EVALUATION OF RECONSTITUTED HIGH DENSITY LIPOPROTEIN AS AN ANTICANCER DRUG DELIVERY PLATFORM

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

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December 2009

ACKNOWLEDGEMENTS

I thank my mentor for his encouragement and guidance over the course of this project. I also thank my committee members, Dr. Basu, Dr. Kim, Dr. Nair, and Dr. Cammarata. Many thanks go out to our laboratory's long-time collaborator, Dr. Walter McConathy. I have had several wonderful people working with me in the lab over the years, Sulabha, Paranape, Sabitha Buttreddy, Maninder Malik, etc. I would like to thank Dr. Caffrey's lab for advice regarding protein iodination protocols. I also thank Dr. Jerry Simecka's research group for their advice and assistance with animal studies; they gave invaluable input into this project. I also thank Dr. Monte Krieger at MIT for the gift of the ldl A7 and mSRBI-transfected cell lines, as well as Dr. Michael Oda at the Children's Hospital Oakland Research Institute for the Apo A-1-expressing clone and his advice. I also must thank my family and friends, particularly my husband, Robert, for all their support. This project was partially funded by the National Science Foundation Project Score fellowship and the National Institute of Health Neurobiology of Aging Training Grant.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	ii
LIST OF TABLES	v
LIST OF ILLUSTRATIONS	vi
CHAPTER I	

INTRODUCTION	1
--------------	---

Chemotherapy Drug Delivery Vehicles	3
a. Liposomes	4
b. Lipoproteins	5
HDL Metabolism and Selective Lipid Uptake Mechanism	7
a. Apolipoprotein A-1 and HDL Formation	7
b. Scavenger Receptor Class B, Type I and	
Selective Lipid Uptake	9
c. HDL, SR-BI and Cancer	10
Project Goals	11
Note on Materials Used in the Dissertation	13
References	38

CHAPTER II

PREPARATION OF RECONSTITUTED HIGH DENSITY LIPOPROTEIN

Preface	44
Summary	45
Introduction	46
Materials and Methods	48
Results	51
Discussion	54
References	72

CHAPTER III

CHARACTERIZATION OF RECONSTITUTED HIGH DENSITY LIPOPROTEIN

73
74
75
76
81
84
102

CHAPTER IV

DELIVERY MECHANISM OF RECONSTITUTED HIGH DENSITY LIPOPROTEIN

Preface	105
Summary	106
Introduction	107
Materials and Methods	109
Results	112
Discussion	114
References	124

CHAPTER V

CONCLUSIONS AND DISCUSSION

127
128
131
134
154

LIST OF TABLES

CHAPTER II

Table 1: Components of Drug Delivery Vehicles for Paclitaxel	59
Table 2: Results of Different Emulsifiers/Solvents for Paclitaxel	65
Table 3: Comparison of Chemical Composition of Different	
rHDL/PTX formulations	70
CHAPTER III	
Table 1: Composition of rHDL/PTX particles	86
Table 2: Comparison of IC50 values for selected cancer cell lines with rHDL/PTX and free PTX	96
CHAPTER V	
Table 1: Chemical Composition of rHDL and Sparks HDL	137

LIST OF ILLUSTRATIONS

CHAPTER I

Figure 1: Structure of paclitaxel	14
Figure 2: Structure of liposomes	16
Figure 3: Comparison of Lipoprotein Sizes	18
Figure 4: Localization of a hydrophobic drug in the core region of a generic lipoprotein	20
Figure 5: Conformation of Apolipoprotein A-1	22
Figure 6: Transformation of the discoidal precursor HDL to its stable, spherical configuration	24
Figure 7: Structure of Scavenger Receptor, Class B, Type I	26
Figure 8: Expression of SR-BI in Various Cancer Cell Types	28
Figure 9: Expression of SR-BI in Leukemia Cell Lines	30
Figure 10: Expression of SR-BI in Breast Cancer Cell Lines	32
Figure 11: Expression of SR-BI in Ovarian Cancer Cell Lines	34
Figure 12: Illustration of rHDL structure and selective anticancer drug delivery by rHDL/drug complexes	36
CHAPTER ΙΙ	

CHAPTER II

Figure 1: Tissue Distribution of Taxol vs. rHDL/Taxol	56
Figure 2: Incorporation of Paclitaxel into Drug Delivery Vehicles	61

Figure 3: Dist	ribution of Paclitaxel between Liver and Blood n the Mouse	63
Figure 4: KBr I	Density Gradient Ultracentrifugation of Different rHDL/PTX preparations	67
CHAPTER II	Ι	
Figure 1: Rete	ention of paclitaxel (PTX) vs. sodium cholate	88
Figure 2: Dist	ribution of the radioactivity of [14C] paclitaxel	90
Figure 3: Elec	tron micrograph of the negatively stained reconstituted high density lipoprotein/paclitaxel nanoparticles	92
Figure 4: Cyto	otoxicity of the reconstituted high-density lipoprotein (rHDL)/paclitaxel (PTX) nanoparticles	94
Figure 5: Wei	ght loss incurred by female C57Bl/6 mice after three consecutive injections of paclitaxel	98
Figure 6: Wei	ght loss data (mean±SD) combined for days 4–7	100
CHAPTER IV	T	
Figure 1: Incr	eased Uptake of Cholesteryl esters and Paclitaxel by SR-BI transfected cells	116
Figure 2: SR-	BI is Expressed in Prostate Cancer Cell Lines	118
Figure 3: Mec	hanism of Paclitaxel Uptake in PC3 Prostate Cancer Cell Line is Receptor-Mediated	120
Figure 4: Pacl	itaxel Uptake by the OVCAR-3, ovarian cancer cell Line	122
CHAPTER V		
Figure 1. Com	nparison of Anti-Tumor Activity of Sonicated (Sparks) or Cholate Dialysis rHDL/PTX in MDA-MB-435 Tumor-Bearing Mice.	139
Figure2: Acti	vity of rHDL/PTX in MDA-MB-435 Tumor-Bearing Mice	141
Figure 3: Elec	ctron Microscopy of rHDL Vehicle	143

Figure 4:	Expression of SR-BI in Brain Cancer Cell Lines	145
Figure 5:	Cytotoxicity of rHDL/PTX vs. PTX in Normal Human Astrocytes	147
Figure 6:	Comparison of Cytotoxicity of rHDL/PTX vs. PTX in U118-MG Glioblastoma Cell Line	149
Figure 7:	Tissue Distribution of Alexa 555-siRNA complexed with rHDL	151

CHAPTER I

INTRODUCTION

The war on cancer was declared by President Richard Nixon in 1971, but after almost four decades, cancer is edging out heart disease as the leading cause of death in America. The variability of human cancers and recurrence of tumors are all factors in the difficulty of cancer treatment. The limitations of treatment are the side effects of cancer therapy, tumor drug resistance, and accessibility of drugs to tumor tissue. The classic target of the original chemotherapy agents were cells with a high growth rate. These chemotherapy agents kill tumor cells, but also target proliferating cells such as hair follicle and gut epithelial cells. Targeting of normal proliferating cells leads to the most common side effects of cancer therapy include nausea, alopecia, anemia, and leukopenia. Besides common side effects, there are often drugspecific side effects such as cardiotoxicity induced by doxorubicin (Adriamycin) therapy. Paclitaxel, which is an anticancer agent found in the bark of the Pacific yew tree, targets tubulin and stabilizes microtubules. Microtubule stabilization prevents microtubule disassembly during mitosis and eventually induces apoptosis. Paclitaxel (Figure 1) is a high molecular weight compound (MW = 853.9 g/mol) with an octanol/water partition coefficient (logP) of 3.5 (1). Due to its water insolubility, paclitaxel is formulated into Taxol[®], in which the drug is combined with the solubilizer Cremophor EL/ethanol. The drug-specific side effects of Taxol® include neutropenia, neuropathy, and hypersensitivity reactions, including anaphylaxis. Abraxane, a paclitaxel-serum albumin complex, has reduced side effects and a higher therapeutic index than Taxol ® (2). However, the stability of Abraxane can be called into question, as this formulation must be used within 8 hours of reconstitution for intravenous administration.

As our understanding of cancer biology expands, new agents target different aspects of tumor cells. Due to the over-expression of certain receptors, some drugs target hormone and growth receptors, either as antibodies such as herceptin or antagonists such as tamoxifen. The blood supply needed by tumors is also being targeted by angiogenesis inhibitors such as Avastin. Gene therapy to replace a mutated tumor suppressor or knockout an oncogene is also considered avenues for novel therapies. While an attractive future avenue for cancer therapy, gene delivery has numerous obstacles to surmount including the large size of genes and their targeting to tumors. The knockout of genes, important for the survival of cancer cells, can be accomplished through the use of RNA interference. However, appropriately designed carrier systems may be needed for siRNA therapy, to overcome their high negative charge that hinders the crossing of the cell membranes.

Tumor drug resistance is often a major factor in the failure of conventional cancer chemotherapy and the recurrence of tumors. Cancer drugs that attempt to diffuse through the plasma membrane of cancer cells are pumped out the cell by ABC transporters. These ABC transporters (normally important in nutrient transport) are over-expressed in some cancer cells, becoming drug efflux pumps, preventing apoptosis and cell death induced by chemotherapy. The ABC transporters involved in drug resistance have been named Multi-Drug Resistance (MDR1) or Multidrug Resistance-associated Protein (MRP1) (3, 4). Changing the delivery of

chemotherapy agents from passive diffusion to targeted delivery to specific cell-surface receptors may facilitate the bypassing of drug efflux pumps.

Delivery of chemotherapy agents is also hindered by first-pass metabolism by the liver and the reticulo-endothelial system. These mechanisms limit the amount of drug available for delivery to tumor tissues. Chemotherapy agents are often unable to fully penetrate tumor tissue, even though the vasculature of solid tumors is usually leaky. Drug delivery vehicles may protect anti-cancer agents from uptake by the liver and macrophages and thus allow a higher proportion of injected drug to reach tumor tissues. Drug delivery vehicles are also of a small enough diameter have been considered advantageous as they may become trapped in the leaky vasculature of tumors, thus resulting in increased delivery.

Chemotherapy Drug Delivery Vehicles

Drug delivery vehicles can enhance cancer chemotherapy by reducing toxicity, targeting tumors, and bypassing drug efflux pumps. Because most chemotherapy drugs are hydrophobic, most vehicle systems are lipid-based. Although novel vehicles such as quantum dots, carbon nanotubes, and gold-coated nanoparticles are on the forefront of drug delivery research, the majority of research has been achieved with lipid-based vehicles, such as liposomes and lipoprotein-based nanoparticles.

Liposomal Delivery Systems

Because liposomes currently are the most frequently utilized drug delivery vehicles in cancer chemotherapy, their characteristics and performance will be briefly discussed here. Liposomes were first described in 1961 by the British hematologist A.D. Bangham (5). Unlike micelles, which are surrounded by a lipid monolayer, liposomes are bilayer structures with the hydrophobic tails oriented inward to sequester water and the hydrophilic heads of the lipids oriented outward. The hydrophilic heads interact with the aqueous environment, which surrounds the liposome and lie within the core. The liposome was proposed as a drug delivery vehicle to facilitate the usage of lipophilic drugs, which are encapsulated by the lipid bilayer shell. Besides providing solubilization, liposomes can also alter the pharmacokinetics of drugs, offering slow or controlled release. Advances in liposome research are further refining drug delivery with targeting, either achieving site avoidance or tumor targeting (6). Targeting can be passive or active, leading to accumulation in the diseased tissue; this is known as the Enhanced Permeability and Retention Effect (EPR) (7).

The factors leading to a successful formulation of a liposomal drug are circulation time, vesicle size, drug leakage, and reticulo-endothelial system (RES) saturation (6). Longer circulation time is generally preferred. Liposomes have limitations as some are rapidly taken up by the reticulo-endothelial system and degraded with half-lives on the order of 60 minutes(8). Liposome formulations encapsulated with polyethylene glycol created "Stealth" coating (Figure 2A), greatly increasing the half-life of the particles (\approx 1 day) (9). Liposomes are being further refined as drug delivery vehicles with the addition of ligands or antibodies for targeting (Figure 2B). However, targeted liposomes are challenged by ligand insertion and retention into liposomes and maintaining extended circulation times (6).

A smaller vesicle size is a favorable factor for liposomal formulations as most liposomes have an average diameter of 100-200 nanometers. Competition for liposome uptake by the liver (Kupffer cells) or tumor can be decreased by RES saturation (6) to promote increased accumulation of the drug in the tumor tissue, compared to the non-encapsulated drug.

Lipoprotein-Based Drug Delivery Systems

Lipoproteins have a hydrophobic inner core of cholesteryl esters and triglycerides and an outer shell of free cholesterol, phospholipid, and apoproteins. Here the more stable cholesterol transporting lipoprotein classes, Low density lipoproteins (LDL) and high density lipoproteins (HDL) will be considered as potential drug delivery agents. The main apolipoprotein (in LDL) is Apolipoprotein B100 (apo B100). The apolipoproteins found on HDL are Apolipoprotein A-I, (apo A-I), apo A-II, apo A-IV, apo C-III, and apo E (10). Unlike liposomes, the core of lipoproteins is an interior hydrophobic compartment for the transport of water-insoluble lipid components (Figure 4). They have a structural arrangement favorable for the transport of hydrophobic drugs (11).

As cholesterol carriers and lipoprotein-based drug delivery vehicles, HDL and LDL have similar lipid components and are of similar size, shape and structure. In comparison to liposomes, lipoproteins are approximately 10-fold smaller (10-20 nm), which is highly favorable for a drug delivery formulation (Figure 3). In contrast to liposomes, human LDL and HDL have half-lives of 3 and 4-5 days, respectively (12-14). Again, a longer circulation time is a highly favorable factor for a drug delivery vehicle.

Both native LDL and HDL have been experimented with as drug carriers, however, practical and safety considerations have prevented them from being developed toward clinical

applications. LDL receptors have been shown to have increased activity and expression in various cancer cell lines (15). However, low density lipoprotein is the less favorable candidate for a lipoprotein-based nanoparticle. The major protein component of LDL is apo B-100, with a molecular weight of 513kDa. There are difficulties in purifying and solubilizing apo B-100, which has a tendency to aggregate. Another consideration is that any drug encapsulated into LDL must be able to withstand the acidic conditions in the lysosome during endocytosis, the uptake mechanism for LDL (16).

Several anthracycline derivatives were tested with an LDL-based vehicle; the drug incorporation and stability of this particle was not satisfactory (17,18). Studies are being conducted into a synthetic nano-LDL that contains a lipophilic peptide that can interact with the LDL receptor causing endocytosis. (19) This nano-LDL particle is formulated to carry either imaging agents or chemotherapy drugs. However, these studies have numerous stability and safety concerns and are preliminary at this time.

High density lipoprotein has been shown to naturally associate with hydrophobic compounds in the blood. It has been known that Vitamin E complexes with HDL for transport in the plasma (20). When plasma distribution measurements have been made, it has been observed that antibiotic compounds (63, 64) and immunosuppressants (65,66) distribute to the HDL fraction of the plasma. Recently, a study found that cholesterol-linked siRNA complexed with HDL after plasma incubation (21). Numerous examples in the literature can be found of synthetic HDL nanoparticles as carriers for antifungals (22), imaging agents (23), and cancer chemotherapy drugs(24,67).

High Density Lipoprotein Metabolism and Selective Lipid Uptake Mechanism

Apolipoprotein A-1 and HDL Formation

The protein mainly involved in HDL biogenesis and cholesterol transport is Apo A-I. Apo A-I is a polypeptide of 243 amino acids, which forms a series of amphipathic α -helices. The first domain of the protein forms a globular structure of α -helices, which is thought to interact and be buried in the phospholipid outer shell. There are ten amphipathic α -helices, proposed to be approximately 11-22 residues long(25). Studies on the 3-dimensional structure for Apo A-1, show the protein is in a belt-like conformation. HDL usually contains 2-4 molecules of Apo A-1 and the apoproteins align their helices in an antiparallel fashion (Figure 5) (26). Mutational analyses have shown that it is not single amino acid residues, but the entire amphipathic α -helix that facilitates cholesterol efflux and lipid delivery to form HDL (27).

Although the majority of work done in this project used Apo A-1, derived from human plasma, the isolation procedures are complicated and hindered by low yield. The current supply of Apo A-1, used for this study, is from a recombinant system purified from extracts of a transformed *E coli* strain. This optimized bacterial expression has a yield of 100 mg per liter of bacterial culture (28). The clone contains a hexahistidine tag for purification on a nickel-agarose column. The hexahistidine tag is removed via cleavage with a thrombin-sepharose resin.

Nascent HDL is released from the liver as a discoidal, immature HDL containing phospholipid, apolipoproteins, and some free cholesterol. In the circulation, it is remodeled into mature, spherical HDL through the interaction of Apo A-I and lecithin-cholesterol acyl transferase (LCAT) (Figure 6). LCAT catalyzes the reaction esterifying cholesterol. Next, HDL interacts with an ABC transporter (ABCA1), to facilitate

cholesterol removal from the peripheral tissues. Peripheral cholesterol is delivered by HDL to

the liver, in a mechanism known as reverse cholesterol transport. Extensive

study of HDL in rodents has found that delivery of cholesteryl esters occurs into the liver and steroidogenic tissues, such as ovaries and adrenal glands. It appears that delivery in humans also is also to the liver and steroidogenic organs. Cholesteryl esters may also be transferred to lower density lipoproteins such as LDL through the action of plasma proteins like cholesterol ester transfer protein (CETP) and eventually taken up by the liver via endocytosis of LDL (29) Cholesterol ester transfer to LDL may actually be the dominant pathway for cholesterol removal from peripheral tissues.

The mechanism of uptake for delivery of cholesteryl esters is different than uptake for other lipoproteins, (e.g. LDL). While, LDL interacts with the LDL receptor and subsequently undergoes endocytosis and lysosomal degradation, HDL interacts with its receptor to deliver cholesteryl esters via *selective lipid uptake*. The selective uptake process involves the delivery of cholesteryl esters via a yet uknown mechanism *without whole particle uptake and degradation*. The HDL receptor that takes up cholesterol from HDL is known as Scavenger Receptor, Class B, Type I (SR-BI) (30).

Scavenger Receptor, Class B Type I and Selective Lipid Uptake

Scavenger Receptor, Class B Type 1 is a 509 amino acid, 82kDa glycoprotein. It contains a short C-terminal cytoplasmic domain, two transmembrane domains (one N-terminal, the other C-terminal), and an approximately 403 amino acid extracellular loop of unknown structure (Figure 7). (31, 32) Selective lipid uptake has been found to result from the residues in the extracellular loop, not the cytoplasmic tails or transmembrane domains. (33,34) An anti-apoptotic signal transduction pathway has been found associated with the 45-residue C-terminal

cytoplasmic tail of SR-BI through a phosphoinositide 3-kinase/Akt/AP-1 pathway. (35) A splicing variant also exists known as SR-BII, which has an identical extracellular loop, but a distinct C-terminal cytoplasmic tail(36). Homologs of SR-BI have been found in the mouse (37), human (38), pigs (39), turtles, chicken, frogs (40) and salmon (41). Human SR-BI is also known as C36 and LIMP II-analogous protein 1 (CLA-1)(38). SR-BI binds several species of lipoprotein particles, HDL, LDL, VLDL, and oxidized LDL; SR-BI also binds anionic phospholipids, implicating it in the disposal of apoptotic cells (28).

Three models have been proposed for the mechanism of selective lipid uptake. It is generally agreed that it is a two-step mechanism. First, Apo A-I binds to SR-BI in a fast step. The second rate-limiting step is the specific uptake of the core component of cholesteryl esters (42). How SR-BI accomplishes this second step is heavily debated. One model proposes that HDL undergoes a type of endocytosis known as transcytosis or retroendocytosis. This mechanism, also used by transferrin for delivering iron, involves internalization of the particle without degradation, delivery of cholesteryl esters, and the extracellular release of depleted HDL. (43) The second model proposed that HDL docks at SR-BI and fuses with the plasma membrane to deliver its cholesteryl esters to the cytoplasm. (34,44) The third model has proposed that SR-BI creates a hydrophobic pore that excludes water to allow the cholesteryl esters to enter the cell by a non-aqueous pathway. (45) It is possible that all are SR-BI-mediated mechanisms that occur in different tissues.

In polarized hepatic cells, SR-BI appears to facilitate retroendocytosis (46). In hepatic and gastrointestinal cells, SR-BI does interact with an accessory protein, PDZK1, which maintains SR-BI cell surface localization and selective lipid uptake (47). However, studies showed that SR-BI alone, reconstituted into liposomes, was

necessary and sufficient to accomplish selective lipid uptake (48). In other cell types, PDZK1 is not expressed, so selective lipid uptake does not appear to occur by the same mechanism.

SR-BI has been shown to form dimers and higher-order oligomers in rodent steroidogenic tissues and a SR-BI-transfected COS-7 cell line (49). Selective lipid uptake in adrenal cells occurs in microvillar channels through the action of SR-BI alone. In this tissue, SR-BI forms a hydrophobic channel for the passage of cholesteryl esters from HDL (50). SR-BI has also been localized in microvillar channels in ovarian granulosa cells and Leydig cells in rodents (51, 52). By forming a hydrophobic channel, SR-BI allows cholesteryl esters to follow their concentration gradient, which coincides with the fact that SR-BI directs both cholesterol uptake and efflux (53). Forming a hydrophobic channel for the passage of cholesteryl esters could be a steroidogenic tissue-specific mechanism.

HDL, SR-BI and Cancer

Hypocholesteremia has been observed as a complication of many cancers (54). Although it is usually attributed to a decrease in LDL cholesterol, a study by Fiorenza et al, found that 50% of the decrease was in HDL cholesterol (55). Corroborating evidence from a recent study by Muntoni et al found decreased total cholesterol and HDL cholesterol in many different types of malignancies (56). Tumor cells have been suggested to have higher demand for cholesterol due to increased proliferation. Several studies have found increased cholesteryl ester uptake and accumulation and high SR-BI expression in breast cancer cell lines (57), hepatocarcinoma cell lines (58), and testicular tumors (59). Subsequently, it has been shown in primary breast tumors that SR-BI is highly expressed in these cells (Figure 10) as compared to normal breast tissue (36). Our laboratory has previously reported high expression levels of SR- BI (Figure 8) in breast and prostate cancer cell lines (60), leukemia cell lines (Figure 9) and ovarian cancer cell lines (Figure 11).

Project Goals

Based on its structure, rHDL is expected to have many advantages as a drug delivery platform, shielding water-insoluble drugs from the aqueous environment and targeting the drugs to tumor tissue via the overexpression of SR-BI receptors (Figure 12). <u>Hypothesis:</u> Reconstituted high density lipoprotein-drug complexes have similar chemical and physical characteristics to native HDL. These particles can serve as efficient drug delivery vehicles and thus exhibit *in vitro* and *in vivo* anticancer activity. This anticancer activity occurs via selective lipid uptake, which is mediated through an HDL receptor (CLA-1 or SR-BI) mechanism.

The first objective of this project was to formulate a reconstituted high density lipoprotein that could carry Cremophor-free paclitaxel. Because paclitaxel is highly waterinsoluble, I searched for the optimal solvent or detergent and particle formation methodology. There are two common methods for rHDL formation, sonication or detergent (cholate) dialysis. The following chapter of this dissertation will discuss the experimental results of the solvents, detergents, and methods used.

The second objective of this project was to determine if rHDL/paclitaxel was chemically and physically similar to native HDL and if it also exhibited anticancer activity. The rHDL delivery vehicle is produced via a modified cholate dialysis protocol. We determined the chemical composition of our reconstituted HDL and found the content to be similar to native HDL. I examined the shape and size of rHDL and compared its properties to mature native HDL

for delivery of paclitaxel through selective uptake. Subsequently, I evaluated its capacity for delivery of paclitaxel through selective uptake to cancer cells. The physical characteristics of rHDL/paclitaxel (rHDL/PTX) were determined by gel chromatography, gel electrophoresis and electron microscopy.

The cancer models used in this object of the project included prostate, breast, and ovarian cancers. These are all cancers of reproductive tissues and are steroid hormoneproducing. The criteria for use in these studies was the level of expression of SR-BI, which has been shown to be higher in these cancers. Pharmacokinetic parameters, including the 50% Inhibitory Concentration (IC₅₀) and Maximum Tolerated Dose (MTD), were determined for our rHDL/PTX vehicle. The IC₅₀ for rHDL/PTX was tested in a variety of cancer cells lines, ranging from breast to prostate and ovarian. The first *in vivo* experiment is the determination of the maximum tolerated dose (MTD) of rHDL/paclitaxel in C57Black female mice. In the literature, MTD is defined as the dose at which no death and 15% weight loss occurs. The treatment dosages for tumor-bearing mice will proceed from the results of the MTD experiments.

The third objective of this project was to determine if the cellular uptake of paclitaxel from rHDL was mediated through an HDL receptor mechanism. I first examined the expression of SR-BI in prostate cancer cell lines and a non-tumorgenic prostate cell line. Next, I looked at paclitaxel uptake from rHDL in SR-BI-transfected ldl A7 cell line. The parental ldl A7 cell line is derived from the Chinese Hamster Ovary (CHO) cell line and expresses minimal amounts of SR-BI receptor.

To determine if paclitaxel uptake was indeed selective, I used radiolabeled tracers on different components of rHDL to track the fate upon incubation with prostate cancer cells. I

then performed competition experiments with native HDL to determine if the selective uptake of paclitaxel was HDL receptor-mediated.

Note on Materials Used in the Dissertation

Chapter II consists of unpublished data. Chapter III represents already published data (McConathy, W, et al.; *Anti-Cancer Drugs* 2008;). The data in Chapter IV has been accepted for publication (Mooberry, L., et al.; Receptor mediated uptake of paclitaxel from a synthetic high density lipoprotein nanocarrier; *Journal of Drug Targeting*; In Press). Chapter III and IV were modified from the published versions for stylistic purposes. Chapter V contains additional studies that are being prepared for publication.

Figure 1. Structure of paclitaxel. (2α,4α,5β,7β,10β,13α)-4,10-bis(acetyloxy)-13-{[(2R,3S)-3-(benzoylamino)-2-hydroxy-3-phenylpropanoyl]oxy}-1,7-dihydroxy-9-oxo-5,20-epoxytax-11-en-2-yl benzoate has a molecular weight of 853.9 g/mol and is derived from the Pacific yew tree. Reproduced from Mastropaolo, D., Camerman, A., Luo, Y., Brayer, G.D., and Camerman, N.; (1995) "Crystal and Molecular Structure of Paclitaxel (Taxol);" PNAS 92; 6920-6924 (1).



Figure 2. Structure of Liposomes (A) Schematic of a liposome showing basic structure (right) and a PEGylated "Stealth" liposome (left). (B) Schematic of a Targeted liposome. Antibody linkage occurs through biotin-avidin (left) or maleimide-polyethylene glycol (MAL-PEG) linkage (right). Reproduced from Pastorino, F., Marimpietri, D., Brignole, C., Di Paolo, D., Pagnan, G., Daga, A., Piccardi, F., Cilli, M., Allen, T.A., and Ponzoni, M.; (2007); "Ligand-Targeted Liposomal Therapies of Neuroblastoma;" Curr. Med. Chem.; 14; 3070-3078 (9).





Figure 3: Comparative Diagram of Lipoprotein Sizes. In this diagram, the sizes of HDL (10 nm) and LDL (20 nm) can be compared to the average size of a liposome (100nm). Reproduced from Wilson, P. W.F.; <u>Atlas of Atherosclerosis</u> 2nd Edition; Philadelphia; Current Medicine Group; 2000 (68).



Figure 4. Localization of a hydrophobic drug (paclitaxel) in the core region of a generic lipoprotein. Reproduced from WEINBERG RB: Lipoprotein metabolism: hormonal regulation. *Hosp. Pract*. (Off Ed.) (1987) 22 (6):223-227, 230, 233-234. (61)



Adapted from Weinberg, RB. Hosp Pract 1987, 22:223-227

Figure 5. Conformation of Apolipoprotein A-1. The structure of Apo A-1 showing the amphipathic helices that make up the double-belt conformation. These helices are in antiparallel alignment. Reproduced from Li L, Chen J, Mishra VK, Kurtz JA, Cao D, Klon AE, Harvey SC, Anantharamaiah GM, and Segrest JP, (2004). Double belt structure of discoidal high density lipoproteins: molecular basis for size heterogeneity. J Mol Biol.; 343(5):1293-311 (25).







Figure 6. Transformation of the discoidal precursor (nascent) HDL to its stable, spherical configuration via the accumulation of cholesteryl esters, catalyzed by lecithin–cholesterol acyltransferase. Reproduced from ALEXANDER ET, BHAT S, and THOMAS MJ *et al.* (2005) Apolipoprotein A-I helix 6 negatively charged residues attenuate lecithin-cholesterol acyltransferase (LCAT) reactivity. Biochemistry 44 (14):5409-5419. HDL: High-density lipoprotein; LCAT: Lecithin–cholesterol acyltransferase. (62)



Figure 7. Structure of Scavenger Receptor, Class B, Type I. In this diagram, the extracellular loop is depicted with N-glycosylation sites, the transmembrane domains, and the N- and C-terminal cytosolic tails with PDZK1 binding site. Reproduced from Rhainds D, Brissette L, (2004). The role of scavenger receptor class B type I (SR-BI) in lipid trafficking. defining the rules for lipid traders. Int J Biochem Cell Biol.; 36(1):39-77. (32)



Figure 8. Expression of SR-BI in a Selection of Cancer Cell Lines.

Lane 1: T47D human breast ductal carcinoma. Lane 2: PC3 human prostate carcinoma. Lane 3: OV1063 human ovarian carcinoma. Lane 4: MCF7 human breast adenocarcinoma. Lane 5: DU145 human prostate carcinoma. Lane 6: HeLA human cervical carcinoma. Lane 7: normal human fibroblasts. Reproduced from Lacko, A.G., Nair, M., Paranjape, S., Johnson, S., McConathy, W.J.; (2002); "High Density Lipoprotein Complexes as Delivery Vehicles for Anticancer Drugs;" Anticancer Res. 22; 2045-2049. (60)


Figure 9. Expression of SR-BI in Leukemia and Other Cancer Cell Lines

Lane1: Normal Lymphocytes. Lane 2: K549 human lung carcinoma. Lane 3: HL60 human promyelocytic leukemia. Lane 4: SUP B15 Human B cell precursor leukemia. Lane 5: 32D lymphoblasts, mouse myeloid cells. Lane 6: ldl mSR-BI, SR-BI transfected CHO-derived cell line. Lane 7: DU145, human prostate cancer. Lane 8: B16 mouse melanoma. Lane 9: Molecular weight standards. Lane 10: Empty.



Figure 10. hSR-B1/CLA-1 expression in breast cancer and adjacent noncancerous cells. (**A**) reverse transcription-PCR analysis of CLA-1 mRNA expression in breast cancers. **Top panel**, CLA-1; **bottom panel**, β-actin. *Lanes 1, 3, 5,* and 7 and *Lanes 2, 4, 6,* and 8 show results from noncancerous mammary tissue and breast cancers, respectively. (**B**) CLA-1 protein expression in breast cancers. *Lanes 1, 3, 5,* and 7 and *Lanes 2, 4, 6,* and 8 show results from noncancerous mammary tissue and breast cancers, respectively. (**B**) CLA-1 protein expression in breast cancers. *Lanes 1, 3, 5,* and 7 and *Lanes 2, 4, 6,* and 8 show results from noncancerous mammary tissue and breast cancers, respectively. (**C**) immunohistochemical localization of CLA-1 in breast cancer. Immunoperoxidase staining of CLA-1 is present in the cytoplasm of the breast cancer (*right panel*), and tissue stained with control IgG was negative (*left panel*). Reproduced from Cao, W.M., Murao, K., Imachi, H., Yu, X., Abe, H., Yamauchi, A., Niimi, M., Miy-auchi, A., Wong, N.C.W., and Ishida, T.,; (2004); "A Mutant High-Density Lipoprotein Receptor Inhibits Proliferation of Human Breast Cancer Cells;"Cancer Res.; 64; 1515-1521. (35)



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Figure 11. Expression of SR-BI in Ovarian Cancer Cell Lines RT-PCR showing expression of SR-BI in various tissues. **Top panel:** Normal mouse tissues. SR-BI expression can only be seen in liver. **Bottom panel:** HeyA8 drug-sensitive ovarian cancer cell line. SKOV-3 drug-resistant ovarian cancer cell line. A2760-Par ovarian cancer cell line. IGROV ovarian cancer cell line. HeyA8-MDR drug-resistant ovarian cancer cell line. RMG2 drug-resistant ovarian cancer cell line. A2780-CP20 drug-resistant ovarian cancer cell line.



Figure 12. Illustration of rHDL structure and selective anticancer drug delivery by

rHDL/drug complexes. A model is shown of paclitaxel delivery from rHDL via HDL receptor.

Adapted from Steinberg; Science; 1996; 271; 460-461. (44)



REFERENCES

1. Mastropaolo, D., Camerman, A., Luo, Y., Brayer, G.D., and Camerman, N.; (1995) "Crystal and Molecular Structure of Paclitaxel (Taxol);" PNAS 92; 6920-6924

2. Ibrahim NK, Desai N, Legha S, Soon-Shiong P, Theriault RL, Rivera E, Esmaeli B, Ring SE, Bedikian A, Hortobagyi GN, and Ellerhorst JA, (2002). Phase I and pharmacokinetic study of ABI-007, a cremophor-free, protein-stabilized, nanoparticle formulation of paclitaxel. *Clin. Cancer Res.* (2002) **8** (5): 1038 -1044.

3. Ambudkar SV, Kimchi-Sarfaty C, Sauna ZE, and Michael M Gottesman MM, (2003). P-glycoprotein: from genomics to mechanism. Oncogene 22, 7468–7485.

4. Kruh GD, and BelinskyMG, (2003). The MRP family of drug efflux pumps. Oncogene 22, 7537–7552

5. Bangham AD, Standish MM, and Watkins JC, (1965). Diffusion of univalent ions across the lamellae of swollen phospholipids. J Mol Biol.; 13(1):238-52.

6. Gabizon, A.A., Shmeeda, H., and Zalipsky, S.; (2006); "Pros and Cons of the Liposome Platform in Cancer Drug Targeting;" Journal of Liposome Research;" 16; 175-183

7. Maeda H, (2001). SMANCS and polymer-conjugated macromolecular drugs: advantages in cancer chemotherapy. Adv Drug Deliv Rev.; 46(1-3):169-85.

8. Senior J, Gregoriadis G, (1982). Stability of small unilamellar liposomes in serum and clearance from the circulation: the effect of the phospholipid and cholesterol components. Life Sci. 1982 Jun 14;30(24):2123-36

9. Pastorino, F., Marimpietri, D., Brignole, C., Di Paolo, D., Pagnan, G., Daga, A., Piccardi, F., Cilli, M., Allen, T.A., and Ponzoni, M.; (2007); "Ligand-Targeted Liposomal Therapies of Neuroblastoma;" Curr. Med. Chem.; 14; 3070-3078.

10. Assmann G, von Eckardstein A, and Funke H, (1993). High density lipoproteins, reverse transport of cholesterol, and coronary artery disease. Insights from mutations. Circulation.; 87(4 Suppl):III28-34.

11. Lacko AG, Nair M, Prokai L, and McConathy WJ, (2007). Prospects and challenges of the development of lipoprotein-based formulations for anti-cancer drugs. Expert Opin Drug Deliv.; 4(6):665-75.

12. Langer T, Strober W, and Levy RI, (1972). The metabolism of low density lipoprotein in familial type II hyperlipoproteinemia. J Clin Invest.; 51(6):1528-36.

13. Blum CB, Levy RI, Eisenberg S, Hall M 3rd, Goebel RH, and Berman M, (1977). High density lipoprotein metabolism in man. J Clin Invest.; 60(4):795-807.

14. Illingworth DR, Connor WE, and Alaupovic P, (1981). High density lipoprotein metabolism in a patient with abetalipoproteninemia. Ann Nutr Metab.; 25(1):1-10.

15. Vitols S, Angelin B, Ericsson S, Gahrton G, Juliusson G, Masquelier M, et al, (1990). Uptake of low density lipoproteins by human leukemic cells in vivo: relation to plasma lipoprotein levels and possible relevance for selective chemotherapy. Proc Natl Acad Sci U S A; 87:2598–2602.

16. Goldstein JL, Brown MS, (1977). The low-density lipoprotein pathway and its relation to atherosclerosis. Annu Rev Biochem.; 46:897-930.

17. Masquelier M, Vitols S, Palsson M, Mars U, Larsson BS, and Peterson C, (2000). Low density lipoprotein as a carrier of cytostatics in cancer chemotherapy: study of stability of drug-carrier complexes in blood. J. Drug Target; 8 (3): 155 -164.

18.Masquelier M, Tirzitis G, Peterson CO, Pålsson M, Amolins A, Plotniece M, Plotniece A, Makarova N, Vitols SG, (2000). Plasma stability and cytotoxicity of lipophilic daunorubicin derivatives incorporated into low density lipoproteins. Eur. J. Med. Chem.; 35 (4): 429 -438.

19. Nikanjam M, Blakely EA, Bjornstad KA, Shu X, Budinger TF, and Forte TM, (2007). Synthetic nano-low density lipoprotein as targeted drug delivery vehicle for glioblastoma multiforme. Int J Pharm.; 328(1):86-94.

20. Sundaram GS, London R, Manimekalai S, Nair PP, and Goldstein P, (1981). alpha-Tocopherol and serum lipoproteins. Lipids.; 16(4):223-7.

21. Wolfrum C, Shi S, Jayaprakash KN, Jayaraman M, Wang G, Pandey RK, Rajeev KG, Nakayama T, Charrise K, Ndungo EM, Zimmermann T, Koteliansky V, Manoharan M, and Stoffel M, (2007). Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. Nat Biotechnol.; 25(10):1149-57.

22. Oda MN, Hargreaves PL, Beckstead JA, Redmond KA, van Antwerpen R, and Ryan RO, (2006). Reconstituted high density lipoprotein enriched with the polyene antibiotic amphotericin B. J Lipid Res.; 47(2):260-7

23. Corbin IR, Chen J, Cao, W, Li H, Lund-Katz S, and Zheng G, (2007). Enhanced cancertargeted delivery using engineered high-density lipoprotein-based nanocarriers. J Biomed. Nano.; 3; 367-376.

24. Lou B, Liao XL, Wu MP, Cheng PF, Yin CY, Fei Z, (2005). High-density lipoprotein as a potential carrier for delivery of a lipophilic antitumoral drug into hepatoma cells. World J Gastroenterol.; 11(7):954-9.

25. Li L, Chen J, Mishra VK, Kurtz JA, Cao D, Klon AE, Harvey SC, Anantharamaiah GM, and Segrest JP, (2004). Double belt structure of discoidal high density lipoproteins: molecular basis for size heterogeneity. J Mol Biol.; 343(5):1293-311.

26. Silva RA, Hilliard GM, Li L, Segrest JP, Davidson WS, (2005). A mass spectrometric determination of the conformation of dimeric apolipoprotein A-I in discoidal high density lipoproteins. Biochemistry.; 44(24):8600-7.

27. Williams DL, de La Llera-Moya M, Thuahnai ST, Lund-Katz S, Connelly MA, Azhar S, Anantharamaiah GM, and Phillips MC; (2000). Binding and cross-linking studies show that scavenger receptor BI interacts with multiple sites in apolipoprotein A-I and identify the class A amphipathic alpha-helix as a recognition motif. J Biol Chem.; 275(25):18897-904.

28. Ryan RO, Trudy M. Forte TM, and Oda MN, (2003). Optimized bacterial expression of human apolipoprotein A-I. Prot. Expr. Pur. 27; 98–103.

29. Barter PJ, and Hopkins GJ,(1983). Relative rates of incorporation of esterified cholesterol into human very low density lipoproteins and low density lipoproteins. In vitro studies of two separate pathways. Biochim Biophys Acta.; 751(1):33-40.

30. Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, and Krieger M. (1996) Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science.; 271(5248):518-20

31. Krieger, M.; Annu. Rev.Biochem. 1999; 68; 523-558.

32. Rhainds D, Brissette L, (2004). The role of scavenger receptor class B type I (SR-BI) in lipid trafficking. defining the rules for lipid traders. Int J Biochem Cell Biol.; 36(1):39-77.

33. Gu X, Trigatti B, Xu S, Acton S., Babitt J, and Krieger M, (1998). The efficient cellular uptake of high density lipoprotein lipids via scavenger receptor class B type I requires not only receptor-mediated surface binding but also receptor-specific lipid transfer mediated by its extracellular domain. J Biol. Chem.; 273; 26338-26348. 34. Connelly, M.A., de la Llera-Moya, M., Monzo, P., Yancey, P.G., Drazul, D., Stoudt, G., Fournier, N., Klein, S.M., Rothblat, G.H., and Williams, D.L, (2001). Analysis of chimeric receptors shows that multiple distinct functional activities of scavenger receptor, class B, type I (SR-BI), are localized to the extracellular receptor domain. <u>Biochemistry</u>; 40; 5249-5259.

35. Cao, W.M., Murao, K., Imachi, H., Yu, X., Abe, H., Yamauchi, A., Niimi, M., Miyauchi, A., Wong, N.C.W., Ishida, T.; <u>Cancer Research</u>; 2004; 64; 1515-1521.

36. Webb NR, Connell PM, Graf GA, Smart EJ, de Villiers WJ, de Beer FC, and van der Westhuyzen DR; (1998) SR-BII, an isoform of the scavenger receptor BI containing an alternate cytoplasmic tail, mediates lipid transfer between high density lipoprotein and cells. J Biol Chem.; 273(24):15241-8

37. Acton SL, Scherer PE, Lodish HF, and Krieger M; (1994) Expression cloning of SR-BI, a CD36-related class B scavenger receptor. J Biol Chem.; 269(33):21003-9.

38. Calvo D, Gómez-Coronado D, Lasunción MA, and Vega MA; (1997) CLA-1 is an 85-kD plasma membrane glycoprotein that acts as a high-affinity receptor for both native (HDL, LDL, and VLDL) and modified (OxLDL and AcLDL) lipoproteins. Arterioscler Thromb Vasc Biol.; 17(11):2341-9.

39. Goti D, Hrzenjak A, Levak-Frank S, Frank S, van der Westhuyzen DR, Malle E, and Sattler W; (2001) Scavenger receptor class B, type I is expressed in porcine brain capillary endothelial cells and contributes to selective uptake of HDL-associated vitamin E. J Neurochem.; 76(2):498-508.

40. Duggan AE, Marie RS Jr, and Callard IP; (2002) Expression of SR-BI (Scavenger Receptor Class B Type I) in turtle (Chrysemys picta) tissues and other nonmammalian vertebrates. J Exp Zool.; 292(5):430-4.

41. Kleveland EJ, Syvertsen BL, Ruyter B, Vegusdal A, Jørgensen SM, and Gjøen T; (2006) Characterization of scavenger receptor class B, type I in Atlantic salmon (Salmo salar L.). Lipids.; 41(11):1017-27.

42. Williams DL, Temel RE, and Connelly MA; (2000) Roles of scavenger receptor BI and APO A-I in selective uptake of HDL cholesterol by adrenal cells. Endocr Res.; 26(4):639-51.

43. Silver, D.L., Wang, N., and Tall, A.R.; (2000). Defective HDL particle uptake in ob/ob hepatocytes causes decreased recycling, degradation, and selective lipid uptake. <u>Journal of Clinical</u> <u>Investigation</u>; 105; 151-159.

44. Steinberg, D, (1996). A docking receptor for HDL cholesterol esters. Science; 271;460-461.

45. Rodrigueza, W.V., Thuahnai, S.T., Temel, R.E., Lund-Katz, S., Phillips, M.C., Williams, D.L.; <u>The Journal of Biological Chemistry</u>; 1999; 274; 20344-20350.

46. Wustner, D., Mondal, M., Huang, A., Maxfield, F.R.(2004). Different transport routes for high density lipoprotein and its associated free sterol in polarized hepatic cells. Journal of Lipid <u>Research</u>; 45; 427-437.

47. Kocher O, and Krieger M; (2009) Role of the adaptor protein PDZK1 in controlling the HDL receptor SR-BI. Curr Opin Lipidol.; 20(3):236-41.

48. Liu B, Krieger M. (2002) Highly purified scavenger receptor class B, type I reconstituted into phosphatidylcholine/cholesterol liposomes mediates high affinity high density lipoprotein binding and selective lipid uptake. J Biol Chem.; 277(37):34125-35.

49. Reaven E, Cortez Y, Leers-Sucheta S, Nomoto A, and Azhar S, (2004). Dimerization of the scavenger receptor class B type I: formation, function, and localization in diverse cells and tissues. J Lipid Res.; 45(3):513-28.

50. Connelly, MA, (2009). SR-BI-mediated HDL cholesteryl ester delivery in the adrenal gland. Molecular and Cellular Endocrinology 300 (2009) 83–88.

51. Reaven E, Lua Y, Nomoto A, Temel R, Williams DL, van der Westhuyzen DR, and Azhar S; (1999). The selective pathway and a high-density lipoprotein receptor (SR-BI) in ovarian granulosa cells of the mouse. Biochim Biophys Acta.; 1436(3):565-76.

52. Reaven E, Zhan L, Nomoto A, Leers-Sucheta S, and Azhar S; (2000). Expression and microvillar localization of scavenger receptor class B, type I (SR-BI) and selective cholesteryl ester uptake in Leydig cells from rat testis. J Lipid Res.; 41(3):343-56.

53. Gu X, Kozarsky K, and Krieger M; (2000). Scavenger receptor class B, type I-mediated [3H]cholesterol efflux to high and low density lipoproteins is dependent on lipoprotein binding to the receptor. J Biol Chem.; 275(39):29993-30001.

54. Markel A, Brook GJ, (1994). Cancer and hypocholesterolemia. Isr J Med Sci.; 30(10):787-93.

55. Fiorenza AM, Branchi A, Sommariva D, (2000). Serum lipoprotein profile in patients with cancer. A comparison with non-cancer subjects. Int. J. Clin. Lab. Res.; 30 (3): 141 -145.

56. Muntoni, S., Atzori, L., Mereu, R., Satta, G., Macis, M.D., Congia, M., Tedde, A., Desogus, A., and Muntoni, S., (2009). "Serum Lipoproteins and Cancer;" Nutr Metab Cardiovasc Dis.; 19(3):218-25.

57. Pussinen PJ, Karten B, Wintersperger A, Reicher H, McLean M, Malle E, and Sattler W, (2000). The human breast carcinoma cell line HBL-100 acquires exogenous cholesterol from high-density lipoprotein via CLA-1 (CD-36 and LIMPII analogous 1)-mediated selective cholesteryl ester uptake. Biochem J.; 349(Pt 2):559-66.

58. Wadsack C, Hirschmugl B, Hammer A, Levak-Frank S, Kozarsky KF, Sattler W, and Malle, (2003). Scavenger receptor class B, type I on non-malignant and malignant human epithelial cells mediates cholesteryl ester-uptake from high density lipoproteins. Int J Biochem Cell Biol.; 35(4):441-54.

59. Arenas MI, Lobo MV, Caso E, Huerta L, Paniagua R, and Martín-Hidalgo MA, (2004). Normal and pathological human testes express hormone-sensitive lipase and the lipid receptors CLA-1/SR-BI and CD36. Hum Pathol. 200; 35(1):34-42.

60. Lacko AG, Nair M, Paranjape S, Johnson S, and McConathy WJ.; (2002). High density lipoprotein complexes as delivery vehicles for anticancer drugs. Anticancer Res.; 22(4):2045-9.

61. Weinberg RB, (1987). Lipoprotein metabolism: hormonal regulation. Hosp. Pract.(Off Ed.) 22 (6):223-227, 230, 233-234.

62. Alexander ET, Bhat S, Thomas MJ, Weinberg RB, Cook VR, Bharadwaj MS, Sorci-Thomas M, (2005). Apolipoprotein A-I helix 6 negatively charged residues attenuate lecithin-cholesterol acyltransferase (LCAT) reactivity. Biochemistry 44 (14):5409-5419.

63. Kennedy AL, Wasan KM, (1999) Preferential distribution of amphotericin B lipid complex into human HDL3 is a consequence of high density lipoprotein coat lipid content. J Pharm Sci.; 88(11):1149-55.

64. Yeganeh MH, McLachlan AJ, (2002). In-vitro distribution of terbinafine in rat and human blood. J Pharm Pharmacol.; 54(2):277-81.

65. Kwong M, Sivak O, Kwong EH, Wasan KM, (2001). Cyclosporine A transfer between highand low-density lipoproteins: independent from lipid transfer protein I-facilitated transfer of lipoprotein-coated phospholipids because of high affinity of cyclosporine a for the protein component of lipoproteins. J Pharm Sci.; 90(9):1308-17.

66. Zahir H, Nand RA, Brown KF, Tattam BN, McLachlan AJ, (2001). Validation of methods to study the distribution and protein binding of tacrolimus in human blood. J Pharmacol Toxicol Methods. 2001; 46(1):27-35.

67. Bijsterbosch MK, Schouten D, van Berkel TJ (1994). Synthesis of the dioleoyl derivative of iododeoxyuridine and its incorporation into reconstituted high density lipoprotein particles. Biochemistry.; 33(47):14073-80.

68. Wilson, P. W.F. (2000). <u>Atlas of Atherosclerosis</u> 2nd Edition; Philadelphia; Current Medicine Group.

CHAPTER II

PREPARATION OF RECONSTITUTED HIGH DENSITY LIPOPROTEIN

PREFACE

Toxic side effects remain a serious concern during cancer chemotherapy, including the toxicity associated with some of the solubilizing agents currently in use (e.g. Cremophor). Novel methods of drug delivery for chemotherapy provide not only improved solubility but also enhanced targeting for anti-cancer agents. The first objective of this project was to formulate a reconstituted high density lipoprotein that could carry Cremophor-free paclitaxel. I found that HDL isolated from human plasma could act as a carrier for paclitaxel. With the appropriate solvent and formation method, the optimal formulation could be found, thus removing Cremophor as a concern. Because paclitaxel is highly water-insoluble, either an organic solvent or an amphipathic compound as a detergent had to be used. There are two common methods for rHDL formation, sonication or detergent dialysis. During sonication, the lipid aggregates are disrupted via mechanical means to form a lipid emulsion. In detergent dialysis, the lipid/drug ingredients of rHDL are mixed together in a dry film, followed by the addition of apo A-I and the detergent sodium cholate in an aqueous buffer. Subsequently, dialysis is used to remove the detergent and to coalesce the ingredients into a reconstituted HDL configuration. To determine if the paclitaxel was successfully encapsulated into the rHDL, density-gradient ultracentrifugation was used. Here, the radioactive paclitaxel is expected to float with the lipoprotein band if it remains incorporated into the rHDL complex. In this chapter, the experimental results of the solvents, detergents, and methods used are discussed.

SUMMARY

A new approach for drug delivery to malignant tumors has been developed by encapsulating lipophilic chemotherapeutic agents into reconstituted high density lipoproteins (rHDL). These rHDL complexes resemble normal HDL and they markedly improve the solubility and transportability of otherwise poorly soluble drugs. The purpose of the present study was to employ human HDL and rHDL, as drug delivery vehicles for paclitaxel, to minimize the potential immune response associated with intravenous therapy. HDL is capable of directly exiting the circulation and entering the tissues where the core components are taken up by cells.

INTRODUCTION

Two of the major concerns associated with cancer chemotherapy are the systemic toxicity of the chemotherapeutic agents and their water insolubility. These side effects include nausea, alopecia, weight loss, and sloughing of the gastrointestinal tract. In addition, some of the most frequently used chemotherapeutic agents, including adriamycin (doxorubicin), have serious toxicities associated with their use. For example, doxorubicin therapy can result in cumulative cardiotoxicity and Taxol ®, due to its Cremophor EL component, is also highly toxic.

This project involves the development of a drug delivery system using reconstituted high density lipoprotein (rHDL). High density lipoprotein is the densest, but has the smallest diameter of the lipoproteins (8-12 nm). It can pass into the sub-vascular tissue, unlike many liposomes, which are too large. Furthermore, HDL has a unique cholesteryl ester delivery mechanism, termed selective lipid uptake via a receptor-mediated process. The HDL receptor is known as Scavenger Receptor, Class B, Type I (SR-BI) in rodents and CD36 and LIMP II analogous protein (CLA-1) in humans. Anticancer drugs packed into the core of the rHDL may also be taken up through the mechanism of selective lipid uptake.

These reconstituted HDL (rHDL) particles contained Taxol, were efficiently taken up by cancer cells and were as toxic to cancer cells as the commercial Cremophor/Taxol preparations. Immunoblotting and competition studies demonstrated drug uptake by several cancer cell lines from the rHDL/taxol complex apparently occurred by an SR-BI type receptor mediated mechanism (1).However, due to the low amount of paclitaxel incorporated, I investigated a more

optimal formulation. The first approach used HDL isolated from human plasma to solubilize paclitaxel. I found that it appeared to solubilize un-emulsified paclitaxel (Sigma). This is already an improvement over current formulations that employ the toxic solubilizing agent Cremophor. However, due to concerns for using plasma-derived products, an artificial HDL capable of solubilizing paclitaxel had to be found.

The solubilization of paclitaxel in a rHDL particle was attempted with various organic solvents and detergents. Eventually, three approaches were tried in parallel. The first approach utilized Cremophor EL, but in much lower amounts, approximately 10-fold less than in the commercial formulation. The second approach utilized the Vitamin E tocopherol polyethylene glycol succinate (TPGS) as an emulsifier for paclitaxel (2); other groups have reported TPGS has anticancer activity (3). The last approach used a modified cholate dialysis procedure (4).

MATERIALS AND METHODS

<u>Materials</u> Egg yolk phosphatidyl choline, free cholesterol, cholesteryl oleate, potassium bromide (KBr), paclitaxel (PTX) were purchased from Sigma-Aldrich chemicals. [¹⁴C]paclitaxel was purchased from Sigma. Apolipoprotein A-I (apo A-I) was provided by ZLB Bioplasma. Vitamin E TPGS was provided by Eastman Chemical Company, Kingsport TN. <u>Taxol Distribution in Mouse Tissue</u> Female BALB-c mice 6-8 weeks of age (Harlan) were injected via tail vein with 200 mL Taxol in 1X PBS or 200 mL rHDL/Taxol (130,000-160,000 cpm; 5 mice per group). In the second study, Taxol and human HDL/PTX were used. At specific timepoints after injection, the mice were sacrificed humanely and their blood and organs were harvested. The tissue was minced and a sample dissolved in Tissue Solubilizer (Amersham) following manufacturer's protocol. Before scintillation counting, the tissue sample was decolorized with 30% H_2O_2 . The counts were calculated for the whole organ and all organ counts were totaled. The ratio was calculated as the organ count / total organ count. All animal studies were reviewed and approved by the IACUC.

Preparation of Reconstituted HDL Drug Complex

Mix 1.8mg Egg Yolk Phosphatidyl Choline(EYPC) in chloroform with 0.45mg free cholesterol in ethanol, 0.9mg cholesteryl oleate and 300µg of Taxol (or dissolved paclitaxel) & 130,000 cpm [14C] paclitaxel. Dry the mixture under N2 to form a thin film. Subsequently 5ml of Sonication Buffer (10mM Tris, 0.1M NaCl, 1mM EDTA pH 8.0) was added to the lipid-drug film. Sonicate the mixture for 60 min at 60°C. Reduce the temperature to 42°C and add 10mg of Apo A-1

dissolved in 3M guanidine-HDL and continue sonication for another 30 minutes. Collect the HDL-sized fraction by KBr-density gradient ultracentrifugation. Measure cholesterol, protein and paclitaxel and pool the fractions. Dialyze against 1X PBS overnight. Store at 4°C.

Preparation of rHDL/PTX from human HDL and paclitaxel

Human HDL was purified from plasma by dodecylamine-agarose (DDA) chromatography as described previously (7). Paclitaxel (300 μ g, dissolved in CHCl₃) was mixed with 130,000 cpm [14C] paclitaxel and dried under N₂. Three milliliters of either purified human HDL or bovine serum albumin (5 mg/mL by protein) were added and mixed. The drug-vehicle mixture underwent freeze-thaw cycling with an initial incubation at 37°C, then two freeze-thaw cycles at -20°C and 37°C.

Potassium Bromide Density Gradient Ultracentrifugation

A 3 milliliter sample of rHDL was used for KBr Density Gradient Ultracentrifugation (. One milliliter fractions were collected from each sample. The density was estimated by measuring the mass of a 100µL sample from each fraction. HDL-sized fractions were collected from density \approx 1.063 to 1.21 g/mL (Fractions 7, 8, 9).

Chemical Compositional Analysis

Protein concentration of the collected fractions was measured with Bradford protein reagent (Bio-Rad). Total cholesterol and phospholipid were determined by colorimetric assays (DMA). Paclitaxel and Taxol were measured with by the specific activity of [14C] paclitaxel (Sigma-Aldrich).

Vitamin E Assay

Ethanol was mixed and vortexed with a volume of sample in a 1:1 ratio to precipitate protein. The sample was centrifuged at 3,000 rpm for 5-10 minutes and the supernatant collected. 50 microliters of 4 mg/mL bathophenanthroline (in ethanol) was added to a 96-well microplate, then 100 microliters of sample supernatant was added. 50 microliters of 0.6mg/mL ferric chloride (in ethanol) and 50 microliters of 85% phosphoric acid were added. The absorbance was read at 536 nm on a Bio-Rad microplate reader (8). A 40 mg/ml stock solution of Vitamin E TPGS was used to form the standard curve.

RESULTS

It has been previously reported that rHDL particles containing Taxol were able to facilitate the uptake of the drug by cancer cells (1). Further investigation was needed into the pharmacokinetics of an rHDL/Taxol vehicle. Studies carried out in mice showed no significant differences in the bio-distribution of Taxol® and rHDL/Taxol (Figure 1). These results can possibly be explained by the remaining amount of Cremophor left in the complex. When assayed, it was found that the ratio of Cremophor to paclitaxel remained at 100:1 with the concentrations of Cremophor and paclitaxel at 1.71 mg/ml and 0.017 mg/ml, respectively. Due to the low paclitaxel concentration, the high Cremophor concentration, and the similar bio-distribution results, a search began for a new formulation with lowered or absent Cremophor. I investigated whether human HDL isolated from plasma could act as a vehicle for Taxol/paclitaxel (PTX). The experimental approach involved freeze-thaw cycling as described by Castile and Taylor(5). I compared the rHDL/Taxol & human HDL/PTX (Table 1) to a recent formulation involving serum albumin as a solubilizer/carrier for Taxol/Paclitaxel (6). The particles containing ¹⁴C taxol, were isolated via preparative ultracentrifugation and then dialyzed to remove the potassium bromide. Although serum albumin had high incorporation of Taxol, upon dialysis, the incorporation dropped markedly. Serum albumin is not a stable carrier for paclitaxel (Figure 2), therefore it has little potential as a drug delivery vehicle to cancer cells. Reconstituted HDL formed a com

plex with Taxol, but not with paclitaxel. The most promising results were with human plasma HDL and paclitaxel (Figure 2).

I repeated the tissue distribution studies with the human HDL/PTX formulation. I did not examine the tissues as exhaustively as in the initial study, but only concentrated on the liver and plasma. As in the rHDL/Taxol studies, the primary organ of PTX uptake was the liver. However, paclitaxel concentration was 10-fold higher in the plasma when injected as a component of the HDL complex, as compared to the free Taxol®. I found that the Taxol had 97% in the liver and 3% in the plasma 30 minutes after injection. In contrast, HDL/paclitaxel had 62% in the liver and 38% in plasma 30 minutes after injection. Similar results were found 60 minutes after injection (Figure 3).

Because human HDL is a product derived from human plasma, there are concerns about its use as a drug delivery vehicle. Subsequently, a number of studies have been conducted to develop an appropriate drug formulation that would not include Cremophor EL in the solubilization of PTX. The encapsulation and incorporation of paclitaxel in the rHDL was tested and measured by density gradient ultracentrifugation. The rHDL/PTX has a buoyant density similar to that of native HDL (d = 1.063-1.21 g/mL), which is usually fractions 7, 8, and 9 (Figure 4A). Because a current paclitaxel formulation using serum albumin as an emulsifier is undergoing clinical trials, I attempted serum albumin as an initial solubilizer before incorporating PTX into the rHDL particles as well as the organic solvent, acetone. (9). These approaches were unsuccessful; no macromolecular rHDL/PTX could be recovered after ultracentrifugation (Figure 4B). The paclitaxel was found in fractions corresponding to a density >1.22 g/mL. The results of the solvents and detergents tried for rHDL/PTX formulation are summarized in Table 2.

A derivative of Vitamin E, known as tocopherol polyethylene glycol succi-

nate(TPGS), had been used to form paclitaxel-containing nanoparticles(2). Furthermore, other groups reported anticancer activity by Vitamin E(3). When used as an emulsifier for paclitaxel, we achieved an rHDL/PTX with a similar buoyant density to native HDL (Figure 4C). Furthermore, rHDL/PTX/TPGS incorporated about 45-46% of the starting paclitaxel. We measured the concentration of TPGS remaining after particle formation by a colorimetric Vitamin E assay and found 1.2 to 2 mg/mL of TPGS. This concentration is similar to that found for Cremophor in the rHDL/Taxol formulation. The concentrations of the lipid and protein components of rHDL/PTX/TPGS are listed in Table 4.

Three different approaches were compared to formulating rHDL/PTX. First, a rHDL particle was made that contained paclitaxel emulsified in a 10-fold lower Cremophor concentration. The second approach formed an rHDL/PTX by a modified cholate dialysis procedure, which had been used previously to form rHDL particles containing a model compound, dilauryl flourescein (unpublished results). The last approach was rHDL/PTX using Vitamin E TPGS. Comparing the chemical composition and loading capacity (Table), the reduced Cremophor rHDL/PTX incorporated the least amount of PTX and cholate dialysis incorporated the most. Because the Vitamin E TPGS formulation also had a low amount of phospholipid than would be found in native HDL, this particle probably resembles a liposome or micelle. The optimal formulation for a HDL-based drug delivery vehicle was the modified cholate dialysis procedure; future studies were conducted with this protocol.

DISCUSSION

I have been able to utilize human HDL and rHDL as drug delivery vehicles for paclitaxel. Bovine serum albumin while capable of initially solubilizing Taxol and paclitaxel, was not stable upon dialysis and filter sterilization as its drug content dropped over 10-fold. Our data suggest that high density lipoprotein (HDL) is a better drug delivery vehicle than serum albumin because the drug/lipoprotein complex exhibited greater stability than the albumin/drug complexes. The tissue distribution experiment was repeated using the human HDL/paclitaxel (PTX) formulation. It was found that paclitaxel in this form remained in the plasma of the mice at 10-fold higher concentration than free Taxol. This is an important finding because the toxic emulsifier Cremophor has been removed. Also, the liver is the site of drug metabolism; by encapsulating paclitaxel in HDL, more active, unmetabolized drug could be available for tumor uptake. This may enable the drug to reach higher concentrations in the tumor tissue.

The uptake of HDL core components is mediated by Scavenger Receptor Class B Type 1 (SR-BI), which is found mainly in the liver, steroidogenic tissues, and macrophages. In addition, evidence suggests that cancer cells overexpress HDL receptors because the increased need for cholesterol in rapidly proliferating systems. The tissue distribution studies were critical, because the rHDL could deliver the paclitaxel to the liver and steroidogenic tissues via SR-BI.

However, when experiments on the tissue distribution of Taxol [®] were performed in mice, no differences could be found between groups injected with free Taxol [®] or rHDL/Taxol. In the second study, paclitaxel uptake by the liver was reduced when delivered by HDL. In addition, this mechanism could play an important role in overcoming the drug resistance of cancer cells. By the interaction of apolipoprotein A-1 and SR-BI, drugs that are delivered to the cancer cells may bypass the P-glycoproteins and other membrane transporters associated with drug resistance.

Further investigation into rHDL/PTX by cholate dialysis is necessary. The composition of the particle must be optimized. The assumption was made that because rHDL/PTX had the same buoyant density as native HDL, it is of similar size and shape. It is not known whether rHDL/PTX is discoidal like nascent HDL or spherical like mature HDL. Electron microscopy can confirm the size and shape of rHDL/PTX. Further animal studies must be performed as well. Removing the Cremophor should improve the toxicity profile for rHDL/PTX in comparison to Taxol. After determining safe dosages, rHDL/PTX can be used to treat tumorbearing mice to investigate the efficacy of the drug delivery vehicle.

Current research into the delivery of hydrophobic drugs has focused on the use of liposomes to solubilize and transport therapeutic agents in the bloodstream. These data suggest that the HDL based drug delivery system is capable of extending the maximum plasma concentration in addition to facilitating the solubility of hydrophobic drugs. Further potential advantages of the new drug delivery system involve targeting via chemical modifications of the protein or lipid constituents of the rHDL complex, in addition to overcoming the resistance of certain tumors against anti-cancer drugs.

Figure 1: Tissue Distribution of Taxol vs. rHDL/Taxol. After I.V. injection, mice were sacrificed and tissues harvested at the specified timepoints. Data are expressed as Mean \pm S.D. (n=5 mice/group). Significant differences were not found between the Taxol and rHDL/Taxol groups.





Table 1: Components for Paclitaxel Drug Delivery Vehicles. Solutions of bovine serum albumin and human HDL were made to a protein concentration of 5 mg/mL before incubating with drug. Freeze-thaw cycling conditions: 37°C, 60 minutes; -20°C, 60 minutes; repeated twice.

Name	Drug Vehicle	Paclitaxel Formulation
Alb/PTX	Serum Albumin	Paclitaxel (Sigma)
Alb/Taxol	Serum Albumin	Taxol
rHDL/PTX	rHDL	Paclitaxel (Sigma)
rHDL/Taxol	rHDL	Taxol
hpHDL/PTX	human plasma HDL	Paclitaxel (Sigma)
hpHDL/Taxol	human plasma HDL	Taxol

Figure 2. Incorporation of Paclitaxel into Drug Delivery Vehicles. Labeling of preparations correspond to the protocols found in Table 2. HDL-sized fractions were isolated by potassium bromide density gradient ultracentrifugation and dialyzed against 1X PBS, overnight. Paclitaxel incorporation was tracked by radiolabel. Data are expressed as mean \pm S.D., n = 4, from 2 independent preparations.



Figure 3: Distribution of Paclitaxel between Liver and Blood in the Mouse. Mice (5 per group) were sacrificed 30 to 60 minutes after I.V. injection and the liver and plasma harvested. I found that the HDL/PTX concentration in the plasma was 10-fold higher compared to Taxol. Data are expressed as Mean \pm S.D. (* represents p-value < 0.002).


Table 2: Reconstituted HDL with Alternate Solvents and Detergents for Paclitaxel. Three organic solvents were used to make an rHDL/PTX. In all 3 cases, the paclitaxel did not incorporate into the particle. A non-ionic detergent, n-octyl glucoside was not able to keep paclitaxel in solution. Serum albumin was tried due to reports that it complexes with paclitaxel. It was thought to potentially facilitate loading into the rHDL.

Emulsifier/Solvent	Result		
Ethanol	Low Incorporation		
Acetone	Low Incorporation		
Isopropanol	Low Incorporation		
10% Serum Albumin	Low Incorporation		
N-octyl glucoside	PTX Did Not Dissolve		

Figure 4: KBr Density Gradient Ultracentrifugation of Different rHDL/PTX preparations.

Each preparation was subjected to ultracentrifugation to determine PTX incorporation into

rHDL. One mL fractions were collected and measured for [¹⁴C]-paclitaxel, protein and choles-

terol. The fractions, corresponding to HDL lie in density range (1.063 to 1.21 g/ml) or Fraction

7, 8, and 9.

(A) rHDL/PTX by Cholate Dialysis. Data is expressed as Mean \pm S.D. of 2 representative preparations.

(B) rHDL/PTX dissolved with acetone. Data is expressed as Mean \pm S.D. of 2 representative preparations.

(C) rHDL/PTX Solubilized by Vitamin E TPGS. Data is expressed as Mean \pm S.D. of 3 representative preparations.



В





С

Table 3: Comparison of Chemical Composition of Different rHDL/PTX formulations. All

concentrations are in mg/mL. TC, total cholesterol; CE, cholesteryl oleate; FC, free (unesterified) cholesterol; PC, phosphatidyl choline; PTX, paclitaxel; rHDL, reconstituted high-density lipoproteins.

- (1): rHDL/Taxol: Original formulation
- (2) rHDL with Reduced Cremophor
- (3) rHDL/PTX with Vitamin E TPGS as emulsifier
- (4) rHDL/PTX by Cholate Dialysis

	Preparation	Units	РТХ	apo	ТС	CE	FC	PL	Cholic
				A-1					Acid
1	Sonication	mg/mL	0.017	1.14	0.33	0.24	0.09	4.3	0.00
2	Sonication/Less Cremophor	mg/mL	0.083	0.25	0.025	0.01	0.015	0.33	0.00
3	Sonication/TPGS	mg/mL	0.16	1.16	0.04	0.02	0.02	0.22	0.00
4	Cholate Dialysis	mg/mL	0.45	1.65	0.14	0.05	0.09	1.35	0.35

REFERENCES

1. Lacko AG, Nair M, Paranjape S, Johnson S, and McConathy WJ.; (2002). High density lipoprotein complexes as delivery vehicles for anticancer drugs. Anticancer Res.; 22(4):2045-9.

2. Mu L, and Feng SS; (2002). Vitamin E TPGS used as emulsifier in the solvent evaporation/extraction technique for fabrication of polymeric nanospheres for controlled release of paclitaxel (Taxol). J Control Release.; 80(1-3):129-44.

3. Youk HJ, Lee E, Choi MK, Lee YJ, Chung JH, Kim SH, Lee CH, and Lim SJ; (2005). Enhanced anticancer efficacy of alpha-tocopheryl succinate by conjugation with polyethylene glycol. J Control Release.; 107(1):43-52.

4. Jonas A.; (1986). Reconstitution of high-density lipoproteins. Methods Enzymol.; 128:553-82.

5. Castile JD, and Taylor KM; (1999). Factors affecting the size distribution of liposomes produced by freeze-thaw extrusion. Int J Pharm.; 188(1):87-95.

6. Paál K, Müller J, and Hegedûs L.; (2001). High affinity binding of paclitaxel to human serum albumin. Eur J Biochem.; 268(7):2187-91.

7. Jahani M, Huttash RG, and Lacko AG; (1980). A novel chromatographic method for the preparation of high density lipoproteins. Prep Biochem.; 10(4):431-44.

8. Fabianek, J., DeFillipi, J., Rickards, T., and Herp, A., (1968) "Micromethod for Tocopherol Determination in Blood Serum," Clinical Chemistry, 14 456-462.

9. Dosio F, Arpicco S, Brusa P, Stella B, and Cattel L. (2001) Poly(ethylene glycol)-human serum albumin-paclitaxel conjugates: preparation, characterization and pharmacokinetics. J Control Release. 76(1-2):107-17.

CHAPTER III

EVALUATION OF SYNTHETIC/RECONSTITUTED HIGH-DENSITY LIPOPROTEINS AS DELIVERY VEHICLES FOR PACLITAXEL

Reproduced from McConathy WJ, Nair MP, Paranjape S, Mooberry L, and Lacko AG, (2008). <u>Anticancer Drugs</u>; 19(2):183-8

PREFACE

The original cholate dialysis procedure produced phospholipid/Apo A-I discs, which is a precursor to mature spherical HDL. Mature spherical HDL is the form that interacts with SR-BI to deliver cholesteryl ester through selective lipid uptake. A mature HDL particle has high protein content (approximately 50-60%), due to the existence of two to four molecules of Apo A-I. Our rHDL delivery vehicle is produced via a modified cholate dialysis protocol. We determined the chemical composition of our reconstituted HDL and found the protein content is similar. I must ensure the rHDL has a shape and size similar to mature native HDL for delivery of paclitaxel through selective uptake.

Pharmacokinetic parameters, including the 50% Inhibitory Concentration (IC_{50}) and Maximum Tolerated Dose (MTD), must be determined for the rHDL/PTX vehicle. In the literature, MTD is defined as the dose at which no death and 15% weight loss occurs. Any studies in tumor-bearing mice base the treatment dosage on the MTD.

SUMMARY

Reconstituted (synthetic) high-density lipoprotein particles carrying paclitaxel (rHDL/PTX) were prepared with substantially higher PTX content than reported earlier. The rHDL/PTX complexes seemed to be primarily spherical nanoparticles when examined via electron microscopy, with a constant composition, molecular weight and exceptional stability even after ultracentrifugation and storage for up to 6 months. The rHDL/PTX nanoparticles had superior cytotoxicity against several cancer cell lines (MCF7, DU145, OV1063 and OVCAR-3), the half maximal inhibitory concentration (IC50) having been found to be 5–20 times lower than that of the free drug. Studies with mice showed that the rHDL/PTX nanoparticles were substantially better tolerated than the corresponding dosages of either Taxol or Abraxane.

INTRODUCTION

A number of challenging factors, including poor water solubility, toxic side effects and drug resistance, have long interfered with the efficacy of cancer chemotherapeutic agents. Lipoproteins have been considered appropriate drug-delivery vehicles capable of overcoming these problems [1], owing to their structural features [2], biocompatibility and targeting capability via receptor mediated mechanisms [3]. Our laboratory focused on the preparation and evaluation of reconstituted high-density lipoprotein (rHDL)/drug complexes, primarily for the purpose of enhancing the delivery of anticancer agents [4–6]. These more recent efforts, reported here, resulted in substantially more enhanced paclitaxel (PTX)-carrying capabilities, physical/chemical properties and in-vivo tolerability than our previously reported formulation [4].

MATERIALS AND METHODS

Materials

Sodium cholate, egg yolk phosphatidyl choline (PC), free cholesterol, cholesteryl oleate, potassium bromide (KBr) and PTX were purchased from Sigma-Aldrich chemicals (St Louis, Minnesota, USA). [14C]PTX was purchased from Sigma. Abraxane was a gift from Dr Ray Page, Department of Pharmacology, University of North Texas Health Science Center, Texas, USA (UNTHSC). For the initial phase of these studies, the apolipoprotein A-I (apo A-I) was provided by ZLB Bioplasma (Berne, Switzerland).

For the latter portion of the studies, the apo A-I was isolated from human plasma as described by Brewer et al. [7].

Methods

Preparation of reconstituted (synthetic) high-density lipoprotein/paclitaxel complex with sodium cholate dialysis

The cholate dialysis procedure used was based on the procedures described earlier for discoidal rHDL particles [8,9]. After testing a variety of formulations of ingredients, a mixture of PC in CHCl3 with free cholesterol, cholesteryl oleate and PTX was prepared with a molar ratio of apo A-I:cholesterol:cholesteryl oleate:PC=1 : 5 : 1.3 : 115. This formulation was based on preliminary studies, after varying the levels of the respective components to optimize the incorporation of PTX into rHDL. The lipid mixture (PC, C and cholesteryl oleate) and 2mg of PTX were dried under N2 to a thin film and dispersed in 60 ml of dimethylsulfoxide [(DMSO)

Sigma-Aldrich] and 1.4 ml of buffer (10 mmol/l Tris, 0.1 mol/l KCl and 1mmol/l EDTA; pH 8.0; Sigma-Aldrich). About 140 ml of sodium cholate [100 mg/ml stock in 0.15 mol/l NaCl, 0.003 mol/l KCl and 0.15 mol/l KH2PO4, pH 7.4, designated as phosphate buffered (0.008mol/l Na2HPO4, 0.002mol/l KH2PO4) saline (PBS; 0.14mol/l NaCL; 0.01 mol/l KCl); pH 7.4] was added, to produce mixtures with a final PC to the cholate molar ratio of B1 : 1.6. Apo A-I (12.7 mg/ml) in 0.4 ml of PBS was added to this mixture, and the final volume was adjusted to 2ml with PBS. The lipid/protein/cholate mixture was then incubated for 12 h at 4°C, followed by dialysis against 2 L of PBS for 2 days, with three buffer changes. Using [3H]cholate as a tracer, we determined that after 48 h of dialysis, <2% of the cholate remained in the rHDL/drug preparations. The PTX-containing rHDL fractions were isolated by single-step density gradient ultracentrifugation [10,11], and were dialyzed overnight against PBS, pH 7.4. These rHDL preparations can be stored at 41C for at least 60 days.

Preparative ultracentrifugation

KBr was added to 3ml of rHDL/drug preparation, to achieve a density of 1.22 g/ml. The sample was placed in a centrifuge tube, and KBr solutions of decreasing density were layered on top of the sample as follows: 1 ml, 1.22 g/ml; 4 ml, 1.063 g/ml and 3 ml, 1.019 g/ml [10,11]. The layered sample was subsequently centrifuged for 24 h at

39 000 rpm in a Beckman Optima LE 80K (Beckman Coulter Inc., Fullerton California, USA) ultracentrifuge at 4°C in a swinging bucket rotor (SW 40). Fractions (1 ml) were collected from the top and analyzed for protein, phospholipids and cholesterol. Fractions were designated as follows and analyzed as described earlier [12]: those corresponding to very low-density lipoprotein [fractions 1–2, buoyant density (d)<1.007 g/ml]; to intermediate density lipoprotein (fraction 3, d=1.007–1.02 g/ml); to low-density lipoprotein (fractions 4–6, d=1.020–1.060 g/ml);

to HDL2 (fractions 7–8, d=1.060–1.15 g/ml); to HDL3 (fractions 9–10, d=1.15–1.235 g/ml) and to lipoprotein-deficient serum (fractions 11–12, d>1.235 g/ml).

Electron microscopy

After dialysis against a buffer containing 0.125 mol/l ammonium acetate, 2.6 mmol/l ammonium carbonate and 0.26 mmol/l EDTA (pH 7.4), the isolated rHDL samples were negatively stained with 2% sodium phosphotungstate (pH 7.2) and placed on Formvar/ carbon-coated 200-mesh nickel grid support films (Ted Pella Inc., Reading, California, USA), as previously described by Forte et al. [13]. The particles were visualized (magnification of 50 000) using a Zeiss 910 (Carl Zeiss SMT Inc., Peabody, Massachusetts, USA) transmission electron microscope. The photographs obtained were enhanced, and the particle diameter was determined with Adobe Imageready CS2 (Adobe Systems Inc., San Jose, California, USA) software. For the estimation of the diameter of the rHDL/PTX nanoparticles, 50 images with distinct margins and symmetrical shapes were measured.

Cell culture

Established cancer cell lines were obtained from the American Type Culture Collection (Manassas Virginia, USA) and grown according to the procedures provided by the American Type Culture Collection, employing the respective recommended media, including the mixture of 1% penicillin and streptomycin and 10% fetal bovine serum (Gibco/Life Technologies, Invitrogen Corp, Carlsbad, California, USA). All the cells were grown in 75-cm2 flasks and split, using 0.25% trypsin to release the cells from the medium, once 80–90% confluency was reached. Cells were counted and plated onto 96-well microtiter plates, 1 day before being used for assays.

Assessment of the cytotoxicity of the reconstituted (synthetic) high-density lipoprotein/paclitaxel complexes

The cytotoxic effect of the rHDL/PTX preparations on cancer cells was assessed by the MTT assay [14]. Cells were plated in 96-well plates (5000 cells/well) in their respective media. Next day, the monolayers were washed with PBS (pH 7.4) twice, and then incubated at 37°C for 24 h with rHDL/PTX complexes in serum-free media. The following day, 25 \Box 1 of MTT (1 mg/ml) was added to each well and incubated for 3 h at 37°C. Plates were centrifuged at 1200 rpm for 5 min. The medium was removed, the precipitates were dissolved in 200 ml of DMSO and the samples were read at 540nm in a

microtiter plate reader.

Determination of reconstituted (synthetic) high-density lipoprotein components

Total cholesterol (Thermo DMA, Arlington, Texas, USA), free (unesterified) cholesterol and phospholipids (Wako Pure Chemical Industries Ltd, Osaka, Japan) were determined by the respective enzymatic reagent kits, using microtiter plate assays [15,16]. The concentration of cholesteryl esters was obtained as the difference between total and free cholesterol values. Protein determinations were carried out using Bradford reagent kits supplied by Bio-Rad Laboratories (Hercules, California, USA).

Molecular weight determination

The molecular weight of the rHDL/PTX complex was determined by fast protein liquid chromatography on a 10×300mm Superose 6 (Amersham Biosciences, Piscataway, New Jersey, USA) column using apoferritin, alcohol dehydrogenase and serum albumin as standards. The molecular weight of the rHDL/Ptx complex was calculated on the basis of the principles described by Ackers [17].

Determination of the maximum tolerated dose

Female C57BL6 mice (6–8 weeks, 18–21 g, Harlan) were housed, no more than four/cage, in accordance with the Institutional Animal Care and Use Committee guidelines. The mice were given standard mouse chow and water ad libitum, and the bedding was changed once a week. The mice were held for 1–2 days before the injection schedule began. Subsequently, the mice were randomized and grouped the day before injection. The injection volume in all the dosage groups was 1.5 ml via the intraperitoneal route, and injections were administered on days 1, 2 and 3. The dosage for Taxol injection was 30 mg/kg (n=6), for Abraxane, 70 mg/kg (n=6) and for rHDL/PTX, 100 mg/kg (n=6). A control group was injected with the rHDL vehicle (n=4). The weights and the health of the mice were monitored for 30 days. Weighings were performed once a day for the first 7 days and twice a week for the remaining monitoring period. These studies were approved by the Animal Use Committee of the UNTHSC.

Statistical methods

Statistical analysis was carried out to evaluate the data, when applicable. Accordingly, the data in Table 2 and Fig. 5 represent mean±standard error of the mean (SEM).

RESULTS

Formulation of reconstituted (synthetic) high-density lipoprotein/drug

A series of formulation studies were conducted to establish the optimal composition of the rHDL particles, to accommodate the maximum amount of PTX/particle. These studies were based on the protein (apo A-I) concentration of the rHDL complex, the largest component of these particles (Table 1). The preliminary data established the most favorable mixture of egg yolk phosphatidyl choline, cholesterol and cholesteryl oleate of the rHDL/PTX particles, which has been detailed under Methods (preparation of the rHDL/PTX complex).

Figure 1 shows the pattern of incorporation of PTX into the rHDL particles. The loading of PTX into the rHDL particles seemed to be maximal when about 2 mg of PTX was added to the reaction mix that contained the optimal amounts of the other ingredients. At this point, the incorporation efficiency of the drug was about 50%, with the maximal incorporation of 1mg of PTX corresponding to a 2-mg initial load.

Characterization of the reconstituted (synthetic) high-density lipoprotein/paclitaxel complexes

The PTX incorporation represented about 10% of the total mass of the rHDL complex (Table 1). Based on the estimated molecular weight of rHDL (~170 000), approximately 25 PTX molecules were incorporated into the rHDL/PTX complexes/particle. The overall composition of these rHDL/PTX complexes showed some resemblance to that of native human

HDL, although their PC content was substantially higher and their cholesterol content substantially lower than that of their circulating counterpart. During preliminary studies, the optimal PTX incorporation was observed with relatively low cholesterol-containing rHDL particles.

The retention of PTX within the rHDL complex vs. the retention of sodium cholate is shown in Fig. 1. The data show an approximately 60% retention of the PTX vs. a less than 5% retention of the sodium cholate, on the basis of the starting concentrations before dialysis. The rHDL/PTX particles were further characterized by ultracentrifugation and electron microscopy. In Fig. 2, the ultracentrifuge pattern of the rHDL/PTX preparation shows that the synthetic HDL/drug complex had flotation properties very similar to those of native HDL, as indicated by the arrows for the peak distribution of native human HDL and low-density lipoprotein. These flotation properties were maintained after 6 months' storage at 4°C. The lower peak, representing the stored sample, is due to the application of correspondingly lower starting material for the ultracentrifuge run.

Figure 3 shows the electron microscopy of the rHDL/PTX particles on phosphotungstate negative staining. These data show that, although some of the rHDL/PTX represent discoidal particles, the majority of the structures are spherical, with a mean diameter of 11.4 ± 3.1 nm (range 7.4–20.7 nm).

Assessment of the cytotoxicity of the reconstituted (synthetic) high-density lipoprotein/paclitaxel complexes

The MTT assay, as described under Methods, was used in these studies. First the MCF7 breast cancer cell line was used to evaluate the cancer cell-killing potential of the rHDL/PTX nanoparticles (Fig. 4). This was followed by a more detailed assessment using several cancer cell lines (Table 2). The progressively cytotoxic effect of the rHDL/PTX

82

nanoparticles with increasing drug concentrations is shown in Fig. 4. Clearly, the rHDLdelivered PTX was much more effective than the free PTX in this regard. A broader view of several cancer cell lines in Table 2 shows that the IC50 values for the rHDL-encapsulated PTX were 5–20-fold lower than those for the free PTX.

Animal studies

The purpose of these studies was to compare the respective tolerance levels of mice toward rHDL/PTX, Abraxane and Taxol. To design an effective protocol, the concentrations of PTX used were the established respective MTDs (30 mg/kg for Taxol and 70 mg/kg for Abraxane). The dosage for rHDL/PTX was chosen to exceed those of both the other formulations because better tolerance of this formulation by the mice was anticipated. The results of these experiments (in Fig. 5) show the time course of the mean weight change for the three groups of PTX-treated mice and the controls. On the basis of these data, the rHDL/PTX formulation proved to be markedly superior to both Abraxane and Taxol, as the 15% weight loss that occurred had been reached or exceeded at the 30-mg/kg dose for Taxol and at the 70-mg/kg dose for Abraxane. In contrast, the rHDL/PTX formulation did not reach the 15% weight loss even at the 100-mg/kg dose level of PTX. These data (in Fig. 5) also show that the initial weight loss (during the first 3-day period) might have been partially due to the trauma associated with the injections. Therefore, the weight changes for each treated group were combined for days 4-7 and presented as mean±SD (Fig. 6). The weight loss in the rHDL/PTX group was significantly less than in the Taxol group (P<0.0005), whereas it was substantially, but not significantly, lower than in the Abraxane group (P=0.084).

83

DISCUSSION

Poor solubility, toxic side effects and limited antitumor potential have consistently hampered the development of new chemotherapeutic agents. This study involves a practical approach to employ rHDL as drug-delivery agents for cancer chemotherapy. Lipoprotein-based formulations have long been thought of as attractive candidates for delivering anticancer drugs to tumors [18,19], primarily because of their biodegradable components and the potential for receptor-mediated uptake of the transported drug by endocytosis [20] or by the selective uptake of core components [21]. The overexpression of lipoprotein receptors by malignant cells and tumors has been widely reported [22–24], including the selective delivery of the toxic anticancer agents to tumors [25], further enhancing the utility of lipoprotein-based delivery platforms in cancer chemotherapy. In addition, recent studies show that lipoproteins can be targeted to specific tumors by attaching homing ligands to their surface components [26]. For these and other reasons, our efforts have focused on developing a synthetic HDL-type nanoparticle that is particularly suitable for the delivery of anticancer agents to tumors [4–6].

This report includes the description of a reconstituted rHDL/drug complex with superior drug-carrying capacity, compared with our earlier model [4]. In addition, we report, for the first time, the generation of spherical particles containing ingredients of human HDL via the cholate dialysis method (without the use of physical force). The spherical configuration of the rHDL/drug nanoparticles is an essential feature enabling enhanced drug loading and stability, an essential requirement for a practical formulation to be used for cancer chemotherapy.

Several attempts have been reported, over the last several decades, to use lipoprotein-type structures as drug delivery vehicles [27–29]. No reports are, however, available of these formulations being employed in a clinical setting or even undergoing phase I trials. The main obstacle in developing successful lipoprotein-based formulations has likely been the availability of the respective apolipoprotein components (apo B-100 and apo A-I) needed for these lipoprotein/drug formulations in commercially suitable quantities. This situation has, however, changed recently, with the development of processes for producing recombinant apo A-I via bacterial [27] or plant vehicles (http://www.sembiosys.com/Main.aspx?id=15), improving the prospects for preparing synthetic HDL-type drug-delivery vehicles. Additional attempts have been reported to overcome this problem by using small peptides as surrogates for apolipoprotein B-100 [28–30].

The data presented in this study open the way for further in depth investigations, to explore the antitumor potential of rHDL and other lipoprotein-based formulations, and to take advantage of the receptor/tumor-targeting potential of these nanoparticles [26].

Table 1. Composition of rHDL/PTX particles (weight %) prepared by the described cholate dialysis method. The data represent the mean ± SEM of three determinations. CE, cholesteryl oleate; FC, free (unesterified) cholesterol; PC, phosphatidyl choline; PTX, paclitaxel; rHDL, reconstituted high-density lipoproteins.

	Protein	PC	FC	CE	РТХ
Distribution	33.5 ± 1.1	58.8 ± 1.1	1.3±0.3	0.34±0.15	9.9+0.7
(%)					

Figure 1. Retention of paclitaxel (PTX) vs. sodium cholate by the reconstituted high-density lipoprotein (rHDL)/PTX complex during the dialysis process. Data are expressed as Mean ± S.D. of 3 independent preparations.



Figure 2. Distribution of the radioactivity of [14C]paclitaxel (PTX), incorporated into reconstituted high-density lipoprotein (rHDL), along with unlabeled PTX (see Methods) upon density gradient ultracentrifugation. The peak representing the rHDL/PTX preparation after 6 months' storage is smaller because approximately half of the original amount was used for the post-6-month centrifugation. LDL, low-density lipoprotein. Representative example.



Figure 3. Electron micrograph of the negatively stained reconstituted high density lipoprotein/paclitaxel nanoparticles.



Figure 4. Cytotoxicity of the reconstituted high-density lipoprotein (rHDL)/paclitaxel (PTX) nanoparticles vs. free PTX estimated by the MTT assay (see Methods).



Table 2. Comparison of IC50 values for selected cancer cell lines with rHDL/PTX and free PTX. The cells were exposed to increasing concentrations of the respective drug preparations for

 24 h before the performance of MTT cell viability assay. PTX, paclitaxel; rHDL, reconstituted

 high-density lipoproteins.

Cell lines used	IC ₅₀ for rHDL/PTX (µmol/I)	IC ₅₀ for PTX (µmol/l)
OV1063	1.2	14.2
OVCAR 3	14.1	70.3
DU145	1.8	14.2
MCF7	0.6	14.2

Figure 5. Weight loss incurred by female C57Bl/6 mice after three consecutive injections of paclitaxel (PTX) as Taxol, Abraxane and the reconstituted high-density lipoprotein (rHDL)/PTX formulation, respectively. Results are presented as the mean weight loss for each group treated with PTX (Taxol, Abraxane and the rHDL/PTX formulation, respectively) and the controls (rHDL only).



Figure 6. Weight loss data (mean±SD) combined for days 4–7 for each paclitaxel (PTX)treated group [Taxol, Abraxane and reconstituted high-density lipoprotein (rHDL)/PTX].


REFERENCES

1. Counsell RE, Pohland RC. Lipoproteins as potential site-specific delivery systems for diagnostic and therapeutic agents. J Med Chem 1982; 10:1115–1120.

2. Kader A, Pater A. Loading anticancer drugs into HDL as well as LDL has little effect on properties of complexes and enhances cytotoxicity to human carcinoma cells. J Control Release 2002; 80:29–44.

3. Firestone RA, Pisano JM, Falck JR, McPhaul MM, Krieger M. Selective delivery of cytotoxic compounds to cells by the LDL pathway. J Med Chem 1984; 27:1037–1043.

4. Lacko AG, Nair M, Paranjape S, Johnson S, McConathy WJ. High density lipoprotein complexes as delivery vehicles for anticancer drugs. Anticancer Res 2002; 22:2045–2049.

5. Lacko AG, Nair M, McConathy WJ. Lipoprotein nanoparticles as delivery vehicles for anticancer agents. In: Amiji M, editor. Nanotechnology in cancer therapy. Boca Raton: CRC Press; 2007. pp. 777–785.

6. Lacko AG, Nair M, Paranjape S, Mooberry L, McConathy WJ. Trojan horse meets magic bullet to spawn a novel, highly effective drug delivery model. Chemotherapy 2006; 52:171–173.

7. Brewer HB, Ronan R, Meng M, Bishop C. Isolation and characterization of apolipoprotein A-I, A-II and A-IV. Methods Enzymol 1986; 128:223–246.

8. Matz C, Jonas A. Micellar complexes of human apolipoprotein A-I with phosphatidylcholines and cholesterol prepared from cholate-lipid dispersion. J Biol Chem 1982; 257:4535–4540.

9. Jonas A. Reconstitution of high density lipoproteins. Methods Enzymol 1986; 128:553–582.

10. Kelley JL, Kruski AW. Density gradient ultracentrifugation of serum lipoproteins in a swinging bucket rotor. Methods Enzymol 1986; 128:170–181.

11. Terpstra AH,Woodward CJ, Sanchez-Muniz FJ. Improved techniques for the separation of serum lipoproteins by density gradient ultracentrifugation. Anal Biochem 1981; 111:49–57.

12. Loney WW, Kudchodkar BJ, Weis S, Clearfield MB, Shores J, Lacko AG. Evaluation of gemfibrozil therapy: predictive response from lipoprotein subfraction analysis. Am J Ther 1997; 9–10:301–309.

13. Forte T, Norum KR, Glomset JA, Nichols AV. Plasma lipoproteins in familial lecithin: cholesterol acyltransferase deficiency: structure of low and high density lipoproteins as revealed by electron microscopy. J Clin Invest 1971;50:1141–1148.

14. Arnould R, Dubois J, Abikhalil F, Libert A, Ghanem G, Atassi G, et al. Comparison of two cytotoxicity assays – tetrazolium derivative reduction (MTT) and tritiated thymidine uptake – on three malignant mouse cell lines using chemotherapeutic agents and investigational drugs. Anticancer Res 1990; 10:145–154.

15. Urrutia-Rojas X, Menchaca J, Wadley W, Ahmad N, Lacko AG, Bae S, et al. Cardiovascular risk factors in Mexican Americans at risk for type 2 diabetes mellitus. J Adolescent Health 2004; 34:290–299.

16. Kieft KA, Bocan TM, Krause BR. Rapid online determination of cholesterol among plasma lipoproteins after high performance gel filtration chromatography. J Lipid Res 1991; 32:859–866.

17. Ackers GK. Analytical gel chromatography of proteins. Adv Protein Chem 1970; 24:343–446.

18. Gal D, Ohashi M, MacDonald PC, Buchsbaum HJ, Simpson ER. Low density lipoprotein as a potential vehicle for chemotherapeutic agents and radionucleotides in the management of gynecologic neoplasms. Am J Obstet Gynecol 1981; 139:877–885.

19. Peterson C, Masquelier M, Rudling M, Soderberg K, Vitols S. Lipoproteins, malignancy, and anticancer agents. Targeted Diagn Ther 1991; 5:175–200.

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

21. Cao WM, Mura K, Imachi H, Yu X, Abe H, Yamauchi A, et al. Mutant high density lipoprotein receptor inhibits proliferation of human breast cancer cells. Cancer Res 2004; 64:1515–1521.

22. Markel A, Brook GJ. Cancer and hypocholesterolemia. Isr J Med Sci 2004; 30:787–793.

23. Vitols S, Angelin B, Ericsson S, Gahrton G, Juliusson G, Masquelier M, et al. Uptake of low density lipoproteins by human leukemic cells in vivo: relation to plasma lipoprotein levels and possible relevance for selective chemotherapy. Proc Natl Acad Sci U S A 1990; 87:2598–2602.

24. Goncalves RP, Rodrigues DG, Maranhao RC. Uptake of high density lipoprotein (HDL) cholesteryl esters by human acute leukemia cells. Leuk Res 2005; 29:955–959.

25. Lacko AG, Nair M, Paranjape S, Mooberry L, McConathy WJ. 2006 Receptor mediated uptake of anti-cancer drugs from reconstituted high density lipoproteins (rHDL). Presented at High Density Lipoproteins: From Basic Science to Therapeutic Applications, Parma, Italy, a satellite conference for the XIV International Symposium on Atherosclerosis.

26. Zheng G, Chen J, Li H, Glickson JD. Rerouting lipoprotein nanoparticles to selected alternate receptors for the targeted delivery of cancer diagnostic and therapeutic agents. Proc Natl Acad Sci U S A 2005; 102: 17757–17762.

27. Ryan RO, Forte TM, Oda MN. Optimized bacterial expression of human apolipoprotein A-I. Protein Expr Purif 2003; 27:98–103.

28. Baillie G, Owens MD, Halbert GW. A synthetic low density lipoprotein particle capable of supporting U937 proliferation in vitro. J Lipid Res 2002; 43:69–73.

29. Owens MD, Baillie G, Halbert GW. Physicochemical properties of microemulsion analogues of low density lipoprotein containing amphiphatic apoprotein B receptor sequences. Int J Pharm 2001; 228:109–117.

30. Nikanjam M, Blakely EA, Bjornstad KA, Shu X, Budinger TF, Forte TM. Synthetic nanolow density lipoprotein as targeted drug delivery vehicle for glioblastoma multiforme. Int J Pharm 2007; 328:86–94.

CHAPTER IV

RECEPTOR MEDIATED UPTAKE OF PACLITAXEL FROM A SYNTHETIC HIGH DENSITY LIPOPROTEIN NANOCARRIER

PREFACE

There are several examples of drugs being complexed with synthetic or reconstituted HDL. What has not been known is whether drugs complexed with rHDL interact with the HDL receptor, SR-BI and are taken up through selective uptake. Many studies that examine selective lipid uptake use the ldl A7 and ldl A7 [mSR-BI] cell lines. This cell line is derived from Chinese Hamster Ovary cells and does not express LDL receptor or SR-BI. Because the core components are taken up selectively, radiolabeled cholesteryl ester and paclitaxel was used in our rHDL particle to quantify uptake. Because the protein component remains extracellular in selective uptake, but is internalized in endocytosis, radioactive iodine was crosslinked to Apo A-1 for tracking and determining internalization.

SUMMARY

The purpose of these studies was to determine the mechanism(s) whereby paclitaxel, is taken up by cancer cells, once encapsulated into synthetic/reconstituted high density lipoprotein (rHDL). The uptake of paclitaxel (PTX) was found to be facilitated by the scavenger receptor type B-1 (SR-B1)when drug-loaded rHDL particles were incubated with cells that express the SRB1 receptor. Studies with double-labeled, PTX containing rHDL nanoparticles showed that prostate cancer (PC-3) cells incorporated PTX primarily via a selective (SR-B1 type) uptake mechanism. In the presence of a10-fold excess of plasma HDL, PTX uptake decreased to 30% of the control. These findings suggest that the incorporation of lipophilic drugs by cancer cells from rHDL nanoparticles is facilitated by a receptor mediated (SR-B1) mechanism.

SIGNIFICANCE

Recent reviews on cancer therapy have progressively emphasized the benefits of the targeted delivery approach for anticancer drugs (1). It has been shown that anticancer agents can be targeted to cancer cells that generally express a high level of lipoprotein receptors, by encapsulation into reconstituted high density lipoprotein (rHDL) nanoparticles. The findings of the studies presented here suggest that targeted therapy via lipoprotein delivery vehicles may be an important and timely approach for cancer chemotherapy.

INTRODUCTION

Barriers to effective systemic drug delivery include poor solubility, first-pass metabolism by the liver, and toxic side effects of many pharmaceutical formulations. Numerous attempts have been made to overcome these problems by utilizing several novel delivery strategies (1, 2). Nevertheless, recent reviews on the outcome of even some of the most aggressive chemotherapy regimens, demonstrate only limited advances toward the cure of malignancies (3). Perhaps targeted delivery of anti-cancer agents (1) and individualized therapy will offer more dramatic advances toward curing cancer in addition to markedly improve the quality of life of patients undergoing treatment (4).

Lipoproteins have long been considered as effective drug transporting vehicles because their structure contains a shielded core, suited to accommodate lipophilic drugs, including anti-cancer agents (5). Therefore, pharmaceutical preparations involving lipoprotein components will likely result in formulations with extended residence time in the circulation. Furthermore, the uptake of cholesterol carrying lipoproteins (low density lipoproteins; LDL and high density lipoproteins; HDL) have been proposed to be providers of lipid membrane constituents to cancer cells to allow their high rate of proliferation (6, 7). Accordingly, it has been demonstrated that the expression of both LDL and HDL receptors are enhanced in cancer cells (8, 9). Clinical data collected from hundreds of cancer patients are consistent with this concept (10-12). Lipoproteins thus have the potential to exceed the performance of most currently employed drug delivery systems, including

liposomes, because of their relatively small size and their versatility in targeting (13.Lacko et al, 2007).

We have developed biocompatible nanoparticles made up of high density lipoprotein constituents that are able to stably incorporate lipophilic drugs, including PTX, presumably into their core compartment (14). In this communication, data are presented to show that PTX, encapsulated in reconstituted/synthetic high density lipoprotein (rHDL) nanoparticles, is taken up by cancer cells via a receptor mediated mechanism that is identical to the path that facilitates the uptake of cholesteryl esters from high density lipoproteins. The increased scavenger receptor type B1 (SR-BI) expression and cholesterol uptake by cancer cells suggest that effective targeted drug delivery via reconstituted high density lipoprotein (rHDL) formulations should be clinically feasible.

MATERIALS AND METHODS

Cell Culture

PC3 cells (ATCC) were maintained in DMEM/F-12 media supplemented with 10% Fetal Bovine Serum in 75-cm2 flasks. The cell lines ldl A7 and ldl A7 [mSRBI] (15) were grown in Ham F-12 media, supplemented with 2 mM glutamine, 5% Fetal Bovine Serum, 1% Penicillin/Streptomycin, and 300 µg/mL G-418 for ldl A7 [mSR-BI] only. PZ-HPV7 cells were grown in Keratinocyte-SFM media (Invitrogen) supplemented with 0.05 µg/mL Bovine Pituitary Extract and 5 ng/mLEGF. LNCap and DU145 cell lines were grown in RPMI 1640 media supplemented with 10% Fetal Bovine Serum, 2 mM glutamine, and 1% Penicillin/Streptomycin.

Paclitaxel Uptake in ldl A7 and ldl A7 [mSR-BI] cells

Plasma HDL was isolated by potassium bromide density gradient ultracentrifugation (16, 17). The fractions at density 1.063 g/mL to 1.21 g/mL were collected and dialyzed against 1X PBS, pH 7.4 at 4°C overnight. The dialysate was concentrated to 3.95 mg/ml of protein (measured by BCA assay). The cell lines ldl A7 and ldl A7 [mSR-BI] were plated at 150,000 cells/well in 24-well plates and incubated at 37°C for 24 hours. Each well was washed twice with 1X PBS, pH 7.4 and serum-free Ham's/F-12 media was added for 1.5 hours. After serum- starvation, rHDL/CE or rHDL/Ptx was added in the absence or presence of 10-fold excess (by protein) of plasma HDL. After 3 hours, the media was removed and the wells washed once with 1X PBS, pH 3.0 and once with 1X PBS, pH 7.4. 200 μ L of 0.5 M NaOH was added to lyse the cells. The protein concentration of the cell lysate was measured by BCA assay.

Radiolabeled paclitaxel or cholesteryl ester in the cell lysates was measured by scintillation counting.

Western Blotting

All cell lines were grown to confluency and were removed from the flask with scraping and Cell Extract Buffer (50mM Tris pH 8.0, 150mM NaCL, 0.02% Sodium Azide, 1 µg/mL Aprotinin, 100 µg/mL PMSF, and 1% NP-40). Cells were lysed by sonication and protein concentration of the extracts was determined by BCA protein assay (Pierce). 10µg of cell extract protein was separated on SDSPAGE and transferred to nitrocellulose membrane (Bio-Rad). The membrane was probed with anti-SRBI antibody (Novus Biologicals) and visualized with antirabbit peroxidase-linked secondary antibody (Pierce). The β -actin loading control was probed with anti- β actin antibody (Cell Signaling Technology, Inc.).

Apolipoprotein A-I Iodination

Apo A-I was labeled with 125 Iodine (Amersham) using IODO-Beads Iodination Reagent (Pierce), following manufacturer's protocols. The unreacted iodine was removed using Biogel P-2 (Bio-Rad). The iodinated Apo A-I was mixed with unlabeled Apo A-I and used to form rHDL/Ptx that was also labeled with [¹⁴ C]-paclitaxel (Sigma). The procedure for rHDL/Ptx formation was as described (13). Proper diameter of the [¹²⁵ I]-rHDL/Ptx was confirmed using 4-20% non-denaturing gradient gel electrophoresis (Bio-Rad).

Folic acid conjugation of apo A-I

2mg folic acid was dissolved in 250µl of anhydrous DMSO and incubated with 5- fold molar excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide for 30 minutes at 23°C in the dark (18). 500µg of Apo A1 was dialyzed against 0.1 M NaH2PO4, 0.1 M boric acid, pH 8.5. A 10fold molar excess of carbodiimide-incubated folic acid was then added to the protein solution, and incubated at 23°C for 1 hour. Unchanged material was separated from the labeled protein using a Sephadex G -25 column equilibrated in PBS, pH 7.4. FA-conjugated Apo A1 was used to prepare the rHDL as described. Paclitaxel uptake was measured as described earlier (8).

Binding/Uptake Studies

PC3 cells were plated on 24-well plates at a concentration of 120,000 cells/well and incubated overnight at 37°C. The cells were serum-starved for 30 minutes with serum-free DMEM/F-12 media. Media containing rHDL/Ptx only or rHDL/Ptx with a 10-fold excess of Apo A-I, Apo A-I/PC discs, or HDL₃ was added and the cells were incubated at 37°C for 90 minutes. The media was removed and the cells were washed twice with 1X PBS, pH 3.0 and once with 1X PBS, pH 7.4 to remove any extracellularly bound rHDL. The cells were lysed with 0.5M NaOH and counted for ¹²⁵Iodine and ¹⁴C.

Statistical Analyses

Data are expressed as mean \pm S.D. Statistical analyses were performed using Student's t-test.

RESULTS

The purpose of these studies was to determine the role of lipoprotein receptors in the uptake of paclitaxel (PTX) from a lipoprotein-type drug carrier system. I initially used the parental and the SR-BI-transfected ldl A7 cell lines (15), to determine whether PTX uptake from rHDL was correlated with SR-BI expression. I found that the uptake of both CE and PTX were 3-fold higher by the SR-B1 over-expressing (ldl A7) cells (Figure 1) compared to the control. Subsequently, I examined PTX uptake by prostate cancer (PC3) cells from rHDL by using a double-labeled procedure slightly modified from the method of Benoist et al (19). The PC3 cells have been shown to have a relatively high expression of the SR-BI receptor (8, Figure 2A). Double-labeled rHDL/PTX particles were prepared containing [14C] -paclitaxel in the rHDL core and [125I] labeled apo A-I. When examined by non-denaturing gradient gel electrophoresis, these [125I]-labeled rHDL/PTX complexes showed essentially the same mobility as unlabeled rHDL/PTX, intermediate between HDL2 and HDL3 (Figure 2B).

Using the method of Acton et al (20), the selective (SR-B1 mediated) uptake of PTX from the rHDL complexes was estimated by subtracting the protein (whole particle) uptake, based on 125I (protein) incorporation, from the total uptake, based on the 14C incorporation by the PC-3 cells. These studies showed that 82% of the total incorporated PTX was taken up via a selective (SRB1 mediated) mechanism by the PC-3 cells (Figure 3A).

To obtain additional data regarding the nature of the encapsulated PTX uptake by PC-3 cells, competition studies were performed with a 10-fold excess of apo A-1, discoidal rHDL, and

native HDL3 respectively. While apo A-1 alone was unable to inhibit PTX uptake from rHDL, both discoidal rHDL and native HDL3 inhibited paclitaxel uptake to about 30% of control rHDL/Ptx uptake levels (Figure 3B). These data strongly suggest that paclitaxel uptake from rHDL/Ptx is mediated through an SR-BI type HDL receptor.

We have explored another receptor-mediated drug uptake mechanism from nanoparticles involving the folic acid receptor (21). Accordingly, folic acid was conjugated to the Apo A-1 component of the rHDL nanoparticles, to form FA-rHDL/PTX. The folic acid receptor has been found to be over-expressed in ovarian cancer cells and tumors, including the drugresistant ovarian cancer cell line, OVCAR-3 (22). When PTX uptake was compared from FArHDL/PTX and rHDL/PTX in OVCAR-3 cells, the PTX incorporated was about 5-fold higher from the folate conjugated rHDL nanoparticles than Taxol®, and 2-fold higher that of the rHDL encapsulated PTX respectively (Figure 4). These findings thus suggest that the folic acid receptor (and perhaps numerous other cell surface receptors) may be taken advantage of to facilitate the uptake of anti-cancer agents for therapeutic purposes.

DISCUSSION

The cholesterol carrying lipoproteins, HDL and LDL, have similar features. Both are of relatively small size, biocompatible, with similar lipid components. HDL is smaller (11 nm vs. 20 nm), has a higher protein-to-lipid ratio, and is known as the "good cholesterol" carrier. Both native LDL and HDL have been experimented with as drug carriers (21,13), however, practical and safety consideration have prevented them from being developed toward clinical applications. Synthetic LDL has also been studied and was found to be effective in delivering drugs or imaging agents to glioblastoma cells (23, 24). Nevertheless, this approach has been judged to be premature due a number of concerns, including lack of homogeneity (23) and to potential antigenicity and drug leakage (25) of the sLDL particles.

It has been previously reported that cancer cell lines show an enhanced expression of the SR-BI receptor (8). Subsequently, Cao et al showed that breast tumor cells markedly overexpressed the CLA-1 receptor compared to normal cells (26). Several other studies have also found increased SR-BI expression in breast cancer cell lines (27), hepatocarcinoma cell lines (28), and testicular tumors (29). From the Western blot shown in Figure 2, minimal expression of SR-BI was seen by PZ-HPV7, a non-tumorigenic prostate cell line, compared to the three prostate tumor cell lines, LNCap, DU145, and PC-3. These studies are in concert with our current observations that selective delivery of anti-cancer agents via the rHDL delivery model is feasible as normal peripheral tissues are expected to have minimal expression of SR-BI and thus marginal uptake of the anti-cancer drug from the rHDL carrier (6). Recent studies have shown that tumor tissue avidly incorporated anti-cancer agents, when delivered via the rHDL vehicle while only a marginal amount of the payload was taken up by the liver and none by any of the peripheral tissues (30).

Other studies have been performed with native LDL particles labeled with folic acid residues for delivery of imaging agents and anticancer drugs (21). These folic acid conjugated LDL particles have shown that the delivery of FA-LDL occurred only through the FA receptor and not via the LDL receptor (21). The higher expression of the folate receptor by malignant tissues (31) apparently contributes to the enhanced drug uptake by cancer cells from the FA-rHDL/PTX particles (Figure 4). Furthermore, this strategy may provide the opportunity for conjugating a wide range of other targeting molecules to rHDL and thus may open the way for individualized therapy.

The frontier of cancer therapy is individualized treatment with drug formulations utilizing highly specific targeted delivery. Reconstituted high density lipoproteins offer many advantages as drug delivery agents because they mimic the native HDL particle in size and in the selective uptake of core components. Individualized therapy may be thus achieved by the screening of tumors for specific receptor or surface antigen expression and then attaching the appropriate targeting agent to Apo A-1 to produce a highly effective rHDL vector for drug delivery.

Figure 1. Increased Uptake of Cholesteryl esters and Paclitaxel by SR-BI transfected cells. Uptake of radiolabeled cholesteryl ester (CE) and radiolabeled paclitaxel (Ptx) from rHDL were measured in ldl A7 (open bars) and ldl A7 [mSR-BI] (checked bars) cell lines in the absence or presence of 10-fold excess of plasma HDL. Uptake of CE was significantly higher (p<0.05) in the transfected ldl A7 [mSR-BI] as well as uptake of Ptx (p<0.001). Data are expressed as mean \pm S.D., (n=4).



Figure 2. SR-BI is Expressed in Prostate Cancer Cell Lines, But Minimally in Normal Prostate Cells.

(A) 10 μ g of cell extract protein were run on SDS-PAGE and blotted for SR-BI. A band representing SR-BI(82kDa) can be seen above the 75kDa marker in PC3, DU145, and LNCap. β -actin was used as loading control.

(**B**) The electrophoretic mobility of rHDL is found to be similar to plasma HDL. 10 μ g of sample was run on 4-20% polyacrylamide gel electrophoresis.



В



A

Figure 3. Mechanism of Paclitaxel Uptake in PC3 Prostate Cancer Cell Line is Receptor-Mediated. (A) Counts from [125I]-labeled Apo A-1 were used to determine paclitaxel taken up by internalization. The difference between total and internalized paclitaxel is the paclitaxel taken intracellularly by a selective mechanism. (B) When 10-fold excess of Apo A-1, discoidal Apo A-1/PC complexes, and plasma HDL3 were incubated with rHDL/Ptx, it decreased Ptx uptake by 20% (p>0.05), 72%(p>0.0001, and 70%(p>0.0001) respectively. Data are expressed as mean ± S.D., (n=4).





А



Figure 4. Paclitaxel Uptake by the OVCAR-3, ovarian cancer cell Line. Following protocols previously published in (Lacko, et al, 2002), uptake was measured by tracking radiolabeled paclitaxel in a drug-resistant ovarian cancer cell line. Paclitaxel uptake from rHDL is 2-fold higher than Taxol and 5-fold higher with targeted folate-rHDL. Data are expressed as mean \pm S.D., n = 3 wells.



REFERENCES

1. Das, M., Mohanty, C. and Sahoo, S.K. (2009). Ligand-based targeted therapy for cancer tissue. Expert Opin. Drug Deliv. *6*, 285-304.

2. Fenske, D.B., Chonn, A. and Cullis, P.R. (2008). Liposomal nanomedicines: an emerging field. Toxicol. Pathol. *36*, 21-29.

3.Richardson, D.L., Backes, F.J., Seamon, L.G., Zanagnolo, V., O'Malley, D.M., Cohn, D.E., Fowler, J.M. and Copeland, L.J. (2008). Combination gemcitabine, platinum, and bevacizumab for the treatment of recurrent ovarian cancer. Gynecol. Oncol. *111*, 461-466.

4. Hamilton S.R. (2008). Targeted therapy of cancer: new roles for pathologists in colorectal cancer. Mod Pathol. Suppl 2:S23-30.

5. Counsell R.E, and Pohland R.C. (1982) Lipoproteins as potential site-specific delivery systems for diagnostic and therapeutic agents. J. Med. Chem. 25, 111-125.

6. Lacko A.G, Nair M, Paranjape S, Mooberry L. McConathy W.J. (2006) Trojan horse meets magic bullet to spawn a novel, highly effective drug delivery model. Chemotherapy 52, 171 - 173.

7. Vitols S. (1991). Uptake of low-density lipoprotein by malignant cells-possible therapeutic applications. Cancer Cells. *3*, 488-95.

8. Lacko, A.G., Nair, M., Paranjape, S., Johnson, S. and McConathy, W.J. (2002). High density lipoprotein complexes as delivery vehicles for anticancer drugs. Anticancer Res. 22, 2045-2049.

9. Vitols S, Angelin B, Ericsson S, Gahrton G, Juliusson G, Masquelier M, Paul C, Peterson C, Rudling M, Söderberg-Reid K, et al. (1990). Uptake of low density lipoproteins 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, 2598-602.

10. Dessi S. Batetta B. Pulisci D. Accogli P, Pani P, Broccia G. (1991) Total and HDL cholesterol in human hematologic neoplasms. Int. J. Hematol. 54, 483-486.

11. Fiorenza A.M. Branchi A. Sommariya D. (2000). Serum lipoprotein profile in patients with cancer. A comparison with non-cancer subjects. Int. J. Clin. Lab. Res. 30, 141 -145.

12. Markel A. Brook G.J. (1994). Cancer and hypocholesterolemia. Isr. J. Med. Sci. 30,787 -793.

13. Lacko AG, Nair, M, L. Prokai, McConathy WJ. (2007). Prospects and challenges of the development of lipoprotein based drug formulations for anti-cancer drugs. Expert Opinion on Drug Delivery 4, 665-675.

14. McConathy, W.J., Nair, M.P., Paranjape, S., Mooberry, L. and Lacko, A.G. (2008). Evaluation of synthetic/reconstituted high-density lipoproteins as delivery vehicles for paclitaxel. Anticancer Drugs *19*, 183-188.

15. Acton, S.L., Scherer, P.E., Lodish, H.F., and Krieger, M. (1994). Expression cloning of SR-BI, a CD36-related class B scavenger receptor. J Biol Chem.; 269; 21003-9.

16. Kelley, J.L. and Kruski, A.W. (1986). Density gradient ultracentrifugation of serum lipoproteins in a swinging bucket rotor. Methods Enzymol. *128*, 170-181.

17. Redgrave, T.G., Roberts, D.C. and West, C.E. (1975). Separation of plasma lipoproteins by density-gradient ultracentrifugation. Anal. Biochem. *65*, 42-49.

18. Leamon, C.P. and Low, P.S. (1993). Membrane folate-binding proteins are responsible for folate-protein conjugate endocytosis into cultured cells. Biochem. J. *291*, 855-860.

19. Benoist, F., Lau, P., McDonnell, M., Doelle, H., Milne, R. and McPherson, R. (1997). Cholesteryl ester transfer protein mediates selective uptake of high density lipoprotein cholesteryl esters by human adipose tissue. J. Biol. Chem. 272, 23572-23577.

20. Acton, S., Rigotti, A., Landschulz, K.T., Xu, S., Hobbs, H.H. and Krieger, M. (1996). Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science 271, 518-520.

21. Zheng, G., Chen, J., Li, H. and Glickson, J.D. (2005). Rerouting lipoprotein nanoparticles to selected alternate receptors for the targeted delivery of cancer diagnostic and therapeutic agents. Proc. Natl. Acad. Sci. U. S. A. *102*, 17757-17762.

22. Yamada, A., Taniguchi, Y., Kawano, K., Honda, T., Hattori, Y. and Maitani, Y. (2008). Design of folate-linked liposomal doxorubicin to its antitumor effect in mice. Clin. Cancer Res. *14*, 8161-8168.

23. Nikanjam, M., Blakely, E.A., Bjornstad, K.A., Shu, X., Budinger, T.F. and Forte, T.M. (2007). Synthetic nano-low density lipoprotein as targeted drug delivery vehicle for glioblastoma multiforme. Int. J. Pharm. *328*, 86-94.

24. Nikanjam, M., Gibbs, A.R., Hunt, C.A., Budinger, T.F. and Forte, T.M. (2007). Synthetic nano-LDL with paclitaxel oleate as a targeted drug delivery vehicle for glioblastoma multiforme. J. Controlled Release *124*, 163-171.

25. Corbin, I.R. and Zheng, G. (2007). Mimicking nature's nanocarrier: synthetic low density lipoprotein-like nanoparticles for cancer-drug delivery. Nanomedicine *2*, 375-380.

26. Cao, W.M., Murao, K., Imachi, H., Yu, X., Abe, H., Yamauchi, A., Niimi, M., Miyauchi, A., Wong, N.C. and Ishida, T. (2004). A mutant high-density lipoprotein receptor inhibits proliferation of human breast cancer cells. Cancer Res. *64*, 1515-1521.

27. Pussinen, P.J., Karten, B., Wintersperger, A., Reicher, H., McLean, M., Malle, E. and Sattler, W. (2000). The human breast carcinoma cell line HBL-100 acquires exogenous cholesterol from high-density lipoprotein via CLA-1 (CD-36 and LIMPII analogous 1)-mediated selective cholesteryl ester uptake. Biochem. J. *349*, 559-566.

28. Wadsack, C., Hrzenjak, A., Hammer, A., Hirschmugl, B., Levak-Frank, S., Desoye, G., Sattler, W. and Malle, E. (2003). Trophoblast-like human choriocarcinoma cells serve as a suitable in vitro model for selective cholesteryl ester uptake from high density lipoproteins. Eur. J. Biochem. *35*, 451-462.

29. Arenas, M.I., Lobo, M.V., Caso, E., Huerta, L., Paniagua, R. and Martin-Hidalgo, M.A. (2004). Normal and pathological human testes express hormone-sensitive lipase and the lipid receptors CLA-1/SR-BI and CD36. Hum. Pathol. *35*, 34-42.

30. Lacko AG, Shahzad S, Nair M, Mangala LS, McConathy WJ, Lopez-Berestein G, Sood AK (2009). Targeted delivery of drugs via biocompatible nanoparticles (Abstract). NanoIsrael 2009, Jerusalem, Israel.

31. Weitman, SD, Lark RH, Coney LR, Fort DW, Frasca V, Zurawski VR, Jr., Kamen BA (1992). Distribution of the Folate Receptor GP38 in Normal and Malignant Cell Lines and Tissues. Cancer Res. *52*, 3396-3401.

CHAPTER V

CONCLUSIONS AND DISCUSSION

The studies in the following section are the continuation of the *in vivo* evaluation that began with the tissue distribution and Maximum Tolerated Dose studies. They were not completed at the time of publication of the Material in Chapter III and so are included here.

ADDITIONAL STUDIES

A comparison was made between an HDL-type particle made by a different process than cholate dialysis and rHDL. This HDL was formed by the mechanical disruption of the lipid film by sonication (1). The drug chosen for incorporation was cyclosporin A, an immunosuppressant compound. The lipid formulation was different, using 1-palmitoyl 2-oleoyl phosphatidylcholine and no cholesterol, free or esterified. This process was used to form paclitaxel-containing particles, here referred to as "Sparks HDL". I compared the chemical composition of our rHDL vehicles, containing paclitaxel and cyclosporine A, to the Sparks HDL vehicle (Table 1). efficacy of these paclitaxel vehicles was tested in nude mice bearing MDA-MB-435 breast cancer tumors. Due to the low incorporation of paclitaxel and low treatment dose, Sparks HDL did not cause any tumor suppression in these mice (Figure 1). However, a dose of rHDL/PTX at 80% of Maximum Tolerated Dose (80 mg/kg) caused a 60% suppression of the tumor in that treatment group as compared to the untreated controls (Figure 2), which is comparable to results obtained with Taxol®.

DISCUSSION

In this project, I have shown that a synthetic high density lipoprotein can be reconstituted from lipid and protein components, also incorporating a model chemotherapy drug, paclitaxel. It has been proposed that drugs with a logP value of greater than 2 could be capable of incorporating into rHDL (2). By performing basic organic chemistry, fatty acids could be attached to drug agents, making them more hydrophobic and more easily incorporated into rHDL. For example, the incorporation of paclitaxel into a synthetic LDL particle was <20%, but increased to 48.5% when paclitaxel oleate was used (3). Many chemotherapy agents that were set aside due to solubility problems could be rescued with the rHDL vehicle, increasing the host of agents to battle cancer.

It has been assumed that the paclitaxel is located in the hydrophobic core of the rHDL particle, however this is not definitive. The loss of paclitaxel content in the rHDL system may be due to association of the drug with the exterior phospholipid bilayer, instead of the core. Further studies are planned to determine the location of the drug in the interior or associated with the exterior. Exploration of the uptake mechanism of core components from rHDL vehicles by observing the trafficking of the fluorescently labeled cargo from rHDL by real-time fluorescence microscopy may coincide with structural studies.

Throughout these studies, we have found that the expression of SR-BI is minimal in normal tissues and enhanced in cancerous tissues. The Scavenger Receptor Class B Type I has the potential to be a bio-marker. It is possible that as tumor cells proliferate and become more aggressive and invasive, that the SR-BI expression will correlate with the degree of malignancy. To explore the use of SR-BI as a bio-marker, the expression of SR-BI must be examined in a series of breast tissue cell lines, ranging from normal, pre-cancerous, malignant, to invasive.

Research projects often focus on only one type of cancer; in this project, cancers of the reproductive tissues were studied. Breast, ovarian, and prostate cancer are all steroidogenic tissues and as such have expanded cholesterol pathways compared to other non-hormone producing tissues. A future avenue of exploration could be to explore whether cholesterol taken up by cancers of reproductive tissues is used for hormone production, instead of proliferation.

In the maximum tolerated dose studies, the intraperitoneal (I.P.) route was chosen to allow a greater volume of drug to be injected into the mouse. Tissue distribution studies comparing the I.P. versus intravenous routes have found increased circulation time for drugs by the I.P. route (4). C57Black mice were chosen for the MTD studies due to the fragility and expense of nude mice. The MDA-MB-435 breast carcinoma cell line and female nude mice were chosen as the *in vivo* model. Although SR-BI expression was not examined in the MDA-MB-435 model, this breast cancer cell line has been known as a ductal carcinoma, similar to T47D. Initial studies into the expression of SR-BI in cancer cell lines found moderate levels of the receptor in T47D (5). However, a recent controversy exists over the identification of MDA-MB-435 as a melanoma cell line rather than a breast cancer cell line (6). Recent publications report that MDA-MB-435 is of breast cancer origin, but is a member of a basal-type subgroup. It has

been suggested that MDA-MB-435 has some features and genetic mutations similar to melanoma, leading to the misclassification (7, 8).

If a selective uptake mechanism of a wider range of pharmaceutical agents is supported then anticancer drug uptake via rHDL delivery may be able to bypass of the ABC family of transporters and multi-drug resistance of many cancers. We have found an increase in drug delivery to the OVCAR-3 drug-resistant ovarian cancer cell line when formulated into rHDL/Ptx. When folic acid was cross-linked to Apo A-1, drug delivery was further targeted to the ovarian cancer cells. Future studies will examine the anti-tumor activity of FA-linked rHDL/Ptx in tumor-bearing mice.

Other studies were performed by a group using native LDL particles labeled with folic acid residues for delivery of imaging agents and perhaps anticancer drugs (9). These folic acid derivatized LDL particles have shown that the delivery of FA-LDL occurred only through the FA receptor and not via the LDL receptor. Although the mechanism of uptake for FA-rHDL/Ptx was not investigated, the mechanism of uptake for the FA receptor occurs by potocytosis through caveolae (10). This mechanism provides yet another path for the selective uptake of drugs via the SR-BI receptor because caveolin and SR-BI have been shown to colocalize (11). Furthermore, linking other targeting molecules to rHDL may thus present additional opportunities for selective uptake as SR-BI does not have a specific binding site on Apo A-1 that may be disrupted by cross-linking, but recognizes the amphipathic helical nature of Apo A-1 (12). Alternatively, the folic acid labeled rHDL particles may also deliver their drug load to the target cells via receptor mediated endocytosis via the folic acid or other targeted receptors.

FUTURE DIRECTIONS

High Density Lipoprotein Structural Studies

This is the first report of spherical reconstituted HDL particles made by cholate dialysis (13). In an rHDL that only contained cholesteryl ester, without drug, electron microscopy clearly shows a spherical particle (Figure 2). The original cholate dialysis procedure produced discoidal HDL particles that were then modified by the enzyme LCAT (14). The mechanism by which the transformation of nascent discoidal HDL becomes mature spherical HDL is not fully understood. Using our synthetic rHDL and recombinant Apo A-1, amino acid residues can be changed to allow linkage of fluorescent tags. Greater understanding of the transformation to mature HDL may be achieved through structural studies of our rHDL particle.

Reconstituted HDL as a Chemotherapy Delivery Vehicle for Brain Tumors

Brain cancer is unique, because tumors in specific areas of the brain commonly occur within certain age groups. For example, medulloblastoma and neuroblastoma occur in children, while astrocytoma and glioblastoma usually occur in adults 60 years and older. Most types of brain tumors are difficult to treat and have high mortality rates. Due to the blood-brain barrier, chemotherapy treatment occurs by extremely painful spinal taps or the limited number of drugs that can pass the barrier. A drug delivery vehicle that can pass through the blood-brain barrier would be beneficial for brain cancer therapy.

Three lines of evidence indicate that an HDL-based delivery system for chemotherapy drugs may impact brain cancer. The first report found that cholesteryl esters were located only in brain tumor cells; no cholesteryl esters were found in healthy brain tissue. (15) Secondly, another report showed that an in vitro blood-brain barrier model transported HDL and HDL-associated α -tocopherol. (16) Lastly, a group has reported that the HDL receptor, SR-BI, mediated phospholipid uptake from HDL in primary porcine cerebral epithelial cells(17). HDLassociated molecules that originate with plasma HDL could possibly cross the blood-brain barrier.

In these preliminary studies, the SR-BI expression was explored via immunoblotting. The following brain cancer cell lines were chosen, neuroblastoma (SK-N-SH), medulloblatoma (D283 Med), astrocytoma (SW 1088), and glioblastoma (U-118 MG). There was minimal expression in normal human astrocytes; in contrast, considerable expression was seen in all brain cancer cell lines (Figure 3). When cytotoxicity studies were performed in normal human astrocytes, I found that even at the highest drug concentration, rHDL/paclitaxel caused almost no toxicity (96% cell viability via MTT assay) (Figure 4). In comparison, free paclitaxel (in DMSO) caused 25% toxicity at the highest concentration used. Incorporating paclitaxel into rHDL lessened the toxicity to normal cells. However, when similar cytotoxicity studies were performed in the U118 MG glioblastoma cell line, the opposite trend occurred. At the highest concentration used, rHDL/PTX reduced the cell viability to <50%, but paclitaxel (in DMSO) only reduced the cell viability to 75% (Figure 5). These studies need to be further explored with expanded drug concentrations. The comparison of the toxicity of rHDL/PTX could also be compared to both Taxol[®] and paclitaxel, because there is a contribution to cytotoxicity from the Cremophor EL component. SR-BI expression and trafficking of

rHDL/PTX across the endothelial cells that make up the blood brain barrier must be examined before rHDL can be further tested as a viable drug delivery vehicle for brain cancer.

Reconstituted HDL for Delivery of RNA Interference

RNA interference (or RNAi) blocks a particular gene through the use of short interfering RNA (siRNA), a 21-nucleotide sequence targeting a particular gene. Once across the cancer cell membrane, the siRNA interacts with the RNA-induced Silencing Complex (RISC) to shut down translation of the messenger RNA (18). Knockdown of genes important to the tumor cells has the potential for treating each cancer according to its genetic profile, resulting in individualized therapy. However, RNAi chemotherapy has unique challenges due to the degradation of RNA by the ubiquitous nucleases in the body (19). A delivery system is needed for protection of the RNA and direct targeting to the tumor.

This rHDL particle has been used to complex with siRNA to generate a drug platform for the delivery of siRNA to ovarian cancer cells. The rHDL is able to incorporate approximately 70-80% of the starting siRNA into the complex. However, there is not any chemical composition or physical characterization of these rHDL/siRNA particles yet. Neither is there any *in vitro* transfection data for the rHDL/siRNA in ovarian cancer cell lines. Although transfection studies were performed and a trend appeared that extended the knockdown past that observed with a standard transfection reagent, transfection by rHDL/siRNA needs to be optimized. Studies are also being planned that will combine rHDL/siRNA vehicles with the targeting capabilities of folic acid.

Recent tissue distribution studies carried out in tumor-bearing nude mice showed that a fluorescently labeled siRNA incorporated into rHDL was delivered mainly to the tumor

and to a much smaller extent to the liver, while all other peripheral tissues showed no uptake (Figure 6). These studies are in concert with our observations that selective delivery of anticancer agents via the rHDL delivery model is feasible as normal peripheral tissues are expected to have minimal expression of SR-BI.

CONCLUSIONS

The factors that are favorable for a drug delivery vehicle are circulation time, vesicle size, drug leakage, and reticulo-endothelial saturation. Reconstituted high density lipoprotein has advantages that meet all of these factors. The circulation time of human HDL is 4- to 5-fold that of a PEGylated Stealth liposome. Native human HDL has a spherical shape and a diameter approximately 10-fold smaller than the average liposome. From electron microscopy studies, rHDL/PTX has a diameter of 11.4 nm and a spherical shape resembling native HDL. No drug leakage upon density gradient ultracentrifugation was observed after 6 months of storage of the rHDL/PTX nanoparticles, indicating stability of the complexes. Tissue distribution studies showed less uptake by the liver of HDL/PTX compared to the current chemotherapy formulation, Taxol®, which could signify reduced competition between the liver and tumor.

Other advantages of the rHDL/PTX nanoparticle are the capacity to carry various water-insoluble compounds, as demonstrated by our model compound, paclitaxel. Compounds can even be modified to become more hydrophobic through fatty acylation and thus, more suitable for the rHDL carrier. Explorations could be made to design an rHDL nanoparticle to incorporate multiple drugs, leading to additive and synergistic effects. However, this platform is not limited to hydrophobic compounds; rHDL has now been adapted as a vehicle for siRNA,

which holds great promise for cancer therapy. This nanoparticle is not a vehicle for a single drug, but a platform with the potential to act as a carrier for multiple drug classes.

The reduced toxicity to normal tissues observed in the Maximum Tolerated Dose studies, but enhanced toxicity to cancer cells is another benefit of the rHDL platform. Selective uptake from HDL mediated through SR-BI is a unique mechanism for drug delivery. Data presented here, showed that a drug incorporated into the rHDL vehicle, is taken intracellularly through a similar mechanism. Finally, the ability to modify Apo A-1, the protein component of the rHDL platform, to carry targeting molecules leads to new avenues of drug delivery. This targeting and delivery were observed with folic acid-linked rHDL/PTX, which showed increased delivery of PTX to a drug-resistant ovarian cancer cell line. Preliminary studies also showed that rHDL/PTX treatment of tumor-bearing mice could repress tumor growth compared to untreated controls.

This project focused on targeting and toxicity to cancers of reproductive tissue origin, such as prostate, breast, and ovarian. The standard strategy of treatment is often Taxol® for these types of cancers. These cancers all showed significant expression of the HDL receptor, SR-BI, which has been observed in other cell lines than the ones selected for this project. Besides, high proliferation rates, these cancers also are steroid-hormone producing tissues, which may amplify their demand for cholesterol. The examination of SR-BI expression in cancer was expanded to include leukemia cell lines and brain cancer cell lines, providing a potential opportunity for chemotherapy by rHDL/drug nanoparticles. Just as different drugs may be incorporated into rHDL, rHDL is a platform that has the potential to deliver drugs to multiple types of cancers.

The main strides in prevention of cancer deaths have been made by early detection; latestage and recurrent cancers still have high mortality rates. Because cancer is such an individualized disease, with similar types of cancer expressing different oncogenes, mutations, and survival factors, individualized therapy is the future of cancer chemotherapy. By examining the profile of each cancer to find drug sensitivities, RNA interference targets and receptor expression, rHDL vehicles can be designed with chemotherapy agents, specific siRNA, and attaching ligands or antibodies to target cancer cell receptors. Reconstituted high density lipoprotein has the capacity to be an ideal vehicle for individualized targeted therapy.
Table 1. Chemical Composition of rHDL and Sparks HDL. Drug incorporation was measured with radiolabeled paclitaxel and cyclosporine A. rHDL is made by our cholate dialysis procedure; Control is the particle made by the Sparks procedure. All concentrations are in mg/mL.

	Drug	Protein	PL	ТС
rHDL/PTX	0.37±.02	2.41±.06	4.53±.07	0.16±.01
Control/PTX	0.034±.001	0.42±.02	0.32±.03	N/A
rHDL/Cyc A	0.41±.004	2.7±.08	4.77±.28	0.15±.01
Control/CycA	.058±.002	0.058±.002	0.74±.04	N/A

Figure 1. Comparison of Anti-Tumor Activity of Sonicated (Sparks) or Cholate Dialysis rHDL/PTX in MDA-MB-435 Tumor-Bearing Mice. Mice were treated with 2X I.P. injections of PTX preparation when tumors reached \approx 125mm₃. Tumors were measured twice weekly with calipers. Data represents tumor suppression from Day 17 after treatment. 50% of mice in both Sparks HDL groups died after injection. 1: Untreated Control (n =5). 2: 80 mg/kg rHDL/PTX (n=5). 3: 0.12 mg/ml rHDL/PTX (n=4). 4: 0.12 mg/mL Sparks HDL/PTX (n = 2). 5. rHDL Vehicle (n=4). 6. Sparks HDL Vehicle (n =2).



Figure 2. Activity of rHDL/PTX in MDA-MB-435 Tumor-Bearing Mice. Mice were implanted with MDA-MB-435 cell line and treated with 2X intraperitoneal (I.P.) injection of PTX preparation when tumor reached $\approx 125 \text{ mm}_3$. Tumors were measured twice weekly with calipers. Data represented as Mean, n = 5.



Figure 3. Electron Microscopy of rHDL Vehicle. $200 \ \mu g$ of rHDL vehicle was negatively stained with phosphotungstate and viewed under electron microscopy at a magnification of 50,000X. Scale is located in lower left corner.



Figure 4: Expression of SR-BI in Brain Cancer Cell Lines. 10 µg of cell extract protein were loaded in each lane. Lane 1: U118 MG glioblastoma cell line. Lane 2: HTB12 astrocytoma cell line. Lane 3: HTB 185: medullablastoma cell line. Lane 4: SK-N-SH neuroblastoma cell line. Lane 5: Normal human astrocytes.

U118MG HTB12 HTB185 SK-N-SH Astrocytes



Figure 5. Cytotoxicity of rHDL/PTX vs. PTX in Normal Human Astrocytes. 5,000 cells/well were seeded into 96-well microplates and grown overnight. rHDL/PTX and free paclitaxel (in DMSO) were added and incubated for 24 hours. Cell viability was measured with the MTT assay. (dark gray bars, rHDL/PTX; light gray bars, PTX (DMSO); n= 6 wells)



Figure 6. Comparison of Cytotoxicity of rHDL/PTX vs. PTX in U118-MG Glioblastoma

Cell Line. 5,000 cells/well were seeded into 96-well microplates and grown overnight. rHDL/PTX and free paclitaxel (in DMSO) were added and incubated for 24 hours. Cell viability was measured with the MTT assay. (Black bars, rHDL/PTX; Light gray bars, PTX (DMSO); n = 5 wells)



Figure 7. Tissue Distribution of Alexa 555-siRNA complexed with rHDL.

- (A) Tissue samples from SKOV3 tumor-bearing nude mice. Top panel: Hematoxylin & eosin staining. Middle panel: Merged confocal microscopy of 5 μg IV-injected rHDL/siRNA. Bottom panel: Merged confocal microscopy of 5 μg IP-injected rHDL/siRNA.
- (B) Tumor samples from SKOV3 tumor-bearing nude mice. Left panel: Hematoxylin & eosin staining. Middle panel: Merged confocal microscopy of 5 µg IV-injected rHDL/siRNA. Right panel: Merged confocal microscopy of 5 µg IP-injected rHDL/siRNA.

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153

REFERENCES

1. Braschi S, Neville TA, Vohl MC, Sparks DL, (1999). Apolipoprotein A-I charge and conformation regulate the clearance of reconstituted high density lipoprotein in vivo. J Lipid Res.; 40(3):522-32

2. Lacko AG, Nair M, Prokai L, McConathy WJ, (2007).Prospects and challenges of the development of lipoprotein-based formulations for anti-cancer drugs. Expert Opin Drug Deliv.; 4(6):665-75.

3. Nikanjam M, Gibbs AR, Hunt CA, Budinger TF, and Forte TM, (2007). Synthetic nano-LDL with paclitaxel oleate as a targeted drug delivery vehicle for glioblastoma multiforme. J Control Release.; 124(3):163-71.

4. Shimada T, Nomura M, Yokogawa K, Endo Y, Sasaki T, Miyamoto K, Yonemura Y, (2005). Pharmacokinetic advantage of intraperitoneal injection of docetaxel in the treatment for peritoneal dissemination of cancer in mice. J Pharm Pharmacol.; 57(2):177-81.

5. Lacko AG, Nair M, Paranjape S, Johnson S, McConathy WJ, (2002). High density lipoprotein complexes as delivery vehicles for anticancer drugs. Anticancer Res.; 22(4); 2045-2049.

6. Rae JM, Creighton CJ, Meck JM, Haddad BR, Johnson MD, (2007). MDA-MB-435 cells are derived from M14 melamona cells—a loss for breast cancer, but a boon for melanoma research. Breast Cancer Res Treat; 104(1), 13-19.

7. Chambers AF, (2009). MDA-MB-435 and M14 cell lines: Identical but not M14 melanoma? Cancer Research; 69(13); 5292-5293.

8. Hollestelle A, Schutte M, (2009). Comment Re: MDA-MB-435 and M14 cell lines: Identical but not M14 melanoma? Cancer Research.

9. Zheng G, Chen J, Li H, Glickson JD, (2005). Rerouting lipoprotein nanoparticles to selected alternate receptors for the targeted delivery of cancer diagnostic and therapeutic agents. Proc Natl Acad Sci U S A; 102(49):17757-62.

10. Ritter TE, Fajardo O, Matsue H, Anderson RG, Lacey SW, (1995). Folate receptors targeted to clathrin-coated pits cannot regulate vitamin uptake. Proc Natl Acad Sci U S A.; 92(9):3824-8.

11. Babitt J, Trigatti B, Rigotti A, Smart EJ, Anderson RG, Xu S, and Krieger M (1997).

Murine SR-BI, a high density lipoprotein receptor that mediates selective lipid uptake, is N-glycosylated and fatty acylated and colocalizes with plasma membrane caveolae. J Biol Chem.; 272(20):13242-9

12. Williams DL, de La Llera-Moya M, Thuahnai ST, Lund-Katz S, Connelly MA, Azhar S, Anantharamaiah GM, and Phillips MC; (2000). Binding and cross-linking studies show that scavenger receptor BI interacts with multiple sites in apolipoprotein A-I and identify the class A amphipathic alpha-helix as a recognition motif. J Biol Chem.; 275(25):18897-904.

13. McConathy WJ, Nair MP, Paranjape S, Mooberry L, and Lacko AG, (2008). Evaluation of synthetic/reconstituted high-density lipoproteins as delivery vehicles for paclitaxel. Anticancer Drugs.; 19(2):183-8

14. Jonas A, (1986). Reconstitution of high density lipoproteins. Methods Enzymol; 128:553–582.

15. Tosi MR, Bottura G, Lucchi P, Reggiani A, Trinchero A, Tugnoli V, (2003). Cholesteryl esters in human malignant neoplasms. Int J Mol Med.; 11(1):95-8

16. Balazs Z, Panzenboeck U, Hammer A, Sovic A, Quehenberger O, Malle E, Sattler W, (2004). Uptake and transport of high-density lipoprotein (HDL) and HDL-associated alpha-tocopherol by an in vitro blood-brain barrier model. J Neurochem.; 89(4):939-50.

17. Sovic A, Balazs Z, Hrzenjak A, Reicher H, Panzenboeck U, Malle E, Sattler W, (2004). Scavenger receptor class B, type I mediates uptake of lipoprotein-associated phosphatidylcholine by primary porcine cerebrovascular endothelial cells. Neurosci Lett. 2004 Sep 16;368(1):11-4.

18. Arnaud CH, (2006). Delivering RNA interference. Chemical Engineering News; 84(46); 16-23.

19. Liszewski K, (2008). Conquering RNAi delivery challenges. Gene Eng Biol News; 28; 1-4.