Targeted Nanoparticles for the Treatment of Neuroblastoma

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ABSTRACT

Neuroblastoma (NB) is one of the most frequently diagnosed tumors in infants and children. However, the mechanism by which it is initiated and subsequently develops on the molecular and cellular level is yet to be fully elucidated. Its wide spectrum of clinical presentation has baffled physicians and biomedical scientists alike. The variant called high risk neuroblastoma (HRNB) is extremely resistant to the currently available drug regimes. Despite the recent advances in anti-cancer agents and the use of multi-modality therapy for the treatment of HRNB the morbidity and mortality in this group of patients continues to remain high.

The purpose of our project was to find novel alternative therapeutic approaches by encapsulating known anti-NB agents in a lipoprotein based formulation to achieve selected, targeted delivery of these drugs to HRNB tumors. We wanted to enhance the therapeutic efficacy of these drugs that have shown encouraging results in pre-clinical trials but have so far exhibited an adverse pharmacokinetic profile precluding their systemic application. Our laboratory has been working for the last several years on a novel drug delivery platform by encapsulating drugs into the core of high density lipoprotein (HDL) type nano-particles. Using this strategy, we encapsulated all-trans retinoic acid (ATRA), fenretinide (FR) and valrubicin into reconstituted HDL (rHDL) nanoparticles and subsequently evaluated some of their physical and chemical properties and their anti-NB potential. Further, we tested the efficiency of an apolipoprotein mimetic peptide called 5-A peptide as a component of rHDL particles and compared its efficiency with apolipoprotein A-1 (Apo-A1). The 5-A peptide offers numerous advantages over the Apo-A-1 both in terms of cost of production as well as manufacturing time.
After successfully encapsulating the drugs, we characterized them and tested their cytotoxic potential on various cancerous cell lines. We also conducted cell uptake studies to test our hypothesis of tissue targeting and selective uptake of rHDL nano-particles mediated by the scavenger receptor type B1 (SR-B1). We conducted a pilot study on nude mice in which we administered rHDL containing fluorescent dye intravenously in mice xenografted with NB tumors and took subsequent images to track its distribution in the body. Our results demonstrate that it is possible to encapsulate ATRA, FR and valrubicin into rHDL preparations with a predictable efficiency; these nano-particles show a dose dependent cytotoxic effect on NB cell lines. We anticipate that the results of our studies will facilitate the application of liposomal nano-particles and these novel drugs in the treatment of HRNB in the future.
Acknowledgement

This dissertation would not have been possible without the guidance and the help of several individuals who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study. First and foremost, my utmost gratitude to Dr. Andras Lacko, whose support and encouragement I will never forget. Dr. Lacko has been my inspiration as I hurdled the obstacles in the completion this research work and has helped me equally in the pursuit of my medical career. I would also like to thank Dr. Paul W. Bowman for his valuable guidance and for showing faith in me and also for providing us with important financial support.

I would also like to thank Dr. Maya Nair, Dr. Rhonda Roby and Dr. Laszlo Prokai who gave me important feedback throughout this project. Thanks are also due to Dr. Sharad Singhal and Sanjay Thamake because of their enormous help during the in vivo studies. This work would not have been possible without the constant assistance and encouragement of my colleagues Dr. Nirupama Sabnis, Dr. Linda Mooberry, and Mr. Chintan Trivedi.

Last but not the least; I would like to thank my family and friends for their support throughout my educational career. Their unflinching courage and conviction will always motivate me. Most importantly I would like to thank God for his mercy, grace, strength and love.
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CHAPTER I
INTRODUCTION TO PROJECT

Neuroblastoma (NB) is the most common extra cranial solid tumor diagnosed in children (Tonini & Pistoia, 2006). It is a cancer that forms in the sympathetic nerve tissues which is a part of the autonomic nervous system. The malignant growth usually originates from the adrenal glands but it may also occasionally initiate in the neck, chest or spinal cord. About half of the cases occur in children, younger than two years of age, and occasionally it is diagnosed prenatally. By the time of diagnosis, the tumors usually metastasize to other parts of the body. NB is a disease with an extremely variable course – some rapidly fulminating forms can kill within months while at the other end of the spectrum the tumors can be self remitting. Although Stage 1 and Stage 2 tumors are localized and well differentiated and can be successfully treated by surgical resection, patients with Stage 3 and Stage 4 tumors are usually disseminated already at diagnosis and therefore have very poor prognosis due to inadequate response to conventional treatment (Maris & Matthay, 1999). A variant of the disease known as high risk neuroblastoma (HRNB) presents with a particularly poor prognosis. The ineffectiveness of therapy (usually a combination of chemotherapy and radiotherapy) is primarily due to drug resistance present in the tumors and the dose limiting toxicity of the treatment. Consequently, there has been little or no improvement in the therapeutic intervention for HRNB over the last several decades.

Targeted drug delivery has been an important focus of recent research, especially regarding cancer chemotherapy. Various investigators have tried to achieve selective delivery of highly toxic drugs without concomitant damage to normal tissues. It has been suggested that lipoproteins have structural
properties that enable them to serve as drug delivery vehicles because of their ability to incorporate hydrophobic drugs into their micellar core and subsequently facilitate the cellular uptake of drugs via receptor mediated mechanisms. It was suggested over 30 years ago by Counsell and Pohland (Counsell & Pohland, 1982) that lipoproteins could deliver the ‘magic bullet’ via targeted chemotherapy and yet these macromolecular lipid/protein complexes have been largely overlooked as drug delivery agents despite their potential for receptor mediated targeting of cancer cells and tumors.

Our laboratory has developed a novel drug delivery strategy for the chemotherapy of NB employing reconstituted/synthetic (high density lipoprotein-like) nano-particles which can be utilized to carry anti-cancer drugs (McConathy, Nair, Paranjape, Mooberry, & Lacko, 2008; McConathy et al., 2011; Mooberry, Nair, Paranjape, McConathy, & Lacko, 2010; Shahzad et al., 2011). This concept is based on the rationale that cellular uptake of the core content of high density lipoproteins (HDL) is mediated by SR-B1 receptors (Calvo, Gómez-Coronado, Suárez, Lasunción, & Vega, 1998) which are particularly strongly expressed by cancer cells and tumors. Recently, several cancer cell lines have been shown to have a high expression of SR-B1 receptors to facilitate the selective uptake of the anti-cancer agents from the rHDL nano-particle drug carrier. During preliminary studies, several anti-cancer drugs were successfully encapsulated by our laboratory and work is currently underway to demonstrate the efficacy of drug delivery and anti-tumor potential in vivo in a NB model. If successful, this drug delivery strategy should markedly enhance the efficiency of parenteral administration of anti-neuroblastoma agents, lower systemic toxicity (and side effects), and thus, substantially improve the quality of life and the prognosis for HRNB patients.

We propose a targeted drug delivery system for NB chemotherapy using reconstituted/synthetic lipoproteins (rHDL) nanoparticles. This system makes use of the SR-B1 receptors (a type of scavenger
receptor) to deliver anti-cancer agents in a highly selective manner to malignant cells and tumors, leaving normal tissues largely unaffected (Figure 4). We use drugs with favorable properties that are encapsulated in nanoparticles that resemble high density lipoproteins. The rHDL nanoparticles have been shown to be effective in suppressing ovarian and breast tumor development (Corbin et al., 2007).

The major limitation of the current chemotherapy approach for treating NB is the systemic toxicity of anti-cancer drugs. Because essentially all anti-cancer drugs work by killing cells a certain level of residual toxicity is unavoidable. Consequently some “collateral damage” is likely to occur and thus some non cancerous cells are bound to be damaged or killed along with the malignant growth. Avoidance of systemic toxicity is paramount in the pediatric age group because their immature hepatic and renal systems preclude the use of anti-cancer drugs at a high dosage. Selective, tumor specific delivery of drugs would greatly enhance anti-cancer activity especially if combined with reduced toxicity to normal tissues and organs systems. In addition to achieving site specific delivery of drugs, it is anticipated that the size and biocompatibility of rHDL nanoparticles will prevent the clearance of the drug via the reticulo-endothelial system and thus increase the drug’s half-life in circulation when compared to parenteral administration of the drug alone.

HDLs exist in biological systems composed of an outer shell and a core region; the latter being occupied by hydrophobic components. The major ingredients of naturally occurring HDLs are phospholipid, cholesterol (free and esterified) and apolipoprotein. The amphipathic structure of phospholipid provides the basic framework for these macromolecular lipid/protein complexes. As shown in Figure 5, the hydrophilic heads of phospholipids orient to the exterior and hydrophobic tails are protected by being stabilized in the interior. Interspersed between the phospholipids are the apolipoprotein-A molecules which are also essential as ligands for the receptor mediated uptake of the
core content of HDL (cholesteryl esters). Initially, HDLs emerge from the liver and intestine as discoidal shaped particles while the subsequent incorporation of cholesterol and its esterification give rise to the rapidly growing core region, yielding an ultimately spherical structure.

Our laboratory has developed a process for the preparation of rHDL nanoparticles as carriers of anti-cancer agents (McConathy et al., 2008). Subsequently, we have described the physical and chemical properties of these nanoparticles (McConathy et al., 2008). In the present study, we are using the same concept to evaluate drugs with demonstrated cytotoxic potential against NB subsequent to encapsulation into rHDL nanoparticles. For our initial studies we have chosen two retinoic acid derivatives namely - ATRA and FR and an anthracyclin derivative called valrubicin.

Clinical studies have supported the concept of targeting specific tissues via lipoprotein carriers, including a recent case control study of 519 patients with various types of solid tumors and 928 controls, where the serum lipid profile in cancer patients was markedly altered (Muntoni et al., 2009). The total cholesterol values were significantly lower, due mainly to low levels of HDL-C (high density lipoprotein cholesterol), while LDL-C was generally unchanged. These and several other studies suggest that lipoprotein receptors (especially the HDL receptor) are highly active in malignant cells and thus may be used to facilitate the delivery of anti-cancer agents.

Development of human NB is believed to be due to an arrest in the differentiation of the neural crest sympathoadrenal progenitor cells. However, neuroblastoma, as well as the derived cell lines, maintains the potential for terminal differentiation and regression (Tonini & Pistoia, 2006). In fact, among all the human tumors, NB has the highest rate of spontaneous regression, and malignant or even metastatic tumors have been reported to mature spontaneously to a benign tumor known as ganglioneuroma. The extreme variation in the clinical course of this disease has led to a growing interest
towards NB differentiation *in vitro*. It has been demonstrated that many agents, including retinoic acid (RA), can induce neurite out-growth, increase membrane excitability and neurotransmitter enzymes, change cell surface antigens, reduce tumorigenicity of various NB cell lines. We therefore decided to begin our studies with (ATRA), a well known drug for preventing relapse and maintaining remission in NB patients subsequent to the initial intensive therapy. Retinoic acid is the biologically active derivative of vitamin A or retinol and is known to regulate cell growth and differentiation and can reverse malignant cell growth. In recent years, there has been a substantial rise in the use of retinoic acid for treatment of NB that generated a lot of interest in research. The exact cellular mechanism of the action of retinoids is still not fully understood while they are being increasingly used as an important treatment modality for preventing the relapse of NB throughout the world.

Fenretinide, or HPR (N-4-hydroxylphenyl retinamide), another retinoic acid derivative, has been shown to possess considerable anti cancer activity against NB and against other cancer cell lines. Unlike retinoic acid which differentiates cancer cells FR acts via a different mechanism and can actually kill the cancer cells (Lotan, 1995). Most of our initial studies employed ATRA instead of FR because both have similar physical and chemical properties and incorporation of ATRA can be easily tracked by a using radio-labeled drug. It has been demonstrated that many cancer cells in general have a higher expression of SR-B1 receptors and a higher uptake of liposomal nano-particles as compared to the normal tissue, although specific receptor characteristics of NB cells have yet to be reported.

Valrubicin, or N-Trifluoroacetyladriamycin-14-valerate (AD 32), is an analogue of adriamycin and it has shown promising anti tumor activity during pre-clinical studies (Israel, Modest, & Frei, 1975). Its current therapeutic use, however, is restricted due to poor water solubility and toxic side effects. It is used intravesicularly for cancers of the urinary bladder and for some metastasized gynecological cancers
(Kuznetsov, Alsikafi, O'Connor, & Steinberg, 2001). We intend to test the feasibility of a targeted delivery option for valrubicin and its anti-cancer potential against NB.
CHAPTER II
Experimental Design and Methods

Refer to Figure 3 for an overview of the experimental design.

Preparation of reconstituted high density lipoprotein (containing ATRA) complex

Cholate dialysis was based on the procedures described for discoidal rHDL particles (Figure 5) (Jonas, 1986; Matz & Jonas, 1982). After testing a variety of formulations of ingredients, a mixture of phospatidyl choline (PC) in CHCl₃ with free cholesterol (C), cholesteryl oleate and ATRA 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 ATRA into rHDL. The lipid mixture (PC, C and cholesteryl oleate) and 2 mg of ATRA were dried under nitrogen to a thin film and dispersed in 60 mL of dimethylsulfoxide [(DMSO) Sigma-Aldrich] and 1.4 mL of cholate dialysis buffer (10 mM Tris, 0.1 M KCl and 1 mM EDTA; pH8.0; Sigma-Aldrich, St. Louis, Missouri). Approximately 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 approximately 1: 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 41°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 final preparations. The ATRA containing
rHDL fractions were isolated by single step density gradient ultracentrifugation, and were dialyzed overnight against PBS, pH 7.4. These rHDL preparations can be stored at 41°C for 60 days.

**Determination of reconstituted (synthetic) high-density lipoprotein components**

Total cholesterol, free (unesterified) cholesterol and phospholipids were determined by the respective enzymatic reagent kits (Wako Pure Chemical Industries Ltd, Osaka, Japan), using microtiter plate assays (Urrutia-Rojas et al., 2004). The concentration of cholesteryl esters was obtained as the difference between total and free cholesterol values. Protein determinations were carried out using BSA™ protein assay kits supplied by Thermo scientific (Rockford, Illinois, USA).

**Analyzing the size and uniformity of nano-particles using electron microscopy**

After dialysis against a buffer containing 0.125 M ammonium acetate, 2.6 mM ammonium carbonate and 0.26 mM EDTA (pH 7.4), the isolated rHDL samples were negatively stained with 2% sodium phosphotungstate (pH 7.2) and placed on Formvar stabilized carbon-coated 200-mesh nickel grid support films (TedPella Inc., Redding, California, USA) (Forte, Norum, Glomset, & Nichols, 1971). 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. Refer to Figure 28 and Figure 29 for electron microscopy images of a sample of nano-particles containing ATRA.
**Dynamic light scattering**

Dynamic light scattering (DLS) technology is used to determine the particle size and polydispersity index. Particles suspended in a dispersing fluid are subject to random collisions with the thermally excited molecules of the dispersing fluid resulting in Brownian motion. The velocity and direction of the resulting motion are random but the velocity distribution of a large number of mono-sized particles averaged over a long period will approach a known functional form, in this case the size distribution of the particles. For this purpose the Nanotrac particle size analyzer (Microtrac, York, Pennsylvania) was used.

**TIRF microscopy**

In order to visualize the nanoparticles and see how they behave in a fluid environment we use the method of total internal reflection florescence microscopy (TIRFM). The DiI dye (1, 1’-dioctadecyl-3, 3,3’3’-tetramethylindocarbocyanine perchlorate, chemical formula: C_{59}H_{89}ClN_{2}O_{4}) was used for this experiment and specific amount of dye was encapsulated into the recombinant liposomes using the same protocol as previously described. A favorable octanol water partition coefficient and superior fluorescent properties makes DiI the dye of choice for easy encapsulation and visualization. A TIRFM uses an evanescent wave to selectively illuminate and excite fluorophores in a restricted region of the specimen immediately adjacent to the glass-water interface. The evanescent wave is generated only when the incident light is totally internally reflected at the glass-water interface. The evanescent electromagnetic field decays exponentially from the interface, and thus penetrates to a depth of only approximately 100 nm into the sample medium. Thus the TIRFM enables a selective visualization of surface regions. Refer to Figure 30 for the actual TIRFM image.
Cell culturing

Established cancer cell lines (SMS-KCNR and SK-N-SH) were obtained from the Child Oncology Group (COG) and grown according to the procedures provided by the COG, employing the respective recommended media, including the mixture of 1% penicillin and streptomycin and 10% fetal bovine serum (Life Technologies, 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 the flasks were 80% to 90% confluent. Cells were counted and plated onto 96-well microtiter plates 1 day before being used for assays.

Cell uptake assays

Cells in confluent flasks of 75-cm² were plated in 24 well plates with careful counting so that the same number of cells and same amount of media was present in each well. These wells were then used to assess the receptor mediated uptake of drugs from rHDL. SMS-KCNR cells were plated on a 24-well plate at a concentration of 120,000 - 150,000 cells/well and incubated overnight at 37 °Celsius. The cells were serum starved for 30 minutes with serum-free Isckov’s media. Media containing rHDL/ATRA only or rHDL/ATRA with serial dilutions of native HDL was added and the cells were incubated at 37° Celsius for 90 minutes. The media was removed and the cells were washed twice with PBS, pH 3.0 and once with PBS, pH 7.4 to remove any extracellularly bound rHDL. The cells were lysed with 0.5M NaOH and ³H radiation count was measured (Mooberry et al., 2010). Since native human HDL is the natural ligand of SR-B1 receptor it is expected that by increasing the concentration of native HDL in wells the receptor mediated uptake of tritiated ATRA from rHDL will be reduced due to mutual competition among the two ligands (Figure 10). The media was then removed from the wells and
cells from each well detached by using NaOH. The radiation count was then measured in each well using the scintillation counter.

**Comparing Apo-A1 and apolipoprotein mimetic peptides.**

Apolipoprotein A1 is the major ingredient of native and recombinant HDLs and is obtained from human sources by a time consuming process and is hence very expensive. In contrast, apolipoprotein mimetic peptides can be commercially prepared by a faster and cheaper method. They may therefore prove to be extremely important when a large scale production of rHDL is desirable as in case of pharmaceutical companies. Another important goal of my project was to assess the efficiency of other apolipoprotein mimetic peptides in making recombinant HDL nanoparticles and to study their chemical composition and physical characteristics. Also we want to investigate if these nanoparticles can be used as potential drug delivery vehicles with the same efficacy. Natural Apolipoprotein A1 has eight amphipathic helices and they are essential for functioning of HDL as a ligand. Most apolipoprotein mimetic peptides are short synthetic peptides that typically contain at least one amphipathic helix (Navab et al., 2005). Our initial studies with 5A peptide gave encouraging results and hence we are pursuing it further.

**In vivo imaging studies**

Since an important part of our hypothesis is based on the tissue selective delivery of drugs hence we wanted to see if such a targeted delivery can actually be achieved in live animal models too. We chose HPHH dye because of its favorable physical and chemical properties and its ease of encapsulation. Further the excitation and emission wavelengths were in the appropriate range for us to visualize the dye after an intravenous injection in the animal. We chose nude mice for our experiments so that tumors can
be easily grown in them. A fixed number of SMS-KCNR cells were injected subcutaneously in mice and they were followed until they developed sufficiently large tumors. The rHDL containing fluorescent dye were then injected in the tail vein and images were taken at specific time points after the injection to assess the distribution and tissue specific concentration of the encapsulated dye. Unfortunately few initial injections The IVIS 200 series (Caliper Life Sciences, Hopkinton, Massachusetts, USA) were used for imaging and anesthesia in mice experiments. All the mice were given proper care during the study and were euthanized according to the protocol.
CHAPTER III

Results

Encapsulation efficiency of ATRA

On gradually increasing the amount of ATRA applied, we observed an increase in incorporation until a plateau was reached at about a concentration of 3000 µg/mL of ATRA. The maximum incorporation was at 1500 µg/mL. However, because the uniformity in size of the nanoparticles was reduced beyond 1250 µg/mL, we adopted the drug application range of 750-1250 µg/mL for the preparation of ATRA containing rHDL nanoparticles.

Estimation of particle size

DLS analysis was done to obtain the information regarding size distribution of the nano-particle preparations. Most batches were in the desirable size range (approx 50-150 nm) and were relatively monodispersed (Table 1). On increasing the applied ATRA beyond 1250 µg/mL, we noted progressive precipitation of the drug. Results of DLS analysis are shown in Figures 14 through 19.
### Table 1: Average Size and dispersion of ATRA nano-particles using DLS

<table>
<thead>
<tr>
<th>Nanoparticle type</th>
<th>Particle size in nm (Å)</th>
<th>S.D (σ)</th>
<th>Polydispersity Index (σ²/Å²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10ATRA+10rATRA</td>
<td>102.2</td>
<td>31.8</td>
<td>0.06</td>
</tr>
<tr>
<td>20ATRA+10rATRA</td>
<td>93.8</td>
<td>38.70</td>
<td>0.08</td>
</tr>
<tr>
<td>30ATRA+10rATRA</td>
<td>89</td>
<td>25.3</td>
<td>0.05</td>
</tr>
<tr>
<td>50ATRA+10rATRA</td>
<td>160</td>
<td>90.6</td>
<td>0.10</td>
</tr>
<tr>
<td>100ATRA+10rATRA</td>
<td>97.1</td>
<td>44.2</td>
<td>0.09</td>
</tr>
<tr>
<td>120ATRA+10rATRA</td>
<td>167</td>
<td>160</td>
<td>0.25</td>
</tr>
</tbody>
</table>

### Uptake suppression studies

In order to test the involvement of SR-B1 receptors in the uptake of ATRA from rHDL we used tritiated ATRA for the estimation of cellular uptake of drug. The uptake of the core components of both rHDL and native human HDL is mediated by the SR-B1 receptors (Mooberry et al., 2010) as both of them have similar overall structures. These studies were performed with approximately 150,000 cells in each well. Equal amounts of serum free media and tritiated ATRA in rHDL were added to each well. Increasing concentration of native HDL was added to the respective wells (except the control that had no HDL) to study the impact of HDL on the uptake of ATRA by the NB cells. These data show that by progressively increasing the concentration of native HDL the uptake of tritiated ATRA from the rHDL nanoparticles was suppressed (Figure 10 and Figure 20).
**Efficacy of Apo-A1 vs. 5-A peptide**

Our studies to compare Apo-A1 with 5-A peptide showed that for ATRA both of them had a similar efficiency of encapsulation and so we decided to use the 5A peptide for our future studies.

**Ultracentrifugation Studies**

In order to understand how the rHDL particles will interact with serum in the circulation, we mixed a known amount of radioactive rHDL and human serum in two test tubes. One tube was ultracentrifuged immediately while the other one was incubated for 24 hours and then ultracentrifuged. We wanted to see how the tritiated ATRA in the rHDL particles distribute themselves in the various fractions. Results showed that there was a shift in radioactivity from fractions [11, 12] to fractions [3, 4] and [6, 7]. The exact interpretation of this experiment however is unclear. Refer to Figures 23, 24 and 25 for our experimental setup and results.
Valrubicin nanoparticles integrity and size

Size and dispersion of valrubicin rHDL particles was assessed using dynamic light scattering as well as electron microscopy and all the samples were found to be monodispersed. Transmission electron microscopy of the particles showed that they were spherical in shape without too many agglomerates.

Valrubicin nanoparticles composition

Table 2: Compositions of Valrubicin np prepared using Apo-A1 and 5A peptide

<table>
<thead>
<tr>
<th></th>
<th>Protein (mg/mL)</th>
<th>Chol. (mg/mL)</th>
<th>Phospho. (mg/mL)</th>
<th>Drug (mg/mL)</th>
<th>Total wt (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Using 5A peptide</strong> <em>(2000 µg Val)</em></td>
<td>1.021</td>
<td>0.126</td>
<td>7.63</td>
<td>0.5625</td>
<td>9.3395</td>
</tr>
<tr>
<td><strong>Using Apo A</strong> <em>(2000 µg Val)</em></td>
<td>1.213</td>
<td>0.174</td>
<td>9.36</td>
<td>0.60</td>
<td>11.347</td>
</tr>
</tbody>
</table>
Valrubicin incorporation measurement

Incorporation of drugs into nanoparticles was measured using the optical properties of valrubicin. A strong peak of absorbance is observed at a wavelength of 530 nm. A comparison between the intensity of the pre-dialysis with the post-dialysis sample is done to obtain the percentage of valrubicin incorporated.

Table 3: Absorbance of standard samples of valrubicin

<table>
<thead>
<tr>
<th>µg/mL</th>
<th>absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25</td>
<td>0.012428</td>
</tr>
<tr>
<td>2.5</td>
<td>0.024348</td>
</tr>
<tr>
<td>5.0</td>
<td>0.050072</td>
</tr>
<tr>
<td>10</td>
<td>0.091610</td>
</tr>
</tbody>
</table>

\[ y = 0.009x + 0.002 \]
### Table 4: Drug Incorporation in valrubicin nano-particles

<table>
<thead>
<tr>
<th></th>
<th>Absorbance</th>
<th>µg/mL</th>
<th>Weighed according to final volume</th>
<th>Percentage incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val (2000 µg) + <strong>peptide</strong> before dialysis</td>
<td>0.06723</td>
<td>7.225539</td>
<td>13.00597</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.074336</td>
<td>8.01508</td>
<td>11.30126</td>
<td><strong>86.89289</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Absorbance</th>
<th>µg/mL</th>
<th>Weighed according to final volume</th>
<th>Percentage incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val (2000 µg) + <strong>apoA</strong> before dialysis</td>
<td>0.0985</td>
<td>10.70001</td>
<td>19.26002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.085848</td>
<td>9.294243</td>
<td>16.07904</td>
<td><strong>83.48402</strong></td>
</tr>
</tbody>
</table>
Chemical composition of nanoparticles

Refer to Figures 31, 32 and 33 for a pictorial presentation of composition of ATRA containing nanoparticles.

Table 5: Sample containing 750 µg/mL ATRA initially

<table>
<thead>
<tr>
<th>Protein (mg/mL)</th>
<th>Chol. (mg/mL)</th>
<th>Phospho. (mg/mL)</th>
<th>Drug (mg/mL)</th>
<th>Total wt (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X peptide (30 + 5)</td>
<td>0.6089</td>
<td>0.384</td>
<td>6.1891</td>
<td>0.5625</td>
</tr>
<tr>
<td>2X peptide (30 + 5)</td>
<td>0.4954</td>
<td>0.149</td>
<td>5.0725</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Table 6: Sample containing 1250 µg/mL ATRA initially

<table>
<thead>
<tr>
<th>Protein (mg/mL)</th>
<th>Chol. (mg/mL)</th>
<th>Phospho. (mg/mL)</th>
<th>Drug (mg/mL)</th>
<th>Total wt (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X peptide (50 + 5)</td>
<td>0.7271</td>
<td>0.0736</td>
<td>6.5193</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Storage and stability of nanoparticles

An important issue to be addressed when considering large scale production of rHDL nanoparticles is their ability to be stored for a longer period. We still need to conduct more studies to see if lyophilization and reconstitution are efficient procedures for storing liposomal nano-particles for a long term. Currently we store them in glass tubes at a temperature of 4 degree Celsius protected from light. We redialysed the samples that had been stored for a month in the refrigerator and analyzed them for drug retention and integrity of their shape and size. We found that the particles had the same size distribution as before but the drug content had reduced to 50-60% of the original. We still need to do more studies to analyze whether the loss of drug was due to leaking out of intact nano-particles or due to the nano-particles falling apart. Refer to Figures 34, 35, 36 and 37 for DLS analysis of the abovementioned samples.

Table 7: Retention of drug in two samples stored over 1 month

<table>
<thead>
<tr>
<th></th>
<th>1500 µg/mL (30 µl) ATRA nanoparticles</th>
<th>2500 µg/mL (100 µl) ATRA nanoparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug content initially</td>
<td>7719.6</td>
<td>14902</td>
</tr>
<tr>
<td>Drug content 1 month later</td>
<td>3930.6</td>
<td>7734</td>
</tr>
</tbody>
</table>
Cytotoxicity assay of FR in SMS-KCNR cell line

Table 8: Result of MTT assay done to evaluate the effect of drug

<table>
<thead>
<tr>
<th>µg/mL</th>
<th>control</th>
<th>15 µg/mL</th>
<th>30 µg/mL</th>
<th>60 µg/mL</th>
<th>120 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>µMolar</td>
<td>control</td>
<td>40 µMolar</td>
<td>80 µMolar</td>
<td>160 µMolar</td>
<td>320 µMolar</td>
</tr>
<tr>
<td>absorbance</td>
<td>0.16575</td>
<td>0.164571</td>
<td>0.159571</td>
<td>0.131667</td>
<td>0.1255</td>
</tr>
<tr>
<td>Percentage cell viability</td>
<td>100</td>
<td>99.28895</td>
<td>96.27236</td>
<td>79.4369</td>
<td>75.71644</td>
</tr>
</tbody>
</table>

Refer to Figure 38 for the graphical representation of the results of MTT assay.

Calculations

Initial stock - 50 mg/mL = 50 µg/µl
30 µl of stock in 2 mL of pre-dialysis sample = 1500µg/2mL = 750µg/mL
Incorporation efficiency for the sample - 80% = 600µg/mL = 0.6 mg/mL = 0.6 µg/µl

MTT- 5µl in 200 µl well
25µl in 1 mL => 15 µg/mL

Mol. Wt of fenretinide: 392 gm
MTT concentration- 15000µg/1000mL = 15 mg/l = 0.00003827 Molar = 38.27 µMolar

Cytotoxicity assay of FR in Glioblastoma cell line

Table 9: Result of MTT assay done to evaluate the effect of drug

<table>
<thead>
<tr>
<th>% cell viability</th>
<th>100</th>
<th>62.95</th>
<th>51.82</th>
<th>45.17</th>
<th>43.93</th>
<th>44.13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug (µg/mL)</td>
<td>control</td>
<td>30</td>
<td>60</td>
<td>120</td>
<td>240</td>
<td>300</td>
</tr>
<tr>
<td>Drug (µMolar)</td>
<td>control</td>
<td>80</td>
<td>160</td>
<td>320</td>
<td>640</td>
<td>800</td>
</tr>
</tbody>
</table>

Refer to Figure 39 for the graphical representation of the results of MTT assay.

In-vivo imaging study

Refer to Figures 40 to 46 for the results of in-vivo imaging study.
CHAPTER IV
Discussion

Neuroblastoma is the most common neuroectoderm derived solid tumor of pediatric age and one of the most intriguing aspects of this disease is its broad spectrum of clinical behavior (Miller, 1995) (London et al., 2005). Some of these patients undergo spontaneous regression or may gradually differentiate into benign ganglioneuromas (D'Angio, Evans, & Koop, 1971; A. E. Evans, Gerson, & Schnaufer, 1976; Haas, Ablin, Miller, Zoger, & Matthay, 1988). The Stage 1 and Stage 2 of the disease (Brodeur et al., 1993) can often be cured with surgery alone (Alvarado et al., 2000; Perez et al., 2000) while patients with disseminated cancer respond well to treatment with chemotherapy and surgery (Bowman et al., 1997; Schmidt et al., 2000). The heterogeneous nature and variation in clinical outcome depends upon various clinical and biophysical factors like Stage and history of disease as well as patient age and genetics (Brodeur et al., 1993; A. E. Evans, D'angio, Propert, Anderson, & Hann, 1987; Shimada et al., 1999). Most of the patients who are older than one year age and who have advanced stage of the disease die from it despite an intensive multimodality therapy (Matthay et al., 1999).

Staging and risk stratification

The current international staging system of NB and the pre-treatment classification of risk groups is shown in Appendix 1 (Cohn et al., 2009) and Appendix 2 (Brodeur et al., 1993).
**High Risk Neuroblastoma - a clinical enigma**

According to the revised international classification at least 40% of the newly diagnosed patients of NB are designated to the high risk group, based on adverse features such as age greater than 18 months at presentation, presence of disseminated disease, unfavorable histological features, and amplification of the MYCN oncogenes (Park, Eggert, & Caron, 2008). The treatment of this group of patients currently consists of a coordinated sequence of chemotherapy, surgery and radiation (Matthay et al., 1999; Pearson et al., 2008). Most patients have an extensively metastasized disease even at the time of presentation and hence resection of primary tumor is rarely performed. Instead, these patients receive intensive multi-agent chemotherapy as the induction phase which is designed to substantially reduce the overall disease burden and facilitate the later resection of primary tumor which is performed after numerous chemotherapy courses. The chemotherapeutic agents consist of varying combinations of alkylating agents, anthracyclins, platinum compounds, and epipodophyllotoxins. After the intensive induction therapy is completed, the next step usually consists of one or more course of high dose chemotherapy with autologous hematopoietic stem cell support. Agents such as carboplatin, etoposide and melphalan are used during this high dose chemotherapy. Once the patients recover from the acute effects of consolidation, they receive focal radiotherapy to the primary tumor site as well as any residual metastatic sites that are found at the completion of induction. Finally, six courses of the differentiating agents such as isomers of RA are given to eradicate the remaining tumor cells which are present in at least half of all the patients who are considered completely remitted by imaging criteria.
As described above the treatment regime of NB is very extensive, complex and long term. The usual duration of treatment varies from 1 to 2 years and is fraught with drug toxicity issues and complications. According to globally reported data, even after this aggressive treatment less than 35% of children are likely to achieve long term cure (Matthay et al., 1999; Pearson et al., 2008; Zage, Kletzel, Murray, Marcus, Castleberry, Zhang, London, & Kretschmar, 2008b). Treatment failure arises most often from the setting of minimal residual disease following high dose chemotherapy. Although prolonged disease stabilization can be obtained in some children following tumor recurrence, almost all of the children who relapse eventually die from disease progression (Lau et al., 2004). Even patients who appear to have achieved a cure with initial therapy remain at risk for later developing long term complications related to treatment such as hearing loss, cardiac dysfunction, infertility and second malignancies (Laverdière et al., 2005). The above mentioned observations clearly highlight the need of more effective and less toxic therapies.

Many new modalities of treatment are currently under research to overcome some of the stated problems. Despite advances in treatment and improvement in survival for patients with HRNB over the last three decades, significant obstacles still remain. First of all, 20-50% of patients have soft tissue or osteomedullary NB that is refractory to induction chemotherapy (Modak & Cheung, 2010). Recent advances in second line chemotherapy, immunotherapy and targeted therapy have only marginally improved the morbidity and mortality of this group while cure remains out of reach for most. Secondly, most of the patients who achieve remission relapse in the bone marrow or less commonly in soft tissue sites. Therefore, newer strategies using non-cross resistant therapies are required to increase the rate of remission in this group of patients.
Our approach

The aim of this project was to evaluate the feasibility of using rHDL nanoparticles for encapsulating anti-cancer agents that with therapeutic potential against NB. We started our project with the known differentiating agents like RA (vitamin A). There are two forms of this drug, 13-cis and all-trans retinoic acid, which possess differentiating properties with minor variations. This drug is used to prevent the relapse of NB after an intensive chemotherapy with other cytotoxic drugs, has brought patient to remission. RA is then given for a period of many months to years to differentiate any remaining cancerous cells in the body.

Addressing the issues of encapsulation

The reason we chose the ATRA is because of its structural similarities to FR and because it has a lipophilic nature which makes it suitable for encapsulation. After initial challenges with solubility, we were able to dissolve the drug in DMSO and use it for encapsulation. We performed numerous studies to evaluate the efficiency of incorporation of ATRA using our current protocol. Since a radioactive variant of ATRA is commercially available, it was possible to use radio-tagged rHDL and compare the radioactivity of pre- and post-dialysis samples to assess the efficiency of incorporation into nanoparticles. We gradually increased the concentration of ATRA applied initially to optimize incorporation into the nanoparticles and minimum precipitation of drug. We estimated from our initial experiments that a range of 0.75 to 1.25 mg/mL of ATRA was most suitable for an efficient incorporation.
After establishing a workable protocol for encapsulating ATRA we analyzed the characteristics of these particles. Using dynamic light scattering we measured the mean particle size and their polydispersity index. The particle size for our selected range of drug concentration was in the range of 50 nm to 150 nm which is good for intravenous administration and most of our samples were dispersed along a single peak. Then we analyzed the chemical composition of these nano-particles using standard protein, cholesterol and phospholipid assays. The drug content of our preparation attained a maximum of up to 9-10% with best efficiency. The cholesterol content in our preparations was less than 1.5% which further makes it favorable for intravenous use since earlier commercially available liposomal preparations of anti-cancer drugs were not very popular due to their severe adverse effect on the lipid profile of the patients.

After ATRA we decided to encapsulate FR and valrubicin, both of which are not currently used therapeutically but have demonstrated considerable cytotoxic effect on cancerous cell lines in in-vitro studies. Fenretinide is a synthetic retinoid derivative similar to ATRA but with a cytotoxic effect on Neuroblastoma cells in contrast to ATRA which only induces differentiation of the cancerous cells. Fenretinide has dose related cytotoxicity against NB cell lines in vitro and it acts in part by increasing the intracellular levels of ceramide (Lovat et al., 2003). A phase 1 trial with FR demonstrated that although its toxicities are quite modest, feasibility was a significant issue due to a capsular formulation that is difficult to administer to small children. This observation then led to NANT trials of intravenous FR, as well as a new orally administered lipid matrix formulation called Lym-X-Sorb, which are designed to achieve a more favorable pharmacokinetic profile. The lipid formulation available in the market however has its own adverse effects as mentioned earlier which need to be addressed. Since a
radioactive form of FR is not commercially available so we used its optical properties to measure the percentage incorporation.

Encapsulation of valrubicin was also measured in the same way by comparing the absorbance at its specific wavelength. The chemical composition was then determined using the same standard assays as described for ATRA. Achieving successful encapsulation of all three selected drugs was an encouraging step for us because we could now try their cytotoxic effect on different models. Also a combination of these drugs can be used to achieve an even higher cell killing effect more than individual drugs. Such a combination therapy will be more useful for patients who are on multiple drug regimes during their chemotherapy, especially if the chemotherapeutic agents have similar lipophilicity.

Problems in physical characterization of nanoparticles

Physical characterization of liposomal nano-particles is quite challenging due to its not so rigid shape and structure. We therefore used both light diffraction and electron microscopy to estimate the size of our rHDL nano-particles. The dynamic light scattering is based on the principal that a beam of photons is scattered by the particles present in the media and this scattering is directly proportional to the sixth power of the diameter of particles. The error in this method however is huge as there are always some particles and agglomerates in the sample which are much bigger than the average size of nano-particles and hence they affect the overall result greatly due to the aforementioned exponential relation. Electron microscopy of the samples was done to directly visualize and estimate the size of nano-particles. The standard preparation for EM imaging requires the sample to be dried on tungsten wires and the process
has the potential to shrink the nanoparticles due to osmolarity variations. However, using both EM imaging and dynamic light scattering can provide an estimate of the range of the particle sizes.

**Effect of rHDL on cancer cells**

MTT assays were done on SMS-KCNR cell line of NB which was obtained from the Child Oncology Group. Cell viability assays done on NB gave favorable results suggesting a dose dependent cell killing effect on cancer cells. A liposomal preparation of FR will not only help in overcoming the problems associated with intravenous delivery of this drug but will also provide some degree of tissue selectivity due to the proposed receptor mediated uptake.

**In-vivo imaging studies**

An important aim of our study was to see how rHDL nanoparticles behave inside live animal and to try to see if they demonstrate any tissue selective localization. 5 nude mice with xenografts of NB were injected with rHDL containing HPPH dye and sequential images were taken at specific time points. Unfortunately a couple of injections did not enter the tail vein and instead went subcutaneously which caused some difficulties during the study and in later interpretation of results. We waited for a couple of weeks in those mice who had received subcutaneous injections for the drug to wash off completely (as confirmed by the imager) before trying another injection. Since only two injections were truly successful and one of those two mice died before completing all the preset time points so a comparative analysis is impossible from the data obtained. In one of the mice that were injected with HPHH np made using
peptide, we saw considerable tissue localization of the dye in the tumor. And there was also indication that liposomal HPPH stays in the circulation for a much longer time as compared to free HPHH dye.

**Future Directions**

Some pilot MTT assays were also done on glioblastoma which is another major cranial tumor of the pediatric age group. A dose dependent cytotoxicity was seen on these cells as well, which is an encouraging indication and should be further explored since glioblastoma is a major killer in pediatric age group and its treatment just like NB is full of complications. Our future aims include direct estimation of SR-B1 receptor expression on various NB cell lines, checking the effect of SR-B1 selective inhibitors on drug uptake, encapsulation of a combination of drugs in the single rHDL preparation and conducting more extensive in-vivo studies with animals having NB xenografts. We anticipate that the results of our studies will facilitate the application of liposomal nano-particles and these novel drugs in the treatment of NB.
## Appendix 1: International Neuroblastoma Risk Group (INRG) Consensus Pretreatment Classification

<table>
<thead>
<tr>
<th>INRG Stage</th>
<th>Age (months)</th>
<th>Histological Category</th>
<th>Grade of Tumor Differentiation</th>
<th>MYC N</th>
<th>11q Aberration</th>
<th>Ploidy</th>
<th>Pretreatment Risk Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1/L2</td>
<td></td>
<td>GN maturing; GNB intermixed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A Very low</td>
</tr>
<tr>
<td>L1</td>
<td></td>
<td>Any, except GN maturing or GNB intermixed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B Very low</td>
</tr>
<tr>
<td>&lt;18</td>
<td></td>
<td>Any, except GN maturing or GNB intermixed</td>
<td>Differentiating</td>
<td>NA</td>
<td>no</td>
<td></td>
<td>D low</td>
</tr>
<tr>
<td>&gt;18</td>
<td></td>
<td>GNB nodular; neuroblastoma</td>
<td>Poorly differentiated or undifferentiated</td>
<td>NA</td>
<td>No</td>
<td></td>
<td>H Intermediate</td>
</tr>
<tr>
<td>M</td>
<td>&lt; 18</td>
<td></td>
<td></td>
<td>NA</td>
<td>Hyperploid</td>
<td></td>
<td>F Low</td>
</tr>
<tr>
<td></td>
<td>&lt; 12</td>
<td></td>
<td></td>
<td>NA</td>
<td>Diploid</td>
<td></td>
<td>I Intermediate</td>
</tr>
<tr>
<td></td>
<td>12 to &lt; 18</td>
<td></td>
<td></td>
<td>NA</td>
<td>Diploid</td>
<td></td>
<td>J Intermediate</td>
</tr>
<tr>
<td></td>
<td>&lt; 18</td>
<td></td>
<td></td>
<td>Amp</td>
<td></td>
<td></td>
<td>O High</td>
</tr>
<tr>
<td></td>
<td>≥ 18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P High</td>
</tr>
<tr>
<td>MS</td>
<td>&lt;18</td>
<td></td>
<td></td>
<td>NA</td>
<td>No</td>
<td></td>
<td>C Very low</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td></td>
<td>Q High</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Amp</td>
<td></td>
<td></td>
<td>R High</td>
</tr>
</tbody>
</table>

Pretreatment risk group H has two entries. 12 months = 365 days; 18 months = 547 days; blank field = “any”; diploid (DNA index ≤ 1.0); hyperdiploid (DNA index > 1.0 and includes near-triploid and near-tetraploid tumors); very low risk (5-year EFS > 85%); low risk (5-year EFS > 75% to ≤ 85%); intermediate risk (5-year EFS ≥ 50% to ≤ 75%); high risk (5-year EFS < 50%). GN, ganglioneuroma; GNB, ganglioneuroblastoma; Amp, amplified; NA, not amplified; L1, localized tumor confined to one body compartment and with absence of image-defined risk factors (IDRFs); L2, locoregional tumor with presence of one or more IDRFs; M, distant metastatic disease (except Stage MS); MS, metastatic disease confined to skin, liver and/or bone marrow in children <18 months of age; EFS, event-free survival.
Appendix 2: International neuroblastoma staging system

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stage 1</strong></td>
<td>Localized tumor with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumor microscopically (nodes attached and removed with the primary tumor may be positive)</td>
</tr>
<tr>
<td><strong>Stage 2A</strong></td>
<td>Localized tumor with incomplete gross excision; representative ipsilateral nonadherent lymph nodes negative for tumor microscopically</td>
</tr>
<tr>
<td><strong>Stage 2B</strong></td>
<td>Localized tumor with or without complete gross excision, with ipsilateral nonadherent lymph nodes positive for tumor. Enlarged contralateral lymph nodes must be negative microscopically</td>
</tr>
<tr>
<td><strong>Stage 3</strong></td>
<td>Unresectable unilateral tumor infiltrating across the midline(^a), with or without regional lymph node involvement; or localized unilateral tumor with contralateral regional lymph node involvement; or midline tumor with bilateral extension by infiltration (unresectable) or by lymph node involvement</td>
</tr>
<tr>
<td><strong>Stage 4</strong></td>
<td>Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver, skin, and/or other organs (except as defined for Stage 4S)</td>
</tr>
<tr>
<td><strong>Stage 4S</strong></td>
<td>Localized primary tumor (as defined for Stage 1, 2A, or 2B), with dissemination limited to skin, liver and/or bone marrow(^b) (limited to infants &lt;1 year of age)</td>
</tr>
</tbody>
</table>

Multifocal primary tumors (e.g., bilateral adrenal primary tumors) should be Staged according to the greatest extent of disease, as defined previously, followed by subscript “M”.

\(^a\) The midline is defined as the vertebral column. Tumors originating on one side and “crossing the midline” must infiltrate to or beyond the opposite side of the vertebral column.

\(^b\) Marrow involvement in Stage 4S should be minimal, that is, less than 10% of total nucleated cells identified as malignant on bone marrow biopsy or on marrow aspirate.

More extensive marrow involvement would be considered to be Stage 4. The MIBG scan (if done) should be negative in the marrow.
Figure 1: Structure of fenretinide

Figure 2: Structure of valrubcin
Figure 3: Experimental design
Figure 4: Selective uptake of cholesterol esters from HDL particles (Steinberg, 1996)
Figure 5: Detailed structure of recombinant HDL particle (outer shell is made up of phospholipids, unesterified cholesterol and proteins while core is made up of anti-cancer drug)

Figure 6: Ingredients and procedure for making nanoparticles

1) Free Cholesterol, Cholesterol Esters, Phosphatidyl choline, Retinoic Acid (radioactive and non radioactive)
2) Vaporize using nitrogen, add Na Cholate and Cholate dialysis Buffer
3) Dialyzed for 48 hours in Phosphate Buffer Solution.
4) Collect the solution and analyze.
Figure 7: Functioning of the light scattering machine
(Courtesy: http://www.microtrac.com/ProductsTechnology/NanotracerParticleSizeAnalyzer/NanotracerTechnology.aspx)

Figure 8: Hypothetical dynamic light scattering of two samples: Larger particles on the top and smaller particle on the bottom (Courtesy: Wikipedia)

Figure 10: Competitive inhibition of drug uptake
Figure 11: simplified structure of Apo-A1 protein showing interconnected helices and 5-A peptide showing two helices connected by a peptide bond.
Figure 12: Hypothetical discoidal HDL model with Apo A-1 shown as wrapping the whole structure together (Shih, Sligar, & Schulten, 2009)
Figure 13: Efficiency of encapsulation of ATRA in rHDL particles
Figure 14: Dynamic light scattering from 500 µg ATRA rHDL particles

Figure 15: Dynamic light scattering from 1000 µg ATRA rHDL particles
Figure 16: Dynamic light scattering from 1500 µg ATRA rHDL particles

Figure 17: Dynamic light scattering from 2500 µg ATRA rHDL particles
Figure 18: Dynamic light scattering from 5000 µg ATRA rHDL particles

Figure 19: Dynamic light scattering from 6000 µg ATRA rHDL particles
Figure 20: Suppression of radioactive ATRA uptake after adding native HDL
Figure 21: Comparison between encapsulation efficiency of peptide and apoA
(using 1000 µg ATRA)
<table>
<thead>
<tr>
<th></th>
<th>initial ATRA content</th>
<th>final ATRA content</th>
</tr>
</thead>
<tbody>
<tr>
<td>using peptide</td>
<td>1500 µg</td>
<td>1043.2 µg</td>
</tr>
<tr>
<td>using ApoA</td>
<td>1500 µg</td>
<td>1071.9 µg</td>
</tr>
</tbody>
</table>

**Figure 22: Comparison between encapsulation efficiency of peptide and apoA (using 1500 µg ATRA)**
Figure 23 fractions of the ultracentrifuge
Figure 24: Comparison between radioactivity counts of 2 samples of rHDL particles -
1) Immediately after mixing with human serum
2) After 24 hour of incubation with human serum

Figure 25: Protein count of the same two ultracentrifuge samples
Figure 26: Dynamic light scattering from valrubicin rHDL containing Apo-A1 and 2000 µg drug

Figure 27: Dynamic light scattering from valrubicin rHDL containing 5A peptide and 2000 µg drug
Figure 28: Electron microscopy image of ATRA rHDL sample showing spherical nanoparticles

Figure 29: Contrast enhanced electron microscopy image of ATRA rHDL sample showing spherical nanoparticles
Figure 30: TIRF image showing nanoparticles containing fluorescent dye (TIRF also gives real time clips of the sample where we can appreciate the Brownian movement of the rHDL particles)
Figure 31: composition of ATRA nanoparticles made with 1X peptide

Figure 32: composition of ATRA nanoparticles made with 2X peptide
Figure 33: composition of ATRA nanoparticle containing 2500 µgm drug
Figure 34: Dynamic light scattering from sample of ATRA rHDL (containing 1500 µg drug) immediately after preparation

Figure 35: Dynamic light scattering from the same sample after one month of storage
Figure 36: Dynamic light scattering from a sample of ATRA rHDL (containing 5000 µg drug) immediately after preparation

Figure 37: Dynamic light scattering from the same sample after one month of storage
Figure 38: Percentage cell viability of NB cells after 24 hours of exposure

Figure 39: Percentage cell viability of Glioblastoma cells after 24 hours of exposure
Figure 40: Dorsal image of a mouse- immediately after injecting the florescent dye in tail vein
Figure 41: Dorsal image of a mouse- 48 hours after injecting the florescent dye in tail vein
Figure 42: Dorsal image of a mouse- 68 hours after injectng the florescent dye in tail vein
Figure 43: Dorsal image of a mouse - 94 hours after injecting the fluorescent dye in tail vein
Figure 44: Dorsal image of a mouse- 114 hours after injecting the florescent dye in tail vein
Figure 45: Regions where the average radiant efficiency was measured and compared
Figure 46: Comparison between average radiant efficiency from tumor vs. control in the mouse that was injected with HPPH containing rHDL np made using 5A-peptide.
CHAPTER VI

References


