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EVALUATION OF RECONSTITUTED HIGH-DENSITY LIPOPROTEIN AS A DRUG DELIVERY SYSTEM IN DRUG-SENSITIVE AND DRUG-RESISTANT OVARIAN CANCER CELL LINES

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INTRODUCTION

Cancer is characterized by uncontrolled, abnormal growth of cells that may spread to other tissues through the blood stream or the lymphatic system, where they start new (metastatic) cancers. Depending on their origin, cancers can be divided generally into three broad groups: 1) Carcinomas - cancers derived from cells that line various tissues, 2) Sacrcomas - those derived from the underlying supporting tissue, 3) Hematologic tumors - the origin of the cells in this type of tumors is the bone marrow or the lymphatic system.

The treatment of choice for the majority of malignancies is chemotherapy promoting the invasion of malignant lesion by cytotoxic agents that destroy cancer cells. Multi-drug resistance (resistance to cytotoxic agents), solubility and the toxic side effects of the chemotherapeutic agents remain a serious concern in the management of advanced ovarian carcinoma, resulting in poor survival figures of 10-20% among these patients [11-14, 16].

Multidrug Resistance in Cancer:

Drug resistance is one of the major problems in cancer chemotherapy as many tumors develop resistance to anticancer drugs during therapy. About 40-45% of the malignant tumors may develop resistance to chemotherapeutic agents. In women, about 4% of deaths occur due to ovarian cancer and, in more than 90% of patients with

metastatic disease, the failure of the treatment is due to drug resistance [7] as some of the tumors become resistant to a broad range of drugs.

Malignant cells in cancerous tumors may be either drug-sensitive or drug-resistant. Drug-sensitive cells are killed during chemotherapy, while some of the drug-resistant cells survive. The subsequent tumor growth then primarily represents resistant cells that are likely to survive chemotherapy [2]. Drug resistance can be implemented via several different mechanisms, primarily attributed to a group of membrane proteins that function as energy-dependent efflux pumps that expel chemotherapeutic drugs from the tumor cells [2]. Subsequently, the intracellular concentration of the drug falls below a cell-killing threshold [3], thus allowing tumor cells to survive and grow, resulting in poor response to chemotherapy. The other mechanisms by which drug resistance occur are, alteration in the amount of target receptor or enzyme due to gene amplification, decreased conversion of a drug to its active form, decreased affinity of the target receptor or enzyme for the drug, and repair mechanisms adapted by the cells to the effect induced by the drug.

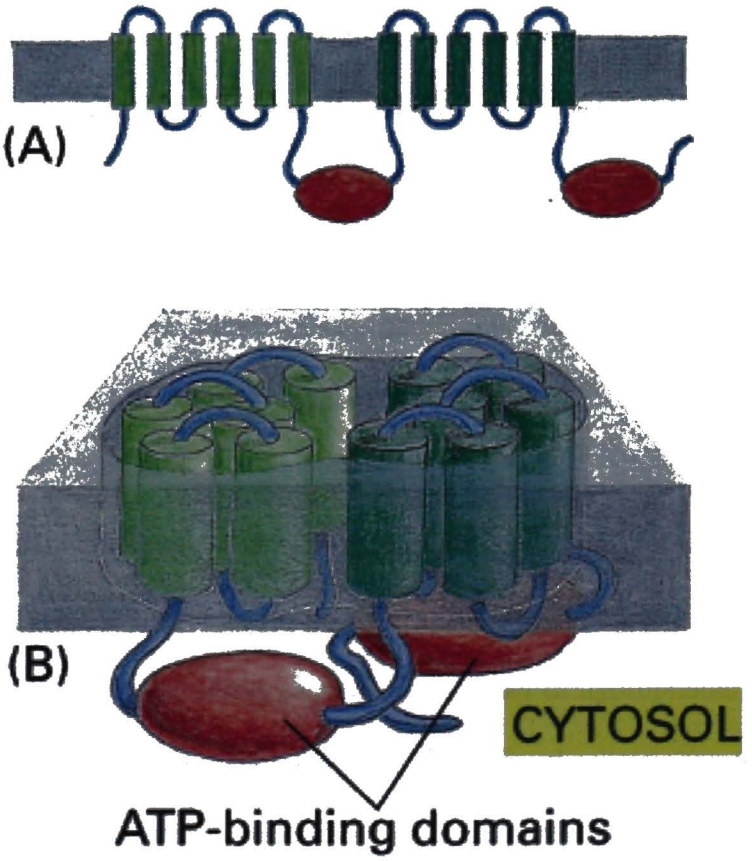
Regardless of the mechanism, eventually the drug expelled by the membrane-bound pump mechanism exceeds that of the influx of the drug into the cells so that the intracellular concentration of the drug will progressively decrease, rendering chemotherapy less effective and may promote the development of additional resistance to that drug. These ATP-dependent efflux pumps belong to the superfamily of ATP Binding Cassette (ABC) proteins and are also referred to as MDR (Multi Drug Resistance)

proteins. The main players in this category are P-Glycoprotein (P-gp, MDR1, ABCB1), Multidrug resistance protein (MRP1, ABCC1), and the mitoxantrone resistance protein (MXR/BCRP, ABCG2), the major one among these being P-gp. Another important feature of these efflux pumps is their wide substrate specificity [3]. A number of structurally unrelated hydrophobic compounds act as substrates for these pumps. The structure of a typical ABC transporter protein consists of two membrane-bound domains and two nucleotide-binding domains as shown in figure1 A & B.

Figure 1(A). Topology diagram of a typical ABC transporter

(B). Hypothetical arrangement of the polypeptide chain in the membrane

(Molecular Biology of the Cell, 3rd Edition, Alberts et.al.)



Each membrane-bound pumping unit consists of six transmembrane domains. The nucleotide-binding domains bind ATP and hydrolyze it for the generation of ATP necessary to drive drug efflux from the cells. In mammals, a single gene encodes the two nucleotide-binding domains and two membrane-bound domains of P-gp [5]. This gene is referred to as MDR1 gene. The MDR1 gene has been identified as the gene responsible for multidrug resistance in human tumor cells. This gene is overexpressed and amplified in many of the multidrug-resistant cell lines. The product of this gene, P-gp, is a 170 KDa transmembrane protein [1], that is over expressed in the plasma membrane of tumor cells during multi-drug resistance. P-gp is a transporter of hydrophobic compounds that include most of the anticancer agents [3, 17-19]. Mechanism of resistance due to P-gp is the most widely observed. It has also been shown that P-gp not only increases drug efflux, but also reduces drug influx into the cytosol. The increased drug efflux due to these ATP-dependent efflux pumps leads to reduced accumulation of drug molecules within the cells and is thus associated with poor response to chemotherapy in tumor cells.

To overcome these problems of chemotherapy, various drug delivery systems including liposomes and lipoproteins have been proposed [6].

Lipoproteins:

Lipoproteins are heterogeneous complexes of lipids and proteins. They transport lipids from the site of their synthesis or absorption to peripheral tissues through the systemic circulation. They also act as carriers of a number of hydrophobic compounds in the circulation. Lipoproteins, in general, consist of a nonpolar lipid core of triglycerides and cholesteryl esters surrounded by an envelope of polar phospholipids, free cholesterol

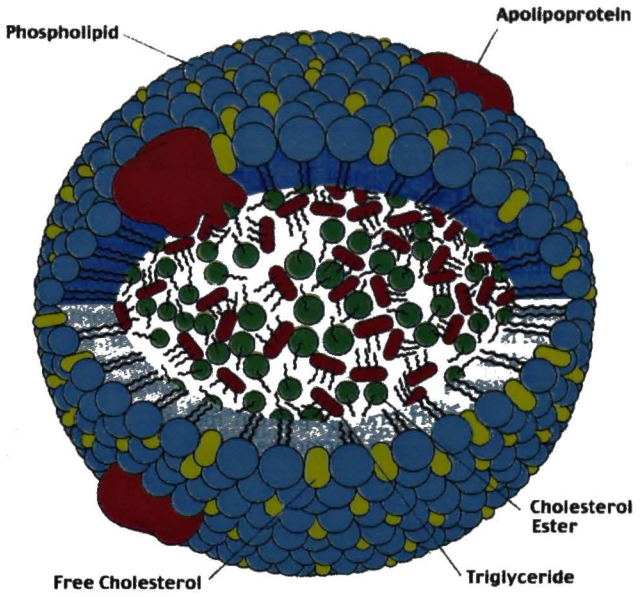
and proteins (apolipoproteins). The major phospholipids located at the surface layer of the lipoprotein particles are phosphatidylcholine, phosphatidylserine, sphingomyelin and phosphatidylethanolamine. There are five classes of lipoproteins according to their buoyant density: chylomicrons, very low- density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). Their lipid and protein contents differ depending on their respective physiological functions. The complexes with lower lipid content relative to the protein have higher densities and vice versa [4]. Different classes of lipoproteins may be separated by ultracentrifugation.

High-Density Lipoproteins (HDL):

High-density lipoproteins (HDL) are one among five main classes of lipoproteins in the bloodstream. They are the most abundant lipoproteins in the circulation, with the particle number being 10-20 fold more than the number of all other lipoproteins together [10]. The average high-density lipoprotein particle has a smaller size with a hydrophobic lipid core of cholesteryl esters and triglycerides surrounded by a surface monolayer of phospholipids, unesterified cholesterol and apolipoprotein AI.

HDL is the smallest and densest of the plasma lipoproteins [9]. The diameter of HDL ranges between 8-11nm [8]. The density of HDL particles ranges from 1.063-1.210 g/ml. The molecular weight of HDL ranges from 200-400 KDa. The composition of HDL is 55% - protein, 24% - phospholipids, 2% - free cholesterol, 15% cholesteryl esters, and 4% - triacylglycerols by percent weight.

Figure 2. General structure of a lipoprotein particle

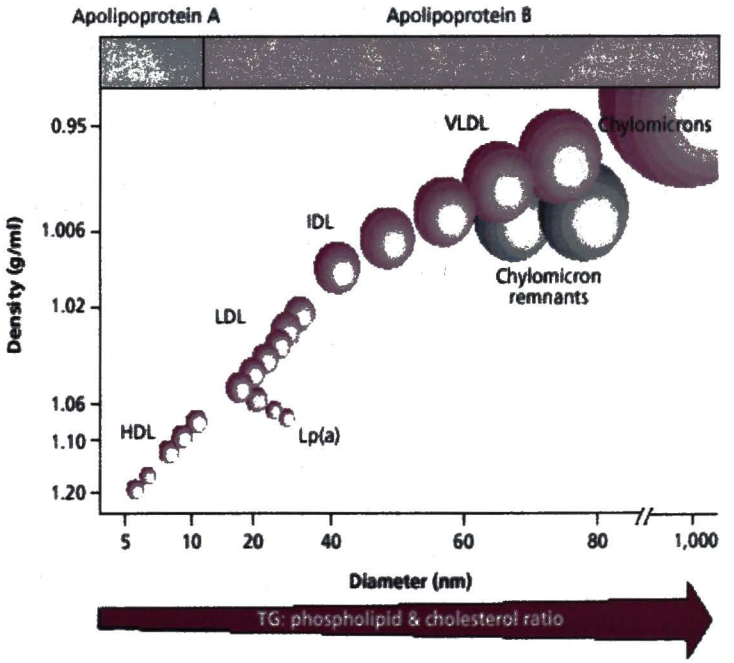


Apolipoproteins provide structural integrity to the particle and are involved in the secretion of the lipoprotein. They are also ligands for a number of receptors. The major protein in HDL is apolipoprotein AI, which comprises of 70% of the protein mass. It is synthesized in the liver and intestine. The second major apolipoprotein being apolipoprotein AII, which accounts for approximately 20% of the protein mass and it is synthesized in the liver. HDL plays a key role in reverse cholesterol transport, which is the transport of cholesterol from the peripheral tissues back to the liver where it is excreted into bile. In this process, excess cholesterol in the peripheral tissues moves into nascent HDL where it is esterified by lecithin cholesterol acyl transferase (LCAT).

Advantages of rHDL as a drug delivery system:

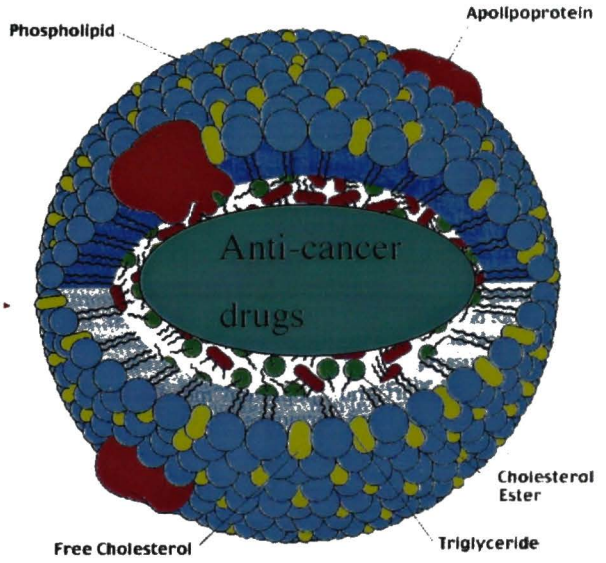
Despite the many recent improvements in the application of cancer by chemotherapy (11-14, 16), drug resistance, solubility and toxic side effects remain a serious concern in the management of advanced stage malignant tumors, including ovarian carcinoma. To overcome these problems encountered during chemotherapy, improved drug delivery systems including liposomes and lipoproteins have been developed [6]. It has been shown that efficient drug delivery by liposomes was inversely related to the diameter of the particle. Because the diameter of HDL is almost five-times smaller than that of the smallest liposomes [6], it may easily penetrate the vascular and extravascular tissues lining the systemic circulation.

Figure 3. Size of different lipoprotein particles



The average high-density lipoprotein particle has a smaller size (figure 3) with a hydrophobic lipid core, which creates an ideal target for the incorporation of hydrophobic drugs (figure 4).

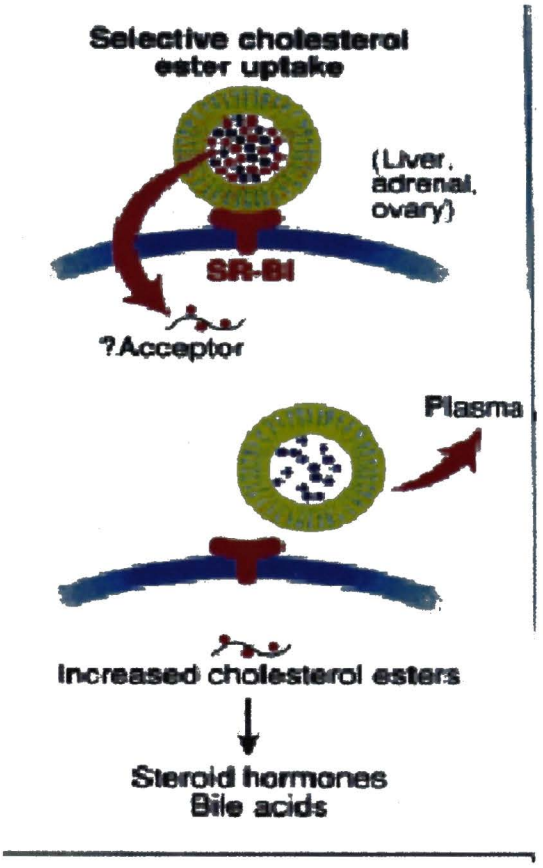
Figure 4. General Structure of a Lipoprotein showing the place for anticancer drugs (in the core region)



Normal and tumor cells take up the hydrophobic core components of HDL particles for their proliferation by specific cell surface receptors (SR-B1 type) (figure 5). This is one of the major advantages of the rHDL based drug delivery.

Figure 5. Mechanism for the cellular uptake of HDL core components

(from: Steinberg, D. Science, 1996 271:460-1)

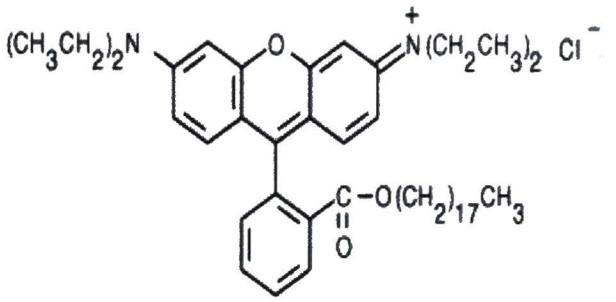


Because of these characteristics, HDL could serve as a hydrophobic drug transporter and hence, reconstituted high-density lipoprotein (rHDL) particles have been developed to act as delivery vehicles for cancer chemotherapeutic agents [6]. These rHDL particles resemble the plasma HDL and may have marked advantages in overcoming drug resistance during cancer treatment.

Octadecyl Rhodamine B Chloride (ORB):

ORB is a derivative of Rhodamine B, which is a substrate for P-gp. Like its parent compound, ORB is also a fluorescent hydrophobic compound and poorly soluble in water. It has high affinity for phospholipids and is used as a membrane probe in many experiments due to its lipophilic properties.

Figure 6. Structure of Octadecyl Rhodamine B Chloride



Molecular Formula:

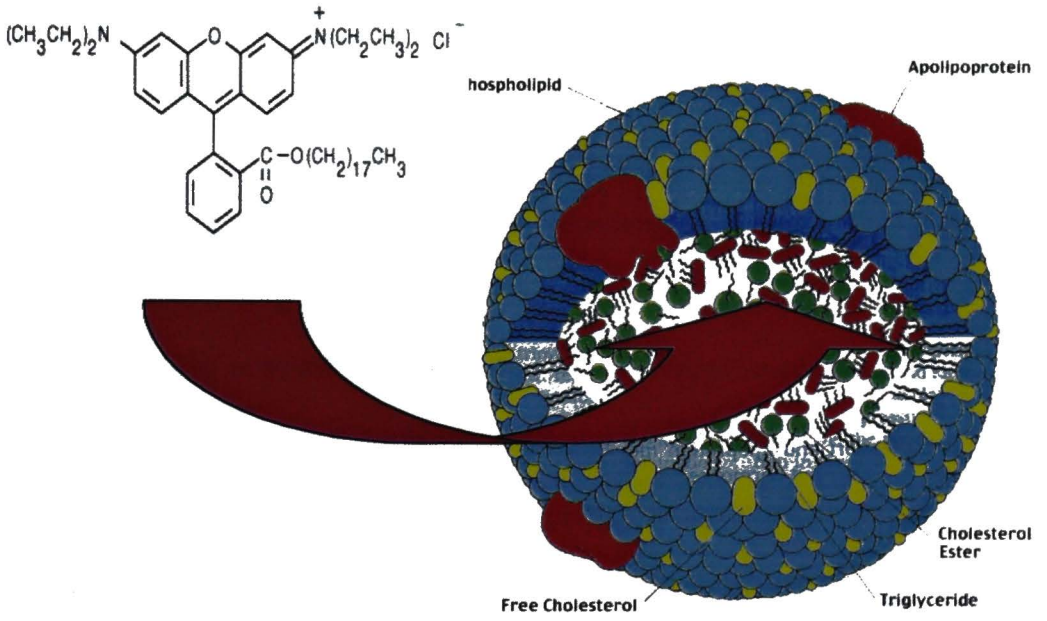
$C_{46}H_{67}ClN_2O_3$

Molecular Weight: 731.50

It is structurally related to Rhodamine-123 that was shown to be effective as a chemotherapeutic agent in animal models due to its selective mitochondrial cytotoxicity. The mechanism of ORB is thought to be that once it is delivered to cancer cells by reconstituted high-density lipoproteins, the hydrophobic ORB is hydrolyzed liberating the rhodamine component that exerts its cytotoxic effect. Because of its hydrophobicity, we

assumed it would be a good candidate for encapsulation into the lipophilic core of the rHDL particles.

Figure 7. Figure showing the incorporation of Octadecyl Rhodamine B Chloride into the core of a lipoprotein particle.



OBJECTIVE

The objective of this research project is to determine whether a reconstituted high-density lipoprotein (rHDL) drug delivery system can reduce or overcome drug resistance in ovarian cancer cells. The model compound to used as a drug for this project is Octadecyl

Rhodamine B chloride (ORB). This objective was assessed by doing uptake and efflux studies in drug-resistant and drug-sensitive ovarian cancer cell lines to compare the amount of ORB being expelled. Because the energy-dependent efflux pumps responsible for multi-drug resistance are over-expressed and amplified in resistant cells, they should expel a higher amount of the drug from the cells. The rHDL system is anticipated to reduce drug resistance by releasing the drug directly inside the cells bypassing the efflux pumps, it is anticipated that the efflux of ORB would be less when it is delivered with rHDL.

SIGNIFICANCE

The selective uptake of the HDL core components by tumor cells is a potential advantage of the rHDL drug delivery system because the drug packed into the core of rHDL is expected to be released intracellularly through receptor-mediated (SR-B1 type) mechanism, by-passing the transmembrane efflux pumps that expel the cytotoxic drugs. Many of the chemotherapeutic drugs are hydrophobic (poorly soluble in water). The rHDL drug delivery vehicle is designed to solve this problem by solubilizing otherwise poorly water-soluble chemotherapeutic agents for injection into the systemic circulation [6]. Often, high doses of chemotherapeutic agents are required to kill the cancer cells and to suppress tumor growth. These doses also affect the normal cells around the tumor. Since the rHDL targeting system is receptor-mediated (SR-B1) and since these receptors appear to be overexpressed in tumor cells, the toxic side effects to the normal cells may thus be reduced.

MATERIALS AND METHODS

Cell Culture:

The cell lines selected for the proposed studies are OV1063 (drug-sensitive), SK-OV-3 and OVCAR-3 (drug-resistant), all from ATCC. All of these are human ovarian carcinoma cell lines. OV1063 cells will be grown in RPMI 1640 medium containing 10% of Fetal Bovine Serum (FBS). OVCAR-3 cells will be grown in RPMI 1640 medium (ATCC Catalog No. 30-2001) containing 20% FBS and 0.01mg/ml Bovine Insulin. SK-OV-3 cells will be grown in McCoy's 5a medium (ATCC Catalog No. 30-2007) containing 10% FBS. Two other cell lines were also used - ldlmSRB1 and ldlA7 cells. These are Chinese Hamster Ovary (CHO) cells. ldlmSRB1 cells are transfected with mouse SR-B1 receptor. ldlmSRB1 cells will be grown in Ham's/F-12 medium with Geneticin antibiotic and ldlA7 cells in Ham's/F-12 medium.

The above cell lines were cultured in 75 cm^2 flasks in their respective media. Trypsin-EDTA (0.05%) was added to detach the cells from the bottom of the flasks. Then, their respective media were added to the flasks to inhibit trypsin digestion. The cells and the media were spun at 4000g, 4°C for 7 minutes, 30 seconds. The supernatant was removed, and the cells were resuspended in the complete media. An aliquot of the homogeneous suspension of the cells was taken to count the number of cells on a hemacytometer (Fisher Scientific Catalog No. 0267110) and the required cell number was plated in multi-well plates with the complete media.

Preparation of rHDL/Octadecyl Rhodamine B Chloride (rHDL/ORB):

Egg-yolk Phosphatidyl Choline (1.8 mg) (Sigma) in chloroform and 300μg of Octadecyl Rhodamine B Chloride (Molecular Probes) in Dimethyl Sulfoxide (DMSO) were mixed in a glass tube and dried the components into a thin film under nitrogen for 2 minutes. Free cholesterol (0.45mg) in chloroform and 0.9mg of Cholesteryl oleate in chloroform, both from Sigma, were added to the same glass tube and the mixture was dried under nitrogen for 2 more minutes. Dissolved the film in 5 ml of Sonication buffer (10mM Tris, 0.1M KCl, 1mM EDTA, pH adjusted to 8.0) by vortexing for 8 minutes. Sonicated the mixture in a Branson sonicator using a microtip, under the stream of nitrogen for 60 minutes at 60°C in a water bath. Reduced the temperature of the water bath to 42°C and added 4mg of Apolipoprotein AI, mixed and resumed sonication for 30 minutes at 42°C. Then, dialyzed the mixture in a porous tube (Molecular weight cut off - 3500), from BioDesign Dialysis Tubing™ against 137mM NaCl, 4.3mM Na_2HPO_4, 1.2mM KH_2PO_4, 2.7mM KCl, pH 7.4 (PBS - Phosphate Buffered Saline) at 4°C for overnight. The tube was wrapped in an aluminum foil and stored at 4°C until use (within a week).

Ultracentrifugation:

3ml of rHDL/ORB will be taken in a 13ml ultracentrifuge tube. KBr (0.4mg/ml) was dissolved in the sample. The sample was layered with different density solutions in the following order, 1ml of 1.22g/ml, 4 ml of 1.063g/ml, 3ml of 1.019g/ml and fill the tube with deionized water. The tubes will be balanced and ultracentrifuged for 24 hours at 39000rpm and 4°C in a swinging bucket rotor. Then, 1ml fractions were collected from the top to isolate particles with the floatation properties of HDL.

Preparation of ORB:

Took 300μg of Octadecyl Rhodamine B Chloride in DMSO in a glass tube and dried the solution into a thin film under low nitrogen for 2 minutes. Dissolved the film first in 200 μl of DMSO, then added 4.8 ml of serum-free medium and vortexed.

Cellular Uptake and Efflux study:

The cells (7,500 cells/well) were plated in a 96-well plate with the complete medium. After 24 hours, the medium was removed from the wells and the cells were washed once with PBS. The cells were then incubated with different concentrations of ORB and rHDL/ORB for 2 hours at 37°C in the presence of 5% CO_2. A control with no ORB or rHDL/ORB was maintained. After 2 hours of incubation, the medium and drug (ORB or rHDL/ORB) was removed from the wells and the cells were washed once with PBS. Then, phenol-red free DMEM (Dulbecco's Modified Eagle Medium) was added and measured the fluorescence at an excitation wavelength of 530/25nm and an emission wavelength of 590/35nm to measure the uptake of ORB and rHDL/ORB. The plate was then incubated with the phenol-red free DMEM for different time points (15 minutes, 30 minutes and 1 hour) at 37°C in the presence of 5% CO_2. After incubation for each time point, 50μl of the medium was removed from each of the wells and added to an empty 96-well plate. The fluorescence was measured again at the same wavelength like above to measure the efflux of ORB and rHDL/ORB from the cells.

Competition Study of rHDL with HDL_3 as the competitor:

The cells (7,500 cells/well) were plated in a 96-well plate with the complete medium. After 24 hours, the medium was removed from the wells and the cells were

washed once with PBS. All the cells were then incubated with the same concentration of the rHDL/ORB and increasing concentration of the competitor (HDL_3) for 2 hours at 37°C in the presence of 5% CO_2. A control with rHDL/ORB and no competitor as well as no rHDL/ORB and no competitor was maintained. After 2 hours of incubation, the drug and the medium were removed from the wells and the cells were washed once with PBS. Then, 0.5M NaOH was added to lyse the cells. The lysed cells were spun in a centrifuge at 1200g, 4°C for 5 minutes and the supernatant was taken into another plate. The fluorescence was measured at an excitation of 530/25nm and an emission wavelength of 590/35nm to see the uptake of rHDL/ORB in the presence of increasing concentration of a competitor.

Fluorescence Microscopy:

Cells were cultured on glass cover slips. After reaching the desired confluence, the cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature. The cells were then washed with 1X PBS and mounted on slides using fluorosave (CalBiochem) and then examined under a fluorescence microscope. Dapi staining of the nucleus was done by fixing the cells with methanol:acetone(1:1) at -20°C for 10 minutes and then incubating the cells in Dapi (400 nM) for 30 minutes at 37°C. The coverslips were then washed and mounted on slides using fluorosave (CalBiochem) and then examined under a fluorescence microscope.

RESULTS

The purpose of this study was to perform uptake and efflux studies of ORB in drug-sensitive (OV1063) and drug-resistant (SK-OV-3 and OVCAR-3) cells. Experiments were carried out by delivering ORB to the cells with and without rHDL to compare the amount of ORB taken up and expelled by the respective cells. Because serum contains lipoproteins, the experiments were also done in the absence of serum to show the specific effect of the rHDL vehicle. Because the efflux pumps responsible for multi-drug resistance are overexpressed and amplified in resistant cells, the efflux of ORB should be higher in these cells compared to the drug-sensitive cell line, OV1063. But, since our rHDL system is designed to reduce drug resistance by releasing the drug directly inside the cell bypassing the efflux pumps, the efflux of ORB should be less when it is delivered with rHDL.

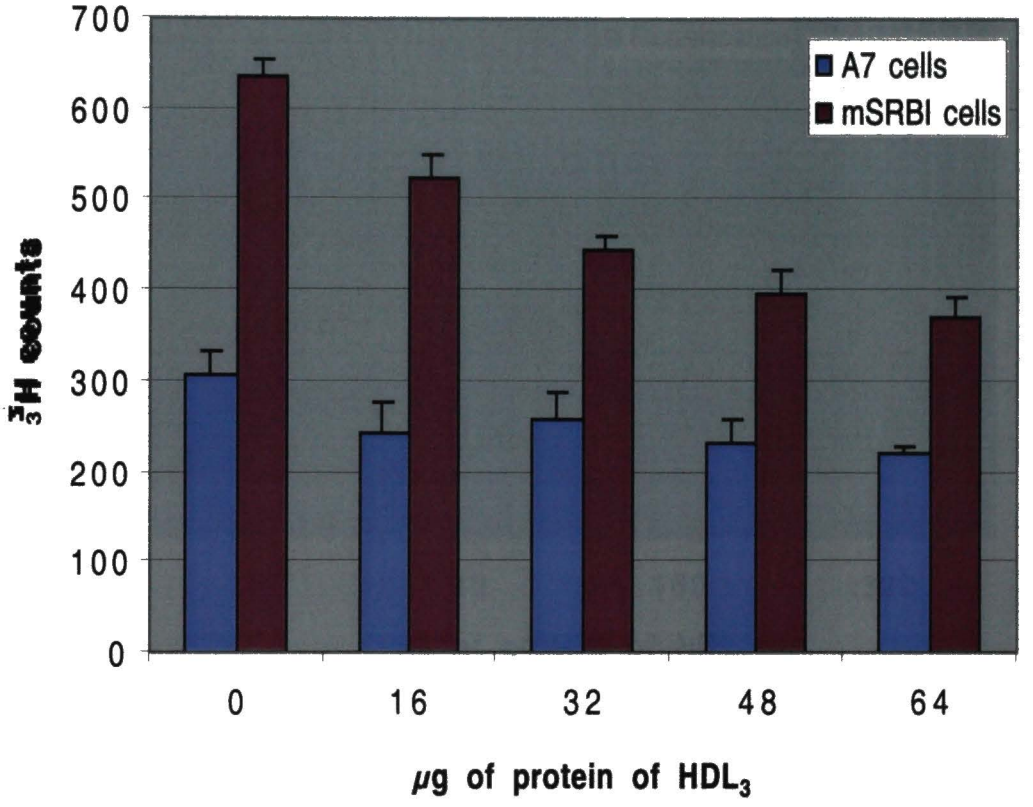
Competition Study of rHDL with HDL_3 [20] as the competitor:

The competition study of ^{3}H-cholesteryl oleate labeled rHDL (^{3}H/rHDL) was done in ldlmSRB1 and ldlA7 cells to see the uptake of ^{3}H/rHDL in the absence and presence of increasing concentrations of a competitor (in this case, HDL_3), which competes for the same SR-B1 receptor as ^{3}H/rHDL.

Figure 8 shows that, with the increasing concentration of HDL_3 (competitor), the uptake of ^{3}H/rHDL decreased in mSRB1 cells indicating that both ^{3}H/rHDL and HDL_3 are competing for the same SR-B1 receptor, whereas on the other hand, in normal A7 cells, which express minimal level of SR-B1 receptors, there wasn't any significant difference in the uptake of ^{3}H/rHDL with the increasing concentration of HDL_3.

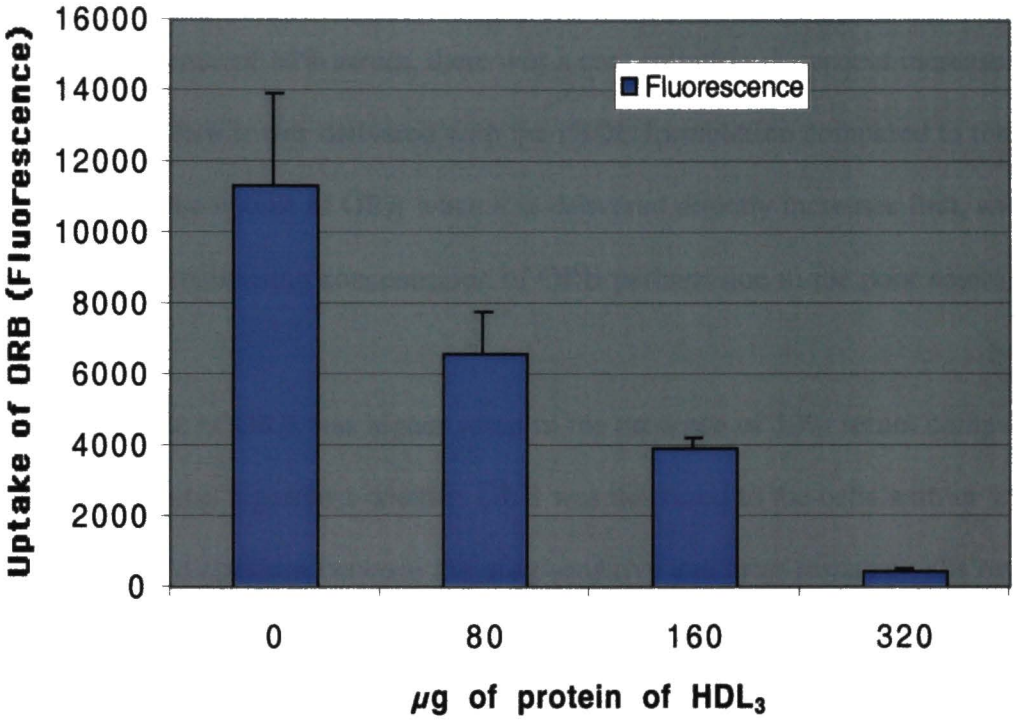
The competition study was again done in OV1063 (drug-sensitive) cells to determine the uptake of rHDL/ORB with increasing concentration of HDL_3 as the competitor. Figure 9 shows that with the increasing concentration of HDL_3, the uptake of rHDL/ORB decreases.

Figure 8. Competition Study of ^{3}H/rHDL with increasing concentration (16, 32, 48, 64 μg of protein) of HDL_3 as the competitor in ldlmSRB1 and ldlA7 cells.



The data in Figure 8 show that in mSRB1 cells, with the increasing concentration of HDL_3, the uptake of ORB decreases. This decrease is statistically significant ($p < 0.05$). The decrease in the uptake of ^{3}H/rHDL in ldlmSRB1 (SR-B1 overexpressed) cells with increasing concentration of the competitor suggests the involvement of SR-B1 receptor.

Figure 9. Competition study of rHDL/ORB with HDL_3 as the competitor in OV1063 cells.



The data in figure 9 shows that, with increasing concentration of HDL_3, the uptake of rHDL/ORB decreases.

Uptake of Octadecyl Rhodamine B Chloride with rHDL (rHDL/ORB) in drug-sensitive (OV1063) and drug-resistant (SK-OV-3 and OVCAR-3) cancer cell lines:

The uptake and efflux studies of ORB were done in the above three cell lines with rHDL/ORB and ORB alone. In all the three cell lines (figures 10, 12, 14), both in the presence and absence of 10% serum, there was a concentration-dependent increase in the uptake of ORB when it was delivered with the rHDL formulation compared to the ORB alone. Whereas the uptake of ORB when it is delivered directly increases first, and then plateaus with the increasing concentration of ORB perhaps due to the poor solubility of ORB.

The uptake of ORB was higher when in the presence of 10% serum compared to no serum conditions, regardless whether ORB was delivered to the cells with or without rHDL vehicle. Comparison between the drug-sensitive and drug-resistant cells revealed that the uptake of ORB in drug-sensitive (OV1063 cells) was higher than the drug-resistant (SK-OV-3 and OVCAR-3) cells. There was no significant difference in the uptake between the two resistant cells (figures 12, 14).

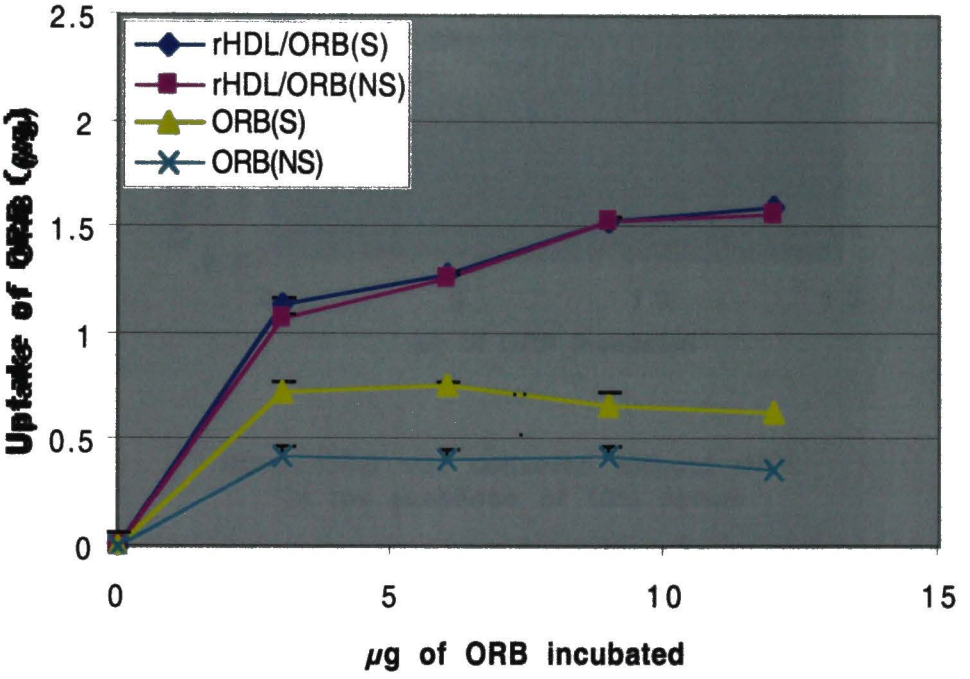
These measurements are taken by reading the fluorescence of ORB when the intact cells were suspended in the medium.

Efflux of Octadecyl Rhodamine B Chloride with rHDL (rHDL/ORB) from drug-sensitive (OV1063) and drug-resistant (SK-OV-3 and OVCAR-3) cancer cell lines:

In drug-sensitive and drug-resistant cell lines, both in the presence and absence of serum, the efflux of ORB from the cells was lower when it was delivered with rHDL compared to ORB alone (figures 11, 13, 15). There was concentration-dependent in the

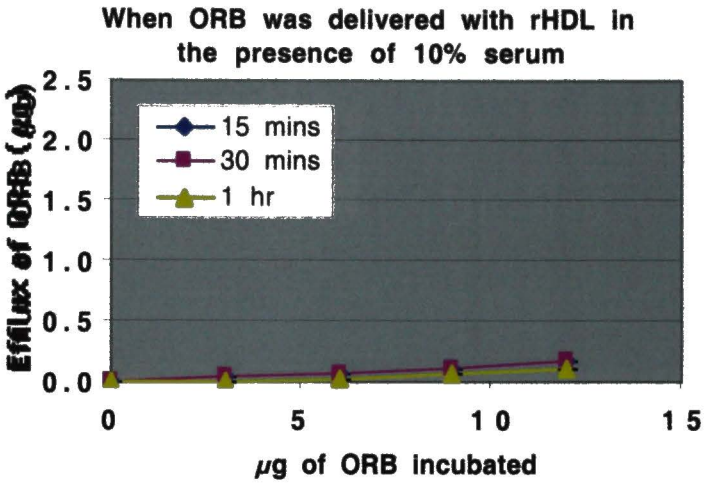
increase in the efflux of ORB from the cells when it is delivered with or without rHDL. There is almost no significant difference in the efflux of ORB whether in the presence or absence of 10% serum.

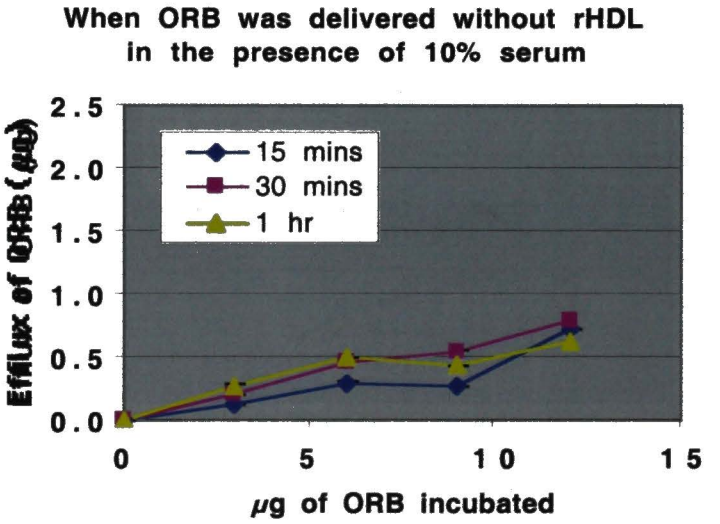
Figure 10. Uptake of ORB in OV1063 cells incubated with increasing concentration of rHDL/ORB and ORB alone under 10% serum and no serum conditions for 2 hours at 37°C.



The data in figure 10 shows that the uptake of ORB is more in both serum and no serum conditions when it is delivered to the cells with rHDL (blue - serum and pink - no serum) compared to the free ORB delivery (yellow - serum and turquoise - no serum).

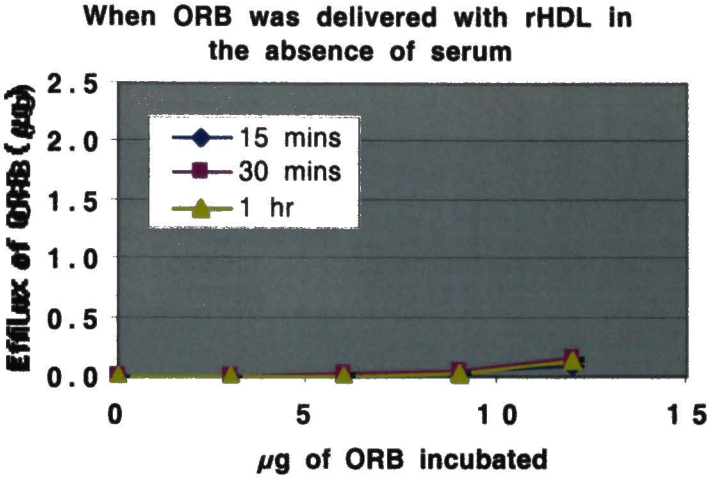
Figure 11(a and b). Efflux of ORB in OV1063 cells at different time points (blue - 15 mins, pink - 30 mins, yellow - 1 hr) under 10% serum conditions.

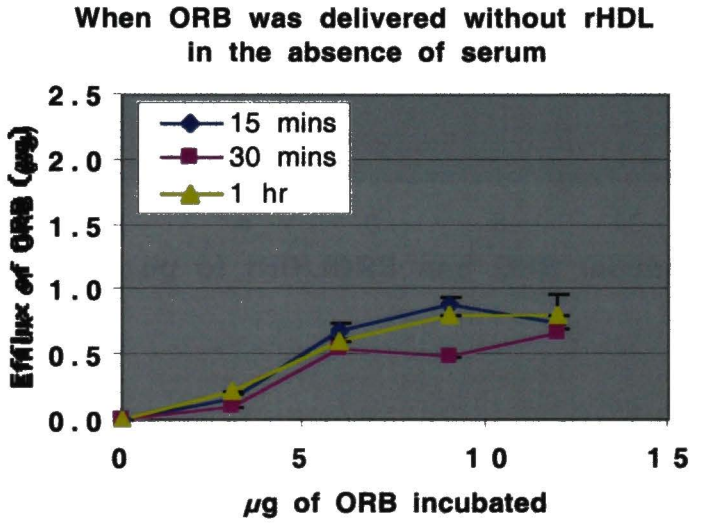




The data in figures 11(a and b) shows that the efflux of ORB is more when it is delivered to the cells without rHDL compared to rHDL delivery.

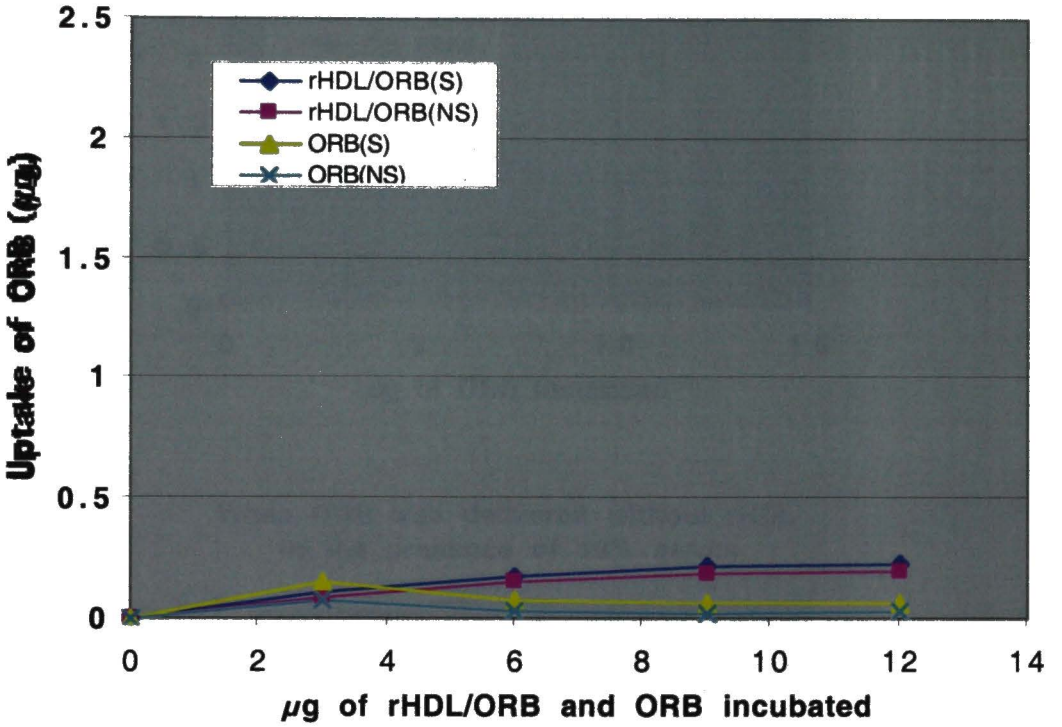
Figure 11(c and d). Efflux of ORB in OV1063 cells at different time points (blue - 15 mins, pink - 30 mins, yellow - 1 hr) under no serum conditions.





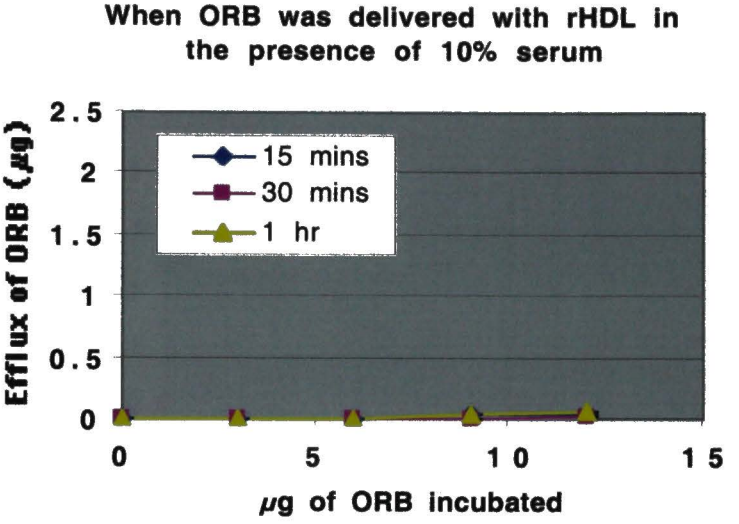
The data in figures 11(c and d) shows that the efflux of ORB is more when it is delivered to the cells without rHDL compared to rHDL delivery.

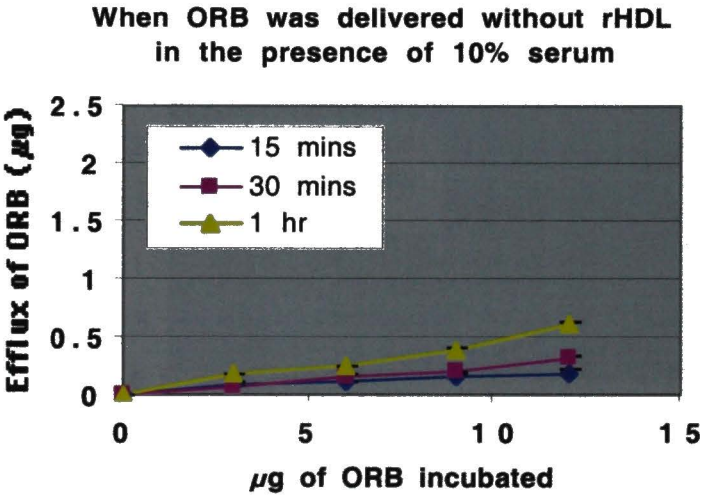
Figure 12. Uptake of ORB in SK-OV-3 cells incubated with increasing concentration of rHDL/ORB and ORB alone under 10% serum and no serum conditions for 2 hours at 37°C.



The data in figure 12 shows that the uptake of ORB is more in both serum and no serum conditions when it is delivered to the cells with rHDL (blue - 10% serum and pink - no serum) compared to the free ORB delivery (yellow - 10% serum and turquoise - no serum).

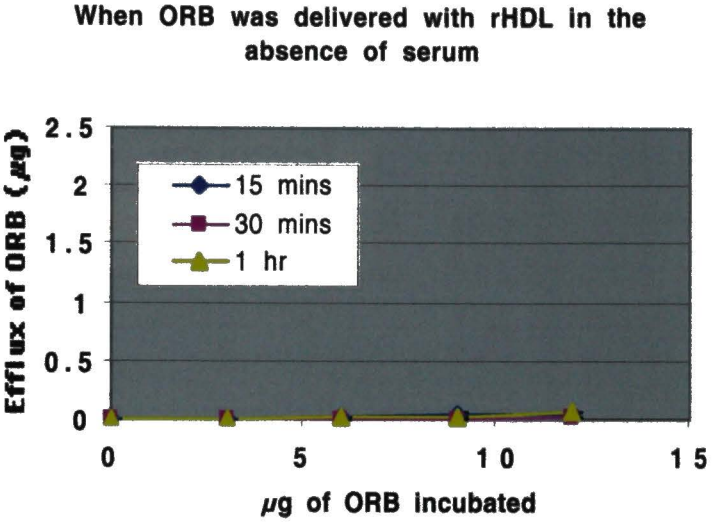
Figure 13(a and b). Efflux of ORB in SK-OV-3 cells in the presence of 10% serum at different time points points (blue - 15 mins, pink - 30 mins, yellow - 1 hr).

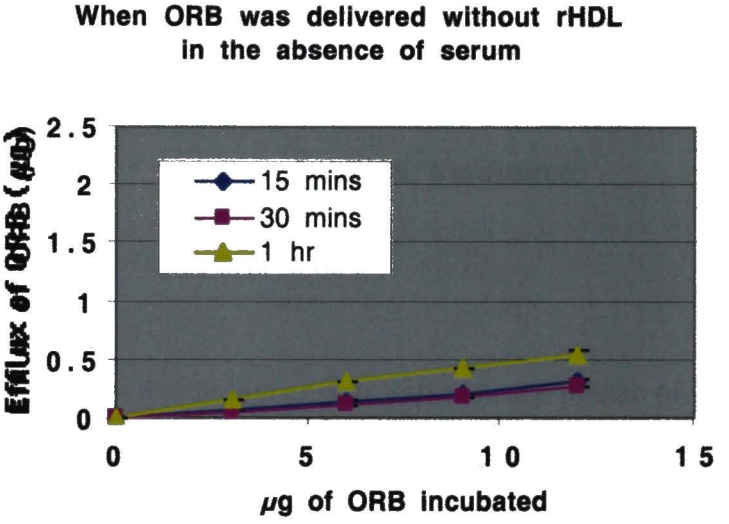




The data in figures 13(a and b) shows that there is a concentration-dependent and time-dependent increase in the efflux of ORB when it is delivered to the cells as ORB alone compared to rHDL/ORB.

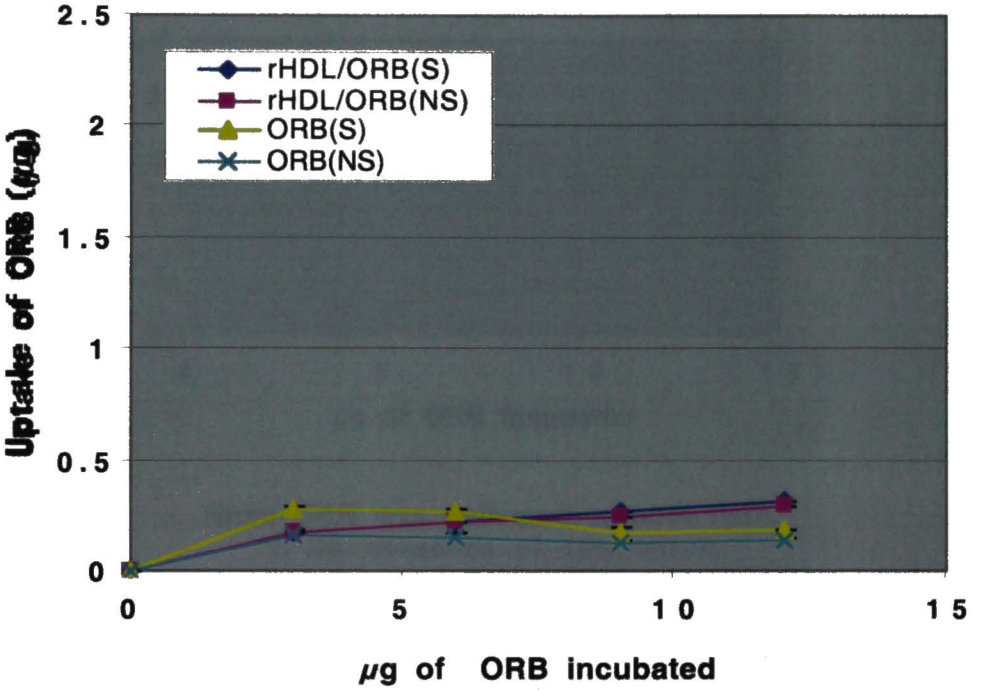
Figure 13(c and d). Efflux of ORB in SK-OV-3 cells in the absence of serum at different time points points (blue - 15 mins, pink - 30 mins, yellow - 1 hr).





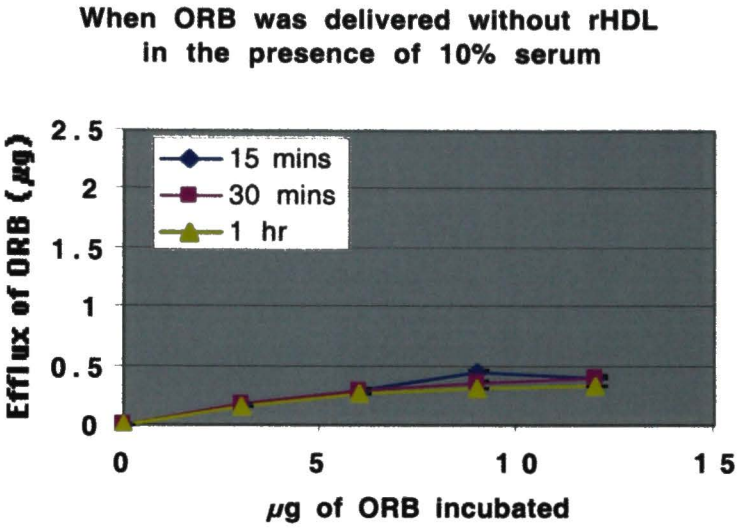
The data in figures 13(c and d) shows that there is a concentration-dependent increase in the efflux of ORB when it is delivered to the cells as ORB alone compared to rHDL/ORB.

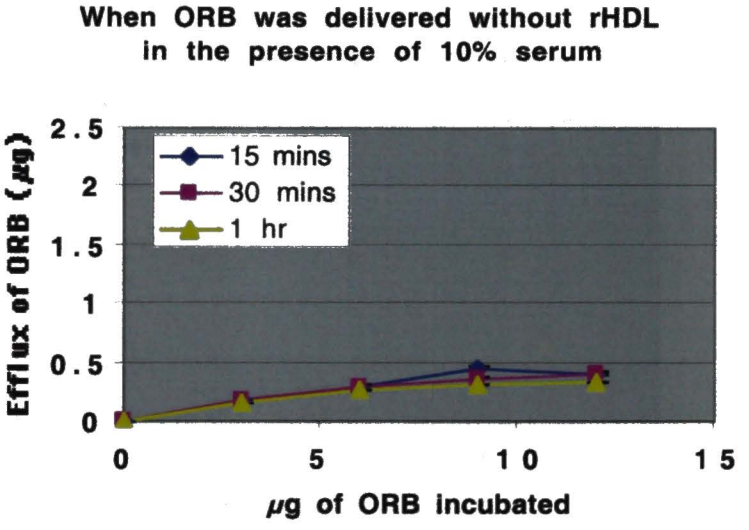
Figure 14. Uptake of ORB in OVCAR-3 cells incubated with increasing concentration of rHDL/ORB and ORB alone under 10% serum and no serum conditions for 2 hours at 37°C.



The data in figure 14 shows that at higher concentration, the uptake of ORB is more in both serum and no serum conditions when it is delivered to the cells with rHDL (blue - 10% serum a,d pink - no serum) compared to the free ORB delivery (yellow - 10% serum and turquoise - no serum).

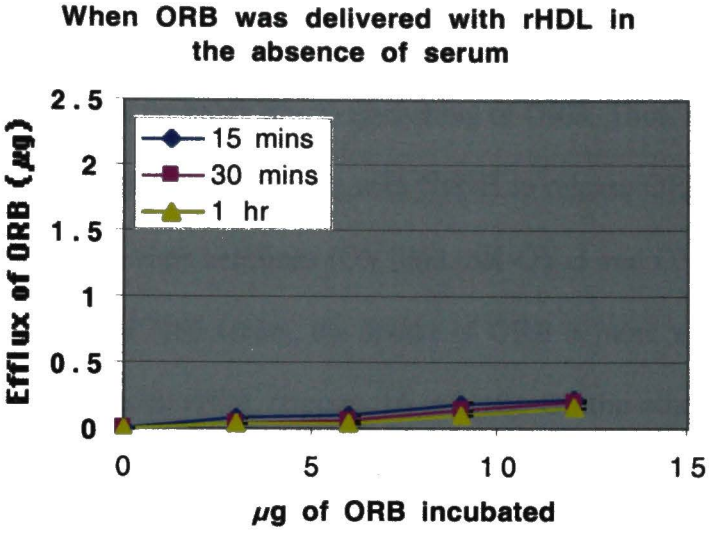
Figure 15(a and b). Efflux of ORB in OVCAR-3 cells at different time points (blue - 15 mins, pink - 30 mins, yellow - 1 hr) in the presence of 10% serum.

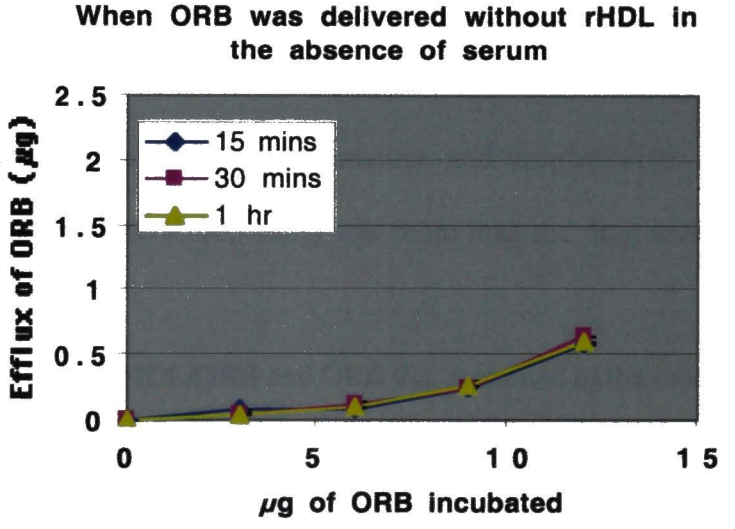




The data in figure 15(a and b) shows that at different time points, the efflux of ORB is more when it is delivered to the cells without rHDL.

Figure 15(c and d). Efflux of ORB in OVCAR-3 cells at different time points (blue - 15 mins, pink - 30 mins, yellow - 1 hr) in the absence and 10% serum.





The data in figures 15(c and d) shows that there is a concentration-dependent increase in the efflux of ORB in when it is delivered to the cells as ORB alone compared to rHDL/ORB.

Uptake of Octadecyl Rhodamine B Chloride with rHDL (rHDL/ORB) in cancer cell lines when cells are lysed with NaOH:

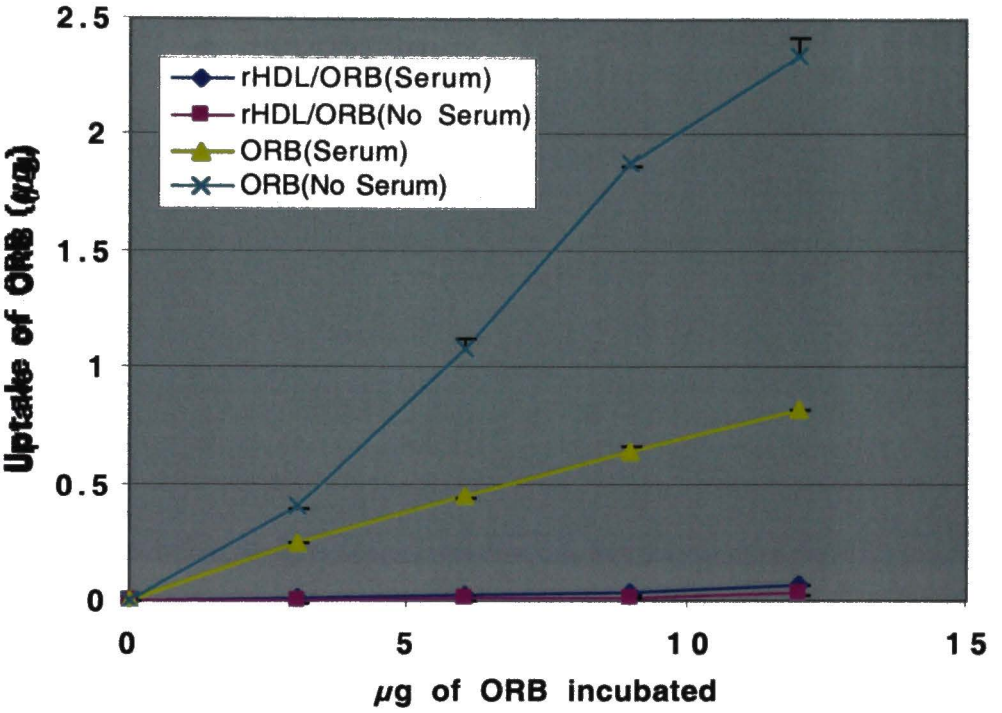
In the previous uptake experiments, efflux of ORB from the cells was more than the uptake. This may be probably due to quenching of ORB. Thus, the experiments were repeated again, this time lysing the cells with NaOH to release ORB and then measured the fluorescence. In all three cell lines (OV1063, SK-OV-3 and OVCAR-3), both in the presence and absence of 10% serum, the uptake of ORB is more when it is delivered to the cells directly than with rHDL (figures 16, 17, 18). On the other hand, there is very little uptake when ORB is delivered with rHDL, both in the presence and absence of 10% serum. When uptake is compared in the presence and absence of 10% serum in the case of ORB alone, the uptake in the absence of serum was more than in the presence of serum.

Comparing between the drug-sensitive and drug-resistant cells, the uptake of ORB in drug-sensitive (OV1063 cells) was more than the drug-resistant (SK-OV-3 and OVCAR-3) cells.

The amount of rHDL/ORB and ORB that remained in the medium after 2 hours of incubation with the cells (uptake) was measured in OVCAR-3 cells (figure 20, which shows that, in the case of rHDL/ORB both in the presence and absence of serum, and in the case of ORB in the presence of serum, most of ORB stays in the medium. But, in some cases, the concentration of ORB is more than what is actually added. So, it is very unclear of the effect of different environments (e.g., PBS, Serum) on the measurement of the fluorophore.

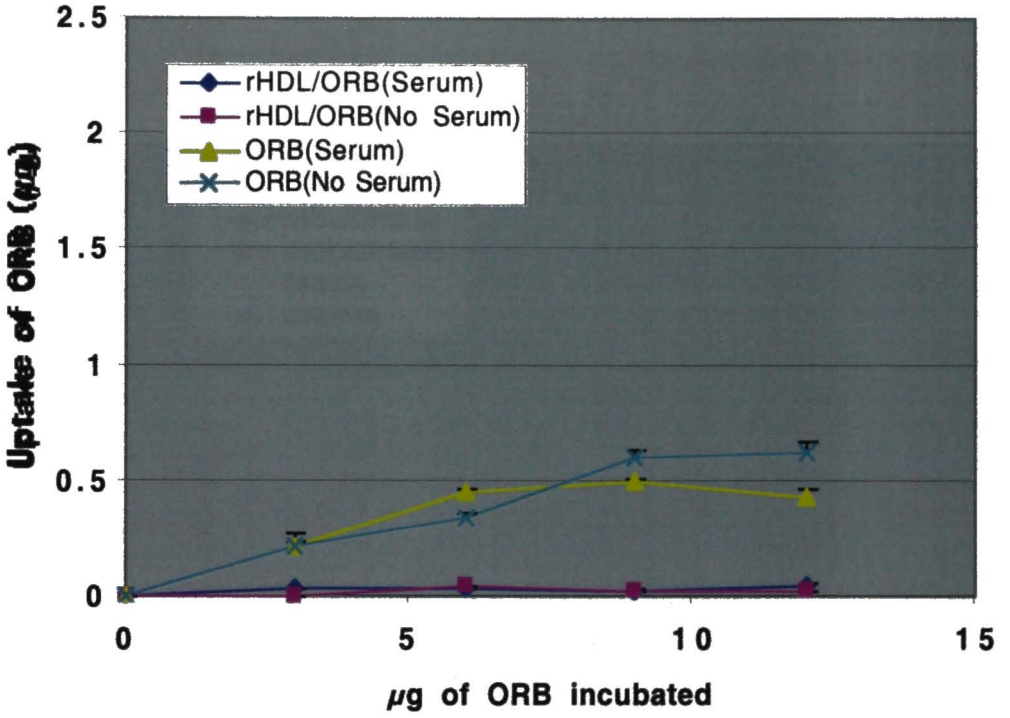
Fluorescence microscopy pictures (figure 21) show that in both the cases, where ORB is delivered with or without rHDL, it appears that ORB may be accumulating in the cytosol of the cells.

Figure 16. Uptake of ORB in OV1063 cells incubated with increasing concentration of rHDL/ORB and ORB alone under 10% serum and no serum conditions for 2 hours at 37°C and lysed with NaOH.



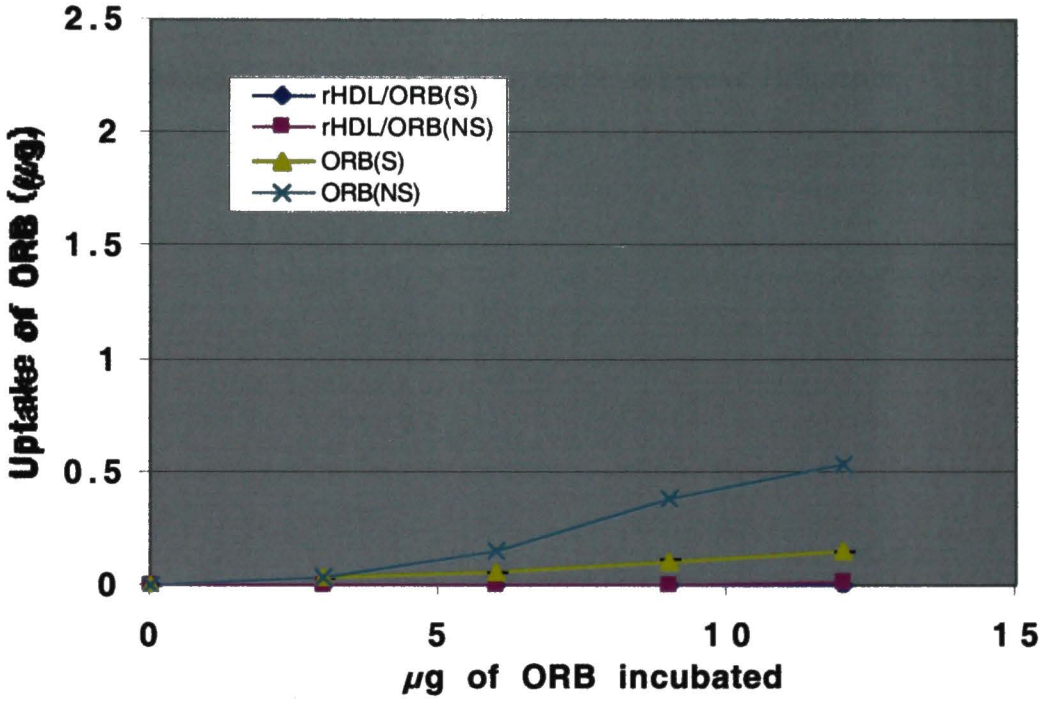
The data in figure 16 shows that the uptake of ORB is more in both serum and no serum conditions when it is delivered to the cells without rHDL (yellow - serum, turquoise - no serum) compared to rHDL/ORB (blue - serum, pink - no serum).

Figure 17. Uptake of ORB in SK-OV-3 cells incubated with increasing concentration of rHDL/ORB and ORB alone under 10% serum and no serum conditions for 2 hours at 37°C and lysed with NaOH.



The data in figure 17 shows that the uptake of ORB is more in both serum and no serum conditions when it is delivered to the cells without rHDL (yellow - serum, turquoise - no serum) compared to rHDL/ORB (blue - serum, pink - no serum).

Figure 18. Uptake of ORB in OVCAR-3 cells incubated with increasing concentration of rHDL/ORB and ORB alone under 10% serum and no serum conditions for 2 hours at 37°C and lysed with NaOH.

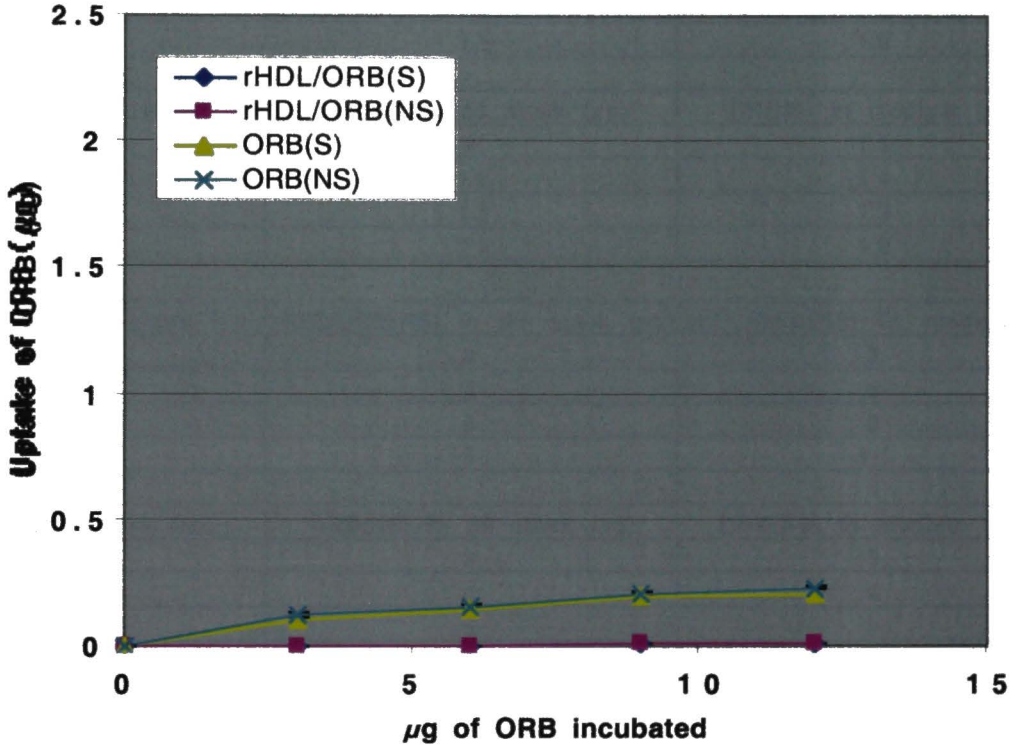


The data in figure 18 shows that the uptake of ORB is more in both serum and no serum conditions when it is delivered to the cells as a free drug (yellow - 10% serum and turquoise - no serum) compared to rHDL/ORB (blue - 10% serum and pink - no serum).

Uptake of Octadecyl Rhodamine B Chloride with rHDL (rHDL/ORB) in OV1063 cells when ORB is extracted with Butanol (Butanol extraction) [12]:

In this uptake study, ORB is extracted with butanol and then measured. Figure 19 shows that, both in the presence and absence of serum, the uptake of ORB is more when it is delivered to the cells directly than with the rHDL vehicle. There is almost no difference in the uptake whether in the presence or absence of 10% serum.

Figure 19. Uptake of ORB in OV1063 cells incubated with increasing concentration of rHDL/ORB and ORB alone under 10% serum and no serum conditions for 2 hours at 37°C. ORB was extracted with Butanol.



The data in figure 19 shows that the uptake of ORB is more in both serum and no serum conditions when it is delivered to the cells as a free drug (yellow - 10% serum and turquoise - no serum) compared to rHDL/ORB (blue - 10% seum and pink - no serum).

Figure 20. Amount of rHDL/ORB and ORB leftover in the medium after 2 hours of uptake in the presence and absence of serum.

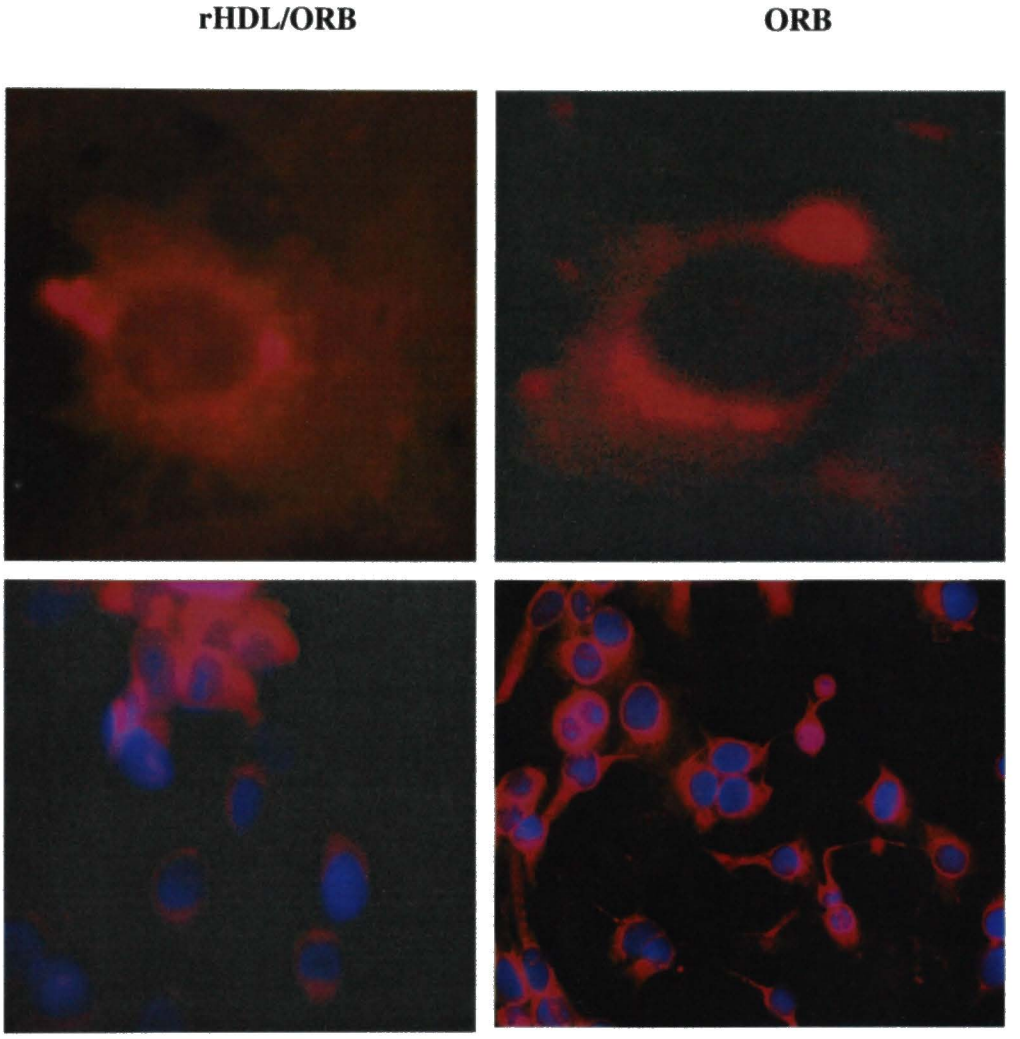
rHDL/ORB in stock (μg)	rHDL/ORB(S) in dil stock (μg)	rHDL/ORB(S) in medium (μg)
4	4	4
7	8	7
11	11	10
14	15	12
ORB in stock (μg)	ORB(S) in dil stock (μg)	ORB(S) in medium (μg)
3	8	11
5	16	14
8	19	13
10	22	13

rHDL/ORB in stock (μg)	rHDL/ORB(NS) in dil stock (μg)	rHDL/ORB(NS) in medium(μg)
4	2	3
7	4	6
11	6	9
14	8	11
ORB in stock (μg)	ORB(NS) in dil stock (μg)	ORB(NS) in medium (μg)
3	3	3
5	4	4
8	5	5
10	6	6

Diluted stock has PBS, ORB or rHDL/ORB and medium with either serum or absence of serum.

The data in figure 20 shows that most of rHDL/ORB (both in the presence and absence of serum) and ORB alone (in the presence of 10% serum) stays in the medium than ORB. But, in some cases, the concentration of ORB is more than what is actually added. So, it is very unclear of the effect of different environments (e.g., PBS, Serum) on the measurement of the fluorophore.

Figure 21. Fluorescence microscopy to show the localization of rHDL/ORB and ORB in OVCAR-3 cells.



The pictures in figure 21 show that, in both the cases, when ORB is delivered with and without rHDL, it appears that ORB may be accumulating in the cytosol.

DISCUSSION

The reconstituted HDL (rHDL) drug delivery system has been developed to reduce the problems associated with the cancer treatment like drug resistance, solubility and toxic side effects to the normal cells. rHDL complexes are chosen to act as delivery vehicles due to their smaller size, hydrophobic core and receptor-mediated uptake of HDL core components by cells. The mechanism is based on the fact that rapidly dividing cancer cells take up HDL core components to satisfy their cholesterol requirements by specific cell surface receptors (SR-B1 type receptors). Hence, the chemotherapeutic drug delivered to the cells as a component of rHDL will be taken up by the cells through these receptors. Our lab previously has showed, that this SR-B1 receptor is present to a minimal level in normal cells whereas it is overexpressed in tumor cells. So, the side effects to the normal cells are minimal. Earlier studies in our lab showed that taxol (a chemotherapeutic drug) when packed into our rHDL complexes was efficiently taken up by cancer cells.

The model candidate chosen for this project is Octadecyl Rhodamine B Chloride (ORB). To summarize the results of this project, there was concentration-dependent increase in the uptake of ORB when it was delivered to the cells with rHDL. When ORB is delivered directly (without rHDL), the uptake first increased, and plateau with increasing concentration of ORB. On the other hand, there was almost no efflux when ORB is delivered with rHDL and there is concentration-dependent increase in the efflux

when it is delivered directly. And the efflux of ORB was more than the uptake. All these measurements were taken by directly reading the fluorescence of ORB when it is inside the cells. These results have shown repeatability in both drug-sensitive (OV1063) and drug-resistant (SK-OV-3 and OVCAR-3) cells.

It is known that fluorescent compounds have the problem of self-quenching which could be solved by diluting the sample. The plateau in the uptake of ORB when it is delivered directly may probably be due to quenching of ORB. And in this case, this problem cannot be solved because it cannot be diluted. So, these experiments were again repeated in drug-sensitive and drug-resistant cell lines, this time by lysing the cells with NaOH to release ORB. But, when ORB was measured this time, the results were all opposite to what we observed without lysing the cells. The uptake increased with concentration when ORB was delivered directly than with rHDL and correspondingly, there was efflux from the cells. It was not sure whether NaOH releases ORB from the cells or not. So, the uptake study was again done in OV1063 cells, this time extracting ORB with butanol [15]. But, the results were similar to the results obtained by lysing the cells with NaOH. It is very unclear about the effect of different environments (with and without rHDL) in different solvents on the fluorophore. These results need to be further evaluated by future studies.

Limitations/Alternative approaches:

In addition to quenching, ORB has a major problem in measuring its fluorescence under different environments as shown in uptake studies with different solvents. So, perhaps

using radioactively labeled ORB or other resistance inducing drugs may be used to resolve this problem.

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