipathic molecules, might well alter the conformation and the binding capacity of the apoprotein to the LDL receptor. The effects of additives such as drug molecules on the function of the particle is to some extent dependent on changes in the relationship between the apoprotein and the particle, and they may be vital to the success of targeting.

1.7.3.2. Partitioning of drugs both into core and surface monolayer: The lipoproteins in solution represent a discrete colloidal lipid phase possibly in the form of a microemulsion. Lipid soluble materials should readily partition into the core of the lipoprotein particle. Indeed, it appears that a range of lipid-soluble materials has this ability to partition into lipoprotein particles; simple partitioning does not seem to have been utilized to any great extent to transfer drugs into lipoproteins except for LDL. Daunomycin (DNM) has been bound to LDL through the incubation of LDL with glass beads coated with the drug [Iwanik *et al.*, 1984]. The drug was found to be present both in the internal core of the particle and on the surface [Iwanik *et al.*, 1984]. Studies of the selectivity of anthracyclines for negatively charged PL membranes [Burke *et al.*, 1988] were consistent with a mode of binding involving both hydrophobic and electrostatic interactions, in agreement with the findings of Henry *et al.*, [1985]. The electrostatic interactions involve the amino group of the sugar moiety and the ionized phosphate groups of the PL, hydrophobic interactions with the tetracycline ring and the hydrocarbon interior of the bilayer.

Molecules that are taken up into the monolayer also have the possibility of altering the distribution of the particle by [a] altering the surface charge or [b] adding new receptors to the surface. This technique has been used for the synthesis of Tris-Gal-Chol, which interacts with galactose receptors [Kempen *et al.*, 1984]. In the lipoprotein pathway, it is known that exchange of surface constituents [apoproteins and PL] takes place among lipoprotein species [Tall and Small, 1980]. Material may move from the original lipoprotein in which it was incorporated to another. Additionally, the apoprotein in the surface layer

may be affected by exchanges in the monolayer composition, which would undoubtedly alter the physicochemical properties of this layer [Schroeder, 1979] and might diminish any targeting specificity that the lipoprotein possesses.

1.8. Incorporation methods

One of the fundamental problems in utilizing LDL-mediated targeting is the development of a satisfactory drug incorporation method. Generally, the drug incorporation method into LDL must meet two basic requirements:

[1] formation of a stable, nonleaking LDL-drug conjugate and

[2] retain the natural integrity of the apo B protein.

The second requirement restricts the experimental conditions [pH, dielectric constants, temperature, use of metal ions, exposure to air, vortexing, etc.] [Fong. *et al.*, 1985]. Even small changes in parameters such as pH and dielectric constants may alter the three dimensional structure of apo B in such a way that LDL will lose its receptor recognition properties. Four main methods for the incorporation of drugs into LDL can be distinguished.

1.8.1. Reconstitution methods

The delipidation group of incorporation methods involves a more rigorous handling of the lipoprotein. LDL can be freeze dried in a starch [Schouten *et al.*, 1988, Glass *et al.*, 1985; Soltys *et al.*, 1982] or sucrose [Forester *et al.*, 1983] "frame" and subsequently extracted with organic solvents, followed by reconstitution with the drug [Lundberg, 1987].

Other delipidation methods utilize detergents [Rifici *et al.*, 1984] or enzymes [Fidge. *et al.*, 1985] to prepare soluble apo B-PL conjugates that can be reconstituted with the particular drug [Masquelier *et al.*, 1986; Krieger *et al.*, 1979]. The delipidation methods are not very efficient and LDL particles with only limited amounts of incorporated drug result. Recovery of apo B was usually less than 40% and the reconstituted LDL-drug conjugates were larger than native LDL and passed through 0.8 μm filters but not 0.45 μm filters [Shaw *et al.*, 1987]. Moreover, the method is very tedious and the physiological behavior of the reconstituted LDL needs to be monitored extensively, because modification can occur easily.

1.8.1.1. Solvent extraction: This method was first reported by Krieger *et al.* [1978]. The procedure includes lyophilization and heptane extraction of LDL, mixing the resulting apo B PL preparation with drug and extracted LDL neutral lipids, and finally solubilization of reconstituted LDL-drug conjugates by addition of buffer followed by purification by centrifugation and filtration. The conjugate remained stable during dialysis, density gradient centrifugation, and gel filtration. The reconstituted LDL retained its ß-mobility on agarose gel electrophoresis and its ability to be precipitated by both an antibody to native LDL and by heparin manganese. The Krieger method has been used to incorporate a variety of hydrophobic compounds into the core of LDL, including dioleyl methotrexate [Krieger *et al.*, 1979], 25-hydroxy-cholesteryl oleate [Krieger *et al.*, 1978], cholesteryl nitrogen mustard [phenesterine] [Firestone *et al.*, 1984], and pyrene coupled to a derivative of cholesteryl oleate [Mosley *et al.*, 1981].

A major limitation of this method is that the LDL-drug conjugate was more rapidly removed from plasma after IV injection than native LDL [Masquilier *et al.*, 1986]. This was overcome by using sucrose instead of starch and a plasma disappearance rate equal to that of native LDL was obtained [Masquilier *et al.*, 1986]. However, the amount of AD32 incorporated in LDL was lower with this modified Krieger method [100 molecules/LDL particle] than with the original one [400 molecules/LDL particle]. The recovery of drug in the final preparation in this modified method was low [Shaw *et al.*, 1987; Lundberg, 1987].

1.8.1.2. Detergent solubilization: Detergents [*e.g.*, sodium deoxycholate] can be used for the delipidation and solubilization of the water insoluble apo B from LDL [Atkinson and Small, 1986]. The apo B-detergent conjugate was then isolated by gel filtration and used for reassembly of LDL with a neutral lipid [or drug] microemulsion. Such a method has been applied to the incorporation of cholesteryl oleate [Lundberg and Suminen, 1984] and a cytotoxic steroid mustard carbamate [Lundberg, 1987] into the core of the reconstituted LDL particle. Detergent solubilization methods are rather tedious for routine LDL-drug conjugation. The conjugates prepared by this method are less than satisfactory since upon aging at 4°C the turbidity of the preparations slowly increased. Complete detergent removal was not successful. The method was not considered useful since the stability of the LDL-drug conjugates was poor and the removal of detergent was not complete. However, by these procedures an optically clear preparation of reassembled LDL was obtained, which was stable as determined by density gradient ultracentrifugation and gel filtration. The LDL

particles had a mean diameter of 22 nm and exhibited ß-migration during agarose electrophoresis. *In vivo*, a considerable portion of a reconstituted LDL preparation was cleared rapidly from the plasma [in mice], indicating uptake by the RES [De Smidt and Van Berkel, 1990].

1.8.1.3. Enzymatic digestion: Enzymatic delipidation of LDL [Lundberg, 1987] was proposed as a more gentle method. The CE core was hydrolyzed with sterol ester hydrolase [EC 3.1.1.13] in the presence of egg yolk phosphotidylcholine [EYPC] vesicles and albumin in order to bind the reaction products, free cholesterol and free fatty acid. *In vitro* results were encouraging in terms of stability and cytotoxicity. However, no *in vivo* studies have been performed.

1.8.2. Transfer/Contact methods

The essential step in the contact method is physicochemical transfer of drug molecules from a solid surface into LDL particles [e.g., the wall of a glass tube, glass beads or small siliceous earth crystals such as Celite 545] [Seki *et al.*, 1985]. The procedures involve evaporation to dryness of the drug in organic solvent as a thin film on the support and then incubation with a lipoprotein preparation in the presence of the necessary preservatives such as antioxidants, antimicrobial agents, dark room, and inert gas. After completed incubation [usually at 37°C] the lipoprotein-drug conjugate can be purified by Sephadex G-120-15 column chromatography [Iwanik *et al.*, 1984] or by centrifugation [3000

rpm for 10 min] if Celite 545 is employed [Seki *et al.*, 1985]. For sterilization and removal of aggregated complexes a 0.20 μm filter can be used.

This method is increasingly used to incorporate drugs [Shaw *et al.*, 1987; Seki *et al.*, 1985]. The successful partitioning of a number of different structured drugs into LDL or modified LDL has been reported [Shaw *et al.*, 1987]. They include hexadecylmethotrexate [55 molecules/LDL], AD 32 [100 molecules/LDL], muramyltripeptidephosphatidyl ethanolamine [140 molecules/AcLDL], arabinofuranosylcytosine-5'-[n-hexadecylphosphate [90 molecules /LDL], and DNM [6 molecules/LDL]. However, contact methods apparently can only be used for drugs that exhibit the right physicochemical characteristics for partitioning into the LDL.

This method is technically simple and rapid. The native integrity of the LDL molecules and stability of the drug is restored. The biological behavior of the conjugates seem to be acceptable both *in vitro* [Iwanik *et al.*, 1984] and *in vivo* [Seki *et al.*, 1985]. The dry film method meets the requirements for simplicity and rapidity that must be placed on any practically useful method.

1.8.3. Miscellaneous transfer

Hynds *et al.* [1985] performed a simple exchange between LDL and a drug-lipid microemulsion. Up to 30 % of the chlorambucil used was recovered in LDL by this method. When the inhibition of protein synthesis was tested on human glioma cells in culture, the conjugate was found to be more effective than chlorambucil itself. A similar technique was

used to incorporate 9-methoxy-ellipticin into LDL [Via *et al.*, 1982]. The drug was incorporated into a dimethylphosphotidyl choline, cholesteryl oleate stabilized microemulsion, and the latter fused with human LDL. The LDL-drug conjugate was more effective than the free drug to kill L1210 and P388 leukemic cells *in vitro.*

1.8.4. Direct/Aqueous addition

This is the simplest of the incorporation methods. Conjugation of drug and lipoprotein was accomplished simply by addition of an aqueous solution of the drug to the lipoprotein preparation, followed by isolation of the conjugate by dialysis, gel filtration and ultrafiltration. Such measures gave incorporation of 15-450 molecules of aclacinomycin A per LDL particle [Rudling *et al.*, 1983], 3-5 Dox per high density lipoprotein [HDL] [Shaw *et al.* 1987], and approximately 130 photofrin II per LDL [Candide *et al.*, 1986]. This method can only be used on a limited extent number of drugs since a delicate balance between lipophilicity and water solubility is required. Unfortunately, the rapid and easy incorporation of drugs into the lipoprotein is usually accompanied by a high rate of leakage of the drug from the carrier [Rudling, *et al.* 1983]. In addition, drugs conjugated with lipoprotein by aqueous addition will undergo a fast transfer to other lipoproteins and cells [Van Berkel. 1990]

1.8.5. Facilitated transfer

This is a relatively unexploited method. The exchange of strongly lipophilic lipids,

like TAG and CEs, from lipoproteins to cultured cells in plasma free medium is extremely slow [Ekman and Lundberg, 1987]. However, the addition of transfer mediators like solvents [Fielding and Feilding, 1991; Fielding *et al.*, 1979] or lipid transfer proteins [Tall, 1986] can appreciably increase the transfer rate. A corresponding approach may be applicable also to lipophilic drugs and LDL particles.

Dimethylsulfoxide [DMSO], has been used for the incorporation of labeled CEs into lipoproteins and has also been tested for incorporation of AD 32 into LDL, but with inferior results compared to that obtained by the modified Krieger method. Use of the cholesteryl ester transfer protein [CETP] present in plasma [Tall, 1986, Blomhoff *et al.*, 1984] has been unsuccessful in transferring two drug molecules, dioleoyl fluoxouridine and dioleoyl methotrexate, from dry film into LDL particles in one study [De Smidt and Van Berkel, 1990b]. However. the method has been used to label acetylated LDL with [^{14}C] cholesteryloleate 49 and to incorporate [^{3}H] cholesteryloleyl ether into lactosylated LDL [Bijsterbosch and Van Berkel, 1990]. The method has proven to be successful for endogenous molecules like cholesteryl oleate. Far less experience has been gained with the incorporation of foreign molecules like drugs. The structural requirements of various compounds to serve as a substrate for the transfer proteins have to be defined more explicitly in order to comment on the potential wider application of this method.

1.9. Statement of reserach problems

Anticancer drugs could, more discriminatingly, be delivered to neoplastic tissues by means of LDLs. However, two major obstacles to this strategy have been identified. The first imperfection is the low efficiency of drug loading into the core of LDL particles without disrupting its innate integrity; and the second is the possible exchange or transfer of loaded drug[s] among lipoprotein subspecies in plasma. According to the existing loading techniques, only 5-100 drug molecules with MWs less than 300 can be accommodated into an LDL particle in its endogenous form. Drug molecules, if attached to the surface of LDL particles simply due to hydrophobic interactions, may be displaced spontaneously by the excess amount of PL in biological membranes and lipoproteins in the extracellular fluids. In addition, drug[s] may undergo exchange and transfer among lipoprotein subspecies by lipid transfer protein[s] even if drugs have very hydrophobic properties [Morton, 1990; Wasan, 1990-96]. All these factors will contribute to decrease the drug concentration at tumor site[s]. Moreover, merely giving a higher dose of a LDL-drug conjugate with a low drug concentration cannot be practical at killing tumors due to saturation of the LDL receptor pathway as mentioned earlier. Therefore, loading large quantities of hydrophobic drugs into the oily core of LDL is imperative to achieve a therapeutically effective concentration and targeting effect. To accomplish this, the exploitation of biochemical approach[es] are required to establish an effective anticancer drug targeting system.

1.10. Rationale of these studies

1.10.1. Selection of drug candidates

Dox, with a broad spectrum of antitumor activity, continues to be of major importance in cancer treatment [Neidle, 1992; Arcamone, 1980; Arcamone *et al.*, 1974; 1975] and tends to be the standard against which new drugs are judged [Neidle, 1992]. It is of value, either alone or in combination, in the treatment of lymphatic leukemia, lymphomas, breast cancer, genitourinary tumors, epidermoid carcinomas, and soft tissue carcinomas. Unfortunately, many of the low growth fraction carcinomas common in older patients [lung, breast, colerectal, and bladder tumors, for example] are poorly responsive to this agent. Dox produces a range of dose dependent toxic reactions such as, cardiotoxicity, myelosuppression, and gastrointestinal toxicity. These dose dependent side-effects are related to widespread biodistribution of this drug in the non-tumorous tissues in addition to tumor tissues. The most commonly used formulation is Dox·HCl, even though liposomal formulations are currently in clinical use [Janknegt, 1996]. I speculate if Dox association with lipoproteins could be increased, it would decrease its distribution to plasma albumin resulting in decrease cardiotoxicity and increase cytotoxicity.

Polyiodinated cholesteryl iopanoate [CI] has been proposed as a nonhydrolyzable CE analogue and as a radiotracer for *in vivo* studies regarding LDL uptake. When drug and CI are incorporated together into LDL particles, *in vivo* uptake of LDL-drug conjugates in different tissues could be determined by quantification of CI inside the tissues. Unlike

naturally occurring CEs, this compound is not a substrate for CE hydrolase and can therefore be trapped intracellularly. This will help to determine total CI uptake from CI loaded LDL. Since an insect lipid transfer particle [LTP] could load a large amount of CE into LDL, I speculate that a substantial amount of CI could be incorporated into LDL for the development of an *in vivo* tracer for LDL uptake studies. It can be developed into a diagnostic agent for early detection of tumors. In conclusion, I propose to use Dox and CI in my studies to incorporate into LDL particles.

1.10.2. Development of analytical methods

I have radiolabelled CI. Radiochemical methods such as gamma counting are available for its quantification. High performance liquid chromatography [HPLC] method is different from radiochemical methods in respect to its potential to quantitate the integrity, stability or purity of a drug. I developed a reverse phase HPLC methods for the quantification of CI. Furthermore, I propose that a colorimetric method for Dox could be developed by the addition Cu^{2+} using bicinchoninic acid [BCA] protein assay kit. The enhancement could make the colorimetric method comparable to the spectrofluorometric method. If successful, this new method is expected to be technically simple and cost-effective.

1.10.3. Drug association to lipoproteins

There is increasing evidence that lipoproteins may be important in the binding of very

lipophilic and/or basic compounds [Danon and Chen, 1979]. The interaction of lipophilic compounds with plasma lipoproteins has been shown to influence the pharmacokinetics and organ distribution of a number of lipophilic compounds. It has been demonstrated that the interaction of several compounds, including amphotericin B [Amp B] [Wasan *et al.*, 1990-1996; Wasan and Lopez-Berestein, 1993b] with plasma lipoproteins modifies their pharmacokinetics, tissue distribution, and pharmacological activity. Wasan *et al.*, [1994a-c] have demonstrated that Amp B initially associates with the HDL fraction upon incubation in plasma. Moreover, cyclosporin A enters the hydrophobic core regions of lipoproteins where it may be distributed according to its high octanol /water partition coefficient [log P> 2.8] [Yang and Elmquist, 1996; Lemaire and Tillement, 1982; Tillement *et al.*, 1978].

Studies have shown that anthracyclines are bound to human plasma protein to an extent of 50-85% [Eksborg *et al.*, 1982]. The binding of lipoproteins and different anthracycline analogs could be ascribed to physicochemical determinants of lipophilicity [Chassany *et al.*, 1996; 1994]. In conclusion, the binding of certain classes of drugs to lipoproteins indicates that lipophilic compounds can be incorporated in lipoproteins, especially LDL particles. To test the preference of Dox for LDL, I propose to study *ex-vivo* plasma distribution of Dox.

1.10.4. Physicochemical factors and drug loading

I explained earlier that lipophilic compounds readily partition into the core of the lipoprotein particle. However, simple partitioning does not seem to produce a significant

driving force to facilitate transfer of the drugs into LDL. Contact methods apparently can only be used for a limited amount of drugs that exhibit the right physicochemical characteristics for partitioning into the LDL. Hynds *et al.* [1984] performed a simple exchange between LDL and a drug-lipid microemulsion. A similar technique was used by Samadi-Baboli *et al.*[1990; 1989]. In this technique, drug was incorporated into a stabilized microemulsion, and the latter was fused with LDL. Based on these findings, I hypothesized that drug molecules if suspended with the aid of a suitable wetting agent or incorporated in the liposome would be more readily incorporated into the LDL when compared to the dry film method. Liposomal formulation of drugs was used to further investigate LDL-drug interactions and their effect in drug loading. Several factors affecting drug loading into LDL particles, such as, temperature, incubation time, stoichiometry of LDL-drug conjugates, were optimized to determine favorable loading condition[s].

1.10.5. Physicochemical characterization of LDL-drug conjugates

The success of LDL-mediated approach could be ascertained from findings of advanced biochemical and biophysical techniques, such as, sodium dodecyl sulphate-polyacrylamide gel electrophoresis [SDS-PAGE], electron microscopy [EM], and differential scanning calorimetry [DSC]. *In vitro* characterization is imperative to have insight into the *in vivo* behavior of LDL-drug conjugates. I, therefore, propose to characterize the LDL-drug conjugates using SDS-PAGE, EM, and DSC.

1.10.6. Use of biological transfer catalysts

A number of loading techniques have been discussed earlier. With all of these techniques, the key question is whether it is possible to incorporate a lipophilic drug into the core of a lipoprotein particle, in sufficient concentration, and without disrupting the native integrity of LDL. Exploiting a suitable promoting factor has been considered essential for successful drug loading into LDL particles. An insect lipid transfer particle will be examined as a promoting factor in the loading strategy and the rationale for this is discussed here.

The movement of hydrophobic materials associated with lipoproteins in the circulatory system is a dynamic process that is mediated by transfer factor[s]. In humans, a 67 kDa glycoprotein called CETP mediates the exchange and transfer of CE and PL among lipoproteins [Tall, 1986]. Human microsomal TAG transfer protein [MTP] is responsible for TAG lipid movement in VLDL formation within the lumen of microsomes [Wilson *et al.*, 1991; Breiter *et al.*, 1991]. In insects, lipid transfer between insect lipoproteins [lipophorins] is accomplished by a transfer factor found in hemolymph and other tissues. In the tobacco hornworm, *Manduta sexta*, a high MW protein called [LTP] was discovered to be a multifunctional lipid transfer catalyst [Ryan 1986a-b]. Preliminary experiments suggest that part of LTP has a functional similarity to a subunit of human MTP. It has been suggested that one of the three subunits of LTP [apoLTP III, 55 KDa] might function like protein disulfide isomerase [PDI], which is involved in the process of protein folding and unfolding [Wetterau *et al.*, 1992; 1991]. This mechanism may be responsible for the carrying capacity of LTP for its substrates.

The rate of spontaneous exchange of lipophilic molecules between lipoproteins is slow [Ekman and Lundberg, 1987]. However, the addition of transfer mediators like CETP [Tall, 1986] can appreciably increase the transfer rate. A corresponding approach may be applicable to incorporate lipophilic drugs into LDL particles although CETP was not successful in enhancing drug incorporation into LDL particles [de Smidt and Van Berkel, 1990]. The reason for this failure could possible be the high substrate specificity of the human CETP. Compared with human CETP, insect LTP has unique characteristics. LTP can transfer lipids almost unidirectionally from one lipoprotein to another. The rate of this transfer process is much faster than that of human CETP. LTP was found to be efficient in loading hydrophobic compounds into a tightly packed LDL particle. The various substrates transferred by LTP include diacylglycerol [DAG], TAG, PL, cholesterol and CE, and hydrocarbon wax [Liu *et al.*, 1991; Singh *et al.*, 1991; Ryan, 1990; Ryan. *et al.*, 1990;]. The lack of substrate specificity of LTP provides a good ground to speculate that the transfer of hydrophobic anticancer agents into LDL may be possible using LTP as a transfer catalyst. When human LDL was used to accept the lipids associated with other lipoprotein subspecies, the capacity of LDL as a lipid sink was greatly enhanced by LTP [Ryan *et al.*, 1990]. It was found that each LDL particle could bear an additional 600 DAG molecules [MW 665] inside the lipid core without altering its basic structure [Liu and Ryan, 1991]. It is likely that the eight intramolecular disulphide bridges in the apo B protein [Innerarity *et al.*, 1979], which has 4536 amino acids, forms a protein matrix for the LDL particle, may undergo significant conformational changes by an unfolding/refolding process in order to accept additional lipids

into its core. If this holds true, the risk of dilution of the LDL-drug conjugate, via exchange, or redistribution of the drug into the circulatory LDL lipid pool would be reduced.

1.10.7. Biological evaluation of LDL-drug conjugates

Our long term goal is to establish LDL as a targeted carrier in cancer chemotherapy. Before the onset of a preclinical trial in an appropriate animal model, evaluation of a formulation in a suitable tissue culture model is essential. Biological evaluation of this formulation in cell cuture was important to verify my hypothesis that LDL-Dox conjugates were internalized by a receptor mediated endocytosis process by cells. Therefore, this new LDL-Dox formulation would be more effective than existing formulations of Dox. I propose to evaluate different formulations of Dox in a well characterized tumor cell line, HeLa. In addition, a modification of LDL by acetylation was found to be effective at selectively targeting cancer and/or infections involving macrophage cells (Shaw *et al.*, 1988; 1987]. I hypothesize that if LDL is modified by acetylation, when drug is incorporated into this modified LDL, the modified LDL-drug conjugates will be, preferentially, taken up by a mouse Mϕ cell line, J774.A1.

1.10.8. Drug interference in protein assay method

In LDL based pharmacologic and experimental therapeutic research, it is often necessary to quantitate the apo B protein associated with LDL-drug conjugates. Common methods for the assay of proteins in biological fluids include Lowry, Bradford, and the BCA

assay methods. All of these methods are potentially interfered with by a variety of chemical components, including drugs, buffers, and metal ions. It is imperative that the method used to determine protein concentrations is not interfered with by any component other than protein. Before selecting any colorimetric protein assay method, I propose to test all drug candidates for any possible interference in the analytical method. This forms the basis for drug interference studies using common protein assay kits.

1.11. Specific objectives of these studies

To examine LDL as a potential targeting carrier for cytotoxic agents in cancer chemotherapy, my specific goals were as follows:

A. Physicochemical characterization of LDL-drug conjugates.

A.1. To investigate hydrophobic binding of Dox with human lipoproteins and investigate the suitability of Dox as a candidate for loading in LDL.

A.2. To load Dox into LDL and evaluate drug incorporation techniques in terms of incubation conditions [temperature, time, stoichiometry of LDL-drug conjugates], process parameters [the dry film method with or without wetting agents], and formulation parameters [liposomal, aqueous suspension or solution of drug formulations].

A.3. To characterize LDL-drug conjugates using physicochemical and advanced techniques such as SDS-PAGE, EM, and DSC.

B. To optimize variables in enhancement of Dox and CI loading using insect LTP. Human CETP was also considered as a possible transfer factor.

C. To evaluate the cytotoxicity of LDL-Dox conjugates compared to corresponding conventional formulation, Dox·HCL in saline *in vitro* on tumor cells; HeLa cells, using [3-[4,5-dimethylthiazoyl-2-yl]-2,5-diphenyl-tetrazolium bromide], [MTT],

assay.

D. To modify LDL via acetylation and *in vitro* characterization of modified LDL-Dox conjugates physicochemically and biologically in a Mϕ cell line, J774.A1.

E. To assess drug interference in protein assay using conventional kits [Lowry, Bradford, and the BCA assay kits].

CHAPTER 2:

EVALUATION OF FACTORS AFFECTING DRUG LOADING INTO LDL PARTICLES

2.1. Introduction

A number of particulate systems such as antibodies [Mori and Huang, 1995; Edwards and McIntosh, 1986], microspheres [Jalil and Nixon, 1992; Juliano, 1985], liposomes [Allen, 1995; 1994; Mayhew *et al.*, 1984] and other macromolecules [Duncan, 1992; Sezaki and Hashida, 1985] have been proposed as targeting carriers to deliver cytotoxic drugs. There are many obstacles to the successful delivery of drugs using these carrier moieties. Ideally, the drug-carrier conjugate must avoid unwanted uptake *in vivo* by the RES. This is especially important for a carrier designed to target non-RES cells, such as tumor cells of non-RES origin. Cells of the RES are extremely effective in removing foreign materials from the blood. These carriers [>100 nm] are rapidly removed from the circulation in a single pass through the liver [Illum *et al.*, 1984]. These particulate carriers must also have the correct surface characteristics to avoid opsonization, complement activation, and uptake by the RES [Davis and Illum, 1986, Poste, 1983]. The particulate carriers must also be able to escape from the vasculature in order to reach their site of action; particles [>50 nm] are unable to achieve this except in the liver, spleen, and bone marrow where the endothelial cells of the blood capillaries are discontinuous. Also, the large scale manufacturing of artificial carrier systems poses another obstacle for their potential application in therapy with

regards to the safety, validation, reproducibility, and scaling up. This implies that an endogenous particle [<50 nm] with natural abundance and availability, and a capacity to carry drugs, may solve many problems related to artificial carrier systems such as immunocompatibility, targeting and large scale manufacturing. An endogenous cholesterol carrier, LDL, has been proposed as one such carrier system to deliver cytotoxic agents. The potential of LDL as a drug carrier has been discussed earlier [see Chapter 1]. However, despite many efforts, no practical regime of a cell selective delivery of a LDL-drug conjugate has so far been marketed. Failure to obtain stable, therapeutically effective LDL-drug conjugates, is one of the major reasons.

I have mentioned earlier that the physico-chemical factors of drugs may influence their entrapment into LDL particles since the transfer of drugs into LDL particles is accomplished by either by means of physical diffusion or a partitioning of the drug between the different phases of the LDL. Hydrophilic drugs will not attach to the lipoproteins or diffuse into the lipid core of LDL. Lipophilic drugs are, therefore, good candidates for this approach of drug delivery.

An anthracyclic derivative, Dox, was considered in these studies [Figure 2.1]. As indicated earlier in Chapter 1, the clinical potential of Dox is often limited by its toxicity, and considerable efforts have been made in recent years to find new derivatives or delivery vehicles for Dox with a more favorable therapeutic index [Lameh *et al.*, 1988; Seshadri *et al.*, 1986; Isreal *et al.*, 1985; Isreal and Potti, 1981; Isreal and Modest, 1977; Arcamone *et al.*, 1974; 1975]. To achieve site specific drug delivery of Dox to tumor cells, attempts have

been made to load Dox or its lipophilic analogues in LDL [Westesen *et al.*, 1995; Masquelier *et al.*, 1985; Shaw *et al.*, 1984]. Although *in vitro* data for cytotoxic agents using LDL were encouraging, once tried *in vivo* the efficacy was not as promising [Tokui *et al.*, 1995; De Smidt and Van Berkel, 1990]. In the present study, attempts were undertaken to load Dox in a favorable way so that the LDL-drug conjugates would follow the normal metabolic fate of LDL *in vivo*.

In these studies, the effects of different physical factors to enhance drug loading into LDL particles were examined in terms of incubation time, temperature, and LDL-drug molar ratios [stoichiometry]. The dry film method was most efficient in terms of generating LDL-drug conjugates which are similar to native LDL *in vitro* and *in vivo* [De Smidt and Van Berkel, 1990]. This method was considered to evaluate the effect of different surfactants and additives in drug loading. Additionally, a comparison was made among different formulations of Dox such as aqueous solution or liposomal preparation to examine their effects on drug loading into LDL particles. In short, I investigated different physico-chemical factors which influence drug loading into LDL particles. The physico-chemical characterization of LDL-drug conjugates using advanced biochemical and biophysical techniques such as SDS-PAGE, DSC, EM will be discussed in the proceeding chapter.

2.2. Materials and methods

2.2.1 Materials

Dox [Dox·HCl, Adriamycin ¶] was purchased from Adria Laboratories Ltd. [Mississauga, Canada]. High purity EYPC, oleic acid, Spans, Tweens [20, 40, 60 and 80], Celite 545, Triton-X were purchased from Sigma Chemical Co. [St. Louis, MO]. All HPLC grade solvents were from Fisher Scientific [Ottawa, Canada]. Two buffers were used. Phosphate-buffered saline [PBS] which consisted of 137 mM NaCl, 3mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, adjusted to pH 7.4 with 2M NaOH [Fisher Scientific]. Dialysis buffer [Tris] containing 150 mM NaCl, 50 mM Tris, and 0.3 mM EDTA, was adjusted to pH 7.4 with 2M HCl [Fisher Scientific].

2.2.2. Preparation of LDL samples

Fresh human plasma was obtained from the Canadian Red Cross [St. John's, Canada]. To avoid multiple enzymatic degradations, a cocktail [2 mL sodium azide 2.5%, 0.5 mL benzamide 1M, 2.5 mL phenylmethylsulfonylfluoride [PMSF], 0.2 M] was immediately added to 500 mL of the plasma as described before [Edelstein and Scanu, 1986]. LDL [density 1.019-1.063 g/mL] was isolated from plasma by density gradient ultracentrifugation in KBr using a L8-70M ultracentrifuge [Beckman, Fullerton, CA] with a Ti-60 zone rotor at 37,000 rpm and 8°C for 40 hours. To remove excess KBr, the LDL was dialyzed against the dialysis buffer for 24 hours followed by filtration through a Millipore membrane filter

[0.20 μM pore size] [Milford, MA]. The LDL was stored at 2-8°C for no more than two weeks before use.

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2.2.3. Analysis of LDL

The LDL protein content in native or LDL-drug conjugates was determined by the Bradford method using bovine serum albumin [BSA] as the standard. Cholesterol and TAG were measured using Sigma reagent kits based on procedure numbers 352 and 336 of Sigma respectively. Lipoprotein purity was assessed by SDS-PAGE [see Chapter 3, section 3.2.3]. The presence of a single band was considered to indicate the absence of any additional proteins as contaminants or degradation by-products.

2.2.4. HPLC assay of Dox

The Dox concentration in LDL was measured by reverse phase HPLC. The Beckman HPLC system consisted of two 110 pumps, one Detector 166, one Autosampler 105 and in-line System Gold software for running the system and for quantification [Beckman, Fullerton, CA]. A Phenomenex C_{18} Bondclone [3.9X150 mm] with a guard column [3.9X30 mm] was used [Phenomenex, Torrance, CA]. Dox was eluted with acetonitrile: 40 mM phosphate buffer, [28.5:71.5, v/v] pH 4.0 at a flow rate of 1 mL/min [Brown *et al.*, 1981, Eksborg *et al.*, 1978; Isreal *et al.*, 1978]. The compound was assayed by a UV detector at 254 nm. Standard curves were made using standard solutions of Dox in the mobile phase ranged 0.20 to 10 μg/mL. Five to 50 μL of samples were injected in duplicate. All

measurements were based on peak area response of the drug as calculated using the system Gold Beckman software for HPLC. Standard curves were made in every set of experiments to ensure the accuracy of the analysis.

2.2.4.1. General extraction method: Fifty μL DoxHCl [3.45 mmoles] was made alkaline with 100 μL 0.05 N NaOH and extracted with 2 mL chloroform. The precipitated portion was removed by centrifugation at 1000x g for 5 minutes. The extraction was carried out three times followed by centrifugation as mentioned before. The combined organic layers were evaporated to dryness under a stream of nitrogen. The dried sample was then reconstituted with the appropriate solvent to make standard Dox solutions.

To extract Dox from biological samples such as plasma or LDL, 50 μL NaoH [0.5 M] was added to 1 mL samples. Acetonitrile-0.1 M H_3PO_4 [4 : 1] [0.5 mL] was then added to the samples [0.5 mL] in a stoppered centrifuge tube; the mixture was vortex-mixed for 30 seconds then centrifuged at 1000x g for 5 min to pellet the precipitated protein. The supernatant [5 to 50 μL] was chromatographed and the peak areas were compared with those from standards prepared by the addition of drug to a centrifuged LDL- or plasma-acetonitrile-0.1 M H_3PO_4 [5:4:1] mixture.

The extent of recovery of Dox from plasma and LDL [Isreal *et al.*, 1978], was determined by comparing the slope of the regression line of the plasma/LDL extracted Dox with that obtained in the standard solution of Dox.

$$\% \ Recovery = 100 \ X \ \frac{Slope \ of \ plasma \ extracted \ Dox}{Slope \ of \ the \ standard \ Dox}$$

2.2.4.2. Data analysis: Calibration curves of peak area vs concentration of Dox were analyzed by linear regression analysis to obtain the equation of best fit. The correlation coefficient of regression, [R^2], was calculated. Interday variations in the analytical precision were assessed by using a two-way ANOVA regression model with the calculation of p values. Intra-day comparisons were done by a Student's t-test and by calculating the p values. When $p>0.05$, the difference in the mean values of the groups [two groups in student's t-test] was not considered statistically significant.

2.2.5. Determination of partition coefficient [P]

The method described in Bijsterbosch *et al.*, [1994] was followed. Aliquots of 10 μg/mL of extracted Dox or Dox·HCl was dried in a 4 mL stoppered glass vial. Then, 1 ml 1-octanol and 1 mL PBS buffer [pH 7.4] were added, and the mixtures were shaken for 16 hours at room temperature by a Wrist Action Shaker [Burrell, Pittsburgh, PA]. Samples of the octanol and the aqueous phase were then assayed by HPLC, and the P [concentration in octanol/concentration in PBS] was calculated.

2.2.6. Incorporation of drugs into LDL

The dry film method was modified to enhance the efficiency of drug loading.

Dox·HCl [3.45 mmoles] was converted to free Dox with NaOH [0.05 M] and then extracted with chloroform. An aliquot of the chloroform solution of free Dox was dried down under a stream of nitrogen gas in a test tube. When the chloroform was completely removed, the drug formed a thin dry film at the bottom of each test tube. LDL was then added and the mixture was incubated in the dark with continuous gentle shaking at 37°C for up to 24 hours. In another experiment, Dox·HCL was directly added to LDL for incubation [direct addition method]. The LDL-drug conjugates were isolated by a single ultracentrifugation step at a density of 1.063 g/mL at 40,000 rpm for 40 hours using a 60 Ti or 75 Ti Beckman rotors. The LDL-drug band at the top was then aspirated and subjected to dialysis for 24 hours to remove KBr and free drug. The LDL-drug conjugates were filtered through a 0.20μm Millipore filter and stored at 2 to 8°C for no more than two weeks before use. The identification of the LDL was accomplished by a protein, a cholesterol and a TAG assay. The concentrations of Dox was determined by the HPLC method as described above.

2.2.7. Use of wetting agents

A number of wetting agents were examined for their effects on the drug loading efficiency of Dox. First, a wetting agent, i.e., Tween 20 was added to the chloroform solution of Dox. The mixture was thoroughly agitated with a vortex mixer, the solvent was evaporated under a stream of nitrogen, and saline [0.9% NaCl] was added such that the Tween 20 constituted less than 3% of the final volume. The solution was vortexed again and placed briefly under nitrogen to remove any residual chloroform. LDL was then added to the

drugs suspended with Tween 20 and incubated as mentioned in the dry film method. Other wetting agents including different grades of Tweens and Spans, Triton X, or surface increasing agent, i.e., Celite 545 were examined using the same protocol.

2.2.8. Preparation of multilamellar vesicles [MLV]

The preparation of homogenous and multi-layered vesicles of EYPC:Dox [15:1] mixture in Tris buffered solution, [145 mM NaCl, 10 mM Tris-HCl, pH 4.0], was done by extrusion of emulsions. A chloroform/methanol mixture [1:1, v/v] containing Dox and EYPC was evaporated to dryness under nitrogen at 40-45°C. The lipid-drug film was evaporated for at least 2 hours. Subsequently, glass beads and the hydration medium [pH 4.0] were added. Nitrogen was passed through the hydration buffer for about 15 min. The film was vortexed three times with 1 minute intervals at 55°C and left, after complete dispersion, in a refrigerator for one night. At this stage, 1 mL of the dispersion contained about 40 μmol of phospholipid [EYPC] and 2.5 mg of Dox. This suspension was incubated with LDL to make LDL-Dox conjugates. The liposomes, mainly MLV, were sized by sequential extrusion through a double polycarbonate membrane filter, with pore diameters of 0.22 μm [Millipore] under nitrogen pressures of up to 15,000 psi. This was done at least 10 times to obtain homogenous MLV according to Olsen *et al.* [1982].

2.2.8.1. Separation of free Dox: Free Dox [nonliposome associated Dox] was removed by dialysis at 4°C. The dialysis membranes [MW cut off, MWCO, 12,000-14,000]

were soaked with deionized water for at least 15 minutes and rinsed extensively before use. After this, less than 10% of the total amount of Dox was in the free form. All liposome dispersions were kept protected from light at 4-6°C under N_2. Freshly prepared liposomes were used for loading experiments.

2.2.8.2. Determination of loading capacity: The separation of neutral liposomes was achieved by ultracentrifugation for 3 hours at 40,000 rpm using a Beckman 75Ti rotor. After destruction of the liposomes by addition of Triton X-100 and subsequent heating, Dox was assayed by HPLC. Phospholipid content was determined according to the procedure of Rouser *et al.* [1966].

2.2.8.3. Stability of liposomes: The ratio of free Dox to liposome associated Dox was measured as described above. In the case of plasma incubations, freshly prepared liposome dispersions were mixed at a 1:1 ratio with human plasma and incubated at 37°C. Chemical stability was monitored using HPLC.

2.2.9. Distribution of Dox over plasma [lipo]proteins: Sequential flotation ultracentrifugation

For the redistribution studies, Dox·HCl [100 μg] was added to 2 mL of human plasma and the solution was incubated for 4 hours at 37°C under N_2 while shaking continuously. In a separate study, plasma was preincubated with oleic acid [20 mg/mL of plasma] for 2

hours before incubation with plasma. After the incubations were complete, the samples were transferred to polycarbonate centrifuge tubes. Their solvent densities were adjusted to 1.006 g/mL by KBr. Following centrifugation [L7-65 Beckman Instrument] with a 75 Ti rotor at 50,000 rpm for 17.5 hours at 8°C the VLDL-rich and VLDL-deficient fractions were recovered. Following this initial spin the VLDL-deficient fraction was adjusted to a solvent density of 1.063 g/mL with KBr and respun at 40,000 rpm for 17.5 hours at 8°C to separate the LDL-rich fraction from the rest of the fractions containing HDL and LPDP. The latter fraction was adjusted to a solvent density of 1.21 g/mL with KBr and respun at 50 000 rpm for 21.4 hours at 8°C to separate the HDL and LPDP fractions. All isolated lipoproteins and lipoprotein deficient fractions were dialyzed against the dialysis buffer [2 L] for 18 hours before analysis. The MWCO of the dialysis tubing used was 12,000-14,000.

2.3. Results

2.3.1. Chemical analysis of lipoprotein

No significant difference between native LDL and LDL-drug conjugates were found in terms of protein, total cholesterol, and TAG content. The results from LDL preparations used in this study in Table 2.1 are in agreement with reported literature data (Westesen *et al.*, 1995).

2.3.2. HPLC analysis of Dox

Typical chromatograms are shown in Figure 2.2a to 2.2.c. Figure 2.2a shows the chromatogram of Dox in mobile phase, and Figure 2.2b shows Dox after being extracted from plasma and Figure 3.2c shows the chromatogram of a Dox spiked human LDL sample. The retention time of Dox was 4.77 minutes. A calibration curves for Dox is shown in Figure 2.3. Peak areas obtained with human plasma samples with the five different concentrations of Dox over the concentration range of 10 to 300 ng/10 μL were linearly related to the spiked concentrations. The equation of best fit for the line was:

$$Y = -0.206 + 1.67 \times 10^{-3} X \qquad R^2 = 1.00$$

Where Y is the peak area of Dox and X is the Dox concentration [Figure 2.2]. The coefficient of determinations ranged from 99.7 to 99.9%, and the CV of the slope determined on five separate occasions was 2.02 % [Table 2..3]. The minimum detection limit for Dox was found to be 5 ng [10 μL from 0.5 μg/mL standard solutions]. The peak height detected at this minimum concentrations was more than three times that of the noise.

The % recovery was calculated [n=5] and found to be more than 92% from plasma and 94% from LDL. Peak areas obtained with LDL spiked with four and five different concentrations of drug over the standard concentration range [Table 2.2] were linearly related to the spiked concentrations. The reproducibility of the method was determined by both intra- and inter-day variability studies [Table 2.4]. The %CV's for the interday variability were 4.19, 1.25, and 3.67 for the spiked Dox of 1, 5, and 10 μg/mL respectively. A two-way

ANOVA regression model showed no significant difference [p>0.05] between determinations performed from day to day, over fifteen days. The intra-day % CV [n=6] for three test samples was 6.35, 3.29 and 3.77, respectively. Performance of one way ANOVA on intraday measurements showed no significant variations [p>0.05]. Thus, the HPLC method described here was statistically valid and proven appropriate for my experimental protocols.

2.3.2. P of candidate drugs

The octanol/PBS partition coeffcient (P) gives a measure of the lipophilicity of the candidate drugs. Dox [2.5 ±0.12, P values are expressed in mean ±SD of three determinations] in the free form was found to be more hydrophobic [>5 fold] than the salt form [Dox·HCl] [0.052 ±0.03]. This difference in their lipophilicities should result in a different affinity for LDL.

2.3.3. Method of drug loading

I have compared the incorporation rate of Dox into LDL particles by different loading methods. The incorporation was performed by the contact method. I have loaded Dox [average 35 molecules/LDL particles] more efficiently into LDL compared to its close analogue, daunomycin [DNM] [7 molecules/LDL particle] [Shaw *et al.*, 1987]. Figure 2.4 shows the efficiency of density ultracentrifugation to separate LDL-drug conjugates obtained by the dry film method from the incubation mixture. In addition, dialysis was used to

remove excess free drug from the LDL-drug conjugates after incubation or ultracentrifugation [Figure 2.5]. The essential step of this loading method is the partitioning of the drug from a solid surface to the lipoprotein. The contact area can be greatly enlarged by using glass beads or Celite, the latter being used for this series of experiments. All conjugates were successfully prepared by the dry film method and were passed through 0.20 μm filters with negligible losses of apo B.

2.3.4. Factors influencing drug loading

2.3.4.1. Incubation time: I studied the effect of incubation time on Dox loading [Figure 2.6]. Drug loading is mainly a partition or diffusion mechanism which is a time dependent phenomenon. I investigated the effect of drug loading on incubation time up to 24 hours. It was found that drug loading reached a plateau after 5-6 hours. In the literature, different incubation times were reported ranging from 2 to 24 hours [Tokui *et al.*, 1994; Hossaini *et al.*, 1994; Schultis *et al.*, 1991; Vitols *et al.*, 1990; Samadi-Baboli *et al.*, 1990; Shaw *et al.*, 1987; Masquilier *et al.*, 1986]. I observed that about 50% of the drug was incorporated within the first 30 minutes and an additional 25% of drug was loaded in the next 90 minutes of incubation [Figure 2.6]. This suggests that the strength of the strong hydrophobic interactions of the drug with LDL may be the rate limiting factor in drug loading. A further time dependent improvement [25% of total loading] of drug loading occurred in the next 2-4 hours which was assumed to be mainly due to partitioning and diffusion processes of the drugs into LDL. Since approximately 70% of the drug was

incorporated within 4 hours, an incubation time of 4 to 6 hours was adopted for subsequent loading experiments. I suggest that incubation time may be a factor which needs attention in order to optimize loading parameters.

2.3.4.2. Incubation temperature: Most drug loading studies reported were conducted at the normal physiological temperature of 37°C. The use of physiological temperature is relevant as the final preparations will encounter this temperature *in vivo*. Temperature was expected to be a factor influencing the drug loading into LDL, because increased temperature results in an increase in molecular motion and subsequently influences the interaction between drug molecules and LDL. Also, temperature has significant influence on the fluidity of the LDL particles, and as a result, affects the molecular dynamics of the particles. I tested drug loading at different temperatures including 4°, 22°, 37°, and 60°C [Figure 2.7]. A further increase in temperature was not considered as apo B protein was found to undergo irreversible denaturation at 80°C [Prassl *et al.*, 1995]. Figure 2.8 shows DSC thermograms of native LDL which starts to denature at temperature above 45°C. As speculated, a higher drug loading with a higher temperature was observed [Figure 2.9]. This observed effect could best be attributed to the thermal transition behavior of LDL components at different temperatures as will be discussed in the next chapter, section 3.3.4.

2.3.4.3. Use of Wetting agents. Drug loading using the dry film method was not efficient. I hypothesized that drug transfer from an aqueous solution or suspension would

be better compared to drug transfer from a solid surface into LDL particles. To make an aqueous suspensions of lipophilic Dox, I used a variety of wetting agents and examined their comparative efficiency in loading Dox into LDL particles. All of the wetting agents resulted in a higher incorporation rate than the dry film method [Figure 2.9]. However, I observed significant differences in the incorporation rate of drug as a function of the nature of the wetting agent. The rate of Dox incorporation ranged from 7 μg/mg LDL protein to 30 μg/mg LDL protein corresponding to an average of approximately 6 to 32 molecules of Dox incorporated by LDL particles [Figure 2.8]. The results obtained with Tweens in formulating Dox prior to their incubation with LDL were equivalent or superior [in terms of recovery of Dox and LDL protein] to those obtained with other agents such as sodium deoxycholate, Spans, and Triton X-100. The loading efficiency with different grades of Tween was not statisically significant [$p>0.05$]. However, the latter experiments were performed using Tween 20 as the wetting agent. Tween 20 is being used in a variety of pharmaceutical formulations to suspend drug and is an approved excipient for parenteral use [Leyland, 1994].

2.3.4.4. Use of liposomal preparations: Liposomes were used as a delivery vehicle to load LDL with Dox to see whether a better loading in LDL particles could take place. Liposomes, mainly multilamellar vesicles [MLV], were made without cholesterol [unstable liposomes] so that they would release their contents once in contact with LDL particles [Storm *et al.*, 1989]. MLV were chosen as the encapsulated aqueous volume is of

minor importance to achieve a high loading capacity as Dox associated with the lipid bilayers [Gabizon, 1995; 1989; Rahman et al., 1989; Crommelin and Bloois, 1983; Goormaghtigh *et al.*, 1980; Goldman *et al.*, 1978]. The preparation of MLVs was first reported by Bangham and coworkers [Bangham *et al.*, 1965]. Their method has proven to be very popular and suitable for the encapsulation of a variety of substances. MLV were considered appropriate for my studies as they had higher incorporation efficiency in loading lipophilic drugs [Basu, 1994]. The extrusion method was adopted to obtain a uniform size distribution of liposomes. An acidic hydration medium was chosen for the preparation of Dox-liposomes as pH 4.0 is favorable for extrusion compared to pH 7.4 [Crommelin *et al.* 1983] and and it was reported that the decomposition rate of liposomal Dox is much higher at pH 7.4 than at pH 4.0 [Beijnen *et al.*, 1986; Janseen *et al.*, 1985]. Dialysis is a common procedure to remove free drug from the liposomal drug, even though this procedure is time-consuming, it is simple and effective and was used in this study. The following aspects concerning the stability of the liposomes were examined: [1] retention of entrapped contents; [2] influence of plasma on the release rate of Dox from liposomes; [3] chemical stability of the encapsulated drug.

Aggregation occured during storage at 4-6°C for over a period of 4 weeks. The absence of electrostatic repulsion is likely to account for the tendency of the liposome to aggregate. About 35% of the amount of drug was lost over a 4-week period [Table 2.6]. Plasma induced leakage was rather high for the liposomes. The highest incorporation was achieved when this liposomal preparation was incubated with LDL [Figure 2.10]. This is

likely because of the better interactions of liposomes with LDL. The phospholipid layers of MLVs interact because of their instability with the LDL and drug is presumably released on the surface or in the vicinity of LDL, from where it diffuses easily into the core of LDL [Vingerhoeds *et al.*, 1994]. This study demonstrated the use of liposomes as a lipid drug donor in drug loading studies with LDL. The highest amount of drug incorporation was possible with the MLV preparations [Figure 2.10] and this method may be expanded to incorporate lipophilic drugs into LDL particles.

2.3.5. Stoichiometry of LDL-drug conjugates

To study the stoichiometry of the incorporation procedure, I added different amounts of drug to fixed amounts of freshly isolated LDL [Figure 2.11]. As the amount of drug was increased, the drug/LDL protein ratio of the conjugate increased until a plateau was approached at approximately 30 μg Dox per mg LDL [32 drug molecules per LDL particle] [n=5] [Figure 2.9]. In all cases, the stoichiometry of LDL drug levels was confirmed after reflotation at a density of 1.063 g/mL. HPLC analysis revealed that the compound did not dissociate nor degrade and constituted more than 95% of the incorporated drug. This study indicates the need to optimize the molar ratio of a drug and LDL to maximize the loading efficiency.

2.3.6. Stability of LDL-drug conjugates after ultracentrifugation and dialysis

The colloidal stability of the LDL-drug conjugates was excellent and no change in

particle size or any aggregation was noted during storage of sterile preparations at 4°C for several months. However, for this study, all preparations were used within 1 week and during this period of time no change in physical characteristics was noted.

LDL-drug conjugates showed good physical stability as examined by density gradient ultracentrifugation [Figure 2.4], in accordance with the results obtained with Dox. The recovery of apo B in the final LDL-drug preparations was very good [~90%]. The values [mean ± SEM] for the recovery of Dox was satisfactory: 84 ± 4% [n=4]. In fact, the high recovery of drug is an important advantage of the contact method.

When the LDL-Dox preparation was subjected to ultracentrifugation, a uniform peak was found [Figure 2.4]. In various preparations the incorporation of Dox varied from 0.2 to 0.6% of the added drug.

2.3.7. Plasma protein binding of Dox: sequential flotation ultracentrifugation

In order to analyze the distribution of Dox in plasma, the compound was incubated with 2 mL of freshly prepared human plasma for 4 hours at 37°C. The solution was then analyzed by density gradient ultracentrifugation [Figure 2.12]. Approximately 78% of Dox was recovered in the fraction at density>1.21 g/mL [lipoprotein deficient, albumin rich fraction]. The other 22% had distributed over the lipoprotein-containing fractions.

Pretreatment of human plasma with oleic acid and subsequent incubation with Dox resulted in a significant decrease from 78 to 33% in Dox in the fraction having d>1.21 g/mL [$p<0.01$] [Figure 2.12]. The HDL- and LDL-containing fractions show a 3-fold increase in

Dox activity, and the VLDL/chylomicron-containing fractions [d<1.006] show more than 2.5-fold change in Dox content [Figure 2.12]. A concentration dependent increse in redistribution pattern was observed with preincubation with different amount of oleic acid. With 1 mg oleic acid preincubation, the effect was less pronounced [data not shown] than that achieved with 3 mg oleic acid preincubation [Figure 2.12]. The redistribution of Dox in different plasma lipoproteins by oleic acid demonstrated that Dox had preference for lipoproteins.

2.4. Discussion

The use of the LDL receptor pathway has not been tested adequately in site specific drug delivery due to lack of a suitable loading procedure to prepare a stable LDL-drug conjugate [Firestone, 1994]. A number of loading methods have been described [Lundberg, 1991]. The major limitation of the methods is either instability of LDL-drug conjugates or low incorporation efficiency. Extensive processing of LDL via reconstitution was reported to increase drug incorporation during their loading [Shaw *et al.*, 1987]. These include extraction of the lipid core by use of organic solvents, detergents, or enzymes, and the use of microemulsions to reconstitute LDL particles [Samadi-Baboli *et al.*, 1990; Masquelier *et al.*, 1986]. These reconstituted particles were found to be larger in size [more than 45 nm, twice the size of native LDL] and less stable than native LDL. The dry film method was considered to be the method of choice and was used for the first time in human trials with vincristine. [Filipowska *et al.*, 1992; Breeze *et al.*, 1994]. However, organic solvents were

used in these studies which is viewed as a disadvantage because of the possibility of protein denaturation.

Wetting agents were used to incorporate very hydrophobic compounds such as CI [De Forge *et al.*, 1991]. I attempted to develop a better method of incorporating drug molecules into LDL particles. An aqueous suspension or solution of a drug was found to be better compared than solid drug in loading experiments with the LDL. I speculated that this was due to better interactions and bonding of drugs with the LDL. In the dry film method, only a limited surface area is available for interactions with LDL and diffusion of drugs from the solid surface is limited. In this case, the interaction of a drug and the LDL should be stronger than the interaction of the drug with the attached materials such as glass or polycarbonate to allow the drug to diffuse from the dry film surface into the core of LDL particles. However, when drugs are in solution or suspended in a medium, a large increase in surface area is achieved and diffusion of drugs from the media into the LDL is more favorable. In the dry film method, drug diffusion into the LDL occured mainly from the monolayer of the dry film whereas in solution or suspension, drug diffusion occurred from all sides.

Liposomes are lipid vasicles like LDL and therefore their interactions are assumed to be thermodynamically more favorable. When the Dox was incorporated into MLV and the latter was incubated with LDL, drug particles were presumably released on the surface of LDL particles from where they could diffuse into the core or intercalate inside LDL particles. Liposomes were unstable in nature as they disintegrates when in contact with lipoproteins. Expulsion of drugs from the liposome in the vicinity of LDL may be the main

reason for higher drug loading efficiency. However, stealth or stable liposomes, which are stable in plasma, may not serve as drug donors for LDL.

The physical properties of the LDL-drug complexes were identical to native LDL. In this study, the Dox loading using a modification of the dry film or contact method has been significantly improved. My procedure does not involve reconstitution or the use of organic solvents in the final preparation. The simple incubation of drug solutions or suspensions with LDL gave incorporation only of 0.2% to 0.6% of the drug added. In addition to higher incorporation efficiency, LDL-drug preparations were stable.

Drug incorporation involves incubation of drug and LDL for a defined period of time. Various incubation times were reported in the literature for different compounds, as mentioned above. I was interested in seeing how incubation time could influence drug loading. If drug loading is time dependent it may give insights into the hydrophobic interactions or diffusion of drugs into LDL particles. Incubation time is important clinically for a more beneficial fast loading technique in which the patient's own LDL may be infused back with the loaded drug [plasmapheresis].

Temperature is another of the major factors which influences drug loading and all my loading experiments were carried out at physiological temperature. Temperature may also affect apo B interactions with the LDL receptor by modulating the physical properties of lipids and/or protein. To observe the influence of temperature on LDL lipids and proteins I used DSC and found no significant alterations in LDL-drug conjugates due to drug loading below 40°C. This will be discussed in more detail in Chapter 3, section 3.3.8.

In order to examine the distribution of Dox over the different plasma lipoproteins, drug was incubated with human plasma [direct addition method] and the mixture was subsequently analyzed by density gradient ultracentrifugation. Seventy-eight percent of the compound was recovered in the LPDP, and 22% associated with HDL, LDL, and VLDL/chylomicrons. As albumin, which is one of the main proteins in the LPDP fraction, is known to bind a variety of endogenous and exogenous compounds, I tried to examine whether a redistribution of Dox association with albumin can be done *in vitro*. Albumin is known to have high affinity sites for fatty acids [Goodman, 1958]. After saturation of these binding sites by pretreatment of human plasma with a solution of oleic acid and subsequent incubation with Dox, a significant decrease [from 78 to 33%] in the amount of Dox recovered in the albumin density fraction was observed. This was accompanied by a strong increase [from 22 to 67%] in the amount of Dox associated with HDL and LDL. The data suggest that an equilibrium exists between Dox bound to fatty acid binding sites on albumin and lipoproteins present in plasma. The equilibrium can be influenced by compounds that compete for the fatty acid binding sites on albumin. This data suggest that drug distribution among lipoprotein subspecies could be achieved; this redistribution pattern may help to diminish adverse effects or increase the therapeutic activity of some drugs. For example, if Dox association with plasma albumin can be refashioned to LDL or other lipoproteins, it will help to diminish the cardiotoxicity of Dox which is related to the rapid association of Dox with plasma albumin *in vivo*. Simultaneously, Dox delivery to cancer cells will possibly be increased. In short, this study demonstrated the significant role of various physical

enhancement factors that affect incorporation of drug molecules in LDL.

Table 2.1. Chemical composition of LDL and LDL-Dox conjugates

	Concentration Ratio's [a]		
Sample	Cholesterol	TAG	Number of drug molecules/LDL
[mg of protein/mL]			Mean [SD] [n=5]
Literature[b]	1.8	0.2	
LDL-native [3.15]	1.1	0.3	
LDL-Dox[c] [4.55]	2.0	0.6	35 [10]

a. Concentration [mg/mL] ratios of cholesterol or TAG, to protein

b. Westensen *et al.*, 1995.

c. The conjugate was made by the contact method with the aid of Tween 20.

Table 2.2. HPLC analytical profiles of Dox

HPLC parameters	Mean values (n≥5, CV≤10%)
Retention time [minutes]	4.77
Detection limit [μg/mL]	0.25
Linear range [μg/mL]	0.5 to 20
% Recovery from LDL samples	>94
% Recovery from plasma samples	> 90

Table 2.3. Calibration coefficient of variation of Dox in HPLC

Conc	1	2	5	10	20	Slope	R^2
µg/mL			Peak areas				
Day 1	0.386	0.597	1.252	2.890	8.844	1.774	0.97
Day 2	0.377	0.587	1.438	3.931	8.516	1.742	0.99
Day 5	0.379	0.582	1.425	3.943	8.147	1.667	0.99
Day10	0.379	0.567	1.476	3.973	8.149	1.667	0.99
Day15	0.374	0.595	1.481	3.867	8.262	1.684	0.99
Mean	0.371	0.583	1.45	3.916	8.149	1.666	0.99
SD	0.012	0.12	0.03	.048	.083		
CV[%]	3.2	2.1	2.1	1.2	1		

Table 2.4. Inter- and intraday variations of Dox

Spiked amount [μg/mL]	1	5	10
Inter-day variation [individual values, peak area]			
Monday	0.95, 0.97	4.92, 4.85	23.52, 24.22
Tuesday	0.96, 0.98	4.95, 4.78	24.85, 25.12
Wednesday	0.99, 1.0	4.90, 4.93	23.56, 24.89
Thursday	1.01, 1.1	4.95, 4.90	25.15, 25.56
Friday	0.98, 0.99	5.00, 4.95	26.5, 25.42
Mean [SD] [n=10]	0.99 [0.04]	4.41 [0.06]	24.87 [0.91]
%CV [n=10]	4.19	1.25	3.67
Two-way ANOVA p value	0.119	0.086	0.457
Intra-day variation [peak area]			
All samples	0.95, 0.96, 0.98	4.92, 4.87, 5.12	23.56, 24.51, 25.10
Friday	1.12, .98, .97	4.65, 4.75, 4.89	24.50, 25.84, 26.23
Mean [SD] [n=6]	0.99 [0.6]	4.87 [0.16]	25.11 [0.95]
%CV [n=4]	6.35	3.29	3.77
ANOVA, p value	0.29	0.12	0.18

Table 2.5. Physico-chemical properties of liposomal Dox

Loading capacity[a]		26 ± 4 mmol Dox/mol PL
Stability[b] at 4-6°C	1 day	5 ± 4% leakage
	1 week	20 ± 10% leakage
	4 weeks	40 ± 15%leakage
Stability[b] in plasma at 37°C	15 minutes	15 ± 5% leakage
	60 minutes	30 ± 10% leakage

[a] Values represent the amount of Dox bound per mole phospholipids [EYPC] in the final product.

[b] Leakage criteria are related to the amount of liposome-bound Dox at day 0 or at the start of the incubation.

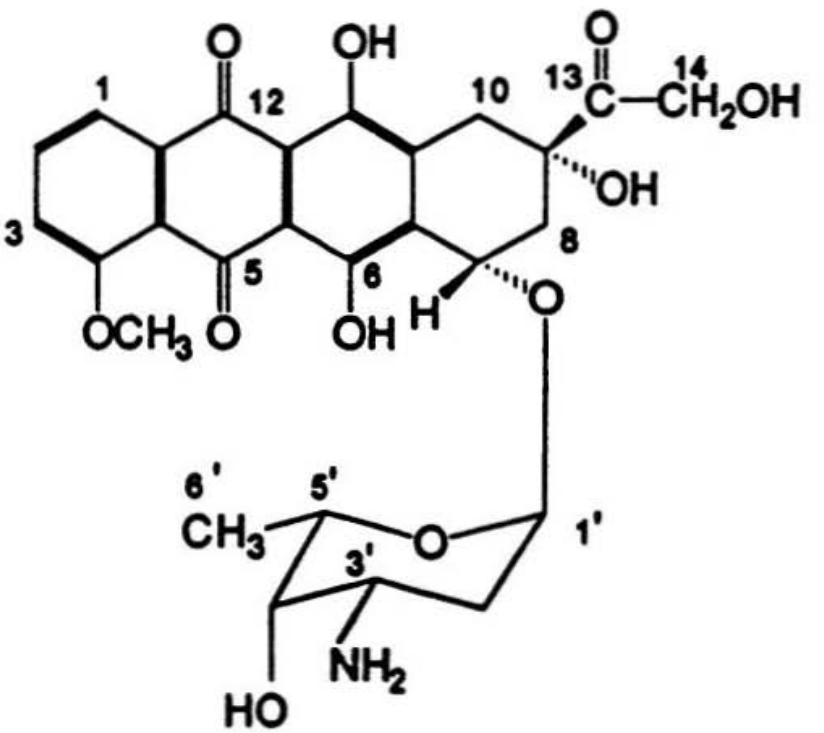


Figure 2.1. Chemical structure of Dox.

Figure 2.2. Chromatograms of Dox in mobile phase using HPLC.

Figures 2.2a. Dox in mobile phase,

Figure 2.2b. Dox extracted from plasma, and

Figure 2.2c. Dox extracted from LDL.

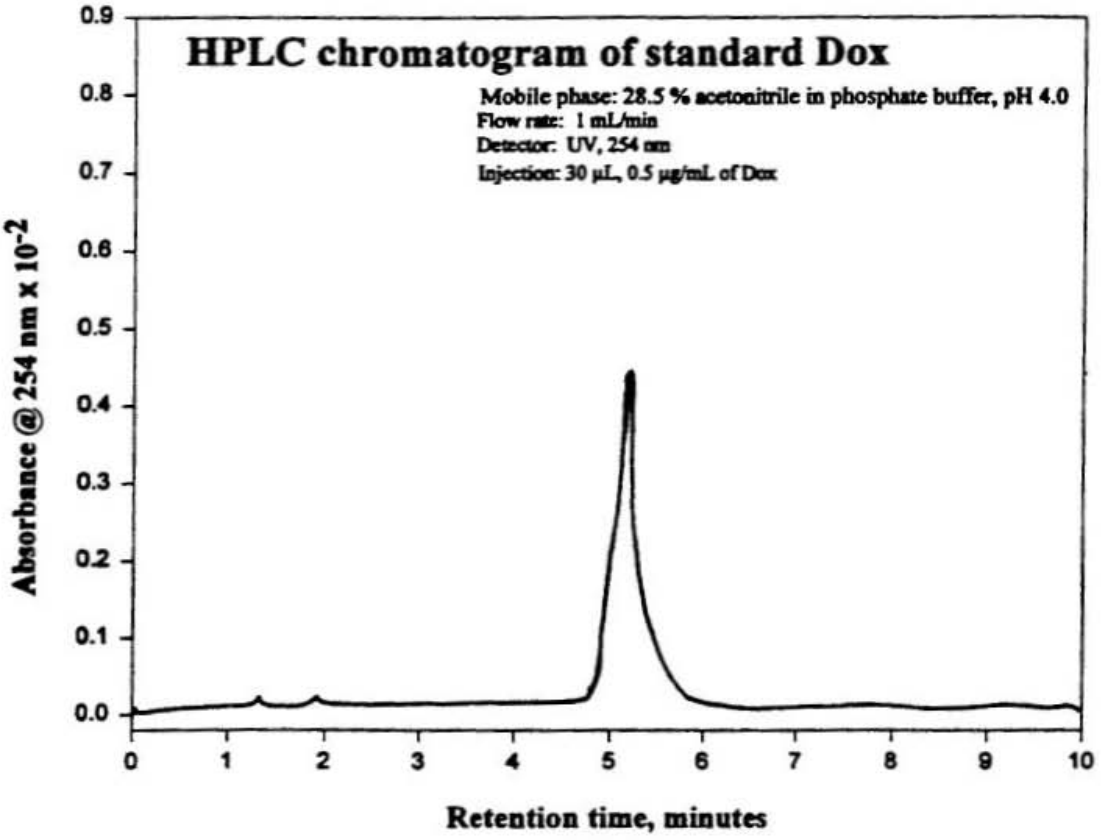

HPLC chromatogram of standard Dox
Mobile phase: 28.5 % acetonitrile in phosphate buffer, pH 4.0
Flow rate: 1 mL/min
Detector: UV, 254 nm
Injection: 30 µL, 0.5 µg/mL of Dox
Absorbance @ 254 nm x 10-2
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
7
8
9
10
Retention time, minutes

Figure 2.2a

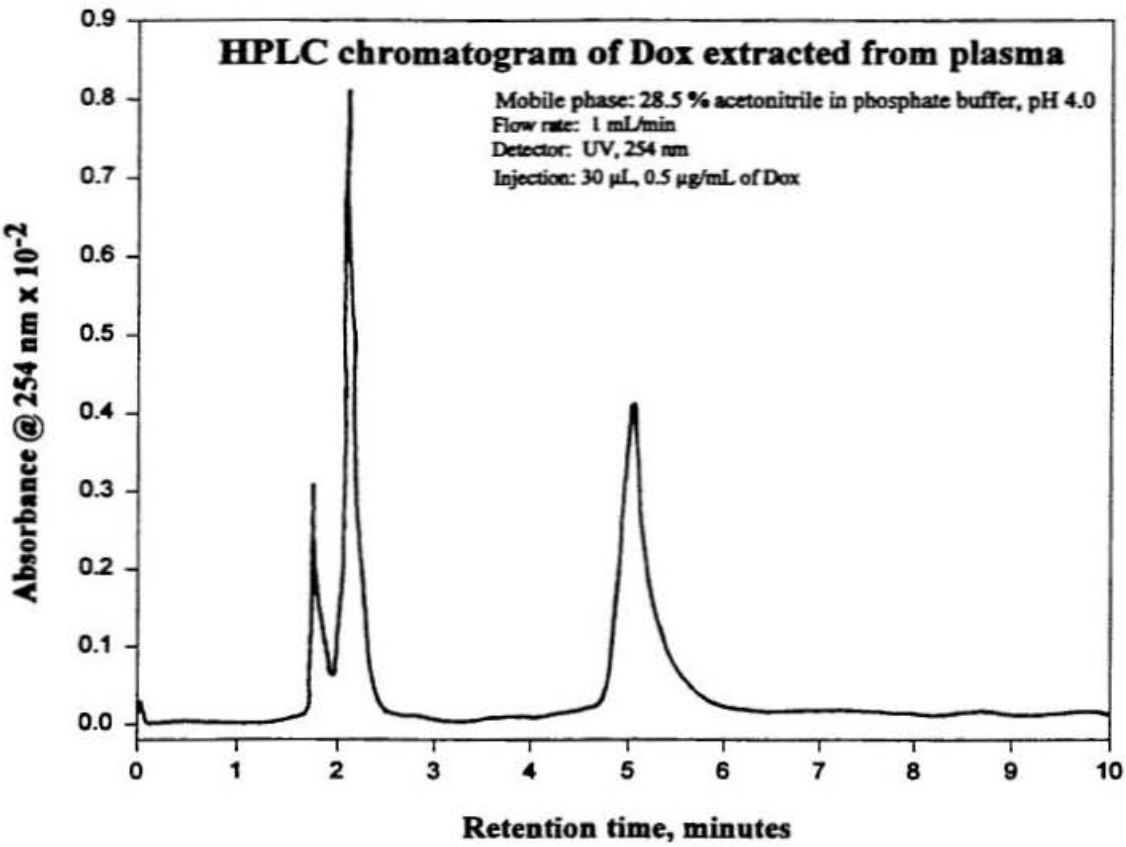

HPLC chromatogram of Dox extracted from plasma
Mobile phase: 28.5 % acetonitrile in phosphate buffer, pH 4.0
Flow rate: 1 mL/min
Detector: UV, 254 nm
Injection: 30 µL, 0.5 µg/mL of Dox
Absorbance @ 254 nm x 10-2
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
1
2
3
4
5
6
7
8
9
10
Retention time, minutes

Figure 2.2b

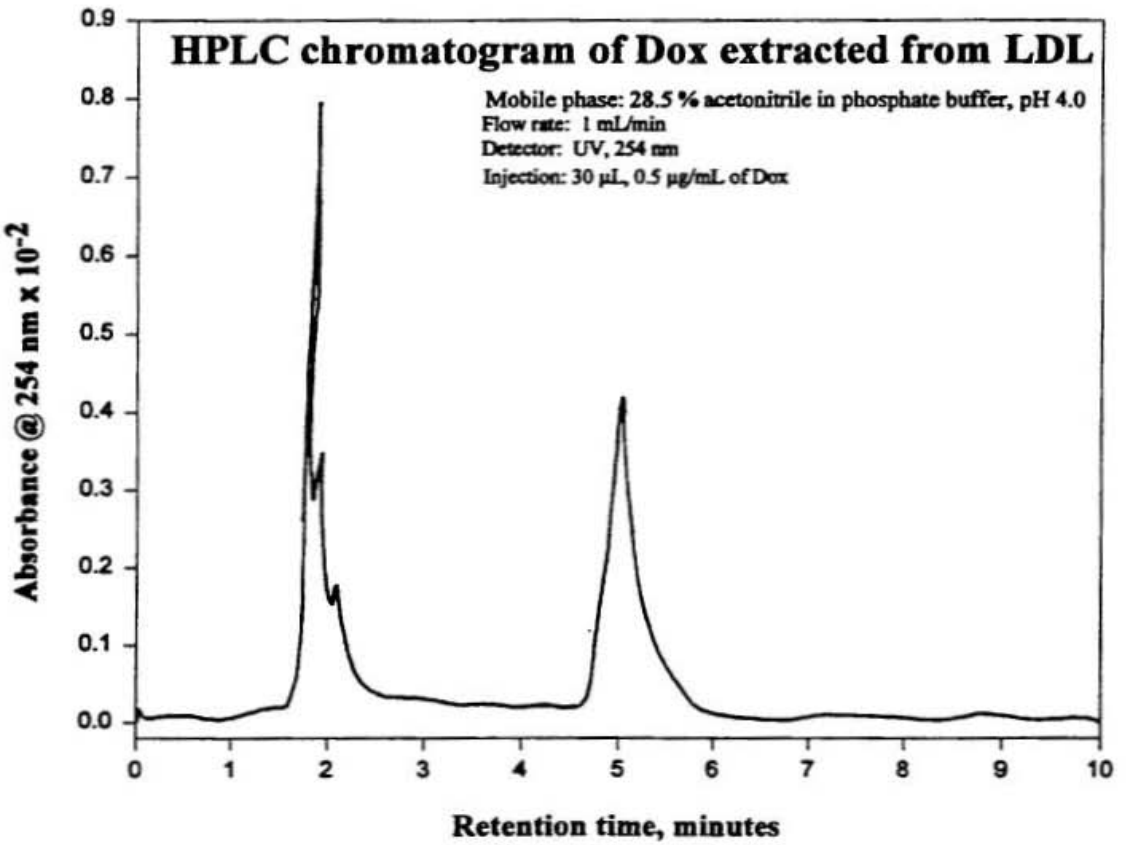

HPLC chromatogram of Dox extracted from LDL
Mobile phase: 28.5 % acetonitrile in phosphate buffer, pH 4.0
Flow rate: 1 mL/min
Detector: UV, 254 nm
Injection: 30 µL, 0.5 µg/mL of Dox
Absorbance @ 254 nm x 10-2
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
1
2
3
4
5
6
7
8
9
10
Retention time, minutes

Figure 2.2c

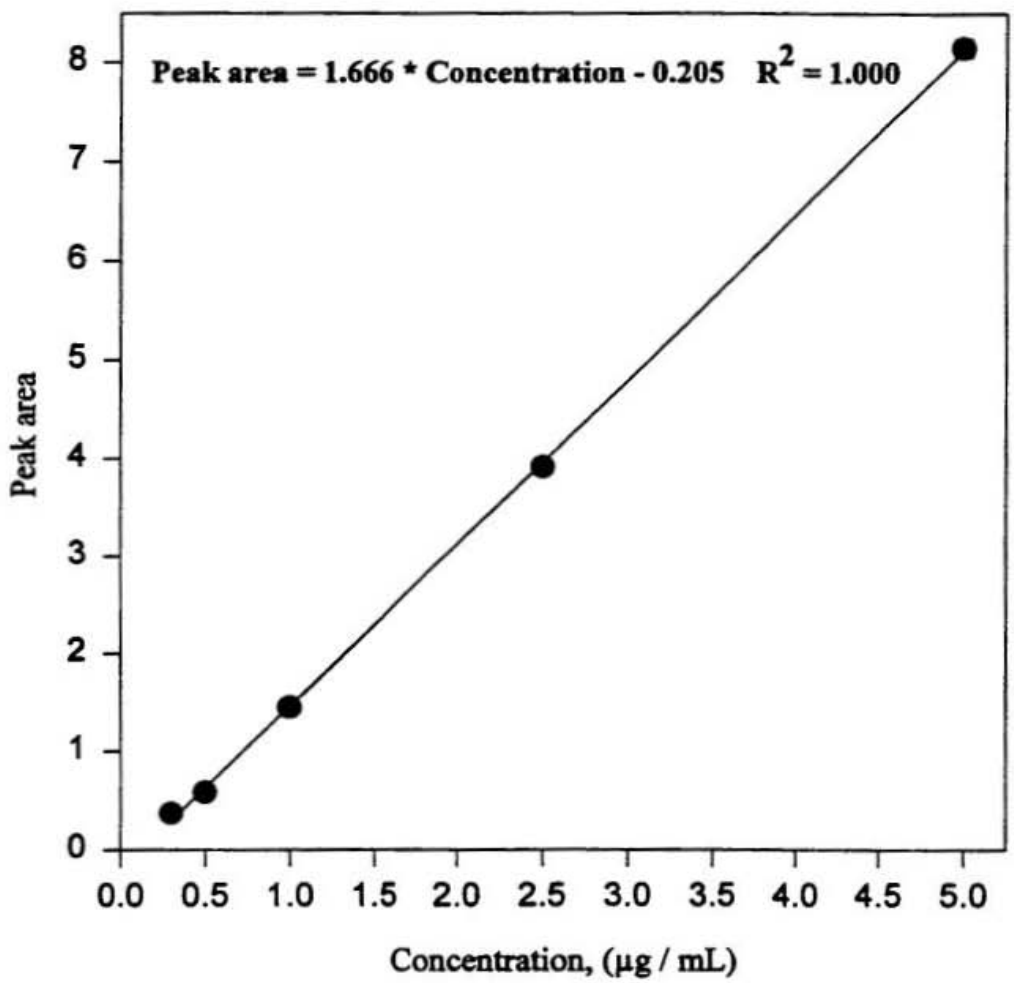


Figure 2.3. Calibration curve of peak area versus concentration of spiked standard solutions of Dox. Each point represents the mean ±SD of five determinations.

Figure 2.4. Stability of LDL-Dox conjugates after ultracentrifugation. Density ultracentrifugation of solutions of Dox that were incubated with LDL according to the contact method with Tween 20. After density ultracentrifugation at 40,000 rpm, 8°C, for 30 hours in a 75Ti (Beckman rotor) 0.5 mL fractions were collected from top to the bottom of the tube. Each fraction was assayed for Dox and protein concentration using HPLC and the Bradford method, respectively. Each point represents the mean ± SD of three determinations.

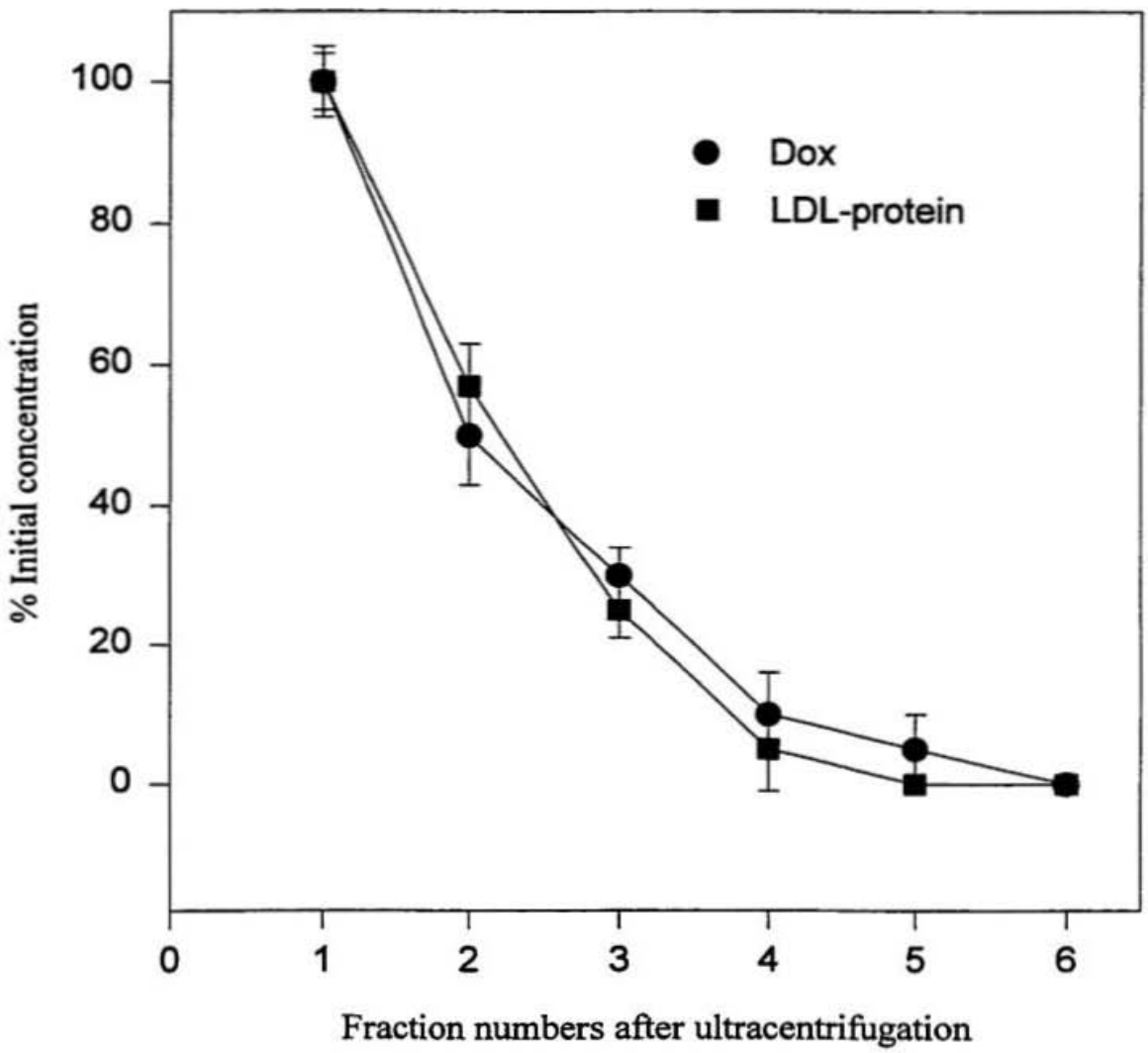
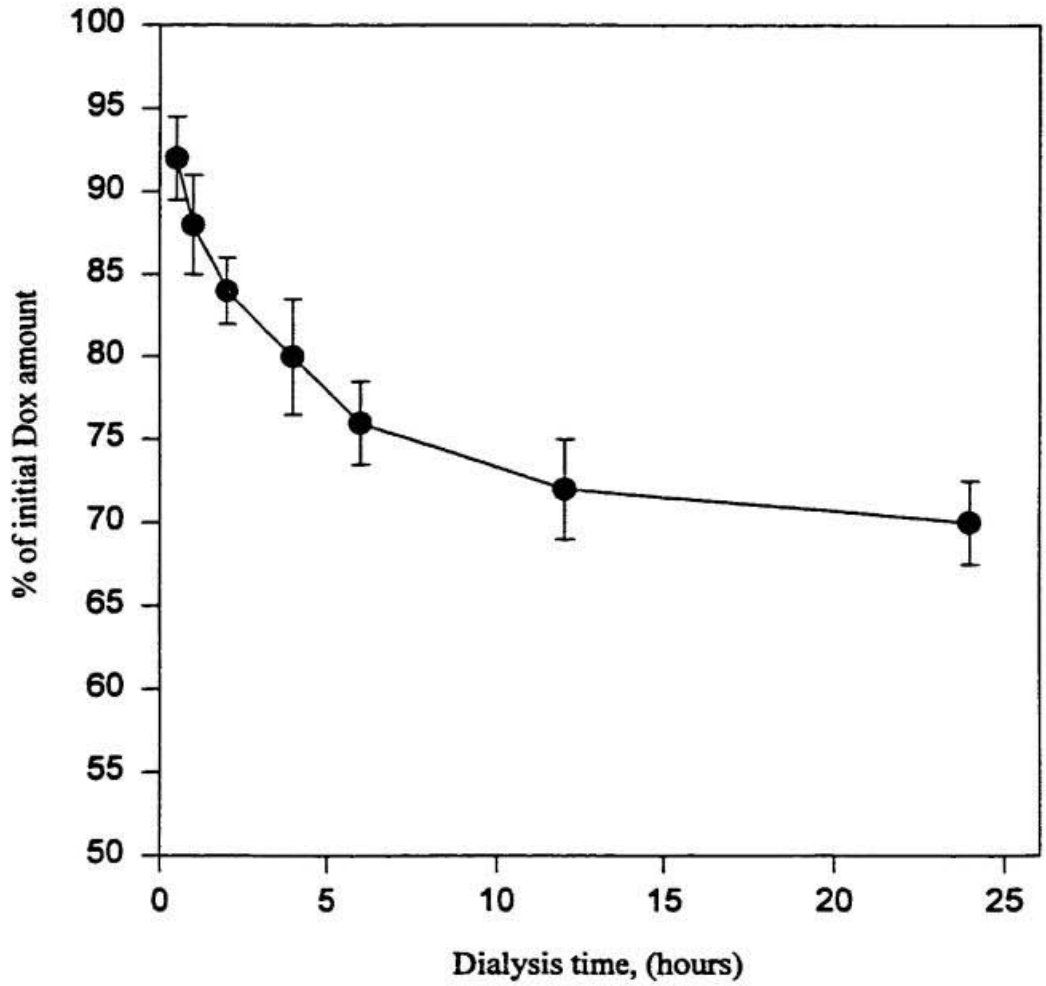

100
80
60
40
20
0
% Initial concentration
Dox
LDL-protein
0
1
2
3
4
5
6
Fraction numbers after ultracentrifugation

Figure 2.5. Effect of dialysis on the stability of LDL-Dox conjugates. LDL-Dox prepared as mentioned in Figure 2.3. The top fraction was colllected and aliquots of 0.5 mL were subjected to dialysis for the indicated time and withdrawn for drug and protein assay were performed using HPLC (for Dox) and Bradford assys. Dialysis was performed at 2-8 °C against 2 L of dialysis buffer. The unbound drug or salt was removed during dialysis. Each point represents the mean ±SD of three determinations.


100
95
90
85
80
75
70
65
60
55
50
% of initial Dox amount
0
5
10
15
20
25
Dialysis time, (hours)

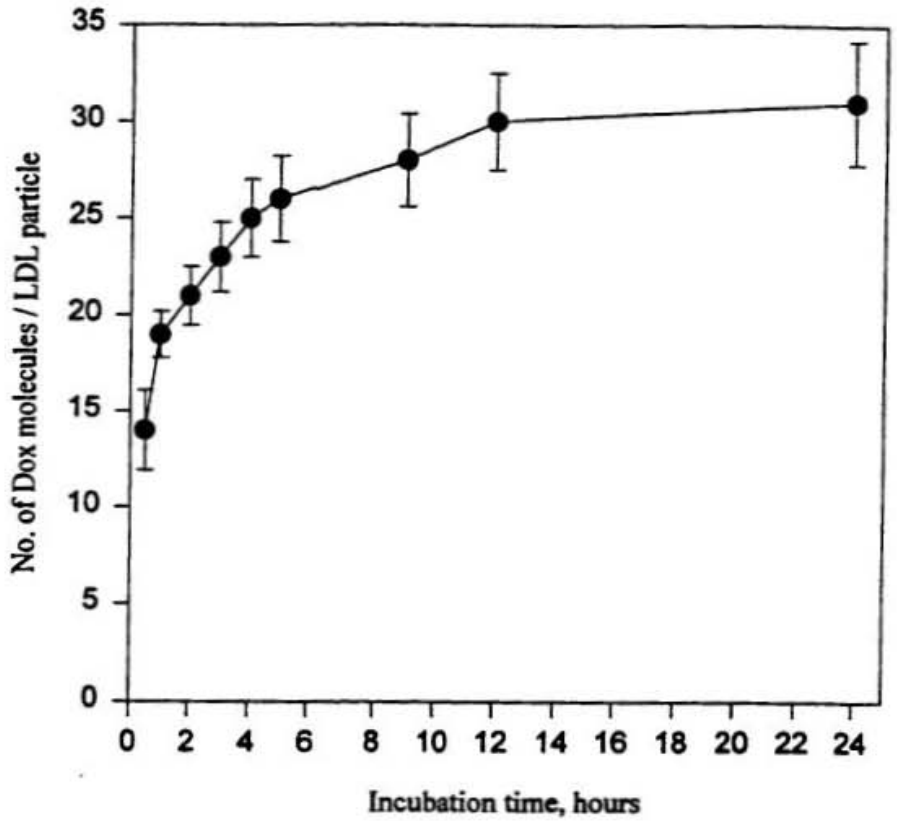
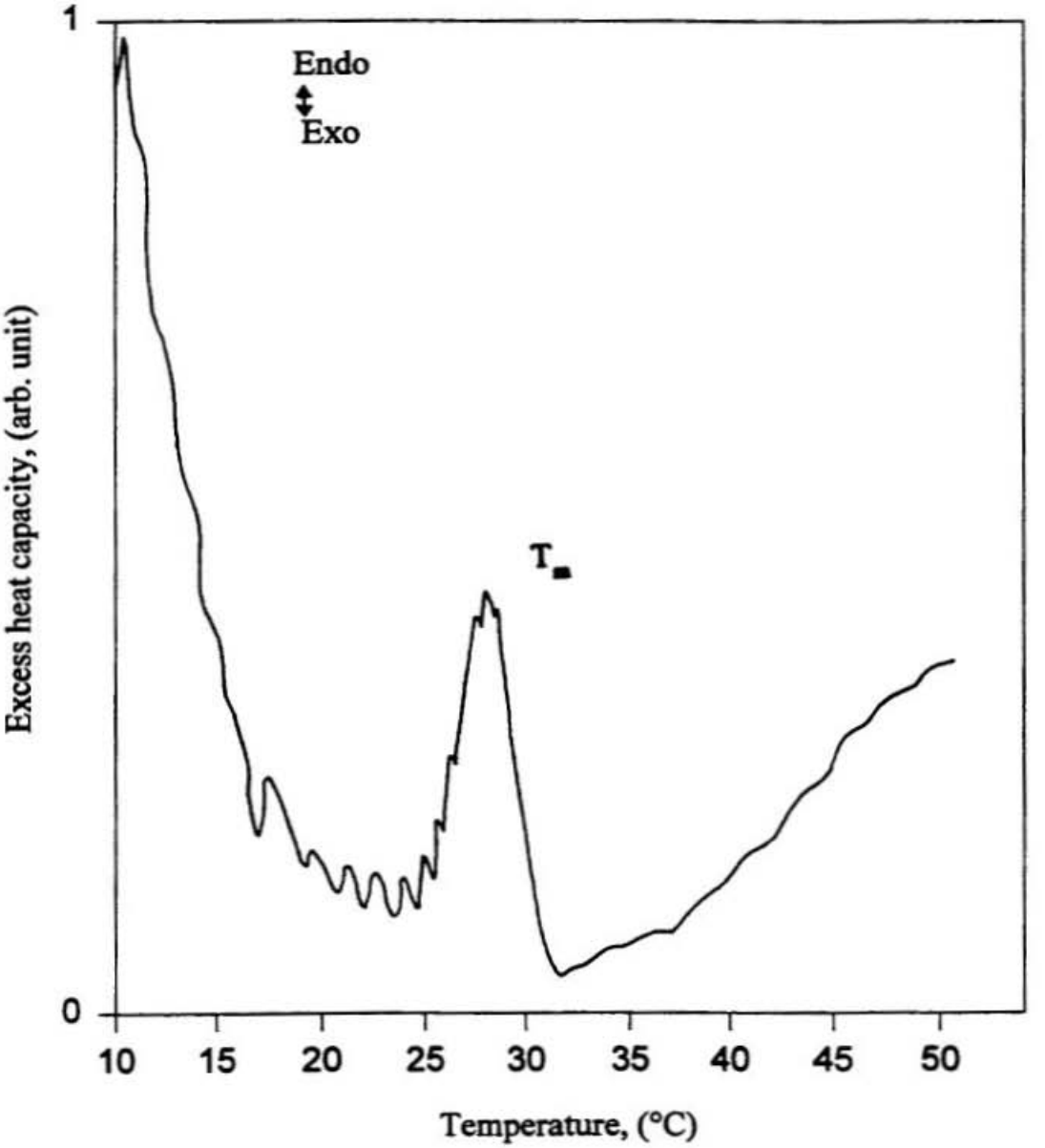


Figure 2.6. Effect of incubation time on Dox loading. Five hundred μg Dox coated on a glass vials were suspended with Tween 20 (>3% of final concentration) and were incubated with 500 μg LDL up to 24 hours at 37°C. After incubation, LDL-Dox conjugates were isolated from the free drug by ultracentrifugation followed dialysis. Drug and protein assays were performed as mentioned in Figure 2.5. Each point represents the mean ±SD of three determinations.

Figure 2.7. Differential scanning calorimetry of native LDL. Samples were scanned at 0.5°C / min from 10 to 50°C. DSC sensitivity was the same for all runs. For all samples, the protein concentration was more than 1.50 mg / mL. Buffer baselines were subtracted. The data were normalized to the protein concentration. Similar DSC thermograms were obtained (Similar T_m and variable transition enthalpy for samples) for LDL-Dox conjugates. Thus, DSC thermograms for LDL-Dox were not shown.


1
Endo
Exo
Excess heat capacity, (arb. unit)
T_m
0
10
15
20
25
30
35
40
45
50
Temperature, (°C)

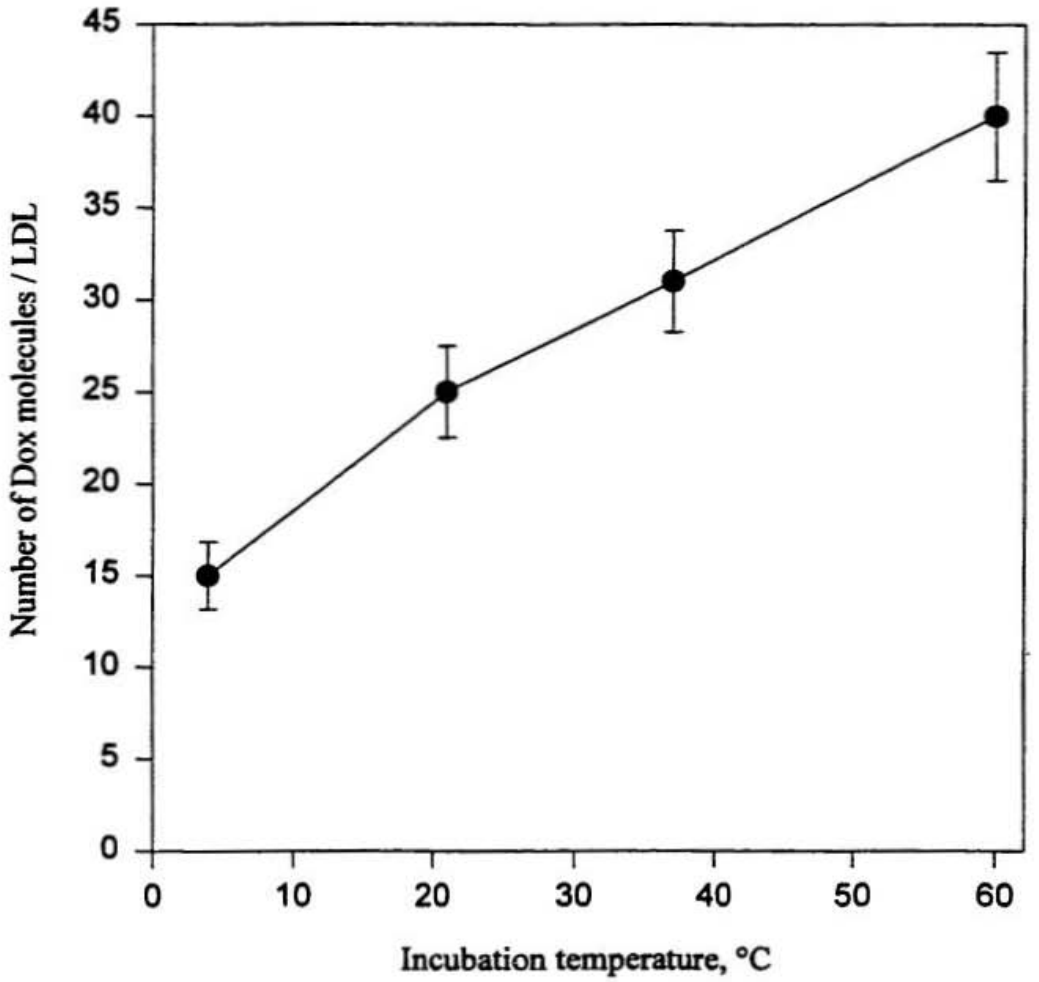


Figure 2.8. Effect of incubation temperature on Dox loading. For explanation see Figure 2.6. Each point represents the mean ±SD of three determinations.

Figure 2.9. Relative incorporation efficiency of detergents in loading Dox in LDL using contact method. Control indicates the dry film method. In the contact method, the dry film residue was suspended with the agents indicated before incubation. Incubation conditions were mentioned in Figure 2.6. Each point represents the mean ±SD of three determinations.

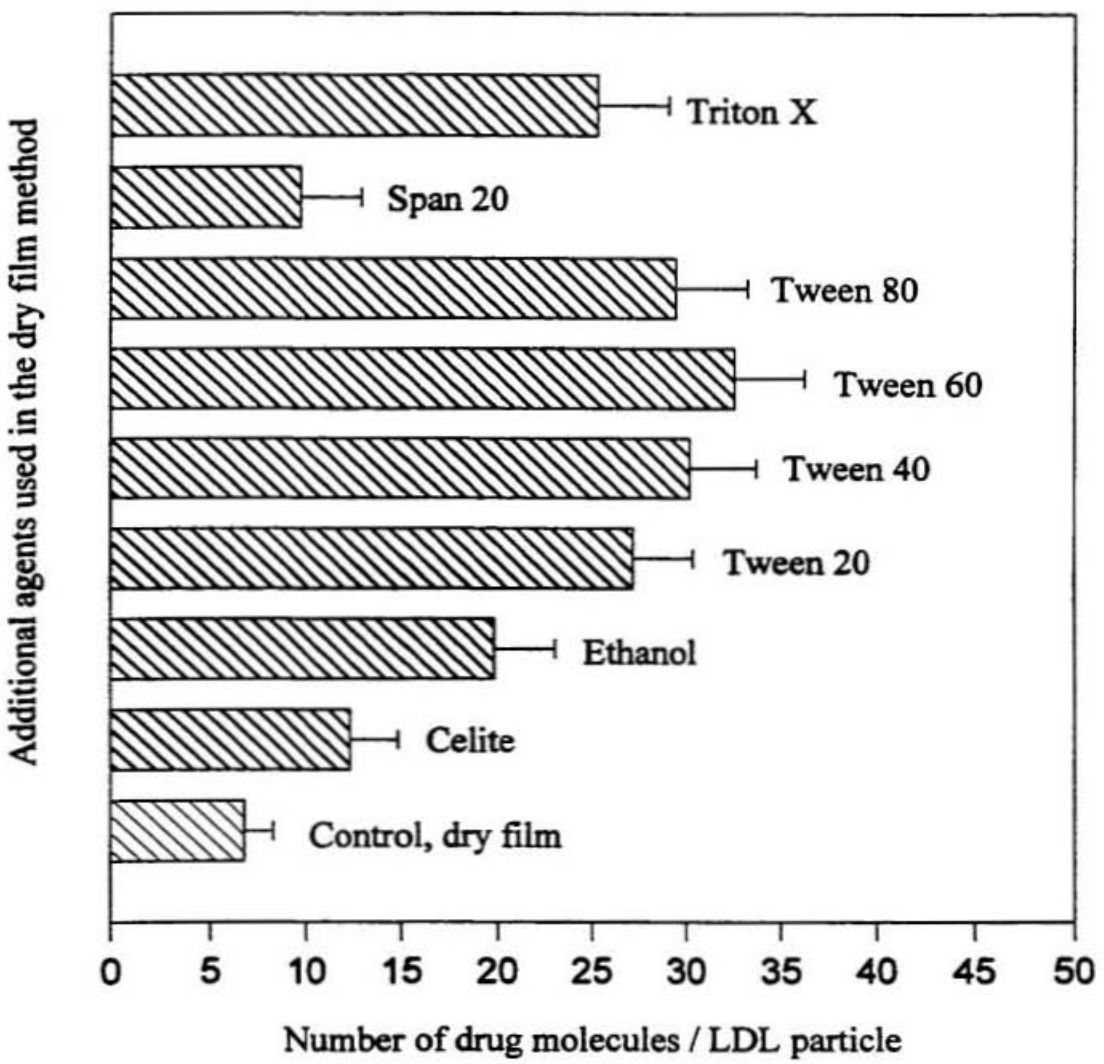
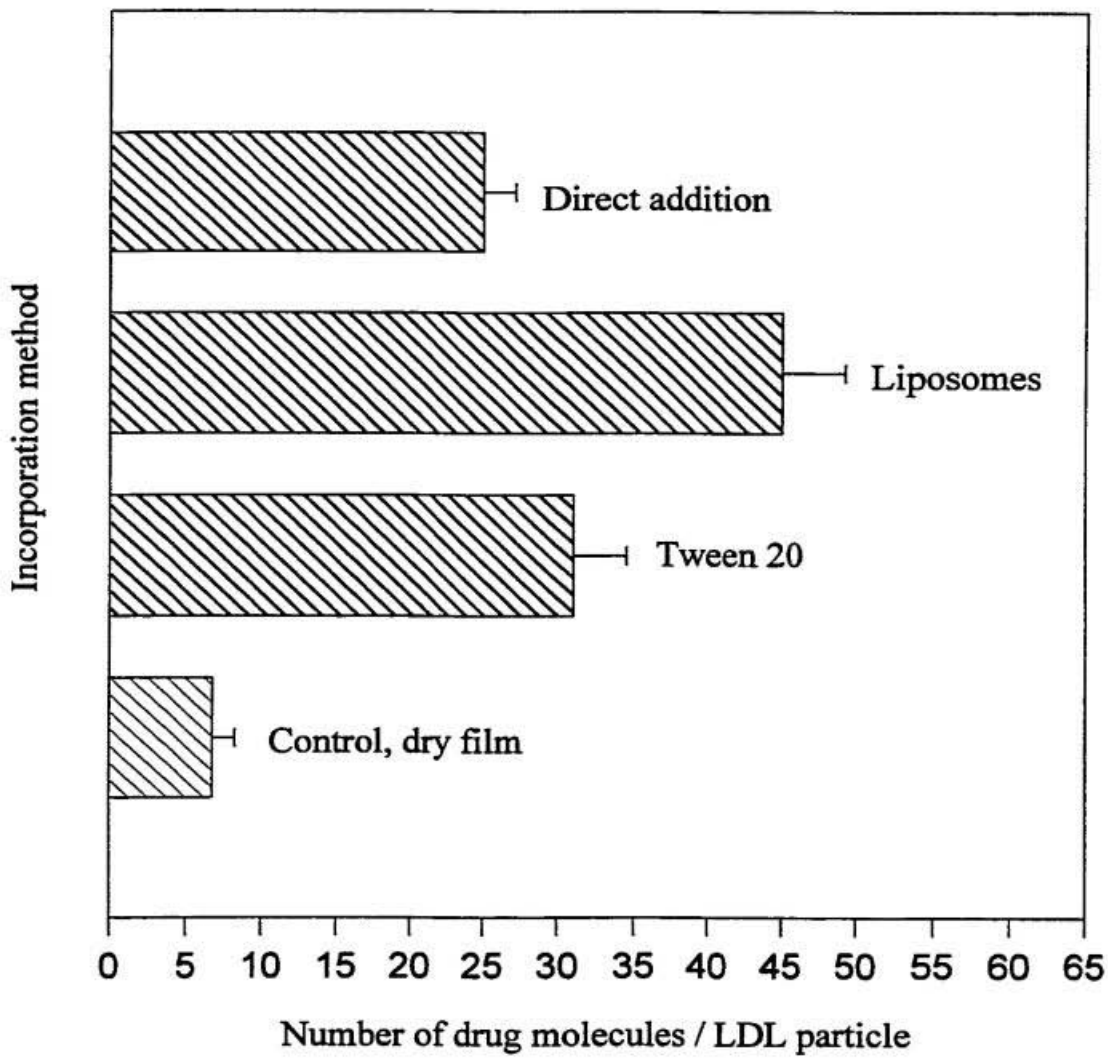

Additional agents used in the dry film method
Triton X
Span 20
Tween 80
Tween 60
Tween 40
Tween 20
Ethanol
Celite
Control, dry film
0
5
10
15
20
25
30
35
40
45
50
Number of drug molecules / LDL particle

Figure 2.10. Relative incorporation efficiency of different methods in loading Dox in LDL particles. See material and methods for details. The dry film and the contact method with Tween 20 were described before. Liposomal Dox was made using EYPC (Dox:EYPC=1:40) by the extrusion method and free dox was isolated from liposomal Dox by dialysis method. Drug loading efficiency of liposome was 24%. DoxHCL or liposomal Dox was directly added to the LDL (the direct addition method) and was incubated according to the conditions described in Figure 2.6. Each point represents the mean ±SD of five determinations.


Incorporation method
Direct addition
Liposomes
Tween 20
Control, dry film
0 5 10 15 20 25 30 35 40 45 50 55 60 65
Number of drug molecules / LDL particle

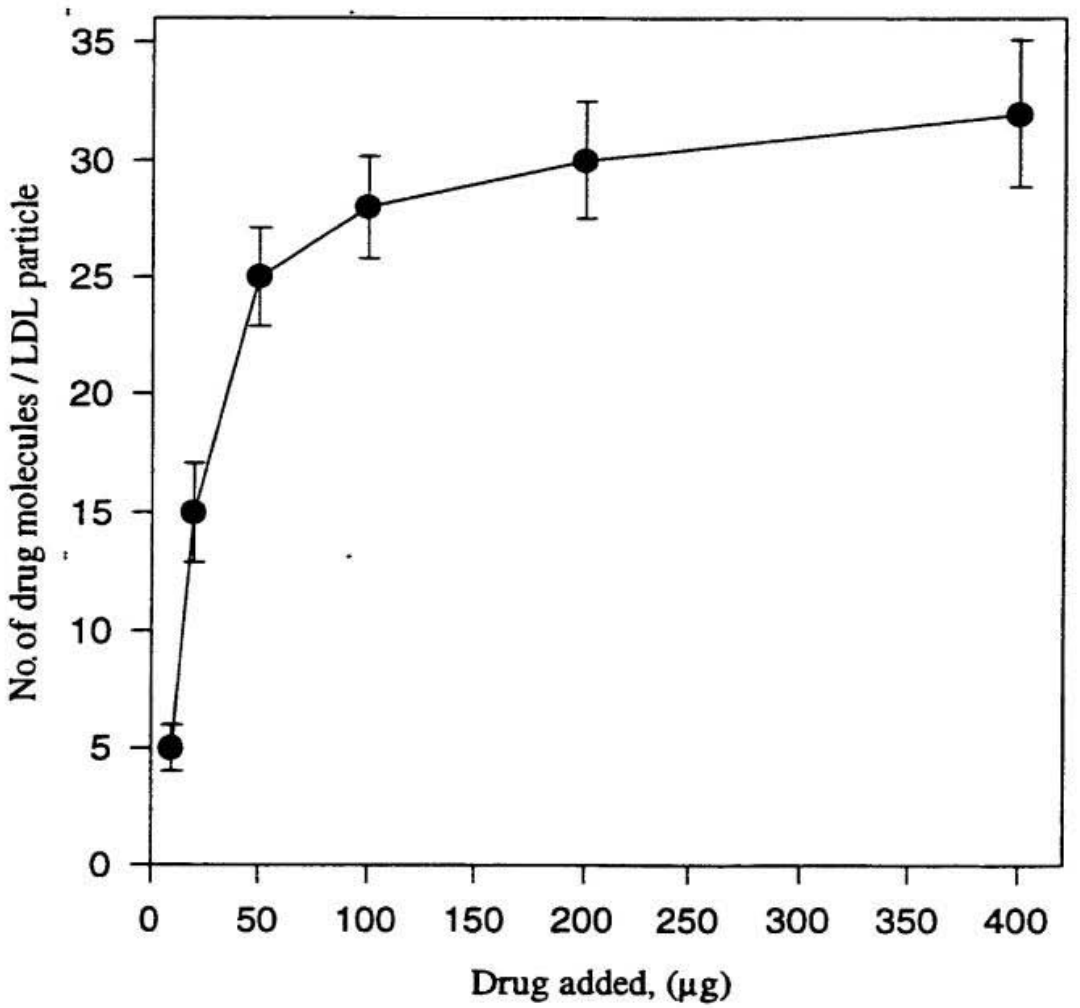
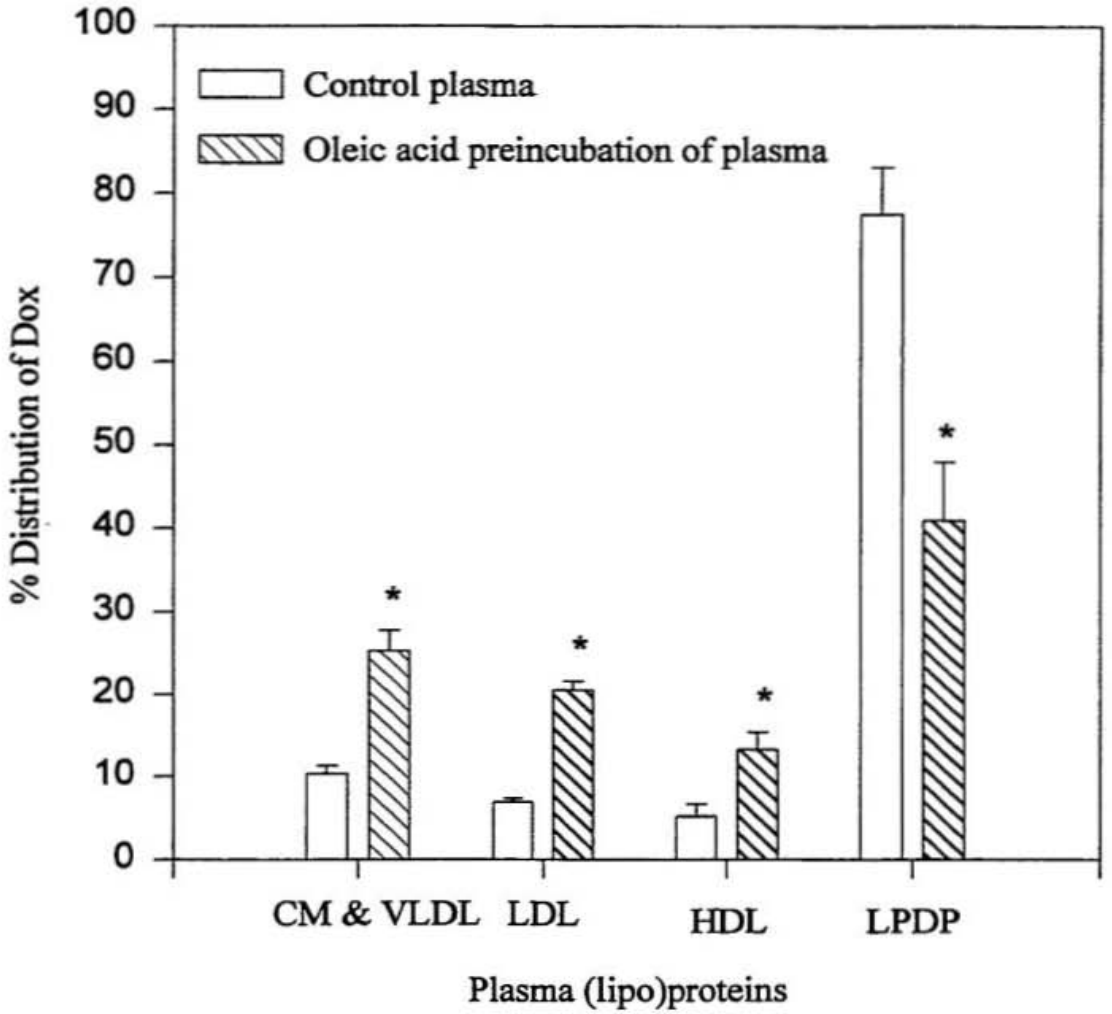


Figure 2.11. Stoichiometry of LDL-Dox conjugates. LDL-Dox conjugates were prepared using Dox and constant amounts of LDL by the contact method using Tween 20 at 37°C (see Figure 2.6). Each point represents the mean ±SD of five determinations.

Figure 2.12. Effect of preincubation of human plasma with oleic acid on the association of Dox with plasma (lipo)proteins. LDL-Dox conjugates were incubated for 4 hours at 37°C with 2 mL of human plasma (normal), or with 2 mL of human plasma that was preincubated with 3 mg oleic acid. After the incubation, different lipoprotein fractions were sequentially isolated from plasma using sequential floatation ultracentrifugation. All lipoprotein fractions were analyzed for Dox content by HPLC. Each point represents the mean ±SD of three determinations. The asterisk (*) indicates statistically significant difference between two groups when analyzed by student's t-test [$p<0.05$].


100
90
80
70
60
50
40
30
20
10
0
% Distribution of Dox
Control plasma
Oleic acid preincubation of plasma
*
*
*
*
CM & VLDL
LDL
HDL
LPDP
Plasma (lipo)proteins

CHAPTER 3:

PHYSICO-CHEMICAL CHARACTERIZATION OF LDL-DRUG CONJUGATES

3.1. Introduction

The suitability of LDL as a carrier for Dox was tested further by investigating the interactions of the LDL-Dox conjugates with plasma lipoproteins. I have demonstrated that Dox can be loaded into the lipid core of LDL particles. If the LDL-Dox conjugates are unstable in plasma, the released free drug will be rapidly distributed to plasma albumin. Therefore, a targeting effect will not be achieved. In addition, Dox loaded inside the LDL should not undergo leakage, or redistribution, or exchange with lipoproteins present in plasma. This kind of premature release or leakage of the drug would make the LDL-mediated approach null and void. I tested the carrier capacity of LDL in which LDL-Dox conjugates would be incubated with plasma and the drug distribution between different lipoprotein fractions would be monitored using sequential flotation ultracentrifugation in conjunction with HPLC.

Extensive processing during drug loading was reported to influence physico-chemical properties, surface properties, and the receptor affinity of LDL [Shaw *et al.*, 1987]. If their native integrity is changed, they would be recognized by the RES. Thus, it is important that LDL retains its native properties even after drug incorporation. With the aid of different biophysical techniques, it is now possible to characterize LDL molecules and establish their

native integrity. For example, the size and the physical properties of the LDL-drug conjugates can be evaluated using EM and DSC. By using electrophoresis, the native integrity of apo B protein in terms of protein degradation can be evaluated. In Chapter 2, I have studied Dox loading in LDL. In this chapter, LDL-Dox conjugates were studied using SDS-PAGE, EM and DSC.

3.2. Materials and methods

3.2.1. Materials

Human plasma, LDL, Dox, LDL-Dox conjugates were described in Chapter 2.

3.2.2. Carrier capacity of LDL-Dox conjugates in plasma

In order to examine the carrier potential of LDL for Dox, LDL-Dox conjugates were incubated with plasma for 2 hours at 37°C. After incubation, the distribution of Dox over plasma lipoproteins was determined by sequential flotation ultracentrifugation as described in Chapter 2 [section 2.10].

3.2.3. SDS-PAGE

The electrophoretic mobility of LDL and LDL-drug conjugates was examined by SDS-PAGE. A 5% gel was run according to the method of Laemmli [1970] and stained with Coomassie Brilliant Blue R-250 using Mini-PROTEAN II electrophoresis cell [BioRad,

Hercules, CA].

3.2.4. EM

The particle size of native and LDL-drug conjugates was measured by EM. LDL-drug conjugates were prepared according to the method of Forte *et al.* [1968] This was performed on a Philips EM 300 by Lisa Lee and Howard Gladney [EM unit, Medical School Laboratories, Memorial University of Newfoundland, Canada]. Samples [20-40 μg/mL] were negatively stained with 1% uranyl acetate and photographed at magnifications [calibrated] of 10,000 or 50,000 or 75,000. The diameters of lipoproteins were measured on 3x enlarged photographic prints.

3.2.5. DSC

Both native LDL and LDL-Dox conjugates were run according to the procedure of Keough *et al.* [1991] on a Microcal 2 [model MC-2, Amherst, MA] operating at a scan rate of 0.5°C/min. The protein concentrations of all samples were maintained at 1.78 mg/mL. By integrating the areas under the transition curve using Microcal software, transition enthalpies were readily determined.

3.3. Results

3.3.1. Carrier capacity of LDL of Dox in plasma

Ideally LDL-Dox conjugates should not release drug, which would then be distributed in plasma. Such stability of the LDL-Dox conjugates was tested by incubation of LDL-drug conjugates in human plasma. More than 75% of the added drugs [Dox] in the form of LDL-drug conjugates was found in the LDL fractions of plasma following a 2 hour incubation at 37°C [Figure 3.1]. Ultracentrifugation of the plasma at different densities was carried out to isolate different lipoprotein fractions; free drug quantities in all those fractions were analyzed by HPLC. All other fractions contained a low quantity of drugs [less than 15%] [Figure 3.1]. These results suggest that the drug, in the LDL particle, remained non-exchangable in plasma. This is especially important in respect to potential applications of LDL-drug conjugates in therapy in which LDL-drug conjugates will not dissociate or exchange in the circulatory plasma before being taken up by cancer cells.

3.3.2. Electrophoretic mobility

The electrophoretic mobilities [a single band] of LDL-drug conjugates were similar to the mobility of native LDL on 5% polyacrylamide gels, indicating that particles had an identical size [Figure 3.2]. SDS-PAGE did not show any apo B fragmentation. This suggests that the drug loading procedure did not induce any protein degradation.

3.3.3. EM

The mean diameter and the size distribution of LDL-drug conjugates compared to native LDL was determined by EM. Negative staining of the different LDL systems with uranylacetate revealed a homogenous size distribution for the native LDLs and the drug-loaded systems [Figure 3.3]. Particle size measurements revealed that the conjugate prepared by methods used in this study had size close to native LDL [Figure 3.4] while LDL-drug particles prepared according to Kriger *et al.* [1978] were reported to be larger [~45 nm].

Isolated particles appeared to be spherical in shape, but adjacent particles were anisometrically deformed. The mean particle diameters were obtained from photographs [magnification x 75000] after another x 1.5 magnification by copying. Average measurement of 100 particles for each sample yielded a mean diameter of 22 nm for native LDLs [20.43-22.52 nm], and LDL-Dox [20.34-22.61 nm] [Figure 3.4].

3.3.4. DSC

I monitored the thermal transition of LDL lipids by DSC which gave some insight into the effect of temperature on drug loading as well as the site of the drug in the LDL [Table 3.1]. LDL cholesteryl esters undergo a thermotropic transition close to physiological temperatures, 30°C [Figure 3.5]. Below the melting temperature, [Tm], which is 25°C, the CE exist in a radial smectic state, while they exist in a liquid state above Tm [Deckelbaum *et al.*, 1977]. The fact that the Tm depends on the core TG content suggests that the physical state of the core lipids will change above Tm. This may explain why drug diffusion increases

with an increase in temperature above Tm. However, if drug resides in the core and disrupts the binding of core lipids, a different transition may be speculated.

Typical DSC thermograms of LDL samples alone [Figure 2.7] and in the presence of Dox, were studied. A broad transition of core lipids was observed in the range of 24-27°C with all preparations [Figure 3.5]. This non-cooperative transition behavior is inherent to LDL samples. The apparent change in transition enthalpy of LDL samples due to drug loading could be explained in terms of their miscibility with core lipids. When Dox was loaded in the LDL, a decrease in transition enthalpy was observed [Table 3.1]. This result suggests that Dox intercalates with LDL core lipids and exists as a separate entity in the core lipid. Since Dox is not miscible, it perturbs the packing of core lipids which start to loose some of their transition enthapy. This may be the reason for the low transition enthalpy of core lipids of LDL-Dox conjugates. The packing of core lipid is very important as it may influence the surface structure of LDL and hence interaction of apo B with cell membrane LDL receptors. An altered LDL surface structure could also affect the interaction of LDL with tissues by receptor independent mechanisms as well as its susceptibility to *in vivo* modification.

Cholesterol increases the order of the surface PLs of native LDL. Philips and Schumaker [1989] have shown that approximately 70% of the total unesterified cholesterol is present on the LDL surface. These cholesterol molecules interact particularly strongly with LDL phospholipids in part because they have a high content of saturated PLs and sphingomyelin. ApoB and core lipids have a major influence on the surface order. An

analysis of the amino acid sequence of apo B shows that there are numerous domains throughout its sequence which can interact with the lipid domain. An analysis of the lipid binding properties of proteolytic fragments of apo B has shown that lipid binding regions are widely distributed within the protein. In my studies the native integrity of LDL was restored as no degradation products appeared on gel for LDL preparations [Figure 3.4].

The incubation temperature at 37°C seemed to have no adverse effect on physical properties of LDL. Nevertheless, drug molecules were found to have loaded efficiently into the core of LDL particles as evident from their transition enthalpy. Whether this drug binding will affect receptor binding properties of LDL could be examined *in vitro* by biological evaluations of the conjugates in tissue cultures. My DSC results with different samples reconfirmed previous findings. The magnitude of this change is similar to that observed during the liquid crystalline to crystalline phase transition of PL bilayers. At higher temperatures, for example, 60°C, a higher loading of drugs was achievable [Figure 2.8]. This was probably due to a more fluid nature of LDL and more molecular motions of the drug during Brownian diffusion into the LDL particles.

I conclude that neither the conformation of apo B nor its ability to bind to the LDL receptor is likely to be affected by temperatures below 40°C. Drugs were loaded at least in the core of LDL particles.

3.4. Discussion

The use of LDLs as drug carriers is dependent on drug loading that does not drastically altering their structure. The LDL-drug conjugates must not be recognized by the RES, and the receptor mediated uptake by cells must not be affected, since the main aim in the use of LDL as drug carriers is their uptake by cancer cells. For this reason, I have monitored the structural characteristics of the drug-loaded LDL and some of their dynamic properties, as well as the state of apo B. The major finding of the present study is that, about 0.3 weight (wt) % of drug can be incorporated without any detectable disturbance of the structure of the LDLs. The leakage of drugs from LDL-drug conjugates was not significant. These findings imply that LDL-drug conjugates will be effectively recognized as native LDL by the apo B receptors that are enriched on tumor cells. Once recognized, the LDL-drug conjugates should be internalized and release the incorporated drugs intracellularly. In this way, a targeting effect may be achieved [see Chapters 5 and 6].

To observe the influence of temperature on LDL lipids and proteins I used DSC and found no significant alterations in LDL-drug conjugates due to drug loading at temparatures below 40°C. The LDL particle provides several sites for the insertion of drug molecules, depending on their lipid solubility. The core is by far the best site because of its capacity and ability to shield the drug from the extracellular enzymes. A second site for drug intercalation is the PL monolayer. Agents that have both polar and lipid soluble components may partition in the monolayer, between the apolar core and the aqueous environment. However, in this location they would be less well protected from plasma hydrolases and water. Further, they

may be able to stimulate the immune system to remove the LDL-drug conjugate from the circulation. My DSC results showed that Dox perturbs the thermal transition of core LDL lipids. This suggests that the drug was loaded inside the core of LDL.

The LDL used in this study had an average diameter of 21.9 ± 1.21 nm [n>100 particles], in agreement with previously published results [Tucker and Florence, 1983]. After drug incorporation, the average LDL diameter also did not increase significantly. The amount of drug incorporated in LDL did not change its size significantly. This implies that the incorporation of drug does not greatly deform LDL structure.

The present study showed that apo B protein of the LDL preparations retained its native conformation when the drug was incorporated by the procedure described above. The LDL receptor-mediated endocytosis of cytotoxic LDL-drug conjugates might provide distinct advantages over the trapping of antineoplastic drugs in liposomes. The main reason for this statement is that liposomes are subjected to destruction by blood components, primarily by lipoproteins [Wasan *et al.,* 1996] and to a fast clearance from the circulation by the RES [Janknegt, 1996; Basu, 1994]. Since the loading of the particles with drug molecules does not influence the conformation of the protein component of the LDL, this preparation is expected to follow the same *in vivo* fate as native LDL particles in animal or human studies. This is very important, as the use of LDLs as drug carriers depends upon the preservation of the particles' characteristics, such as receptor recognition and RES avoidance. The present study indicates that, in favorable cases, such as loading with the lipophilic drug, the characteristics of the LDL structure and its dynamic properties are entirely preserved despite

the incorporation of significant amounts of drug [about 0.5 wt %]. In conclusion, it is possible to load Dox efficiently into LDL particles without perturbing some of the physico-chemical properties of LDL.

Table 3.1. Calorimetric parameters of LDL preparations*

Sample	Tm (CE), °C	ΔH_{cal} (KJ/Kg of apo B)
LDL	25	6.473
LDL-Dox	26.6	1.474

* The values are the average of several heatings; the experimental error is within ±10%. This refers to the transition of core lipid peak.

Figure 3.1. **Carrier capacity of LDL for Dox.** LDL-drug conjugates were incubated with plasma for 2 hours at 37°C. Different lipoprotein fractions were isolated using sequential ultraflotation method and drugs were analyzed by HPLC methods. Plasma was sequentially adjusted to d=1.006, 1.063, or 1.121 g/mL by KBr and ultracentrifuged for 40 hours at 8°C for sequential isolation of CM + VLDL (d=1.006), LDL (d=1.063), and HDL (d=1.21). Drug was assayed using HPLC methods. The total amount of drugs recovered in different fractions was considered 100%. Each value represents the mean ±SD (n=3).

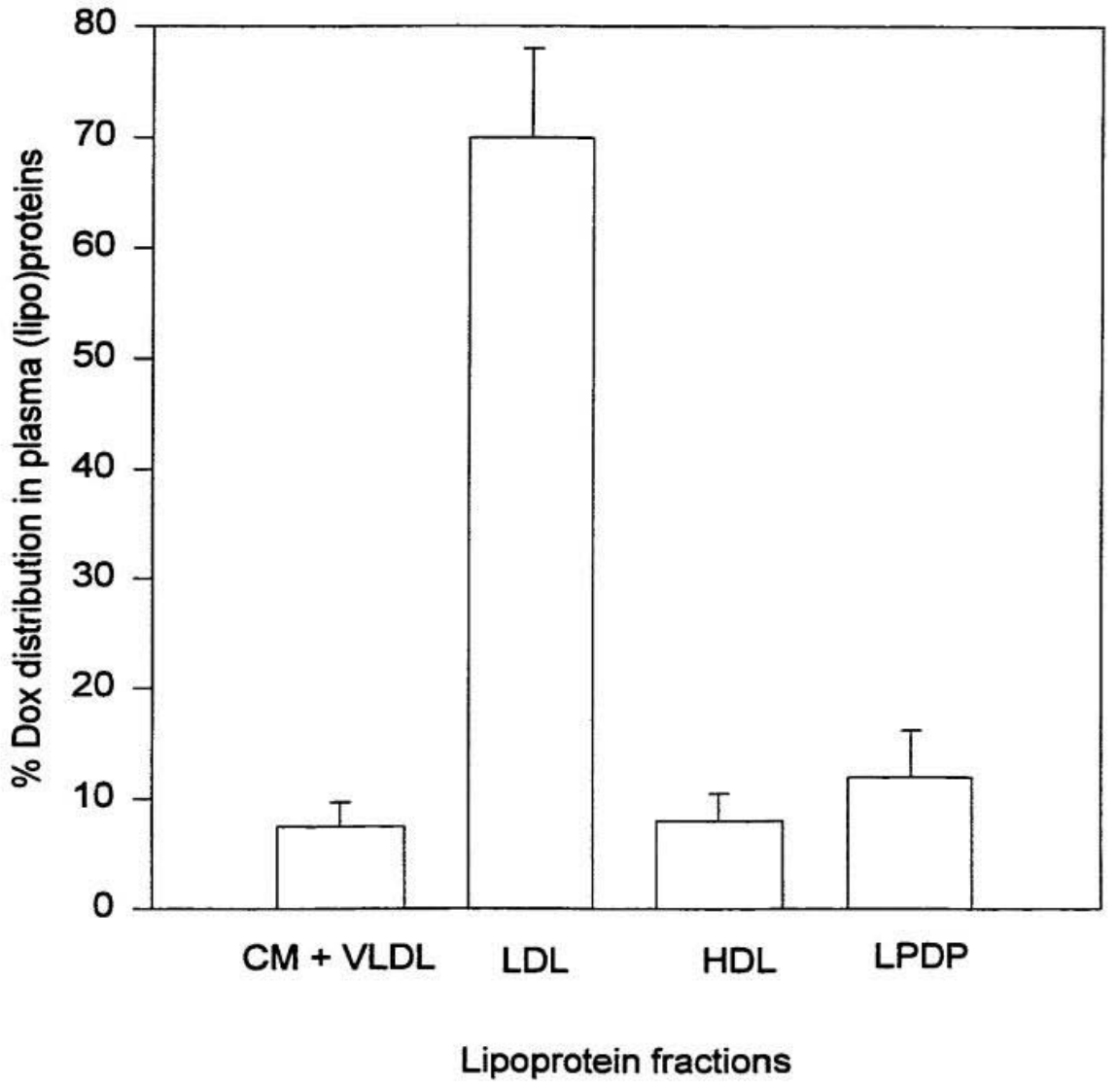

80
70
60
50
40
30
20
10
0
% Dox distribution in plasma (lipo)proteins
CM + VLDL
LDL
HDL
LPDP
Lipoprotein fractions

Figure 3.2. SDS-PAGE of LDL, and LDL-drug conjugates. A 5% acrylamide gel loaeded with LDL samples was electrophoresed at 30 mA constant current for 30 minutes. The positions of a broad range of MW standards from BioRad are shown with arrow marks. Samples were incubated for 4 hours at 37°C. The LDL-Dox conjugates were separated by ultracentrifugation followed by dialysis. After dialysis, samples were ubjected to SDS-PAGE. Lane 1, broad range MW markers; Lane 2, native LDL (5 μg); Lane 3, LDL-Dox conjugates [5 μg protein and 0.15 μg Dox] and Lane 4, LDL-CI conjugates [discussed in Chapter 4] [LDL-drug conjugates were prepared by the contact method with Tween 20].

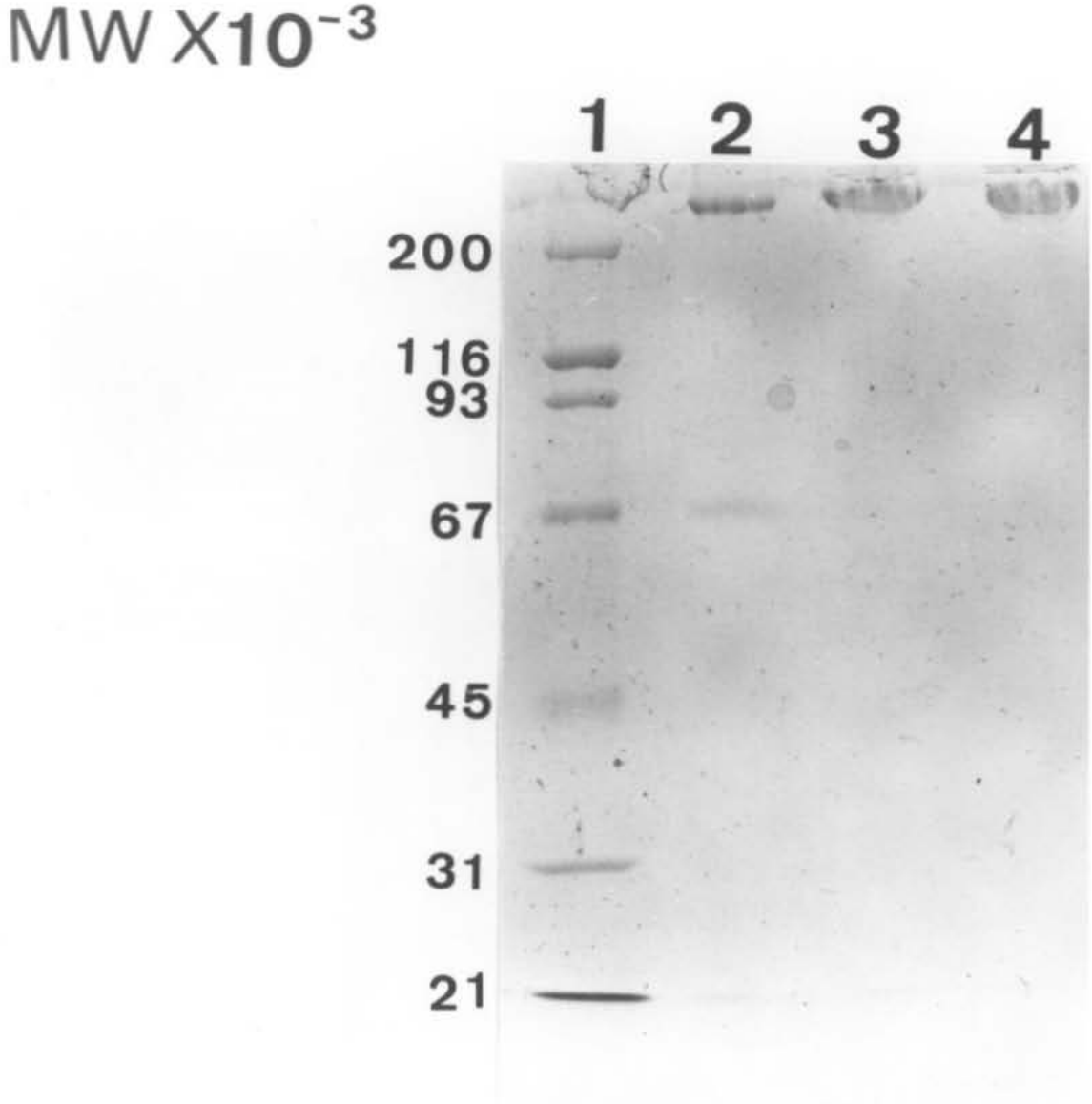

MW X10⁻³
1
2
3
4
200
116
93
67
45
31
21

Figure 3.3. Electron micrograph of LDL preparations. The LDL preparations (10-40 μg protein/mL) were applied to carbonformvar membranes and negatively stained with 2% phosphotungstate solution. They were examined on a Philips 300 instrument at a magnification 75,000 x, the bar represent 100 nm. Panel A, native LDL; Panel B, LDL incubated with Tween 20; Panel C, LDL incubated with Dox·HCl [the direct addition method]; Panel D, LDL incubated with Dox with Tween 20 [the contact method].

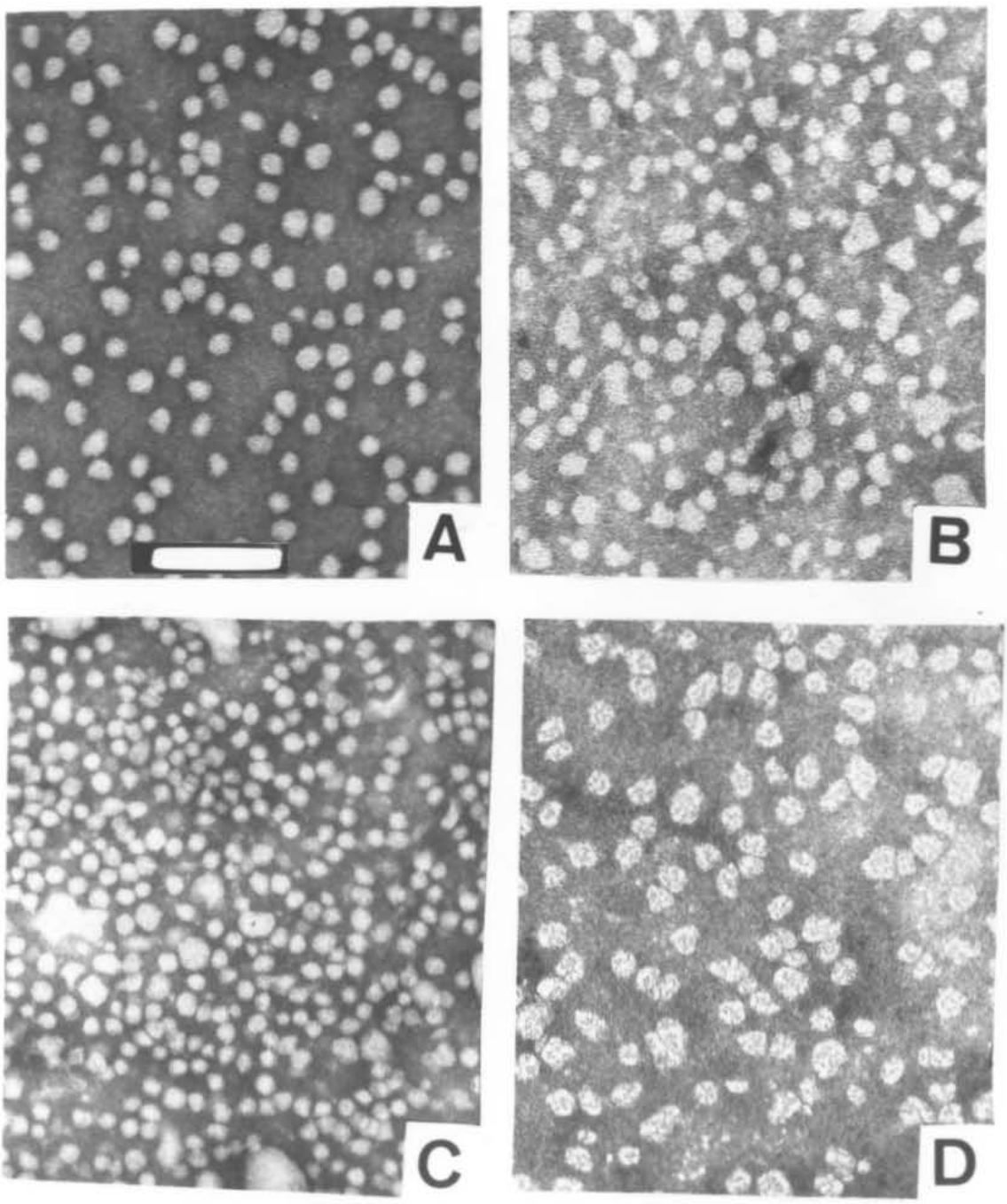

A
B
C
D

Figure 3.4. Particle size of LDL preparations measured by EM. Samples were negatively stained with 1% uranyl acetate and photographed at magnifications, 75,000 x 3. Results were mean diameter ±SD of at least 100 particles diameter.

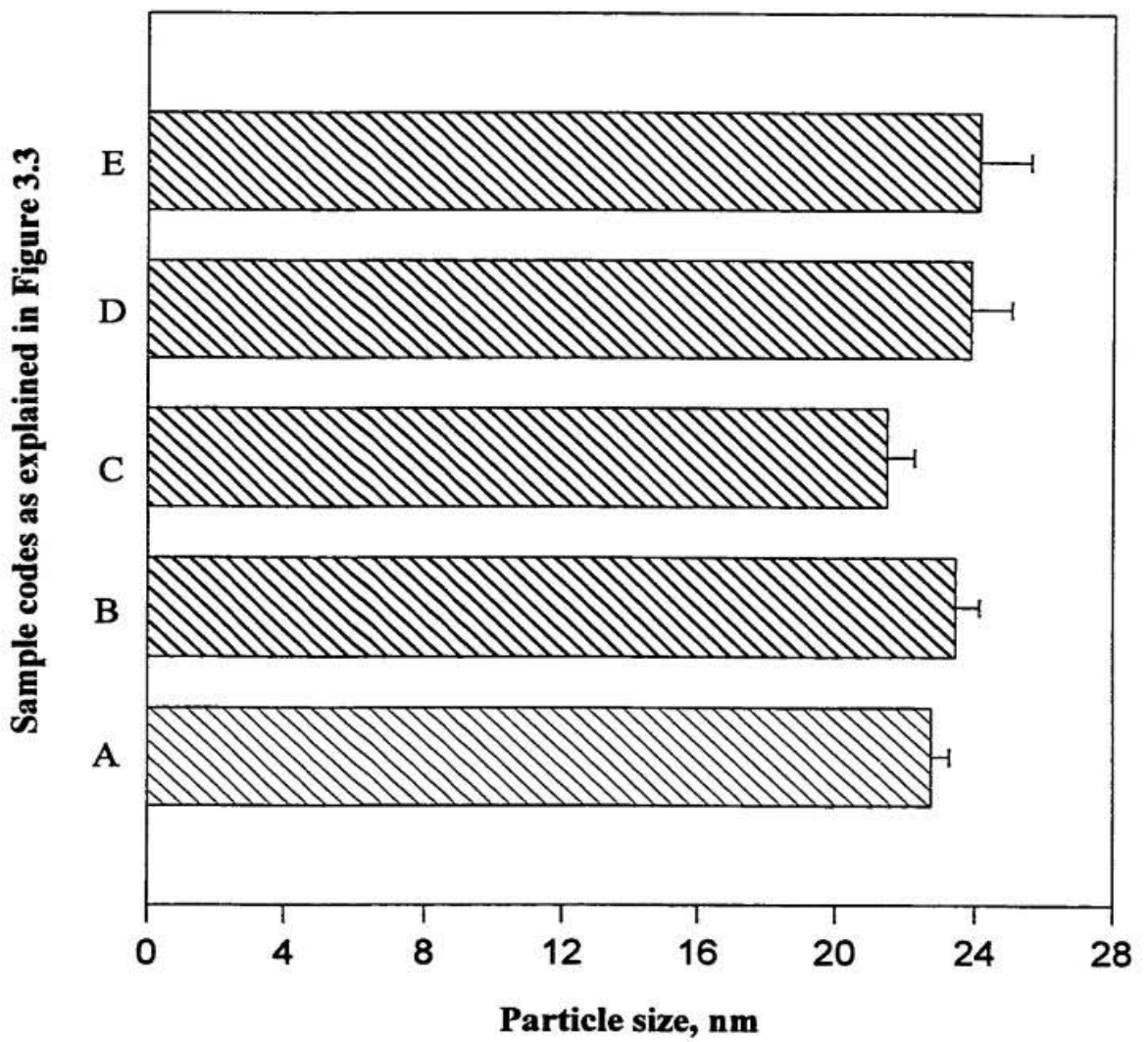
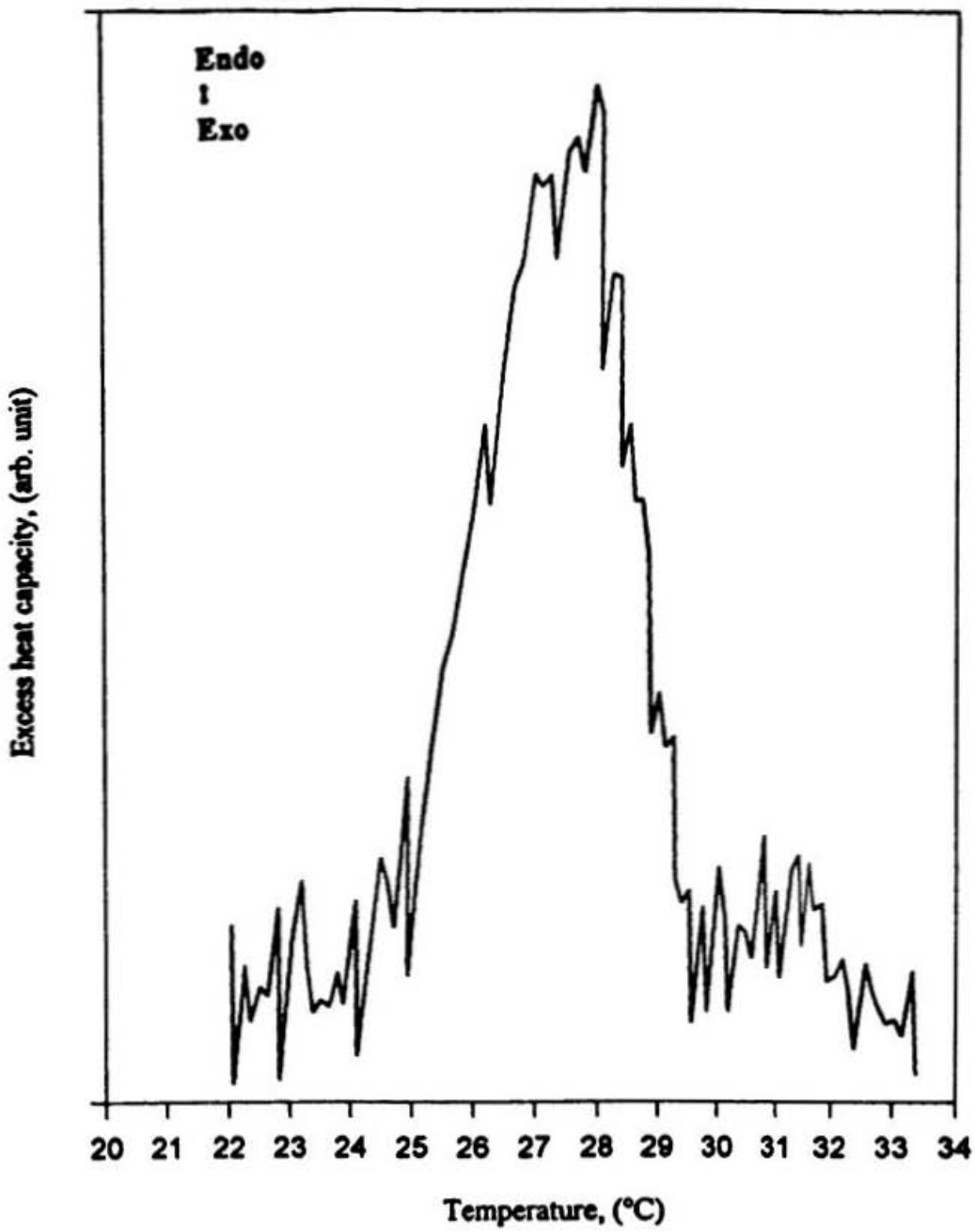

Sample codes as explained in Figure 3.3
E
D
C
B
A
0
4
8
12
16
20
24
28
Particle size, nm

Figure 3.5. Differential scanning calorimetry of LDL-Dox conjugates. LDL was incubated with Dox for 4 hours at 37°C (the directaddition method). LDL-Dox conjugates were separated from the incubation mixture by ultracentrifugation followed by dialysis. Samples were scanned at 0.5°C / min from 10 to 50°C. DSC sensitivity was the same for all runs. For all samples, the protein concentration was more than 1.50 mg/mL. Buffer baselines were subtracted. The data are normalized to the protein concentration. Similar DSC thermograms were obtained (Similar T_C and variable transition enthalpy for samples) for all other LDL-drug conjugates.


Endo
↕
Exo
Excess heat capacity, (arb. unit)
20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
Temperature, (°C)

CHAPTER 4:

EVALUATION OF BIOLOGICAL TRANSFER CATALYSTS IN ENHANCING DRUG LOADING INTO LDL PARTICLES

4.1. Introduction

I have demonstrated earlier that drug incorporation into LDL particles can be increased by modulating physico-chemical factors and by designing lipophilic prodrugs. Still, a further increase in loading efficiency without significantly changing the integrity of LDL particle is desirable. In this section, I investigated the potential of biological transfer catalysts to further improve drug loading.

Biological transfer catalysts are widely distributed in both invertebrates and vertebrates. Among the catalysts, only lipid transfer proteins are considered in this study. These catalysts facilitate the transfer of a variety of lipid components among lipoprotein particles *in vivo*. LDL may act as donor or acceptor for these proteins. My primary aim was to load cytotoxic drugs in an endogenous lipid particle, LDL. Any foreign molecule, if transported by these proteins, is expected to follow similar transfer kinetics as the natural endogenous substrates. However, these transfer proteins in higher animals, like humans, are substrate specific, presumably to meet higher and specific biological demands of more complex biochemical systems [Tsuchida *et al.*, 1995; Ryan, 1990]. In contrast, invertebrates' transfer proteins are generally less selective due to simpler biochemical systems. To investigate the potential of biological transfer catalysts to transfer cytotoxic drugs in LDL

from a drug reservoir, transfer proteins both from humans and insects were selected for this study.

Two different lipid transfer proteins have been identified in human plasma. One is known as CETP [Ohnishi *et al.*, 1990; Morton, 1990; Morton and Zilversmit, 1982;] and the other is phospholipid transfer protein [PLTP] [Tollefson *et al.*, 1988; Tall *et al.*, 1983]. While PLTP catalyzes only PL transfer between plasma lipoproteins, CETP catalyzes the transfer of CE, TAG, and PL. When two different cytotoxic compounds, dioleoyl-floxuridine and dioleoyl-methotrexate, were tested to determine their potential transport into LDL by these transfer proteins from a drug reservoir, a negligible or low incorporation efficiency was observed [Lundberg 1992; De Smidt and Van Berkel, 1990]. In order to examine the apparent selectivity of CETP against foreign compounds, I chose to use this protein in my studies.

Recently, insect lipid transfer proteins [LTP] have been investigated for their role in transferring different lipid components among lipophorins [insect lipoproteins] and human lipoproteins [Singh et al., 1992]. One such protein has been isolated from the hemolymph of the tobacco horn worm, *M. sexta* [Ryan, 1990a; 1986a,b]. This protein can facilitate net vectorial transfer of lipid mass among lipoprotein particles. Evidence of LTP-mediated net transfer has been obtained from studies with insect hemolymph lipophorins [Ryan *et al.*, 1990a; 1986a,b; Ando *et al.*, 1990], apoprotein-stabilized TAG/PL microemulsions [Ando *et al.*, 1990], human HDL [Ryan *et al.*, 1992; Silver *et al.*, 1990],and human LDL [Singh *et al.*, 1992] as substrates. Net lipid transfer occurs from the lower density lipophorin to the

higher density lipophorin in the presence of LTP, producing, at equilibrium, a single lipoprotein population intermediate in density between the starting lipoproteins [Ryan *et al.*, 1986a,b; 1985]. LTP has also been shown to catalyze net transfer of PL and TAG from a human apoprotein A-I-stabilized PL/triolein microemulsion to human LDL [Ando *et al.*; 1988]. Facilitated net transfer of CE, free cholesterol, and PL occurred to a much lower extent than DAG net transfer, indicating that DAG is the preferred substrate for this protein. However, LTP was found to transfer a number of endogenous particles with a variety of different chemical structures. This suggests that LTP mediated transport is not substrate-specific. I wanted to extend this approach to the loading of foreign compounds such as cytotoxic drugs into LDL particles.

Insect LTP appears to be distinct from other plasma lipid transfer catalysts described to date. First, it exists as a very high density lipoprotein of high MW [Mr> 670, 000]. LTP has three apoprotein components and 14% lipid in the native particle [Ryan *et al.*, 1988]. Although it has been shown that the lipid component of LTP is in equilibrium with that of potential donor or acceptor lipoproteins [Ryan *et al.*, 1990], the precise role of individual LTP apoproteins is not clear. A second distinguishing feature of LTP is its propensity to catalyze unidirectional net lipid transfer versus homo or hetero exchange of lipid. In contrast, human CETP catalyzes reciprocal random exchange of CE and TGA [Ohnishi *et al.*, 1995]. LTP catalyzed net transfer establishes an altered final equilibrium lipid distribution that results in changes in the total mass of lipid associated with lipoprotein substrates rather than redistribution of lipid classes via a simple exchange process.

LTP may provide a useful method to alter the core content of lipoproteins depending on the donor and acceptor composition. I proposed to load hydrophobic foreign compounds via LTP mediated transfer into human LDL. The reaction may produce drug enriched lipoprotein particles as stable end products. In an attempt to understand the catalytic properties of biological transfer catalysts I have examined the ability of human and insect transfer proteins to catalyze transfer between drugs and human LDL. In addition to Dox, I proposed to load a CE analog, CI, into the LDL particle. This is greatly simplified by the use of radiolabeled derivatives of CI [e.g. ^{125}I-CI]. The utility of compounds of this type has been amply illustrated by Stein *et al.* [1988]. I adopted the radioiodination procedure to radiolabelled CI according to the procedure of Weichert *et al.*, [1986]. The radioiodination of this compound will help to quantify this compound even when CI incorporation is very low. In short, the potential utility of biological transfer catalysts as tools to transfer drugs into human LDL is investigated in this chapter.

4.2. Materials and methods

4.2.1. Materials

LTP and high density lipophorin [HDLp] from *M. sexta* were gifts from Professor Robert O. Ryan, University of Alberta, Edmonton, Canada. Human LDL and LPDP were isolated from plasma by using sequential flotation ultracentrifugation method described earlier [Chapter 2, section 2.10]. Dox was also described in Chapter 2 (section 2.1).

Nonradioactive CI was a gift from Professor Raymond E. Counsell [the University of Michigan, Ann Arbor, MI]. The ^{125}I used was a no-carrier-added solution of $Na^{125}I$ [5 mCi/0.1 mL] in reductant-free 0.1 N NaOH obtained from DuPont Canada Ltd. [Ontario, Canada]. Tetrahydrofuran [THF] was distilled from $LiAlH_4$ under helium immediately prior to use. Unless otherwise noted, starting materials, reactants and solvents were obtained commercially and were used as such or purified and dried by standard means.

All radioiodination reactions were conducted inside a plexiglass glove box vented with a radio-iodine trap. Thin layer chromatography [TLC] analyses were performed on Merck silica gel GF254 plates. The plates were monitored by UV fluorescence, or staining with iodine vapor. The solvent system was hexane : ethyl acetate, 5:2 for CI. Column chromatography was performed on Merck silica gel-60 [230-400 mesh] eluted with the same solvent system. An HPLC system in conjunction with radioactive counting was used to determine radiochemical purity and specific activity of CI was calculated. The HPLC system was described in Chapter 2 [section 2.4].

4.2.2. Preparation of ^{125}I-CI

The radioiodination of CI was carried out using an isotope exchange reaction in pivalic acid as described by Weichert *et al.*, 1986 [Figure 2.4]. Briefly, 4-80 mg of CI was placed in a 2 mL plasma vial which was then sealed with a teflon-lined rubber septum and an aluminum cap. Freshly distilled THF [100-200 µL] and aqueous $Na^{125}I$ [10-50 µL] were added in succession via a microliter syringe and a gentle stream of N_2 was applied to remove

the solvents. When the residue appeared dry, the seal was removed and solid pivalic acid [5-20 mg, dried by azeotrope with toluene and distilled under nitrogen] was added. The vial was resealed and partially immersed in a preheated [155-160°C] oil bath. When the isotope exchange reaction was essentially complete [usually 1-2 hours], the reaction vial was allowed to cool and anhydrous THF [200 μL] was added with a glass syringe and the vial swirled gently. A TLC test was performed with 1-2 μL of sample and the remaining contents were transferred to the top of a silica gel-60 column [1 X 10 cm] and subsequently eluted with hexane/ethyl acetate [5:2] as the solvent system. Chemical purity was determined by HPLC and radiochemical purity was determined by HPLC in conjunction with γ-counting using an automatic gamma counter [LKB Wallac 1277 Gammamaster, Turku, Finland]. The ^{125}I-CI was then stored at 4°C in a lead casing. The specific activity of the compound [cpm/μg of CI] was counted as follow:

$$\textit{Specific Activity} = \frac{\textit{calculated cpm of the eluent by the } \gamma \textit{ counter}}{\textit{amount detected by HPLC, } (\mu g)}$$

4.2.3. HPLC assay of CI

A new reverse phase HPLC procedure was developed to determine CI. The principle HPLC conditions are outlined in Table 2.1. A concentration range given in Table 2.1 was used to make a calibration curve. All standard solutions were in chloroform. Samples were

extracted from plasma or LDL by a direct precipitation method using chloroform. The extent of recovery of drugs from LDL was determined as described in Chapter 2 [section 2.4.1].

4.2.4. LTP assay

The principle of LTP assay is based on the catalytic transfer of CEs from HDLp [donor] to LDL [acceptor]. LDL has limited capacity for CEs and overloading of CE by LTP destabilizes the LDL which aggregates and forms a turbid solution. This turbidity is proportional to the amount of CE transferred by LTP. This turbidity is measured at 405 nm and used for LTP assay. A time dependent CE transfer is expected which will plateau after several hours depending on the catalytic efficiency of LTP. Also, this transfer is found to be dependent on the concentration of LTP [Singh *et al.*, 1992]. The protocol reported by Singh *et al.*, [1992] was used with minor modifications. In brief, standard assays were conducted in 96-well microtiter plates. Unless otherwise specified, human LDL [50 μg protein] was incubated with *M. sexta* HDLp [250 μg protein] for 12 hours at 37°C in the presence and absence of LTP. The final volume of incubation was 0.2 mL in PBS. During incubation the plates were read every 30 minutes on a plate reader fitted with a 405-nm filter. Control samples lacking LTP were run in parallel and the absorbance at 405 nm of the control samples was subtracted from that of LTP-containing incubations to obtain the LTP-induced absorbance change.

4.2.5. Preparation of LDL-drug conjugates using LTP

Two different methods were used for incorporation of lipophilic prodrug into LDL using LTP. In all cases experiments were performed in duplicate or triplicate with appropriate controls. Control samples contained 0.9% NaCl instead of LTP.

4.2.5.1. Contact method: Lipophilic CI or free Dox dissolved in chloroform were added to glass tubes. The solvent was evaporated under N_2 at room temperature; the drug was thus coated on the solid surface. After complete evaporation of all solvent, LDL was added followed by LTP or PBS. The mixture was incubated at 37°C for at least 12 hours under N_2 with continuous shaking. In certain cases, a drop of ethanol or Tween 20 was added prior to the addition of LDL and LTP to suspend drugs.

4.2.5.2. Direct addition: This method was used for Dox·HCl, because Dox is water-soluble. Appropriate amounts of drug and LDL were added followed by the final addition of LTP. Incubation conditions were similar to the contact method.

4.2.6. CETP mediated drug loading

This method is based on a method previously described by Blomhoff *et al.*, [1984]. Briefly, 50 μL [50 μg] of drugs [CI, and Dox] to be incorporated in LDL were dissolved in chloroform and the solvent was evaporated under a stream of N_2 to dryness in a glass tube. The residue was then dissolved in 50 μL of acetone followed by addition of 500 μL LPDP.

After evaporation of the acetone with N_2, 300 μL of PBS was added. The LPDP-drug preparation was then incubated with 500 μg of LDL at 37°C for 4 hours in the dark with constant gentle shaking. In the case of DoxHCl, Dox was directly added to LPDP followed by incubation with LDL as mentioned before.

4.2.7. Isolation and evaluation of LDL-drug conjugates

Once the incubations were completed, the incubation mixtures were adjusted to a density of greater than 1.063 g/mL with KBr and centrifuged at 40,000 rpm in a Ti60 Beckman rotor for 20-30 hours at 8°C. After ultracentrifugation, the LDL-drug conjugates were collected from the top of the tube and extensively dialyzed overnight as described before. The assay methods for the determination of the protein concentration [Bradford method] and the drug concentration [HPLC or liquid scintillation or gamma counting] were reported earlier in Chapters 2 and 3. The integrity of LDL-protein was determined by SDS-PAGE [Chapter 3, section 3.2.3]. Particle size of the particles were measured by EM [Chapter 3, section 3.2.4].

4.2.8. UV-visible scanning

To investigate the location of a drug, UV-visible scans were made of the free drug and LDL-drug conjugates in the UV-visible range using a UV-visible spectrophotometer. Only LDL-Dox was considered in this study. Two standard scans were made: [1] DoxHCl in PBS buffer and [2] free Dox in chloroform. The spectra from LDL-Dox conjugates were

compared with these two standard spectra. Dox spectra in the two different solvents were considered to be complimentary to Dox in the outer aqueous environment and on the inner lipid environment of LDL. Dox, if present in the aqueous surface layer, is expected to give a spectra similar to the Dox·HCl spectra taken in PBS buffer. However, Dox, if present in the core, is expected to have a spectrum complimentary to the Dox spectrum in chloroform.

4.2.9. Statistical analysis

Statistical analysis was performed between two groups using the student's t-test and the p value was calculated. Differences between two groups were considered significant if $p > 0.05$. All data are expressed as the mean ± SD.

4.3. Results

4.3.1. Preparation of ^{125}I-CI

CI was successfully radio-labelled using ^{125}I [Figure 4.2]. Radiochemical yield for CI was found to be excellent [>95 %]. The specific activity of the compound was 2000 cpm/μg CI.

4.3.2. HPLC assay of CI

The chromatograms of different analogs in standard solutions are shown in Figures

4.3a and 4.3b. Typical calibration curve for CI is shown in Figure 4.4. The equations of best fit for the lines of CI was calculated. The coefficient of determinations ranged from 98 - 100 %, and the CV of the slope determined on the five separate occasions was less than 5 %.

Peak areas obtained with plasma or LDL spiked with four and five different concentrations of drug over the standard concentration range [Table 4.1] were linearly related to the spiked concentrations. In all cases, direct addition of chloroform to LDL or plasma was sufficient to extract more than 95% of the drug from spiked LDL samples. The minimum detection limit for the drug was 10 ng [10 μL from 1 μg/mL standard solutions] and was suitable for my studies. The peak height detected at this minimum concentration was more than three times that of the background noise. The reproducibility of the method was determined by both intra- and inter-day variability studies [Table 4.2]. Statistical analysis of CI showed no statistically significant inter-day or intra-day variations of the samples [p values in all cases were found to be> 0.05] [Table 4.2].

4.3.3. Biological assay of LTP

In a typical LTP assay, initial quantity of lipid acceptor [LDL] is incubated with large excess amount of lipid donor [HDLp] in the absence [control] and presence of LTP. If LTP is catalytically functional, lipids will be transferred from the donor to the acceptor. When the capacity of acceptor [LDL] is reached the excess amount of lipids will promote the aggregation of LDL and the solution will become turbid. The development of turbidity can be spectrophotometrically quantified. In the absence of LTP, or if LTP is denatured, no lipid

movement occurs and the solution will remain clear. My assay to verify the catalytic activity of LTP is a modification of a method reported earlier [Singh *et al.*, 1992]. The development of sample turbidity was observed in terms of reaction time and LTP concentration [Figures 4.5 and 4.6]. The results showed that control lipoprotein incubations lacking LTP had a baseline absorbance, which did not change as a result of incubation, of about 0.25 at 405 nm. When catalytic amounts of LTP were added, there was a time-dependent increase following an initial lag phase, in sample absorbance at 405 nm. This increase was linear for periods up to 10 hours and reached a plateau for longer time periods [15 to 24 hours], suggesting a reaction end point had been reached. When either LDL or HDLp was omitted from incubations containing LTP no increases in absorbance were observed. As expected there was a concentration dependent increase in absorbance at 405 nm due to the turbidity of the solution was observed [Figure 4.6]. On the other hand, control incubations lacking LTP did not show an increased absorbance at 405 nm. The control absorbance was found to be 0.025 throughout the time period [Figure 4.5]. These data suggest that LTP was catalytically effective. However, the catalytic efficiency was less than expected when compared with the results of Singh *et al.*, [1992]. LTP catalytic activity is also temperature dependent and the greatest efficiency was reported at 37°C [Singh *et al.*, 1992]. Based on these data, I proposed to use 50 μg of LTP per mg of LDL protein and long incubation times of 9-15 hours for studies involving drug loading.

4.3.4. Physicochemical properties of LDL-drug conjugates

The efficiency of incorporation of drugs into the LDL was evaluated by density

ultracentrifugation, SDS-PAGE, and EM as discussed in Chapter 3. All LDL-drug conjugates showed only one band indicative of apo B of LDL [Figure 4.7]. The particle size of most LDL-drug conjugates [24 ± 2 nm] change significantly when compared to native LDL [21 ± 1 nm] [Figure 4.8 and 4.9]. However, the change in particle size is small in magnitude.

4.3.5. Contact method

The dry film method is good in generating stable LDL-drug conjugates as indicated in Chapter 3. The essential step of this method is the partitioning of drug from a solid surface to the lipoprotein. This method was not found to be very effective in loading drugs in LDL particles. Interestingly, when LTP was used to enhance drug loading, a 3- to 5-fold increase in drug loading was observed for Dox [Figure 4.10]. This effect was not observed with CI. Figure 4.10 shows that LTP has potential to enhance drug loading at least 2 to 5 fold. However, further optimization of this method was necessary to enhance loading with more hydrophobic compounds such as CI. This prompted me to modify the dry film method. I considered that increased interaction of LDL and drug was essential for LTP's activity, as the catalyst would act at the interface of the drug molecule and the LDL particle. In order to solve this problem, I proposed to use organic solvents or surfactants to solubilize or suspend the drug molecules before incubating them with LDL and LTP. Results are discussed below.

4.3.6. Organic solvent aided method.

In order to optimize contact between drug and LDL, 50 μL ethanol was added to solubilize the drug before incubation with LDL and LTP. LTP was found to be approximately 2-fold more active in ethanol solubilized than the control without ethanol for CI [Figure 4.10]. A small amount of ethanol was found not to denature apo B protein. No degradation bands appeared on gels after SDS-PAGE and no visual turbidity was apparent [data not shown]. It is worth mentioning that the first clinical trials of VCR were performed using preparations made by the dry film method with the aid of an organic solvent, dichloromethane [Breeze *et al.*, 1994; Filipowska *et al.*, 1992]. The results from human trials indicated that small amounts of organic solvents may not induce significant denaturation to apo B protein of LDL, as LDL-drug conjugates had favorable pharmacological effects.

The dry film method is good in generating stable LDL-drug conjugates as indicated in Chapter 3. The essential step of this method is the partitioning of drug from a solid surface to the lipoprotein. This method was not found to be effective enough in loading drugs in LDL particles. Interestingly, when LTP was used to enhance drug loading, a 3- to 5-fold increase in drug loading was observed for Dox [Figure 4.10]. This effect was not observed with CI. Figure 4.10 shows that LTP has potential to enhance drug loading at least 2 to 5 fold. However, further optimization of this method was necessary to enhance loading with more hydrophobic compounds like CI. I considered that increased interaction of LDL and drug was essential for LTP's activity, as the catalyst would work at the interface of the

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4.3.8. Detergent aided method.

I have already demonstrated that the detergent aided method is more efficient in loading lipophilic drugs into LDL compared to the dry film method. Results from ethanol solubilization were encouraging; however, complete solubilization of lipophilic drugs by

ethanol was not considered as I observed that apo B denatured at higher concentrations. Alternately, to suspend the drug, I attempted to use Tween 20 which was already found effective in loading Dox into LDL. This wetting agent was already found suitable to deliver hydrophobic compounds in suspension. The suitability and safety profile of this detergent is discussed elsewhere in the literature [Leyland, 1994]. To test whether LTP worked better in this system, I have used CI. A 3- to 5-fold difference in loading efficiency was observed [Figure 4.11].

4.3.9. Direct addition

This method generates stable LDL-drug conjugates. It has been reported that the LDL-drug conjugates were found to leak from the lipid core or to be loosely attached to the PL outer-layer in preparations using this method [Firestone, 1994; Shaw *et al.*, 1987; Lundberg, 1987]. However, in my experience with cell culture studies [see Chapters 5 and 6], I found the LDL-drug conjugates were stable enough to generate cytotoxic effects in cultured cells indistinguishable from the effects of LDL-drug conjugates made by the dry film method. The direct addition method was found suitable to load Dox·HCl. With LTP, the loading efficiency was improved at least 3- to 4-fold [Figure 4.11]. In one case, the loading efficiency for Dox in the presence of LTP was found to be 22-fold higher than that of the control without LTP. This result indicates the importance of modifying incubation conditions in enhancing drug loading in LDL.

4.3.10. Enzymatic transfer via CETP

Figure 4.12 shows the relative incorporation efficiency of drugs using this method compared to the contact method with or without LTP. Uniform LDL-drug conjugates could be generated without any further improvement to the loading efficiency of the compounds. This once again confirms the previous finding of low incorporation efficiency of CETP [De Smidt and Van Berkel, 1990b]. Compared to the dry film method, no significant incorporation was observed [Figure 4.10]. I conclude that CETP was not effective in enhancing drug loading into LDL under my experimental protocols.

4.3.11. Location of drug

UV-visible scans were carried out in two different solvents to compare the spectra of LDL-Dox conjugates. I hypothesized that Dox in LDL would have corresponding spectra either to the spectra of Dox in chloroform or Dox·HCl in PBS. Dox in the two different environments behaves differently. There are two different environments in LDL, the central lipid core and the outer aqueous PL monolayer. UV-visible spectra of LDL-Dox conjugates were similar to those of Dox in chloroform. Drugs were loaded into the oily core of LDL. UV-visible spectra of Dox and LDL-Dox are shown in Figure 4.13. The spectra #1 and #2 of panel A were the Dox/LDL-Dox in the absence and in the presence of LTP. Clearly, LTP induced significant increases in the amounts of Dox associated with LDL in comparison to conventional spontaneous transfer. The increases ranged from 3-4 fold. With further refining of the conditions [see below], more Dox can be packaged into LDL. When the

spectra of Dox/LDL-Dox were compared to that of Dox·HCl in saline, a peak at about 543 nm became evident in Dox/LDL [#1 and #2] while Dox·HCl [#3 and #4] only showed a very weak shoulder [panel B]. To explore the possible contribution of this peak in Dox/LDL-Dox samples, the spectra of different concentrations of both the Dox base in chloroform and the Dox·HCl in PBS buffer have been scanned [panel C]. The spectra of Dox in chloroform [#5, #6 and #7], had 543 nm absorption peaks, while Dox·HCl solutions in PBS buffer [#8 and #9] only showed shoulders at this wavelength. These results suggested that Dox associated with LDL was more like the Dox dissolved in chloroform than in aqueous medium. The Dox might be dissolved in the oily core of LDL. This is the preferred location where Dox would not be leaked out in the general circulation while the surface bound compounds would be quickly displaced or exchanged by a very large amount of PLs presented in all the biomembranes and lipoproteins. The localization of Dox in the core was also predicted from my DSC study discussed previously in Chapter 3 [section 3.7].

4.4. Discussion

The pivalic acid exchange radioiodination of CI using a previously reported method [Weichert *et al.*, 1986] was successful. The HPLC procedure reported here was found suitable in my experimental protocol. Recoveries of drug from LDL samples were excellent [>95%].

I have discussed the fact that substrate transferred by LTP is nonspecific [Singh *et al.*,

1992; Singh and Ryan, 1991; Liu and Ryan, 1991; Ryan, 1990; Ryan *et al.*, 1990]. This lack of substrate specificity provides a good basis for transferring various hydrophobic cytotoxic drugs to LDL by LTP. In my studies, I have chosen a variety of structurally dissimilar molecules as substrates for LTP. All the compounds were hydrophobic in nature and Dox.HCl was ionic in nature. Both lipophilic and ionic substrates were reported for LTP. In the biological system, [DAG] had the highest preference for transport by LTP. In my studies, there was no preference was observed for CI although CI was structurally very similar to CE. In all the cases, the drug loading efficiency was found to be 2- to 5-fold higher with LTP compared to control. CI was found to be better candidates. CI due to its close similarity with CE was considered a better substrate. In addition, its lipophilic nature presumably made it more interactive with the transfer protein. The transport of Dox.HCl by this protein indicated that there was a preference for ionizable species. One possible explanation could be better interactions of ionic drugs with ionic PL outer layer. Since the drugs are available at the interface because of ionic interaction, they are more accessible to LTP. If this holds true, then it explains why there was no dramatic difference in drug loading efficiency by LTP. Results indicate that drug molecules should have sufficient hydrophobicity to be loaded into LDL by LTP.

The contact method is based upon the passive partitioning of drug from a solid surface into the LDL. The transfer protein method, described previously for the incorporation of radiolabelled cholesteryl oleate into lipoproteins [Blomhoff *et al.*, 1984], utilizes transfer enzyme activity present in plasma, mainly, CETP. Experimental conditions

for drug incorporation with these methods were tolerable to preserve native integrity of the LDL particle because drug incorporation was either spontaneous or protein-facilitated. Dry film procedures produce LDL-drug conjugates with low incorporation efficiency. Moreover, incorporated drug may not be associated with the inner core of the LDL particle. Such a 'loose" incorporation was reported earlier with benzo[a]pyrene-LDL complexes obtained by a similar procedure [Remsen and Shireman, 1981].

The CETP catalyzed incorporation method was very successful [up to 80% incorporation efficiency] in the case of incorporation of radiolabelled cholesteryl oleate. Moderate selectivity, by a factor of 5 to 6, was observed in the CETP-catalyzed transfer of CE over TAG between plasma lipoproteins. On the other hand, the transfer of CE by CETP was highly selective over the negligible transfer of TAG, by a factor of 60 to 500, between microemulsions with LDL size [Ohnishi *et al.*; 1994-1995]. The presence of free cholesterol in these microemulsions reduced slightly the rate of CE transfer but had no effect on TAG transfer. Other surface active reagents such as cholic acid, Triton X-100 and Tween 20, did not have an effect on the TAG transfer either [Ohnishi *et al.*, 1995]. From this discussion it is clear that the CETP procedure was selective even for endogenous molecules. In my studies, density ultracentrifugation showed that drugs cannot be incorporated into LDL with this procedure, confirming a high degree of structural specificity which explains why foreign particles were not transported by this protein. In addition, most of the mammalian proteins are found to be highly selective and even stereospecific for their substrates [Ryan, 1990; Ohnishi *et al.*, 1995].

In contrast to CETP studies, LTP can transfer lipids unidirectionally from one lipoprotein to another [Singh and Ryan, 1991; Ryan *et al.*, 1990; 1986]. The rate of this transfer process is much faster than that of human CETP [Singh *et al.*, 1992]. Preliminary experimental evidence suggests that part of LTP has a functional similarity to human MTP. It has been suggested that one of the three subunits of LTP [apo LTP-III, 55 kDa] might function like protein disulfide isomerase involved in the process of protein [subunit] folding and unfolding [Breiter *et al.*, 1991]. If this is true, it would explain why LTP is so efficient in loading hydrophobic compounds into a tightly packed LDL particle. Unlike CETP, LTP was effective in transferring drug molecules. The advantage of such transfer is that the drug will be transported to the core of the LDL.

In the present study I have examined the capacity of LDL particles to accept cytotoxic molecules as well as the substrate specificity of LTP-mediated transfer. The results provide evidence that the drug incorporation into LDL can be significantly increased and reveal a potentially useful and improved method whereby LDL-drug conjugates can be produced from LDL *in vitro*.

Table 4.1 HPLC analytical profiles of CI

Profile	CI*
Mobile phase	22% THF in acetonitrile
UV detection [wave length, nm]	254
Retention time, [minutes]	8.65
Detection limit, [μg/mL]	0.25
Linear range, [μg/mL]	1.0 to 50
% Recovery from LDL samples	>95%

* Values are the mean with CV less than 10%.

Table 4.2 Inter- and intraday variations of CI

Spiked amount [µg/mL]	25	50	100
Inter-day variation [individual values, peak areas]			
Day 1	23.65, 24.15	49.15, 48.62	92.42, 97.85
Day 2	28.75, 25.05	47.25, 48.25	93.65, 98.75
Day 5	23.55, 24.75	49.95, 51.25	102.26, 99.95
Day 7	25.10, 25.46	48.75, 51.12	98.0, 99.25
Day 15	26.25, 25.47	49.58, 52.26	97.75, 98.25
Mean [SD] [n=10]	25.20 [1.5]	49.60 [1.54]	97.8 [2.87]
%CV [n=10]	5.95	3.10	2.93
Two-way ANOVA			
p value	0.605	0.074	0.244
Intra-day variation [peak areas]			
All samples	23.10, 24.52,25.27	46.53, 47.52, 49.84	92.53, 97.50, 103
Friday	23.51, 25.42, 26.25	48.5, 50.10, 52.24	96.10, 95.52, 105
Mean [SD] [n=6]	24.85 [1.2]	449.10 [2.0]	97.7 [5.25]
%CV [n=4]	4.85	4.07	5.37
p value	0.49	0.19	0.797

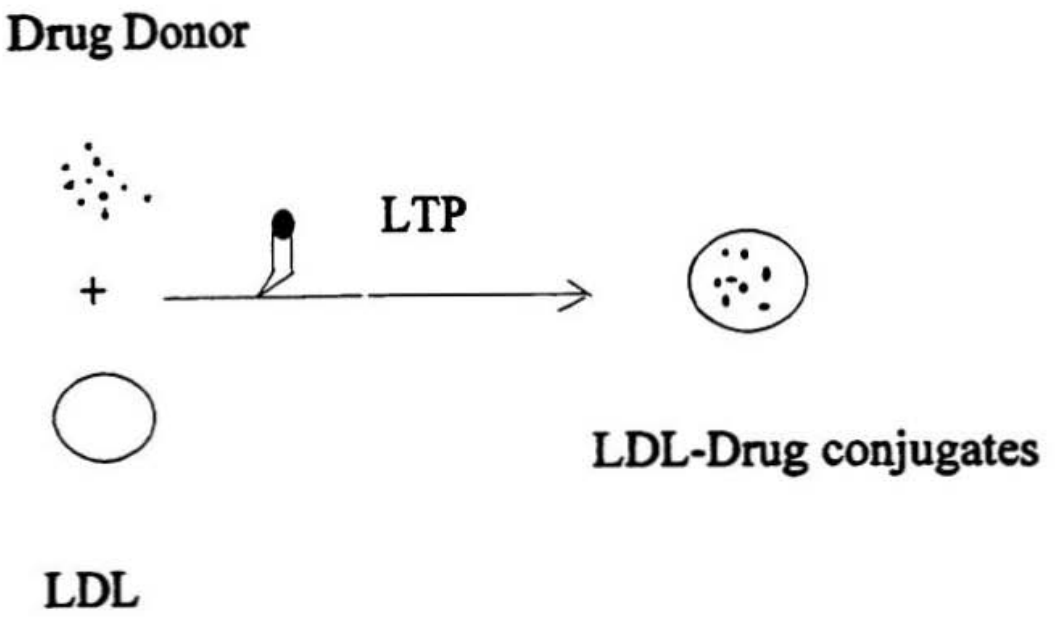


Figure 4.1. Schematic diagram of LTP facilitated drug transfer.

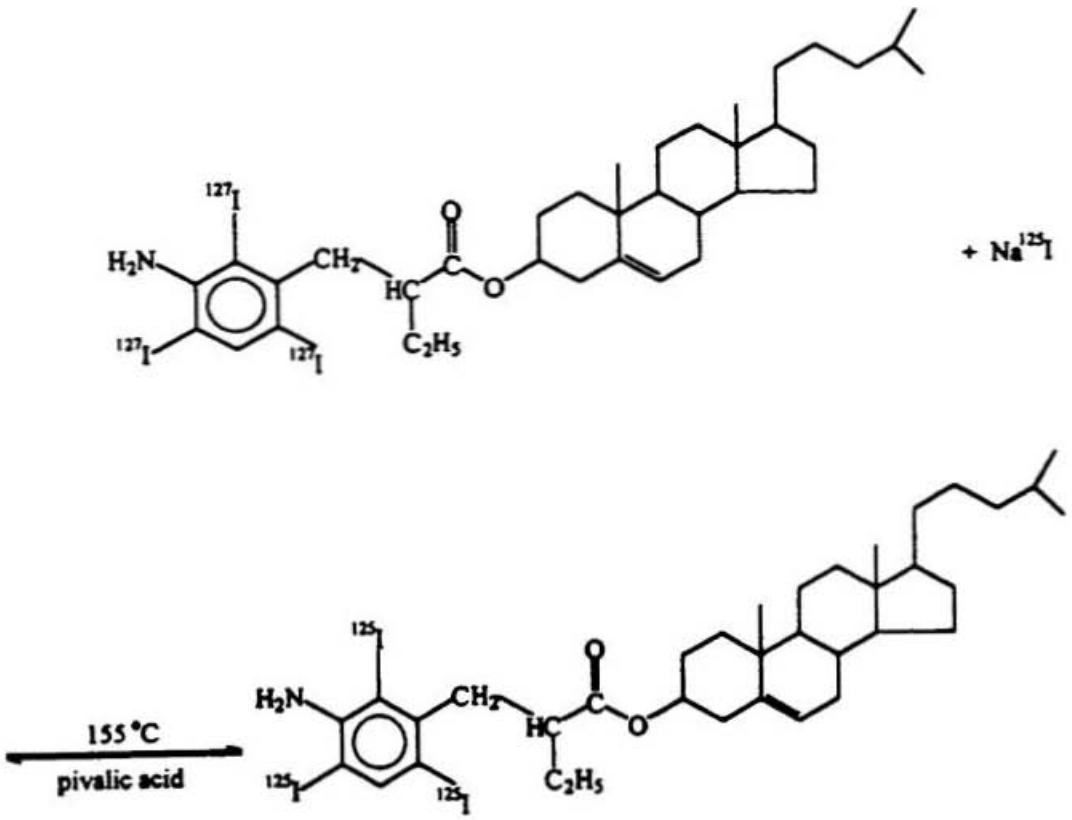


Figure 4.2. Radioiodination of CI. CI was radioiodinated by an isotope exchange reaction with $Na^{125}I$ in a melt of pivalic acid according to the procedure of Weichert et al., [1985]. The reaction was complete in an hour at 155°C with no decomposition of CI. The radiochemical yield was more than 95% with a specific activity of over 2000 cpm/μg CI.

Figure 4.3. HPLC chromatograms of CI in chloroform.

Figures 4.2a, Standard CI in chloroform;

Figure 4.2b, CI in LDL extracted in chloroform [recovery of CI is more than 95%].

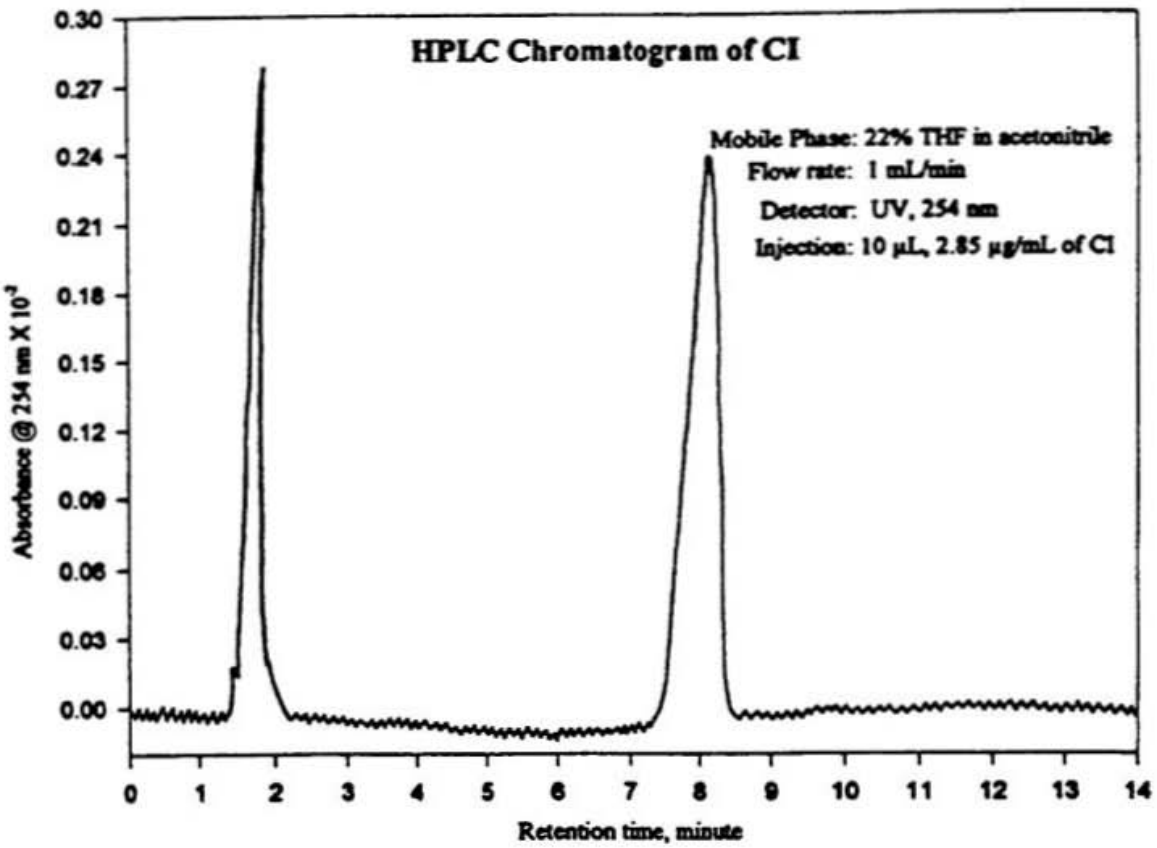

HPLC Chromatogram of CI
Mobile Phase: 22% THF in acetonitrile
Flow rate: 1 mL/min
Detector: UV, 254 nm
Injection: 10 µL, 2.85 µg/mL of CI
Absorbance @ 254 nm X 10⁻³
0.30
0.27
0.24
0.21
0.18
0.15
0.12
0.09
0.06
0.03
0.00
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Retention time, minute

Figure 4.2a

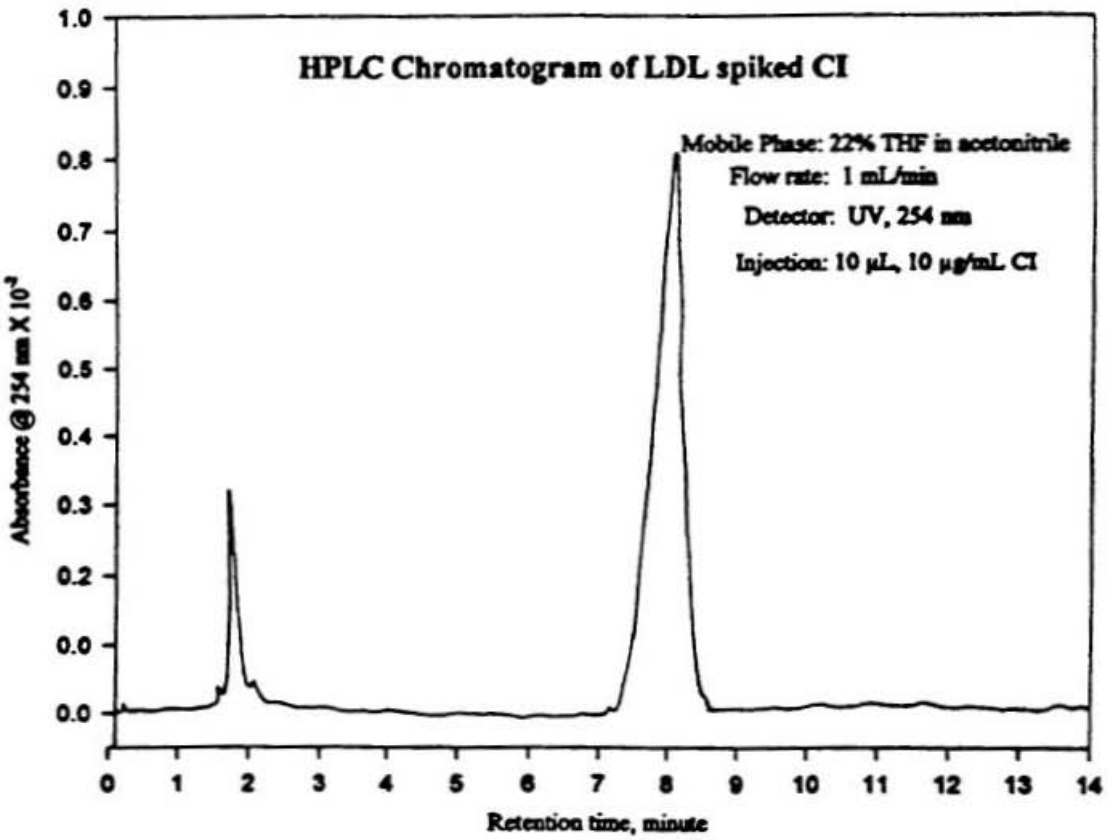

HPLC Chromatogram of LDL spiked CI
Mobile Phase: 22% THF in acetonitrile
Flow rate: 1 mL/min
Detector: UV, 254 nm
Injection: 10 µL, 10 µg/mL CI
Absorbance @ 254 nm X 10⁻³
Retention time, minute
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.0
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 4.2b

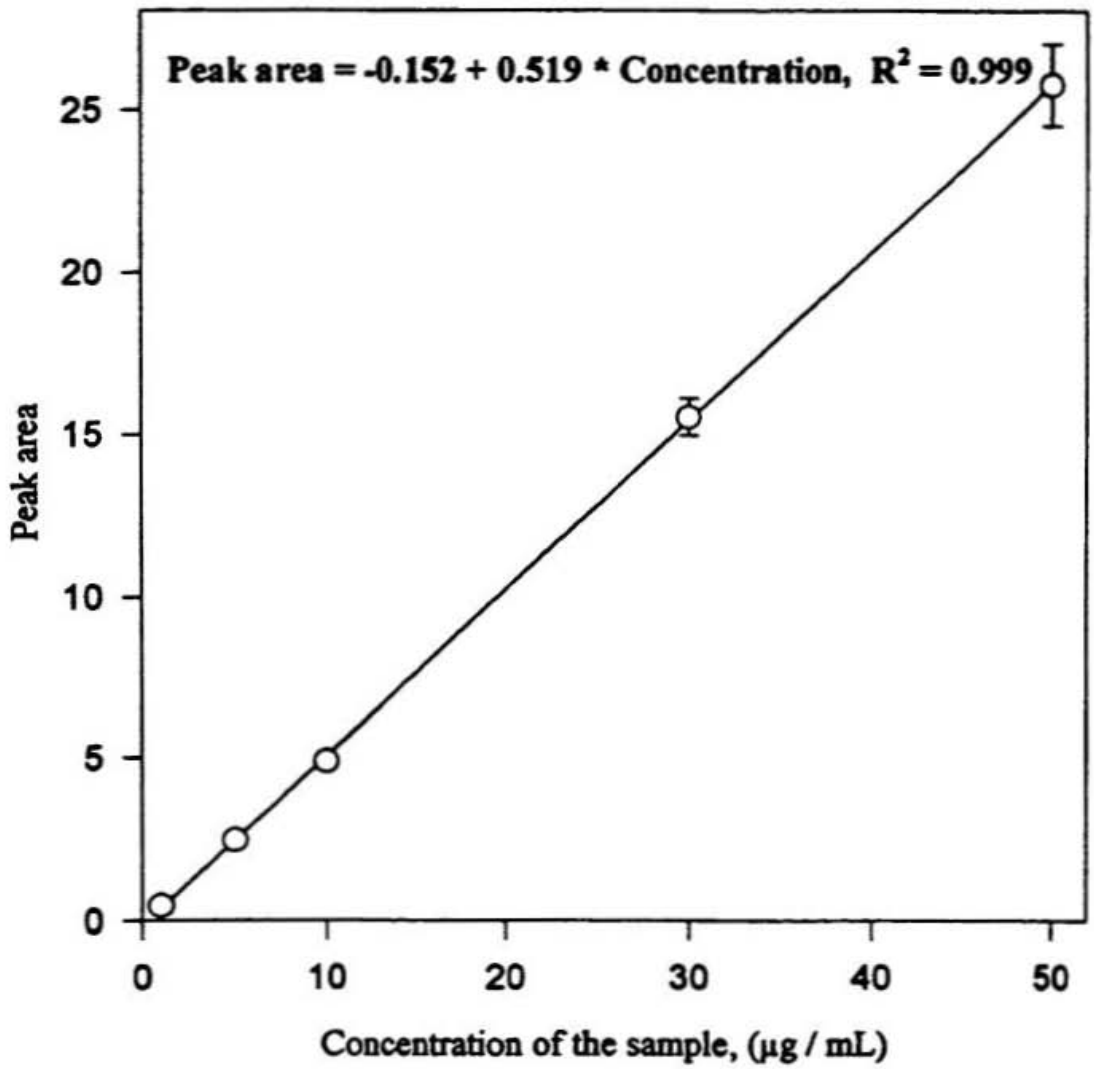


Figure 4.3. **Calibration curve of peak area versus concentration of spiked standard solutions of CL** Each point represents the mean ±SD of four determinations.

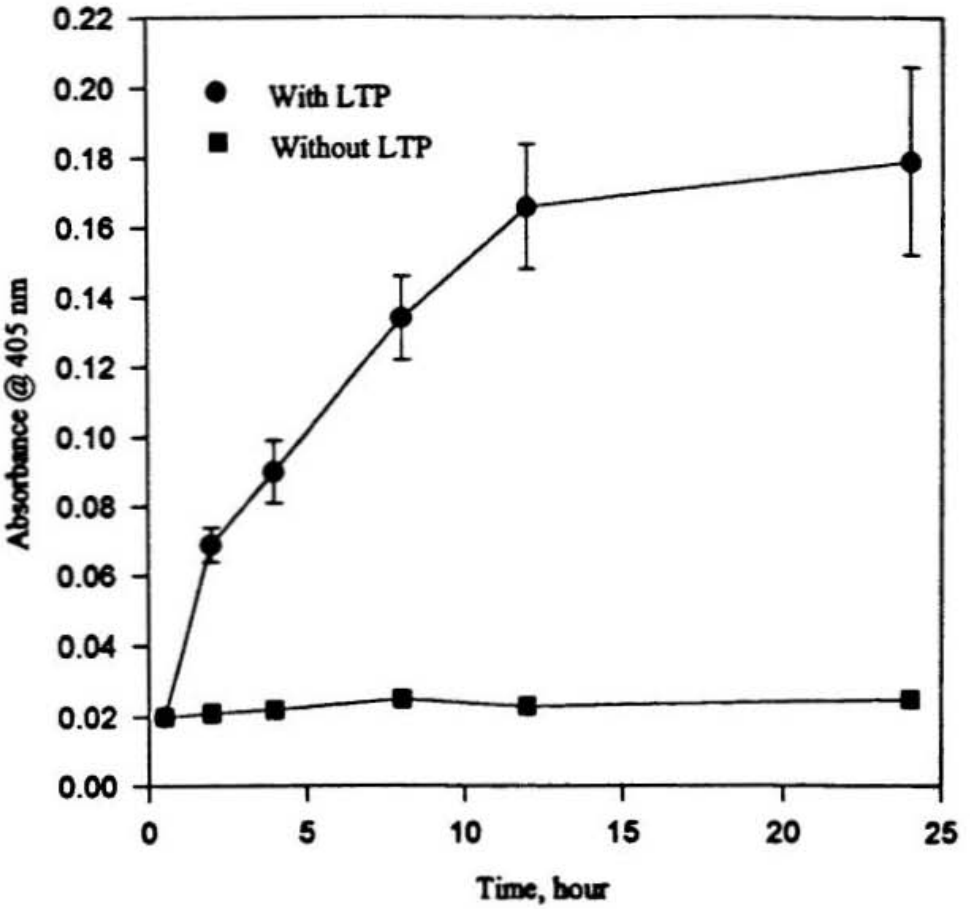


Figure 4.5. **The effect of incubation time on LTP-mediated lipoprotein sample turbidity.** HDLp [250 μg of protein] and LDL [50 μg protein] were incubated in the presence of 2 μg LTP for indicated times at 37°C. Following incubation, sample absorbance at 405 nm was determined on a microtiter plate reader. The results shown are the mean ±SD of three determinations.

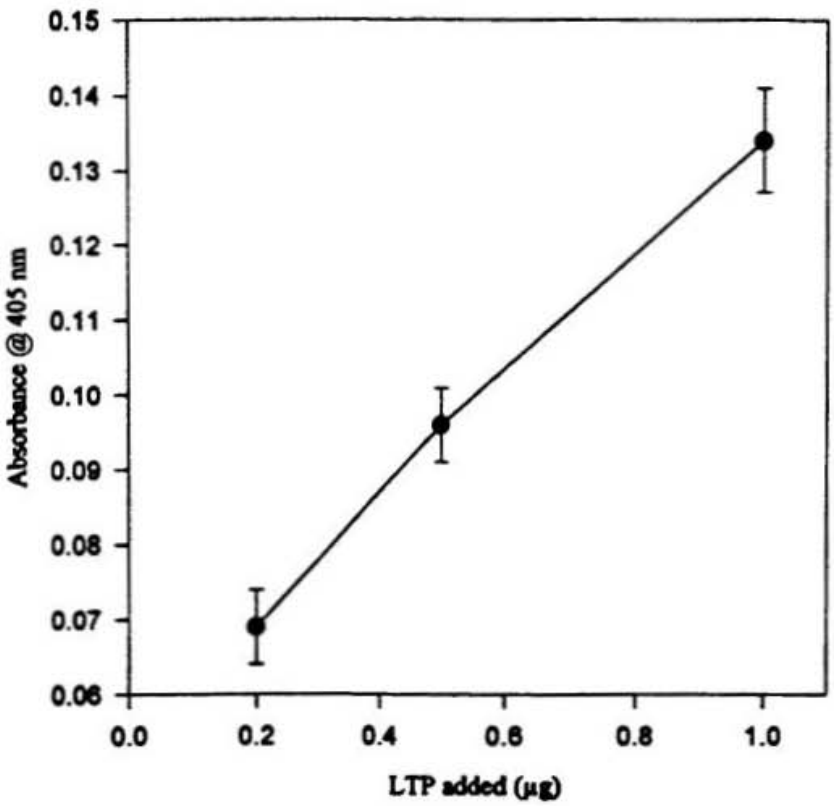


Figure 4.6. The effect of LTP concentration on lipoprotein sample absorbance at 405 nm. HDLp [250 µg protein] and LDL [50 µg protein] were incubated with given amount of LTP for 60 min at 37°C. Following incubation the 405-nm absorbance of each sample was determined. The results shown are the mean±SD of three determinations.

Figure 4.7. **SDS-PAGE of native LDL and LDL-drug conjugates.** A 5% gel was run according to the method of Lammelli [1970]. Lane 1. Control LDL during incubation, and Lane 2. LDL-Dox conjugates. Twenty μg of LDL protein were run in the gel for each sample. In all cases, the LDL-Dox or LDL-CI conjugate produced only one band indicating no degradation of LDL during loading [data not shown].

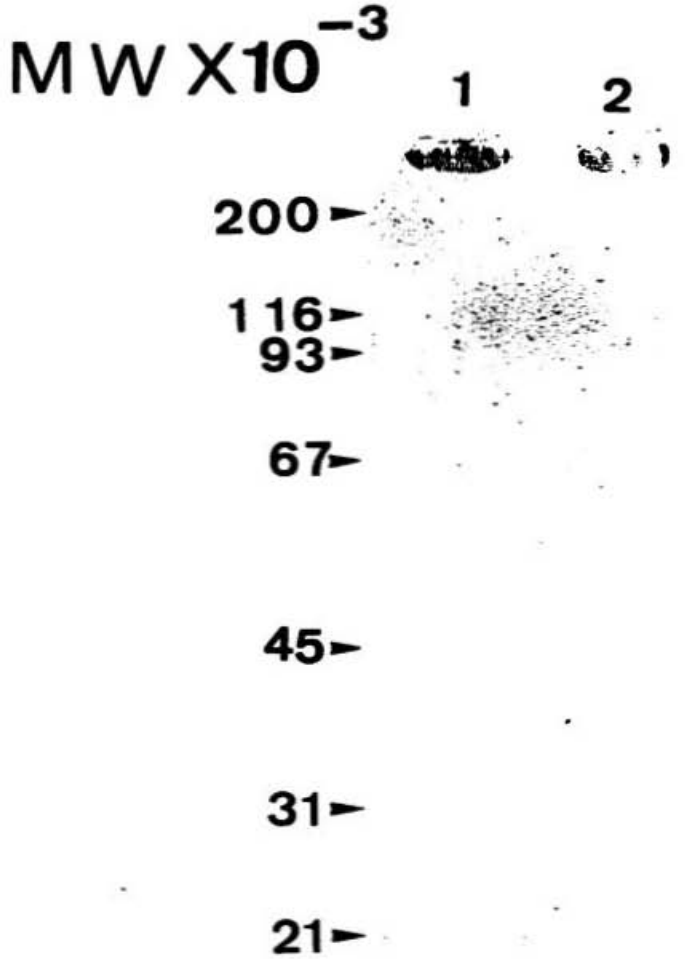

MW X10^{-3}
1
2
200
116
93
67
45
31
21

Figure 4.8. Electron micrographs of LDL preparations. Panel A. Native LDL; B. LDL-Dox [prepared by the direct addition method]; C. LDL-Dox; and D. LDL-CI [C and D was prepared by the contact method with Tween 20]. The LDL preparations were applied to carbonformvar membranes and negatively stained with 2% phosphotungstate solution. They were examined on a Philips EM 301 instrument at a magnification of 75,000X.

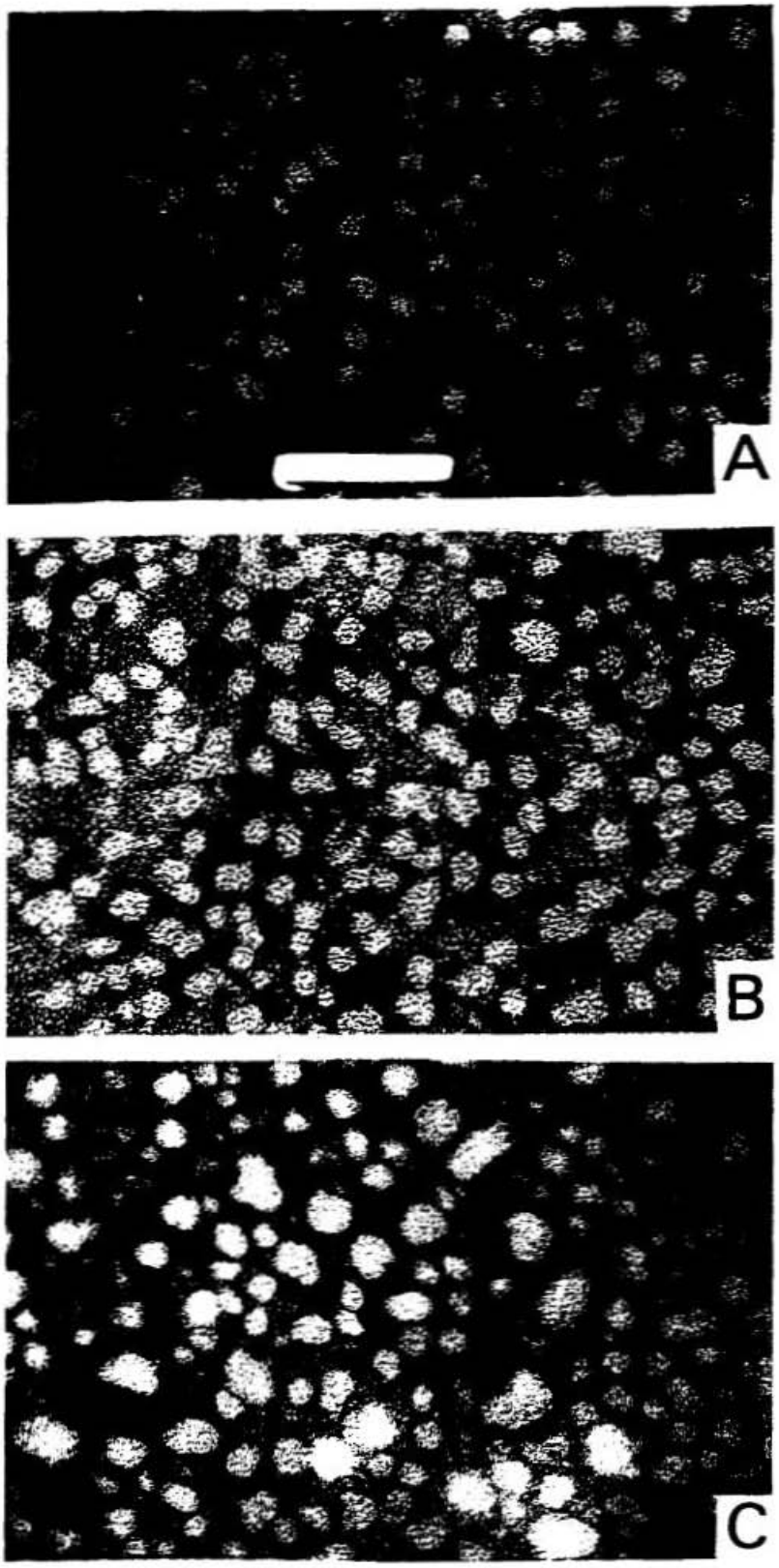

A
B
C

Figure 4.9. Particle size measurement of LDL samples by EM. The LDL preparation were applied to carbonformvar membranes and negatively stained with 2% phosphotungstate solution. They were examined on a Philips EM 301 instrument at a magnification of 75,000x. The diameter of 100 particles were calculated and the results are expressed as the mean of diameter ±SD.

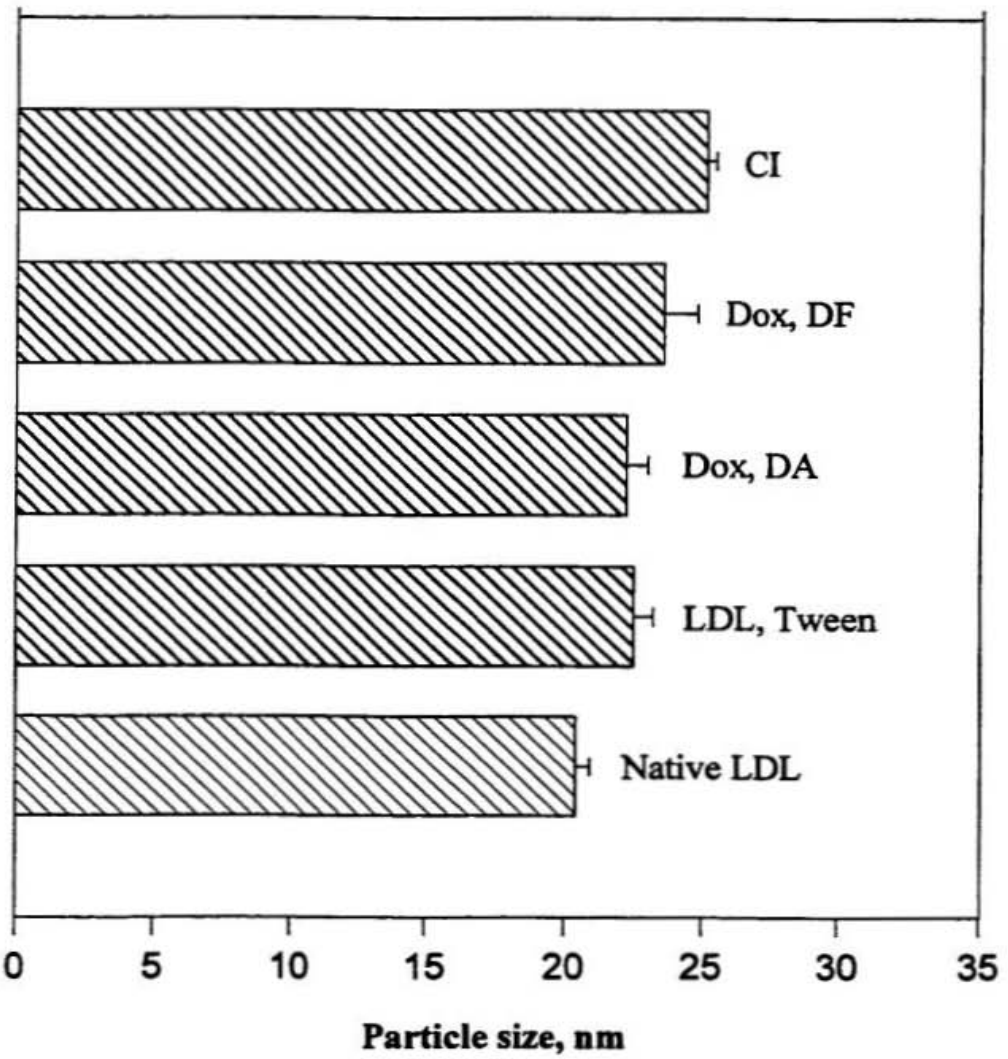
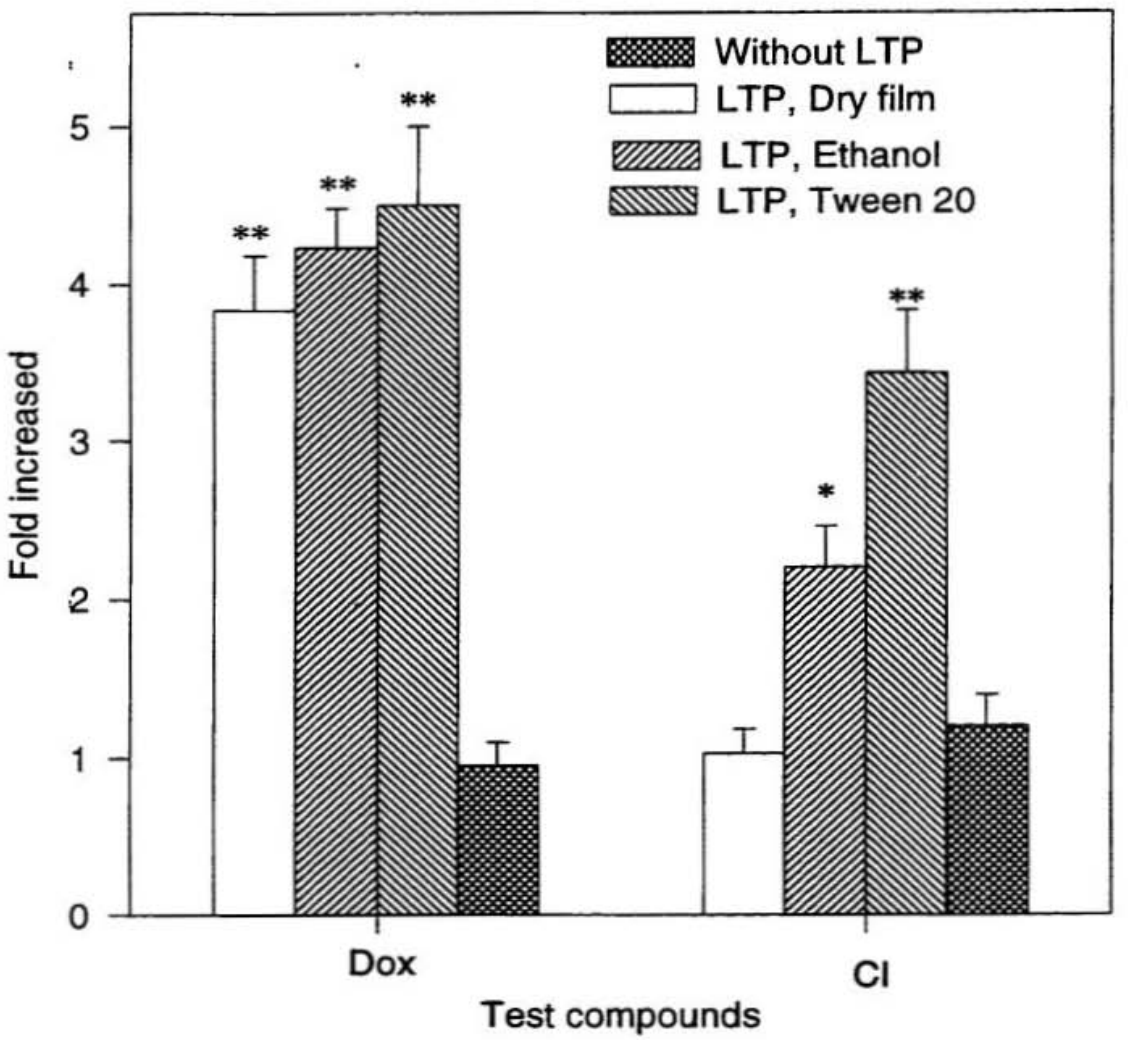

CI
Dox, DF
Dox, DA
LDL, Tween
Native LDL
0
5
10
15
20
25
30
35
Particle size, nm

Figure 4.10. Loading efficiency of LTP in contact methods. A. Dry film method: Drugs [Dox, or CI] were dried down on a test tube from a chloroform solution [dry film] and LDL was added in the presence or absence of LTP. B. Organic solvent aided method: the dry film of the drug was solubulized in few drops of ethanol and incubated with LDL in the presence or absence of LTP. C. Detergent aided method: the dry film of the drug was suspended with Tween 20 [> 3% of the total volume] and incubated with LDL in the presence and absence of LTP. In all cases, incubation followed in the dark at 37°C for 12 hours under gentle shaking. The results are mean ±SD of at least three determinations. The asterisks indicate statistically significant difference (single, $p<0.05$, double, $p<0.001$) compared to control without LTP.


Without LTP
LTP, Dry film
LTP, Ethanol
LTP, Tween 20
Fold increased
0
1
2
3
4
5
**
**
**
*
**
Dox
Cl
Test compounds

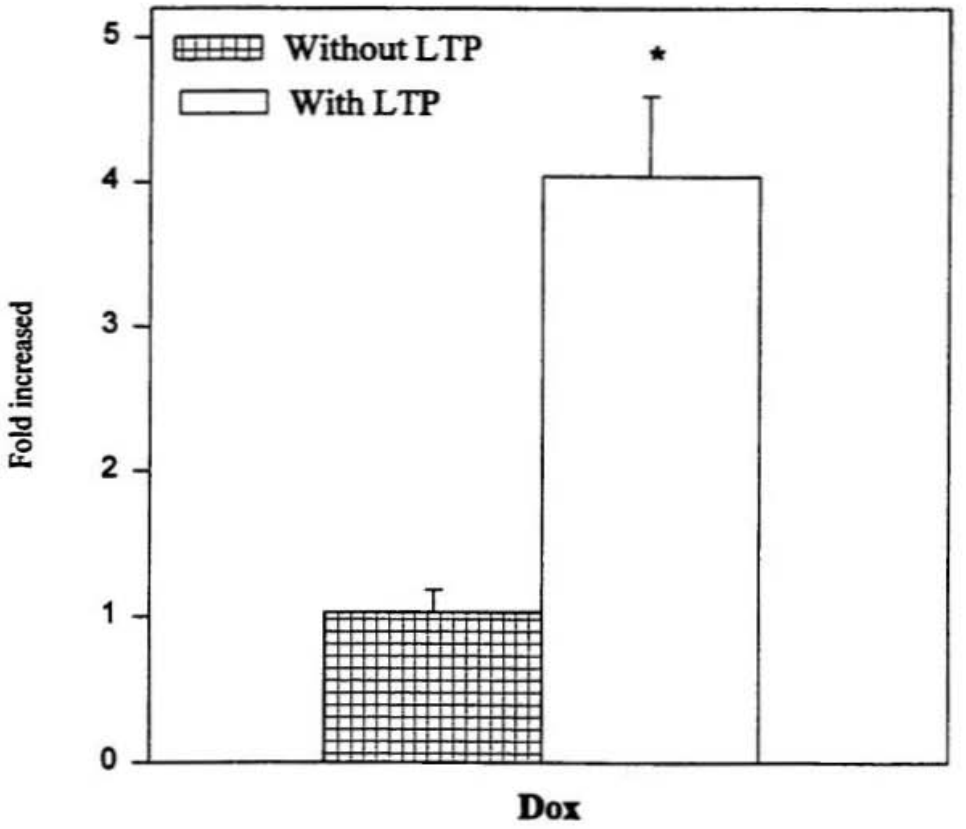


Figure 4.11. Loading efficiency of LTP in the direct addition method. Drug [Dox.HCl in normal saline] were directly added to LDL samples in the presence or absence of LTP. Incubation followed at 37°C for 12 hours in the dark under gentle shaking. The amount of drug incorporated into LDL in the presence and absence LTP was calculated and shown as mean ±SD of three determinations.

Figure 4.12. Loading efficiency of LTP and CETP in incorporating drugs in LDL. Drug incorporation in LDL using LTP was described in Figures 4.7 and 4.86. Drug incorporation using CETP was performed according to Blomhoff *et al.*, [1984]. Fifty μg drugs [Dox or CI] were dried down from their chloroform solutions with N_2. The resultant residue was dissolved in 50 μL of acetone and 500 μL of LPDP was added. After evaporation of acetone with N_2, 300 μL of PBS was added. For DoxHCl, 50 μg drugs were directly added to 500 μL of LPDP followed by the addition of 300 μL of PBS. The LPDP-drug preparation was then incubated with 500 μg of LDL at 37°C for 4 hours in the dark. The amount of drug incorporated into LDL in the presence of LTP or CETP was calculated and shown as the mean ±SD of three determinations. The double asterisks indicate statistically significant difference ($p>0.5$) compared to control without LTP.

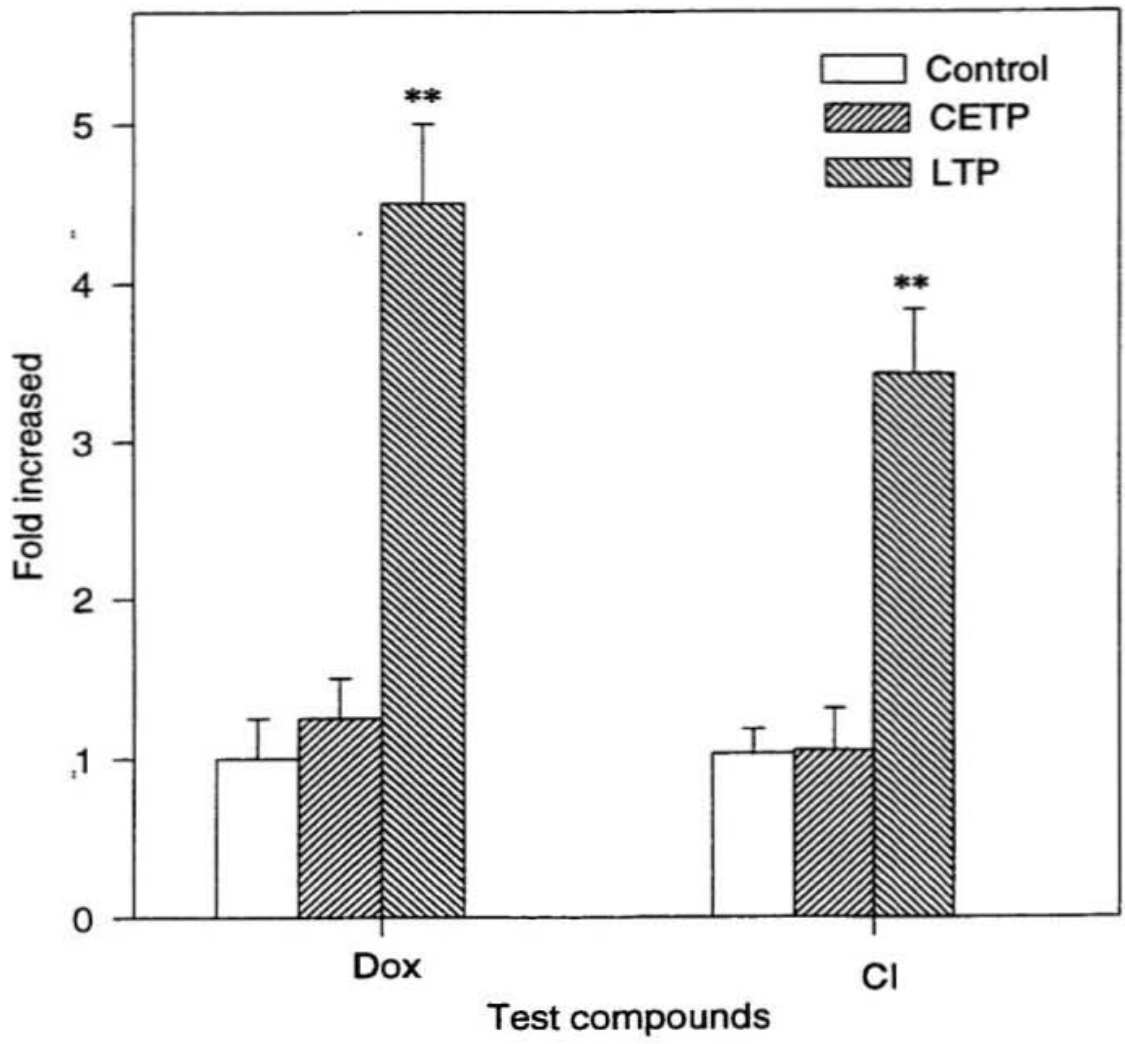
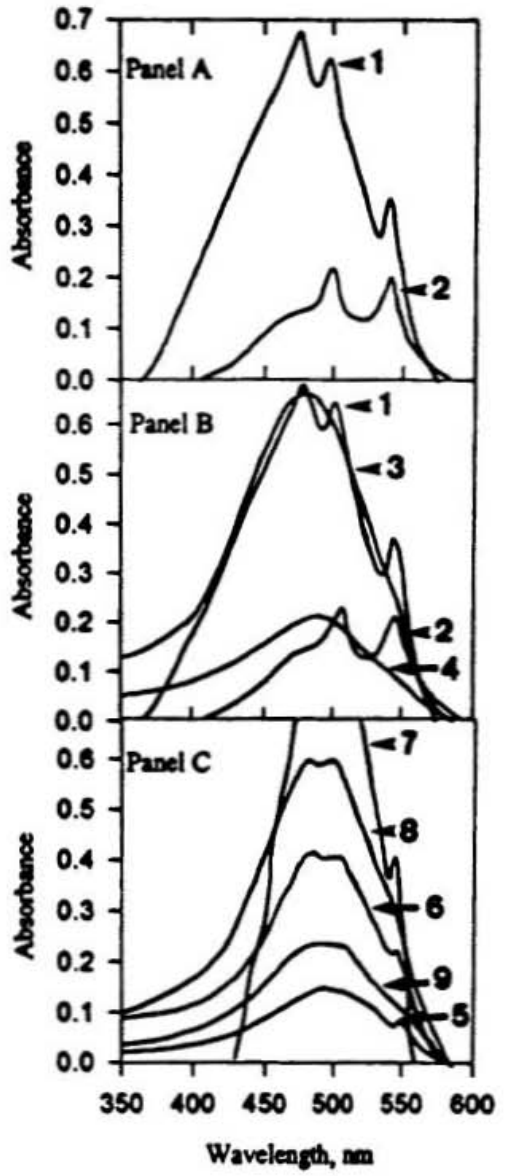

Control
CETP
LTP
**
**
Fold increased
0
1
2
3
4
5
Dox
Cl
Test compounds

Figure 4.12. UV-visible scans of Dox and LDL-Dox conjugates. Dox·HCl was dissolved in PBS or free Dox was dissolved in chloroform. Dox·HCl and Dox were scanned after subtracting scans of PBS and chloroform [controls], respectively. LDL-Dox conjugates was scanned after subtracting LDL scan taken as control. Panel A, LDL-Dox conjugates with (1) or without (2) LTP; panel B, additionally Dox.HCl in PBS solution at different concentrations (3 and 4); and panel C, free Dox in $CHCl_3$ (5,6,7) and Dox.HCl in PBS (8,9 in addition to 3,4). Note that a shoulder around 540 nm when Dox is in $CHCl_3$.


Panel A
Panel B
Panel C
Absorbance
Wavelength, nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
350
400
450
500
550
600
1
2
3
4
5
6
7
8
9

CHAPTER 5:

IN VITRO DELIVERY OF CYTOTOXIC DRUGS INTO TUMOR CELLS VIA THE LDL RECEPTOR PATHWAY

5.1. Introduction

The physico-chemical characterization of LDL-drug conjugates was described in Chapters 2 and 3. I have demonstrated that LDL-conjugates are biochemically and biophysically similar to native LDL. In this chapter, I discuss the biological characterization of both free and LDL-Dox conjugates in a human cervical cancer cell line, HeLa.

The main pathway of cellular recognition and uptake of LDL is mediated by the LDL receptor, which is predominantly present on tumor cells. This cellular uptake mechanism can be up- and down-regulated in response to cellular cholesterol supply and demand [Soutar and Knight, 1990]. If the LDL is modified, its uptake by receptor mediated processes will be less than expected in tumor cells. It must be remembered that LDL can be modified by manipulations, such as minimal oxidation [Berliner *et al.*, 1990; 1986], storage, and vortexing [Lougheed *et al.*, 1991], making it possible that some experiments with purified LDL may inadvertently involve a degree of modification. This minute modification of LDL may not be detectable by the physicochemical techniques. However, such modifications can easily be distinguished by cells in culture [Figure 1.4]. Biological evaluation of the LDL-drug conjugates in an appropriate tumor model may further identify whether or not any changes have occurred during the loading experiments. In my studies, the uptake of the

LDL-drug conjugates were evaluated by their cytotoxic effect on HeLa cells. HeLa cells were chosen because they have previously been shown to specifically bind LDL by a receptor-mediated pathway [Lestavel-Delattre *et al.*, 1992; Johnson *et al.*, 1983]. If LDL is modified during the loading experiment, a decrease in uptake via LDL receptors by the cell will be observed. I also proposed to compare the cytotoxicity of free drugs or LDL-drug conjugates in HeLa cells as a measure of their potency. Cell cytotoxicity determination using MTT is an acceptable method and widely used in cancer research. This assay is based on the metabolic reduction of a soluble tetrazolium salt, MTT, by the mitochondrial enzyme activity of viable tumor cells into an insoluble colored formazan product. This product is measured spectrophotometrically after dissolution in DMSO [Ford *et al.*, 1989; Alley et al., 1988; Carmichael *et al*,. 1987]. The colorimetric assay has the advantages of being safer, less costly and simpler than the radiometric assays.

I hypothesize that Dox will be selectively transported by the carrier, LDL, into the cell much faster than the free drug, which does not have any carrier. Free drugs will be transferred inside the cell only by diffusion. If this is true, the cytotoxicity of the LDL-drug conjugate will be more pronounced compared to the free drug and will be proportional to the efficiency of the carrier in transferring the drug inside the cell. The cellular uptake of LDL by the receptor mediated processes are subject to up- and downregulation, as described in Chapter 1, section 1.5.3. If the cytotoxicity of the LDL-drug conjugate can also be similarly modulated in cells, this will strengthen the hypothesis that LDL carried the molecules inside the cells. To prove my hypothesis, this chapter describes cell cytotoxicity studies of Dox in

HeLa cells using MTT. Additionally, the carrier efficiency of LDL to deliver Dox and into the cells was compared with carrier-free or existing formulations of free Dox.

5.2. Materials and methods

5.2.1. Materials

Human LDL, LPDP, and Dox were described in Chapters 3 and 4. MTT [M 2128] and DMSO were purchased from Sigma [St. Louis, MO]. Filters [0.2 μm] were from Millipore. MTT was dissolved in PBS at a concentration of 0.5 mg/mL, sterile filtered and stored in a dark environment at 4°C for up to a maximum of 1 week. Drug or LDL-drug conjugate dilutions were prepared in the cell culture medium [range, 0.001- 40 μg/mL].

5.2.2. Preparation of LDL-Drug conjugates

Dox-LDL drug conjugates were prepared using the direct addition method as mentioned in Chapters 3 and 4. The LDL conjugates were fully characterized using biochemical and biophysical techniques as detailed in those chapters.

5.2.3. Cell culture

HeLa cells, were gifts from Professor Alan Pater [Faculty of Medicine, Memorial University of Newfoundland, St. John's, Canada]. Cells were grown in Dulbecco's modified Eagle's medium [DMEM] supplemented with 2 mM L-glutamine, 0.1 mM nonessential

amino acids, 0.08 % [w/w], 2 % sodium bicarbonate, 10 % heat inactivated fetal calf serum [FCS] [GIBCO] plus 0.2 mg/mL streptomycin and 200 IU/mL penicillin G. Cells were maintained at 37°C and gassed with 4% [v/v] CO_2 in air. The doubling time was 27 ± 1.5 hours [mean ± SEM]. For all experiments, exponentially growing cells were used.

5.2.4. Determination of cytotoxicity

Cytotoxicity of free drug or LDL-drug formulations was conducted by the method of Ford *et al.*, [1989] and Carmichael *et al.*, [1987] with minor modifications.

1. Under sterile conditions, 100 µL of the cell suspension [5 x 10^4 cells] harvested from log-phase growth were seeded into 96 flat well bottomed plates. Three types of media were employed, standard media as listed above, media containing LPDP instead of FCS and the FCS supplemented media with 15µg/mL of an extra LDL.
2. The plates were incubated for 24 hours as above.
3. Medium was removed from the cells and test dilutions were added in 100 µL fresh medium. Under sterile conditions, medium was aspirated by a microtitre pipette from the wells and doubling dilutions of Dox or LDL-Dox conjugates were added in a volume of 100 µL to each well. The test was performed in quadruplicate for each dilution, with appropriate control wells that received 100 µL medium or LDL only. The plates were incubated for 48 hours [step 2].
4. The medium was aspirated as discussed above and each well washed twice with sterile PBS [200 µL] at RT. One hundred µL of medium was then added to each well

and a 24 hour recovery period followed.

5. Following removal of the medium by aspiration under sterile conditions after recovery, MTT solution [100 μL] was added to each well. A 4 hour incubation period in the humidified incubator at 37 °C followed.

6. After incubation, medium was aspirated from each well, DMSO [100 μl/well] was added, the plates gently shaken and the absorbance of the well measured at 570 and 630 nm using a Biotek EL310 EIA plate reader. Absorbance at 630 nm was used as the reference wavelength for detecting artifacts in the plastic plates and was subtracted from the 570-nm values. The mean absorbance for these wells was subtracted from the absorbance values in the other wells. Tests using LDL and LPDP were conducted in the same manner.

8. Results from the plate reader were expressed as follows:

$$\%\ \textit{Cell survival at each dilution} = \frac{\textit{Mean absorbance at each dilution}}{\textit{Mean control absorbance}} \times 100$$

9. A dose response curve of %cell survival [ordinate] against drug concentration [abscissa] was constructed. The IC_{50} value was calculated from the plot.

5.2.5. Statistical analysis

For statistical evaluation of the data, one way ANOVA for multiple groups and the t-test for two groups was performed to compare between/among groups with the calculation of p values. All values are expressed in mean ±SEM.

5.3. Results

5.3.1. Physico-chemical characterization of LDL-drug conjugates

I have characterized the LDL-Dox conjugates as mentioned in Chapters 3 and 4. The drug loading capacity of the conjugates were complimentary to my previous results as mentioned in Figure 2.11 and Table 5.1. Similar results were obtained when the samples were analyzed by SDS-PAGE [as shown in Figure 3.4], EM [as shown in Figures 3.3 and 3.4], and DSC [as shown in Figure 4.7] and Table 5.1.

5.3.2. Cytotoxicity of LDL-drug conjugates

The cytotoxicity of Dox, and LDL-Dox conjugates, was studied in terms of %cell survival in HeLa cells. The dose response curves are plotted in Figure 5.1. Figure 5.1 shows the cytotoxicity of different formulations of Dox on Hela cells. Free Dox in its existing formulations was the least effective compared to LDL-Dox formulations. The cytotoxic parameter, IC_{50} value, for Dox was found to be 1 μg/mL which is at least 6-fold higher than any LDL formulations. IC_{50} values for different formulations were found to be 0.055, 0.142, and 0.173 μg/mL in down-, normal, and up-regulated cells [Table 5.2] [one way ANOVA p value<0.05]. There was a statistically significant difference in up- and down-regulated cells [paired t-test p values for up- and down regulated cells compared to normal cells were<0.5]. Theoretically, cellular uptake of LDL should be down-regulated by fasting cells overnight. Cells were incubated in LPDP instead of FCS to upregulate LDL receptor activity

in cells. After fasting, when LDL was supplied in the form of LDL-drug conjugates, cellular uptake of LDL-drug conjugates were increased relative to normal cells. The comparative cellular uptake of LDL-drug conjugates could be inferred using the cytotoxicity profiles of the different formulations. If the cellular uptake of the cytotoxic drug is increased, a correspondingly increased cytotoxic effect should be observed. Indeed, the IC_{50} value of Dox in up-regulated cells were 2.58-fold higher than normal cells [$p<0.05$]. Likewise, the cellular uptake of LDL could be down-regulated by incubating cells in the presence of excess LDL. A higher IC_{50} value for LDL-drug conjugates in down-regulated cells is expected compared to the normal cells. A slightly higher but statistically significant [t-test $p<0.05$] IC_{50} value [0.173 μg/mL] in up-regulated cells was observed compared to normal cells [0.142 μg/mL] for Dox [$p<0.05$]. This implies that upregulation of the cell is a more effective way in increasing LDL receptor activity. For the LDL-Dox formulation, the highest cytotoxic effect was observed in up-regulated cells [IC_{50} = 0.055 μg/mL] which is almost 20-fold more cytotoxic than free Dox [IC_{50} = 1.0 μg/mL] [$p<0.001$]. Free Dox had no significant effect in up- or down regulated cells when compared to normal cells [$p> 0.05$] [data not shown]. The native LDL in the same LDL protein concentration range had no effect on cell growth. The difference in cytotoxic effects is more pronounced among the LDL formulations at lower concentrations [Figure 5.1].

It can thus be noted that the cytotoxic activity of the LDL conjugates is 6-fold higher than that of free drug depending on the cellular concentrations of LDL receptors. The IC_{50} of Dox fell 18-fold when transported within LDL, illustrating the ability of the LDL to

potentiate the action of free drug.

5.4. Discussion

The results presented in this study support the findings that HeLa cells internalized LDL drug preparations by receptor-mediated processes [Lestavel-Delattre et al., 1992; Johnson et al., 1983]. If the drug is tightly bound to LDL, then excess native LDL should compete with the conjugate for binding to the LDL receptor and, hence, reduce uptake and toxicity of the conjugate. The LDL receptor mediated uptake was confirmed by the up- and downregulation effects caused by a large excess of LDL or LPDP in the media in addition to normal constituents. I have demonstrated that this receptor mediated process could be used to deliver cytotoxic agents to cells. This method is especially important for drugs which need to be transported inside the cells to exert their cytotoxic effects. Dox, is unique in inducing cytotoxicity even without entering inside the cells [Tritton and Yee, 1983]. If the drug does not enter the cell, cytotoxicity to cells is negligible. Free Dox is effective in inducing cytotoxicity in cells without actually entering the cells. However, I inferred from my data that Dox action inside cells, perhaps by DNA intercalating, inducing of topoisomerase II mediated DNA cleavage, inhibiting of background DNA cleavage, seems to be more effective than its action at the cell surface [Bodley *et al.*, 1989]. This view is in agreement with previous findings related to the site of action of Dox [Bodley *et al.*, 1989]. I propose that LDL can be used to deliver drugs more effectively into the cell.

The LDL receptor-mediated endocytosis of cytotoxic LDL-drug might provide

distinct advantages over the trapping of antineoplastic drugs in artificial carriers. The main reason for this statement is that artificial carriers are subjected to destruction by blood borne components, primarily by lipoproteins [Rohrer *et al.*, 1990] and to fast clearance from the circulation by the RES [Suits *et al.*, 1989]. Since the loading of the particles with drug molecules does not influence the receptor recognition of the protein component of LDL, this preparation is expected to follow the *in vivo* fate of native LDL particles in animal or human studies. This is very important, as the use of LDL as a drug carrier depends on the preservation of particle characteristics such as receptor recognition and RES avoidance. If the physical properties of the core lipids of LDL is disturbed during drug loading, e.g., a change in fluidity of core, a premature drug leakage into the systemic circulation may occur and much of the drug will be released before the LDL-drug conjugates reach the target site. In the case of LDLs, the fluidity of the core is therefore important as it influence the drug release kinetics and thereby determines if the carrier can be used for any drug targeting or if only the depleted carrier will reach the target tissue [Westesen *et al.*, I995]. The present study indicates that in favorable cases, such as loading with the anticancer drug, the characteristics of the LDL structure and its dynamic properties are preserved in addition the incorporation of significant and effective amounts of drug. This present study also demonstrates that cytotoxic drugs can be modified, incorporated into LDL and selectively delivered to cells by LDL receptors *in vitro*.

It is essential to realize that the effective use of LDL as a targeting vehicle in medical practice will require the use of a drug substance with high cytotoxic activity. Under normal

in vivo conditions, the LDL receptor can be down-regulated by the native LDL in the blood stream [Goldstein *et al.*, 1977]. LDL-drug conjugates are diluted by the native LDL, which will compete for the binding sites on the receptor. This problem might, in part, be dealt with by downregulation of receptors in patients as discussed in Chapter 1. I demonstrated that downregulation of LDL receptors does not significantly effect cytotoxicity of LDL-drug conjugates. This has been demonstrated in human studies by Filipoeska *et al.*, [1992]. However, in light of these considerations, it is obvious that the 10-fold increase in cytotoxic activity obtained by the new formulations presented in this study, is a significant progress in the field of drug delivery targeting using LDL or lipid emulsions.

Table 5.1. Physico-chemical characteristics of LDL-Dox conjugates used in HeLa cells

Features of LDL-drug conjugates	Mean value ±SEM
Drug loading capacity [μg of drug/mg of LDL protein]	47.06 ± 3.10
Ratio of cholesterol to protein	1.7 ± 0.20
Apo B integrity from SDS-PAGE [no. of bands observed]	Single
Particle size from EM, [nm]	22.12 ± 0.12
Transition enthalpy [KJ/kg of protein] calculated from DSC thermograms]	1.474 ± 0.11

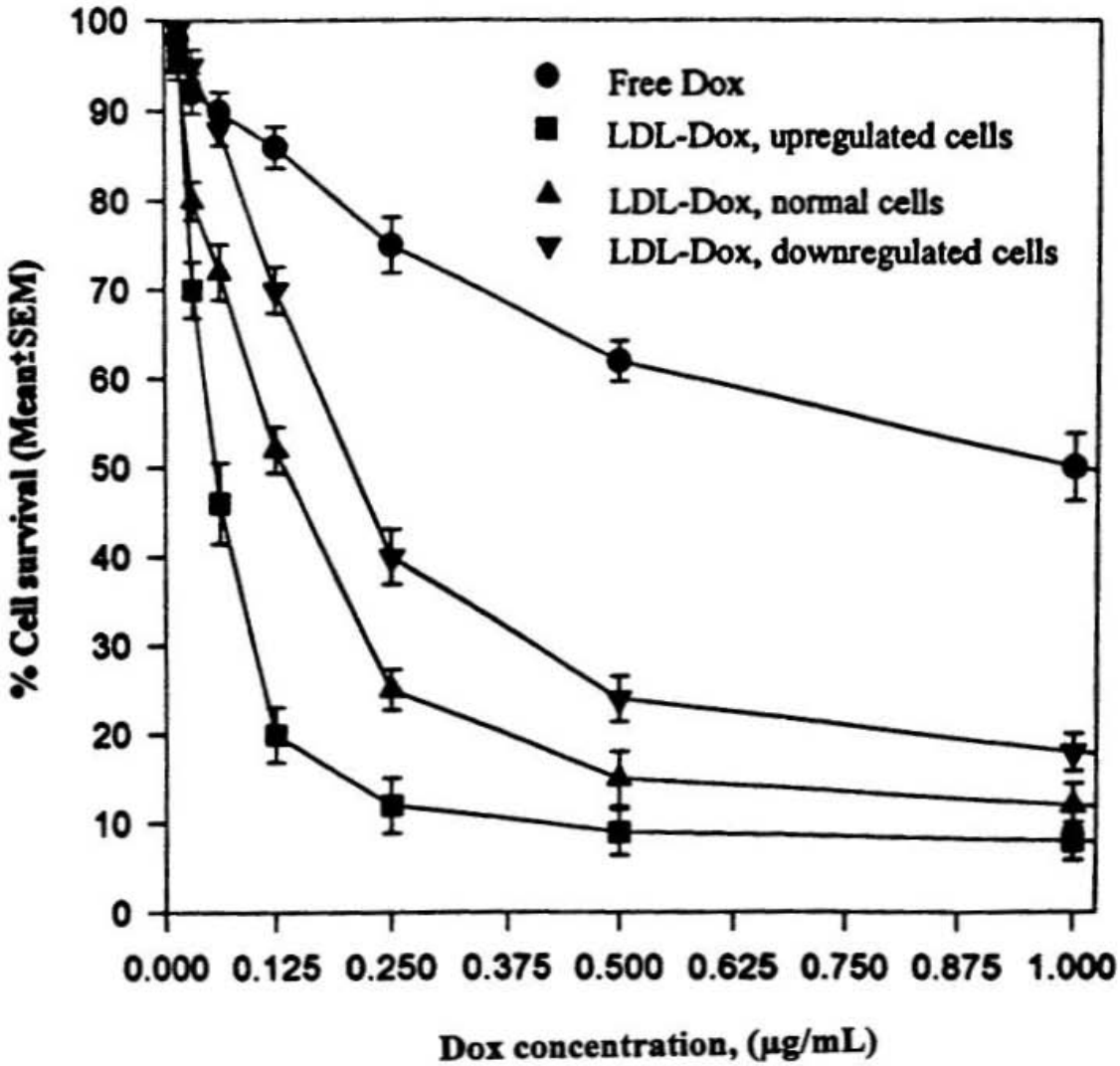
Table 5.2. IC_{50}[a] values for cytotoxicity of HeLa cells treated with free Dox or LDL-Dox conjugates

Formulations	IC_{50}[b], [μg/mL] in HeLa cells		
	Normal	Up-regulated	Down-regulated
Free Dox	1.00 ± 0.2	0.85 ± 0.13	1.12 ± 0.121
LDL-Dox conjugates	0.142 ± 0.01	0.055 ± 0.01	0.173 ± 0.04

[a] Concentration required to reduce cell survival of HeLa cells to 50 %.

[b] 5×10^4 cells were plated in 96 wells sterile microtitre plates and after 24 hour cultures were exposed to varying concentrations of drug formulations for 48 hours. Plates were then rinsed with PBS and 200 μL fresh growth medium was added. After 24 hour cell cytotoxicity was determined with MTT. IC_{50} values were calculated from three separate experiments, each performed in quadruplicate.

Figure 5.1. Cytotoxic effect of the Dox and LDL-Dox conjugates on normal, up- and down-regulated HeLa cells. Cells [5 X 10^4] were incubated at 37°C with the Dox or LDL-Dox conjugates in doubling dilutions. The cells were sampled after 24 hour incubation and followed by washing twice with PBS and cytotoxicity was counted using the MTT assay as mentioned in Materials and methods. The cytotoxicity of Dox in normal, up- and down-regulated cells were comparable and only the cytotoxicity of Dox on normal cells are shown in the figure for clarity. Each point is the mean ± SEM of four determinations in two independent experiments.


100
90
80
70
60
50
40
30
20
10
0
% Cell survival (Mean±SEM)
Free Dox
LDL-Dox, upregulated cells
LDL-Dox, normal cells
LDL-Dox, downregulated cells
0.000
0.125
0.250
0.375
0.500
0.625
0.750
0.875
1.000
Dox concentration, (µg/mL)

CHAPTER 6:

IN VITRO EVALUATION OF MODIFIED LDL-DRUG CONJUGATES ON MACROPHAGES

6.1. Introduction

Macrophages [Mϕ] are bone marrow-derived mononuclear cells with established roles in host protection against facultative and obligate bacterial pathogens [Edwards *et al.*, 1986; Adams and Hamilton, 1984], viruses [Koff *et al.*, 1985; Ishihara *et al.*, 1985], protozoan parasites [Wirth and Kierszenbaum, 1988], and neoplastic cells [Shaw *et al.*, 1988; Adams and Hamilton, 1985,]. They belong to the mononuclear phagocytic system [MPS], which includes the Kupffer cells in the liver, alveolar, splenic, lymph node, and bone marrow Mϕ, tissue histiocytes, and circulatory blood monocytes. However, Mϕ are not constitutively competent to destroy pathogenic organisms or neoplastic cells. They take up lipoprotein-bound cholesterol either by phagocytosis of whole cells or membrane fragments or via receptor-mediated endocytosis of plasma lipoproteins. Although LDL receptors are present on a variety of cell types, normal tissue Mϕ express few receptors for native LDL [Goldstein *et al.*, 1980] and take up native LDL very slowly *in vitro* [Brown and Goldstein, 1983; Goldstein *et al.*, 1979]. In contrast, LDL that has been reacted with acetic anhydride *in vitro* to form AcLDL is taken up rapidly by a scavenger-receptor mediated endocytotic pathway in Mϕ [Shaw *et al.*, 1988; Pitas *et al.*, 1985; Brown and Goldstein, 1983; Johnson *et al.*, 1983; Goldstein *et al.*, 1980; 1979;]. This pathway is functional in Mϕ, blood

monocytes, sinusoidal endothelial cells, and to a lesser extent, microvascular endothelial cells [Pitas *et al.*, 1985; Via *et al.*, 1985; Voyta *et al.*, 1984; Brown and Goldstein, 1983; Nagelkerke *et al.*, 1983] . The *in vivo* function of the AcLDL pathway has been suggested to be the scavenger system for LDLs that are modified by oxidation products of arachidonic acid [Brown and Goldstein, 1983; Goldstein *et al.* 1979] and/or to play a role in Mϕ-induced inflammation [Brown and Goldstein, 1983]

As indicated earlier the scavenger receptor exists on a restricted number of cell types, thereby offering a distinct targeting advantage over the LDL receptor system [Matsumoto *et al.*, 1990; Shaw *et al.*, 1987]. This receptor is a 260 kDa, trypsin-sensitive glycoprotein [Shaw *et al.*, 1987]. These receptors bind negatively charged lipoproteins and participate in the removal of altered lipoproteins [AcLDL] from the circulation [Basu *et al.*, 1976]. LDL will not bind the scavenger receptor and does not interfere with AcLDL uptake by Mϕ [Shaw *et al.*, 1988; 1987; Brown and Goldstein, 1983]. AcLDL has been proposed as a carrier to deliver drugs specifically to Mϕ [Shaw *et al.*, 1987]. Liposomes have also been proposed as a carrier for delivering drugs [such as immunomodulators] in Mϕ diseases [Schwendener *et al.*, 1984; Rahman *et al.*, 1982]. Drug delivery through liposomes to Mϕ does not have any targeting advantages [Poste *et al.*, 1982]. Liposomes interact with a variety of cell types and are therefore nonspecific for Mϕ. Furthermore, they are often unable to pass through the vascular endothelium. Hence they bypass resident tissues or tumor associated Mϕ. As a drug carrier, AcLDL presents some advantages in comparison with liposomes: [1] AcLDL measures 21 nm and is smaller than the majority of liposomes, and therefore may provide

better infiltration into the different tissues than liposomes; [ii] AcLDL binds only with cells presenting the scavenger receptor and does not bind with other cells as do liposomes. This increases the specificity of the target and may decrease the toxicity of the drugs [Hossaini *et al.*, 1994]. Furthermore, it is now possible to incorporate a number of lipophilic drugs into LDL or AcLDL [Hossaini *et al.* 1994; Shaw *et al.* 1987, 1988; Yanovich *et al.*, 1984].

Histocytic malignancies are aggressive neoplastic diseases which affect the cells of the MPS and are usually fatal if untreated. Dox is commonly used in the treatment of histiocytic malignancies [Buzdar *et al.*, 1985]. I have demonstrated that Dox can be loaded in LDL and the LDL-Dox conjugates were 20-fold more effective than free Dox in killing HeLa cells. Since the scavenger receptor system is known to be present primarily on the cells of the Mϕ, it may be possible to achieve selective delivery of antitumor agents to the neoplastic cells in histiocytic malignancies through these receptors. Recently, it has been demonstrated that the scavenger receptor-mediated delivery of DNM elicits selective toxicity towards murine neoplastic cells of macrophage lineage whereas receptor negative cells remain unaffected, both *in vitro* and *in vivo* [Basu *et al.*, 1994; Mukhopadhyay *et al.*, 1993; 1992].

It is important to establish the efficacy of AcLDL-drug conjugates in an appropriate model system as a prelude to determining the feasibility of extending this approach to human malignancies or infections of macrophage origin. In this study, I propose to deliver Dox using AcLDL as the carrier. The efficacy of AcLDL-drug conjugates in terms of targeting potential will examined in a well characterized mouse macrophage cell line, J774.A1. In

addition, normal LDL-drug conjugates will be used to compare the selectivity of modified LDL over normal LDL-drug conjugates.

6.2. Materials and methods

6.2.1. Materials

Dox, LDL, LDL-Dox, MTT, and DMSO have been described in the previous chapters.

6.2.2. Acetylation of LDL

The LDL was acetylated with the repeated addition of acetic anhydride as described by Basu *et al.* [1976] and dialyzed for 36 hours at 4°C against PBS. Turbidity of the solution was removed by centrifugation [1000x g, 10 minutes]. Sterile filtration using the Millipore membrane filter [0.20 μm pore size] was done to make the final preparation. AcLDL was stored at 2-8°C for no more than two weeks before use.

6.2.3. Preparation of AcDL-drug conjugates

The drug loading procedure was essentially the same as described previously in Chapter 2. Dox was incubated by the direct addition method. AcLDL-drug conjugates were characterized by SDS-PAGE, EM, and DSC as described in Chapter 3.

6.2.4. Cell culture

Mouse Mϕ cells, J774.A1, were grown in DMEM supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.08% [w/w] sodium bicarbonate, 10% heat inactivated FCS plus 0.2 mg/mL streptomycin and 200 IU/mL penicillin G. Cells were maintained at 37°C and gassed with 4% [v/v] CO_2 in air. For all experiments, exponentially growing cells were used. Cells were seeded in 96 well microtitre plates at a density of 5 x 10^5 cells/well. Forty eight hours after seeding, the growth medium was replaced by medium containing a 10% human LPDP or LDL [15 μg protein/mL]. These cells were cultured for a further 24 hours and used for cytotoxic studies. Henceforth, J774.A1 cells cultured with LPDP or LDL will be referred to as up- or down-regulated J774.A1 cells, respectively. Normal cells were grown in standard medium as mentioned earlier.

6.2.5. Determination of cytotoxicity

The procedure was essentially the same as described in Chapter 5, section 5.2.4, with minor modifications. Briefly, cells harvested from log-phase growth were seeded into 96-well flat-bottomed plates at 5 x 10^5 cells/well. Free drug, LDL-drug, and AcLDL-drug formulations were then added to the plates at doubling dilutions. After 48 hours exposure to the drugs, the cells were washed twice and fresh media was added. Cells were incubated until the control cells reached confluence. At this point 100 μL MTT solution [0.5 mg/mL] was added to each well and the cells were incubated in the dark for 4 hours at 37°C. After incubation, DMSO [100μL/well] was added, the plates gently shaken and the absorbance of

the well measured at 570 and 630 nm using an ELISA plate reader. Cytotoxicity was measured as described in Chapter 5 [section 2.4].

6.3. Results

6.3.1. Efficiency of drug incorporated into AcLDL

The MW of Dox is 543 dalton [Da]. LDL molecules contain only one apo B protein [Kostner and Laggner, 1989] and its MW is 543 kDa. I calculated that one AcLDL particle incorporated 44 ± 10 molecules of Dox [n=5 assays].

6.3.2. Characterization of AcLDL-drug conjugates

To study the stoichiometry of the incorporation procedure, I added varying amounts of the drug to fixed amounts of AcLDL. Results were similar to that observed for LDL-drug conjugates. As the amount of drug was increased, the drug to AcLDL protein ratio of the complex increased until a plateau at approximately 44 molecules per AcLDL particle for Dox was approached [Figure 6.1]. HPLC analysis revealed that Dox constituted more than 95% of the incorporated drug [Dox] which indicated stability of the Dox during processing and within LDL. This indicates that Dox is as stable in AcLDL as it is in native LDL. The electrophoretic mobility of AcLDL-drug conjugates was similar to AcLDL on denaturing 15% polyacrylamide electrophoresis gel, indicating that both particles had an identical size. No apo B fragmentation in the gel was visualized [Figure 6.2]. This suggests that the loading

procedure did not change the native integrity of the apo B protein. Particle size measurements by EM revealed that the conjugates prepared by the current method had almost the same size as the native LDL [Figure 6.3]. AcLDL-Dox was found to be 23 ± 1.2 nm in diameter. Figure 6.4 shows electron micrographs of native LDL, AcLDL, and AcLDL-Dox conjugates prepared by the indicated methods. All AcLDL or AcLDL-drug conjugates show similar morphologies to native LDL. These results indicate that there is no difference in physicochemical behavior of drug loaded LDL or AcLDL using my experimental protocols.

6.3.3. Cytotoxicity of AcLDL-drug conjugates

The biological activity of AcLDL-drug conjugates was studied *in vitro* in J774.A1 Mϕ. The selectivity of AcLDL-drug conjugates was compared to free drugs and normal LDL-drug conjugates. The dose response curves plotted in Figure 6.5 shows that cytotoxicity of Dox, LDL-Dox, and AcLDL-Dox conjugates in normal cells. AcLDL-Dox conjugates clearly show a much higher activity than the other formulations [Table 6.1]. IC_{50} values obtained for AcLDL-Dox, LDL-Dox, and free Dox formulations were compared [Table 6.1]. AcLDL-Dox conjugates showed at least 3-5 times more cytotoxicity than native LDL-Dox conjugates [$p<0.001$] which was again 7 times lower for cytotoxicity compared to free Dox [$p<0.001$]. The IC_{50} of the Dox fell from 0.04 to 0.026 μg/mL [>15 fold] when transported within AcLDL and to 0.0084 [4.7 fold] when transported in native LDL.

The native AcLDL, which was in the same AcLDL protein concentration range, had no effect on the cell growth. The cytotoxic efficacy is more pronounced at low concentrations

for AcLDL-Dox conjugates.

6.3.4. Uptake of AcLDL-drug conjugates by up- and down-regulated cells

The scavenger receptor mediated uptake was studied by examining the up- and the downregulation cells on the cellular uptake of LDL formulations. Mϕ predominantly express the scavenger receptor which cannot be modulated like the LDL receptor by the use of LDL or LPDP in the media as mentioned in Chapter 5. Since the expression of the LDL receptor is minimal, a significant increase in cytotoxic effects for LDL-drug conjugates by modulating cells via up- or downregulation of receptor would not be expected. Indeed, cells did not show any change in cytotoxicity in the different media for free drugs and AcLDL formulations [Table 6.1]. When the cells were down-regulated no statistically significant difference between the efficacy of Dox or AcLDL-drug conjugates were observed [$p>0.05$]. For the LDL-drug formulation a higher cytotoxicity was observed when cells were up-regulated using LPDP in the growth medium. However, only minimal modulation [1.728 fold] in the IC_{50} values was observed. This suggests that J774.A1 cells express a limited number of the LDL receptors but express predominantly scavenger receptors. The LDL-drug conjugates were more effective than the free drugs, indicating that the LDL receptor pathway is more efficient than the free drug formulations. As expected, a low uptake of the LDL preparations was observed for the down-regulated cells compared to the up-regulated cells.

6.4. Discussion

Much work has been done on the various modifications that can affect LDL, and on their consequences for its recognition, uptake and degradation by Mϕ, endothelial cells and smooth muscle cells. These modifications are thought to be primarily oxidative in origin [Witzman and Steinberg, 1991], and to be mediated by endothelial and smooth muscle cells either via lipoxygenase [Parthasarathy *et al.*, 1989] or iron-catalyzed peroxidation [Heinecke *et al.*, 1984]. The modifications include acylation of lysine ϵ-amino groups [Steinbrecher, 1987], increased levels of lysolecithin [Parthasarathy *et al.*, 1985], increase in thiobarbuturic acid-relative substances [Bonnefont *et al.*, 1989], aggregation of the lipoprotein particles [Hoff *et al.*, 1992] and cross-linking or fragmentation of the apo B polypeptide [Fong *et al.*, 1987].

Modified AcLDL is taken up by cells mainly by two mechanisms: [1] The scavenger receptors present on Mϕ and endothelial cells, which recognize AcLDL that has had a certain proportion of it ϵ-amino groups modified to increase its net negative charge [Zhang *et al.*, 1993]; two variants have been cloned and sequenced [Kodama *et al.*, 1992, Rohrer *et al.*, 1990]; and [2] the apo B-mediated macrophage AcLDL phagocytosis mechanism [Suits *et al.*, 1989], which is thought to take up AcLDL that has been less severely modified and is more likely to aggregate. The two mechanisms do not appear to downregulate and Mϕ can thereby become overloaded with lipid and cholesterol [both esterified and free], eventually becoming foam cells.

It must be remembered that LDL can be modified by manipulations, such as minimal

oxidation [Berliner *et al.*, 1990; 1986], storage and vortexing [Khoo *et al.*, 1988], making it possible that some experiments with purified lipoprotein may involve a degree of modification. Conjugates and complexes of various drugs with LDL [Halbert *et al.*, 1985, Lundberg, 1987] have been postulated as delivery vehicles to tumors but the relative lack of success has been accounted for in the case of complexes by the lack of stability and, in the case of conjugates, by the switch in affinity towards Mϕ [Schultis *et al.*, 1991].

The current study indicates that AcLDL-drug conjugates are effective in delivering lipophilic cytotoxic drugs to Mϕ. Although Mϕ possess receptors for the native LDL, the uptake and degradation of AcLDL occurs at rates approximately 20-fold greater than LDL [Brown and Goldstein, 1983; Goldstein *et al.*, 1979]. However, in my studies, the AcLDL pathway was found to be less effective which was 3.2-fold higher than the native LDL pathway in case of Dox conjugates. This may be due to the difference in cell line or the drug candidates. It was demonstrated that drug loading into the AcLDL particles does not alter its specificity or binding for Mϕ [Shaw *et al.*, 1988]. My demonstration that the cytotoxicity of the AcLDL-drug conjugates was not completely inhibited by native LDL suggests that the interaction of AcLDL occurs by means of the scavenger receptor. Cytotoxicity studies performed with J774.A1 cells demonstrate the ability to induce cytotoxicity by the AcLDL *in vitro*. Delivery of the drugs using native LDL also led to enhanced cytotoxicity. However, the nonspecific uptake of drugs, in the case of the free drug formulations, was found to be least effective compared to native or modified LDL formulations.

Mϕ exist normally in various stages of functional activation as heterogenous

populations within the body [Fogelman *et al.*, 1990; 1980; Johnson *et al.*, 1982; Sorg, 1982; Miller and Morahan, 1982]. Although the specific delivery of cytotoxic agents to nonactivated Mϕ has obvious therapeutic possibilities, delivery to functionally activated Mϕ *in vivo* may also be useful in light of findings that γ-interferon and muramyl dipeptide can act synergistically in activating the tumoricidal properties of mouse Mϕ [Saiki and Fidler,1985].

It is not clear how AcLDL-drug conjugates will function *in vivo* in the reducing the tumor mass and preventing metastasis. However, AcLDL drug conjugates have been found to be more effective in mice on some oncogenic viruses compared to, free drugs, lipophilic derivatives or liposomal preparations [Dietrich *et al.*, 1986; Ikeda *et al.*, 1985; Ishihara *et al.*, 1985; Koff *et al.*, 1985; Canonico *et al.*, 1984; Mashihi *et al.*, 1984; Kotani *et al.*, 1983].

The AcLDL conjugate is completely soluble in aqueous solutions and represents an optically clear, yellow, apoprotein-coated nanoemulsion. The small size and water solubility of the packaged lipophilic drug particle conjugates will provide distinct advantages when tested *in vivo*. Several lines of *in vitro* evidence suggest that the conjugates remain tightly associated, a criterion absolutely essential for use of the LDL-drug conjugates *in vivo*. During cytotoxic assays, the efficacy of AcLDL-drug conjugates was maintained even in the presence of a large excess of LDL. Exchange of drug from AcLDL-drug conjugates to LDL or leakage of drug from AcLDL to the medium should have lowered the cytotoxicity of the AcLDL-drug conjugates. In my studies no such depression of cytotoxic effect was observed. Moreover, I have demonstrated with LDL-drug conjugates in Chapters 2 and 3 that leakage

or exchange of drug from LDL-drug conjugates was nomimal.

I have shown that the AcLDL allows specific targeting to Mϕ. However, the AcLDL is removed from the circulation by the sinusoidal endothelial cells of the liver, spleen, bone marrow, adrenal glands and ovary. The other endothelial cells [of arteries, veins, or capillaries of the heart, testes, kidney, brain, adipose tissue, and duodenum] did not endocytose AcLDL [Soutar and Knight, 1990]. The specific uptake of AcLDL by sinusoidal endothelia could reflect the requirement for cholesterol: in the ovary and adrenal glands for hormone production, in the spleen and bone marrow for blood cell membrane, and in the liver for bile production [Soutar and Knight, 1990]. One has to assume that, *in vivo*, administration of bile acids could decrease the requirement of cholesterol and therefore the clearance of AcLDL by the liver endothelial cells. However, this limitation for targeting drugs to Mϕ can be an advantage if the drug is released from endothelial cells to the liver, spleen and bone marrow cells that contain putative target Mϕ for HIV. In addition, since it was recently shown that human liver endothelial cells are potential target cells for HIV and can play a role in the pathophysiology of AIDS [Sun *et al.*, 1992], one can propose to use AcLDL for anti-HIV drug targeting to these cells. Mϕ are described as susceptible targets, and persistent reservoirs for HIV, and key immunomodulatory elements that control the extent of AIDS [Zhang *et al.*, 1993]. On the other hand, it has been described that efficient viral replication in Mϕ depends on both cell activation and differentiation [Kodama *et al.*, 1990], and this is known to increase the number of scavenger receptors for AcLDL at the cell surface [Rohrer *et al.*, 1990, Suits *et al.*, 1989]. Consequently, the use of the AcLDL

receptor-mediated endocytosis for anti-HIV drug targeting appears attractive. Finally, If the increase of AcLDL endocytosis in some CD4 monocytes reflects functional abnormalities of Mϕ in later stages of the disease, it may be propitious to use AcLDL to carry drugs to these cells to restore or stimulate their functions [Juompan *et al.*, 1995].

AcLDL particles appear to have potential for delivery of cytotoxic agents. A distinct targeting advantage can be gained with the AcLDL receptor as it is not subject to feedback inhibition and resides on a restricted number of cell types, primarily Mϕ, monocytes, and endothelial cells. Enhanced site delivery to Mϕ would be expected to increase the efficacy of the compound in question while simultaneously lowering its toxicity or undesired side effects generated from interactions with non-Mϕ. The AcLDL particles themselves are nonimmunogenic, do not alter the physicochemical stability of the incorporated drug[s], and are capable of associating with a significant quantity of drug. The combination of specificity on the part of both the drug and its carrier provides a useful approach for the therapeutic management of disease states.

In summary, this study has demonstrated that AcLDL, exhibits suitable physical properties can be used to increase the delivery of Dox to Mϕ or macrophage tumors expressing AcLDL receptors, with cytotoxic consequences. More importantly, such increases result in enhanced therapeutic indices of the drugs compared to that achievable with the corresponding free drug. The AcLDL based formulations are expected to be more tolerable because of its similarity in physico-chemical characteristics to native LDL [Filipowska *et al.* 1992]. Thus, the combination of increased drug delivery for LDL and

increased tolerated drug doses for AcLDL based formulations should result in higher therapeutic benefits in cancer and/or AIDS treatment, respectively.

It is reasonable to suggest that significant targeting of AcLDL-Dox, by means of scavenger receptors, to Mϕ and to a lesser extent arterial endothelial cells [Voyta *et al.*, 1984; Fogelman *et al.*, 1980; Brown and Goldstein, 1979] might be anticipated. However, the desired drug efficacy associated with tumoricidal activation by AcLDL-drug conjugates should be highly confined to Mϕ cell types, as these cell types express predominantly scavenger receptors. Consequently, the combination of specificity on the part of both the drug and its carrier for a particular tissue or cell type may provide a useful general approach for drug targeting and the therapeutic management of disease states.

Table 6.1. IC_{50}[a] values for cytotoxicity in J774.A1 cells treated with different formulations

Compound	IC_{50}[b] in J774.A1 cells		
	Normal	Up-regulated	Down-regulated
Free Dox	0.04	0.036	0.044
LDL-Dox conjugates	0.008	0.009	0.005
AcLDL-Dox conjugates	0.003	0.0026	0.0032

[a] Concentration required to reduce cell survival of J774.A1 cells to 50%.

[b] 5×10^5 cells were plated in 96 wells sterile microtiter plates and after 24 hour cultures were exposed to varying concentrations of each drug for 1 hour. Plates were then rinsed with PBS and 200μL fresh growth medium was added. Cell cytotoxicity after 24 hour incubation was determined with MTT. IC_{50} values were calculated from two separate experiments, each performed in duplicate. Values are the mean of four determinations in three independent experiments with a CV less than 20%. Note that the values in normal, up- and down-regulated cells for Free Dox and AcLDL-Dox conjugates are statistically insignificant ($p>0.5$).

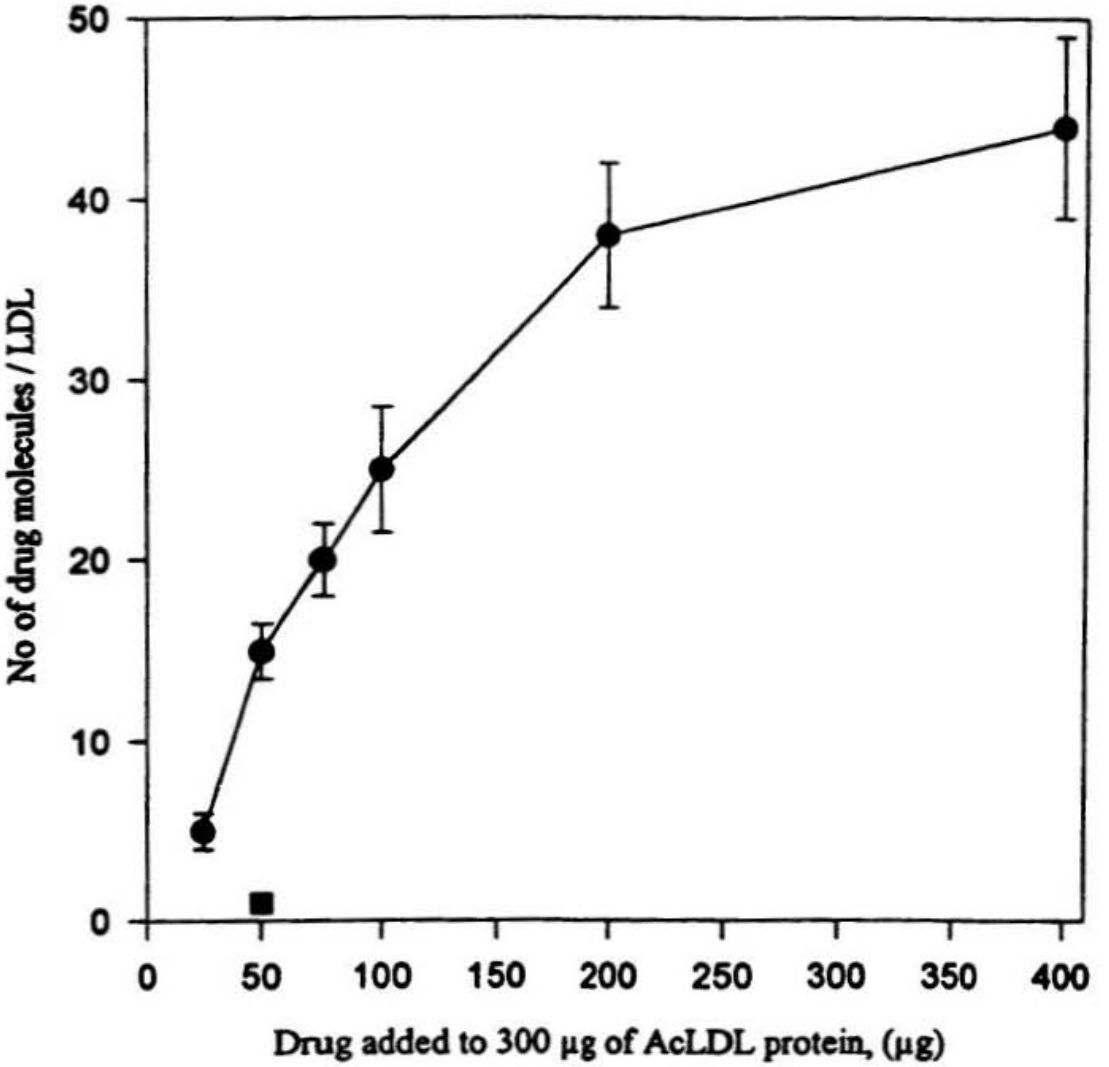


Figure 6.1. **Stoichiometry of AcLDL-Dox conjugates.** The AcLDL-Dox conjugates were prepared using the direct addition method. A constant amount of AcLDL protein (300 µg) was incubated with a variable amount of Dox as indicated. Values are expressed as the mean ±SEM (N=5).

Figure 6.2. Electrophoretic mobility of AcLDL using SDS-PAGE. Lanes 1 and 8: broad range MW markers; Lanes 2 and 3: native LDL; Lanes 4 and 5: AcLDL; and Lanes 6 and 7: AcLDL-Dox conjugates.

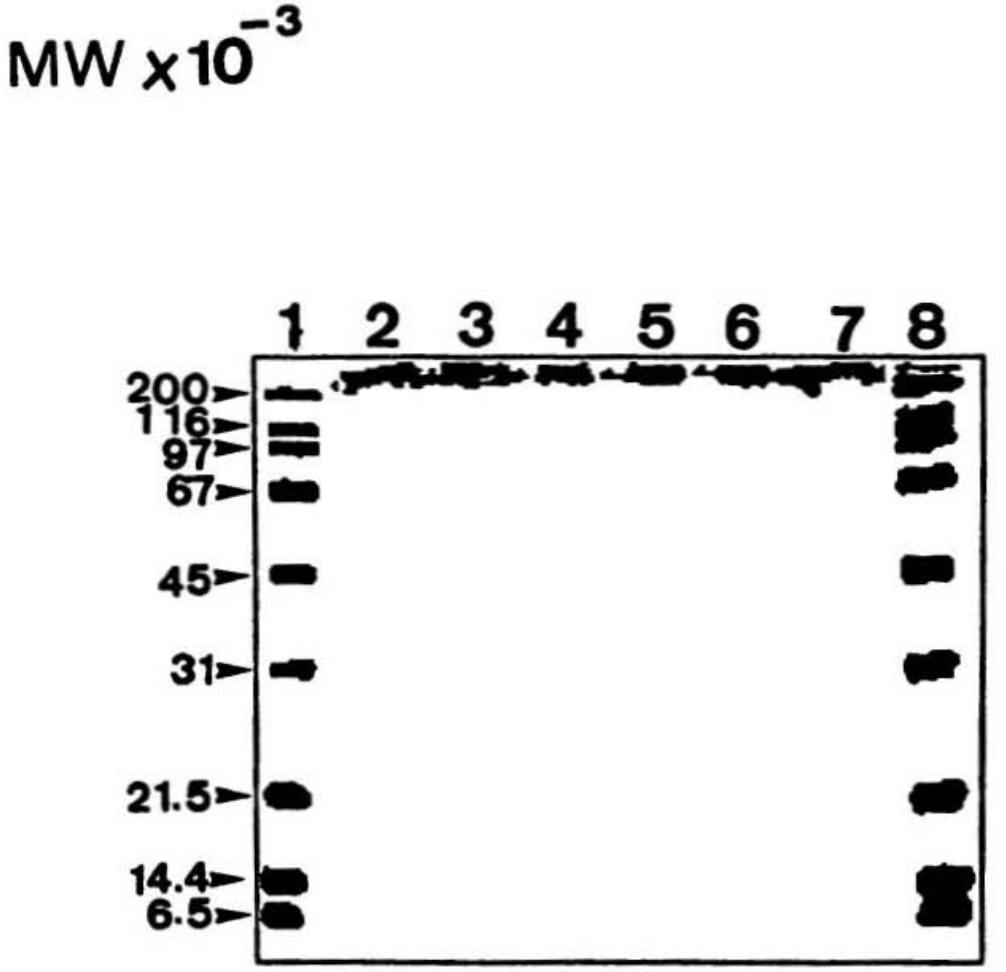
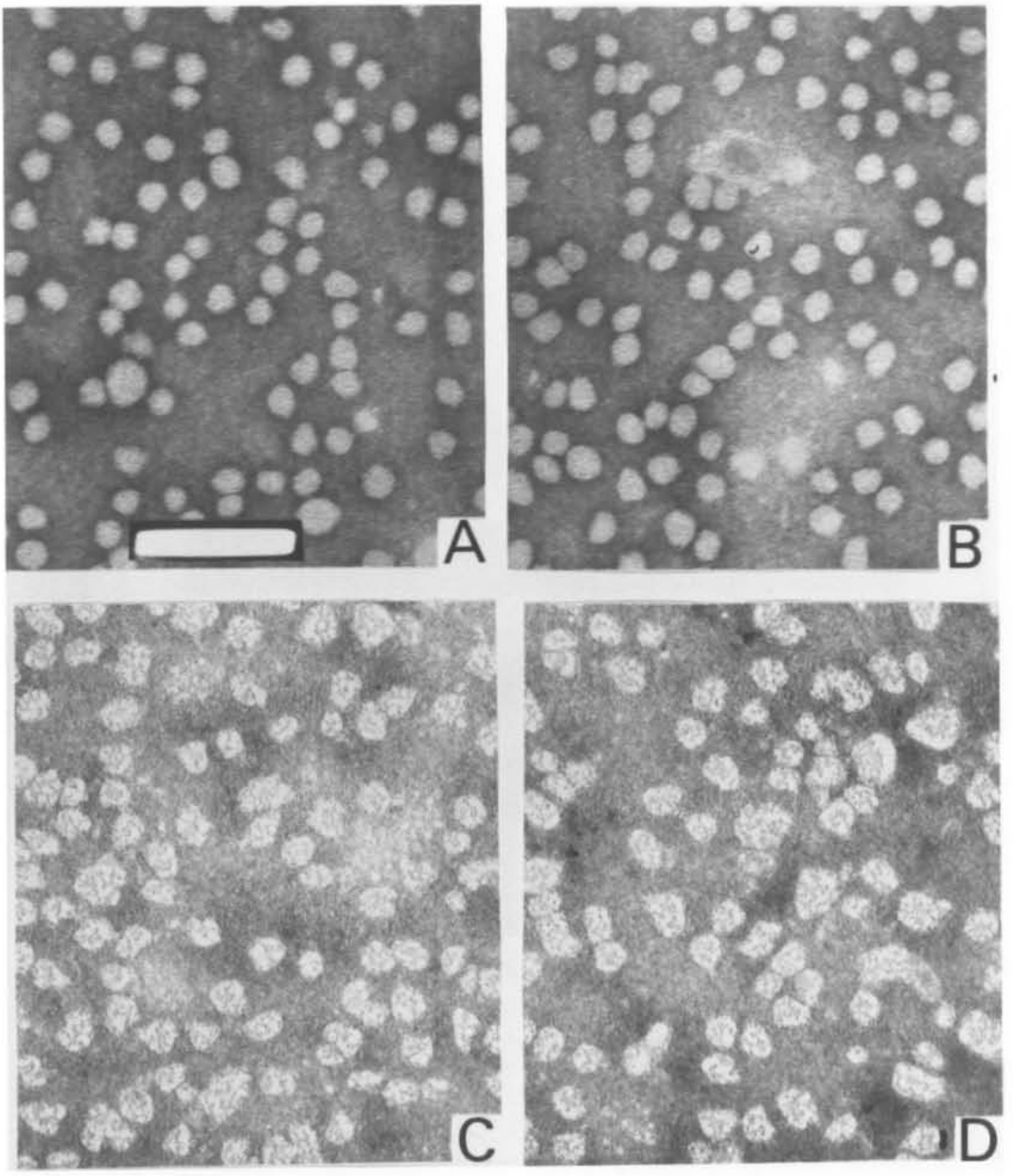

MW x10^{-3}
1 2 3 4 5 6 7 8
200
116
97
67
45
31
21.5
14.4
6.5

Figure 6.3. Electron micrograph of AcLDL preparations. The AcLDL preparations were applied to carbon formvar membranes and negatively stained with 2% phosphotungstate solution. They were examined on a Philips 300 instrument at a magnification of 75,000X. Panel A, AcLDL; panel B, AcLDL-Dox conjugates [the direct addition method]; panel C, AcLDL processed with Tween 20 (control) and panel D, AcLDL-Dox conjugates prepared by the contact method with Tween 20. The bar represents 100 nm.


A
B
C
D

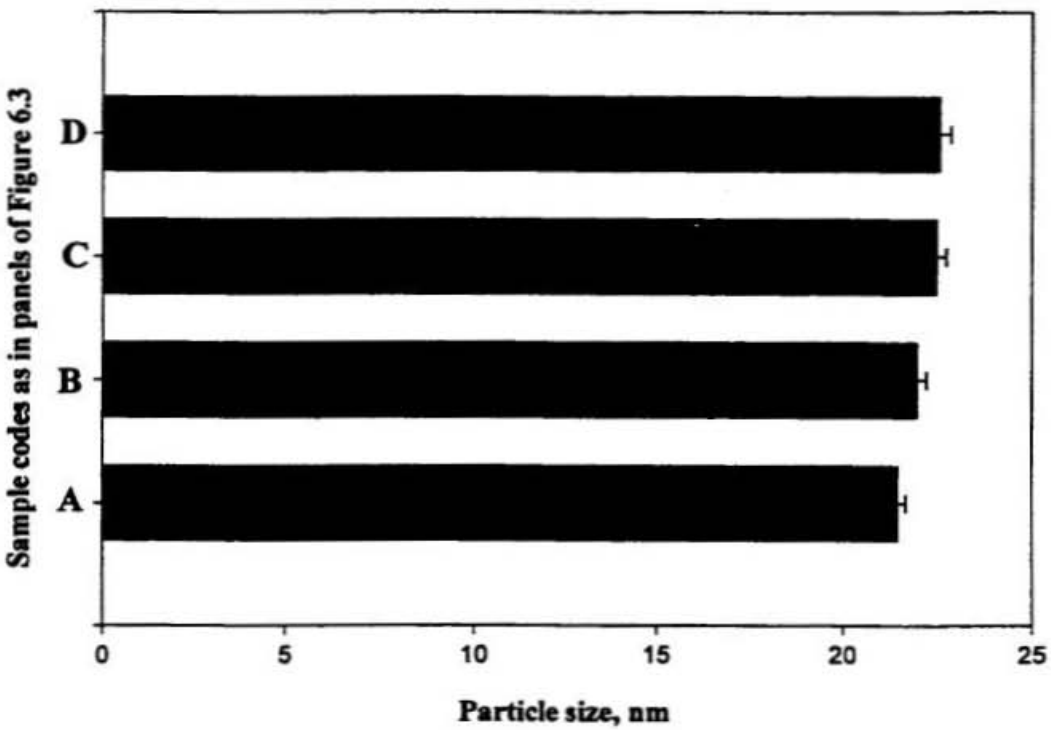
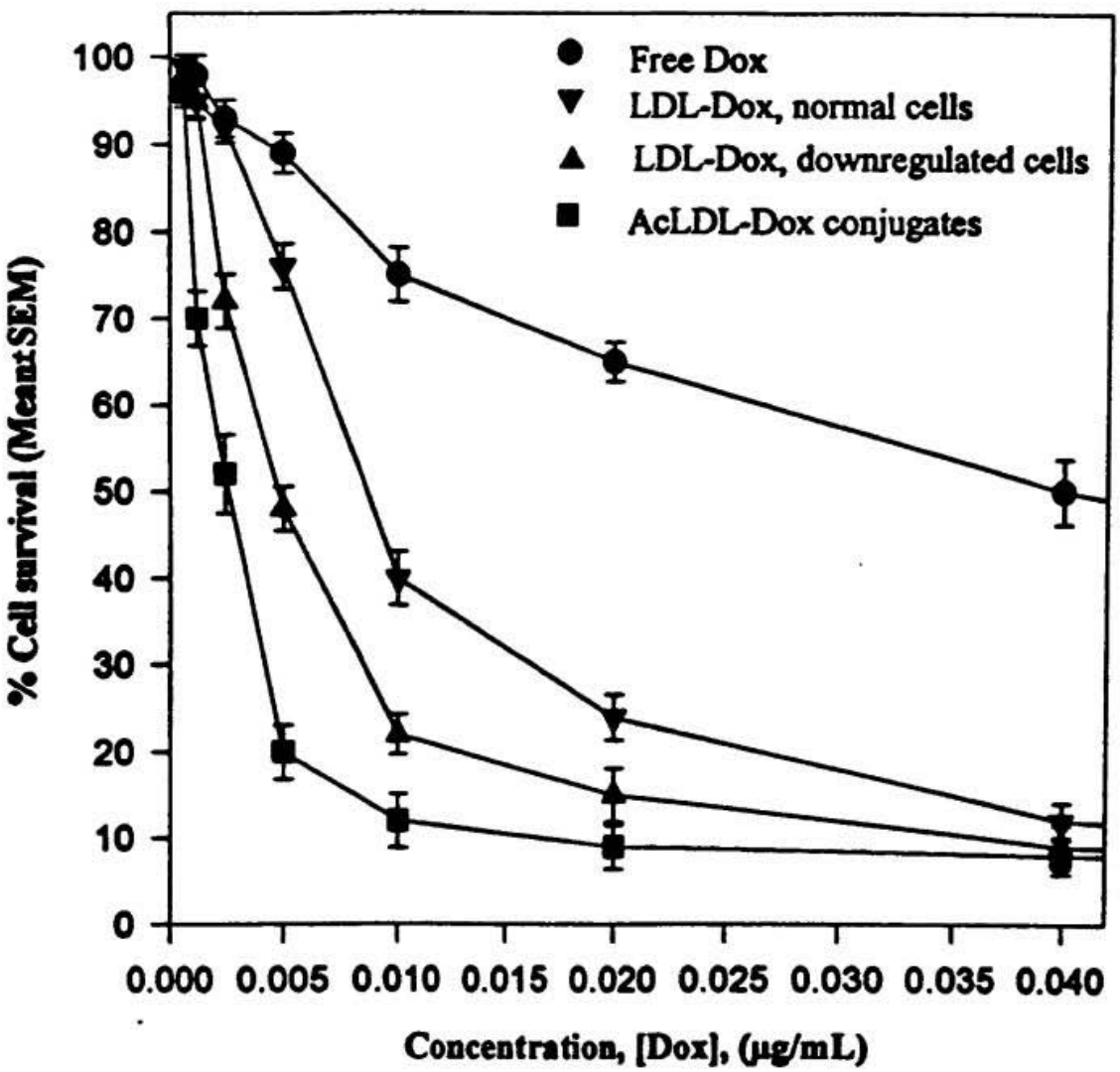


Figure 6.4. Particle size of AcLDL preparations measured by EM. Results are the mean ±SD of at least 100 particles' diameters [see Figure 6.3 for explanation].

Figure 6.5. Dose-response curves of J774.A1 Mϕ control cells after 48-hour treatment with Dox, LDL-Dox conjugates in normal and up-regulated cells. The efficacy of down-regulated cells were statistically nonsignificant compared to normal cells [$p>0.05$], AcLDL-Dox conjugates in normal cells. Dox and AcLDL-Dox had statistically nonsignificant effect on normal, up- or down-regulated cells [$p>0.05$]. % Cytotoxicity was calculated from the MTT assay, as described in section 6.2.5. The data are presented as % of corresponding control cell survival from three independent experiments [mean at each point ±SEM].


Free Dox
LDL-Dox, normal cells
LDL-Dox, downregulated cells
AcLDL-Dox conjugates
% Cell survival (Mean±SEM)
100
90
80
70
60
50
40
30
20
10
0
0.000
0.005
0.010
0.015
0.020
0.025
0.030
0.035
0.040
Concentration, [Dox], (µg/mL)

CHAPTER 7:

DRUG INTERFERENCE IN THE PROTEIN ASSAY METHOD USING BCA[1]

7.1. Introduction

This chapter describes the interference of Dox in the BCA protein assay method. During my drug incorporation studies, I demonstrated the importance of protein quantitation to determine the loading efficiency of a drug into LDL. Any nonprotein molecule, if it interferes with the protein assay, may give an inaccurate protein concentration depending on the degree of interference in the BCA method. This issue is the prime focus point of this study.

Common methods for the assay of proteins in biological fluids include the Biuret [Gornall *et al.*, 1949], Lowry [Peterson, 1979; 1977;Lowry *et al.*, 1951], Coomassie Brilliant Blue G-250 protein dye-binding assay [Bradford] [Sedmak and Grossberg, 1977; Pierce and Suelter, 1977; Bradford, 1976;], and the BCA assay [Wiechelman *et al.*, 1988; Smith *et al.*, 1985]. All of these methods have their advantages and disadvantages [Lleu and Rebel, 1991; Baker, 1991; Vico *et al.*, 1989; Brown *et al.*, 1989; Hill and Straka, 1988; Kessler and Fanestil, 1986; Kirchbaum, 1986; Compton and Jones, 1985; Lea *et al.*, 1984]. Among them, the Lowry method has been used widely for protein quantitation in biological samples. However, complications are encountered due to reagent instability and because many

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[1]A version of this chapter is in press: Abdul Kader and Hu Liu, Clinical Chemistry, (1997)

substances commonly used during protein purification interfere with the Lowry assay [Peterson, 1979]. Thus, alternative protein assays have been developed which simplify protein quantitation and eliminate many of the problems associated with the Lowry method. One alternative procedure developed by Smith *et al.*, [1985] is based on the reduction of Cu^{2+} to Cu^{+} by protein. The Cu^{+} then complexes with bicinchoninic acid [BCA] [Pierce BCA protein reagent], which absorbs light maximally at 540 - 600 nm. This assay is similar to the Lowry method in that both rely on the biuret reaction for generation of a colored complex between peptide bonds and cuprous ions when protein is placed in an alkaline environment containing Cu^{2+} [Bensadoun and Weinstein, 1976; Weichselbaum, 1946]. However, whereas the Lowry method employs Folin-Ciocalteu reagent to enhance the color response of the Biuret reaction, the BCA assay utilizes the sodium salt of BCA, a highly specific chromophore for Cu^{+}. The high specificity and stability of the bicinchoninate chromophore has permitted the development of a simplified, one step protein determination with exceptional tolerance to nonionic detergents and simple buffer salts [Kanshal and Barnes, 1986]. Nevertheless, hydrogen peroxides [Baker, 1991], sucrose and detergents [Minamide and Bamburg, 1990; Hill and Straka, 1988], biogenic amines [Tracy and Jean, 1991], choloropromazine, penicillins, vitamin C, paracetamole [Marshall and Williams, 1992; 1991], or any other compound that can reduce Cu^{2+} to Cu^{+}, will produce the characteristic purple color associated with the binding of Cu^{+} with BCA. It would not be surprising if anthracyclines, which are easily oxidized in alkaline media in the presence of metal ions, would produce the necessary reduction of Cu^{2+} for the formation of a Cu^{+}-BCA complex.

In the Bradford method, the Coomassie blue dye binds primarily to basic and aromatic amino acid residues, especially arginine, and forms a color complex. In this method, interferences may be caused by drug-protein and/or drug-dye interactions. Since the mechanism of color formation is different in the BCA and the Bradford method, both methods were considered for interference studies. The observed interferences are particularly troublesome when attempting to compare protein levels at various stages during protein quantitation in protein-drug conjugates in drug loading studies [Iwanik *et al.*, 1984; Yanovich *et al.*, 1984], distribution studies [Wasan and Morton, 1996; Cusack *et al.*, 1993], in enzyme activity measurements [Mangiapane, 1990], and in cell culture studies to quantify cell protein [Bose *et al.*, 1995; Goldschmidt and Kimelberg, 1989; Tuszynski and Murphy, 1990].

The primary objective of this work was to determine whether an anthracycline, for example, Dox, interferes with the measurement of protein concentrations using the BCA and Bradford assay. To answer this question I also examined the sensitivity and specificity of BCA for Dox.

7.2. Materials and methods

7.2.1 BCA assay

The BCA reagent [No. 23225], BSA [No. 23209] were purchased from Pierce [Rockford, IL] and used according to the instructions supplied with the reagents. Samples of Dox in 0.9 % NaCl or BSA in water were added in a volume of 20 µL to a microtiter

plate. BCA reagent [200 μL] was then added. Color was then allowed to develop for either 2 hours at RT, 30 min at 37°C , or 15 min at 60°C. The microtiter plate was then read in an ELISA plate reader [Model EL 310, Biotek, Vermont, USA].

7.2.2. Bradford method

Protein determinations were performed as described by Bradford [1976], using the dye reagent purchased from Bio-Rad [Ontario, Canada] and BSA standards. Standard procedure for microtiter plates was followed as outlined in the Bio-Rad Protein Assay catalogue.

7.2.3. Statistical analysis

Statistical analysis was performed using least squares linear regression, the student's t test, and ANOVA. Comparison among groups was done by one way ANOVA and the post test Bonferroni method. The Bonferroni p value was used to make all multiple paired comparisons. Differences were considered significant if p was<0.05. All data are expressed as mean ± SE [Standard Error].

7.3. Results

Dox showed a significant interference in the BCA assay, whether assayed in the presence or absence of BSA protein standard [Figure 7.1]. The interference was found to be linear with an immediate appearance of typical assay color without turbidity [even after

further dilutions]. The interference was linear over the standard protein range. The reaction of Dox with the BCA reagent at RT for 2 hours [Figure 7.2] suggests that a wide range of anthracyclines will react with the BCA reagent to produce the characteristic purple color [Table 7.1]. This is based on the assumption that the structure of the different anthracyclines are similar and, as such, they should react with BCA reagent in a similar fashion [Table 8.1]. When compared with BSA protein, the sensitivity of the method towards Dox was found to be higher [33 fold] than for the standard BSA protein. Sensitivity of Dox was found to be 0.016 nmole/mL or 180 ng/mL [n=4, A_{570}=0.009, $p<0.05$ compared to blank], compared to BSA sensitivity [6 μg/mL]. The sensitivity was further confirmed at picomoles/mL range by plotting a calibration curve between 16.53 picomoles/mL and 1.653 nanomoles/mL Dox. The calibration curve [$y = -2.0\ e^{-3} + 2.0577\ e^{-4}\ x$, $R^2 = 0.994$] was found to be linear [n = 4, two-tailed t statistic, $p<0.01$, indicating that the slope was very significantly different than zero] [curve not shown]. There appeared to be a linear increase in absorbance between 0.017 and 1.700 nmoles/mL Dox suggesting that the BCA method could be used to quantitate Dox concentrations that are in the 16nM to 1.600 μM range [Figure 8.3]. However, Dox did not interfere with the Bradford method to quantitate protein [Figure 8.2].

The development of color associated with the reaction of protein with the BCA reagent is slow at RT and enhanced by increasing the temperature and incubation time [Gornall *et al.*, 1949]. Color development with different concentrations of protein for 2 hours at RT [21°C] is equivalent to that developed over 30 min at 37°C and less than that produced after 15 min at 60°C [Smith *et al.*, 1985]. Color development with Dox under

different incubation conditions has been found comparable [$p>0.05$, in all cases when comparison was made by a Bonferroni test among different groups [Figure 8.3]. Color changes produced by the reaction of protein with the BCA reagents continues to develop, at a slow rate, over the next 20 hours when left at RT [Figure 8.4]. I therefore wanted to know whether or not there is any variation in color formation with Dox in the assay protocol under different conditions. At RT, the color development produced by Dox changes rapidly over the first 1.5 hours [Figure 8.4]. There is only a 10-12 % further enhancement of color over the next 20 hours. The color development after 2 hours at RT is similar [$p> 0.05$] to that developed over 30 min at 37°C] [Figure 8.3]. BSA, at a concentration of 0.01 mg/mL did not interfere with the color developed by Dox [Bonferroni $p> 0.05$] compared to the color formation of Dox with the BCA reagent [Figure 8.5]. However, at 0.10 mg/mL and higher, BSA concentrations significantly [Bonferroni $p<0.05$] interfere with the color developed by Dox [Figure 8.5]. Also, Dox concentrations of 1.672 to 33.430 nmoles/mL with different protein concentrations have produced color intensities that are significantly [Bonferroni $p<0.01$ with 1.672 nmoles/mL and $p<0.001$ with the other Dox concentrations when compared to BSA] higher than those due to the protein alone [Figure 8.6]. These results indicate that anthracyclines will significantly alter color development produced by proteins at anthracycline concentrations that exceed 1.6 nmol/mg protein. These studies also demonstrate the feasibility of using the BCA reagent to quantitate Dox at concentrations that are in the 1-1000 nM range.

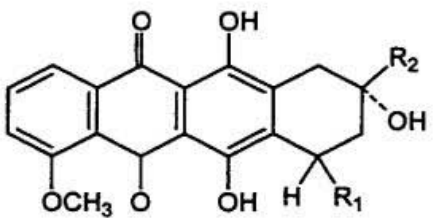
7.4. Discussion

Clinically, it is essential to monitor the nutritional status of a patient under chemotherapy by measuring the total protein concentration of the plasma [Mayhew and Thorn, 1995]. Assessment of chemotherapy-associated nephrotoxicity in patients with cancer can be made by measuring blood and urine protein [Skinner *et al.*, 1991]. The most commonly used dosage schedule for Dox is 6.0 to 7.5×10^{-2} g/m^2 as a single IV injection administered at 21-day intervals. When calculated for an healthy individual, the plasma concentration of Dox or its metabolites is expected to be more than 0.02 g/L [Lentner, 1984]. Therefore, plasma and urinary anthracycline concentrations of anthracyclines including both the parent and the metabolites are expected to be in a range where Dox would affect protein assay by the BCA method. If the BCA method is used in determination of the protein of these biological samples, an increase in protein concentration would be observed. This results may have significant impact on patient's therapy and prognosis. Anthracyclines [Dox, daunomycin, and annamycin and their analogs] have been used in drug loading into lipoproteins, in particular, LDL and HDL [Shaw *et al.*, 1987; Iwanik *et al*, 1984; Yanovich *et al.*, 1984], and their distribution in plasma proteins has been studied [Wasan and Morton, 1996]. To quantitate the drug associated with lipoprotein, it is essential to determine the protein concentrations of the lipoprotein[s] as well. In all cases, the anthracycline concentration has been found higher than 0.22 nmoles and as high as 50 nmoles/mL. Therefore, the concentration of Dox and the ratio of Dox to protein is high enough [for example, 0.24-1.6 μg of drug/mg of protein, 26] to interfere in the color development of

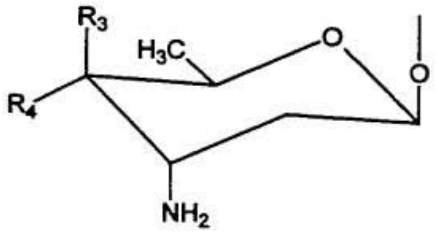
protein in the BCA method. Also, protein concentration is being used as a bench mark to quantitate cells in cell culture studies [Mangiapane, 1990; Goldschmidt and Kimelberg, 1989; Yanovich *et al.*, 1984]. The Dox concentration in cell culture studies was found to be higher [7 to 133.9 nmoles of Dox/mg of cell protein, [Tuszynski and Murphy, 1990] which will interfere with the BCA protein analysis. Anthracyclines have potential adverse reactions and in recent years a number of enzyme activity studies have been carried out to discern the mechanism of various adverse effects [for example, the role of an enzyme, glutathione peroxidase, in oxidation of cardiac muscle and in daunorubicin cardiotoxicity] [Cusack *et al.* 1993, Mangiapane, 1990]. In these studies, protein determinations have to be done to express enzyme activity, for example, glutathione peroxidase activity expressed as nmol NADPH oxidized/min/mg protein, [Cusack *et al.* 1993]. Recently, in a daunorubicin-induced apoptosis study, cells were treated with 1μM daunorubicin to observe morphological alteration of chromatin during apoptosis [Bose *et al.* 1995]. In all of the above situations, anthracyclines have the potential to interfere with the BCA protein assay resulting in inaccurate protein determination.

This inaccurate protein determination may significantly influence the results obtained from different studies related to biochemical pharmacology of anthracyclines. The results from my studies indicate a significant increase in the color development of the BCA reagent with Dox in the BCA method. However, Dox did not interfere with the Bradford assay. These results also suggest that different anthracyclines or their metabolites because of their similar structural moieties [Table 8.1] may interfere in a similar fashion in the BCA and not

in the Bradford method. Anthracyclines, which are widely used in antineoplastic therapy [Arcamone, 1981], would interfere with the BCA protein assay resulting in inaccurate or high protein concentrations. To overcome these interferences with the BCA protein assay method, the Bradford method would be an acceptable alternative.



Anthracyclinone aglycone



Daunosamine sugar (D)

Table 8.1. Dox, its principal metabolites, and daunorubicin

Compound	R_1	R_2	R_3	R_4
Dox	D	$COCH_2OH$	H	OH
Doxol	D	$COCHOHCH_2OH$	H	OH
Doxol aglycon	OH	$CHOHCH_2OH$	-	-
7-OH Dox aglycon	OH	$COCH_2OH$	-	-
7-DeoxyDox aglycon	H	$COCH_2OH$	-	-
Daunorubicin	D	$COCH_3$	H	OH

Figure 7.1. **Dox interference in the BCA protein assay.** Aliquots of 20 μL of sample containing 1.67, 5.02, 8.36, 10.04, 13.38, and 16.72 nmoles/mL of each of the following were assayed: Dox [closed diamond], BSA protein standard [open square], and BSA standard containing Dox [BSA: Dox = 1 : 1] [open diamond]. The data are expressed as the mean ±SEM of quadruplicate determinations.

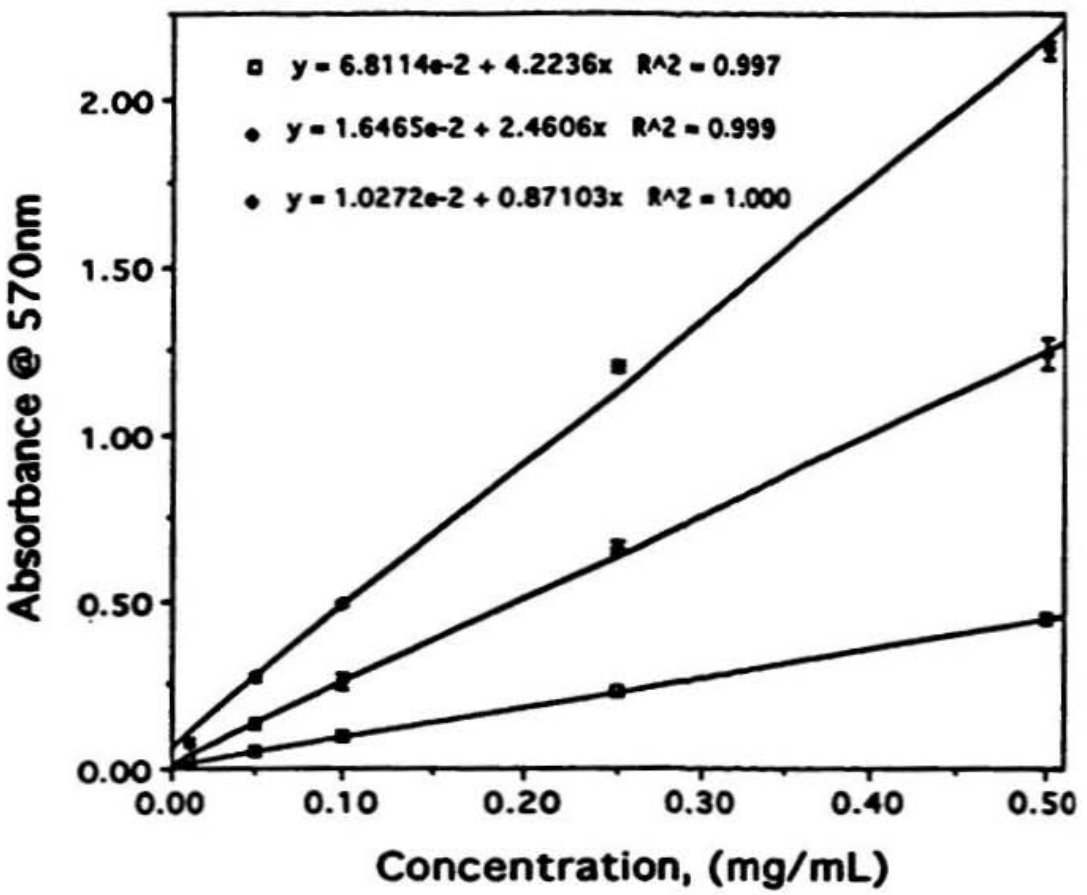

y = 6.8114e-2 + 4.2236x R^2 = 0.997
y = 1.6465e-2 + 2.4606x R^2 = 0.999
y = 1.0272e-2 + 0.87103x R^2 = 1.000
Absorbance @ 570nm
2.00
1.50
1.00
0.50
0.00
0.00
0.10
0.20
0.30
0.40
0.50
Concentration, (mg/mL)

Figure 7.2. Standard calibration curve of Dox with BCA reagent [open diamond] and Coomassie blue [closed diamond]. Concentrations range between 16.725 nM [picomoles/mL] to 1.67256 μM [nmoles/mL]. The data points are the mean ± SE of quadruplicate determinations.

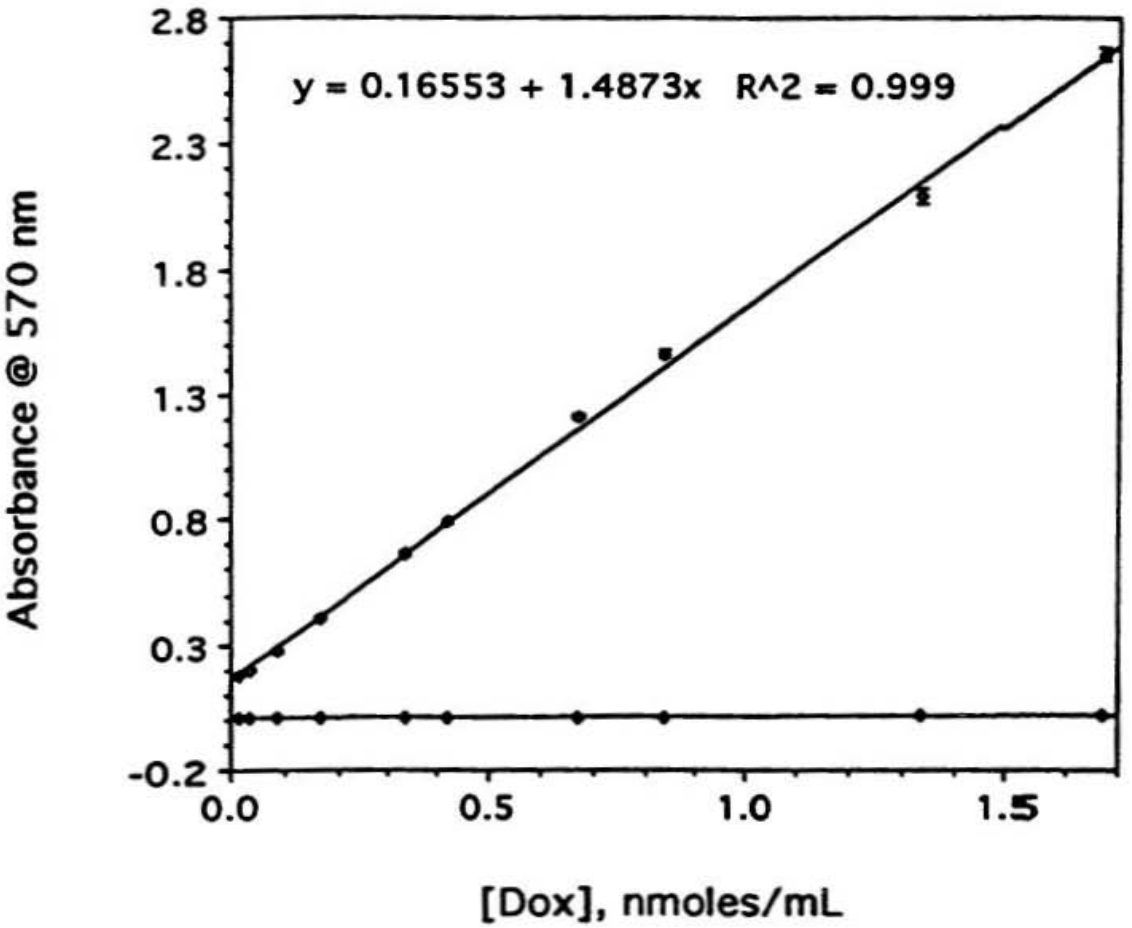

y = 0.16553 + 1.4873x R^2 = 0.999
Absorbance @ 570 nm
2.8
2.3
1.8
1.3
0.8
0.3
-0.2
0.0
0.5
1.0
1.5
[Dox], nmoles/mL

Figure 7.3. Sensitivity of Dox to color development with BCA reagent. Dox was reacted with 200 μL of the BCA reagent at concentrations between 1.67 and 16.72 nmol/mL for either 2 hour at RT [closed square], 30 min at 37°C [closed diamond] or 60°C for 15 min [open square]. Data points represent the mean of quadriplicate determinations ± SE.

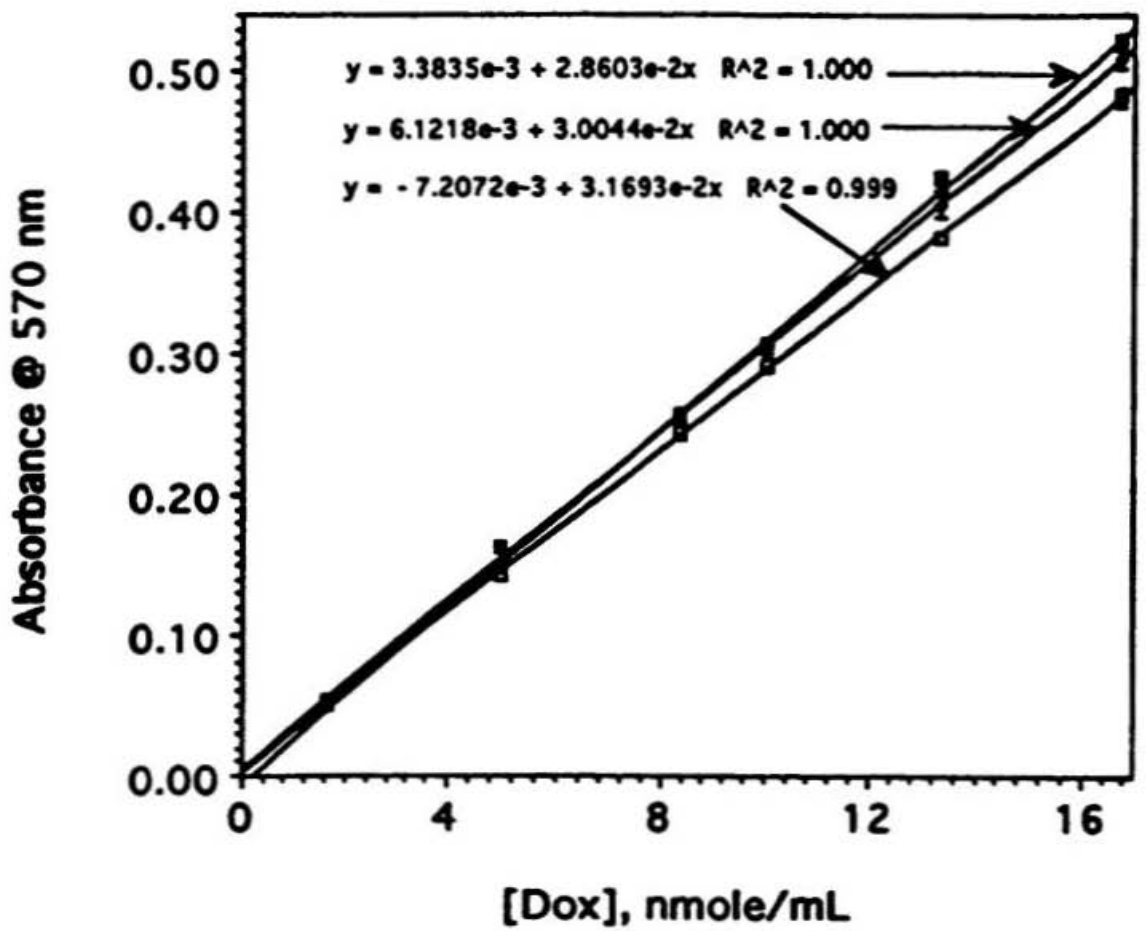

y = 3.3835e-3 + 2.8603e-2x R^2 = 1.000
y = 6.1218e-3 + 3.0044e-2x R^2 = 1.000
y = - 7.2072e-3 + 3.1693e-2x R^2 = 0.999
Absorbance @ 570 nm
0.00
0.10
0.20
0.30
0.40
0.50
0
4
8
12
16
[Dox], nmole/mL

Figure 7.4. Rate of increase in color absorbance produced by Dox. The A_{570} produced by incubation of 200 μL BCA reagent at RT and concentrations from 1.672 [open square], 8.363 [closed square], 16.726 [open circle], 41.814 [open diamond] to 83.63 [closed diamond] nmol/mL was measured after 15, 30, 50, 70, 90, 110, 133, 149, 184, 360, 1320 min. All data points represent the mean of triplicate determinations ± SE.

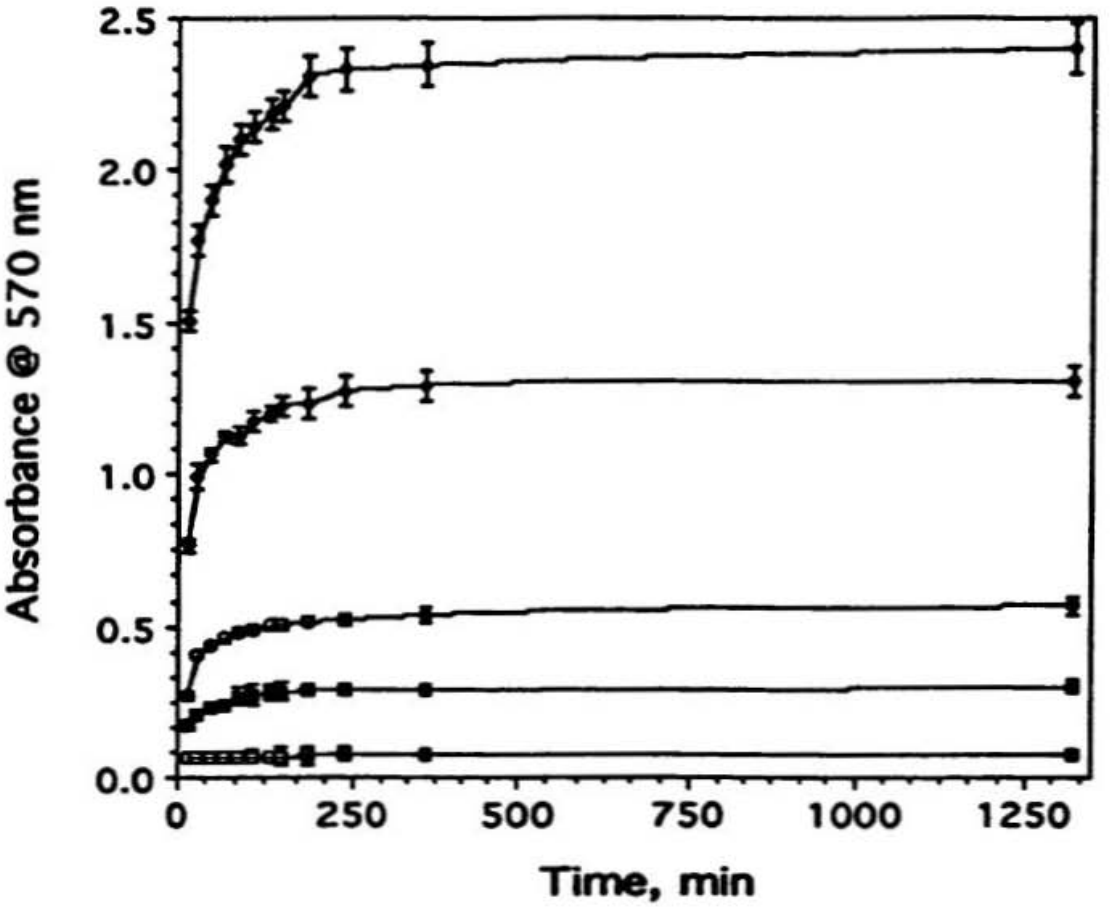

Absorbance @ 570 nm
2.5
2.0
1.5
1.0
0.5
0.0
0
250
500
750
1000
1250
Time, min

Figure 7.5. Color development produced by Dox in the presence of BSA. The color produced by 0 [open square], 0.01 [closed diamond] and 0.10 [closed square] mg/mL BSA protein reacting with 200μL BCA reagent in the presence of 1.672 to 16.72 nmole/0.1 mL Dox after 2 hour incubation at RT. The data are the mean ± SE of quadruplicate determinations.

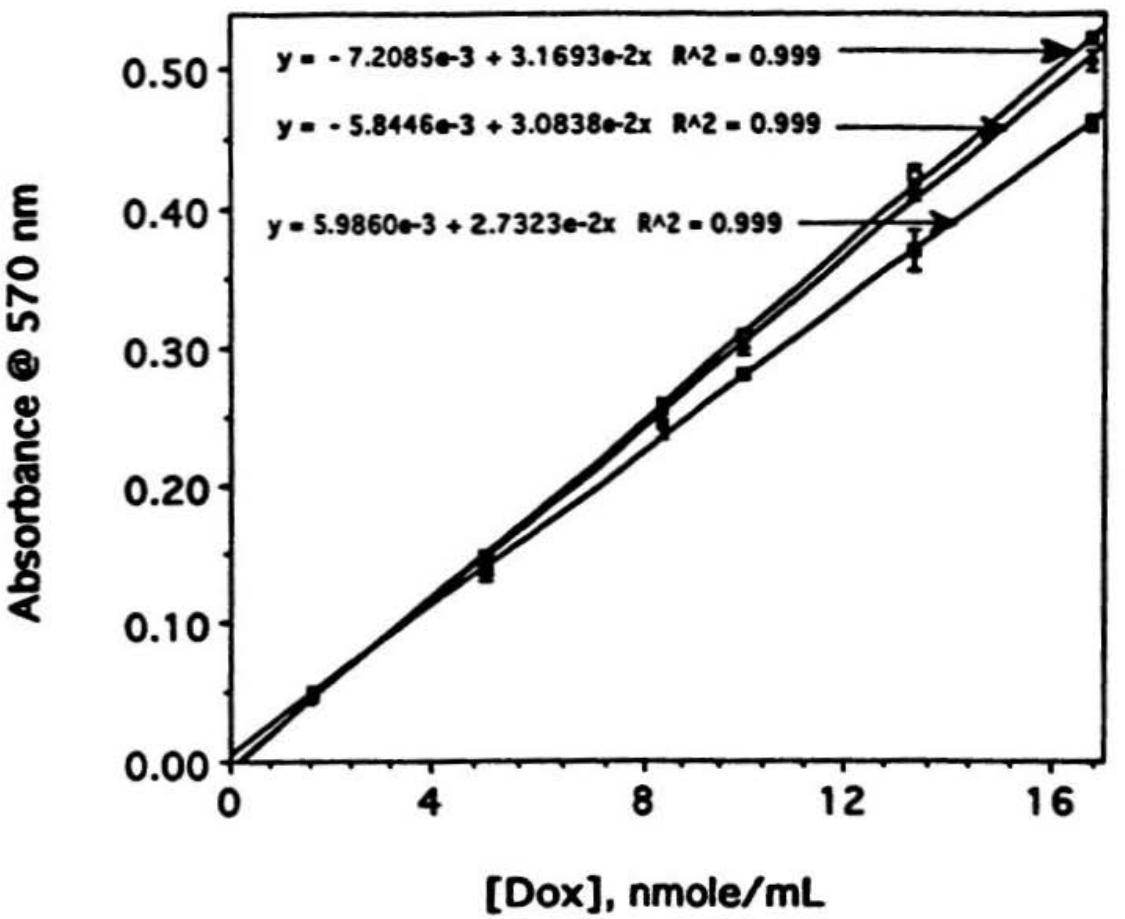
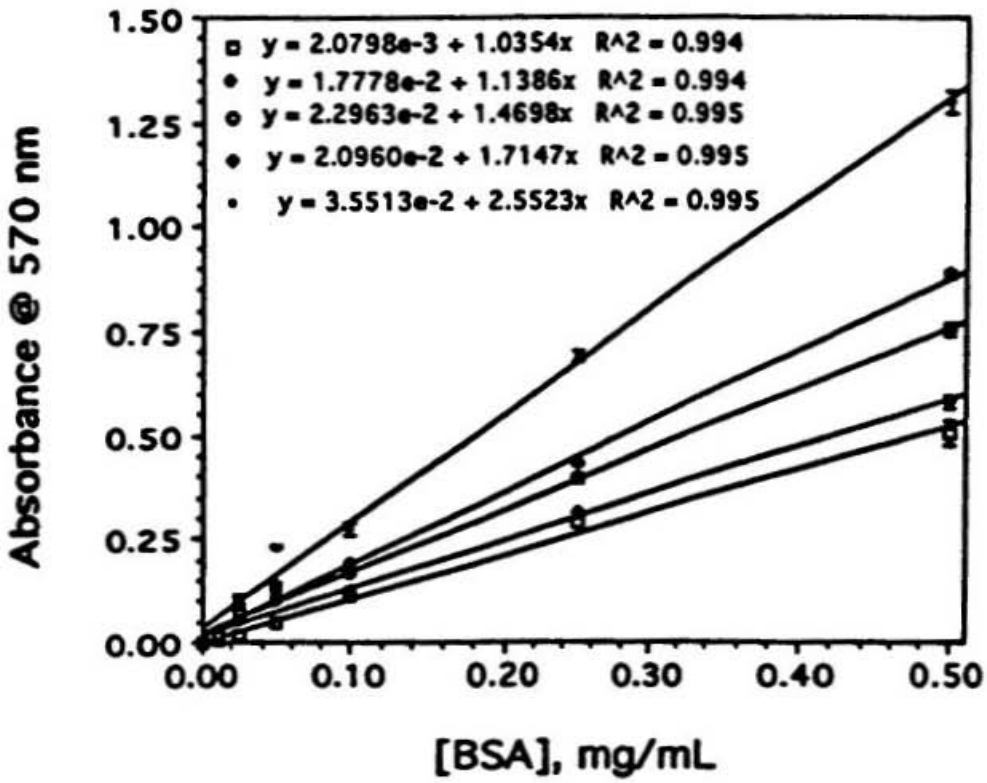

y = - 7.2085e-3 + 3.1693e-2x R^2 = 0.999
y = - 5.8446e-3 + 3.0838e-2x R^2 = 0.999
y = 5.9860e-3 + 2.7323e-2x R^2 = 0.999
Absorbance @ 570 nm
0.00
0.10
0.20
0.30
0.40
0.50
0
4
8
12
16
[Dox], nmole/mL

Figure 7.6. Color development produced by BSA in the presence and the absence of Dox. The BCA reagent was added to various concentrations of BSA protein [0.01 to 0.10 mg/mL] and the color was developed over a 2 hour period at RT. The data points represent the mean ±SE of quadruplicate determinations. Color development was with various concentrations of BSA in the presence of 0 [open square], 1.672 [closed diamond], 8.363 [open circle], 16.73 [open diamond], and 33.43 [closed circle] pmoles/mL of Dox.


y = 2.0798e-3 + 1.0354x R^2 = 0.994
y = 1.7778e-2 + 1.1386x R^2 = 0.994
y = 2.2963e-2 + 1.4698x R^2 = 0.995
y = 2.0960e-2 + 1.7147x R^2 = 0.995
y = 3.5513e-2 + 2.5523x R^2 = 0.995
Absorbance @ 570 nm
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0.00
0.10
0.20
0.30
0.40
0.50
[BSA], mg/mL

CHAPTER 8:

GENERAL DISCUSSION AND FUTURE DIRECTIONS

The study of this thesis has produced some new perspectives on drug targeting using the endogenous LDL. A substantial amount of drug has been incorporated into the native or the modified LDL (AcLDL) and *in vitro* drug targeting to both the tumor cells and the Mϕ has been achieved. The following is a summary of some key points obtained over the course of the study and recommendations for future experiments.

8.1. Physico-chemical principles affecting drug loading into LDL

Physical factors such as incubation time, temperature, stoichiometry of LDL-drug conjugates, and a variety of formulation additives, such as solvents, surface increasing agent, and wetting agents, were studied (Chapter 2). Additionally, different formulations of the drug, such as, solution, suspension, or liposomal preparation were studied and found to have significant effects in drug loading. All these factors have been found responsible for drug loading into LDL particles. I conclude that optimization of the process parameters is imperative to maximize drug loading into LDL particles.

8.2. Physico-chemical characterization of LDL-drug conjugates

The use of LDL as a drug targeting vector requires a highly efficacious drug, able to incorporate into the particle maximally without modifying its structural integrity or receptor

binding properties. The use of biochemical and biophysical techniques provides valuable information regarding the physicochemical characteristics of LDL-drug conjugates. I have incorporated substantial amounts of cytotoxic compounds into LDL or AcLDL particles and showed that the use of SDS-PAGE, EM, and DSC to characterize LDL-drug conjugates provides useful information [Chapters 2,3, and 6]. Additionally, particle size measurement could be done using the quasielastic [Vitols *et al.*, 1990], laser scattering technique [Samadi-baboli *et al.*, 1990], photon correlation spectroscopy [McNeil-Watson and Parker, 1991], or synchrotron radiation solution X-ray scattering [Westesen, 1995]. The site of the incorporated drugs in LDL was examined by UV-visible spectroscopy [Chapter 4]. Additionally, the incorporation sites can be examined by NMR spectroscopy [Westesen *et al.*, 1995], or fluorescence quenching experiments [Sautereau *et al.*, 1995]. The use of CD spectroscopy [Provencher and Glockner, 1981] and/or ^{1}H NMR spectroscpoy [Ala-Korpela *et al.*, 1995] can be applied to examine the apo B integrity after drug loading and may be useful to further examine the conformation of the protein. Especially important, are fluorescence quenching experiments in which a fluorescent drug is used to identify the location of incorporated drug molecules in LDL, because, if the drug is primarily located in the core, it will not exchange or leak from the LDL *in vivo*.

8.3. LTP as an enhancing agent to incorporate drugs into LDL particles

In my studies, LTP was found to be effective in enhancing drug loading from 2- to 5-fold [Chapter 4]. This increase in drug loading is significant as the native integrity of the

LDL particle remained unchanged. However, more studies are needed to exploit better drug candidates for LDL or to design better drug donors such as microemulsion or liposomes for LTP.

8.4. Multiple drug resistance [MDR] studies

MDR can be overcome by delivering drug using LDL. Shaw *et al.*, (1987) showed that LDL-drug conjugates suppressed P-gp activity related to drug resistance. However, no *in vivo* studies have been performed. Recently, Metherall *et al.*, (1996) showed that MDR activity is required for the esterification of LDL-derived cholesterol. I speculate that LDL-drug conjugates will deliver substantial amount of cholesterol to MDR cells and will suppress the activity of P-gp. In a preliminary study, I have seen that LDL preparations have the potential to overcome P-gp mediated drug resistance in a CEM VLB-1000 MDR human leukemic cells (Bradley *et al.*, 1989; Kader and Liu, unpublished data). If this can be shown *in vivo*, it would be an efficient method to overcome MDR in cancer chemotherapy.

8.5. Modification of LDL to target specific cell types

LDL can be modified to selectively target specific type of cells. For instance, I have shown in Chapter 6 that acetylation of LDL lead to deliver cytotoxic drugs primarily to Mϕ. Recently, LDL was modified by lactosylation and the modified LDL was rapidly and specifically internalized by the galactose-specific receptors on Kupffer cells [Bijsterbosch and Van Berkel, 1990; Bijsterbosch *et al.*, 1989]. Later, lactosylated LDL has been proposed

as a potential carrier for the targeted delivery of drugs to Kupffer cells [Bijsterbosch and Van Berkel, 1990; Bijsterbosch *et al.*, 1989]. These modified particles themselves are nonimmunogenic, do not alter the partitioned drug's bioactivity, and are capable of associating significant quantitities of compounds. I speculate that, in the future, LDL can be suitably modified to deliver therapeutic agents specifically to certain cell types depending on the type of modification of the LDL.

8.6. *In vivo* studies

The ultimate goal of the LDL-mediated drug targeting is to elicit a selective *in vivo* effect. I have demonstrated that the LDL-drug conjugates did not show any significant changes in *in vitro* characterization compared to native LDL. However, it should be tested *in vivo* whether the LDL-drug conjugate has maintained its recognition properties. Important parameters for *in vivo* validation of the LDL-drug conjugate are plasma half-life and tissue distribution studies, which can be performed in rats or mice, are therefore (Versluis *et al.*, 1996; De Smidt and Van Berkel, 1990). An additional method for determining if the LDL-drug conjugate is internalized via the LDL-receptor pathway *in vivo* can be provided by selective upregulation of rat liver parenchymal LDL receptors [Harkes *et al.*, 1983]. Pretreatment of rats with high doses of ethinylestradiol establishes a 17-fold upregulation of parenchymal LDL receptors. Compared with control rats, the liver uptake of the reconstituted LDL in the ethinylestradiol-treated rats should be greatly increased if the particle is internalized by the LDL-receptor pathway.

8.7. Human trials

Further experiments are required *in vivo*, probably in nude mice with human tumor xenografts, to determine the affinity of LDL-drug complexes for tumor and normal tissues, to examine the comparative pharmacokinetics of the LDL-drug conjugates relative to free drug and to explore the possibility of a differential therapeutic effect. A small scale clinical trial of LDL-vincristine conjugates in humans has been reported [Filipowska, 1992]. However, thus far, there have been no reports of pharmacokinetic data in humans. Such studies are critical, since a prerequisite for successful drug targeting is that the LDL-drug conjugate is recognized as self; otherwise, the LDL-drug conjugate would be cleared rapidly from the blood stream.

8.8. Distribution studies

Compared to albumin and α_1 acid glycoprotein, lipoproteins have received less attention as potential carrier proteins of drugs in plasma. However, basic drugs such as quinidine and tetracycline [Piafsky, 1980], polyaromatic hydrocarbons such as 2,3,7,8-tetrachlorobenzo-p-dioxin and benzo[a]pyrene [Shu and Bymun, 1983], and probucol [Urien *et al.*, 1984] have been reported to bind to lipoproteins. In addition, the lipoproteins are best known as the carriers of CE [Brown and Goldstein, 1986]. For Dox, I speculate that high binding of the drug with albumin may be the mechanism for its fatal cardiotoxicity. *In vitro*, a redistribution to lipoproteins was achieved using different concentrations of the oleic acid [Chapter 2, section 3.9]. I speculate that a similar redistribution of Dox from plasma

albumin to lipoproteins, if it occurs *in vivo,* may help to decrease or eliminate fatal cardiotoxicity.

REFERENCES

Adams, D.O., and Hamilton, T.A., (1984) The cell biology of macrophage activation. Ann Rev. Immunol. 2: 283-318.

Addleman, W. (1972) Cancer, cholesterol and cholesterylamine. N. Engl. J. Med. 287: 1047-1049.

Ala-Korpela, M., Oja, J., Lounila, J., Jokisaari, J., Savolainen, M.J., and Kesaniemi, Y.A. (1995) Structural changes of lipoproteins by ^{1}H NMR. Chemical Physics Letters. 242: 95-100.

Alberts, A.W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Albers-Schonberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J., and Springer, J. (1980) Mevinolin: a highly potent competitive inhibitor of hydrozymethylglutaryl-coenzyme A reductase and a cholesterol lowering agent. Proc Natl Acad Sci U.S.A.; 77: 3957-3961.

Allen, T.M., Brandeis, E., Hansen, C.B., Kao, G.Y., and Zalipsky, S. (1995) A new startegy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells. Biochim. Biophys Acta 1237: 99-108.

Allen, T.M., Hansen, C.B., Lopes-de-menezes, D.E. (1995a) Pharmacokinetics of long circulating liposomes. Adv. Drug Delivery Rev. 16: 267-284.

Allen, T.M. (1994) Long circulating (sterically stabilized) liposomes for targeted drug delivery. Trends Pharmacol. Sci. 15: 215-220.

Alley, M.C., Scudiero, D.A., Monks, A., Hursey, M.L., Czerwinski, M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Showmaker, R.H., and Boyd, M.R. (1988) Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res. 48: 589-592.

Allison, B.A., Pritchard, P.H., and Levy, J.G. (1994) Evidence of low density lipoprotein receptor-mediated uptake of benzoporphyrin derivative. Cancer Res. 69: 833-839.

Ando, S., Ryan, R.O., and Yakoyama, S. (1990) Lipid transfer catalyzed by insect hemolymph lipid transfer particle between human plasma low density lipoprotein and a triolein/phospholipid microemulsion. Biochim. Biophys. Acta 962: 143-148.

Arcamone, F., Franceschini, G., and Penco, S. (1974) Process for the preparation of adriamycin and adriamycinone and adriamycin derivatives. US Patent 3,803, 124, April 9, 1974.

Arcamone, F., Penco, S., Vigevani, A., Radaelli, S., Franchi, G., Di Marco, A., Casazza, A.M., Dasdia, T., Formelli, F., Necco, A., and Soranzo, C. (1975) Synthesis and antitumor properties of new glycosides of daunomycinone and adriamycinone. J. Med. Chem. 18: 703-

Atkinson, D., and Small, D.M. (1986) Recombinant lipoproteins: implications for structure and assembly of native lipoproteins. Ann. Rev. Biophys. Chem. 15: 403-456.

Bagshawe, K.D., Springer, C.J., and Searle, F. (1988) A cytotoxic agent can be generated at cancer sites. Br. J. Cancer 58: 700-103.

Bakan, D.A., Longino, M.A., Weichert, J.P., and Counsell, R.E. (1996) Physicochemical characterization of a synthetic lipid emulsion for hepatocyte-selective delivery of lipophilic compounds: application to polyiodinated triglycerides as contrast agents for computed tomography. J. Pharm. Sci. 85: 908-914.

Baker, W.L. (1992) Potential interference of hydrogen peroxide in the 2,2'-bicinchoninic acid protein assay. Anal. Biochem. 192: 212-214.

Bakker, W.I.A. (1995) Delivery of antimicrobials to infected tissue macrophages. Adv. Drug delivery Rev. 17: 5-20.

Bangham, A.D., Standish, M.M., and Watkins, J.C. (1965) Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. 13: 238-252.

Bases, R.E., and Krakoff, I.H. (1965) Studies of serum cholesterol levels in leukemia. J. Reticuloendothelial. Soc. 2: 8-14.

Basu, M.K. (1994) Liposomes in drug targeting. Biotech. Genetic Rev. 12: 383-408.

Basu, S., Mukhopadhyay, B., Basu, S.K., and Mukhopadhyay, A. (1994) Enhanced intracellular delivery of doxorubicin by scavenger receptor-mediated endocytosis for preferential killing of histocytic lymphoma cells in culture. FEBS Letters 342:249-254.

Basu, S.K., Goldstein, J.L., Anderson, R.G.W., and Brown, M.S. (1976) Degradation of

cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. Cell Biology 73: 3178-3182.

Beijnen, J.H., Van der Houwen, O.A.G., and Underberg, W.J.M. (1986) Aspects of the degradation kinetics of doxorubicin in aqueous solution. Int. J. Pharmaceut., 32: 123-131.

Bensadoun, A., and Weinstein, D. (1976) Assay of protein in the presence of interfering materials. Anal. Biochem. 70: 241-250.

Berliner, J.A., Territo, M.C., Sevanian, A., Ramin, S., Kim, J.A., Bamshad, B., Esterson, M., and Fogelman, A.M. (1990) Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. J. Clin. Invest. 85: 1260-1266.

Berliner, J.A., Territo, M.C., Sevanian, A., Ramin, S., Kim, J.A., Bamshad, B., Esterson, M. Bernini, F., Tanenbaum, S.R., Sherrill, B.C., Gotto, A.M. Jr, and Smith, L.C. (1986) Enhanced catabolism of low density lipoproteins in rat by lactosaminated Fab fragment J. Biol. Chem. 261: 9294-9299.

Biessen, E.A., Beuting, D.M., Vietsch, H., Bijsterbosch, M.K., and Van Berkel, T.J. (1994), Specific targeting of the antiviral drug 5-iodo 2'-deoxyuridine to the parenchymal liver cell using lactosylated poly-L-lysine J. Hepatol. 21: 806-15.

Bijsterbosch, M.K., Schouten, D., and Van Berkel, T.J. (1994) Synthesis of the dioleoyl derivative of iododeoxyuridine and its incorporation into reconstituted high density lipoprotein particles. Biochemistry 47: 14073-80.

Bijsterbosch, M.K., and Van Berkel, T.J. (1992), Lactosylated high density lipoprotein: A potential carrier for the site specific delivery of drugs to parenchymal liver cells. Mol. Pharmacology 41: 404-11.

Bijsterbosch, M.K., and Van Berkel, T.J.C. (1990) Uptake of lactosylated low density lipoprotein by galactose specific receptors in rat liver. Biochem. J. 270: 233-239.

Bijsterbosch, M.K., Gijsbertus, J.Z., and Van Berkel, T.J.C. (1989) Lactosylated low density lipoprotein: A potential carrier for the site specific delivery of drugs to Kupffer cells. Mol. Pharmacol. 36: 484-486.

Bilheimer, D.W., Goldstein, J.L., Grundy, S.M., Starzl, T.E., and Brown, M.S. (1984) Liver transplantation to provide low-density-lipoprotein receptors and lower plasma cholesterol in a child with homozygous familial hypercholesterolemia. N. Engl. J.

Med. 311: 1658-1664.

Bilheimer, D.W., Grundy, S.M., Brown, M.S., and Goldstein, J.L. (1983) Mevinolin and colestipol stimulate receptor-mediated clearance of low density lipoprotein from plasma in familial hypercholesterolemia heterozygotes. Proc. Natl. Acad. Sci. U.S.A. 80: 4124-4128.

Bilheimer, D.W., Watanable, Y., and Kita, T. (1982) Impaired receptor mediated catabolism of low density lipoprotein in the WHHL rabbit, an animal model of famlial hypercholesterolemia. Proc. Natl. Acad. Sci. U.S.A. 79: 3305-3309.

Bilheimer, D.W., Stimne, N.J., and Grundy, SM. (1979) Metabolic studies in familial hypercholesterolemia. J. Clin. Invest. 64: 524-533.

Blackshear, P.J., and Rhode, T.D. (1989) Implantable infusion pumps for drug delivery in man: theoretical and practical considerations. In: Roerdink, F.H.D., Kroon, A.M. (eds) Drug Carrier Systems. New York, John Wiley, 1989,9, pp. 293-310.

Blomhoff, R., Drevon, C.A., Eskild, W., Helgerud, P., Norum, K., and Berg, T. Clearance of acetyl low density lipoprotein by rat liver endothelial cells. J. Biol. Chem., 259: 8898-8903.

Bodley, A., Liu, L.F., Isreal, M., Seshadri, R., Koseki, Y., Guiliani, F.C., Kirshenbaum, S., Silber, R., and Potmesil, M. (1989) DNA topoisomerase Iimediated interaction of doxorubicin and daunorubicin congeners with DNA. Cancer Res. 49: 5969–5978.

Bogner, J.R., Kronawitter, U., Rolinski, R., Truebench, K., and Goebel, F.D. (1994) Liposomal doxorubicin in the treatment of advanced AIDS-related Kaposi sarcoma. J. Acquired Immune Deficiency Syndrome. 7: 463-468.

Bonnefont, D., Legrand, A., Peynet, J., Emerit, J. Delattre, J., and Galli, A. (1989) Distribution of thiobarbituric acid-reactive substances in lipoproteins and proteins in serum. Clin. Chem. 35: 2054-2058.

Bose, R., Verheij, M., Friedman, A.H., Scotto, K., Fuks, Z., and Kolesnick, R. (1995) Ceramide synthase mediates daunorubicin-induced apoptosis: An alternative mechanism for generating death signals. (1995) Cell 82: 405-414

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the the principle of protein dye binding. Anal. Biochem. 72: 248-254.

Braulke, T., Tippmer, S., Chao, H.J., and von Figura, K. (1990) Insulin-like growth factors I and II stimulate endocytosis but do not affect sorting of lysosomal enzymes in human fibroblasts. J. Biol. Chem. 265: 6650-6655.

Breeze, A.S., Gauci, L., Filipowska, D., Laudanski, T. (1994) Delivery of cytotoxic drugs to cancer patients using low density lipoproteins. J. Control. Release 28: 321-322.

Breiter, D.R., Kanost, M.R., Benning, M.M., Wesenberg, G., Law, J.H., Wells, M.A., Rayment, I., and Holden, H.M. (1991) Molecular structure of an apolipoprotein determined at 2.5 A resolution. Biochemistry, 30: 603-608.

Brewer, H.B. (1994) Lipoprotein metabolism. In: Principles and treatment of lipoprotein disorders, Schettler, G. and Habenicht, A.J.R. (eds.) Springer-Verlag, Berlin. pp.30.

Brodsky, F.M. (1988) Monoclonal antibodies as magic bullets. Pharmaceut. Res. 5: 1-9.

Brouwers, J.R.B.J. (1996) Advanced and controlled drug delivery systems in clinical-disease management. Pharmacy World & Science 18: 153-162.

Brown, R.E., Jarvis, K.L., and Hyland, K.J. (1989) Protein measurement using bicinchoninic acid: elimination of interfering substances. Anal. Biochem. 180: 136-139.

Brown, M.S., and Goldstein, J.L. (1986) A receptor-mediated pathway for cholesterol homeostasis. Science 232: 34-47.

Brown, M.S. and Goldstein, J.L. (1984) How LDL receptors influence cholesterol and atherosclerosis. Sci. Am. 251: 58-66.

Brown, M.S., and Goldstein, J.L. (1983) Lipoprotein metabolism in the microphage. Ann. Rev. Biochem. 52: 223-261.

Brown M.S., Kovanen, P.T., and Goldstein, J.L. (1981) Regulation of plasma cholesterol by lipoprotein receptors. Science 212: 628.

Brown M.S., Kovanen, P.T., and Goldstein, J.L. (1980a) Evolution of LDL receptor concept-from cultured cells to intact animals. Ann. N.Y. Acad. Sci. 348: 48.

Brown, M.S., Basu, S.K., Falck, J.R., Ho, Y.K., and Goldstein, J.L. (1980b) The scavenger cell pathway for lipoprotein degradation: specificity of the binding site that mediates the uptake of negatively-charged LDL by macrophages. J. Supramol. Struct. 13: 67-81.

Brown, M. S., and Goldstein, J.L. (1979) Receptor mediated endocytosis : insights from the lipoprotein receptor system. Proc. Natl. Acad. Sci. U.S.A. 73: 3330-3337.

Brown, M.S., and Goldstein, J.L. (1975) Regulation of the activity of the low density lipoprotein receptor in human fibroblasts. Cell 6:307-316.

Burke, T.G., sartorelli, A.C., and Tritton, T.R. (1988) Selectivity of the anthracyclines for negatively charged model membranes: role of the amino group. Cancer Chemother. Pharmacol. 31: 274-280.

Burton, R., Eck, C.D., and Lloyd, B. (1975), The permeability properties of rat liver lysosomes to nucleotides. Biochem. Soc. Trans. 3: 1251-1254.

Buzdar, A.U., Marcus, C., Smith, T.L. and Blumenschein, G.R. (1985) Early and delayed clinical cardiotoxicity of doxorubicin. Cancer 55: 2761-2765.

Calabreshi, P., and Parks, R.E. In: The Pharmacological Basis of Therapeutics, Gilman, A.G., Goodman, L.S., and Gilman, A., (Eds).; MacMillan; New York 1980.

Cambien, F., Duciemetiere, P., and Richard, J. (1980) Total serum cholesterol and cancermortality in a middle-aged male population. Am. J. Epidemiology 112: 388-394.

Candide, C., Morliere, P., Maziere, J.C., Goldstein, S., Dubertret, L., Reyftmann, J.P., and Polonovosky, J. (1986) In vitro interaction of the photoactive anticancer porphyrin derivative photofrin II with LDL, and its delivery to cultured human fibroblasts, FEBS Lett. 207: 133-138.

Canonico, P.G., Kende, M., Juscri, B.J., and Huggins, J.W. (1984) In vitro activity of antivirals against exotic RNA viral infections. J. Antimicrob. Chemother. 14, Suppl. A. 27-41.

Cardin, A.D., Witt, K.R., Barnhart, C.L., and Jackson RL (1982) Sulfhydryl chemistry and solubility properties of human plasma apolipoprotein B. Biochemistry 21, 4503-4511.

Carmichael, j., Degraff, W.G., Gazdar, A.F., Minna, J.D., and Mitchell, J.B. (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of radiosensitivity. Cancer Res. 47: 943-946.

Carter, S.K. (1975) Adriamycin-a review. J. Natl. Cancer Inst. 55: 1265-1275.

Casida, J.E., Engel, J.L., and Nishizawa, Y. (1966) 3',5'-diester of 5-fluoro-2'-deoxyuridine and thymidine: hydrolysis by esterase in human, mouse, and insect tissue. Biochem. Pharmacol. 15: 627-644.

Catapano, A.L. (1987) Transport of cytotoxic compounds to cells via the LDL receptor pathway. Med. Sci. Res. 15: 411.

Chapman, M.J. (1986) Comparative analysis of mammalian plasma lipoproteins. 128: 70-143.

Chassany, O., Urien, S., Claudepierre, P., Bastian, G., and Tillement, J.P. (1994) Binding of anthracycline derivatives to human serum lipoproteins. Anticancer Res. 14: 1-3.

Chassany, O., Urien, S., Claudepierre, P., Bastian, G., and Tillement, J.P. (1996) Comparative serum protein binding of an anthracycline derivatives. Cancer Chemother. Pharmacol. 38: 571-573.

Chen, Y., Willmott, N., and Florence, A.T. (1987) Comparison of albumin and casein microspheres as a carrier for doxorubicin. J. Pharm. Pharmacol. 39: 978-985.

Clayman, R.V., Bilhartz, L.E., Buga, L.M., Spady, D.K., and Dietschy, J.M. (1986a) Renal cell carcinoma in the Wistar-Lewid rat: a model for studying the mechanisms of a cholesterol acquisition by a tumor in vivo. Cancer Res. 46: 2958-2963.

Cole S.P.C. (1986) Rapid chemosensitivity testing of human lung tumor cells using the MTT assay. Cancer Chemother. Pharmacol. 17: 259-263.

Compton, S.J., and Jones, C.G. (1985) Mechanism of dye response and interference in the Bradford protein assay. Anal. Biochem. 151: 369-374.

Connors, T.A. (1989) Has chemotherapy anywhere to go? Cancer Surv. 8: 693-705.

Counsell, R.E., Ranada, V.V., Blair, R.J., Beierwaltes, W.H., and Weinhold, P.A. (1970) Tumor-localizing agents. IX. Radioiodinated cholesterol. Steroids. 15: 317-328.

Counsell, R.E., Schwendner, S.W., Gross, M.D., Longino, M.A., and McConnell, D.S. (1989) Lipoprotein incorporation enhances radioiodinated cholesteryl ester uptake into steroid hormone-secreting tissues. J. Nucl. Med. 30: 1088-1094.

Counsell, R.E., and Pohland, R.C. (1982) Lipoproteins as potential site specific delivery systems for diagnostic and therapeutic agents. J. Med. Chem. 25: 1115-22.

Counsell, R.E., and Korn, N. (1982) Radioiodinated cholesterol as a radiotracer in biochemical studies. In Receptor-binding radiotracers (Eckelman, W.C., Ed.), CRC press, Boca Raton, FL, pp. 147-162.

Couvreur, P., Roblot-Treupel, L., Poupon, M.F. et al. (1990) Nanoparticles as microcarriers for anticancer drugs. Adv. Drug Deliv. Rev. 5: 209-230.

Crommelin, D.J.A., Slaats, N., and Van Bloois, L. (1983) Preparation and characterization of doxorubicin-containing liposomes. I. Influence of liposome charge and pH of hydration medium on loading capacity an dparticle size. Int. J. Pharmaceut. 16: 79-92.

Crommelin, D.J.A., and Van Bloois, L. (1983) Preparation and characterization of doxorubicin-containing liposomes. II. Loading capacity, long term stability and doxorubicin-bilayer interaction mechanism. Int. J. Pharmaceutics. 17: 135-144.

Crommelin, D.J., Scherphof, G., Storm, G. (1995) Active targeting with particulate carrier systems in the blood compartment. Adv. Drug Delivery Rev. 17: 49-60.

Cusack, B.J., Mushlin, P.S., Voulelis, L.D., Xuande Li, Boucek, R.J. Jr., and Olsen, R.D. (1993) Daunorubicin-induced cardiac injury in the rabbit: A role of daunirubicinol? Toxicol. Appl. Pharmacol. 118: 177-185.

Daemen, T., Hoedemakers, R., Storm, G., and Scherphof, G.L. (1995) Opportunities in targeted drug delivery to Kupffer cells: delivery of immunomodulators to Kupffer cells-activation of tumoricidal properties. Adv. Drug Delivery Rev. 17: 21-30.

Danon, A., and Chen, Z. (1979) Binding of Imipramine to plasma proteins: Effect of hyperlipoproteinemia. Clin. Pharmacol. Ther. 25, 316-321.

Dashti, N., Wolfbauer, G., Koren, E., Knowles, B., and Alaupovic, P. (1984) Catabolism of human low-density lipoproteins by human hepatoma cell line Hep G2. Biochim.Biophys. Acta 794: 374-384.

Kerr, D.J., Hynds, S.A. Shepherd, J., Packard, C.J., and Kaye, S.B. (1988) Comparative cellular uptake and cytotoxicity of a complex of daunomycin-low density lipoprotein in human squamous lung tumour cell monolayers. Biochem. Pharmacol. 37: 3981.

Davis, S.S., Washington, C., West, P., Illum, L., Liversidge, G., Sternson, L., and Kirsh, R. (1987) Lipid emulsions as drug delivery systems. Ann N.Y. Acad. Sci. 507: 75-88.

Davis, S.S., and Illum, L. (1986) Colloidal delivery systems-opportunities and challenges. In: Tomlinson, E., and Davis, S.S., (Eds.). Site specific drug delivery. John Wiley & Sons, NY, London, Sydney, Toronto, pp. 93-110.

De Marre, A., Seymour, L.W., Schacht, E. (1994) Evaluation of the hydrolytic and enzymatic stability of macromolecular mitomycin C derivative. J. Control. Release 31: 89-97.

De Smidt, P.C., and Van Berkel, T.J.C. (1990a) LDL-mediated drug targeting. Crit Rev. Ther. Drug Carriers Sys. 7: 99-119.

De Smidt, P.C. and Van Berkel, T.J. (1990b) Prolonged serum half-life of antineoplastic drugs by incorporation into the low density lipoprotein. 50: 7476-82.

De Smidt, P.C., Bijsterbosch, M.K., and Van Berkel, T.J. (1990c) LDL as a carrier in site specific drug delivery Targeted Diagn. Ther. 3: 355-83. De Smidt, P.C., and Van Berkel, T.J.C. (1992) Characteristics of association of oleoyl derivatives of fluorodeoxyuridine and methotrexate with low density lipoproteins (LDL). Pharmaceut. Res. 9: 565-569.

Deckelbaum, R.J., Shipley, G.G., and Small, D.M. (1977) Structure and interactions of lipids in human plasma low density lipoproteins. J. Biol. Chem. 252: 744-754.

DeConti, R.C., Kaplan, S.R., Papac, R.J., and Calabresi, P. (1973) Continuous intravenous infusions of 5-fluoro-2'-deoxyuridine in the treatment of solid tumors. Cancer, 31: 894-898.

DeForge, L.E., Ruyan, M.K., Schwendner, S.W., Newton, R.S., and Counsell, R.E. (1991) Synthesis and evaluation of radioiodinated cholesteryl ethers as lipoprotein probes. Bioconju. Chem. 2: 254-260.

DeForge, L.E., Schwendner, S.W., DeGalan, M.R., McConnel, D.S., and Counsell, R.E. (1989) Noninvasive assessment of lipid disposition in treated and untreated atherosclerotic rabbits. Pharmaceut. Res. 6: 1011-1016.

DeGalan, M.R., Schwendner, S.W., Gross, M.D., and Counsell, R.E. (1985) Assessment of the antiatherogenic efficacy of a hypolipidemic drug by scintigraphy. In: Current Applications in Radiopharmacology. Proceedings of the Fourth International Symposium on Radiopharmacology (Edited by Billinghurst, M.W.), Pergamon Press, New York, pp. 109-117.

DeGalan, M.R., Schwendner, S.W., Weichert, J.P. and Counsell, R.E. (1986) Radioiodinated cholesteryl iopanoate as a potential probe for the in vitro visualization of atherosclerotic lesions in animals. Pharmaceut. Res. 3: 52-55.

Denizot, F., and Lang, R. (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods 89: 271-277.

Dietrich, F.M., Hochkeppel, H.K., and Lukas, B. (1986a) Enhancement of host resistance against virus infections by MTP-PE, a synthetic lipophilic muramyl peptide I. Increased survival in mice and guinea pigs after single drug administration prior to infection, and the effect of MTP-PE on interferon levels in sera and lungs. Int. J. Immunopharmacol. 8: 931-942.

Dietschy, J.M., and Spady, D.K. (1986b), Regulation of low density lipoprotein levels in animals and man with particular emphasis on the role of the liver. In: Greten, H., Windler, E., and Beisiegel, U., (Eds). Receptor-Mediated Uptake in the Liver. Springer-Verlag, Heidelberg, Germany, pp. 135-152.

Dietschy, J.M., and Spady, D.K. (1984) Regulation of low density lipoprotein uptake and degradation in different animal species. In: Parnham, M.J., and Brune, K., eds. Agents and actions supplements, vol 16, Cologne Atherosclerosis Conference No. 2: Lipids. Stuttgart: Birkhauser Verlag pp. 177-190.

Dresel, H.A., Friedrich, E., Via, D.P., Schettler, G., and Sinn, H. (1985) Characterisation of binding sites for acetylated low density lipoprotein in the rat liver in vivo and in vitro. EMBO J. 4: 1157-1162.

Dubowchik, G.M., and Firestone, R.A. (1995a) Improved cytotoxicity of antitumor compounds deliverable by the LDL pathway. Bioconjugate Chem. 6: 427-439.

Dubowchik, G.M., and Firestone, R.A. (1994) The synthesis of branched steroidal prodrugs of nitrogen mustard for antitumor targeting via reconstituted LDL. Tetrahedron Lett. 35: 4523-4526.

Duncan, R., Cable, H., Llyod, J., Rejmanova, P., and Kopecek, J. (1993) Polymers containing enzymatically degradable bonds. 7. Design of oligopeptide side-chains in poly [N-(2-hydroxypropyl)methacrylamide] copolymers to promote efficient degradation of lysosomal enzymes. Makromol. Chem. 184: 1997-2008.

Duncan, R. (1992) Drug polymer conjugates: potential for improved chemotherapy. Anticancer drugs. 3: 175-210.

Duncan, R., Seymour, L.W., Ulbrich, K., and Kopecek, J. (1988) Soluble synthetic polymers for targeting and controlled release of anticancer agents, particularly anthracycline antibiotics, J. Bioact. Comp. Polym. 3: 4-15.

Edelstein, C. and Scanu, A.M. (1986) Precautionary measures for collecting blood destined for lipoprotein isolation. Methods in Enzymology. 128: 151-155.

Poste, G., Bucana, C., Raz, A., Bugelski, B., Kiesh, R. and Fidler, I.J. (1982) Analysis of the fate of systemically administered liposomes and implications for their use in drug delivery. Cancer Res. 42: 1412-1422.

Edwards, D.C., and McIntosh D.P. (1986) Targeting potential of antibody conjugates. In: Ihler, G.M. (Ed.), Methods of drug delivery. Pergamon Books Ltd, Oxford, pp. 83-120.

Edwards, C.K. III, Hedegaard, H.B., Zlotnik, A., Gangadharam, P.R., Johnston, R.B. Jr., and Pabst, M.J. (1986) Chronic infection due to Mycobacterium intracellular in mice: association with macrophage release of prostaglandin E2 and reversal by injection of indomethacin, muramyl dipeptide, or interferon-gama. J. Immunol. 136: 1820-1827.

Ehrlich, P.A. (1956) General review of the recent work in immunity. In: Himmelweit, F. (Ed.) Collected papers of Paul Ehrlich. Vol. 2. Immunology and cancer research, 90, London: Pergamon Press, pp. 442-447.

Ekman, S., and Lundberg, B. (1987) Transfer of lipids from microemulsions of cholesteryl ester, triglyceride, phosphotidylcholine and cholesterol to human fibroblasts in culture. Biochim. Biophys. Acta 921: 347-355.

Eksborg, S., Ehrsson, H., and Ekqvist, B. (1992) Protein binding of an anthraquinone glycosides with special reference to adriamycin. Cancer Chemother. Pharmacol. 10: 7-10.

Eksborg, S., Ehrsson, H., Andersson, B., and Beran, M. (1978) Liquid chromatographic determination of daunorubicin and daunorubicinol in plasma from leukemic patients. J. Chromatogr. 153: 211-218.

Eley, J.G., Halbert, G.W., and Florence, A.T. (1990) The incorporation of estramustine into

low density lipoprotein and its activity in tissue culture. Int. J. Pharmaceutics. 63: 121-127.

England, R.D., Jones, B.N., Flanders, K.C., Coolican, S.A., Rothgeb, T.M., and Gurd, R.S. (1982) Glucagon carbonyl-terminal derivatives: Preparation, purification, and characterization. Biochem. 21: 940-950.

Esbach, S., Pieters, M.N., Van der Boom, J., Schouten, D., Van der Heyde, M.N., Roholl, P.J., Brouwer, A., Van Berkel, T.J., and Knook, D.L. (1993) Visualization of the uptake and processing of oxidized low density lipoprotein in human and rat liver. Hepatology 18: 537-45.

Faulk, W.P., Taylor, C.G., Yeh, C.J.G., and MacIntyre, J.A. (1990) Preliminary clinical study of transferrin-adriamycin conjugates for drug delivery to acute leukemia patients. Mol. Biother. 2: 57-60.

Faulk, W.P., Hsi, B.L., and Stevens, P.J. (1980) Transferrin and transferrin receptors in carcinoma of the breast. Lancet. 2: 390.

Faust, J., Goldstein, J.L., and Brown, M.S. (1977) Receptor-mediated uptake of low density lipoprotein and utilization of its cholesterol for steroid synthesis in cultured mouse adrenal cells. J. Biol. Chem. 252: 4861-4871.

Feinleib, M. (1983) Review of the epidemiological evidence for a possible relationship between hypocholesterolemia and cancer. Cancer Res. 43: 2503-2507.

Fidge, N.H., and Nestel, P.J. (1985) Identification of the apoproteins involved in the interaction of human high density lipoprotein with receptors on cultured cells. J. Biol. Chem. 260: 3570-3575.

Fidler, I.J., and Kleinerman, E.S. (1994) Clinical application of phospholipid liposomes containing macrophage activators for therapy for cancer metastasis. Adv. Drug Delivery Rev. 13: 325-340.

Fielding, C.J. (1979) Validation of a procedure for exogenous isotopic labelling of lipoprotein triglyceride with radioactive triolein. Biochim. Biophys. Acta 573: 255-265.

Fielding, P.E., Fielding, C.J. (1991) Dynamics of lipoprotein transport in the circulatory system. In Biochemistry of lipids, lipoproteins and membranes, Ed. Vance, D.E.,

Vance, J. Elsevier, Amsterdam.The Netheralnds, pp 427-460.

Filipowska, D., Filipowski, T., Morelowska, B., Kazanowska, W., Laudanski, T., Lapinjoki, S., Akerland, M., and Breeze, A. (1992) Treatment of cancer patients with a low density lipoprotein delivery vehicle containing a cytotoxic drug. Cancer Chemother. Pharmacol. 29: 396-400.

Firestone, R.A., Pisano, J.M., Falck, J.R., Mapaul, M.M., and Krieger, M. (1984) Selective delivery of cytotoxic compounds to cells by the LDL pathway. J. Med. Chem. 27, 1037-1043.

Firestone, R.A. (1994) Low density lipoprotein as a vehicle for targeting antitumor compounds to cancer cells. Bioconjugate chem. 5: 105-113.

Fogelman, A.M. (1990) Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. J. Clin. Invest. 85: 1260-1266.

Fong, L.G., Parthasarathy, S., Witztum, J.L., and Steinberg, D. (1987) Nonenzymatic oxidative cleavage of peptide bonds in apolipoprotein B-100. J. Lipid Res. 28: 1466-1477.

Foon, K.A., Schroff, R.W., and Bunn, P.A. (1985) Monoclonal antibody therapy for patients with leukemia and lymphoma. Dev. Oncol. 38: 139-160.

Ford, C.H.J., Richardson, V.J., and Tsaltas, G. (1989) Comparison of tetrazolium colorimetric and [^{3}H]-uridine assays for in vitro chemosensitivity testing. Cancer Chemother. Pharmacol. 24: 295-301.

Forte, G.M., Nichols, A.V., and Glaeser, R.N. (1968) Electron microscopy of human serum lipoproteins using negative staining. Chem. Phys. Lipids. 2: 390.

Gabizon, A. (1995) Liposome circulation time and tumor targeting: implications for cancer chemotherapy. Adv. drug Delivery Rev. 16: 285-294.

Gabizon, A. (1989) Liposome as a drug delivery system in cancer chemotherapy. In: Roerdink, F.H.D., Kroon, A.M. (eds) Drug Carrier systems. New York: John Wiley; 9: 185-212.

Gal D., Macdonald, P.C., Porter, J.C., and Simpson, E.R. (1981a) Cholesterol metabolism in cancer cells in monolayer culture III. Low density lipoprotein metabolism. Int. J. Cancer 28: 315.

Gal, D., Ohashi, M., Macdonald, P.C., Buchsbaum, H.J. and Simpson, E.R. (1981b) Low Density lipoprotein as a Potential Vehicle for Chemotherapeutic Agents and Radionuclides in the Management of Gynecologic Neoplasms. Am. J. Obstet. Gynecol. 139: 877-885.

Ghanem G.E., Comunale, G., Libert A. et al. (1988) Evidence for alpha-melanocyte-stimulating hormone (α-MSH) receptors on human malignant melanoma cells. Int J Cancer 1988; 41: 248-55.

Ghosh, M.K., and Mitra, A.K. (1991) Effect of 5'-ester modification on the physicochemical properties and plasma protein binding of 5-iododeoxyuridine. Pharmaceut. Res. 8: 771-775.

Gish, D.T., Kelly, R.C., Camiener, G.W., and Wechter, E.J. (1971) Nucleic acids. II. Synthesis of 5'ester of 1-beta-D-arabinofuranosylcytosine possessing antileukemic and immunosuppressive activity. J. Med. Chem. 14: 1159-1162.

Goldman, R., Facchinetti, T,, Bach, D., Raz, A. and Shintzky, M. (1978) A differential interacting of daunomycin, adriamycin and their derivatives with human erythrocytes and phospholipid bilayers. Biochim. Biophys. Acta, 512: 254-269.

Goldschmidt, R.C. and Kimelberg, H.K. (1989) Protein analysis of mammalian cells in monolayer culture using the bicinchoninic assay. Anal. Biochem. 177: 41-45.

Goldstein, J.L., Ho, Y.K., Brown, M.S., Inneararity, T.L., and Mahley, R.W. (1980) Cholesteryl ester accumulation in macrophages resulting from receptor-mediated uptake and degradation of hypercholesterolemic canine beta-very low density lipoproteins. J. Biol. Chem. 255: 1839-1848.

Glodstein, J.L., Ho, Y.K., Basu, S.K., and Brown, M.S. (1979a) Binding site on macrophages that mediated uptake and degradation of acetylated low density lipoprotein producing massive cholesterol deposition. Proc. Natl. Acad. Sci. U.S.A. 76: 333-337.

Goldstein, J.L., Anderson, R.G.W., and Brown,M.S. (1979) Coated pits, coated vesicles and receptor mediated endocytosis. Nature 270: 679.

Goldstein, J.L., and Brown, M.S. (1977) The low-density lipoprotein pathway and its relation to atherosclerosis. Ann. Rev. Biochem. 46: 897-930.

Goodman, D.S. (1958) The interaction of human serum albumin with long chain fatty acid anions. J. Am. Chem. Soc. 80: 3892-3898.

Gornall, A.G., Bardawill, C.J., and David, M.M. (1949) Determination of serum proteins by means of the Biuret reaction. J. Biol. Chem. 177: 751-766.

Greenberg, N., Schumm, D.E., Webb, T.E. (1977) Uridine kinase activities and pyrimidine nucleoside phosphorylation in fluoropyrimidine-sensitive and -resistant cell lines of the Novikoff hepatoma. Biochem. J. 164: 379-389.

Gupta, P.K. (1990). Drug targeting in cancer chemotherapy: a clinical prospective. J. Pharm. Sci. 79: 949-962.

Haberland, M.E., and Fogelman, A.M. (1985) Scavenger receptor mediated recognition of maleyl bovine plasma albumin and the demaleylated protein in human monocyte macrophages. Proc. Natl. Acad. Sci. U.S.A. 82: 2693-2697.

Hadfield, A.F., and Sartolli, A.C. (1984) The pharmacology of prodrugs of 5-fluorouracil and 1-beta-D-arabinofuranosylcytosine. Adv. Pharmacol. Chemother. 20: 21-67.

Halbert, G.W., Stuart, J.F., and Florence, A.T. (1985) A low density lipoprotein-methotrexate covalent complex and its activity against L1210 cells in vitro. Cancer Chemother. Pharmacol. 15: 223-227.

Halperin, G., Stein, O., and Stein, Y. (1986) Synthesis of ether analogs of lipoprotein lipids and their biological application. Methods Enzymol. 129: 816-848.

Hamblin, M.R., and Newman, E.L. (1994) Photosensitizer targeting in photodynamic therapy II. Conjugates of haematoporphyrin with serum lipoproteins. J. Photochem. Photobiol. 26: 147-157.

Harkes, L. and Van Berkel, Th.J.C. (1984) Quantitative role of parenchymal and non-parenchymal liver cells in the uptake of ^{14}C sucrose-labeled low density lipoprotein in vivo. Biochem. J. 224: 21.

Havekes, L., Van Hinsbergeh, V., Kempen, H.J., and Emeis, J. (1983) The metabolism in vitro of human low-density lipoprotein by the human hepatoma cell line hep G2. Biochem J. 214: 951.

Havekes, L.M., Schouten, D., De Wit, E.C.M., Cohen, L.H., Griffioen, M., Van Hinsbergh, V.W.M., and Princen, H.M.G. (1986) Stimulation of the LDL receptor activity in

the human hepatoma cell line Hep G2 by high density serum fractions. Biochem. Biophys. Acta 875: 236.

Havel, R.J., Eder, H.A., and Bragdon, J.H. (1955) The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Inves. 34: 1345-1353

Heggie, G.D., Sommadossi, J.P., Cross, D., Huster, W.J., and Diasio, R.B. (1987) Clinical pharmacokinetics of 5-fluorouracil and its metabolites in plasma, urine and bile. Cancer Res. 47: 2203-2206.

Heidelberger, C., Danenberg, P.V. and Moran, R.G. (1983) Fluorinated pyrimidines and their nucleosides. Adv. Enzymol. 54: 57-119.

Heidelberger, C., Kaldor, G., Mukherjee, K.L., and Danneberg, P.B. (1960) Studies on fluorinated pyrimidines, XI. In vivo studies on tumor resistance. Cancer Res. 20: 903-920.

Heidelberger, C., Chaudhuri, M., Danneberg, P., Mooren, D., Griesbach, L., Duschinsky, R., Schnitzer, R., Pleven, E., and Scheiner, J. (1957) Fluorinated pyrimidines, a new class of tumor inhibitory compounds. Nature. 179: 663-666.

Heinecke, J.W., Rosen, H., and Chait, A. (1984) Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. J. Clin. Invest. 74: 1890-1894.

Henrikksson, P.M., Eriksson, S., Ericsson, M., Rudling, R., Stege, L. Berglund, B. and Angelin. (1989) Hypocholesterolaemia and increased elimination of low-density lipoproteins in metastatic cancer of the prostate. Lancet II: 1178-1180.

Henry, N., Fantine, E.O., Bolard, J., and Garnier-Sullerot A. (1985) Interaction of adriamycin with negatively charged model membranes: evidence of two types of binding sites. Biochemistry 24: 7085-7092.

Hill, H.D. and Straka, J.G. (1988) Protein determination using bicinchoninic acid in the presence of sulfhydryl reagents. Anal. Biochem. 170: 203-208.

Ho, Y.K., Smith, R.G., Brown, M.S., and Goldstein, J.L. (1978), Low density lipoprotein (LDL) receptor activity in human acute myelogenous leukemia cells. Blood 52: 1099.

Hoff, H.F., Whitaker, T.E., and O'Neil, J. (1992) Oxidation of low density lipoprotein leads to particle aggregation an altered macrophage recognition. J. Biol. Chem., 267: 602-609.

Hopkins C, and Trowbridge, I.S. (1983) Internalisation and processing of transferrin and the transferrin receptor in human carcinoma. J. Cell Biol. 97: 508-21.

Hori, K., Suzuki, M., Tanda, S., Satto, S., and Zhang, Q.H. (1993) Functional characterization of developing tumor vascular system and drug delivery. Intl. J. Oncol. 2: 289-296.

Hossaini, H.S., Pierlot, C., Kusnierz, J.P., Parmentier, B., Nizard, F.M., Delattre, S.L., Tartar, A., Fruchart, J.C., Sergheraert, C., and Duriez, P. (1994) Preparation of antiHIV-low density lipoprotein complexes for delivery of anti-HIV drugs via the low density lipoprotein pathways. Biotech. Ther. 5: 69-85.

Houba, P.H.J., Leenders, R.G.G., Boven, E., Scheeren, J.W., Pinedo, H.M., and Haisma, H.J. (1996) Characterization of novel anthracycline prodrugs activated by human β-glucuronidase for use in antibody-directed enzyme prodrug therapy. Biochem. Pharmacol. 52: 455-463.

Hua Yang and Elmquist, W.F. (1993) The binding of cyclosporin A to human plasma: An in vitro microdialysis study. Pharmaceut. Res. 13 : 622-627.

Hynds, S.A., Welsh, J., Stewart, J.M., Jack, A., Soukop, M., Mcardle, C.S., Calman, K.C., Packard, C.J., and Shepherd, J. (1984) Low density lipoprotein metabolism in mice with soft tissue tumors. Biochim. Biophys. Acta 795: 589.

Ikeda, S., Negishi, T. and Nishimura, C. (1985) Enhancement of non-specific resistance to viral infection by muramyl dipeptide and its analogs. Antiviral Res. 5: 207-215.

Illum, L., and Davis, S.S. (Eds) (1987) Polymers in Controlled Drug Delivery. Wright: Bristol, U.K., 1987.

Illum, L., Jones, P.D.E., and Davis, S.S. (1984) Drug targeting using monoclonal antibody-coated nanoparticle. In: Davis, S.S., Illum, L., Mcvie, J.G., and Tomlinson, E. (Eds.) Microspheres and drug therapy: Pharmaceutical, Immunological and medical aspects. Elsevier Science Publishers, Amsterdam, NY, pp: 353-363.

Imba, M., and Johnson, R.K. (1979) Active efflux of daunorubicin and adriamycin in sensitive and resistant sublines of P388 leukemia. Cancer Res. 39: 2200-2203.

Ishihara, C., Hamada, N., Yamamoto, K., Lida, J., Azuma, I., and Yamamura, Y. (1985) Effect of muramyl dipeptide and its stearoyl derivatives on resistance to sendai virus infection in mice. Vaccine 3: 370-374.

Ishitsuka, H., Miwa, M., Takemoto, K., Itoga, A., and Maruyama, H.B. (1980) Probable role of uridine phosphorylase for antitumor activity of 5'-deoxy-5-fluorouridine. Gann, 71: 112-123.

Isreal, M., Pegg, W.J., Wilkinson, P.M., Garnick, M.B. (1978) Liquid chromatographic analysis of adriamycin and metabolites in biological fluids. J. Liq. Chromatogr. 1: 795-809.

Isreal, M., and Modest, E.J. (1977) N-Trifluoroacetyladriamycin-14-alkanoates and therapeutic composition containing same. US Patent 4,035, 566, July 12, 1977.

Isreal, M., Seshadri, R., Koseki, Y., Sweatman, T.W., and Idriss, J.M. (1987) Amelioration of adriamycin toxicity through modification of drug-DNA binding properties. Cancer Treat. Rev. 14: 163-167.

Isreal, M., Seshadri, R., and Idriss, J.M. (1985) N-benzyladriamycin-14-valerate (AD 198), a promising new adriamycin (ADR) analogue. Proc. AM. Assoc. Cancer Res. 26: 220.

Isreal, M., and Potti, P.G. (1981) N-Trifluoroacetyladriamycin-14-O-hemiglutarate and hemiadipate and therapeutic combinations containing same. US Patent 4,299, 822, November 10, 1981.

Iwanik, M.J., Shaw, K.V., Ledwith, B.J., Yanovich, S. and Shaw., J.M. (1984) Preparation and interaction of a low-density lipoprotein: Daunomycin complex with P 388 leukemic cells. Cancer Res. 44: 1206-1215.

Jain, R.K. (1990) Vascular and interstitial barriers to delivery of therapeutic agents in tumors. Cancer Metastasis Rev. 9: 253.

Jalil, R. (1990) Biodegradable poly(lactic acid) and Poly (lactide-co-glycolide) in sustained drug delivery. Drug Dev. Ind. Pharmacy 16: 2353-2367.

Jalil, R., and Nixon, J.R. (1992) Microencapsulation with biodegradable materials. In: Whateley, T.L. (Ed.). Microencapsulation of drugs. Harwood academic publishers, U.S.A., pp 177-188.

Janknegt, R. (1996) Liposomal formulations of cytotoxic drugs. Support Care Cancer 4: 298-304.

Johnson, W.J., Pizzo, S.V., Imber, M.J. and Adams, D.O. (1982) Receptors for maleylated proteins regulate secretion of neutral protease by murine macrophages. Science 218: 574-576.

Jones, M.N., and Hudson, M.J.H. (1933) The targeting of immunoliposomes to tumour cells (A431) and the effect of encapsulated methotrexate. Biochim. Biophys. Acta 1152: 231-242.

Juliano, R.L. (1985) Microparticulate drug carriers In: Borchardt, R.T., Repta, A.J., and Stella, V.J. (Eds.), Directed Drug Delivery. Humana Press, NJ, pp.147-170.

Juliusson, G. and Vitols, S. (1988), Impaired low density lipoprotein receptor activity in chronic B-lymphocyte leukemia cells Eur. J. Haematol. 40: 18-24.

Juompan, L., Puel, J., Fournie, G.J., and Benoist, H. (1995) Study of LDL and acetylated LDL endocytosis by mononuclear cells in HIV infection. Biochim. Biophys. Acta 1272: 21-28.

Kamps, J.A., and Van Berkel, T.J., (1993), Regulation of low density lipoprotein receptors in the human hepatoma cell line Hep G2. Effect of phorbol 12-mristate 13-acetate and low density lipoprotein. Eur. J. Biochem., 213: 989-94.

Kamps, J.A., Van Berkel, T.J., (1992) Complete down regulation of LDL receptor activity in the human hepatoma cell line Hep G2 by beta migrating very low density lipoprotein and non-lipoprotein cholesterol. Different cellular regulatory pools of cholesterol. Eur. J. Biochem. 206: 973-8.

Kamps, J.A., Kuiper, J., Kruijt, J.K., and Van Berkel, T.J. (1991a) Complete down regulation of LDL receptor activity in human liver parenchymal cells by ß-very-LDL. FEBS Lett.,287 (1-2): 34-8.

Kamps, J.A., Kruijt, J.K., Kuiper, J., and Van Berkel, T.J., (1991b), Uptake and degradation of human low density lipoprotein by human liver parenchymal and Kupffer cells in culture. Biochem. J., 276: 135-40

Kane, M.A., Elwood, P.C., Portillo, R.M., Antony, A.C. and Kolhouse, J.F. (1986) J. Biol. Chem. 261: 15625-15631.

Kanshal, V., and Barnes, l.D. (1986) Effect of zwitterionic buffers on measurement of small masses of protein with bicinchoninic acid. Anal. Biochem. 157: 291-294.

Keizer, H.J., and Pinedo, H.M. (1985) Cancer chemotherapy: alternative routes of drug administration. A review. Cancer Drug Del. 2: 147-169.

Keller, H. (1991) Klinisch-chemische Laboradiagnostisk fuer die Praxis; Thieme Verlag: Stuttgart. pp. 294-313.

Kerr, D.J. and Kaye, S.B. (1991) Chemoembolism in cancer chemothrapy. Crit. Rev. Therap. Drug Carrier Sys. 8: 19-39.

Kesaniemi, Y.A., Witztum, J.L., and Steinbrecher U.P. (1983) Receptor-mediated catabolism of low-density lipoprotein in man. Quantitation using glycosylated low density lipoprotein. J. Clin. Invest. 71: 950-959.

Kessler, R.J., and Fanestil, D.D. (1986) Interference by lipids in the determination of protein using bicinchoninic acid. Anal. Biochem. 59: 138-142.

Khoo, J.C., Miller, E., Macloughlin, P., and Steinberg, D. (1988) Enhanced macrophage uptake of low density ipoprotein after self-aggregation. Artheriosclerosis 8: 348-358.

Kim, S. (1993) Liposomes as carriers for cancer chemotherapy. Current status an future prospects. Drugs. 46: 618-638.

King, C.R., Kraus, M.H., DiFiorc, P.P., et al. (1990) Implications of erB-2 overexpression for basic science and clinical medicine. Cancer Biol. 1: 329-37.

Kirschbaum, B. (1986) Use of bicinchoninic acid assay in measuring urinary proteins [letter] Clin. Chem. 32: 72.

Knott, T.J., Rall, S.C., Innerarity T.L., Jacobson, S.F., Urdea, M.S., Wilson B.L., Powell L.M., Pease, R.J., Eddy R., Nakai, H., Byers, M., Priestly, L.M., Robertson, E., Rall, L.B., Betsholtz C., Shows, T.B., Mahley R.W., and Scott, J. (1985) Human apolipoprotein B; Structure of carboxy terminal domains, sites of gene expression, and chromosomal localization. Science 230: 37-43.

Knott, T.J., Pease, R.J., Powell, L.M., Wallis, S.C., Rall, S.C., Inneairity, T.L., Blackhart, B., Taylor, W.H., Marcel, Y., Milne, R., Johnson, D., Fuller, M., Lusis, A.J., Mccarthy, B.J., Mahley, R.M., Levy Wilson, B., and Scott, J. (1986) Complete

protein sequence and identification of structural domains of human apolipoprotein B. Nature 323: 734-738.

Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira, P., and Krieger, M. (1990) Type I macrophage scavenger contains alpha-helical and collagen-like coiled coils. Nature 343: 531-535.

Koff, W.C., Schowalter, S.D., Hamper, B., and Fidler, I.J. (1985), Protection of mice against fetal herpes simplex type 2 infection by liposomes containing muramyl tripeptide. Science 228: 495-497.

Koff, W.C., and Fidler, I.J. (1985) The potential use of liposome-mediated antiviral therapy. Antiviral Res. 5: 179-190.

Koff, W.C., Showalter, S.D., Hampur, B., and Fidler, I.J. (1985) Protection of mice against fatal herpes simplex type 2 infection by liposomes containing muramyl tripeptide. Science 228: 495-496.

Kosmas, C., Linardou, H., Epenetos, A.A. (1993) Review: advances in monoclonal antibody tumour targeting. J. Drug Targeting 1: 81.

Kostner, G.M., and Laggner, P. (1989) Chemical and physical properties of lipoproteins. In: Fruchart, J.C. and and Shepherd J. (eds.) Human plasma lipoproteins, Berlin: Walter de Gruyter, pp:23-51.

Krieger, M. (1986) Reconstitution of the hydrophobic core of low density lipo-proteins. Methods Enzymol. 128: 608-613.

Krieger, M., Mcphaul, M.J., Goldstein, J.L., and Brown, M.S. (1979a), Replacement of neutral lipids of low density lipoprotein with esters of long chain unsaturated fatty acids, J. Biol. Chem. 256: 3845-3853.

Kriger, M., Smith, L.C., Anderson, R.G.W., Goldstein, J.L., Kao, Y.J., Pownall, H.J., Gotto, A.M. Jr, and Brown, M.S. (1979b) Reconstituted low density lipoprotein: a vehicle for the delivery of hydrophobic fluorescent probes to cells. J. Supramol. Struct. 10: 467-478.

Kriger, M., Goldstein, J.L., and Brown, M.S. (1978a) Receptor mediated uptake of low density lipoprotein reconstituted with 25-hydroxy-cholesteryl oleate suppresses 3-hydroxy-3-methylglutaryl-coenzyme A reductase and inhibits growth of human fibroblasts. Proc. Natl. Acad. Sci. U.S.A. 75: 5052-5056.

Krieger, M., Brown, M.S., Faust, J.R., and Goldstein J.L. (1978b) Replacement of exogenous cholesteryl esters of low density lipoprotein with endogenous cholesteryl linoleate: reconstitution of a biologically active lipoprotein particle. J. Biol. Chem. 253: 4093-4101.

Krinick, N.L., and Kopecek, J. (1991) Soluble polymers as targetable drug carriers. In: Juliano, R.L. (ed) Targeted drug delivery. Handbook of experimental pharmacology. Berlin: Springer Verlag, 1991; 100: 105-179.

Kroon, P.A. (1994) Fluorescence study of the motional states of core and surface lipids in native and reconstituted low density lipoproteins. Biochemistry 33: 4879-4884.

Kurz, H., Trunk, H., and Weitz, B. (1977) Evaluation of methods to determine protein-binding of drugs. Arzneim-Forsch Drug Res. 27: 1373-1380.

Laemmli, U.K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lameh, J., Chuang, L.F., Isreal, M., and Chuang, R.Y. (1988) Mechanistic studies on – benzyladriamycin-14-valerate (AD 198), a highly lipophilic alkyl adriamycin analogue. Anticancer Res. 8: 689-694.

Lasic, D.D. (1996) Doxorubicin in sterically stabilized liposomes. Nature 380: 561-562.

Lea, M.A., Grasso, S.V., Hu, J., and Sidler, N. (1984) Factors affecting the assays of histone H1 and polylysine by binding of Coomassie blue G. Anal. Biochem. 141: 390-396.

Lee, F.H. and Hwang, K.M. (1979) Antibodies as specific carriers for chemotherapeutic agents. Cancer Chemother. Pharmacol. 3: 17-24.

Lee, R.J., and Low, P.S. (1995) Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin in vitro. Biochimica et Biophysica Acta 1233: 134-144.

Lemaire, M. and Tillement, J.P. (1982a) Role of lipoproteins and erythrocytes in the in vitro binding and distribution of cyclosporin A in the blood. J. Pharm. Pharmacol. 34: 115-118.

Lemaire, M. and Tillement, J.P. (1982b) The binding characteristics of some adrenergic β-receptor antagonists to human serum proteins. Biochem. Pharmacol. 31: 359-365.

Lestavel-Delattre, S., Martin-Nizard, F., Clavey, V., Testard, P., Favre, G., Doualin, G., Hossaini, H.S., Bard, J.M., Duriez, P., Delbart, C., Soula, G., Lesieur, D., Lesieur, I., Cazin, J.C., Cazin, M., and Fruchart, J.C. (1992) Low density lipoprotein for delivery of an acrylophenone antineoplastic molecule into malignant cells. Cancer Res. 52: 3629-3635.

Leyland, R.L. (1994) Polyoxyethylene sorbitan fatty acid esters. In: Handbook of pharmaceutical excipients (2nd edition), ed. Wade, A., & Weler, P.J. pp. 375-378.

Littman, M.L., Taguchi, T., and Mosbach, E.H. (1966). Effect of cholesterol free, fat free diet and hypocholesterolemic agents on growth of transplantable animal tumors. Cancer Chem. Rep. 50: 25-45.

Liu, F., and Liu, D. (1995) Long circulating emulsions (oil-in-water) as carriers for lipophilic drugs. Pharmaceut. Res. 12: 1060-1064.

Lleu, P.L. and Rebel, G. (1991) Interference of Good's buffers and other biological buffers with protein determination. Anal. Biochem. 192: 215-218.

Lombardi, P., Mulder, M., de Wit, E., Van Berkel, T.J., Frants, R.R., and Havekes, L.M. (1993) Low density lipoproteins are degraded in HepG2 cells with low efficiency. Biochem. J. 290: 509-514

Lothstein, L., Wright, H.M., Sweatman, T.W., and Isreal, M. (1992) N-benzyladriamycin-14-valerate and drug resistance: correlation of anthracycline structural modification with intracellular accumulation and distribution in multidrug resistant cells. Oncol. Res. 4: 341-347.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 165-175.

Lundberg, B. (1994) Cytotoxic activity of two new lipophilic steroid nitrogen carbamates incorporated into low density lipoprotein. Anticancer Drug Design 9: 471-476.

Lundberg, B. (1993) Evaluation of methods for complexing prednimustine to low density lipoprotein. Int. J. Pharmaceutics 99: 275-283.

Lundberg, B., (1992) Assembly of prednimustine low density lipoprotein complex and their cytotoxic activity in tissue culture. Cancer Chemother. Pharmacol. 29: 241-247.

Lundberg, B. (1991) Techniques for complexing pharmacological agents to lipoprotein and

lipid microemulsion. Targeted Diag. Ther. 5: 97-139.

Lundberg, B. (1987) Preparation of drug-low density lipoprotein complexes for delivery of antitumoral drugs via the low density lipoprotein pathway. Cancer Res. 47: 4105-4108.

Lundberg, B., and Suominen, L. (1984) Preparation of biologically active analogs of serum low density lipoprotein. J. Lipid Res. 25: 550-558.3.

Mackichan, J.J. (1989) Protein binding drug displacement interactions: fact or fiction? Clinical Pharmacokinet. 16: 65-73.

Mahley, R.W., Innearity, T.L., Pitas, R.E., Weisgraber, K.H., Brown, J.H., and Gross, E. (1977) Inhibition of lipoprotein binding to cell surface receptors of fibroblasts following selective modification of arginyl residues in arginine rich and beta - apoproteins. J. Biol. Chem. 252: 7279-7287.

Mangiapane, E.H. (1990) The effect of adriamycin on glycerophosphate acyltransferase and lipid metabolism in rat hepatocytes in monolayer culture. Biochem. Pharmaco. 40: 1577-1582.

Mankertz, J., Von Baeyer, H., Rokos, K., Nundel, M., Pauli, G., and Riedel, E. (1995) Cell specific uptake of antiretroviral drugs: AZT coupled to LDL inhibits HIV replication in human macrophages. Int. J. Clin. Pharmacol. Ther. 33: 85-88.

Markel, A., and Brook, G.J. (1994) Cancer and Hypocholesterolemia. Isreal J. Med. Sci. 30: 787-793.

Markwell, M.A.K. (1982) A new solid-state reagent to iodinate proteins: conditions for the efficient labeing of antiserum. Anal. Biochem. 125: 427-432.

Marshall, T., and Williams, K.M. (1991) Drug interference in the Bradford and 2,2'-bicinchoninic acid protein assays. Anal. Biochem. 198: 352-354.

Marshall, T., and Williams, K.M. (1992) Drug interference in the 2,2'-bicinchoninic acid protein assay. Biochem. Soc. Trans. 20(1): 40S

Mashihi, K.N., Brehmer, W., Azuma, I., Lange, W. and Muller, S. (1984) Infect. Immun. 43: 233-237.

Masquilier, M., Vitols, S., and Peterson, C. (1983) Low density lipoprotein as a carrier

of antitumoral drugs: in vivo fate of drug-human low density lipoprotein complexes in mice. Cancer Res. 46: 3842-3847.

Mayhew, S.L. and Thorn, D. (1995) Enteral nutrition support: an overview. American Pharmacy. 35: 47-62.

Mayhew, E., Lazo, R., and Vail, WJ. (1984) Preparation of liposomess entrapment cancer chemotherapeutic agents for experimental in vivo and in vitro studies. In: Gregoriadis, G. (Ed.), Liposome Technology. CRC Press Inc., Boca Raton, pp. 19-32.

Mayor, S., Rothberg, K.G., and Maxfield, F.R. (1994) Sequestration of GPI-anchored proteins in coveolae triggered by cross-linking. Science 264: 1948-1951.

Metherall, J.E., Li, H., Waugh, K. (1996a) Role of multidrug resistance P-glycoproteins in cholesterol biosynthesis. J. Biol. Chem. 271: 2634-2640.

Metherall, J.E., Waugh, K., Li, H. (1996b) Progesterone inhibits cholesterol biosyntheis in cultured cells: Accumulation of cholesterol precursors. J. Biol. Chem. 271: 2627-2633.

Miller, G.A., and Morahan, P.S. (1982) Functional and biochemical heterogeneity among subpopulations of rat and mouse peritoneal macrophages. J. Reticuloendothel. Soc. 32: 124-140.

Milne, R.W., Blanchette, L., Theolis, R., Weech, P.K., and Marcel, Y.L. (1987) Monoclonal antibodies distinguish between lipid dependent and reversible conformational status of human apo B. Mol. Immunol. 24: 435-447.

Minamide, L.S., and Bamburg, J.R. (1990) A filter paper dye-binding assay for quantitative determination of protein without interference from reduced agents or detergents. Anal. Biochem. 190: 66-70.

Mori, A. and Huang, L. (1995a) Targeted Immunoliposomes: potential role in the delivery of cytotoxic drugs. Clin. Immunother. 3: 227-240.

Morton, R.E. and Zilversmit, D.B. (1982) Purification and characterization of lipid transfer proteins from lipoprotein-deficient plasma. J. Lipid Res. 31: 397-406.

Morton, R.G. (1990) Interaction of lipid transfer protein with plasma lipoproteins and cell membranes. Experimentia 46: 552-560.

Morton, R.E., and Zilversmit, D.B. (1983) Interrelationship of lipids transferred by the lipid transfer protein isolated from human lipoprotein-deficient plasma. J. Biol. Chem. 258: 11751-11757.

Mosley, S.T., Goldstein, J.L., Brown, M.S., Falck, J.R., and Anderson, R.G.W. (1981) Targeted killing of cultured cells by receptor-dependent photosensitization. Proc. Natl. Acad. Sci. U.S.A. 78: 5717-5721.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. 65: 55.

Mukhopadhyay, A., Mukhopadhyay, B., Srivastava, R.K., and Basu, S.K. (1992) Scavenger-receptor-mediated delivery of daunomycin elicits selective toxicity towards neoplastic cells of macrophage lineage. Biochem. J. 284: 237-241.

Mukhopadhyay, B., Mukhopadhyay, A., and Basu, S.K. (1993) Enhancement of tumoricidal activity of daunomycin by receptor mediated delivery. In vivo studies. Biochem. Pharmacol. 46: 919-924.

Muller, H.G. (1939) Cholesterol metabolism in health and anemia. Medicine 9: 119-174.

Muller, C.P., Wagner, A.U., Maucher, C., and Steinke, B. (1989) Hypercholesterolemia, an unfavorable feature of prognostic value in chronic myeloid leukemia. Eur. J. Haematol. 43, 235.

Naeff, R. (1996) Feasibility of topical liposome drugs produced on an inductrial scale. Adv. Drug Delivery Rev. 18: 343-347.

Nagelkerke, J.F., Barto, K.P., and Van Berkel, T.J.C. (1983) In vivo and in vitro uptake and degradation of acetylated low density lipoprotein by rat liver endothelial, Kupffer, and parenchyman cells. J. Biol. Chem. 258: 12221-12227.

Nishikawa, O., Yokoyama, S., Okabe, H., and Yamamoto, A. (1988) Enhancement of non-polar lipid transfer reaction through stabilization of substrate lipid particles with apoproteins. J. Biochem. 103: 188-194.

Norata, G., Canti, G., Ricci, L., Nicolin, A.,Trezzi, E., and Catapano, A.L. (1984) In vivo assimulation of low density lipoproteins by a fibrosarcoma tumor line in mice. Cancer Lett. 25: 203.

Ohnishi, T., Tan, C, and Yokoyama, S. (1994) Selective transfer of cholesteryl ester over

triglyceride by human lipid transfer protein between apolipoprotein-activated lipid microemulsions. Biochemistry 33: 4533-4542.

Ohnishi, T., Oikawa, K., Kay, C.M., Yokoyama, S. (1995) Modulation of substrate selectivity in plasma transfer protein reaction over structural variation of lipid particle. Biochim. Biophys. Acta 1254: 117-126.

Okada, H., and Tiguchi, H. (1996) Biodegradable microspheres in drug delivery. Crit. Rev. Ther. Drug Carrier Sys. 12: 1-99.

Oku, N. and Namba Y. (1994) Long circulating liposomes. Crit Rev in Ther. Drug Carrier Sys. 11: 231-270.

Olsen, F., Hunt, C.A., Szoka, F.C., Vail, V.J., and Papahadjopoulos, D. (1979) Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. Biochim. Biophys. Acta. 557: 397-406.

Orenstein, J.M., Meltzer, M.S., Phipps, T. and Gendelman, H.E. (1988) Cytoplasmic assembly and accumulation of human immunodeficiency virus types 1 and 2 in recombinant human colony-stimulating factor-1-treated human monocytes: an ultrastructural study. J. Virol. 62: 2578-2586.

Papahadjopoulos, D., Gabizon, A. (1987) Targeting of liposomes to tumor cells in vivo. Ann. N.Y. Acad. Sci. 507: 64-87.

Park, J.G., Kramer, B.S., Steinberg, S.M., Carmichael, J., Collins, J.M., Minna, J.D., and Gazdar, A.F. (1987) Chemosensitivity testing of human colorectal carcinoma cell lines using a tetrazolium-based colorimetric assay. Cancer Res. 47: 5875.

Parker, K.C., Strominger, J.L. (1983) Localization of the sites of iodination of human β2-microglobulin: quaternary structure implications for histocompatibility antigens. Biochem. 22: 1145-1153.

Parks, J.S., Martin, J.A., Johnson, F.L., and Rudel, L.L. (1985) Fusion of low density lipoprotein with cholesterol ester phospholipid microemulsions. J. Biol. Chem. 260: 3155-3163.

Parthasarathy, S., Steinbrecher, U.P., Barnett, J., Witzum, J.L., and Steinberg, D. (1985) Essential role of phospholipase A2 activity in endothelial cell induced modification of low density lipoprotein. Proc. Natl. Acad. Sci. U.S.A. 82: 3000-3004.

Parthasarathy, S., Wieland, E., and Steinberg, D. (1989) A role for endothelial cell lipoxygenase in the oxidative modification of low density lipoprotein. Proc. Natl. Acad. Sci. U.S.A. 87: 1046-1050.

Pattnaik, N.M., Montes, A., Hughes, B.L., Zilversmit, D.B. (1978) Cholesteryl ester exchange protein in human plasma-isolation and characterization. Biochem. Biophys. Acta 530: 428.

Perez-solar, R., Lopez-berestein, G., and khokhar, A.R., (1989) Design and development of liposome-dependent antitumour agents. In: Lopez-Berestein, G., and Fidler, I.J. (Eds), Liposomes in the Therapy of Infectious Diseases and Cancer, U.C.L.A. Symposia on Molecular and Cellular biology. Vol. 89, A.R. Liss, New York, pp: 59-69.1989)

Peterson, C., Vitols, S., rudling, M., Blomgren, H., Edsmyr, F., and Skoog, L. (1985) Hypocholesterolemia in cancer patients may be caused by elevated LDL receptor activities in malignant cells. Med. Oncol. Tumor Pharmacother. 2: 143.

Peterson, G.L. (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. 83: 346-356.

Peterson, G.l. (1979). Review of the folin phenol protein quantitation method of Lowry, Roseberough, Farr and Randall. Anal. Biochem. 100: 201-220.

Peterson, C., Masquelier, M., Rudling, M., Soderberg, K., and Vitols, C. (1991) Lipoproteins, malignancy and anticancer agents. Targeted Diagn. Ther. (U.S.) 5: 75.

Pierce, J. and Suelter, C.H. (1977) An evaluation of the Coomassie brilliant blue G-250 dye-binding method for quantitative protein determination. Anal. Biochem. 81: 478-487.

Pinedo, H.M. and Peters, G.J. (1988) Fluorouracil: biochemistry and pharmacology. J. Clin. Oncol. 6: 1653-1664.

Pitas, R.E., Boyles, J., Mahley, R.W., and Bissel, D.M. (1985) Uptake of chemically modified LDL in vivo is mediated by specific endothelial cells. J. Cell Biol. 100: 103-117.

Ponec, M., Havekes, L., Kempenaar, J., Lavrijsen, S., Wijsman, M., Boonstra, J., and Vermeer, B.J. (1985) Calcium-mediated regulation of the low density lipoprotein receptor and intracellular cholesterol synthesis in human epidermal keratinocytes.

J. Cell Physiol. 12:, 98.

Ponec, M., Havekes, l., Kempenaar, J., Lavrisen, S., And Vermeer, B.J. (1984) Defective low-density lipoprotein metabolism in cultured, normal, transformed and malignant keratinocytes. J. Invest. Dermatol. 83: 436.

Poste, G., and Kirsh, R. (1983a) Site-specific (targeted) drug delivery in cancer therapy. Biotechnology. 1: 869-878.

Poste, G. (1983b) Liposome targeting in vivo: problems and opportunities. Biology of the cell 47: 19-38.

Poste, G., and Greig, R. (1982a) The experimental and clinical implications of cellular heterogeneity in malignant tumors. J. Cancer Res. Clin. Oncol. 106: 159-170,

Poste, G., and Kirsh, R. (1982b) Liposome encapsulated macrophage activation agents and active non-specific immunotherapy of neoplastic disease. Prog. Clin. Biol. Res. 102: 309-319.

Poste, G. (1982c) Cellular heterogeneity in malignant neoplasms and the therapy of metastases. Ann NY Acad Sci. 397: 34-48.

Poste, G. (1982d) Experimental systems for analysis of the malignant phenotype. Cancer Metastasis Rev. 1: 141-199.

Poste, G., Tzeng, J., Doll, J., Greig, R., Rieman, D., Zeidman, I. (1982e) Evolution of tumor cell heterogeneity during progressive growth of individual lung metastases. Proc. Natl. Acad. Sci. U.S.A.. 79: 6574-6578.

Poznansky, M.J. and Juliano, R.L. (1984) Biological approaches to the controlled delivery of drugs: a critical review. Pharmacol. Rev. 36: 277-336

Prassl, R., Schuster, B., Abuja, P.M., Zechner, M., Kostner, G.M., and Laggner, P. (1995) A comparison of structure and thermal behavior in human plasma lipoprotein(a) an dlow density lipoprotein. Calorimetry an dsmall-angle X-ray scattering. Biochemistry 34: 3795-3801.

Provencher, S.W. and Glockner, J. (1981) Estimation of globular protein secondary structure from circular dichroism. Biochemistry 20: 33-37.

Quesney-Huncus, V., Galick, K.A., Siperstein, M.D., Erickson, S.K., Spencer, T.A., and

Rahman, A., and Schein, P.S. (1989) Use of liposomes in cancer chemotharapy. In: Gregoriadis, G. (ed.) Liposomes as drug carriers. New York: John Wiley, 1988: 381-400.

Ramsen, J.F., and Shireman, R.B. (1981) Effect of low density lipoprotein on the incorporation of benzo-a-pyrene by cultured cells. Cancer Res. 41: 3179-3185.b4

Reddy, B.S. (1981a) Dietary fat and its relationship to large bowel cause cancer. Cancer Res. 41: 3700-3705.

Reddy, B.S. (1981b) Dietary fibre and colon cancer: epidemiologic and experimental evidence. Can. Med. Assoc. 123: 850-856.

Reddy, B.S. (1981c) Diet and excretion of bile acids. Cancer Res. 41: 3766-3768.

Remsen, J.F., and Shireman, R.B. (1987) Effect of LDL on the incorporation of benzo[a]pyrene by cultured cells. Cancer Res. 41: 3179-3185.

Rensen, P.C.N., Van Dijik, M.C.M., Havenaar, E.C., Bijsterbosch, M.K., Krujit, J.K., and Van Berkel, T.J.C. (1995) Selective liver targeting of antivirals by recombinant chylomicrons-a new therapeutic approach to hepatitis B. Nature (Medicine) 1: 221-225.

Rich, M.A., Bolaffi, J.L., Knoll, J.E., Cheong, L, and Eidinoff, M.L. (1958) Growth inhibition of a human tumor cell strain by 5-flurouracil, 5-fluorouridine, and 5-fluorodeoxyuridine-reversal studies. Cancer Res. 18: 731-735.

Rifici, V.A. and Eder, H.A. (1984) A hepatocyte receptor for high density lipoproteins specific for apolipoprotein AI. J. Biol. Chem. 259: 13814-18.

Rivory, L.P., Avent, K.M., and Pond, S.M. (1996) The effects of lipophilicity and protein binding on the hepatocellular uptake and hepatic disposition of two anthracyclines, doxorubicin and iododoxorubicin. Cancer Chemother. Pharmacol. 38: 439-445.

Robert, J. (1980) Extraction of anthracyclines from biological fluids for HPLC evaluation. J. Liq. Chromatogr. 1: 1561-1572.

Robinson, C.J. (1991) Tailoring and targeting fibroblast growth factors. Trends Biotechnol. 9: 141-8.

Rogerson, A., Cummings, J. Willmott, N. and Florence, AT. (1988) The distribution of

9: 141-8.

Rogerson, A., Cummings, J. Willmott, N. and Florence, AT. (1988) The distribution of doxorubicin in mice following administration in niosomes. J. Pharm. Pharmacol. 40: 337-342.

Rohrer, L., Freeman, M., Kodama, T., Penman, M. And Krieger, M. (1990) Coiled coil fibrous domains mediate ligand binding by macrophage scavenger receptor type II. Nature 343: 570-572.

Romer, J. and Bickel, M.H. (1979) A method to estimate binding constants at variable protein concentrations. J. Pharm. Pharmacol. 31: 7-11.

Rose, G., and Shipley, M.J. (1980) Plasma lipids and mortality: A screen error. Lancet 8: 523-526.

Routledge, P.A. (1986) The plasma protein binding of basic drugs. Br. J. Clin Pharmacol, 22: 499-506.

Ruben, R.L., and Neubauer, R.H. (1987) Semiautomated colorimetric assay for in vitro screening of anticancer compounds. Cancer Treat. Rep. 71: 1141.

Rudling, M.J., Angelin, B., Peterson, C.O., and Collins, V.P. (1990a) Low density lipoprotein receptor activity in human intracranial tumors and its relation to the cholesterol requirement. Cancer Res. 50: 483-487.

Rudling, M.J., Reihner, E., Einarsson, K., Ewerth, S., and Angelin, B. (1990b) Low density lipoprotein receptor binding activity in human tissues: quantitative importance of hepatic receptors and evidence for regulation of their expression in vivo. Proc. Natl. Acad. sci. U.S.A.. 87: 3469.

Rudling, M.J., Stahle, L., Peterson, C. and Skoof, L. (1986) Content of low density lipoprotein receptors in breast cancer tissue related to survival of patients. Brit. Med. J. 292: 580-582

Rudling, M.J., Collins, V.P. and Peterson, C. (1983) Delivery of aclacinomycin A to human glioma cells in vitro by the low density lipoprotein pathway. Cancer Res. 43: 4699-4605

Rudling, M.J., Angelin, B., Peterson,C.O., and Collins, V.P. 91990) Low density lipoprotein receptor activity in human intracranial tumors and its relation to the cholesterol

requirement. Cancer Res. 50: 483.

Rutherford, M.S., Futch, W.S. Jr, and Schook, L.B. (1991) Acetylated low density lipoprotein and the delivery of immunomodulators to macrophages. In. Shaw, J.M. (Ed.) Lipoproteins as carriers of pharmacological agents, Marcel Dekker, New York, 1991 pp: 201-223.

Ryan, R.O., Yokoyama, S., Liu, Hu, Czarnecka, Helena, Oikawa, Kim, and Kay, C.M. (1992) Human apolipoprotein A-I liberated from high-density lipoprotein without denaturation. Biochemistry. 31: 4509-4514.

Ryan, R.O., Wessler, A.N., Price, H.M., Ando, S., and Yokoyama, S. (1990a) Insect lipid transfer particle catalyzes bidirectional vectoral transfer of diacylglycerol from lipophorin to human low density lipoprotein. J. Biol. Chem. 265: 10551-10555.

Ryan, R.O., Wessler, N.A., Proce, M.H., Ando, S., Yokoyama, S. (1990b) Insect lipid transfer particle catalyzes bidirectional vectorial transfer of diacylglycerol from lipophorin to human low density lipoprotein. J. Biol. Chem. 265: 10551-10555.

Ryan, R.O., Van Antwerpen, R., Van der Horst, D.J., Beenakkers, A.M.T., and Law, J.H. (1990c) Manduca Sexta lipid transfer particle acts upon a lipophorin to catalyze lipid and apoprotein disproportion. J. Biol. Chem. 265: 546-552.

Ryan, R.O. (1990d) Dynamics of insect lipophorin metabolism. J. Lipid Res. 1: 725-1739.

Ryan, R.O., Howe, A., and Scraba, D.G. (1990e) Studies of the morphology and structure of the plasma lipid transfer particle from the tobacco hornworm, Manduca Sexta. J. Lipid Res. 31: 871-879.

Ryan, R.O., Senthilathipan, K.R., Wells, M.A., and Law, J.H. (1988) Facilitated DAG exchange between insect hemolymph lipophorins properties of Manduca Sexta lipid transfer particle. J. Biol. Chem. 263: 14140-14145.

Ryan, R.O., Wells, M.A., and Law, J.H. (1986a) Lipid transfer protein from Manduca sexta hemolymph. Biochem. Biophys. Res. Comm. 136: 260-265.

Ryan, R.O., Prasad, S.V., Henriksen, E.J., Wells, M.A., and Law, J.H. (1986b) Lipoprotein interconversions in an insect, Manduca Sexta. Evidence for a lipid transfer factor in the hemolymph. J. Biol. Chem. 261: 563-568.

Ryan, R.O., Prasad, S.V., Henrikson, E.J., Wells, M.A., and Law, J.H. (1986d) Lipophorins interconversions in an insect Manduca Sexta. Evidence for a lipid transfer factor in the hemolymph. J. Biol. Chem. 261: 563-568.

Saiki, I., and Fidler, I.J. (1985) Synergistic activation by recombinant mouse interferon-gamma and muramyl dipeptide of tumoricidal properties in mouse macrophages. J. Immunol. 135: 684-688.

Samadi-Baboli, M., Favre, G., Bernadou, J., Berge, D., and Soula, G. (1990) Comparative study of the incorporation of ellipticin-esters into low density lipoprotein (LDL) and selective cell uptake of drug-LDL complex via the LDL receptor pathway in vitro. Biochem. Pharmacol. 40: 203-212.

Santi, D.V., and Danenberg, P.V. (1984) Folates in pyrimidine nucleotide biosynthesis. In: Blakley, R.L., Benkovic, S.J. (eds). Folates and Pterins. John Wiley & Sons, New York. pp. 345-398

Sautereau, A.M., Plenecassagnes, L., Samadi-Baboli, D., Favre, G., Soula, G., and Tocanne, J.F. (1995) Physico-chemical characterization of low density lipoprotein containing a cytotoxic drug: the 2N-methyl-9-oleoyl-ellipticinium. Eur J. Pharm. Biopharm. 41: 136-139.

Schaffer, W.A., Kin, R., Steme, R., Thomer, J., Kim, S.H., and Rine, J. (1989). Genetic and pharmacological suppression of oncogenic mutations in RAS genes of yeast and humans. Science 245: 379-385.

Schouten, D., Van der Kooij, M., Muller, J., Pieters, M.N., Bijsterbosch, M.K., Van Berkel, T.J. (1993) Development of lipoprotein like lipid particles for drug targeting: neo-high density lipoproteins. Mol. Pharmacol. 44: 486-92.

Schroeder, F., Kier, A.B. Olsen, C.D., and Dempsey, N.E. (1984) Correlation of tumor metastasis with sterol carrier protein an dplasma membrane sterol levels. Biochem. Biophys. Res. Commun. 124:P 283.

Schultis, H.W., Von Baeyer, H., Neitzel, H., and Riedal, E. (1991) Preparation of nucleoside-LDL conjugates for the study of cell-selective internalization: stability characteristics and receptor affinity. Eur. J. Clin. Chem. Clin. Biochem. 29: 665-674.

Schultis, H.W., Baryer, H.V., Neitzel, H. and Riedel, E. (1990) Functional characteristics of LDL particles derived from various LDL-apheresistechniques regarding LDL-drug

complex preparation. J. Lipid. Res. 31: 2277-2284.

Schumaker, V.N. and Puppione, D.L. (1986) Sequential flotation ultracentrifugation. Methods in Enzymology, 128: 155-170.

Schwender, R.A., Lagocki, P.A., and Rahman, Y.E. (1984) The effects of charge and size on the interaction of unilamellar liposomes with macrophages. Biochim. Biophys. Acta 772: 93-101.

Sedmak, J.J., and Grossberg, S.E. (1977) A rapid, sensitive, versatile assay for protein using Coomassie brilliant blue G250. Anal. Biochem. 79: 544-552.

Seevers, R.H., Grosiak, M.P., Weichart, J.P., Schwendner, S.W., Longino, M.A., and Counsell, R.E. (1982) Potential tumor- and organ-imaging agents. 23: sterol esters of iopanoic acid. J. Med. Chem. 25: 1500-1503.

Seki, J., Okita, A., Watanabe, M., Nakagawa, T., Honda, K., Tatewaki, N., and Sugiyama, M. (1985) Plasma lipoproteins as drug carriers: pharmacological activity and disposition of the complex of beta-sitosteryl-beta-D-glucopyronoside with plasma lipoproteins. J. Pharm. Sci. 74: 1259-1264.3.78

Seshadri, R., Idriss, J.M., and Isreal, M. (1986) Adriamycin analogues. Preparation and biological evaluation of some thio ester analogues of adriamycin and N-(trifluoroacetyl)-adriamycin-14-valerate. J. Med. Chem. 29: 1269-1273.

Sezaki, H., and Hashida, M., (1985) Macromolecules as drug delivery systems. In: Borchardt, R.T., Repta, A.J., and Stella, V.J. (Eds.), Directed Drug Delivery. Humana Press, NJ, pp. 189-208.

Sezaki, H., Takakura, Y., and Hashida, M. (1989) Soluble macromolecular carriers for delivery of antitumor agents. Adv. Drug Deliv. Rev. 3: 247-266.

Shaw, J.M., Futch, W.S. Jr, and Schook, L.B. (1988) Induction of macrophage antitumor activity by acetylated low density lipoprotein containing lipophilic muramyl tripeptide. Proc. Natl. Acad. Sci. U.S.A. 85: 6112-6116.

Shaw, J.M., Shaw, K.V., Yanovich, S., Iwanik, M., Futch, W.S., Rosowsky, A., and Schook, L.B. (1987) Delivery of lipophilic drugs using lipoproteins. Ann. NY. Acad. Sci. 507: 252-271.

Shepherd, J. And Packard, C.J. (1986) Receptor-independent low-density lipoprotein

catabolism. In: Albers, J.J., and Segrest, J.P. (Eds), Methods of Enzymology. 129. Academic press, New York. pp. 566-590.

Sherwin, R.W., Wentworth, J.A., Cutler, J.A., Hulley, S.B., Kuller, L.H., Stamler, J. (1987) Serum cholesterol levels and cancer mortality in 361, 662 men screened for the multiple risk factor intervention trial. J. Am. Med. Assoc. 257: 943-948.

Shu, H.P., and Nichols, A.V. (1979) Benzo(a)pyrene uptake by human plasma lipoproteins in vitro. Cancer Res. 39: 1224-1230 .

Simpson, E.R., Bilheimer, D.W., Macdonald, P.C. and Porter, J.C. (1979) Uptake and Degradation of Plasma Lipoproteins by Human Choriocarcinoma Cells in culture. Endocrinology 104: 8-16

Singh, T.K.A., Scraba, D.G., and Ryan, R.O. (1992) Conversion of human low density lipoprotein into a very low density lipoprotein-like particle in vitro. J. Biol. Chem. 267: 9275-9280.

Singh, T.K.A., and Ryan, R.O. (1991) Lipid transfer particle-catalyzed transfer of lipoprotein associated diacylglycerol and long chain aliphatic hydrocarbons. Arch Biochem. Biophys. 286: 376-382.

Skinner, R., Pearson, D.J.A., Coulthard, M.G., Skillen, A.W., Hodson, A.W., Goldfinch, M.E., et al. (1991) Assessment of chemotherapy-associated nephrotoxicity in children with cancer. Cancer Chemother. Pharmacol. 28: 81-92.

Skladanowski, A., Konopa, J. (1993) Adriamycin and daunomycin induce programmed cell death (apoptosis) in tumour cells. Biochem. Pharmacol. 46: 375-382.

Sklar, L.A., Craig, I.P., Poenall, J.H. (1981) Induced circular dichroism of incorporated fluorescent cholesteryl esters and polar lipids as a probe of human serum low density lipoprotein structure and melting. J. Biol. Chem. 256: 4286-4292.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., and Provenzano, M.D. et al. (1985) Measurement of protein using bicinchoninic acid. Anal. Biochem. 150: 76-85.

Sorg, C. (1982) Heterogeneity of macrophages in response to lymphokines and other signals. Mol. Immunol. 19: 1275-1278.

Soutar, A.K. and Knight, B.L. (1990) Structure and regulation of the LDL-receptor and its

gene. Br. Med. Bull. 46: 891-916.

Spady, D.K., Turley, S.D., and Dietschy, J.M. (1985a) Receptor-independent low density lipoprotein transport in the rat in vivo. Quantitation, characterization, and metabolic consequences. J. Cin Invest 76: 1113-1122.

Spady, D.K., Turley, S.D., and Dioetschy, J.M. (1985b) Rates of low-density lipoprotein uptake and cholesterol synthesis are regulated independently in theliver. J. Lipid Res. 26: 465-472.

Spady, D.K., Stange, E.F., Bilhartz, L.E., and Dietschy, J.M. (1986) Bile acids regulate hepatic low density lipoprotein receptor activity in the hamster by altering cholesterol flux across the liver. Proc. Natl. Acad. Sci. U.S.A. 83: 1916-1920.

Spiegel, R.J., (1981) The acute toxicities of chemotherapy. Cancer Treat. Rev. 8: 197-207.

Springer C.J., Bagshawe, K.D., Sharma, S.K., et al. (1991) Ablation of human chloriocarcinoma xenografts in nude mice by antobody-directed enzyme prodrug therapy (ADEPT) with three novel compounds. Eur. J. Cancer 11: 1362-6.

Sqalli-Houssaini, H., Pierlot Kusnierz, J.P., Parmentier, B., Martin-Nizard, F., Lestavel-Delatte, S., Tartar, A., Fruchart, J.C., Sergheraert, C,. and Duriez, P. (1994) Preparation of anti-HIV-Low-density lipoprotein complexes for delivery of anti HIV drugs via the low density lipoprotein pathways. Biotech. Therap. 5: 69-85.

Stein, Y., halperin, G., and Stein, O. (1981) The fate of cholesteryl linoleyl ether and cholesteryl linoleate in the intact rat after injection of biologically labeled human low density lipoprotein. Biochim. Biophys. Acta 663: 569-574.

Steinbrecher, U.P., Witztum, J.L., Kesaniemi, Y.A., and Elam, R.L. (1984) Comparison of glucosylated low density lipoprotein with methylated or cyclohexadione-treated low density lipoprotein in the measurement of receptor-independent low density lipoprotein in catabolism. J. Clin. Invest. 71: 960-964.

Steinbrecher, U.P. (1987) Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. J. Biol. Chem. 262: 3603-3608.

Storm, G., Van Bloois, L., Steerenberg, P.A., Van Ellen, E., De Groot, G., and Crommelin, DJA. (1989) Liposome encapsulation of doxorubicin: pharmaceutical and therapeutic aspects . J. Control. Release 9: 215-229.

Suits, A.G., Chait, A., Aviram, M. and Heinecke, J.W. (1989) Phagocytosis of aggregated lipoprotein by macrophages: low density lipoprotein receptor-dependent foam cell formation. Proc. Natl. Acad. Sci. U.S.A. 86: 2713-2717.

Sun, X.M., Webb, J.C., Gudnason, S., Humphries, M., Seed, G.R., Knoght, T.B.L., and Soutar, A.K. (1992) Characterization of deletions in the LDL receptor gene in patients with familial hypercholesterolemia in the United Kingdom. Arterioscl. Thromb. 12: 762-770.

Swanson, C.A., Potischman, N., Barrett, R.J., Berman, M.L., Mortel, R., Twiggs, L.B., Wilbanks, G.D., Hoover, R.N., Brinton, L.A. (1994). Endometrial Cancer risk in relation to serum lipids and lipoprotein levels. Cancer Epidemiology, Biomarkers & Prevention 3: 575-581.

Takatori, T., Koizumi, T., Tokui, T., Mitsuhashi, Y., Shiraishi, A., Tsuruo, T. (1995) Intracellular activation and cytotoxic action of RS-1541 against cultured human tumor cells. Cancer Chemother Pharmacol. 35: 383-290.

Tall, A.R., Abreu, E., and Shuman, J. (1983) Separation of a plasma phosphiolipid transfer protein from cholesteryl ester/phospholipid exchange protein. J. Biol. Chem. 258: 2174-2180.

Tall, A.R., and small, D.M. (1980) Body cholesterol removal: role of plasma high density lipoproteins. Adv. Lipid Res. 17: 1-51.b4

Tall, A.R. (1986) Plasma lipid transfer proteins. J. Lipid Res. 27: 361-367.

Talmud, P.J., Barni, N., Kessling, A.M., Carlsson, P., Darnfors, C., Bjursell, G., Galton, D., Wynn, V., Kirk, H., Hayden, M.R., and Humphries, S.E. (1987) Apolipoprotein B gene variants are involved in the determination of serum cholesterol levels: a study in normol- and hyperlipidaemic individuals. Atherosclerosis 67: 81-89.

Tokui, T., Tokui, Y., Ishigami, M., Tanzawa, K., Ikeda, T., and Komai, T. (1994a) Targeting of an antitumor agent, RS-1541 (palmitoyl-rhizoxin), via low-density lipoprotein receptor. Int. J. Pharmaceutics 110: 277-283.

Tokui, T., Maeda, N., Kuroiwa, C., Sasagawa, K., Inoue, T., Kawai, K., Ikeda, T., Komai, T. (1995) Tumor selective effect of RS-1541 (palmitoyl-rhizoxin) in M5076 sarcoma and host tissues in vivo. Pharmaceut. Res. 12: 370-375.

Tokui, T., Kuroiwa, C., Tokui, Y., Sasagawa, K., Kawai, K., Kobayashi, T., Ikeda, T., Omai,

Tokui, T., Kuroiwa, C., Tokui, Y., Sasagawa, K., Kawai, K., Kobayashi, T., Ikeda, T., Omai, T. (1994b) Contribution of serum lipoproteins as carriers of antitumor agents RS-1541 (palmitoyl rhizoxin) in mice. Biopharm. and Drug Dispos. 15: 93-108.

Tollefson, J.H., Ravnik, S., and Albers, J.J. (1988) Isolation and characterization of a phospholipid transfer protein (LTP-II) from human plasma. J. Lipid Res. 29: 1593-1602.

Tomberg, S.A., Holm, L.E., Cartensen, J.M., Eklund, G.A. (1989) Cancer incidence and cancer mortality in relation to serum cholesterol. J. Natl. Cancer Inst. 81: 1917-1921.

Tomlinson, E. (1991) Design of site specific protein drugs. The Science of the Total Environment 109/110: 9-16.

Tomlinson, E. (1989b) Considerations in the physiological delivery of therapeutic proteins, in Presscott, L.F. and Nimmo, W.S. (Eds), Novel drug delivery and its therapeutic applications, John Wiley & Sons Ltd. Chichester, 1989, Chap 23, pp. 245-262.

Tomlinson, E. (1989a) Selective delivery of protein drugs, in T.J.R. Harris (Ed.), Protein Production, Elsevier Applied Science, Amsterdam, 1989, pp. 207-225.

Tomlinson, E. (1987) Theory and practice of site specific drug delivery. Adv. Drug Delivery Rev. 1: 87-198.

Tracy, L.S., and Jean, D.D. (1991) Interference of biogenic amines with the measurement of proteins using bicinchoninic acid. Anal. Biochem. 195: 14-17.

Tritton, T.R., and Yee, G. (1982) The anticancer agent adriamycin can be actively cytotoxic without entering cells. Science 217: 248-250.

Trouet, A., Masquelier, M., Baurain, R., Campeneere, D.D. (1982) A covalent linkage between daunomycin and proteins that is stable in serum and reversible by lysosomal hydrolases, as required for a lysosomotropic drug-carrier conjugate: in vitro and in vivo studies. Proc. Natl. Acad. Sci. U.S.A. 79: 626-629.

Trouet, A., Deprez-De Campeneere, D., and De Duve, C. (1979) Chemotherapy through lysosomes with DNA-daunorubicin complex. Nature 239: 110-112.

Trowbridge, I.S., and Domingo, D.L. (1981) Antitransferrin receptor monoclonal antibody

and toxic antibody conjugates that effect growth of human tumor cells. Nature 294: 171-173.

Tsomides, T.J., Walker, B.D., and Eisen, H.N. (1991) An optimal viral peptide recognized by CD+T cells binds very tightly to the restricting class I major histocompatibility complez protein on intact cells but not to the purified class I protein. PNAS 88, 11276-11280.

Tsuchida, K., Takeda, H., Mochida, M., Tonosaki, Y., Moribayashi, A., and Maekawa, H. (1995) Lipophorin and lipid transfer protein of *Bombyx Mori*. J. Cellular Biochemistry, 21A. pp. 226.

Tuszynski, G.P., Knight, L.C., Korneki, E., and Srivastava, S. (1983) Labeling of platelet surface proteins with ^{125}I by the iodogen method. Anal. Biochem. 130: 166-170.

Tuszynski, G.P. and Murphy, A. (1990) Spectrophotometric quantitation of anchorage-dependent cell numbers using the bicinchoninic acid protein assay reagent. Anal. Biochem. 184: 189-191.

Van de Water, B., Van Berkel, T.J.C., and Kuiper, J. (1994) Activation of rat Kupffer cells to tumoricidal cells by the immunomodulator muramyl tripeptide-phoaphotidylethanolamine incorporated into the novel drug carrier lactosylated low density lipoprotein. 45: 971-977.

Van Berkel, Th.J.C., and Kuiper, J., (1994), Activation of rat kupffer cells to tumoricidal cells by the immunomodulator muramyl tripeptide-phosphatidylethanolamine incorporated into the novel drug carrier lactosylated low density lipoprotein. 45: 971-7.

Van Berkel, T.J.C. (1993) Drug targeting: application of endogenous carriers for site-specific delivery of drugs. J. Controlled release. 24: 145-155.

Van Berkel, T.J., de Smidt, P.C., Van Dijk, M.C., Ziere, G.J., and Bijsterbosch, M.K. (1990) Drug targeting by endogenous transport vehicles. Biochem. Soc. Transac. 18: 748-50.

Van Berkel, T.J., Krujit, J.K., de Smidt, P.C., and Bijsterbosch, M.K. (1991), Receptor-dependent targeting of lipoproteins to specific cell types of the liver, Targeted Diagn. Ther., 5, 225-49.

Van Berkel, T.J.C. (1993) Drug targeting: application of endogenous carriers for site-specific delivery of drugs. J. Controlled Release 24: 145

Vega, G.L., and Grundy, S.M. (1986) In vivo evidence for reduced binding of low density lipoproteins to receptors as a cause of primary moderate hypercholesterolemia. J. Clin. Invest. 78: 1410-1414.

Vermeer, B.J., Marian, M.D., Wijsman, C., Mommaaskienhuis, A.M., and Ponec, M. (1986) Binding and internalization of low density lipoproteins in SCC25 cells and SV 40 transformed eratinocytes- A morphologic Study. J. Invest. Dermatol. 86: 195-200.

Versluis, A.J., Van Geel, P.J., Oppelaar, H., Van Berkel, T.J.C., and Bijsterbosch, M.K. (1996) Receptor-mediated uptake of low density lipoprotein by B16 melanoma cells in vitro and in vivo in mice. Br. J. Cancer 74: 525-532.

Via, D.P., Craig, I.F., Jacobs, G.W., Van Winkle, W.B., Charlton, S.C., Gotto, A.M. Jr., and Smith, L.C. (1982) Cholesteryl ester rich microemulsions: Stable preotein free analogs of low density lipoproteins. J. Lipid Res. 23: 570-576.3.

Via, D.P., Dresel, H.A., Cheng, S.L. and Gotto, A.M. (1985) Murine macrophage tumors are a source of a 260,000-dalton acetylated low density lipoprotein receptor. J. Biol. Chem. 260: 7379-7386

Vico, F.R., Cayuela, M.M., Peregrin, E.D., and Ramiraz, H. (1989) A procedure for eliminating interferences in the Lowry method for protein determination. Anal. Biochem. 183: 275-278.

Vingerhoeds, M.H., storm, G, and Crommelin, D.J.A. (1994). Immunoliposomes in vivo. Immunomethods 4: 259-272

Vitols, S., Peterson, C., Larsson, O., Holm, P., and Aberg, B. (1992) Elevated uptake of low density lipoproteins by human lung cancer tissue in vivo. Cancer Res. 52: 6244.

Vitols S. (1991) Uptake of low-density lipoprotein by malignant cells-possible therapeutic applications. Cancer Cells 3: 488.

Vitols, S., Angelin, B., Ericsson, S., Gahrton,G., Juliusson, G., Masquilier, M., Paul, C., Peterson, C., Rudling, M., Soderberg-Reid, K., and Tidefelt, U. (1990a) Uptake of low density lipoprotein by human leukemic cells in vivo: relation to plasma lipoprotein levels and possible relevance for selective chemotherapy. Proc. Natl. Acad. Sci. U.S.A. 87: 2598.

Vitols, S., Soderberg-Reid, K., Masquelier, M., Sjostrom, B., and Peterson, C. (1990b) Low density lipoprotein for delivery of a water-insoluble alkylating agent to malignant

cells. In vitro and in vivo studies of a drug-lipoprotein complex. Br. J. Cancer 62: 724-729.

Vitols, S.G., Masquelier, M., and Peterson, C.O. (1985a). Selective uptake of a toxic lipophilic anthracycline derivative by the low density lipoprotein receptor pathway in cultured fidroblasts. J. Med. Chem. 28: 451-454.

Vitols, S., Gahrton, G., Bjorkholm, M. and Peterson, C. (1985b) Hypocholesterolaemia in Malignancy Due to Elevated Low Density Lipoprotein Receptor Activity in Tumor Cells: Evidence from Studies in Patients with Leucaemia. Lancet 2: 1150-1153.

Vitols, S., Gahrton, G. and Peterson, C. (1984a) Significance of the Low Density Lipoprotein Receptor Pathway for the in Vitro Accumulation of Ad-32 Incorporated into LDL in Normal and Leukemic White Blood Cells. Cancer Treatment Reports 68: 515-520.

Vitols, S., Gahrton, G., Ost, A. and Peterson, C. (1984b) Elevated Low Density Lipoprotein Receptor Activity in Leucemic Cells with Monocytic Differentiation. Blood 63: 1186-1193.

Vlassara, H., Brownlee, M., and Cerami, A. (1985) High affinity receptor mediated uptake and degradation of glucose-modified proteins: a potential mehanism for the removal of senescent macromolecules. Proc. Natl. Acad. Sci. U.S.A. 82: 5588-5592.

Voyta, J.C., Via, D.P., Butterfield, C.E., and Zetter, B.R. (1984) Identification and isolation of endothelial cells based on their increased uptake of acetylated low density lipoprotein. J. Cell Biol. 99: 2034-2040

Waller, D.G., and George, C.F. (1989) Prodrugs. Br. J. Clin. Pharmacol. 28: 693-705.

Wang, J., Liu, H., Sykes, B.D., and Ryan, R.O. (1995) Identification and localization of two distinct microenvironments for the diacylglycerol component of lipophorin particles by ^{13}C-NMR. Biochemistry 34: 6755-6761.

Wasan, K.M., Morton, R.E., Rosenblum, M.G., Lopez-Berestein, G. (1994) Decreased toxicity of liposomal amphotericin B due to association of amphotericin B with high density lipoproteins: Role of lipid transfer protein. J. Pharm. Sci. 83: 1000-1010.

Wasan, K.M., and Lopez-Berestein, G. (1994) The interaction of liposomal amphotericin B and serum lipoproteins within the biological milieu. J. Drug Targeting, 7: 373-380.

Wasan, K.M., Vadiei, K., Lopez-Berestein, G, and Luke, D.R. (1990a) Enhancement of the treatment of experimental candidiasis with vascular decongestants. J. Infect. Dis. 161: 562-566.

Wasan, K.M., Vadiei, K., Lopez-Berestein, G, and Luke, D.R. (1990b) pKs, tissue distribution, and toxicity of free and liposomal amphotericin B in diabetic rats. J. Infect. Dis. 162: 211-214.

Wasan, K.M., Brazeau, G.A., Keyhani, A., Hayman, A.C., and Lopez-Berestein, G. (1993) Roles of liposome composition and temperature in distribution of amphotericin B in serum lipoproteins. Antimicrobial Agents and Chemotherapy 37: 246-250.

Wasan, K.M., and Morton, R.E. (1996) Differences in lipoprotein concentrations and composition modify the plasma distribution of free and liposomal annamycin. Pharmaceut. Res. 13: 462-468.

Wasan, K.M., and Perez-Soler, R. (1995) Distribution of free and liposomal annamycin with human plasma is regulated by plasma triglyceride concentrations but not by lipid transfer protein. J. Pharm. Sci. 84: 1094-1100.

Wasan, K.M., Grossie, V.B. Jr., Lopez-Berestein, G. (1994) Concentrations in serum and distribution in tissue of free and liposomal amphotericin B in rats during continuous intralipid infusion. Antimicrobial Agents Chemother. 38: 2224-2226

Wasan, K.M., and Lopez-Berestein, G. (1993) The influence of serum lipoproteins on the pharmacokinetics and pharmacodynamics of lipophilic drugs and drug carriers. Arch. Med. Res. 24: 395-401.

Weichert, J.P., Longino, M.A., Bakan, D.A., Spigarelli, M.G., Chou, T.S., Schwendner, S.W., and Counsell, R.E. (1995) Polyiodinated triglyceride analogs as poetntial computed tomography imaging agents for the liver. J. Med. Chem. 38: 636-646.

Weichert, J.P., Van Dort, M.E., Groziak, M.P., Counsell, R.E. (1986) Radioiodination via isotope exchange in pivalic acid. Appl. Radiat. Isot. 37: 907-913.

Weichselbaum, T.E. (1946) An accurate and rapid method for the determinations of proteins in small amounts of blood serum and plasma. Amer. J. Clin. Pathol. 7: 40.

Weiss, R., Sarosy, G., Clagett-Carr, K., Russo, M., and Leyland-Jones, B. (1986) Anthracycline analogs: the past, present, and future. Cancer Chemother. Pharmacol.

18: 185-197.

Welsh, J., Calman, K.C., Stuart, F., Glegg, J., Stewart, J. M., Packard, C.J., Morgan, H.G., and Shepherd, J. (1982) Low density lipoprotein uptake by tumors. Clin. Sci. 63, 44p.

Westesen, K., Gerke, A., and Koch, M.H.J. (1995) Characterization of native and drug-loaded human low density lipoproteins. J. Pharm. Sci. 84: 139-147.

Wetterau, J.R., Combs, K.A., Mclean, L.R., Spinner, S.N., Aggerback, L.P. (1991) Protein disulfide isomerase appears necessary to maintain the catalytically active structure of the microsomal triglyceride transfer protein. Biochemistry 30: 9728-9235.

Wetterau, J.R., Aggerback, L.P., Bouma, M.E., Eisenberg, C., Munck, A., Hermier, M., Schmitz, J., Gay,G., Radar, D.J., Gregg, R.E. (1992) Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. Science 258: 999-1001.

Wiechelman, K.J., Braun, R.D., and Fitzpatrick, J.D. (1988) Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. Anal. Biochem. 175: 231-237.

Wilkinson, G.R. (1983) Plasma and tissue binding considerations in drug disposition. Drug Metab Rev. 14: 427-465.

Willmott, N., Cummings, J., Stuart, J.F.B., and Florence, A.T. (1985) Adriamycin loaded albumin microspheres. Preparation, in vivo distribution and release in the rat. Biopharm. Drug Dispos 6: 91-104.b4

Wirth, J.J., and Kierszenbaum, F. (1988) Recombinant tumor necrosis factor enhances macrophage destruction of Trypanosoma cruzi in the presence of bacterial endotoxin. J. Immunol 141: 286-288.

Witztum, J.L., and Steinberg, D. (1991) Role of oxidized low density lipoprotein in atherogenesis. J. Clin. Invest. 88: 1785-1792.

Woodle, M.C. (1995) Sterically stabilized liposome therapeutics. Adv. Drug Delivery Rev. 16: 249-265.

Woodle, M.C., and Lasic D.D. (1992) Sterically stabilized liposomes. Biochim. Biophys. Acta. 1113: 171-199.

Word, J.A., Furr, B.J.A., Valeaccia, B. et al. (1989) Prolonged suppression of rat testis function by a depot formulation of Zoladex, a GnRH agonist. J. Androl. 10: 478-486.

Wright, S. and Huang, L. (1989) Antibody-directed liposomes as drug delivery vehicles. Adv. Drug Deliv Rev. 3: 343-389.

Yamaguchi, T., and Mizushima, Y. (1994) Lipid microspheres for drug delivery from a pharmaceutical viewpoint. Crit. Rev. Ther. Drug Carriers Sys. 11: 215-229.

Yang, M.B., Tamargo, R.J., and Brem, H. (1989) Controlled delivery of 1,3-bis(2-choloroethyl)-1-nitrosourea from ethyl vinyl acetate copolymer. Cancer Res. 49: 5103-5107.

Yang, C.Y., Chen, S.H., Gianturco, S.H., Bradley, W.A., Sparrow, J.T., Tanimura, M., Li, W.H. Sparrow, D.A., De Loof, H., Rosseneu, M., Lee, F.S., Gu, Z.W., Gotto, A.M., Chan, L. (1986) Sequence, structure, receptor-binding domains and internal repeats of human apolipoprotein B-100. Nature 323: 738-742.

Yanovich, S., Preston, L., and Shaw, J.M. (1984) Characteristics of uptake and cytotoxicity of a low-density lipoprotein-Daunomycin complex in P388 leukemic cells. Cancer Res. 44: 3377-3382.

Yeh, C.J.G., Taylor, C.G., Faulk, W.P. (1984). Targeing of cytotoxic drug by trnsferrin receptors: Selective killing of acute myelogenous leukemia cells. Protides Biol Fluids. 32: 441.

Yeh, C.J.G. and Faulk, W.P. (1984) Killing of human tumor cells in culture with adriamycin conjugates of human transferrin. Clin. Immunol. Immunopathol. 32: 1-11.

Yoo, J.S.H., Norman, J.O., Joe, C.O., Busbee, D.L. (1986) High density lipoprotein decrease both DNA binding and mutagenicity of R-7, T-8-dihydroxy-T-9, 10-epoxy-7,8,9,10-tetrahydrobenzo-a-pyrene in V79 Chinese hamster cells. Mutat. Res. 159: 83-89.

Zhang, H., Yang, Y. and Steinbrecher, U.P. (1993) Structural requirements for the binding of modified proteins to the scavenger receptor of macrophages. J. Biol. Chem. 268: 5535-5542.

Zyada, L.E., Hassan, H.T., Rees, J.K.H., and Ragab, M. H. (1990) The relationship between

hypocholesterolemia and degree of maturation in acute myeloid leukemia. Hematol. Oncol. 8: 65-69.