REVERSAL OF THE MULTIDRUG RESISTANCE  
BY DRUG COMBINATION  
USING MULTIFUNCTIONAL LIPOSOMES 

Thesis Presented  
By  
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To  
The Bouve’ Graduate School of Health Sciences  
in Partial Fulfillment of the Requirements for the Degree of  
Doctor of Philosophy in Pharmaceutical Sciences  
with specialization in  
Pharmaceutics and drug delivery systems  

NORTHEASTERN UNIVERSITY  
BOSTON, MASSACHUSETTS  

April, 2013
Abstract:

One of the major obstacles to the success of cancer chemotherapy is the multi-drug resistance (MDR) that results due mainly to the over-expression of drug efflux transporter pumps such as P-glycoprotein (P-gp). Highly efficacious third generation P-gp inhibitors, like tariquidar, have shown promising results against MDR. However, P-gp is also expressed in normal tissues like the blood-brain barrier, gastrointestinal tract, liver and kidney. It is therefore important to limit the exposure of P-gp inhibitors to normal tissues and increase their co-localization with anticancer agents in tumor tissues to maximize the efficacy of a P-gp inhibitor. To minimize non-specific binding and increase its delivery to tumor tissues, liposomes, self-assembling phospholipid vesicles, were chosen as a drug delivery vehicle. The liposome has been identified as a system capable of carrying molecules with diverse physicochemical properties. It can also alter the pharmacokinetic profile of loaded molecules which is a concern with both tariquidar and paclitaxel. Liposomes can easily be surface-modified rendering them cell-specific as well as organelle-specific. The main objective of present study was to develop an efficient liposomal delivery system which would deliver therapeutic molecules of interest to tumor tissues and avoid interaction with normal tissues. In this study, the co-delivery of tariquidar and paclitaxel into tumor cells to reverse the MDR using long-circulating cationic liposomes was investigated. SKOV-3TR, the resistant variant of SKOV-3 and MCF-7/ADR, the resistant variant of MCF-7 were used as model cell lines. Uniform liposomal formulations were generated with high incorporation efficiency and no apparent decrease in tariquidar potency towards P-gp. Tariquidar- and paclitaxel- co-loaded long-circulating liposomes showed significant re-sensitization of SKOV-3TR and
MCF-7/ADR for paclitaxel *in vitro*. Further modification of these liposomes with antitumor 2C5 resulted in increased cell association with these cancer cells. The 2C5-modified immunoliposomes, along with unmodified liposomes co-loaded with tariquidar and paclitaxel were tested for their antitumor effects *in vivo*. Significant tumor growth inhibition occurred with combination therapy in resistant as well as sensitive cell lines. However, immunoliposomes failed to increase antitumor effect *in vivo* as spontaneous accumulation of liposomes at added dose may have saturated tumor accumulation.

We were also interested in evaluating physiological factors responsible for the MDR. Spheroids grown *in vitro* provided platform to demonstrate many characteristics of tumor tissues such as cell-cell interaction, a hypoxic core, low pH environment at core and a relevant genetic profile. In this study, spheroids were utilized to evaluate paclitaxel cytotoxicity and to evaluate effects of 2C5 modification on cellular uptake. Lack of cytotoxicity was observed in spheroids treated with paclitaxel alone as well as in combination with tariquidar. Likely explanations could be the presence of cells in diverse cell cycle stages and limited penetration. Also, increased uptake was observed in spheroids when treated with 2C5-modified Rh-labeled liposomes compared to UPC10-modified Rh-labeled liposomes. Such results have clearly demonstrated the importance of using this novel research model in cancer research.
Northeastern University

Bouve’ College of Health Sciences

Dissertation Approval

Dissertation title:
Reversal of the multidrug resistance reversal by drug combination using multifunctional liposomes

Author: Niravkumar R. Patel

Program: Pharmaceutical Sciences

Approval for dissertation requirements for the Doctor of Philosophy in:
Pharmaceutical Sciences with specialization in Pharmaceutics and drug delivery systems

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ACKNOWLEDGEMENTS:

This thesis is a culmination of my aspiration to aid in the cancer research field as well as contributions from my colleagues at Professor Torchilin’s Laboratory. These colleagues deserve a special mention since without them this thesis wouldn’t have been possible. It is a pleasure to convey my gratitude to them all in my humble acknowledgment.

First and foremost, I would like to offer my sincere gratitude to Dr. Vladimir P. Torchilin for his supervision, advice, and guidance from the very early stage of this research as well as providing me with extraordinary mentorship throughout the work. I gratefully acknowledge Dr. D. Mongayt, Dr. W. Hartner and Dr. T. Levchenko for their support, scientific discussions, critical remarks, and other crucial contributions. I am indebted to them more than they know. Many thanks go to Post-doctorate candidates as well as Ph.D. candidates for their support. I express my deepest appreciation for Dr. Carol Paronis, Dr. Michail Sitkovsky, Dr. Robert Schatz and Dr. Slava Epstein for their constructive comments on this thesis. I am thankful that in the midst of all their activity, they accepted to be members of this thesis committee.

Finally, I would also like to thank my wife Dimple Shah, my family, and friends. Throughout all my endeavors, your love, support, guidance and endless patience have been truly inspirational – a simple “thanks” will never suffice.
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1. INTRODUCTION

The efficacy of chemotherapy is often impaired by the development of the multidrug resistance (MDR), which then requires the administration of higher doses of cytotoxic drugs for effective treatments. Multidrug resistance can result due to inherent physiological factors or acquired drug resistant factors. Various tumor tissue properties such as limited tissue penetration, higher interstitial fluid pressure and low pH hypoxic regions at the core contribute to physiological factors responsible for the demonstration of MDR [1-5]. Altered molecular targets, defective apoptotic machinery, over-expression of anti-apoptotic proteins, decreased drug influx as well as increased drug efflux are cellular factors responsible for the acquired drug resistance [6-8]. However, one of the major mechanisms responsible for the acquired drug resistance is the over-expression of the P-gp, a plasma membrane glycoprotein with a molecular weight of 170,000 Daltons. This MDR1 gene product belongs to the ABC (ATP binding cassettes) family and is responsible for the energy-dependent efflux of a variety of drugs including anthracyclins, vinca alkaloids, and taxanes [9-11]. This efflux results in the reduced accumulation of such anticancer drugs inside cancer cells. Therefore, higher doses are required to produce toxicity equivalent before the over-expression of the P-gp [12, 13]. Thus, it appears important to inhibit the P-gp to reverse the MDR and to maintain the accumulation of anticancer drugs in cancer cells. There are a number of molecules identified and developed that reverse the MDR phenomena demonstrated by cancer cells after repetitive drug treatments. However, most of these promising molecules possess inherently undesirable physicochemical properties such as poor solubility, non-specific binding and rapid clearance [11, 14]. To overcome these problems, an efficient delivery system is
required that deliver these molecules to their site of action and decrease their exposure to normal tissues as well as increase their co-localization with anticancer drugs. In this study, liposomes, nano-sized phospholipid vesicles, are used to incorporate paclitaxel, an anticancer agent, and tariquidar, a third generation P-gp inhibitor [15]. Liposomes were formulated with cationic phospholipids to bear a positive charge for improved interaction with cells [16] and liposomes were rendered long-circulating by PEGylation (surface modification with polyethylene glycol) [17]. Liposomes have a distinct advantage of accumulating at the tumor site through enhanced permeability and retention (EPR effect) for passive targeting. Nucleosome specific monoclonal antibody 2C5-modified liposomes were also prepared to evaluate active targeting of liposomes for improved anticancer activity. Along with the molecular mechanisms responsible for the acquired MDR, inherent physiological factors contributing towards the MDR were also studied. To study the effects of various properties possessed by tumor tissue, it was necessary to recreate three dimensional (3D) spheroidal structures in vitro. Methods for efficient production were optimized to evaluate paclitaxel toxicity profile in presence or absence of tariquidar, P-gp blocker, and compared with 2D monolayer culture. Also, the effects of collagenase treatment of paclitaxel on spheroids were also studied.
HYPOTHESES:

1. Long-circulating cationic liposomes co-loaded with tariquidar and paclitaxel will deliver both molecules to cancer tissues with reduced non-specific binding and will achieve significant reversal of the MDR both in vitro and in vivo. Further modification of such liposomes with 2C5 (monoclonal antibody against cancer specific nucleosomes) will result in additional improved reversal of the MDR with reduced non-specific binding.

2. Micro tumor tissues (Spheroids) grown in vitro will provide a platform to evaluate various physiological factors responsible for the multidrug resistance.

To test those hypotheses, the following specific aims were pursued:

1. Prepare and characterize long-circulating cationic liposomes co-loaded with tariquidar and paclitaxel.


3. Evaluate the efficacy of tariquidar towards P-gp by Rh123 efflux study in vitro.

4. Evaluate the reversal of the MDR by determining the IC$_{50}$ values of paclitaxel at 48 hrs in vitro.

5. Prepare and characterize 2C5-modified long-circulating liposomes co-loaded with tariquidar and paclitaxel.

6. Evaluate change in cell association properties of liposomes upon 2C5 modification.

7. Evaluate the reversal of the MDR by determining tumor growth inhibition upon treatment with unmodified and 2C5-modified tariquidar plus paclitaxel-co-loaded liposomes.
8. Optimize a protocol for the formation of consistent spheroids with respect to size, shape and number of cells.

9. Evaluate and compare cytotoxicity of paclitaxel on MCF-7/ADR grown as monolayer and spheroid cultures.

10. Evaluate effect of 2C5 modification on cellular uptake/association in spheroid culture.

11. Evaluate effects of collagenase treatment on the structure of spheroids.

12. Evaluate effects of collagenase treatment on the paclitaxel toxicity profile with or without tariquidar.

The novelty of these formulations involves the possibility of encapsulation and delivery of tariquidar and paclitaxel specifically to cancer tissues by exploiting both passive targeting (EPR effect) as well as active targeting strategies. Likely clinical advantages of such a formulation may include the reversal of the MDR of multiple cancer cell lines to paclitaxel and the reduced non-specific binding of tariquidar which would result in reduction of the systemic toxicity typically observed with free tariquidar administration. Study of tissue structure properties using spheroid culture will enhance our understanding related to physiological conditions in tumors responsible for the development of MDR.
2. BACKGROUND AND SIGNIFICANCE

2.1 Role of Multidrug Resistance in cancer treatment

There are multiple reasons for anticancer drugs to fail in the treatment of cancer. A few of the reasons leading to unsuccessful cancer treatment using chemotherapy include the diversity in pharmacokinetic profile of a chemotherapeutic agent from patient to patient, diversity in the genetic profile amongst patients as well as amongst cancer tissues, location of cancer tissue in body and development of multidrug resistance inherent and/or acquired after even a few doses of chemotherapy [18]. Numerous studies have been carried out to understand the nature and development of multidrug resistance by cancer cells. Such studies indicate that the inherent resistance results from tumor tissue properties such as limited tissue penetration due to cell-cell interaction, higher interstitial fluid pressure and low pH hypoxic region [1-5]. Other mechanisms of inherent drug resistance are altered drug metabolism, genetic changes affecting the capacity of cytotoxic drugs to kill cells and increased repair of DNA damage [19-22]. Acquired multidrug resistance results mainly through the process of cell selection. Detectable tumors of 1 cm$^3$ in patients contain millions of cancer cells out of which a few are likely to be chemoresistant. With initial treatments, most sensitive cancer cells are killed. However, those which are resistant or those with the potential to develop resistance through genetic modification can remain. These remaining resistant cells are likely to produce tumors with a predominant population of resistant cells.

The most commonly encountered mechanism for acquired drug resistance is the over-expression of energy-dependent transporters belonging to the ABC (ATP-binding
cassette) transporters family which results in the efflux of a broad class of hydrophobic cytotoxic drugs [9, 10]. The most frequently encountered over-expressed protein in resistant cancer cells is P-glycoprotein (P-gp) also known as ABCB1 or MDR1 [23, 24]. It possesses broad substrate specificity and is responsible for the efflux of a variety of drugs including anthracyclins, vinca alkaloids and taxanes [9, 10]. This efflux results in the reduced accumulation of an anticancer drug inside cancer cells resulting in the requirement of higher doses to produce toxicity equivalent to the toxicity before the over-expression of the P-gp. It therefore appears important to inhibit P-gp to reverse the MDR in cancer cells for effective treatments. Numerous studies have been undertaken in attempts to re-sensitize cancer cells by silencing P-gp using siRNA (small interfering RNA), by developing cytotoxic compounds that elude P-gp efflux and by inhibiting P-gp function using P-gp inhibitors. Even after discovering potent P-gp inhibitors, non-specific binding and tumor specific targeting have remained challenges. In the present study, co-delivery of tariquidar (XR9576) and paclitaxel either in long-circulating cationic unmodified or 2C5-modified liposomes for the reversal of the MDR were evaluated \textit{in vitro and in vivo}. 
2.2 P-glycoprotein and its inhibitors

Figure 1. P-glycoprotein structure [25]. It contains six-transmembrane domains (TMD) and two intracellularly located nucleotide binding domains (NBD).

P-glycoprotein, a 1280 amino acid long membrane protein with a molecular weight of 170kDa, is an energy-dependent drug efflux pump belonging to the ABC (ATP binding cassette) transporter family. The structure of P-gp contains six transmembrane domains and two ATP binding regions as shown in Figure 1 [26]. Its major function provides a biological barrier to the entry of xenobiotics from the systemic circulation. Its expression in normal tissues such as epithelial cells of the lower gastrointestinal tract (GIT), endothelial cells of brain and testis, the canalicular surface of hepatocytes and renal cells has a significant impact on a drug’s absorption, distribution and excretion. However, a few types of cancer have exploited the effectiveness of the P-gp pumps in preventing drug entry into tumors for their own survival [27]. The multidrug resistance due to the over-expression of P-gp in tumors has been a major obstacle to successful treatment of cancer using chemotherapy. Numerous studies have been carried out to find
P-gp inhibitors to overcome the MDR effect. Depending on their specificity and affinity, P-gp modulators are classified in three generations. The first generation P-gp modulators, which include calcium channel blockers, antibiotics, and other miscellaneous compounds, lacked specificity and were weak inhibitors. These first generation modulators produced P-gp inhibition at relatively high doses which resulted in unwanted pharmacological side-effects [14, 28]. P-gp modulators with higher specificity and increased affinity were later discovered. This, the second generation of modulators included PSC 833[29], GG918 [30] and S9788 [31]. Related studies with second generation modulators indicated the significance of the approach but also revealed the requirement for a more potent and specific P-gp modulator to achieve the successful reversal of the MDR. XR9051, a diketopiperazine derivative, has demonstrated MDR reversal at submicromolar concentration [32]. Experiments involving molecular modeling of XR9051 to improve its potency resulted in the discovery of XR9576 (tariquidar), a potent and specific P-gp inhibitor [15]. Studies evaluating the ability of XR9576 to sensitize a variety of resistant cell lines have shown great promise for reversal of the MDR caused by the P-gp over-expression [33]. There is an increasing concern, however, for the binding of tariquidar to the P-gp present in normal tissues. This off target effect can result in the alteration of pharmacokinetics of other drugs concomitantly taken leading to unwanted toxicity [34]. It would therefore be ideal to limit the exposure of tariquidar to normal tissues and increase its co-localization with cytotoxic drug to tumor tissues to decrease systemic toxicity and increase antitumor activity. To achieve this goal, we proposed the use of liposomes for loading both molecules simultaneously and their delivery to tumor tissues to limit exposure to normal tissues and increase their co-localization for antitumor effect.
2.3. Liposomes as a drug delivery system

Figure 2. Evolution in the structural development of liposomes [35]. (A) Early traditional liposomes have the ability to incorporate hydrophilic molecules (a) inside core and hydrophobic molecules (b) inside lipid bilayer. (B) PEG modified liposomes for greater in vivo circulation time. (C) Antibody modified PEG-coated liposomes for increased active targeting and greater in vivo circulation time.

Liposomes, bilayered lipid vesicles, have been used as a drug delivery system for more than 30 years. Liposomes can be formulated as small unilamellar vesicles with a size distribution around 200 nm, as large unilamellar vesicles with size distribution ranging from 200 nm to 800 nm and as multilamellar vesicles with a size distribution ranging from 500 nm to 5000 nm. All are prepared using biocompatible lipids and possess a low toxicity profile. The structure of liposomes, as shown in Figure 2, also allows them to incorporate hydrophilic drugs in their hydrophilic core as well as hydrophobic drugs in the hydrophobic phospholipid bilayer. Along with numerous advantages like protection of a loaded molecule from degradation, stability upon in vivo
administration as well as a surface easily modified to make them target site-specific, liposomes possess disadvantages including rapid clearance from the systemic circulation and ineffectiveness for oral delivery [36]. An important advance in liposome research was the discovery of the effectiveness of Polyethylene glycol (PEG) chain to protect liposomes from macrophage uptake and subsequent increase of circulation time in vivo [17].

Figure 3. Enhanced permeability and retention effect (EPR effect). Leaky vasculature and poor drainage from the extracellular environment allows increased interstitial accumulation of nanoparticles and thereby increased tumor accumulation of loaded molecules.

In recent years, liposomes have been evaluated for the delivery of cytotoxic drugs with diverse physicochemical properties to tumor tissues. Incorporation of such drugs in liposomes minimizes drug exposure to normal cells. They accumulate in tumor
environment via the associated leaky vasculature and poor interstitial drainage (Figure 3) [36]. This phenomenon is called the enhanced permeability and retention effect (EPR effect) [37-39]. Due to the distinct advantages associated with a longer circulation time, the capability for minimizing exposure to normal tissues of loaded molecules and the increased accumulation in tumor environment upon in vivo administration, we proposed the use of long-circulating cationic liposomes for incorporation of paclitaxel and tariquidar for the reversal of the MDR of cancer cell lines.

### 2.4. Spheroid culture

Monolayer culture and organ culture have been the most popular models of choice for cancer research. In monolayer culture, a single cell suspension can easily be obtained from tumor tissue and grown conveniently in two dimensions (2-D) for in vitro testing with good viability of cells. However, they lack most of the whole tissue characteristics such as cell-cell interaction, a hypoxic core and elevated interstitial fluid pressure, demonstrated by cancer cells in a three-dimensional (3-D) microenvironment [40]. These disadvantages could be overcome using organ culture by the use of pieces of cancer tissue specimens in 3-D. One of the major disadvantages of organ culture is that it is very difficult to obtain specimens in sufficient quantity. Therefore, monolayer cultures are preferred over organ culture. Numerous studies have been carried out using a monolayer culture to evaluate the effects of potential chemotherapeutic molecules on various cancer cells. However, it fails to provide a physiological three dimensional structure which can be used to study vital mechanisms such as cellular response in 3-D as well as tissue penetration of potential molecules or cargoes carrying molecules. This
ultimately has resulted in the lack of correlation between the observed effects in monolayer culture and *in vivo* data [41, 42]. A multi-cellular 3-D spheroid culture has been gaining increased interest as a model of choice to carry out studies previously unfeasible in monolayer culture.

Various attempts at optimization of protocols have been made for the formation of 3-D spheroids (Table 1). However, the tendency for the formation of spheroids varies for different cell lines depending of the method used. It remains necessary to optimize protocols for the formation of spheroids with the model cell lines.

A 3-D spheroid culture provides a unique opportunity to bridge the gap between monolayer culture assays and *in vivo* assays [43]. Spheroids are avascular tumor masses that limit penetration throughout their volume. This limited mass transport in spheroids results in accumulation of metabolic wastes creating conditions somewhat similar to *in vivo* tumors [44-48]. Numerous studies have shown that the gene expression in spheroids is more relevant to an actual tumor tissue than a monolayer culture [48, 49]. Various advantages like cell-cell interaction, relevant gene expression, a hypoxic core and the presence of low pH at core makes a 3-D spheroid culture a useful model to evaluate physiological factors responsible for the MDR.
Table 1. List of various methods currently used for spheroid formation.

<table>
<thead>
<tr>
<th>METHOD</th>
<th>ADVANTAGES</th>
<th>DISADVANTAGES</th>
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<tbody>
<tr>
<td>Spinner flasks [50]</td>
<td>Simple to perform, Massive production, Long-term culture, Dynamic control of culture conditions, Co-culture of different cell types</td>
<td>Require specialized equipment, Variation in size/cell number, High shear force</td>
</tr>
<tr>
<td>Hanging drop [51, 52]</td>
<td>Inexpensive, Well controlled spheroid size, Fast spheroid formation, Co-culture of different cell types, Easy to trace spheroid assembly</td>
<td>Labor intensive, Massive production difficult</td>
</tr>
<tr>
<td>3D Scaffolds [53, 54]</td>
<td>Provide 3D extracellular support</td>
<td>Require specialized equipment for scaffold fabrication</td>
</tr>
<tr>
<td>Pellet culture [55]</td>
<td>Simple to perform, Rapid aggregation of a large number of cells</td>
<td>Shear force, Massive production difficult</td>
</tr>
<tr>
<td>External force enhancement [56]</td>
<td>Rapid cell aggregation</td>
<td>Require specialized equipment and culture conditions</td>
</tr>
<tr>
<td>Non-adhesive liquid overlay [57, 58]</td>
<td>Inexpensive, Simple to perform, Easy to scale up, Bulk production possible</td>
<td>Only forms with certain cell types</td>
</tr>
</tbody>
</table>
3. EXPERIMENTS

We prepared long-circulating cationic liposomes using L-α-phosphatidylcholine (egg, chicken) (EPC), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), cholesterol (CHOL), and polyethylene glycol 2000-phosphatidylethanolamine (PEG2kPE). To increase co-localization of the commonly used anticancer drug, paclitaxel, with the P-gp blocker, tariquidar, and to reverse the MDR, both molecules were incorporated in liposomes simultaneously. The liposomal formulation incorporates both tariquidar and paclitaxel in lipid bi-layer as both molecules are highly lipophilic. To increase the liposome’s association with cells, liposomes were prepared with a cationic zeta potential by addition of the cationic lipid DOTAP. Liposomes were also modified with 2 mol% PEG to increase their *in-vivo* circulation time and decrease macrophage uptake due to the shielding effect of the PEG. Along with these imposed properties, liposomes possessed the ability to accumulate in the cancer tissue environment by the EPR effect as well as a surface which was modified with nucleosome specific 2C5 antibody. These co-loaded long-circulating cationic liposomes were evaluated for their ability to reverse the MDR in the model cell lines towards paclitaxel *in vitro* and *in vivo*. Also, a protocol for the consistent formation of 3-D spheroids *in vitro* using a three dimensional spheroid culture technique was established. The spheroids grown were evaluated for their consistency with respect to size, shape and number of cells. These spheroids were used to determine 2C5-modified liposome uptake, effect of collagenase and the paclitaxel toxicity profile in presence or absence of tariquidar.

The present study was divided into two major parts. In the first part, it was hypothesized that these long-circulating cationic liposomes containing tariquidar and
paclitaxel will result in the reversal of the MDR in model cancer cell lines \textit{in vitro} and \textit{in vivo}. To test this, long circulating liposomes co-loaded with tariquidar and paclitaxel were prepared and characterized. An IC$_{50}$ value of paclitaxel was evaluated for the determination of the reversal of the MDR \textit{in vitro}. The initial results indicated significant reversal of the MDR demonstrated by SKOV-3TR and MCF-7/ADR and encouraged further development of this liposomal formulation. The unmodified PEGylated liposomal formulation relies only on passive targeting to accumulate in tumor environment \textit{in vivo}. Thus, these liposomal formulations were modified with anti-tumor monoclonal antibody 2C5. We hypothesized that the liposomal accumulation in cancer tissue environment will improve significantly with targeted liposomes and will result in reduced non-specific binding to normal tissues and in higher antitumor activity \textit{in vivo}. The results indicated significant improvement in liposomal uptake by cancer cells when modified with 2C5 in monolayer culture in serum-free condition as well as in spheroid culture in complete media. The tariquidar- and paclitaxel- co-loaded long-circulating liposomes produced significant reversal of the MDR \textit{in vivo}. However, modification of 2C5 failed to improve antitumor activity of co-loaded tariquidar and paclitaxel-co-loaded long-circulating liposomes at given doses. No formulations produced any apparent systemic toxicity as body weight remained similar for all treatment groups.

The second part of present study involved the development of 3-D spheroids \textit{in vitro} to evaluate physiological factors responsible for the MDR. As spheroids possess various tumor-like characteristics such as cell-cell interaction, a related gene profile, hypoxic core and elevated interstitial fluid pressure demonstrated by tumor tissues, we hypothesized that the spheroids could be used to evaluate various factors such as kinetic
profile of liposome penetration and uptake profile post 2C5 modification. To test the hypothesis, protocols for the bulk production of consistent spheroids with respect to size, shape and number of cells was optimized. Spheroids were also treated with Rh-labeled unmodified, 2C5-modified and UPC10-modified liposomes to test association/uptake and penetration profiles. Also, the paclitaxel toxicity profile was evaluated in 3D spheroid culture in presence or absence of tariquidar as well as collagenase. Results from such experiments suggested that the penetration in these micro tumor tissues was extremely limited, and drastic differences were observed in paclitaxel toxicity profiles between spheroid culture and monolayer culture. All results from spheroid culture indicated a divergence of results from those of monolayer cultures as well as the importance of using this model in current cancer research.
3.1. Materials and Methods

3.1.1. Materials:

SKOV-3 ovarian cancer cell line and MCF-7 breast cancer cell line were obtained from the ATCC (American type culture collection, Manassas, VA, USA). SKOV-3TR, the taxol resistant variant of SKOV-3, was a kind gift from Dr. Duan Zhenfeng (MGH, Boston, MA). SKOV-3 and SKOV-3TR were cultured in RPMI 1640 media (supplemented with 10% fetal bovine serum and 1% antibiotics as recommended by ATCC) at 37 °C in a 5% CO₂ incubator in all experiments. MCF-7/ADR, the adriamycin resistant variant of MCF-7, was obtained from Dr. Minko. MCF-7 and MCF-7/ADR were cultured in DMEM (supplemented with 10% fetal bovine serum and 1% antibiotics as recommended by ATCC) at 37 °C with 5% CO₂ in all experiments. L-α-phosphatidylcholine (egg, chicken) (EPC), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), cholesterol (CHOL), polyethylene glycol 2000-phosphatidylethanolamine (PEG₃₄PE), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-PE), rhodamine 123 (Rh123) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Tariquidar was a kind gift from Dr. Susan Bates (NCI, Bathesda, MD). FITC-labeled P-gp specific antibody (UIC2) was obtained from Abcam (Cambridge, MA, USA). Cell titer blue reagent was obtained from Promega (Madison, WI, USA). Protein electrophoresis grade Agarose (high gelling temperature) was obtained from Fisher Scientific (NJ, USA), p-nitro phenyl phosphate (pNPP) substrate was obtained from Thermo scientific (IL, USA), Sodium acetate, Triton X-100 and Collagenase from Clostridium Hystolyticum (C5138-100 mg) were obtained from Sigma.
Aldrich (MO, USA). Reconstituted basement membrane (matrigel) (RBM), high concentration and collagenase were obtained from BD Biosciences (MA, USA). Propidium iodide was obtained from Invitrogen (CA, USA) and RNase A was obtained from Quiagen (CA, USA). Accutase was obtained from Innovative Cell Technologies (CA, USA). LDH release assay kit (Cytotox 96 non-radioactive cytotoxicity assay) was obtained from Promega (WI, USA).

3.1.2. Preparation of long-circulating cationic liposomes:

Liposomes were prepared by a thin film hydration method followed by extrusion. Briefly, the lipid components, EPC, CHOL, DOTAP, along with PEG$_{2k}$PE were weighed and dissolved in chloroform at the molar ratio of 64:30:6:2 (EPC:CHOL:DOTAP:PEG$_{2k}$PE). Lipids dissolved in chloroform were dried with a rotary evaporator to form a thin film. The film was subjected to freeze-drying to remove trace levels of organic phase on a Freezone 4.5 Freeze Dry System (Labconco, Kansas city, MO, USA). The lipid film was then hydrated for 30 minutes with the phosphate buffer saline (PBS, 10 mM), pH 7.4 at the final lipid concentration of 2 mg/ml. The liposomal suspension was subjected to bath sonication for 20 min. Liposomes were allowed to rest for 15 min, then extruded through 200 nm pore size polycarbonate membranes using a hand held extruder (Avanti Polar Lipids, Alabaster, AL, USA) to obtain small unilamellar vesicles.
Table 2. Lipid composition for various liposomal formulations.

<table>
<thead>
<tr>
<th>Liposomal Formulations</th>
<th>Lipid Composition</th>
<th>Molar Ratio</th>
<th>Lipid concentration (mg/ml)</th>
<th>Tariquidar (µg/ml)</th>
<th>Paclitaxel (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty liposomes</td>
<td>EPC:CHOL:DOTAP:PEG₉kPE</td>
<td>64:30:6:2</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>XR liposomes</td>
<td>EPC:CHOL:DOTAP:PEG₉kPE</td>
<td>64:30:6:2</td>
<td>2</td>
<td>8.62</td>
<td>-</td>
</tr>
<tr>
<td>PCL liposomes</td>
<td>EPC:CHOL:DOTAP:PEG₉kPE</td>
<td>64:30:6:2</td>
<td>2</td>
<td>-</td>
<td>11.38</td>
</tr>
<tr>
<td>XRPCL liposomes</td>
<td>EPC:CHOL:DOTAP:PEG₉kPE</td>
<td>64:30:6:2</td>
<td>2</td>
<td>8.62</td>
<td>11.38</td>
</tr>
</tbody>
</table>


While preparing tariquidar- and paclitaxel-loaded liposomes, drugs were added to chloroform along with lipids before preparing the thin lipid film. Combined equimolar quantities of paclitaxel and tariquidar were kept at 1% w/w final lipids as shown in Table 2.
3.1.3. Characterization of long-circulating cationic liposomes:

The resulting liposomal formulations were characterized with respect to their size distribution, zeta potential and loading efficiency at given quantity for tariquidar and paclitaxel.

3.1.3.1. Size distribution and zeta potential analysis:

The size distribution and zeta potential of liposomes were measured using the Zetaplus (Brookhaven Instruments Corporation, Holtsville, NY, USA). Briefly, 50 µl of a liposome formulation was resuspended in 1.5 ml of distilled water, and size distribution and zeta potential were measured using these diluted samples according to the manufacturer’s protocol. To evaluate stability of liposomal formulations, size distribution was measured once every week up to four weeks. All liposomal formulations were stored at 4 °C purged with nitrogen gas.

3.1.3.2. Loading efficiency analysis:

Loading efficiency of long-circulating cationic liposomes for paclitaxel and tariquidar was evaluated with reversed phase HPLC technique using the Xbridge C₁₈ (4.6mmX250mm) column (Waters corporation, Milford, MA). An ammonium acetate buffer (10 mM, pH 4); acetonitrile (40:60) was used as a mobile phase. The running time for each injection was kept at 8 minutes and the injection volume was kept at 50 µl. A standard curve was produced for paclitaxel and tariquidar using 0.5 µg/ml, 1 µg/ml, 2 µg/ml, and 4 µg/ml concentrations while measuring absorbance at 227 nm. For loading efficiency determination, liposomal formulations were centrifuged at 10,000 rpm for 10
min to precipitate non-entrapped drugs. From the supernatant, 150 µl of liposomal formulation was taken, and lysed with 850 µl of acetonitrile: ammonium acetate (10 mM, pH 4) (60:40), and subjected to HPLC analysis.

3.1.4. P-glycoprotein expression:

SKOV-3, SKOV-3TR, MCF-7 and MCF-7/ADR cells were allowed to grow in T12.5 flasks until 80% confluency. Cells were detached from the flask using a mechanical cell scraper and centrifuged to form a cell pellet. The cell pellet was resuspended in PBS (10 mM, pH 7.4). Cells were counted and adjusted to get 300,000 cells/300 µl of PBS. Cells were then treated with 5 µl of FITC-labeled antibody against P-glycoprotein (UIC2). Cells were incubated for 1 hr on ice according to the manufacturer’s protocol. After the incubation, cells were centrifuged and washed twice with ice-cold PBS and analyzed using FACS for the green fluorescence intensity (FL1). Untreated cells were taken as controls for all cell lines.

3.1.5. Rhodamine123 (Rh123) uptake:

Rh123 is a known and established fluorescent substrate of P-gp. It is frequently used to monitor P-gp activity. To measure change in tariquidar potency towards P-gp after liposomal incorporation, Rh123 uptake was evaluated after treatment with either free tariquidar or tariquidar loaded liposomes.

SKOV-3, SKOV-3TR, MCF-7 and MCF-7/ADR cells were seeded in T75 flasks at a density of 500,000 cells/flask. Cells were then allowed to grow until 80% confluency, then trypsinized and centrifuged to form a cell pellet. The cell pellet was
resuspended in media at $10^6$ cells/ml. Cells were treated with tariquidar alone or with tariquidar-loaded liposomes at a 100 nM final concentration of tariquidar for 1 hr at 37°C. Cells were then incubated with Rh123 at 0.2 µg/ml for 1 hr. Cells treated only with Rh123 were taken as control. Cells were centrifuged and washed with ice cold PBS (10 mM, pH 7.4). Cells were fixed with 4% formalin and kept on ice until further FACS analysis of Rh123 intensity.

3.1.6. Multidrug resistance reversal:

SKOV-3 and SKOV-3TR cells were seeded in 96-well microtiter plates at a density of 3000 cells/well 24 hrs before treatment. MCF-7 and MCF-7/ADR cells were seeded in 96-well plates at a density of 5000 cells/well 24 hrs before treatment. These cells were treated with tariquidar, tariquidar-loaded liposomes, empty liposomes, paclitaxel-loaded liposomes, and tariquidar-paclitaxel-co-loaded liposomes at various concentrations of paclitaxel and allowed to grow for 48 hrs. After incubation, cells were washed with fresh media and cytotoxicity was measured using Cell Titer Blue assay according to the manufacturer’s protocol. Briefly, washed cells were incubated for 2 hrs with 100 µl of fresh media containing 20 µl of Cell Titer Blue reagent. After the incubation, plates were analyzed for the fluorescent signal at 530 nm excitation and 590 nm emission wavelengths. Cells treated with vehicle (PBS) were taken as controls to calculate % cell death and the IC$_{50}$ values were calculated from three separate experiments.
3.1.7. Preparation of long-circulating cationic immunoliposomes:

Preparation of immunoliposomes was divided in two segments. Initially long-circulating cationic liposomes were prepared using the thin film hydration method followed by 2C5-modification using pNP-PEG-PE linker with a procedure described below.

3.1.7.1. Preparation of long-circulating cationic liposomes:

Long-circulating cationic liposomes were prepared in a similar fashion as described in section 3.1.2. Briefly, the lipid components, EPC, CHOL, DOTAP, along with PEG2kPE were weighed and dissolved in chloroform at the molar ratio of 64:30:6:2 (EPC:CHOL:DOTAP:PEG2kPE). Lipids dissolved in chloroform were dried with a rotary evaporator to form a thin film and subjected to freeze-drying to remove any trace level of organic phase on a Freezone 4.5 Freeze Dry System (Labconco, Kansas City, MO, USA). Lipid film was hydrated for 30 minutes with PBS (10 mM, pH 7.4) at the final lipid concentration of 5 mg/ml. The liposomal suspension was subjected to bath sonication for 20 min. Liposomes were allowed to rest for 15 min to overcome any structural defects. Liposomes were then extruded through 200 nm pore size polycarbonate membranes using a hand held extruder (Avanti Polar Lipids, Alabaster, AL, USA) to obtain small unilamellar vesicles. While preparing tariquidar- and paclitaxel-loaded liposomes, drugs were added to chloroform along with lipids before preparing thin lipid film. A combined amount of equimolar quantities of paclitaxel and tariquidar was kept at 1% w/w final lipids. While preparing Rh-labelled liposomes, the molar ratio was kept at 63:6:30:2:1 for EPC:DOTAP:CHOL:PEG2kPE:Rh-PE. Rh-PE was added along with other lipids before
formation of the lipid film. The surface of liposomes was modified with 2C5 using pNP-PEG-PE linker.

### 3.1.7.2. Preparation of immunoliposomes with 2C5 modification:

To attach antibodies to the liposome surface, the antibody was conjugated with pNP-PEG-PE. Briefly, pNP-PEG-PE was synthesized and purified with a well-established method [59]. Using a rotary evaporator, a thin film was produced for pNP-PEG-PE. For further removal of trace levels of organic phase, the thin film was subjected to freeze drying for at least 30 min. A pNP-PEG-PE film was hydrated in 50 µl citrate buffer saline (pH 5) containing 10 mg/ml octyl glucoside for 15 min with occasional vortexing. This resulted in formation of pNP-PEG-PE micelles. Antibody solution prepared in phosphate buffer (pH 9) was then incubated with 40 times molar excess of pNP-PEG-PE for 4 hrs at room temperature ensuring pH stays above 8.5. The reaction allowed 2C5 attachment with activated PEG terminus with simultaneous hydrolysis of non-reacted pNP groups. The 2C5-PEG-PE conjugate spontaneously formed loose micelles due to the presence of lipid residue in the conjugate. To modify liposomes with 2C5, loose micelles (Ab-PEG-PE) were incubated with preformed liposomes at 169 mg ab/mmol PL for 24 h at 4°C at final PL concentration of 2.96mM, followed by the dialysis (300,000 Dalton membrane cut-off size) against PBS, pH 7.4, for 24 hrs at 4°C to remove non-reacted groups. This resulted in formation of 2C5-modified long-circulating liposomes. To normalize additional pegylation while modifying with 2C5, pNP-PEG-PE loose micelles were incubated with pre-formed liposomes and are labeled as 7% PEG-liposomes or pNP liposomes.
3.1.8 Characterization of long-circulating cationic immunoliposomes:

3.1.8.1 Size distribution and zeta potential analysis:

All liposomal formulations were characterized for their size distribution, zeta potential and loading efficiency for tariquidar as well as paclitaxel as described in section 3.1.3.

3.1.8.2 Antibody activity determination:

Activity of immunoliposomes toward nucleohistones was determined using an ELISA. Briefly, the 96-well microtiter plate was pretreated with 50 µl/well of 40 µg/ml poly-L-lysine (PLL) solution in Tris-buffered saline, TBS (50mM Tris-HCl, 150mM NaCl, pH 7.4) overnight at 4 °C. The PLL solution was discarded and any uncoated surface well was blocked with 200 µl of TBST-Casein (TBS containing 0.05% w/v Tween-20 and 2 mg/ml casein) for 1 h at room temperature (RT) to prevent non-specific binding. Each well was then coated with 50 µl of 40µg/ml mononucleosomes in TBST-Casein for 1 h at RT. Wells were washed three times with 200 µl of TBST, and incubated with 50 µl of serial dilutions of 2C5 in TBST-Casein for 1 h at RT. After incubation, wells were washed as before and incubated with 50 µl/well of 1:5000 dilution of goat anti-mouse IgG peroxidase conjugate in TBST-Casein for 1 h at RT. Wells were washed again as before, and each well was incubated with 100 µl of enhanced Kblue TMB peroxidase substrate for 15 min. The microtiter plate was then read at a dual wavelength of 620 nm with the reference filter at 492 nm using a Labsystems Multiscan MCC/340 microplate reader installed with GENESIS-LITE windows based microplate. UPC10
(non-specific antibody) was used as a negative control. A standard curve for 2C5 and UPC10 was produced at different concentration from 20 µg/ml, 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml, 0.625 µg/ml and 0.3125 µg/ml. Fix amount of immunoliposomes were added to measure immunoreactivity against nucleohistones.

3.1.9. Cellular uptake/association of immunoliposomes in monolayer:

The aim of this study was to evaluate change in cellular association of liposomes to SKOV-3, SKOV-3TR, MCF-7 and MCF-7/ADR cells after modification with monoclonal antibody 2C5 and UPC10. SKOV-3, SKOV-3TR, MCF-7 and MCF-7/ADR cells were grown in 6-well plates until 80% confluent. Cells were detached from flask using Accutase. Cells were centrifuged and washed with 1% BSA in PBS. Approximately 250,000 cells/ml were then incubated with Rh-labelled liposomes non-modified, 2C5-modified and UPC10-modified at final amount of 100 µg/ml of phospholipid for 1 hr and 4 hr. After incubation, cells were washed again with 1% BSA in PBS followed by centrifugation at 2500 RPM for 3 min. Cells were resuspended in 1% BSA in PBS and analyzed with FACS for red fluorescence (FL-2 channel).

3.1.10. Multidrug resistance reversal by immunoliposomes:

The aim of this study was to evaluate change in cytotoxicity of liposomes co-loaded with paclitaxel and tariquidar upon modification with 2C5 and UPC10. SKOV-3 and SKOV-3TR cells were seeded in 96-well plates at a density of 3000 cells/100 µl/well 24 hrs before treatment. After 24 hrs, cells were treated with tariquidar-loaded liposomes, paclitaxel-loaded liposomes, or tariquidar-paclitaxel-co-loaded liposomes either
unmodified (PEG normalized), 2C5-modified or UPC10-modified at various concentrations of paclitaxel for 4 hrs. After the desired period of incubation, cells were washed and incubated with drug free media and allowed to grow for 48 hrs. Cytotoxicity was measured using Cell Titer Blue assay according to the manufacturer’s protocol. Briefly, washed cells were incubated for 2 hrs with 100 μl of fresh media containing 20 μl of Cell Titer Blue reagent. After the incubation, plates were analyzed for the fluorescent signal at 530 nm excitation and 590 nm emission wavelength. Cells treated with vehicle only were taken as controls to calculate % cell death and the assay was carried out in triplicate.

3.1.11. Tumor growth inhibition study:

The aim of this study was to evaluate tumor growth inhibition upon treatment with unmodified and 2C5-modified long-circulating liposomes co-loaded with paclitaxel and tariquidar in SKOV-3 and SKOV-3TR.

Nude mice, about 6–8 weeks old, were injected with 2.5 X 10^6 cells (200 μl of 1:1, PBS:Matrigel) in right flank subcutaneously in 30 mice (6 groups, n=5) with SKOV-3 and in 30 mice (6 groups, n=5) with SKOV-3TR cells. When tumors reached 100 mm^3, mice were treated with PBS, XR liposomes, PCL liposomes, 2C5-empty liposomes, XRPCL liposomes, or 2C5-XRPCL liposomes. Each animal was given paclitaxel at 3 mg/kg/3day and tariquidar 3 mg/kg/3day. Mice were monitored every two days for changes in body weight and tumor growth. Tumor volume was assessed by measuring two perpendicular lengths with vernier calipers and using formula (L X W^2)/2, where L is the longest length and W is perpendicular to L. Mice were sacrificed when tumors
reached 1000 mm³. Tumors from all groups were dissected and tumor weight was measured.

3.1.12. P-gp activity at the blood brain barrier:

To determine P-gp activity at blood brain barrier level, nude mice 6 to 8 weeks old were divided in four groups (n=8) and injected with PBS, tariquidar dissolved in Chromophore:Ethanol (50:50) diluted in isotonic solution, or tariquidar-loaded liposomes at final concentration of 4 mg/kg intravenously. After 2 hrs, all groups were injected with Taxol (Paclitaxel in Chromophore:Ethanol 50:50) at the final concentration of 6 mg/kg intravenously. After 1 hr and 4 hr, mice were injected IP with Ketamin:Xylazine solution and blood was allowed to drain dissecting abdominal aorta. Brain tissue was then isolated, washed and stored at -80°C until further analysis.

To determine paclitaxel in brain tissue, tissue samples were homogenized in 1 ml PBS (10 mM, pH 7.4) on ice. Homogenate was then extracted by agitation with 2 ml of tertiary butyl methyl ether for 1 hr at RT following centrifugation at 5000 rpm for 10 minutes. Supernatant is then collected, evaporated under nitrogen stream and freeze dried for 2 hr. To determine the amount of paclitaxel in freeze dried content, it is reconstituted in acetonitrile:water (60:40) and subjected to HPLC analysis. Standard curve of paclitaxel is obtained from homogenates spiked with known quantities of paclitaxel.
3.1.13. Formation of spheroids using non-adhesive liquid overlay technique:

Each well in 96-well plate is coated with 50 μl of sterile 1.5% Agar prepared in media supplemented with 1% antibiotic solution at 60 °C and allowed to cool down atleast 45 minutes before seeding cells. MCF-7/ADR cells were grown in T75 until 80% confluent. Cells were then detached using typeisin and centrifuged to get cell pellet. Cells were suspended in complete media at final concentration of 10^5 cells/ml. 10,000 cells/100 μl/well were added to previously agar coated well and centrifuged at 1500 rcf for 15 min. Cells were allowed to aggregate and to grow spheroid for several days. Every 4 days, media was replenished with 50 μl of fresh media. Pictures were taken to assess growth of spheroids over a period of 9 days. To determine consistency in size and shape, pictures of the spheroids were taken using Nikon Eclipse E400 microscope.

3.1.14. Validation of phosphatase assay:

There are numerous methods available to determine viable cells in monolayer culture, out of which Cell titer-Blue cell viability assay is the most frequently used. However, the use of this method would be inappropriate to determine viable cells in spheroid culture as it is not validated by manufacturer in spheroids. Therefore, phosphatase assay was evaluated to determine viable cells in spheroid culture [60]. The amount of phosphatase, cytoplasmic enzyme, is directly proposal to the viable cells. Cells present in spheroids can be rapidly lysed with 1% Triton X-100 to release phosphatase enzyme. Phosphatase when incubated with p-nitrophenyl phosphate (pNPP), converts pNPP into p-nitrophenol. After incubation, addition of 1 N sodium hydroxide creates alkaline conditions to convert p-nitrophenol to p-nitrophenolate. P-nitrophenolate has
strong absorbance at 405 nm wavelength. Absorbance at 405 nm wavelength is directly proportional to p-nitrophenolate which corresponds to the amount of phosphatase and the amount of phosphatase is directly proportional to viable cells.

To determine the working range of phosphatase assay with respect to the number of cells for MCF-7/ADR, the cells were grown in monolayer till 70% confluent. Cells were then trypsinized and centrifuged at 1400 rpm for 4 minutes to get cell pellet. Cells were resuspended in PBS (10 mM, pH 7.4) followed by cell counting using trypan blue exclusion method. Cell suspension was adjusted to 2 X 10^6 cells/ ml with PBS. Cells were adjusted at different cell density ranging from 100,000 cells/100 μl, 50,000 cells/100 μl, 25,000 cells/100 μl, 12,500 cells/100 μl, 6,250 cells/100 μl, 3,125 cells/100 μl, 1,562 cells/100 μl, 781 cells/100 μl, 390 cells/100 μl, 185 cells/100 μl, and 92 cells/100 μl of PBS (n=8). Immediately, 100 μl of freshly prepared phosphatase assay buffer (2 mg/ml of pNPP in 0.1 M sodium acetate pH 4.8 with 1% triton X-100) was added to each well followed by 90 minutes incubation at 37 °C. After incubation, reaction was stopped with 10 μl of 1 N sodium hydroxide. Absorbance was read within 10 minutes at 405 nm after addition of sodium hydroxide using Biotek – Synergy HT absorbance plate reader.

3.1.15. Determination of size and number of cells:

Spheroids were grown with MCF-7/ADR cells using non-adhesive liquid overlay technique and allowed to grow for 9 days. To determine size, pictures were taken using SPOT advanced software each day and diameter was analyzed. To determine number of cells after day 3, 5 and 7 incubation, spheroids were subjected to phosphatase assay.
Also, the standard curve was prepared using different concentration of cells using phosphatase assay and number of cells were back calculated against standard curve.

3.1.16. Comparison of cytotoxicity assays:

In order to use the phosphatase assay for determining the cell viability in spheroids, it was first compared to two other popular methods of assessing cell viability i.e the Cell titer blue cell viability assay (Promega) and LDH release assay (Promega). To determine cytotoxicity of paclitaxel using all three assays, 5,000 cells / 100 μl / well of MCF-7/ADR cells were seeded 24 hours before treatment. Cells were then treated with various concentrations of paclitaxel and incubated for 48 hours. After incubation cytotoxicity was determined using all three assays according to manufacturer’s protocols.

3.1.17. Cytotoxicity profile of paclitaxel in spheroids:

Spheroids with MCF-7/ADR cells were grown using non-adhesive liquid overlay technique. Day 3 old spheroids were treated with 30 μg/ml, 10 μg/ml, 3.3 μg/ml and 1.1 μg/ml of paclitaxel alone or paclitaxel in combination with tariquidar for 72 hrs. After desired incubation period, cytotoxicity was evaluated using phosphatase assay as described in section 3.1.14 and LDH release assay as described in manufacturer’s protocol.

3.1.18. Effect of collagenase on paclitaxel toxicity in spheroids:

Spheroids with MCF-7/ADR cells were grown using non-adhesive liquid overlay technique. Day 3 old spheroids were treated with 0.1 μg/ml, 1 μg/ml or 2.5 μg/ml of
paclitaxel alone or paclitaxel in combination with tariquidar in presence or absence of 0.5 mg/ml of collagenase for 24 hrs, 48 hrs and 72 hrs. Untreated and 0.5 mg/ml collagenase treated spheroids were taken as control. After desired incubation time, pictures were taken to visualize effects of paclitaxel, tariquidar and collagenase on spheroids.

3.1.19. Cellular uptake/association of immunoliposomes in spheroids:

Spheroids were initiated with MCF-7/ADR cells using non-adhesive overlay technique and allowed to grow for 7 days. Rhodamine labeled long-circulating liposomes unmodified, 2C5-modified and UPC10-modified were prepared using method described in section 3.1.7. Day 7 old spheroids were treated with these formulations at 0.75 mg/ml final lipid concentration in final volume of 150 μl/well. Spheroids were incubated at 37°C for 4 hrs followed by washing with PBS (10 mM, pH 7.4) before analyzing using confocal microscopy for the uptake of liposomes. Z-stack images, in bright field as well as red fluorescence channel, were taken at interval of 10 μm and core of spheroid was analyzed for fluorescence intensity using Image J software.

3.1.20. Statistical analysis:

Size and zeta potential measurements and in vitro studies were carried out in triplicate to obtain mean ± standard deviation (SD). For tumor weight analysis, unpaired Student’s t-test was used. An ANOVA was used to compare body weight of mice treated with various formulations. One-way ANOVA was used to compare tumor volume for various treatments.
4. RESULTS

4.1 Preparation and Characterization of long-circulating cationic liposomes:

Using thin film hydration method, tariquidar and paclitaxel co-loaded long-circulating cationic liposomes were prepared. An ideal liposomal formulation should have size distribution in range from 100 to 500 nm for *in vivo* administration to prevent capillary blockage. Liposomal formulations had a uniform size distribution ranging from 140 to 160 nm (Table 3). We assumed that tariquidar and paclitaxel were incorporated within the lipid bilayer as they both are highly lipophilic. For all the liposomal preparations, zeta potential values ranged from +23 to +32 mV (Table 3). Upon the addition of tariquidar and/or paclitaxel to the formulation, there was no significant change in the liposome size distribution as well as zeta potential.
Table 3. Size distribution and zeta potential of liposomal formulations.

<table>
<thead>
<tr>
<th>Liposomal formulation</th>
<th>Lipid composition</th>
<th>Molar ratio</th>
<th>Size(^b) (nm)</th>
<th>Zeta potential(^b) (mV)</th>
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<tbody>
<tr>
<td>Empty Liposomes</td>
<td>EPC:CHOL:DOTAP:</td>
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<td>137.6 ± 1.2</td>
<td>32.01 ± 0.86</td>
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<td></td>
<td>PEG(_{2k})PE</td>
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<tr>
<td>XR liposomes(^a)</td>
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<tr>
<td>PCL liposomes(^a)</td>
<td>EPC:CHOL:DOTAP:</td>
<td>64:30:6:2</td>
<td>161.3 ± 0.3</td>
<td>23.68 ± 1.71</td>
</tr>
<tr>
<td></td>
<td>PEG(_{2k})PE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRPCL liposomes(^a)</td>
<td>EPC:CHOL:DOTAP:</td>
<td>64:30:6:2</td>
<td>154.1 ± 7.4</td>
<td>23.56 ± 0.82</td>
</tr>
<tr>
<td></td>
<td>PEG(_{2k})PE</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) XR liposomes – tariquidar-loaded liposomes, PCL liposomes – paclitaxel-loaded liposomes, XRPCL liposomes – tariquidar- and paclitaxel-loaded liposomes.

\(^b\) The values are for 5 measurements of the same formulation. Values represent mean ± standard deviation.
To measure the stability of these liposomal formulations during prolonged storage, the size distribution was assayed for four weeks. These liposomal formulations retained their size (< 200 nm) demonstrating their stability upon prolonged storage at 4°C (Figure 4).

![Size distribution of liposomal formulations](image)

Figure 4. Evaluation of liposomal stability. Size distribution of liposomal formulations determined for over a one month period. Values represent mean ± standard deviation.

The formulations were also characterized for their loading efficiency using an HPLC method. Separation of peaks for both molecules was achieved using an acetonitrile:ammonium acetate (10mM, pH 4) (60:40) mobile phase with a C\textsubscript{18} X-bridge column. Retention time for tariquidar and paclitaxel was 3.9 minutes and 5.2 minutes, respectively. The standard curve obtained for paclitaxel and tariquidar from the presented method had an R\textsuperscript{2} values of 0.9999 (n=3) as shown in Figure 5.
Figure 5. HPLC analysis of tariquidar (A), and paclitaxel (B). Standard curve was obtained by injecting concentrations of tariquidar and paclitaxel on a C\textsubscript{18} X-bridge column (4.6 mm X 250 mm). To obtain proper separation of peaks for both compounds, a mobile phase of acetonitrile : ammonium acetate 10 mM, pH 4 (60:40) was used. (n=3).
Loading efficiency for tariquidar and paclitaxel was calculated using these standard curves. The results indicated an incorporation efficiency of approximately 70-75% of drug added to lipid films (Table 4) for individual formulations. (The combined amount of both molecules was kept to 1% w/w of total lipids).

Table 4. Loading efficiency at given quantity of the added drug in long-circulating cationic liposomes.

<table>
<thead>
<tr>
<th>Liposomal formulations</th>
<th>Loading efficiency for tariquidar (%)</th>
<th>Loading efficiency for paclitaxel (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty Liposomes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>XR liposomes</td>
<td>70 - 75%</td>
<td>-</td>
</tr>
<tr>
<td>PCL liposomes</td>
<td>-</td>
<td>70 - 75%</td>
</tr>
<tr>
<td>XRPCL liposomes</td>
<td>70 - 75%</td>
<td>70 - 75%</td>
</tr>
</tbody>
</table>
4.2. P-glycoprotein expression:

SKOV-3, SKOV-3TR, MCF-7 and MCF-7/ADR cells were evaluated for the expression of the P-gp on the cellular surface. Using FITC-labeled P-gp-specific antibody (UIC2), the P-gp expression level was determined by FACS analysis. The analysis showed a ~ 7% increase in the green fluorescence signal (indicated by FL1 channel in FACS) in FITC-labeled antibody-treated SKOV-3 cells compared to untreated SKOV-3 cells. For SKOV-3TR cells, FACS analysis showed ~ 173% increase in green fluorescence signal in FITC-labeled antibody-treated cells compared to untreated SKOV-3TR cells. Also, there was a very slight (~ 2 %) increase in the green fluorescence signal in FITC-labeled antibody-treated MCF-7 cells compared to untreated MCF-7 cells, whereas there was ~ 81 % increase in the green fluorescence signal in FITC-labeled antibody treated MCF-7/ADR cells compared to untreated MCF-7/ADR cells. These results that showed significantly higher expression of P-gp in SKOV-3TR cells and MCF-7/ADR cells compared to SKOV-3 cells and MCF-7 cells, respectively, (Figure 6) indicated that these cell lines were appropriate models to evaluate P-gp inhibitors.
Figure 6: P-glycoprotein expression in SKOV-3 and SKOV-3TR cells (A) and MCF-7 and MCF-7/ADR cells (B). Cells are treated with FITC-labeled antibody against P-gp were analyzed using FACS. Data are presented as mean ± standard deviation. * p < 0.05.
4.3. Rh123 uptake:

SKOV-3, SKOV-3TR, MCF-7 and MCF-7/ADR cells were treated with free tariquidar or XR-liposomes (tariquidar-loaded liposomes) at a final concentration of tariquidar of 100 nM for 1 hr. Untreated cells were taken as controls. This initial treatment was followed by the incubation of cells with Rh123 at the final concentration of 200 ng/ml. SKOV-3 and MCF-7 cells had similar levels of Rh123 uptake by either untreated cells or cells treated with free tariquidar and tariquidar-loaded liposomes (Figure 7 A, B). The unchanged level of Rh123 uptake in sensitive variants was due to an apparent lack of P-gp expression. On the other hand, Rh123 uptake was decreased significantly in SKOV-3TR and MCF-7/ADR without treatment with tariquidar associate with the high expression of P-gp. However, Rh123 uptake was increased significantly when SKOV-3TR and MCF-7/ADR cells were pretreated with tariquidar or tariquidar-loaded liposomes demonstrating effective P-gp inhibition. Also, similar Rh123 uptake level, in free tariquidar treatment or in tariquidar-loaded liposomes treatment, signifies no activity loss for tariquidar towards P-gp upon liposomal incorporation.
Figure 7. FACS analysis of Rh123, P-gp substrate in SKOV-3 and SKOV-3TR cells (A), MCF-7 and MCF-7/ADR cells (B). Cells were either untreated, treated with free tariquidar, or treated with tariquidar-loaded liposomes at the final concentration of tariquidar of 100 nM for 1 hr followed by incubation with Rh123 at 0.2 µg/ml at 37 °C for 1 hr. n=3. Data are presented as mean ± standard deviation. * p < 0.05.
4.4. Multidrug resistance reversal:

SKOV-3, SKOV-3TR, MCF-7 and MCF-7/ADR cells were treated with different formulations at various doses of paclitaxel to determine the IC$_{50}$ value for paclitaxel. Toxicity profiles of tariquidar alone, empty liposomes and tariquidar-loaded liposomes were evaluated to determine the inherent toxicity of tariquidar and liposomes. All three treatments showed little inherent toxicity (<20% cell death) even at doses as high as 100 nM of tariquidar for all four cell lines used as shown in Figure 8 (A, B, C, D).
Cytotoxicity at 48 hrs in SKOV-3

A

Cytotoxicity at 48 hrs in SKOV-3TR

B

Cell death (%) vs. Concentration of tariquidar (nM)

XR alone
Empty liposomes
XR liposomes
Figure 8. Cytotoxicity of tariquidar, empty liposomes and tariquidar-loaded liposomes in SKOV-3 (A), SKOV-3TR (B), MCF-7 (C), and MCF-7/ADR (D) at 48 hrs. Cells were incubated for 48 hrs with tariquidar alone, tariquidar-loaded liposomes and empty liposomes at different concentrations of tariquidar. After incubation, cytotoxicity was evaluated using a Cell Titer Blue assay. Data represents as mean ± standard deviation from three separate experiments.
To determine the extent of reversal of the MDR, SKOV-3, SKOV-3TR, MCF-7 and MCF-7/ADR were treated with paclitaxel alone, paclitaxel liposomes or tariquidar-paclitaxel- co-loaded liposomes at different concentrations of paclitaxel for 48 hrs. After incubation, cytotoxicity was evaluated to determine the IC$_{50}$ values for paclitaxel.

As shown in Figure 9 (A, B), IC$_{50}$ values for paclitaxel were $\sim$ 27 nM and $\sim$ 2750 nM in SKOV-3 and SKOV-3TR cells, respectively, when cells were treated with paclitaxel alone. Thus, a 100-fold higher dose of paclitaxel would be required to produce an equivalent toxicity in SKOV-3TR cells compared to SKOV-3 cells. The IC$_{50}$ values for paclitaxel were $\sim$ 18 nM and $\sim$ 34 nM in SKOV-3 cells and SKOV-3TR cells, respectively, when cells were treated with tariquidar-paclitaxel- co-loaded long-circulating cationic liposomes, clearly demonstrating a reversal of the MDR. The IC$_{50}$ values for paclitaxel were $\sim$ 50 nM and $\sim$ 2300 nM in MCF-7 and MCF-7/ADR cells, respectively, when cells were treated with paclitaxel alone. Thus, a 46-fold higher dose of paclitaxel would be required to produce an equivalent toxicity in MCF-7/ADR cells compared to MCF-7 cells. MCF-7/ADR cells were also sensitized when treated with paclitaxel- tariquidar- co-loaded long-circulating cationic liposomes resulting in IC$_{50}$ values for paclitaxel $\sim$ 30 nM and $\sim$ 32 nM in MCF-7 and MCF-7/ADR cells, respectively. These results indicated that a similar dose of paclitaxel produces an equivalent toxicity in resistant variant compared to the sensitive variant with simultaneous delivery of tariquidar and paclitaxel in long-circulating cationic liposomes, demonstrate a complete reversal of the MDR.
Figure 9. Multidrug resistance. IC$_{50}$ value for paclitaxel after 48 hrs of incubation in SKOV-3 and SKOV-3TR cells (A), MCF-7 and MCF-7/ADR cells (B). n=3. Data represents mean ± standard deviation from three separate experiments. * p < 0.05. Y-axis indicates log values.
In vitro studies showed significant reversal of the MDR in SKOV-3TR and MCF-7/ADR against paclitaxel using long-circulating cationic liposomes co-loaded with tariquidar and paclitaxel (Figure 9). Further development of these liposomes with monoclonal antibody 2C5 was performed to target them specifically to the cancer cells. Tumors in vivo have a diverse cell population ranging from rapidly dividing cells on the outer surface, quiescent cells in the middle area and necrotic cells in deepest core due to lack of oxygen and nutrition. However, many cells are undergoing apoptosis during tumor growth as well. The apoptotic cell population in most types of tumors liberates nucleosomes which bind to neighboring cancer cells [61]. Numerous studies have shown that the monoclonal antibody 2C5 binds to these nucleosomes and allows increased accumulation of 2C5-modified immunoliposomes in the tumor environment [62-67]. We, therefore, proposed to use the 2C5 monoclonal antibody to modify long-circulating cationic liposomes co-loaded with tariquidar and paclitaxel for active targeting of tumor cells. The hypothesis was that the 2C5-modified immunoliposomes will decrease non-specific binding of tariquidar to normal tissues and increase co-localization of both molecules in the tumor environment resulting in increased reversal of the MDR in vivo and inhibition of tumor growth.

To test our hypothesis, 2C5-modified long-circulating cationic immunoliposomes co-loaded with tariquidar and paclitaxel were prepared and characterized. They were tested for their ability to bind nucleohistones using an ELISA. Further, these liposomes were evaluated for changes in cell association characteristics in vitro, for changes in the cytotoxicity profile of paclitaxel in vitro as well as for changes in antitumor activity in vivo.
4.5. Characterization of immunoliposomes:

4.5.1. Size distribution and Zeta-potential of immunoliposomes:

Liposomes, prepared using the thin film hydration method, were modified with 2C5 to generate immunoliposomes using a ‘micelle transfer’ method. After 2C5 modification, liposomes retained their size; however, zeta potential was reduced (Table 5). To normalize additional PEG and zeta potential, Rh-liposomes were also incubated with pNP-PEG-PE micelles without antibody conjugated with them and are labeled as 7% PEG-Rh-liposomes.

Table 5. Size distribution and Zeta potential of different liposomal formulations.

<table>
<thead>
<tr>
<th>Liposome formulations</th>
<th>Size distribution</th>
<th>Zeta potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh-liposomes</td>
<td>191.9 ± 1.4</td>
<td>31.21 ± 1.96</td>
</tr>
<tr>
<td>7% PEG-Rh-liposomes</td>
<td>196.5 ± 1.5</td>
<td>-24.46 ± 1.23</td>
</tr>
<tr>
<td>2C5-Rh-liposomes</td>
<td>201.7 ± 1.1</td>
<td>-25.55 ± 0.91</td>
</tr>
<tr>
<td>UPC10-Rh-liposomes</td>
<td>210.4 ± 1.8</td>
<td>-28.42 ± 0.94</td>
</tr>
</tbody>
</table>

4.5.2. 2C5 activity determination using ELISA:

Liposomal surface was modified with 2C5 using a pNP-PEG₃₄k-PE linker. During modification process, antibody was subjected to different pH environment. Therefore, it was necessary to determine the activity of 2C5 post-modification using ELISA. The 2C5 retained its activity after liposomal modification and was comparable to free 2C5 activity (Figure 10). These results also indicated a high 2C5 reaction yield. A non-specific antibody UPC10, free and liposome-modified, showed no reactivity towards antigen.
Figure 10. 2C5 activity determination using ELISA. Various concentration of 2C5 and UPC10 were incubated with 40 μg/ml of nucleohistones. Liposomes modified with equivalent amount of 2C5 and UPC10 were also incubated with 40 μg/ml of nucleohistones. Activity of 2C5 and UPC10 towards nucleohistones was determined and compared. Data represents mean ± standard deviation.

4.6 Cellular uptake/association study:

2C5-modified Rh-liposomes were evaluated for their effect on cellular association. SKOV-3 and SKOV-3TR cells were incubated with 100 μg/ml of the final lipid concentration for 1 and 4 hr. Cell association was then evaluated using FACS analysis. Modification with 2C5 resulted in significantly higher association of liposomes with SKOV-3 cells as well as SKOV-3TR cells compared to unmodified liposomes or UPC10-modified liposomes (Figure 11). Data suggest that the modification of liposomal formulation with 2C5 may improve therapeutic efficacy of a loaded drug molecule in
vitro as well as in vivo. Therefore, change in cytotoxicity of 2C5-modified liposomal formulations was determined.

![Cell association study SKOV-3 cells](A)

![Cell association study SKOV-3TR cells](B)

Figure 11. Cell association upon 2C5 modification in SKOV-3 (A) and SKOV-3TR (B) cells. Cells were treated with 7% PEG-Rh-liposomes, 2C5-modified or UPC10-modified Rh-labelled liposomes for 1 hr or 4 hr. Cells were then analyzed for rhodamine (Rh) intensity using FACS. * p < 0.05 (2C5-modified liposomes compared to unmodified or UPC10-modified liposome treatment group). Data represents mean ± standard deviation. n=3.
4.7. Multidrug resistance reversal with immunoliposomes:

Modification of long-circulating liposomes with 2C5 monoclonal antibody resulted in increased uptake by cancer cells *in vitro*. Therefore, cytotoxicity of paclitaxel was evaluated when co-delivered with tariquidar in 2C5-modified liposomes and compared with IgG-modified or unmodified liposomes.

Tariquidar in unmodified, 2C5-modified and UPC10-modified liposomes did not show any toxicity up to 1000 ng/ml in SKOV-3 cells (Figure 12 A) as well as SKOV-3TR (Figure 13 A). This was important as it showed that tariquidar or liposomes do not produce any toxicity that contributes when treated in combination with paclitaxel. As can be seen in figure 12 (B), paclitaxel treatment in unmodified, 2C5-modified or UPC10-modified liposomes produced dose-dependent cytotoxicity in SKOV-3 cells. However, there was no significant difference among treatments. Also, paclitaxel treatment in all liposome formulations in SKOV-3TR cells did not produce any toxicity as in this resistant variant as shown in figure 13 (B).

As can be seen in figure 12 (C), treatment of SKOV-3 cells with tariquidar and paclitaxel combination in unmodified, 2C5-modified and UPC10-modified liposomes produced similar toxicity to paclitaxel treatments in liposome formulations as addition of tariquidar did not produce any additional toxicity. Also, no difference between unmodified, 2C5-modified and UPC10-modified tariquidar-paclitaxel co-loaded liposomes was observed. However, tariquidar and paclitaxel co-loaded liposomes showed significantly higher cytotoxicity compared to paclitaxel-loaded liposomes as tariquidar inhibited P-gp and enhanced retention of paclitaxel in SKOV-3TR cells (Figure 13 (C)). 2C5 modification did not enhance cytotoxicity of combination therapy in SKOV-3TR
cells. As 2C5 depends on exposed nucleosomes on cancer cells, lack of improved cytotoxicity could be explained by significantly lower expression of antigen in rapidly growing cells in monolayer culture.
Figure 12. Reversal of the MDR using immunoliposomes in SKOV-3 cells. 3,000 cells/100 μl were seeded in 96-well plate 24 hrs before treatment. Cells were then treated with various concentrations of tariquidar alone (A), paclitaxel alone (B), or tariquidar-paclitaxel combination (C) for 4 hrs in different liposomal formulations followed by incubation in drug-free media for 48 hrs before analyzing for cytotoxicity. Mean ± S.D.
Figure 13. Reversal of the MDR using immunoliposomes in SKOV-3TR cells. 3,000 cells/100 μl were seeded in 96-well plate 24 hrs before treatment. Cells were then treated with various concentrations of tariquidar alone (A), paclitaxel alone (B), and tariquidar-paclitaxel combination (C) for 4 hrs in different liposomal formulation followed by incubation in drug-free media for 48 hrs before analyzing for cytotoxicity. Mean ± S.D.
4.8. Tumor growth inhibition:

Antitumor activity of tariquidar and paclitaxel co-loaded liposomes, either modified with 2C5 or unmodified, on SKOV-3 or SKOV-3TR cell line xenografts was evaluated by tumor growth inhibition. XRPCL liposome treatment significantly inhibited tumor growth of SKOV-3 cells compared to PBS, XR liposomes, and 2C5-empty liposomes (Figure 14A). Tumor growth inhibition was also statistically significant when compared to PCL liposomes for SKOV-3 cells. These results may be the result of resistance acquired by SKOV-3 cells after repetitive treatment with paclitaxel. XRPCL and 2C5-XRPCL liposome treatment resulted in significant tumor growth inhibition of SKOV-3TR cells compared to PBS, XR liposomes, PCL liposomes and 2C5-empty liposomes (Figure 14B). However, tumor growth inhibition was insignificant when comparing XRPCL liposome treated group with 2C5-XRPCL liposomes treated group in SKOV-3 cells as well as SKOV-3TR cells. These results indicated that the addition of 2C5 at 4 mg/kg to liposomal formulation did not result in enhanced antitumor activity in SKOV-3 cells and SKOV-3TR cells as spontaneous accumulation of unmodified liposomes seems saturating the antitumor activity. These results were also confirmed by dissecting mice and isolating tumors to measure tumor weight. As can be seen in figure 15, tumor weights for XRPCL and 2C5-XRPCL liposome treatment were significantly less compared to PBS, XR liposomes, PCL liposomes and 2C5-empty liposomes for SKOV-3 cells as well as SKOV-3TR cells. Also, no significant difference in tumor weight was observed between the XRPCL and 2C5-XRPCL treated groups. Body weight was also observed throughout the study as a measure for treatment toxicities. As can be seen in figure 16, no significant difference was observed among mice treated with PBS.
and those treated with XR liposomes, PCL liposomes, XRPCL liposomes, 2C5-empty liposomes and 2C5-XRPCL liposomes for both groups (SKOV-3 tumors and SKOV-3TR tumors bearing mice) indicating minimum or no toxicity of formulations.
Figure 14. Effect of treatments on tumor growth. Approximately 2.5 X 10^6. SKOV-3 cells (A) and SKOV-3TR cells (B) were injected in right flank of 36 mice. After 7 days, mice were randomly distributed into 6 groups. Mice were injected with PBS, XR liposomes, PCL liposomes XRPCL liposomes, 2C5-empty liposomes or 2C5-XRPCL liposomes every three days until tumors in control group reached 1000 mm^3. Error bars indicate mean ± standard deviation. * p < 0.05. n=5.
Figure 15. Tumor weight analysis. SKOV-3 (A) and SKOV-3TR (B) tumor-bearing mice, after treatment with PBS, XR liposomes, PCL liposomes, 2C5-empty liposomes, XRPCL liposomes and 2C5-XRPCL liposomes, were sacrificed when tumors in control group reached 1000 mm³. Tumors were isolated and weighed. Error bars indicate mean ± standard deviation. *p<0.05 (n=5).
Figure 16. Body weight analysis. SKOV-3 (A) and SKOV-3TR (B) tumor-bearing mice were monitored for changes in body weight throughout the period of treatment with PBS, XR liposome, PCL liposomes, XRPCL liposomes, 2C5-empty liposomes and 2C5-XRPCL liposomes. Error bars indicate mean ± standard deviation. None of the treatment groups were different from the other treatment groups (ANOVA).
Based on the results obtained, we can conclude that the co-delivery of XRPCL in long-circulating liposomes demonstrated enhanced antitumor activity compared to PCL in long-circulating liposomes in SKOV-3 as well as SKOV-3TR bearing mice. XR liposomes and 2C5-empty liposomes showed no tumor inhibiting properties in SKOV-3 as well as SKOV-3TR cells. Formulations used in these study demonstrated minimum or no toxicity on mice determined by the body weight.

4.9. P-gp activity at the blood brain barrier:

To determine P-gp activity at the blood brain barrier, paclitaxel accumulation in brain was determined. Nude mice were injected IV with PBS, tariquidar alone, or tariquidar liposomes at final concentration of 4 mg/kg. After 2 hrs, mice were injected IV with paclitaxel in chramophore:ethanol (50:50) solution at final concentration of 6 mg/kg. To quantify paclitaxel in brain tissue, known amount of paclitaxel was spiked in brain tissue, extracted and analyzed using HPLC (Figure 17). However, paclitaxel was not detected (remained below detection limit) in brain tissue samples of mice injected with paclitaxel at 6 mg/kg pre-injected with either free tariquidar or tariquidar-loaded liposomes (Figure 18).
Figure 17. Paclitaxel standard curve from brain tissue using HPLC. Brain tissues were isolated, weighed and injected with different amount of paclitaxel. Brain tissues were homogenized and extracted with 2 ml of tert. Butyl methyl ether for 1 hr. Supernatant was collected from samples centrifuged at 1500 rcf for 10 min, evaporated and reconstituted in same volume of acetonitrile:water (60:40). Final reconstitute was subjected to HPLC analysis. n=2.

\[ y = 59741x + 8492.1 \]

\[ R^2 = 0.9943 \]
Figure 18. P-gp activity at blood brain barrier. Nude mice were injected with PBS, tariquidar alone, or tariquidar-loaded liposomes at 4 mg/kg for 2 hrs. All mice were then injected with free paclitaxel intravenously at final concentration of 6 mg/kg. After desired incubation, mice were sacrificed and brain tissues were collected to analyze paclitaxel accumulation. n=3. Data represents mean ± standard deviation.
4.10. Formation of Spheroids using non-adhesive liquid overlay technique:

MCF-7/ADR cells were evaluated for the formation of spheroids using non-adhesive liquid overlay method. After the incubation of 10,000 cells/well, the MCF-7/ADR cells initiated formation of consistent spheroids with respect to size, shape and density (Figure 19). However, adding more than 15,000 cells/well or less than 5000 cells/well did not produce consistent spheroids (data not shown). Also, coating of 1.5% agar and centrifugation at 1500 rcf for 15 min was critical to form consistent spheroids. Lower concentration of agar and lower speed did not result in formation of consistent spheroid (data not shown). Intact spheroid forms around day 3 and continues to grow till day 9. However cells start to shed after day 7. Treatment for further experiments therefore will be carried out during exponential growth phase, which was determined to be from day 3 to day 7.
Figure 19. Formation of spheroids using non-adhesive liquid overlay technique. 10,000 MCF-7/ADR cells were seeded in 96-well plate pre-coated with 50 μl of 1.5% agar. Cells were centrifuged at 1500 rcf for 15 min. Cells were then allowed to grow for 9 days for the formation of spheroids.
4.11. Validation of phosphatase assay:

MCF-7/ADR cells were evaluated for the co-relation of the amount of phosphatase enzyme with the number of cells. Phosphatase activity was determined using phosphatase specific substrate pNPP (p-nitro phenyl phosphate). Using the conditions mentioned in procedure, number of viable cells could be determined accurately up to 100,000 cells/well for MCF-7/ADR cells (Figure 20).

![Validation of phosphatase assay](image)

Figure 20. Validation of phosphatase assay. Different numbers of MCF-7/ADR cells were incubated with phosphatase assay buffer according to protocol and allowed to incubate for 90 min. After incubation reaction was stopped using 10 μl of 1 N NaOH and absorbance was read at 405 nm.
4.12. Determination of spheroid size and number of cells:

Spheroids grown with MCF-7/ADR cells using non-adhesive liquid overlay technique were assessed for consistency in terms of size which was determined using SPOT advanced software. As can be seen in figure 21, non-adhesive liquid overlay technique resulted in formation of spheroids in range of 400 to 500 μm range and remained consistent throughout growth period of 7 days.

![Determination of spheroid size](image)

Figure 21. Determination of spheroid size. Spheroids, produced using non-adhesive liquid overlay technique, were assessed for their size using SPOT advanced software over the period of 9 days. Error bars indicate mean ± standard deviation.

Spheroids were also analyzed for consistency in terms of live number of cells present at particular day. To determine so, spheroids were subjected to phosphatase assay on day 3, day 5 and day 7 to determine number of cells. As can be seen in figure 22, cell
growth in spheroid formation was very low compared to growth in monolayer culture. Also, consistency in number of cells was observed through day 7.

Figure 22. Determination of number of cells in spheroids. Spheroids grown with MCF-7/ADR cells using non-adhesive liquid overlay technique were subjected to phosphatase assay to determine number of cells present on day 3, 5 and 7. Error bars present mean ± standard deviation.

4.13. Comparison of cytotoxicity assays:

The cell titer blue assay, phosphatase assay and LDH release assay were evaluated for determination of cytotoxicity of paclitaxel on MCF-7/ADR cells in monolayer culture. The comparison between these three assays of cytotoxicity revealed that the cytotoxicity in monolayer could be determined accurately using any of these methods as shown in fig 23. However, phosphatase assay or LDH release assay were
used for cytotoxicity determination in spheroid culture as they use cell lysate to determine presence of live cells rather than metabolically intact cells.

Figure 23. Comparison of cytotoxicity assays. 5,000 MCF-7/ADR cells were seeded in 96-well plate 24 hrs before treatment with various concentration of paclitaxel. Cells were then incubated for 48 hrs and cytotoxicity was determined using manufacturer’s protocols for cell titer blue assay, phosphatase assay and LDH release assay.
4.14. Cytotoxicity profile of paclitaxel in spheroids:

Day 3 old spheroids grown using non-adhesive liquid overlay technique from MCF-7/ADR cells were treated with paclitaxel or tariquidar-paclitaxel combination at 30 μg/ml, 10 μg/ml, 3.33 μg/ml or 1.11 μg/ml of paclitaxel. Tariquidar concentration was kept similar to paclitaxel concentration. After 72 hrs, cytotoxicity in spheroids was determined using phosphatase assay as well as LDH release assay. As can be seen in figure 24, paclitaxel alone did not produce toxicity more than 30% with dose as high as 30 μg/ml. This was noteworthy, as paclitaxel IC$_{50}$ value for monolayer culture ranged around 2 μg/ml. Also, similar toxicity was observed even after addition of tariquidar as high as 30 μg/ml. Again, IC$_{50}$ for paclitaxel in combination with tariquidar for MCF-7/ADR ranged around 40 ng/ml. These results demonstrated completely altered cancer cell behavior in spheroids upon treatment with paclitaxel in presence or absence of tariquidar from monolayer culture. Lack of toxicity was also confirmed by LDH assay as fraction of LDH was released in supernatant (Figure 25).
Figure 24. Paclitaxel toxicity profile in spheroids by phosphatase assay. Day 3 old spheroids were treated with various concentration of paclitaxel and paclitaxel-tariquidar combination for 72 hrs before analyzing with phosphatase assay. Data represents mean ± standard deviation. n=3.

Figure 25. Paclitaxel toxicity profile in spheroids by LDH release assay. Day 3 old spheroids were treated with various concentration of paclitaxel and paclitaxel-tariquidar combination for 72 hrs before analyzing with LDH release assay. Data represents mean ± standard deviation. n=3.
4.15. Effect of collagenase on paclitaxel toxicity in spheroids:

Spheroid structure was observed to determine paclitaxel toxicity or tariquidar paclitaxel toxicity in combination in presence or absence of collagenase. Paclitaxel alone and paclitaxel in combination with tariquidar, in presence or absence of collagenase did not produce any structural damage to spheroids or inhibition of growth with dose as high as 2.5 μg/ml (Figure 26, 27). These results diverge from paclitaxel toxicity profile observed in monolayer culture of same cells where paclitaxel-tariquidar combination produced IC₅₀ of 50 nM (~42 ng/ml).
Figure 26. Paclitaxel toxicity on spheroids. Day 3 old spheroids were treated with different concentration of paclitaxel in presence of absence of tariquidar.
Figure 27. Paclitaxel toxicity on spheroids in presence of collagenase. Day 3 old spheroids were treated with paclitaxel in presence of absence of tariquidar.
4.16. Cellular uptake/association of immunoliposomes in spheroids:

2C5 monoclonal antibody against cancer specific nucleosomes has previously been shown to enhance cellular uptake and association in many tumor types. Such nucleosomes are exposed on cancer cells from dying cells in solid tumors. Numerous attempts have been carried out to evaluate 2C5 association with cancer cells in monolayer culture. However, increased uptake is only observed when incubation of 2C5-modified nanoparticles and cells are kept in serum free conditions. Few possibilities include presence of low level of nucleosomes in monolayer culture. It is, therefore, effect of 2C5 modification on cellular uptake/association was evaluated in spheroid culture.

Spheroids from MCF-7/ADR cells were grown using non-adhesive liquid overlay technique and allowed to grow for 7 days before treatment with Rh-labelled unmodified, Rh-labelled 2C5-modified and Rh-labelled UPC10-modified liposomes for 4 hrs. After incubation, spheroids were washed twice with PBS and z-stack images were obtained. As can be seen in figure 28 (B), untreated spheroids showed minimum to no background fluorescence. Bright field images for the same spheroid can be seen in figure 28 (A). Spheroids treated with 2C5-modified Rh-labeled liposomes showed higher fluorescence (Figure 31 (B), 32 (B)) compared to 7%-PEG-Rh-labeled liposomes (Figure 29 (B), 30 (B)) as well as controlled antibody UPC10-modified liposomes (Figure 33 (B), 34 (B)). These data were quantified using image J software for pixel density per area and compared for 8th, 9th and 10th z-stack image for all treatments. When compared, 2C5-modified liposomes showed significant higher liposome association compared to unmodified and UPC10 modified liposomes (Figure 35) confirming phenomenon observed in fluorescence images.
Note: All images in this section follows following format for the bright field as well as fluorescent channel.

<table>
<thead>
<tr>
<th>Top of the spheroid</th>
<th>Section 10 μm deep</th>
<th>Section 20 μm deep</th>
<th>Section 30 μm deep</th>
<th>Section 40 μm deep</th>
</tr>
</thead>
<tbody>
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Figure 28 (A). Cellular uptake/associate of immunoliposomes. Z-stack images of untreated spheroids in bright field. Scale bar indicates 100 μm.
Figure 28 (B). Cellular uptake/associate of immunoliposomes. Z-stack images of untreated spheroids in fluorescence channel. Scale bar indicates 100 μm.
Figure 29 (A). Cellular uptake/associate of immunoliposomes. Z-stack images of spheroids treated with 7% PEG-Rh-liposomes in bright field. Scale bar indicates 100 μm.
Figure 29 (B). Cellular uptake/associate of immunoliposomes. Z-stack images of spheroids treated with 7% PEG-Rh-liposomes in fluorescence channel. Scale bar indicates 100 μm.
Figure 30 (A). Cellular uptake/associate of immunoliposomes. Z-stack images of spheroids treated with 7% PEG-Rh-liposomes in bright field. Scale bar indicates 100 μm.
Figure 30 (B). Cellular uptake/associate of immunoliposomes. Z-stack images of spheroids treated with 7% PEG-Rh-liposomes in fluorescence channel. Scale bar indicates 100 μm.
Figure 31 (A). Cellular uptake/associate of immunoliposomes. Z-stack images of spheroids treated with 2C5-Rh-liposomes in bright field. Scale bar indicates 100 μm.
Figure 31 (B). Cellular uptake/associate of immunoliposomes. Z-stack images of spheroids treated with 2C5-Rh-liposomes in fluorescence channel. Scale bar indicates 100 μm.
Figure 32 (A). Cellular uptake/associate of immunoliposomes. Z-stack images of spheroids treated with 2C5-Rh-liposomes in bright field. Scale bar indicates 100 μm.
Figure 32 (B). Cellular uptake/associate of immunoliposomes. Z-stack images of spheroids treated with 2C5-Rh-liposomes in fluorescence channel. Scale bar indicates 100 μm.
Figure 33 (A). Cellular uptake/associate of immunoliposomes. Z-stack images of spheroids treated with UPC10-Rh-liposomes in bright field. Scale bar indicates 100 μm.
Figure 33 (B). Cellular uptake/associate of immunoliposomes. Z-stack images of spheroids treated with UPC10-Rh-liposomes in fluorescence channel. Scale bar indicates 100 μm.
Figure 34 (A). Cellular uptake/associate of immunoliposomes. Z-stack images of spheroids treated with UPC10-Rh-liposomes in bright field. Scale bar indicates 100 μm.
Figure 34 (B). Cellular uptake/associate of immunoliposomes. Z-stack images of spheroids treated with UPC10-Rh-liposomes in fluorescence channel. Scale bar indicates 100 μm.
Figure 35. Cellular associate/uptake of immunoliposomes in spheroids analyzed using Image J software. Day 7 old spheroids were treated with unmodified liposomes, 2C5-modified or UPC10-modified liposomes at final lipid concentration of 0.75 mg/ml for 4 hrs. After incubation, spheroids were washed and z-stack images were taken. Section 8, 9 and 10 from the top were analyzed using Image J software and compared using student-t-test. * p < 0.05 when 2C5-Rh-liposomes compared with untreated, unmodified or UPC10-modified liposomes.
5. Discussion

Acquired and inherent multidrug resistance demonstrated by solid tumors is a primary defense mechanism against chemotherapy exploited by cancer. Out of many possible mechanisms, over-expression of P-gp is one of the most common mechanisms encountered in clinic following chemotherapy responsible for MDR demonstration. Therefore, third generation tariquidar was evaluated for reversal of the multidrug resistance by cancer cells due to over-expression P-gp. As tariquidar is highly lipophilic, liposomes were used as a delivery system to enhance its solubility and to improve biodistribution. Also, it appeared necessary to deliver tariquidar and chemotherapeutic agent paclitaxel simultaneously for maximum effect. For present study, liposomes were co-loaded with tariquidar and paclitaxel to minimize exposure of paclitaxel and tariquidar to normal tissue and increase co-localization of both molecules in tumor tissues as liposomes accumulate in tumor environment using EPR effect. Preparation of pegylated liposomes resulted in formation of stable formulation and with high incorporation efficiency for both molecules. Also, no apparent reduction in activity was observed post liposome loading. These formulations were evaluated for reversal of the MDR by blocking P-gp in vitro in SKOV-3, SKOV-3TR, MCF-7 and MCF-7/ADR cells. Results indicated significant reversal of the MDR by co-delivery using liposomes. Use of these liposomes in vivo only exploits passive targeting by EPR effect. To improve its activity numerous studies have shown preparation of immunoliposomes. Therefore, we prepared 2C5-modified tariquidar-paclitaxel-co-loaded liposomes and evaluated change in efficacy in cell binding. Results showed significant increase in cancer cell association by 2C5-modified liposomes compared to unmodified liposomes in monolayer culture. Later,
formulations were evaluated for their antitumor activity *in vivo* in mice bearing SKOV-3 and SKOV-3TR xenografts. Results indicated significant tumor growth inhibition with tariquidar and paclitaxel co-loaded liposomes compared to paclitaxel liposomes demonstrating significant reversal of the MDR. However, 2C5 modification failed to produce improved antitumor activity. These could have resulted due to lower dose of 2C5 or administration of doses near saturation level. Based on results obtained, we conclude that use of long-circulating liposomes co-loaded with tariquidar and paclitaxel successfully achieved reversal of the acquired drug resistance *in vitro* as well as *in vivo*. Along with co-localization of tariquidar and paclitaxel in tumor tissues, it was also important to minimize exposure of tariquidar and paclitaxel in normal tissues like blood brain barrier. Therefore, P-gp activity at blood brain barrier was determined. However, paclitaxel accumulation in brain remained below detection limit making it difficult to assess P-gp activity.

Along with acquired drug resistance, inherent drug resistance also plays significant role in eluding chemotherapy. Some of the physiological factors contributing towards inherent drug resistance are cell-cell interaction, higher interstitial fluid pressure, hypoxic core and presence of low pH at core. Effects of such parameters cannot be studied using monolayer culture. Therefore, spheroid culture was established to evaluate these parameters. Out of various protocols evaluated, consistent spheroids were produced using non-adhesive liquid overlay technique. Size, shape and number of cells remained consistent throughout. Also, various protocols such as phosphatase assay and preparation of single cell suspension were adapted to be used in 3D culture model. Our major aim was to evaluate and compare cancer cell behavior in monolayer culture and three
dimensional culture. As discussed before, tariquidar and paclitaxel combination completely reversed multidrug resistance in monolayer culture. However, when evaluated in three dimensional spheroids, addition of tariquidar failed to improve cytotoxicity of paclitaxel in MCF-7/ADR spheroids even at very high concentration. Limited penetration and cells present in diverse cell cycle stages may have been responsible for observed lack of toxicity in spheroids. Also, 2C5-modification improved cancer cell association in monolayer culture only in serum free condition. Treatment in serum containing media, 2C5-modified liposomes showed similar uptake as non-specific antibody-modified liposomes. These results may have resulted due to lack of antigen in rapidly growing monolayer culture. However, 2C5-modification significantly improved cancer cell uptake in spheroids, even in complete media as higher concentration of antigen were exposed due to dying cells at core of three dimensional structure. Spheroids provide an excellent platform to study physiological factors responsible the MDR as it demonstrates many tissue like properties. Also, altered cancer cell behavior in three dimensions from monolayer culture makes spheroid culture very important and valuable asset as a cancer research model.
6. BIBLIOGRAPHY


APPENDIX A: Laboratory Safety and Chemical Hygiene training:

Chemical Hygiene Training – part I (right to know):

Northeastern University
Office of Environmental Health & Safety

On October 5, 2009, Niravkumar Patel successfully completed 01: "Chemical Hygiene Training Part 1 - Right To Know" program on the web, answering 80% of the 15 questions correctly (At least a 80% score is required to pass).

For any questions, contact EHS at (617)373-2769 or ehs@neu.edu

Note: To complete this training you must also take Part 2

Record Locator: tw0000000007924 (Session).

Chemical Hygiene Training – part II (Lab safety and hazardous waste management):

Northeastern University
Office of Environmental Health & Safety

On October 7, 2009, Niravkumar Patel successfully completed 02: "Chemical Hygiene Training Part 2 - Laboratory Safety and Hazardous Waste Management" program on the web, answering 100% of the 10 questions correctly (At least a 80% score is required to pass).

For any questions, contact EHS at (617)373-2769 or ehs@neu.edu

Note:

Record Locator: tw0000000007954 (Session).
APPENDIX B: Radiation Safety Training for Workers:

Radiation Awareness Training:
On August 10, 2011, Niravkumar Patel successfully completed 40: "Radiation Awareness Training (for those not working with radioactive materials while working in radiation use lab)" program on the web, answering 100% of the 10 questions correctly (At least a 80% score is required to pass).

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Record Locator: tw0000000015491 (Session).
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Initial Training for People Who Will Be Working with Radioactive Materials:
On August 10, 2011, Niravkumar Patel successfully completed 43: "Radiation Safety Training & Exam for Initial Workers (for people that will be working with radioactive materials)" program on the web, answering 80% of the 10 questions correctly (At least a 80% score is required to pass).

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Refresher Radiation Training for sealed Sources:

On August 10, 2011, Niravkumar Patel successfully completed 46: "Refresher Radiation Training for SEALED sources (e.g. Co-57, Ru-106, PT-197)" program on the web, answering 80% of the 10 questions correctly (At least a 80% score is required to pass).

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Refresher Radiation Training for Unsealed Sources:

On August 10, 2011, Niravkumar Patel successfully completed 47: "Refresher Radiation Training for UNSEALED sources (e.g. H-3, C-14, P-32, S-35, IN-111)" program on the web, answering 80% of the 10 questions correctly (At least a 80% score is required to pass).

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Record Locator: tw0000000015500 (Session).

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APPENDIX C: Investigator Training to Work with Animals:

User Niravkumar Patel

Submitted 2/18/10 3:26 PM

Status Completed

Score 100 out of 100 points

Instructions This test consists of 20 multiple choice and/or True/False questions. You must answer all questions. You will be notified at the end of the test whether you passed (hopefully) or failed. 70% of the questions must be answered correctly to pass. If you fail you must read the training module and take the test again. If you pass, you will be given approval from the NU-IACUC and the DLAM to work with research animals at Northeastern University.

Protocol for the Use of Vertebrate Animals in Instruction or Research at Northeastern University (attached)