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Liposomal Combination Drug and siRNA Delivery to Combat Drug-Resistant Ovarian Cancer

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ABSTRACT

Ovarian cancer is the deadliest gynecological malignancy and the fifth leading cause of cancer death overall. Due to lack of early symptoms, ovarian cancer is most commonly diagnosed in the distant stages, drastically reducing the 5 year survival rate from 92% in early stage diagnoses to 29% in advanced stage cases. This large difference is thought to be linked to the high rate of recurrence and development of drug resistance to chemotherapeutics in ovarian cancer patients. First-line therapy includes a combination of tumor resection surgery and chemotherapy regimen including cisplatin, a DNA-alkylating agent, and paclitaxel, a microtubule stabilization agent. However, treatment becomes more complex upon recurrence due to the development of drug resistance. Drug resistance has been linked to many mechanisms, including efflux transporters, dysregulation of apoptosis, autophagy, cancer stem cells, epigenetics, and the epithelial-mesenchymal transition. Due to the wide variety of mechanisms involved in resistance, developing and choosing effective therapies is extremely complex.

Liposomes demonstrate potential as delivery systems to combat drug-resistance in cancer due to their versatility in loading. Liposomes possess the ability to load multiple therapeutics to re-sensitize resistant cancer cells while simultaneously treating those cells with a chemotherapeutic agent. Here, a liposomal carrier for both paclitaxel and siRNA was designed and synthesized to provide a combinatorial therapy to re-sensitize drug-resistant ovarian cancer cells to paclitaxel and thereby increase the efficacy of paclitaxel. A custom siRNA array was developed, and we identified three

possible gene targets, ABCB1, JAK2, and CFLAR, involved in the development of drug resistance in paclitaxel-resistant OVCAR3-TR ovarian cancer cells.

Two combinatorial, cationic liposome delivery systems were designed and synthesized via the lipid film hydration method. Liposomes were characterized for size, surface charge, stability, and loading efficiencies. We demonstrated efficient loading of paclitaxel and protection of bound siRNA in both liposome formulations. Cellular uptake of the liposomes was confirmed using fluorescence microscopy. Overall, the liposomes show promise in loading both paclitaxel and siRNA to target genes involved in drug-resistance development in ovarian cancer cells.

DEDICATION

To my parents, Karin and Jim Miller, I will forever be thankful for the sacrifices you made for my brothers and I in order for us to chase our dreams. Mom, thank you for your unconditional support and teaching me compassion. Dad, thank you for inspiring my dream of cancer research and teaching me the value of hard work. My accomplishments are reflections of your sacrifices and lessons.

To Matthew, your endless encouragement, support, and love has made the challenges in this journey a little less daunting. I am constantly inspired by your bravery and dedication to help others.

And finally, to all those affected by cancer in my life. Each and every one of your battles has inspired me and instilled in me resilience and perseverance. To my father, to my grandparents, to my extended family members, and to Sherry Nicklaus and Leigh Kelley, thank you for teaching me the definitions of courage and grace.

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CHAPTER ONE

INTRODUCTION AND BACKGROUND

Ovarian cancer is the fifth leading cause of cancer death amongst women and the most lethal gynecological cancer [1]. Over 22,200 new diagnoses and 14,000 deaths are expected in 2018 [1]. The high death rate of ovarian cancer stems from late diagnosis and lack of early symptoms of the disease. Around 60% of ovarian cancer diagnoses occur in the distant or advanced stages of the diseases, resulting in a five-year survival rate of only 21-29% [1]. However, in localized stages, the five-year survival rate is 87-93% [1]. Without diagnostic advancements, there is a significant need to improve upon current treatments to accurately target cancer cells and increase the efficacy of delivered therapeutic agents.

Current diagnostics for ovarian cancer utilize cancer antigen 125 (CA-125) serum levels, transvaginal ultrasonography, or imaging methods including computed tomography (CT) or magnetic resonance imaging (MRI) in order to identify potentially cancerous tissue. CA-125 is the most used diagnostic for ovarian cancer because serum levels of CA-125 are typically elevated in more advanced stages of the disease [2]. However, measuring CA-125 levels alone is often not sufficient in diagnosing ovarian cancer without the aid of other methods [2]. The most problematic characteristics of CA-125 diagnostics include low sensitivity in stage 1 disease and low specificity due to increased CA-125 levels in other cancers and benign conditions, like menstruation,

pregnancy, endometriosis, and pelvic inflammatory disease [3]. Imaging methods, including CT and MRI, have proven to be more reliable than CA-125 in diagnosis of the first recurrence of ovarian cancer when overall survival and progression-free survival were compared [4]. Currently, transvaginal ultrasonography is utilized when a patient has suspected masses on their pelvis to identify the origin [5].

Upon diagnosis, the current standard of care for ovarian cancer treatment is a combination of cytoreductive surgery and chemotherapy [6]. The order in which these treatments occur depends on each patient and is ultimately decided upon by the doctor. Combination carboplatin and paclitaxel remains the standard frontline chemotherapy treatment for advanced ovarian cancer [7]. The time between initial resection and initiation of chemotherapy regimens is a vital factor in predicting the overall survival of women with advanced ovarian cancer, with an increased risk of death when the time period surpasses 25 days [8]. However, despite first-line treatment, around 70% of patients relapse [2]. Recurrent cancer is rarely treated with second-line surgery and the focus shifts to chemotherapy instead because more problems arise when treating a relapse. Alterations in the sensitivity of cancer cells to chemotherapeutics may occur based on prior exposure to each type of therapeutic agent; thus, drug resistance may become a challenge in treating recurrent cancer.

1.1 Drug Resistance Development in Ovarian Cancer

The aggressive nature of advanced ovarian cancer is thought to be correlated to the development of resistance to chemotherapeutics to which the patients are exposed. High recurrence rates are problematic, but they provide an even greater challenge when sensitivity to chemotherapy used in primary treatment decreases during secondary treatment. Various factors and pathways influence the sensitivity of cells to drugs; therefore, resistance cannot be reversed by only targeting one specific area or pathway. In ovarian cancer, the main mechanisms of resistance include membrane transporter activity [9], dysregulation of apoptosis [10], autophagy [11], cancer stem cells [12], epigenetics [13], and the epithelial-mesenchymal transition [14]. However, other unknown underlying factors could also be mediating the development of resistant phenotypes.

1.1.1 Membrane Transporter Activity

Membrane transporters are involved in maintaining an equilibrium influx and efflux of molecules in the cell, where a change in this equilibrium can change the phenotype of the cell [15]. Chemoresistant phenotypes arise when a decrease in influx of molecules is coupled with an increased efflux due to increasingly active membrane transporters, reducing the cellular uptake, intracellular accumulation, and anticancer activity of chemotherapeutics. The most studied and well-known culprits of chemoresistance are ATP-binding cassette (ABC) membrane transporters, which eliminate large molecules in order to protect the cell (Fig. 1.1).

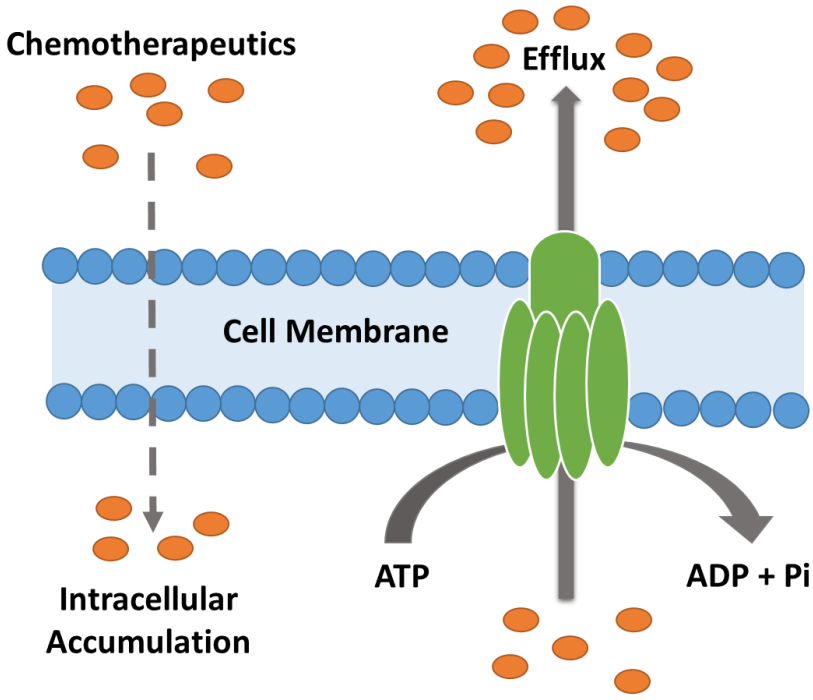


Figure 1.1. ABC membrane transporters and efflux of chemotherapeutics. *A schematic of the efflux mechanism used by ABC membrane transporters (green) embedded in the cell membrane to remove chemotherapeutics (orange) from cancer cells. When membrane transporter activity is upregulated, intracellular accumulation of chemotherapeutics decreases and efflux increases.*

The ABC protein superfamily includes 49 proteins involved in drug resistance from seven subfamilies [16]. Multidrug resistance (MDR) has been linked to upregulation in ABCB1, ABCC1, and ABCG2, as well as many others [17,18]. ABCB1, also known as multidrug resistance protein 1 (MDR1), codes P-glycoprotein (P-gp), a multi-drug membrane transporter [19]. Normal function of P-gp includes the regulation of apoptosis, which can be modified through transporting molecules into or out of the cell

[19]. Overexpression of P-gp led to increased cell protection and decreased stem cell differentiation, thereby, promoting cancerous activity [19].

Expression levels of MDR-related genes in ovarian cancer cells resistant to chemotherapy drugs provide a possible parameter to use to analyze cross-resistance between cell lines. High P-gp expression levels indicate drug resistance in W1 ovarian cancer cell lines resistant to doxorubicin, paclitaxel, and vincristine. BCRP, or ABCG2, expression indicates resistance in topotecan-resistant W1 cells [17]. Cross-resistance discovered between the paclitaxel- and doxorubicin-resistant cell lines could be problematic if doxorubicin is given as a second line treatment for paclitaxel-resistant ovarian cancer. The work of Januchowski and colleagues expands upon earlier studies demonstrating that P-gp, MDR1, MDR2, and LRP drug resistance-associated markers are prognostic factors in ovarian cancer diagnoses [20].

1.1.2 Dysregulation of Apoptosis

Dysregulation of apoptosis pathways in the cell can increase cell survival and avoidance of drug-induced death. Numerous signaling pathways are involved in controlling apoptosis pathways in a cell, including PI3K/Akt and NF-kB. The Bcl-2 family of regulatory proteins mediates cell death by either inhibiting or inducing apoptosis through a balance between pro- and anti-apoptotic proteins. Bcl-2 activates the Akt pathway activity [21], and the NF-kB signaling pathway has been found to be activated by Akt through the presence of mTOR [22]. The upregulation of Bcl-2 anti-apoptotic

proteins is characteristic of cancer and increased proliferation and survival of cancer cells. In paclitaxel-resistant SKOV3 cells, downregulation of Bcl-2 and hTERT increased apoptosis after 6 days of combination treatment with epigallocatechin gallate and sulforaphane [23].

The upregulation of signaling pathways, like PI3K/Akt and NF-kB, has also been found to play an important role in developing resistance to chemotherapy. The development of resistance to cisplatin, paclitaxel, and bevacizumab in ovarian cancer cell lines has been linked to activation of the Akt pathway. In A2780 cisplatin-resistant and CAOV3 cells, blockage of the Akt pathway via gemcitabine treatment inhibited cisplatin-induced Akt activation, and co-treatment with cisplatin completely inhibited the invasion of both cell lines in matrigel [24]. Also, overexpression of Akt in cisplatin-resistant A2780 cells led to an increase in cells escaping the natural killer cells of the immune system and an increase in the expression of anti-apoptotic proteins, CIAP-1 and -2 [25].

In paclitaxel-resistant A2780, SKOV3, and MPSC1 cells, the downregulation of the Akt and NF-kB pathways through tectorigenin treatment resulted in sensitization of cells to paclitaxel and enhanced growth inhibition, compared to paclitaxel treatment alone [26]. Tectorigenin also inhibited the nuclear translocation of NF-kB and the expression of many NF-kB-dependent genes known to be involved in drug resistance, including FLIP, XIAP, Bcl-2, and Bcl-xL [26]. Akt pathway activation in endothelial ovarian cancer cells,

OVCAR-3 and SKOV3 cell lines, demonstrated characteristics of a niche for residual cancerous tissue with resistance to bevacizumab [27].

1.1.3 Autophagy

In addition, autophagy plays a role in the sensitivity of cancer cells to chemotherapy. Autophagy is used by cells to aid in survival under nutrient starvation or other cellular stresses through digestion of their own cellular components to maintain energy. Cells can use autophagy as a protective mechanism against stresses, like the damaging effects of chemotherapy, resulting in decreased anticancer response [28]. Cisplatin-resistant ovarian cancer cells, OV433-CR, demonstrated increased activation of the ERK pathway, which promotes autophagy induction. Inhibition of ERK/MAPK activity decreased autophagy and increased sensitivity of cells to cisplatin-induced death [29]. The inhibition of autophagy in cisplatin-resistant SKOV3 and A2780 cells also increased apoptosis via the overexpression of miR-152, an autophagy-regulating miRNA involved in cisplatin resistance [30].

Reactive oxygen species (ROS) maintenance is vital in all cells in order to respond to environmental changes. When ROS production increases past an allowable threshold, the expected outcome is cell death. However, there is a suspected relationship between autophagy and ROS production. Autophagy is believed to be a protective mechanism when dealing with stress and ROS generation in ovarian cancer cells, increasing as ROS

increases [31]. This extended protection may provide cancer cells a longer time to repair DNA damage from chemotherapy, thus decreasing the efficacy of chemotherapeutics.

1.1.4 Cancer Stem Cells

Autophagy also plays a role in mediating chemoresistance of cancer stem cells (CSCs) [11]. A recent discovery proved that cancer stem cells are involved in the development of drug resistance due to their stem cell-like phenotype (Fig. 1.2). Particularly, their ability to resist apoptosis by DNA damage prolongs the survival of cancer stem cells in cancer tissue [10]. Cancer stem cell phenotypes include characteristic evasion of apoptosis in hypoxic conditions, increased growth potential [32], and increased CSC marker aldehyde dehydrogenase isoform 1 activity [33]. CSCs from the HO8910 cell line demonstrated faster growth and enhanced survival potential in a 3D cell culture as well as increased tumorigenicity in a xenograft mouse model compared to the parental HO8910 cells. Also, CSCs showed increased resistance to 5-fluorouracil, cisplatin, and carboplatin as well as high expression of MDR genes, ABCB1, ABCG2, MMP2, and MMP9 [32]. Recent efforts have explored the mediation of drug resistance via cancer stem cell elimination. In SKOV3-spheroid cells, knockdown of KLF5 is a promising target to eliminate CSCs, shown by a decrease in survivin expression and increase in sensitivity to paclitaxel and cisplatin [34].

Aldehyde dehydrogenase isoform 1 (ALDH1) is an emerging marker, which when used in combination with other stem cell markers, can identify populations of cancer

stem cell populations in ovarian cancer. ALDH1 activity, measured by fluorescently-labeled ALDH1, showed ALDH1-bright cells were more common in ES-2 and CP70 ovarian cancer cell lines and showed increased chemoresistance compared to SKOV3, OVCAR3, and A2780 ovarian cancer cells that did not express high levels of ALDH1 [35]. Tumor samples from 84 subjects indicated the association of cancer stem cell marker ALDH1 with chemoresistance and poor clinical outcomes in epithelial ovarian cancer patients [35].

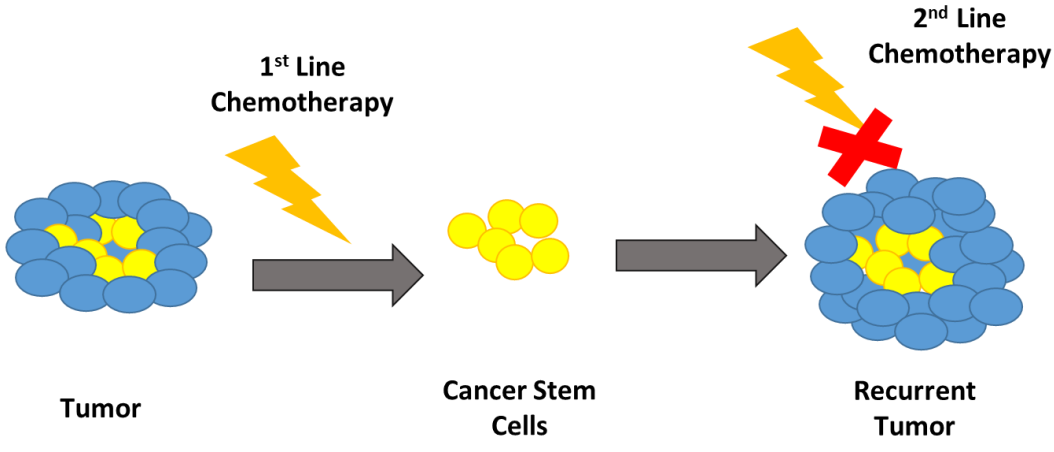


Figure 1.2. Cancer stem cells role in recurrence. *Cancer stem cells (CSCs) survive initial chemotherapy due to increased resistance toward chemotherapeutics compared to other cancer cells. CSCs then proliferate into a recurrent tumor, which can develop resistance toward second-line chemotherapy creating difficulties in the treatment of recurrent cancer.*

1.1.5 Epigenetics

Epigenetics and other changes in the tumor microenvironment, including DNA methylation, histone modification, and microRNA expression, have become greater focuses in the drug resistance mechanisms of ovarian cancer over the last decade [13]. Current studies are exploring various miRNAs and DNA modifications in order to exploit specific pathways involved in the development of chemoresistance in ovarian cancer [36,37]. DNA methylation involves the addition of a methyl group to DNA without changing the sequence, but when methylation occurs in the promoter region of a gene, the gene can be repressed. Hypermethylation of genes is common in drug-resistant ovarian cancer cells, including A2780 cells resistant to cisplatin. In a study of the methylome and gene expression in wild-type and resistant A2780 cells, 13 out of 41 genes were consistently upregulated in the A2780/CP70 cisplatin-resistant ovarian cancer cells compared to parental A2780 ovarian cancer cells, where initially methylated-MLH1, a DNA repair gene, played a role in re-sensitizing cells to cisplatin when demethylated *in vitro* [38]. DNA methylation has been found to interfere with cisplatin-sensitivity in both A2780 and OVCAR3 cells through overexpression of MAFG, a DNA binding transcription factor. MAFG expression can be directly regulated by miR-7, creating a potential target for mediating drug resistance with MAFG and a factor for prognosis in the methylation status of miR-7 [39].

Furthermore, epigenetic silencing, or the process of non-mutational change in gene expression that can be passed down from generation to generation in cells [40],

continues to reveal prognostic biomarkers and potential therapeutic targets in drug-resistant ovarian cancer cells. Epigenetic silencing of miR-199b-5p in cisplatin-resistant A2780 and C13* cells demonstrated activation of the JAG1-mediated Notch1 signaling pathway, encouraging the development of chemoresistance. Downregulation of miR-199b-5p is associated with poor clinical outcomes in ovarian cancer patients; thus, microRNA levels could be a prognostic factor and JAG1 a therapeutic target in cisplatin-resistant ovarian cancer [41].

Similarly, silencing of miR-130b, in cisplatin-resistant A2780 and paclitaxel-resistant A2780 and SKOV3 cells, promote drug resistance through colony stimulating factor 1 (CSF1) targeting. MiR-130b silencing was inversely related to DNA methylation and resistance development. Thus, CSF1 knockdown is a potential treatment to re-sensitize cells to chemotherapy and improve clinical outcomes [36]. Epigenetic silencing of BLU, a tumor suppressor gene, in paclitaxel-resistant ovarian tumor samples led to resistance development, and BLU methylation indicated a shorter progression-free and overall survival in patients. BLU methylation levels may be a promising prognostic biomarker in advanced serous ovarian carcinoma patients [42].

1.1.6 Epithelial-Mesenchymal Transition

Paclitaxel-resistance in A2780 cells induces morphological changes consistent with epithelial-mesenchymal transition (EMT) and an increase in EMT-related biomarkers confirmed by RT-PCR. Activation of the PI3K/Akt pathway is vital to EMT;

thus, when PI3K was inhibited, paclitaxel sensitivity increased and cytoskeleton morphology shifted toward an epithelial phenotype [43]. In A2780 cells resistant to cisplatin, Snail and Slug, EMT transcription factors, contribute to resistance as evidenced by cell morphology changes, from spherical epithelial cells to spindle-like cells with formation of pseudopodia. A whole transcriptome microarray revealed overexpression of E-cadherin transcriptional repressors, Slug, Snail, TWIST2, and ZEB2, and downregulation of E-cadherin in A2780-cis cells compared to the parental A2780 line. Knockdown of Snail and Slug reversed the EMT phenotype and reduced resistance to cisplatin [44]. Snail, Slug, and TWIST upregulation were also linked to platinum resistance and EMT phenotypes in stage III-IV epithelial ovarian tumor biopsies. Activation of the TGF-β pathway led to activation of EMT, and relapse in 70% of patients was associated with increased activation of the TGF-βR2 pathway [45].

CHAPTER TWO

TARGETED DELIVERY SYSTEMS TO COMBAT DRUG RESISTANCE IN OVARIAN CANCER

2.1 Delivery Strategies for Overcoming Drug Resistance

In order to combat different mechanisms of drug resistance development, a variety of therapeutics and delivery strategies have been studied to target and reverse chemoresistance in cancer cells. Currently, in ovarian cancer, the most common methods of reversal are treatment with siRNAs to manipulate the dysregulated genes and inhibitors to target the imbalance of efflux and influx in cells. Anti-resistance therapeutics aim to re-sensitize the cancer cells to chemotherapy drugs so further treatment can be administered to increase progression-free survival of cancer patients.

In order to deliver siRNAs, inhibitors, and chemotherapeutic agents, alone or in combination to chemo-resistant cancer cells, many targeted delivery systems have been developed. Current methods include a variety of nanocarriers to increase the efficacy of chemotherapy drugs and other therapeutic agents while also decreasing negative side effects, like systemic toxicity. The nanocarriers are developed from polymers, lipids, peptides, and other inorganic molecules, as depicted in Figure 2.1.

The delivery systems used for combatting treatment in drug-resistant ovarian cancer are classified into two groups, single agent delivery or combination delivery. Single-agent delivery systems are used to increase the targeting and efficacy of one therapeutic agent. Typically, a drug carrier is used to increase cellular uptake by cancer

cells compared to free drug treatment and decrease off-target effects, limiting the damage to healthy cells. The goal of combination treatment is to maximize the synergistic ability of two compounds to increase anticancer effects while also decreasing adverse side effects of the free therapeutics.

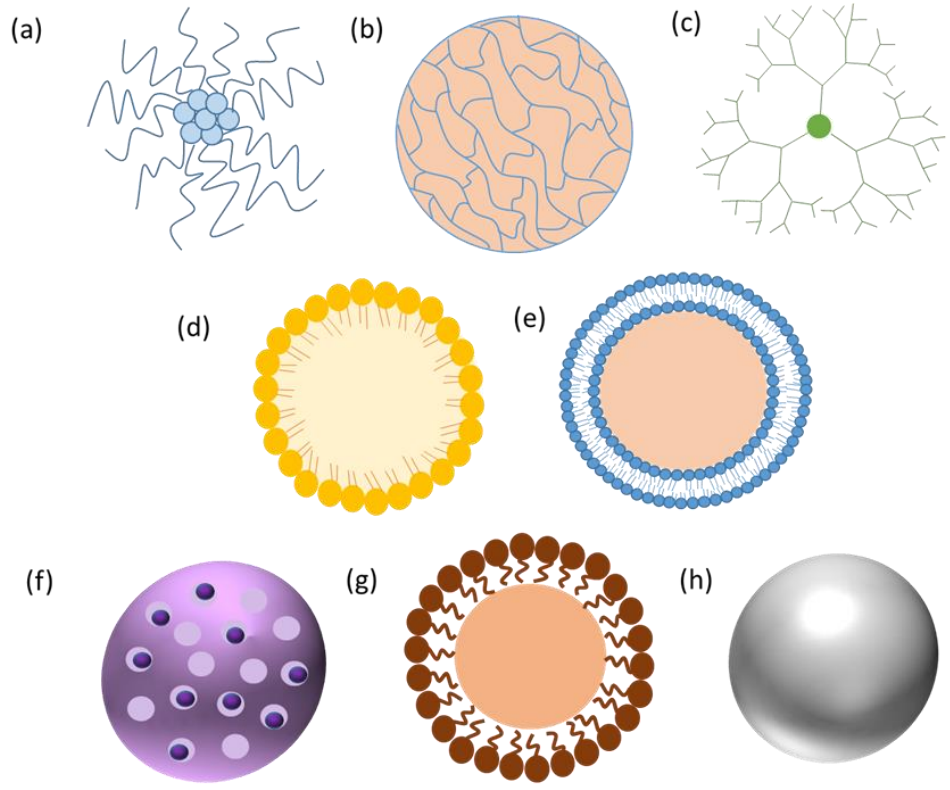


***Figure 2.1*. Nanoparticle Structures.** *Structure of various nanoparticles commonly used in targeted therapies to treat ovarian cancer. (a) Polymeric micelle, (b) Hydrogel, (c) dendrimer, (d) solid lipid nanoparticle, (e) liposome, (f) mesoporous silica nanoparticle, (g) nanoemulsion, and (h) metal nanoparticle.*

Nanoparticles for single-agent use have already performed well in clinical trials. One of the most common, liposomal doxorubicin, is an FDA-approved chemotherapy drug and is widely used in cancer treatment. The benefit of the liposomal form of the drug is increased circulation time compared to free doxorubicin due to protection against destruction by the immune system [46]. Similar to this idea, many current studies seek to improve the efficacy of common chemotherapeutic agents through development of targeted therapies.

2.1.1 Lipid

Lipid-based delivery systems are advantageous due to their drug loading versatility, utility for combination therapies, ability to modify surface characteristics, and decreased toxicity compared to free therapeutics. Drug loading in lipids, especially liposomes, is optimal because hydrophilic and hydrophobic drugs can both be loaded. The hydrophobic tails of lipids are sufficient environments for hydrophobic drugs to reside, while hydrophilic drugs can be loaded into the center of liposomes. In order to combat drug resistance in ovarian cancer cells, the versatility of lipid delivery systems can elicit greater anticancer effects through targeting activity of efflux transporters, cancer stem cells (CSCs), metabolism dysregulation, and hypoxic environments.

Increasing intracellular accumulation of therapeutics remains one of the main goals of nanoparticle delivery systems, thereby decreasing off-target toxicity and

increasing the efficacy of the delivered therapeutics. In drug-resistant ovarian cancer, increasing uptake of therapeutics is necessary to increase the concentration of drugs ultimately reaching the targeted tumor site. Solid lipid nanoparticles loaded with paclitaxel and doxorubicin have exhibited increased cellular uptake into A2780res and SKOV3TR drug-resistant ovarian cancer cells, increasing the anticancer effects of the treatment compared to free drug treatment alone [47,48].

Increase in intracellular uptake has also been demonstrated through the use of surface-modified liposomes for targeting ovarian cancer cells. Folic acid is used to target folate receptors, which are overexpressed on the surface of ovarian cancer cells. One study showed paclitaxel-loaded nanoparticles decorated with folic acid demonstrated increased cellular uptake and inhibited growth and doubling time in SKOV/TAX cells compared to undecorated paclitaxel nanoparticles [49]. The addition of polyethylene glycol (PEG) to lipid nanoparticles also increases the stealth and uptake of the particles into drug-resistant SKOV3 ovarian cancer cells by evading efflux transporters [50].

Due to a relationship between CSC activity and aggressiveness of tumors, decreasing activity of CSCs has gained increasing popularity as a method to target drug resistance development in ovarian cancer cells. A novel, liposomal form of paclitaxel has been found to suppress CSC sub-populations, from drug-resistant ES-2 ovarian cancer cells, when delivered intraperitoneally compared to intravenously administering paclitaxel alone, as indicated by decreased levels of CSC-markers, CD44, CD24, and CD133 [51]. Metabolic reprogramming from glycolysis to oxidative phosphorylation was

also induced through reactivation of p53 in resistant ES-2 cells resulting in better control of tumor growth [51].

The ability to deliver treatments in combination provides a promising method to synergistically enhance the efficacy of therapeutics, especially those used to treat drug-resistance in ovarian cancer. Crosslinked multilamellar liposomes developed to deliver carboplatin and paclitaxel, two of the most common intravenous chemotherapeutics, were shown to increase anticancer effects in NCI/ADR-RES ovarian cancer cells *in vitro* and in OVCAR8 ovarian cancer xenograft mice models [52]. Also, *in vitro* studies showed decreased cancer stem cell activity in NCI/ADR-RES cells, where ALDH+ cells decreased from 57.3% in the untreated control to 30.2% in the resistant cells treated with the combination liposomes [52].

Combination therapies have also been used to mediate resistance by avoiding efflux of chemotherapeutics by p-gp membrane transporters and hypoxia-induced resistance on ovarian cancer cells. Liposomes containing paclitaxel and tariquidar, a third-generation p-gp inhibitor, blocked proliferation and induced G2-M phase arrest in SKOV3-TR and HeyA8-MDR drug-resistant ovarian cancer cells *in vitro*. The combination liposomes reduced tumor weight to 16.9% from 43.2%, in mice xenografts with orthotopic HeyA8-MDR tumors compared to paclitaxel-only liposomes [53]. To address resistance related to hypoxia, liposomes containing doxorubicin and antisense oligonucleotides (ASO) targeting HIF-1α mRNA were delivered to A2780/AD doxorubicin-resistant ovarian cancer cells *in vitro* and athymic nu/nu mice with

A2780/AD subcutaneous tumors *in vivo* [54]. Confocal microscopy and *in vivo* fluorescence imaging identified intracellular localization of the combinatorial liposomes, and RT-PCR and IHC-staining showed decreased expression of HIF-1α when compared to free or liposomal doxorubicin without ASOs. Increased cell death signals were shown through expression of apoptotic proteins, BAX, CASP3, CASP9, and P53, as well as decreased expression of VEGF, identifying a decrease in tumor vascularization [54].

The various modifications of liposomal delivery of therapeutics in order to increase targeting, intracellular uptake, and efficacy of drug-resistant cancer treatments are promising in that the delivery systems can be modified to exploit the different mechanisms of resistance and overexpression of receptors in cancers on an individual basis. Personalized cancer treatment could lead to higher remission rates and lower incidence of drug resistance in secondary treatment. Liposomal therapeutic options show promise in future applications of targeted therapies as few treatments have reached clinical trials.

2.1.2 Polymer

Polymer nanoparticles are the most common synthetic delivery systems utilized in targeted treatments for drug-resistant ovarian cancer. Similar to lipids, polymers possess the ability to increase intracellular accumulation of therapeutics, increase efficacy of treatments via surface-conjugated targeting moieties, deliver synergistic combination agents, and sustain the release of therapeutics. Increased accumulation

and efficacy of paclitaxel and cisplatin in drug-resistant A2780 ovarian cancer cells resulted from single-agent delivery in poly (n-butylcyanoacrylate) nanoparticles compared to free paclitaxel or cisplatin treatment [55,56].

Similar to surface modifications in lipids, the addition of targeting moieties in polymer-based nanoparticles has been shown to increase uptake of therapeutics thereby increasing anticancer effects. Folate-targeted triblock copolymer nanoparticles delivered TLR4 siRNA and paclitaxel to drug-resistant SKOV3 ovarian cancer cells *in vitro*, to re-sensitize cells to paclitaxel and mediate TLR4-driven resistance [57]. Folate has also been used to increase uptake of paclitaxel-loaded PLGA nanoparticles [58]. In addition to folate, biotin is a common targeting ligand used for ovarian cancer due to its affinity for EGF receptors, which mediate resistance via the epithelial-mesenchymal transition. Biotinylated-PAMAM dendrimers exhibited a ten-fold increase in intracellular accumulation of cisplatin in A2780-CP70 cisplatin-resistant ovarian cancer cells compared to free cisplatin treatment alone [59]. Hyaluronic acid is an important targeting moiety for ovarian cancer cells as it targets CD44, a cancer stem cell and drug resistance marker. HA-targeted PEI/PEG nanoparticles were developed to deliver MDR1 siRNA and paclitaxel to OVCAR8TR cells *in vitro* [60]. MDR1 siRNA knockdown increased sensitivity of cells to paclitaxel by decreasing p-gp activity and increased cellular uptake by targeting CD44, compared to untargeted nanoparticles [60].

Combinatorial delivery of anticancer therapeutics is an advantage of the versatility of polymer-based nanoparticle design. Like lipids, paclitaxel and p-gp inhibitor

molecules, tacrolimus and borneol, have been delivered in combination to elicit a greater anticancer effect than either of the drugs alone, by decreasing efflux and increasing intracellular accumulation of paclitaxel in A2780 paclitaxel-resistant ovarian cancer cells *in vitro* [61,62]. The combination of two molecular therapeutics was also demonstrated by co-encapsulation of doxorubicin and rhein in vitamin E-PEG nanomicelles and of paclitaxel and lonidamine in PCL-EGFR targeted NPs. In comparison to free drug treatment, both types of polymer nanoparticles exhibited increased tumor targeting and cytotoxic activity when studied *in vitro* on drug-resistant SKOV3 ovarian cancer cells [63,64]. Combinatorial delivery also includes simultaneous delivery of multiple siRNA, as demonstrated by the delivery of MDR1 and PKM2 siRNA in HA-targeted PEI-PEG NPs to SKOV3TR ovarian cancer cells. The synergistic ability of the dual silencing of multidrug resistance protein and pyruvate kinase isoform 2 showed a 1.3 fold decrease in IC50 value of paclitaxel when drug delivery followed siRNA delivery in SKOV3TR cells, but there was no significant difference in IC50 when delivered to wild-type SKOV3 cells [65].

The most advantageous quality of polymer-based sustained release of therapeutics due to stimuli-responsive or biodegradable characteristics. Sustained release allows the drug to be exposed to the cancer cells for a longer period of time, which leads to increased efficacy of the therapy. pH-responsive polymer nanoparticles exploit the acidic environment of tumors in order to maintain a sustained release of therapeutics. Poloxamer 407 vitamin E-TPGS mixed micelles functionalized with folate

exhibited decreased efflux of doxorubicin in doxorubicin-resistant SKOV3 ovarian cancer cells, where accumulation increased 24% with the addition of poloxamer 407 and 264% with the addition of TPGS, when compared to efflux rates of untreated Dox-resistant SKOV3 cells [66]. The increased accumulation of doxorubicin led to an increase in doxorubicin binding to DNA, increasing cytotoxicity compared to free doxorubicin.

Biodegradable polymers, like polylactic acid (PLA), polyglycolic acid (PGA), or a combination of the two, PLGA, promote sustained release of therapeutics through degradation of the delivery systems. PLGA nanoparticles developed with a poly-dopamine and PEG coating prevented rapid release and clearance of paclitaxel in a BR5FVB1-Akt murine ovarian cancer model [67]. Mice treated with the PLGA NPs showed an 8-fold increase in paclitaxel concentration in the peritoneal cavity than mice treated with free paclitaxel, while the systemic circulation of paclitaxel was three times higher in the free paclitaxel treatment than the nanoparticle treatment [67]. PLGA nanoparticles were shown to sustain release of diphtheria toxin-subunit A suicide genes in chemo-resistant ovarian tumor-bearing SCID mice, inhibiting tumor growth [68]. Chitosan-coated PLA NPs mediated release of p62 siRNA, β5 plasmid, and cisplatin, in C13 cisplatin-resistant ovarian cancer cells *in vitro*, as demonstrated by a 26% decrease in IC50 of cisplatin, compared to only 15.2% with delivery of sP62 alone or 8% with pβ5 [69].

Overall, polymer nanoparticles possess many desirable qualities for targeted therapy options in the reversal and treatment of drug-resistant ovarian cancer. While

qualities like sustained release and specific stimuli response favor polymer systems over lipids, most of the concepts of the two types of delivery systems remain similar. Thus, the addition of targeting ligands, the potential to co-encapsulate therapeutics, and the increase in intracellular accumulation and cytotoxicity are all important to developing a successful targeted therapy for treatment of drug resistance in ovarian cancer.

2.1.3 Inorganic

Beyond lipids and polymers, other nanoparticles have become popular in studying reversal of drug resistance in ovarian cancer, specifically, silica and metal nanoparticles. Mesoporous silica nanoparticles (MSNs) are desirable for the delivery of siRNA specifically to perinuclear targets *in vitro* and *in vivo*. MSNs have been utilized to deliver and knockdown TWIST siRNA to decrease tumor burden in mice and target the epithelial-mesenchymal transition when treated concurrently with cisplatin compared to cisplatin alone [70]. Combination therapy has been attempted with MSNs as well, delivering doxorubicin and Bcl-2 siRNA to control and decrease apoptosis-related resistance. The off-target release of doxorubicin was minimal and the intracellular localization of the agents increased indicating successful management of resistance and increased efficacy of doxorubicin [71].

Metal nanoparticles, such as iron oxide, silver, and copper, are modified with organic materials and loaded with therapeutic agents to treat drug-resistant ovarian cancer. Iron-oxide nanoparticles as delivery systems for doxorubicin have been shown

to sustain slow release of DOX at slightly acidic pHs and avoid drug efflux pumps [72]. In addition, release of doxorubicin from mesoporous iron-oxide nanoparticles has also been studied for combination therapy with hyperthermia, where DOX burst release occurs upon magnetic heating [73]. Lycopene-reduced graphene oxide-silver nanoparticles were developed for co-delivery with trichostatin A. The combined effects of these two agents decreased cell viability in a dose-dependent manner through an increase in apoptosis due to DNA fragmentation and double strand dysfunction [74]. Similarly, phosphorous dendrimers were modified with copper to target BAX pathways. The copper-phosphorus structures increased apoptosis by increasing activation of the BAX signaling pathway [75]. The benefit of using a metal nanoparticle is that therapeutic agents may not be needed as some metals elicit anticancer effects alone.

2.1.4 Theranostic

Lastly, some delivery systems aim to improve treatment and diagnostic abilities in a single therapy by personalization of therapy to meet patient's varying needs. These methods are designated as theranostic approaches in which nanoemulsions of chemotherapeutic drugs are delivered and imaged to predict the success of the therapy in different patients [76]. Nanoemulsions load platinum drugs and doxorubicin, and they are functionalized with folate in order to increase on-target localization of the particles. Targeted nanoemulsions have demonstrated increased cytotoxicity due to efficient cellular uptake than treatment with free chemotherapeutic drugs [77,78].

2.2 Current Clinical Trials

Overall, the versatility of nanoparticle delivery systems in the reversal and treatment of drug-resistant ovarian cancer seems to be a promising quality; however, targeted therapy research is cutting-edge and there has yet to be much translation from the research setting into the clinical setting. Clinical trials for ovarian cancer currently focus on options improving traditional chemotherapy drugs to treat recurrent or resistant cancer through modifications in dosing regimens, differing combinations of drugs, and changing the method of delivery of the drugs, as laid out in Table 2.1. The more recent targeted therapies have yet to become popular in clinical trials due to poor translation of treatments, but a few have been tested as second, third, or last lines of therapy.

Table 2.1.Clinical Trials for Ovarian Cancer Treatment. *Recent clinical trials for ovarian cancer characteristics and phases, with accompanying results.*

Drug(s) In trial	Phase	Delivery Method/Regimen	Drug mechanism(s)
Paclitaxel	I	Novel liposomal platform[79]	Paclitaxel stabilizes microtubules during mitosis and leads to mitotic arrest
Pertuzumab with topotecan or paclitaxel	I	IV pertuzumab every 3 weeks with either topotecan every 3 weeks or paclitaxel weekly[80]	Pertuzumab inhibits ligand-dependent HER2-HER3 dimerization and reduces signaling through PI3K pathway; Topotecan inhibits topoisomerases to cause double strand DNA breakage and cell death
Paclitaxel and carboplatin	II	Cremophor-free polymeric micelles of paclitaxel IV with carboplatin every 3 weeks[81]	Carboplatin inhibits DNA synthesis by causing intra- and inter-strand crosslinking in DNA
Temsirolimus	II	Weekly IV infusions[82]	Temsirolimus inhibits mTOR leading to cell cycle arrest in the G1 phase and inhibiting tumor angiogenesis
Olaparib	II	Oral capsules[83]	Olaparib inhibits PARP enzymes and DNA repair
Pazopanib	II	Daily pazopanib with or without weekly paclitaxel[84]	Pazopanib inhibits tyrosine kinase for antiangiogenic activity
Albumin-bound paclitaxel	II	Cremophor-free nanoparticle of albumin-stabilized paclitaxel IV[85]	Paclitaxel stabilizes microtubules during mitosis and leads to mitotic arrest
Paclitaxel	II	Lipid core nanoparticles weekly IV infusion[86]	Paclitaxel stabilizes microtubules during mitosis and leads to mitotic arrest
Sorafenib with paclitaxel and carboplatin	II	Paclitaxel and carboplatin IV infusions with or without twice daily sorafenib orally[87]	Sorafenib inhibits tyrosine kinases in Raf/MEK/Erk pathways and induces autophagy

2.3 Conclusion and Future Steps

Due to the aggressive malignancy, the limited early diagnostics, and the potential of drug resistance in ovarian cancer, there is a significant need for increased efficacy and specificity in treatment. Without much-needed advancements in diagnostic methods and patient compliance for screenings, the only option to limit the negative effects of ovarian cancer is to improve treatment success. Reversal of drug resistance is currently at the forefront of ovarian cancer research, showing promising results *in vitro* and *in vivo*. Numerous inhibitors and siRNA have been successful in re-sensitizing cells to chemotherapy, including P-gp inhibitors, PARP inhibitors, and siRNA to modify expression in signaling pathways important for cancer proliferation and progression. The success of these inhibitors, while promising, remains low due to the complexity and interdependence of mechanisms protecting the cells from chemotherapeutic agents.

Nanoparticles have become an increasingly popular option to deliver drug resistance reversal agents in combination with chemotherapeutic agents to achieve greater anti-cancer effects without increasing the danger of therapy to patients. Nanoparticles can be developed from lipids, polymers, inorganic materials, or a combination of the molecules in order to decrease toxicity of the drugs being delivered. However, the significance of nanoparticle delivery systems actually lies in the ability to modify particles to increase targeting specificity, increase intracellular accumulation, and decrease premature clearance of the drugs before reaching the targeted area. Translation from *in vivo* studies to clinical trials has been one of the largest obstacles in

producing new treatment options for current ovarian cancer patients. Recently, a few nanoparticle-based therapies have made their way into clinical trials, with hopes that as more research is done on targeted therapies in ovarian cancer models, the number of potential treatments reaching clinical trials will increase.

CHAPTER THREE
RESEARCH AIMS

3.1 OBJECTIVES

Ovarian cancer is the deadliest gynecological malignancy and the fifth leading cause of cancer-related death overall. Around 60% of ovarian cancer cases are diagnosed in the distant stages, where the survival rate is only 21-29% [1]. The aggressiveness of the disease lies in the high rates of recurrence and the development of drug resistance after initial treatment. 70% of ovarian cancer patients will experience a recurrence of their cancer [2], where the development of resistance to prior chemotherapy regimens creates difficulty in determining an appropriate second-line regimen. Currently, the standard treatment of a recurrence is to choose a chemotherapy drug that has not shown cross-resistance with the first regimen. However, some tumors will develop resistance to many chemotherapy drugs. Thus, there is a significant need for therapies to increase the survival rate of drug-resistant ovarian cancer.

The objective of this research is to identify potential gene targets involved in the development of paclitaxel-resistance in ovarian cancer cells. Initial treatment of ovarian cancer includes a regimen consisting of cycles of a platinum-based DNA-alkylating agent, carboplatin or cisplatin, and paclitaxel, a microtubule stabilization agent [88]. Therefore, the effectiveness of secondary treatments containing paclitaxel decreases if paclitaxel-

resistance has developed. The overall goal of this research is to synthesize and characterize cholesterol- and cholesteryl hemisuccinate (CHEMS)-based liposomal combination delivery systems for siRNAs, specific to gene targets with identified involvement in drug resistance in ovarian cancer cells, and paclitaxel to synergistically increase the efficacy of paclitaxel in drug-resistant ovarian cancer.

Through completion of the following aims, I will collect the data necessary to identify potential gene targets involved in the development of paclitaxel-resistance, as well as synthesize and characterize two liposomal carriers with the ability to encapsulate and deliver paclitaxel and siRNAs to drug-resistant ovarian cancer cells. Comparison of the two liposomal delivery systems will determine the potential of substituting CHEMS for cholesterol to incorporate a pH-sensitive compound without compromising the loading and delivery ability of the liposomes.

Aim 1: Identify gene targets involved in the development of paclitaxel-resistance in ovarian cancer cells with a siRNA array. We will design a custom array of siRNAs specific to genes involved in apoptosis, the cell cycle, and drug resistance development in cancer. Through siRNA transfection with the custom array followed by paclitaxel treatment, we will identify the siRNAs responsible for the greatest anticancer effect in paclitaxel-resistant ovarian cancer cells. We hypothesize that the siRNAs which demonstrate the greatest anticancer effect in combination with paclitaxel when compared to treatment with paclitaxel alone, will correlate to genes involved in the development of resistance in ovarian cancer cells.

Aim 2: Synthesize and characterize cholesterol- and cholesteryl hemisucciante-based liposomal carriers, and compare their ability to encapsulate and deliver both paclitaxel and siRNA to drug-resistant ovarian cancer cells. We will synthesize cationic, cholesterol-based liposomes and cholesteryl hemisuccinate-based liposomes and examine the size, surface charge, encapsulation efficiency, siRNA binding, stability, and cellular uptake of the liposomes. We hypothesize that both types of liposomes will be positively charged and uniform in size. We also hypothesize that the liposomes will be stable when paclitaxel is loaded in the hydrophobic region of the lipid bilayer and negatively-charged siRNA is bound to the cationic lipid components. We believe that cellular uptake will be achieved through endocytosis of the cationic liposomes. We believe that cholesteryl hemisuccinate can be substituted for cholesterol in the synthesis of the liposomes without compromising the encapsulation ability or stability of the liposomes.

3.2 Approach

Aim 1:

After determining the IC50 value of paclitaxel in OVCAR3 and OVCAR3-TR, wild-type and drug-resistant ovarian cancer cells, we will evaluate the viability of OVCAR3-TR cells after transfection with the custom siRNA array and 15 nM paclitaxel treatment using an MTS assay. Gene targets will be chosen based on the siRNAs demonstrating the greatest anticancer effects compared to treatment with paclitaxel alone. These chosen

siRNAs will be used as therapeutics bound to the liposomes to re-sensitize cells when delivered in combination with paclitaxel.

Aim 2:

After identification of the gene targets, we will synthesize and characterize the liposomes using dynamic light scattering to determine size and polydispersity index of the particles, and micro-electrophoresis with phase analysis light scattering to find the zeta potential. Then, we will examine the encapsulation efficiency of paclitaxel in the liposomes when loaded with 2.5 μg/mL of paclitaxel. The amount of drug loaded will be determined with high-performance liquid chromatography (HPLC).

After determining paclitaxel loading, we will evaluate the binding/loading of siRNA to the liposomes to quantify the amount of siRNA available for knockdown upon delivery into the drug-resistant cells. Liposomes loaded with fluorescent non-targeting siRNAs allow the use of fluorescence spectrophotometry to determine the amount of siRNA bound to liposomes. siRNA binding will be further studied with a gel shift assay to ensure binding and protection of siRNAs and stability of loaded liposomes.

Cellular uptake will be visualized using immunofluorescence, fluorescent labeling of early endosomes, nuclear counterstaining, and fluorescent siRNA and Nile Red loaded liposomes. OVCAR3-TR cells treated with 100 μM of liposomes will be fixed at 2 hour time points, and fluorescence microscopy will be used to evaluate the intracellular localization of the liposomes.

CHAPTER FOUR

IDENTIFYING GENE TARGETS IN THE DEVELOPMENT OF PACLITAXEL RESISTANCE IN OVARIAN CANCER CELLS

Due to lack of early symptoms, a majority of ovarian cancer diagnoses occur in the distant stages, leading to a low five-year survival rate of about 21-29% [1]. Low survival rates are believed to be linked to recurrence of cancer and development of drug resistance after initial treatment, which involves tumor resection surgery and a chemotherapy regimen, usually including cisplatin or carboplatin, both platinum-based DNA-alkylating drugs, and paclitaxel, a microtubule inhibitor [6,88]. Without advances in current diagnostic methods, there is a significant need to find a therapy that is effective on drug-resistant ovarian cancer.

The underlying intracellular mechanisms responsible for resistance are complex and intertwined, making it difficult to treat the disease by targeting only one mechanism. Thus, combination therapy has been heavily researched as a treatment option for drug-resistant cancer. Combination therapies allow two or more therapeutics to be delivered simultaneously, with the goal of synergistically increasing the efficacy of either treatment alone [89,90]. Therapeutics in combination therapies include chemotherapeutics, inhibitor molecules, and siRNAs. Controlling expression of genes that are believed to be involved in the development of drug resistance with siRNAs could be vital in treating recurrent cancer and re-sensitizing cancer cells to cisplatin, carboplatin, and paclitaxel [91,92]. siRNAs delivered into the cell form an RNA-induced

silencing complex with proteins and specifically bind to target mRNA, interrupting translation and degrading the mRNA [93]. siRNAs can target the various pathways involved in the development of drug-resistance in ovarian cancer by knocking down overexpressed genes coding for p-gp membrane transporters, apoptosis, autophagy, cancer stem cells, epigenetics, and the epithelial-mesenchymal transition. Combination therapies can elicit increased anticancer effects by using siRNAs or inhibitors to re-sensitize cells to chemotherapy drugs and treat the cells simultanously with delivered chemotherapeutics.

Here, a custom siRNA array of gene targets involved in apoptosis and the cell cycle was designed in order to identify genes that may be involved in the development of drug resistance in ovarian cancer cells. The anticancer effect of siRNA transfection followed by paclitaxel treatment was studied in OVCAR3-TR cells. We found five siRNAs had significantly greater anticancer effects compared to treatment with paclitaxel alone.

4.1 MATERIALS AND METHODS

4.1.1 Materials

The custom siRNA array was designed by including siRNAs from apoptosis and cell cycle gene libraries, as well as siRNAs recently shown in literature to play a role in development of drug resistance, and purchased from Dharmacon (Lafayette, CO). Transfection supplies including 5x siRNA buffer, GAPDH siRNA, and Non-targeting #5 siRNA were also purchased from Dharmacon. Lipofectamine RNAiMAX and OPTIMEM

media were both acquired from ThermoFisher Scientific (Waltham, MA) as well as cell culture reagents, fetal bovine serum (FBS), penicillin/streptomycin, and trypsin. The OVCAR3 cell line was obtained from ATCC (Manassas, VA) and the OVCAR3-TR cell line was a generous donation from Dr. George Duran at Stanford University. McCoy's 5A modified media, phosphate-buffered saline (PBS), RNA grade water, and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Pittsburgh, PA). Paclitaxel was obtained from LC Laboratories (Woburn, MA). Thiazolyl Blue Tetrazolium Bromide (TBTB) was purchased from Sigma-Aldrich (St. Louis, MO). Cell Titer One Aqueous Solution Cell Proliferation Assay was acquired from Promega (Madison, WI).

4.1.2 Cell Culture

The human ovarian carcinoma cell lines OVCAR3 and OVCAR3-TR were cultured in McCoy's 5A Modified Medium with 10% FBS and 1% antibiotic (100 IiU/mL penicillin and 100 µg/mL streptomycin). Both cell lines were cultured in an incubator at 37°C with 5% CO_2.

4.1.3 IC50 Studies

The half maximal inhibitory concentration (IC50) value of paclitaxel in OVCAR3 and OVCAR3-TR ovarian cancer cell lines was determined to find the level of resistance in OVCAR-TR cells. Both cell lines were seeded at 35,000 cells/well in two 24-well plates and allowed to attach overnight. Paclitaxel dissolved in DMSO was added to the cells

and allowed to incubate in media containing 10% FBS with a final paclitaxel concentration ranging from 0-10 nM in the OVCAR3 cells and 0-100 nM in the OVCAR3-TR cells for 24 hours. After 24 hours, the cells were washed with PBS three times to ensure the removal of drug and media was replaced for another 48-hour incubation period. After a total of 72 hours post-treatment, the cytotoxicity of paclitaxel was examined using an MTT assay. The media was removed from the cells and Thiazolyl Blue Tetrazolium Bromide was dissolved in PBS at a concentration of 2 mg/mL and added to the cells for a final concentration of 1 mg/mL. The cells were then incubated with TBTB for 4 hours at 37°C. After incubation, the TBTB was aspirated from the cells and formazan crystals were solubilized with 500 µL of DMSO in each well. The absorbance of each well was measured at a wavelength of 540 nm on a Biotek Synergy plate reader (Winooski, VT). Cell viability was normalized to untreated cells and calculated with the equation below:

$$\text{Cell Viability (\%)} = \left(\frac{\text{Absorbance}_{540\ (\text{sample})}}{\text{Absorbance}_{540\ (\text{control})}} \right) \text{x } 100.$$

The ratio of IC50 values in the resistant OVCAR3-TR cells compared to the wild-type OVCAR3 cells was calculated to determine the level of resistance.

4.1.4 siRNA Array for Gene Target Identification

A custom siRNA array was developed by identifying genes involved in apoptosis and the cell cycle, as well as genes recently found to be involved in drug resistance

development, specifically in ovarian cancer. After the IC50 values were determined, an optimal paclitaxel treatment concentration (15 nM) was found through MTT studies for the siRNA array experiment. OVCAR3-TR cells were seeded at 7,000 cells per well in a 96-well plate and incubated overnight to allow for attachment. The cells were transfected with 50 nM siRNA from the custom array and from controls and incubated at 37° C for 24 hours. Five controls were used for the array, GAPDH siRNA, a positive targeting control, Non-targeting #5 siRNA, a non-targeting control, lipofectamine only, free paclitaxel only, and an untreated control. 24 hours post-transfection, the transfection media was removed and the cells were treated with 15 nM paclitaxel and incubated for another 24 hours.

At the 48-hour time point, an MTS cell proliferation assay was performed using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay. The cells were incubated for 2 hours at 37°C with 20 µL of the Cell Titer reagent. After incubation, absorbance was measured using a Biotek Synergy plate reader (Winooski, VT) at an absorbance of 490 nm. Absorbance values were used to calculate cell viability compared to untreated OVCAR3-TR cells using the equation:

$$\text{Cell Viability (\%)} = \left(\frac{\text{Absorbance}_{490\ (\text{sample})}}{\text{Absorbance}_{490\ (\text{control})}} \right) \text{x } 100.$$

The anticancer effects were determined by comparing the viability associated with each siRNA in the array to the viability of cells treated with free paclitaxel only.

Statistical analysis was performed on the viability data from the array to determine significant anticancer effects. Gene targets were chosen based on the highest mean anticancer effects as demonstrated by the lowest viability values.

4.1.5 Statistical Analysis

Quantitative data were presented as mean ± SEM of three independent experiments. Statistical analysis was performed using a Student's t-test or one-way ANOVA, with *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, and a value of $P < 0.05$ was considered statistically significant.

4.2 RESULTS

4.2.1 IC50 Studies

Drug-resistance is identified by an increase in half maximal inhibitory concentration of a drug on cells. The results comparing anticancer activity of paclitaxel in OVCAR3 cells to resistant OVCAR3-TR ovarian cancer cells indicated an increase in IC50 value of paclitaxel in OVCAR3-TR cells. In the OVCAR3 cell line, the IC50 value of paclitaxel was 4.29 ± 0.12 nM. The resistant OVCAR3-TR cells showed a ten-fold increase in paclitaxel IC50 value, 43.10 ± 2.41 nM, indicating a more resistant phenotype toward paclitaxel than the OVCAR3 cells.

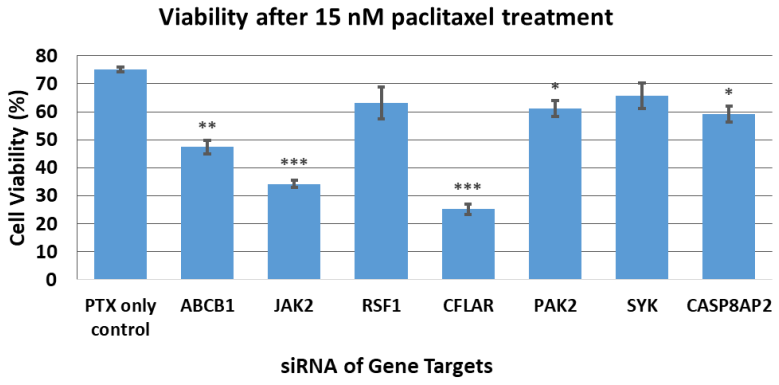
4.2.2 Identification of Gene Targets

A custom siRNA array (Fig. 4.1) was used to identify gene targets that may be involved in the development of drug resistance in ovarian cancer. From the anticancer effects determined via viability data, seven siRNAs demonstrated higher anticancer effects than paclitaxel treatment alone in OVCAR3-TR cells *in vitro*. Out of the seven siRNAs identified, five siRNAs demonstrated significantly higher anticancer activity than paclitaxel treatment alone, including, CASP8AP2, PAK2, ABCB1, JAK2, and CFLAR (Fig. 4.2A-B). The three siRNAs the results in the lowest viability in combination with paclitaxel compared to treatment with paclitaxel alone were chosen as gene targets. ABCB1, which codes for ATP-binding cassette transporters and manages efflux of molecules out of cells, demonstrated the third lowest viability at 49%. JAK2, Janus kinase 2, which plays a role in signaling cell growth, exhibited the second lowest viability at 34%. The lowest viability overall, 29%, was due to silencing CFLAR, an apoptosis regulator protein that inhibits caspase-8 cleavage, thereby inhibiting apoptosis.

BAX	TP53	CASP8	CASP8AP2	CASP9	CASP7	AKT1	PARP1	PARP2	TNF
TNFAIP3	BCL2	BCL2A1	BCL2L1	BCL2L10	BCL2L12	BCL2L13	BCL2L2	BRCA1	BRCA2
CHEK2	MLH1	TP73	WWOX	NEK2	NEK11	KLF5	YEATS4	BCL10	ZEB1
XIAP	PAK2	YAP1	ABCB1	ABCG2	SYK	SOX2	JAK2	RSF1	CFLAR
Untreated	PTX only	Lipofect.	GADPH (+)	NT#5 (-)					

Figure 4.1. Custom siRNA array layout for gene target identification. *A custom siRNA was formed using 40 siRNAs correlating to genes involved in apoptosis or the cell cycle to explore their involvement in the development of drug resistance in OVCAR3-TR ovarian cancer cells. The schematic includes the five controls of the experiment, untreated cells, paclitaxel alone, Lipofectamine, GAPDH targeting control, and NT#5 Non-targeting control.*

A)



B)

BAX	TP53	CASP8	CASP8AP2	CASP9	CASP7	AKT1	PARP1	PARP2	TNF
TNFAIP3	BCL2	BCL2A1	BCL2L1	BCL2L10	BCL2L12	BCL2L13	BCL2L2	BRCA1	BRCA2
CHEK2	MLH1	TP73	WWOX	NEK2	NEK11	KLF5	YEATS4	BCL10	ZEB1
XIAP	PAK2	YAP1	ABCB1	ABCG2	SYK	SOX2	JAK2	RSF1	CFLAR

Legend
GREEN- Viability increased compared to paclitaxel control
YELLOW- Viability decreased compared to paclitaxel, but not significantly
ORANGE- statistically significant with p<0.05
RED- statistically significant with p<0.01

Figure 4.2. Viability of OVCAR3-TR cells after transfection with siRNA array and paclitaxel treatment. *Cell viability of OVCAR3-TR cells (A) as determined by an MTS assay 48 hours after siRNA transfection and 15nM paclitaxel treatment. A heat map was generated based on the significance of anticancer effects (B). Data are mean +/- SEM of 3 independent trials compared to cells treated with paclitaxel, where *P<0.05, **P<0.01, and ***P<0.001 (one-way ANOVA).*

CHAPTER 5

SYNTHESIS AND CHARACTERIZATION OF COMBINATION LIPOSOMES FOR TREATMENT OF DRUG-RESISTANT OVARIAN CANCER

Liposomes are multifunctional nanoparticles and allow for loading of hydrophilic and hydrophobic chemotherapeutics, singularly or simultaneously, for delivery to targeted cancer cells. Liposomal formulations of chemotherapy drugs decrease hypersensitivity and off-target toxicity of chemotherapeutics delivered systemically. By encapsulating paclitaxel, a poorly water-soluble drug, in lipids, the goal is to decrease hypersensitivity and adverse effects due to polyoxyl-35 castor oil, used as a solubilizing vehicle to mediate intravenous delivery of the hydrophobic drug [94]. Many liposomal formulations of paclitaxel have been through clinical trials in recent years and are beginning to gain clinical relevance as treatments for patients with ovarian cancer [95].

Liposomes are not only useful for eliminating the need for harsh solvents or vehicles needed for delivery of poorly water-soluble drugs, but also for their therapeutic loading versatility. Liposomes contain a region where phospholipid tails overlap and create a hydrophobic shell within the membrane bilayer, allowing for hydrophobic molecules to be loaded and protected inside the membrane. The core of liposomes, lined by the hydrophilic heads of phospholipids, creates a suitable environment for loading hydrophilic drugs. Thus, liposomes possess the ability to co-load both hydrophobic and hydrophilic drugs, a characteristic that can be exploited for increasing

the efficacy of chemotherapeutics, especially in difficult to treat cancers [52]. In addition to co-loading drugs, liposomes also can load nucleic acid therapeutics, including plasmid DNA, siRNA, and antisense oligonucleotides, through electrostatic interactions with cationic lipids, to regulate expression of cancer-related genes [96].

The benefits of combination delivery of therapeutics can be further improved by modifying liposomes with surface ligands, antibodies, and stealth components [50,97]. Modifications can assist in targeting and reversing drug-resistance mechanisms in ovarian cancer. Inhibition of drug resistance mechanisms combined with chemotherapy treatment have shown increased anticancer effects in drug-resistant ovarian cancer models compared to chemotherapy alone [98].

To address drug resistance in ovarian cancer, we synthesized cationic, cholesterol- and cholesteryl hemisuccinate-based liposomes in order to deliver paclitaxel in combination with siRNA targeting genes involved in the development of drug resistance. The liposomes were designed to bind negatively-charged siRNA to cationic lipid components and load hydrophobic paclitaxel into the membrane bilayer. Characterization based on size, polydispersity index, and zeta-potential confirmed the formation of cationic liposomes. Efficient loading of paclitaxel and binding of siRNA into the liposomes as well as stability of the loaded liposomes were all demonstrated. Quantification of siRNA in CHEMS-LPs demonstrated lower concentrations than CHOL-LPs in the fluorescence study, however similar siRNA binding was shown in the gel shift assay. Further analysis is needed to determine the loading potential of the CHEMS-LPs.

Cholesterol-based liposomes mediated cellular uptake of Nile Red and fluorescent non-targeting #5 siRNA and CHEMS-LPs mediated uptake of fluorescent non-targeting #5 siRNA into OVCAR3-TR drug-resistant ovarian cancer cells.

5.1 MATERIALS AND METHODS

5.1.1 Materials

All lipid components, DOTAP, DPPC, and DSPE-PEG(2000), a mini hand extruder, and 0.2 μm polycarbonate membranes were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol, cholesteryl hemisuccinate, Nile Red, and chloroform were obtained from Sigma Aldrich (St. Louis, MO). Methanol, glucose, and acetonitrile were purchased from VWR (Radnor, PA). Paclitaxel was acquired from LC Laboratories (Woburn, MA). Fluorescent non-targeting #5 siRNA and non-targeting #5 siRNA were obtained from Dharmacon (Lafayette, CO). Electrophoresis materials including agarose, TAE buffer, ethidium bromide, Ambion PUC19 RNA Ladder, and agarose loading dye were purchased from Fisher Scientific (Pittsburgh, PA). Paraformaldehyde and Triton X-100 were also purchased from Fisher Scientific. NucBlue ReadyProbes stain and Alexa-488 Anti-rabbit secondary antibody were acquired from ThermoFisher Scientific (Waltham, MA). EEA1 rabbit monoclonal antibody was purchased from Cell Signaling Technology (Danvers, MA).

5.1.2 Cell Culture

The human ovarian carcinoma cell line OVCAR3-TR was cultured in McCoy's 5A Modified Medium with 10% fetal bovine serum (FBS), and 1% antibiotic (100iU/mLpenicillin/100ug/mL streptomycin). The cell line was grown in an incubator at 37°C with 5% CO2.

5.1.3 Liposome Synthesis

Cationic liposomes were formed by dissolving lipids at a 25:40:30:4 molar ratio (DOTAP:DPPC:Cholesterol (or CHEMS):DSPE-PEG2000) in a 3:1 (v/v) mixture of chloroform and methanol as previously described [99]. The solvent was evaporated at 40° C for 20 minutes and then the lipid film was allowed to vacuum dry for 2 hours to ensure complete removal of solvent. Fluorescent non-targeting #5 siRNA was added to RNA grade water with 5% glucose (w/v) and used to rehydrate the lipid film. The mixture was then sonicated in an ultrasonic bath for 20 minutes, followed by extrusion through a 0.2 µm filter membrane in a mini-hand extruder. After extrusion, the liposomes were diluted to the desired working concentrations and stored at 4°C. The same process was used to form liposomes containing cholesteryl hemisuccinate in place of cholesterol.

5.1.4 Characterization of Liposomes

Size and polydispersity index of the liposomes at a concentration of 5 mM was determined using Dynamic Light Scattering, while surface charge was determined via

micro-electrophoresis and phase analysis light scattering (M3-PALS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The Zetasizer was maintained at a temperature of 25°C while obtaining measurements. A refractive index of 1.45, an absorbance value of 0.001, and an angle of 173° was used to collect the intensity of scattered light due to the nanoparticles. Intensity-based size distribution plots were generated based on twelve separate measurements.

5.1.5 Liposomal Loading Efficiency of Paclitaxel

To release encapsulated paclitaxel from liposomes, liposomes were disrupted in methanol by centrifuging at 13,000 rpm for 20 minutes prior to HPLC analysis. Loading of paclitaxel into the liposomes was determined using an HPLC system consisting of a Waters Model 2707 Autosampler connected to a 1515 Isocratic HPLC Pump (Waters, Inc., Milford, MA). Samples of 50 µL were injected into a Waters Symmetry C18 (4.6 x 75 mm, 3.5µm) column, with a gradient flow of water and acetonitrile beginning at 66:34 (v/v) at a flow rate of 0.6 mL/min. The UV detector was set to 227 nm. The loading efficiency was determined by comparing encapsulated paclitaxel to the initial amount of paclitaxel dissolved with the lipids using the equation:

$$\text{Loading Efficiency } (\%) = \left(\frac{\text{Amount of drug detected}}{\text{Amount of drug initially loaded}}\right) \times 100$$

5.1.6 siRNA Binding and Stability in Liposomes

Agarose gel electrophoresis was performed to determine the binding and protection of siRNAs in the liposomes. Intact and disrupted liposome samples were loaded into a 2% agarose gel in TAE buffer and ran for 60 minutes at 100V. The gel was stained with ethidium bromide and imaged with a UV illuminator.

Binding of siRNA to liposomes can be estimated by utilizing fluorescent siRNA and measuring the intensity of fluorescence when bound to liposomes. Fluorescence spectroscopy was performed in a Synergy Biotek plate reader (Winooski, VT) to examine the binding of siRNAs to the liposomes. Serial fluorescent siRNA dilutions were used to create a standard curve. Liposome solutions, including both 5 mM and 500 mM stocks, were used as samples and their fluorescence intensities were fit to the standard curve to determine the amount of siRNA bound. Liposome samples disrupted with 0.1% Triton X-100 were also used to examine the siRNA fluorescence intensity without interference from the stable liposomes. Binding efficiency was calculated as:

$$\text{Binding Efficiency } (\%) = \left(\frac{\text{Amount of siRNA detected}}{\text{Amount of siRNA initially added}} \right) \times 100$$

5.1.7 Cellular Uptake of Liposomes

Cellular uptake and intracellular accumulation of cholesterol-containing liposomes loaded with fluorescent non-targeting #5 siRNA and Nile Red were visualized using fluorescence microscopy. OVCAR3-TR cells were seeded at 35,000 cells per well in 24-well plates and incubated to allow attachment overnight. The cells were treated with

liposomes at a final concentration of 50 µM and incubated for 4 or 8 hours. At 4 and 8 hours, the cells were stained with NucBlue ReadyProbes nuclear stain and imaged using an EVOS FL Cell Imaging System (ThermoFisher Scientific, Waltham, MA).

Immunofluorescence assays were performed on OVCAR3-TR cells treated with cholesterol- and CHEMS-containing liposomes loaded with fluorescent non-targeting #5 siRNA and paclitaxel. OVCAR3-TR cells were seeded at 35,000 cells per chamber on 8-chamber collagen I-coated culture slides and incubated overnight for attachment. The cells were then treated with 50 µM liposomes containing cholesterol or CHEMS and incubated for 2 hours. OVCAR3-TR cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. The cells were incubated at 4°C overnight with primary EEA1 antibody solution. Following incubation, cells were washed three times using PBS and incubated with Alexa Fluor 488-conjugated secondary antibody solution. Nuclear material was counterstained with mounting media containing DAPI. Fluorescence microscopy was used to visualize cellular uptake and intracellular accumulation of the liposomes.

5.1.8 Statistical Analysis

Quantitative data were presented as mean ± SEM of three or more independent experiments. Statistical analysis was performed using a Student's t-test.

5.2 RESULTS

5.2.1 Characterization of Liposomes

Two formulations of liposomes were synthesized using the same lipid film hydration method to create liposomes containing cholesterol (CHOL-LPs) and liposomes containing cholesteryl hemisuccinate (CHEMS-LPs) (Fig 5.1). After synthesis, the two types of liposomes were characterized by size, polydispersity index (PDI), and surface charge using DLS and M3-PALS at a concentration of 5 mM of liposomes. The mean particle size of the CHOL-LPs was 114.9 ± 10.35 nm, while the mean size of the CHEMS-LPs was slightly lower at 91.29 ± 7.66 nm (Fig. 5.2A-B). The PDI of the CHOL-LPs, 0.252, was also higher than that of the CHEMS-LPs, 0.214 (Fig. 5.2C). Both values for PDI indicated monodisperse liposomes in solution with minimal aggregation. The mean zeta-potentials of the CHOL-LPs and CHEMS-LPs, +27.6 ± 1.79 mV and +23.2 ± 1.56 mV respectively (Fig. 5.2C), were positive due to the cationic lipid component DOTAP and the ability of CHEMS to be protonated in aqueous solution. Both liposome formulations resulted in uniformly-sized, monodisperse, positively charged particles.

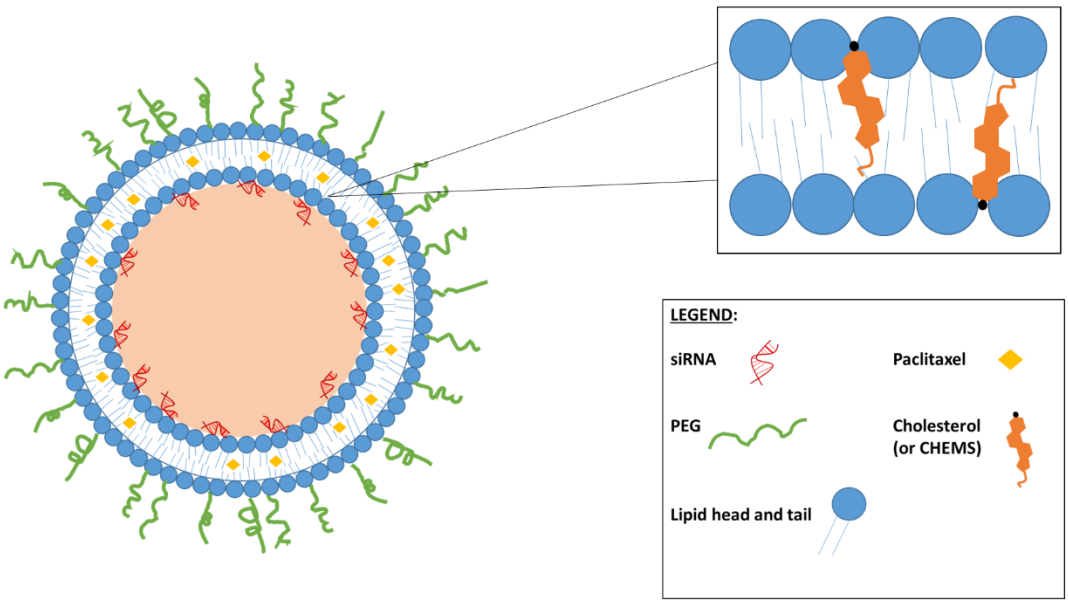
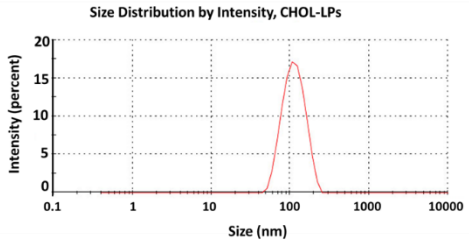
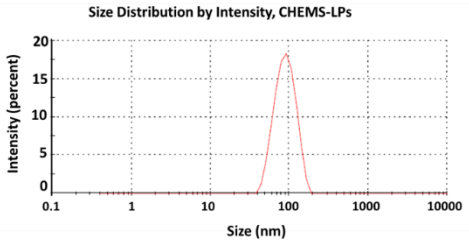


Figure 5.1. Structure and loading of liposomes. *The theoretical structure and loading of PEGylated liposomes containing paclitaxel in the hydrophobic lipid bilayer and siRNA bound to cationic lipid components. In the upper right hand corner, the role of cholesterol or cholesteryl hemisuccinate in the structure of the membrane is depicted.*

A)



B)



C)

Liposome	Particle Size (nm)	PDI	Zeta-Potential (mV)
CHOL-LPs	114.9 ± 10.35	0.251	+27.6 ± 1.79 mV
CHEMS-LPs	91.29 ± 7.66	0.214	+23.2 ± 1.56 mV

Figure 5.2. Size and zeta-potential characterization of liposomes. *Particle size distribution of CHOL-LPs (A) and CHEMS-LPs (B) and PDI (C) were determined using Dynamic Light Scattering and zeta-potential (C) was analyzed using micro-electrophoresis with phase analysis light scattering. Data in (C) are reported as mean ± SEM of 12 measurements.*

5.2.2 Efficient Loading of Paclitaxel into Liposomes

In order to further compare the CHOL-LPs and CHEMS-LPs, the loading efficiency of paclitaxel in the liposomes was analyzed. Paclitaxel was dissolved with the lipids and rehydrated to form liposomes with a final drug concentration of 2.5 μg/mL. HPLC analysis was used to determine the amount of paclitaxel loaded into each type of liposomes, and the loading efficiencies of both types of liposomes were calculated. The CHOL-LPs exhibited a loading efficiency of 80.4%, while the CHEMS-LPs loaded with an efficiency of 79.3% (Table 5.1). Overall, the CHOL-LPs and CHEMS-LPs were comparable in their ability to load paclitaxel.

Table 5.1. Loading efficiency of paclitaxel in liposomes. *Loading of paclitaxel in liposomes was determined via HPLC. The loading efficiency of the liposomes was calculated by comparing the amount of detected paclitaxel to the initial amount of paclitaxel added, 2.5 μg/mL. Data are mean ± SEM of 3 independent trials.*

Liposome	Paclitaxel Concentration (μg/mL)	Loading Efficiency (%)
CHOL-LPs	2.01 ± 0.11	80.4
CHEMS-LPs	1.98 ± 0.07	79.3

5.2.3 Binding and Protection of siRNA in Liposomes

A gel shift assay was performed to examine the siRNA binding capabilities of the CHOL-LPs and CHEMS-LPs. Samples of each liposome at concentrations of 5 mM and

500 mM were examined. siRNA content was also examined after disruption with 0.1% Triton X-100 in order to validate binding of siRNAs in liposomes. Intact and disrupted liposomes at a concentration of 5 mM showed no stained siRNA, likely due to the siRNA concentration being too dilute. Intact 500 mM CHOL-LPs and CHEMS-LPs stained for siRNA inside of the wells, while both disrupted 500 mM liposome samples appeared around the same area of the gel as free siRNA (Fig 5.3). Staining near the well in Lane 10 indicates remaining siRNA bound to liposome components. The disrupted 500 mM CHOL-LPs showed more staining of siRNA than in the disrupted CHEMS-LPs; however, the staining near the well in the disrupted CHEMS-LPs indicates incomplete release of bound siRNA and further studies of the binding potential of CHOL- and CHEMS-LPs are needed.

The amount of fluorescent non-targeting #5 siRNA bound to the liposomes was quantified using fluorescence spectroscopy with a Biotek Synergy plate reader containing a red (530/590) filter cube (Winooski, VT). The concentration of bound siRNA was estimated using a standard curve of serial siRNA dilutions. A standard curve with siRNA and Triton X-100 was also created to account for the interference of Triton X-100 in the fluorescence reading. Intact and disrupted samples of the CHOL-LPs and CHEMS-LPs at a concentration of 500 mM were examined. The 5 mM samples were not analyzed because the concentration of siRNA in the liposomes was too low to be detected, as seen in the gel shift assay. Binding/loading efficiency, as determined by siRNA concentration correlating to intensity of fluorescence, was higher in the CHOL-LPs

than in the CHEMS-LPs (Table 5.2). Fluorescence quenching due to tight interactions between protonated CHEMS and slightly negatively-charged DPPC in the lipid bilayer may have influenced the apparent bound siRNA concentration.

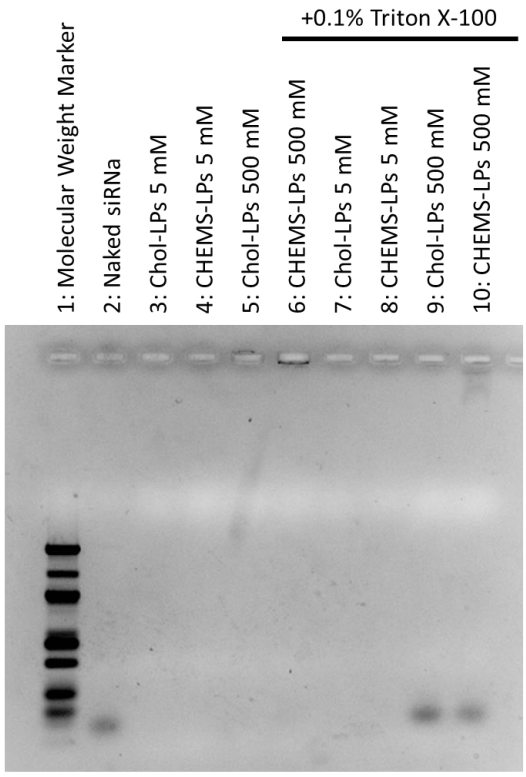


Figure 5.3. Binding and stability of siRNA in liposomes. *Agarose gel electrophoresis demonstrated the binding affinities and liposomal protection of siRNA in CHOL-LPs and CHEMS-LPs. The mobility of siRNA was visualized by staining with ethidium bromide. Lane 1 was loaded with molecular weight marker and lane 2 was loaded with free siRNA. Lanes 3-6 contained samples of intact liposomes, while lanes 7-10 contained liposomes disrupted with 0.1% Triton X-100.*

Table 5.2. Binding/Loading efficiency of siRNA in Liposomes. *siRNA binding/loading was calculated with fluorescence spectroscopy, and siRNA concentrations were determined from fluorescence intensity values fit to a standard curve. Efficiency of binding/loading was calculated by comparing observed siRNA concentration to initial siRNA concentration. Data are mean ± SEM of three independent trials.*

Liposome	LPs (500 mM)	siRNA conc. (nM)	LPs + 0.1% Triton X-100	siRNA conc. (nM)
CHOL-LPs	53%	157.1 ± 1.3	57%	167.6 ± 5.6
CHEMS-LPs	5%	15.8 ± 0.2	2%	5.4 ± 0.2

5.2.4 Cellular Uptake of Liposomes

CHOL-LPs mediated uptake of Nile Red and fluorescent non-targeting #5 siRNA into OVCAR3-TR cells after 4- and 8-hour treatment with 50 µM of liposomes. Fluorescence microscopy was used to visualize the intracellular accumulation of the cholesterol-containing liposomes (Fig. 5.4). As the length of treatment increased, the cellular uptake of the liposomes also increased, as demonstrated by the increase in red fluorescence in the 8-hour treatment compared to the 4-hour treatment.

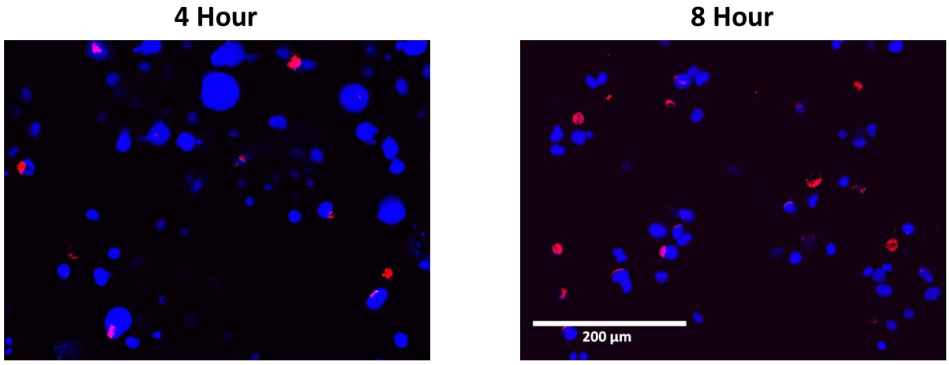


Figure 5.4. Cellular uptake of fluorescently-loaded CHOL-LPs. *OVCAR3-TR cells were incubated with cholesterol-based liposomes loaded with fluorescent non-targeting siRNA (red) and Nile Red at a liposomal concentration of 50 µM and imaged using fluorescence microscopy at 4 hours and 8 hours. NucBlue Live Cell Stain was used to counterstain nuclei (blue).*

Cellular uptake and co-localization of the CHOL-LPs and CHEMS-LPs loaded with fluorescent siRNA was also examined in OVCAR3-TR cells using immunofluorescence. The liposomes are expected to enter the cells through endocytosis due to incorporation of cationic lipids and pegylation, so an early endosomal marker (EEA1) was used to label endosome locations in the cell, as shown by green fluorescence. The CHOL-LPs experienced increased cellular uptake compared to the CHEMS-LPs (Fig. 5.5). However, final conclusions cannot be made about the uptake until further studies are done to analyze the binding efficiency of siRNA in CHEMS-LPs without interference or quenching from any of the lipids. Due to the lack of fluorescent labeling of the liposomes themselves, our uptake visualization is based solely on the fluorescence of siRNA. Co-localization of the CHOL- and CHEMS-LPs with endosomes was analyzed using ImageJ. Little co-localization was visualized in either liposomes (Fig. 5.6), indicating a different

mechanism of cellular uptake than endocytosis, likely due to the ability of cationic lipids to fuse and incorporate into the plasma membrane of cells.

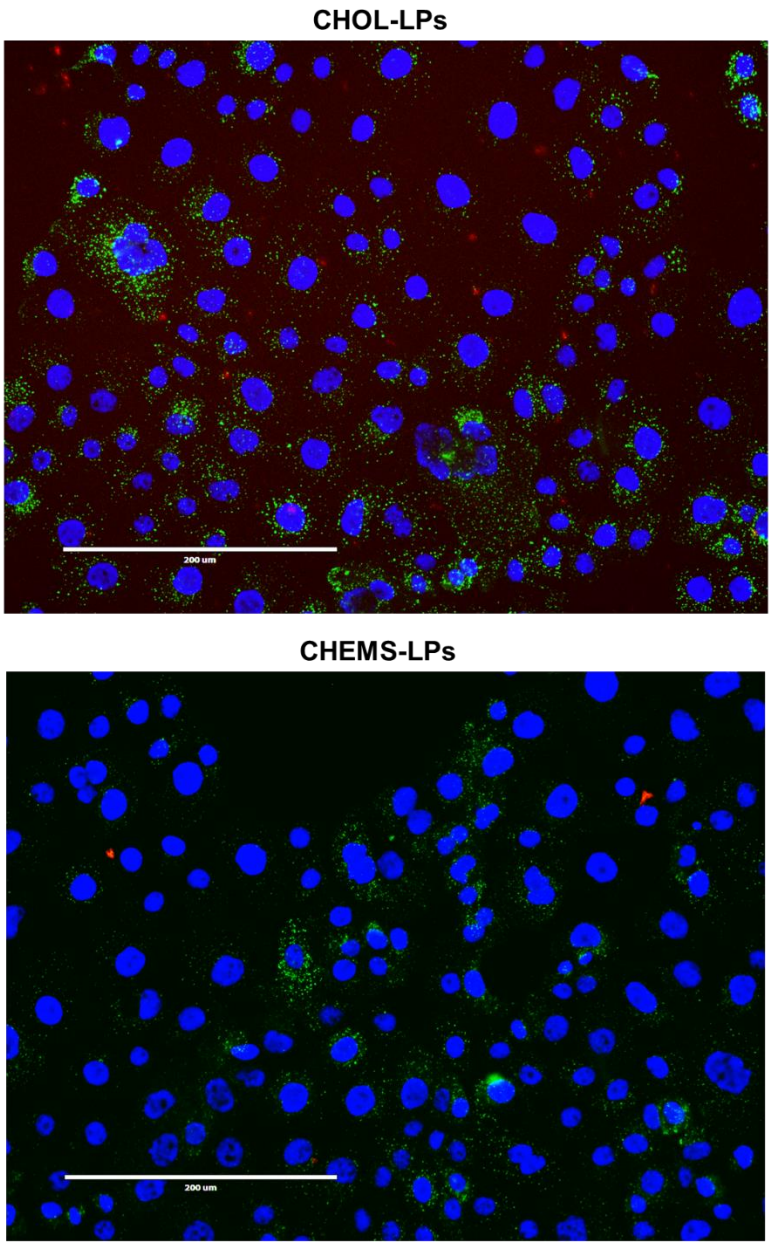


Figure 5.5. Cellular Uptake of CHOL-LPs and CHEMS-LPs via immunofluorescence. *CHOL-LPs and CHEMS-LPs mediated cellular uptake of fluorescent non-targeting #5 siRNA (red) and paclitaxel after 2 hour treatment with 50 μM liposomes. Nuclear content was counterstained with DAPI (blue) and endosomes (green) were labeled using Anti-EEA1 primary and Alexa Fluor 488-conjugated secondary antibodies.*

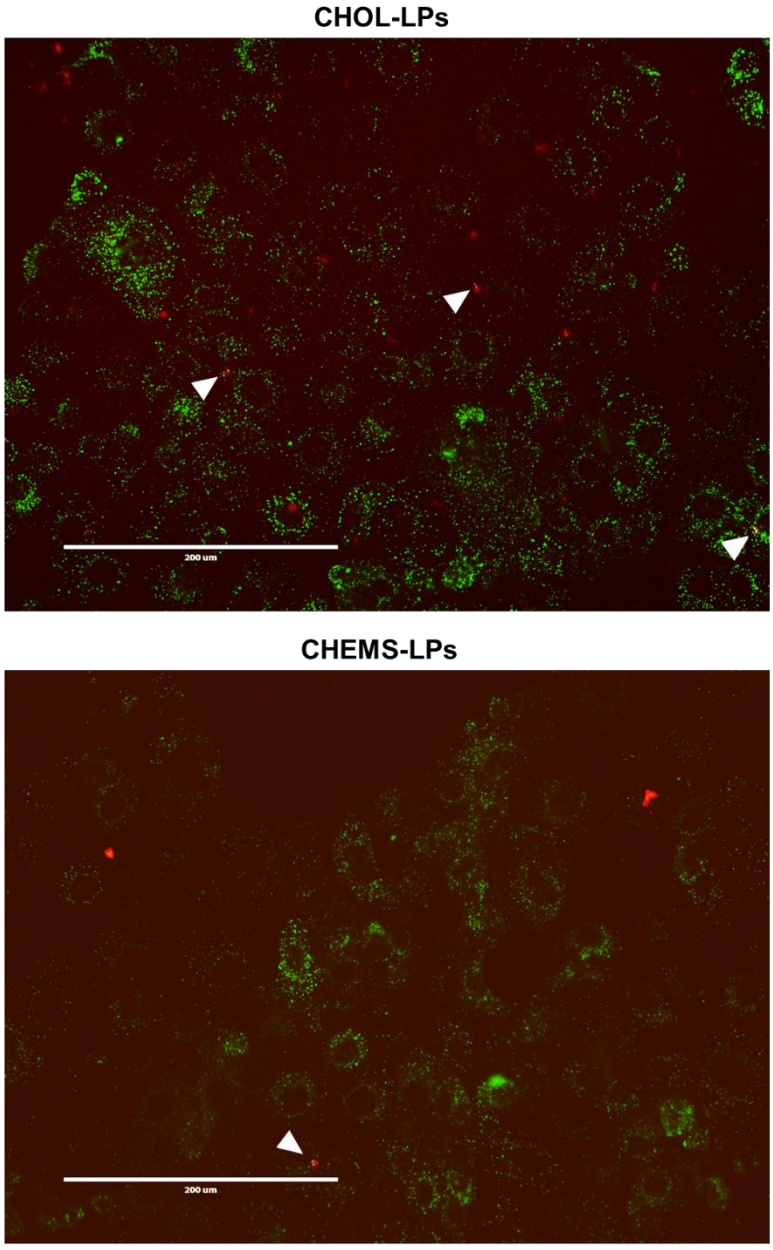


Figure 5.6. Co-localization of CHOL-LPs and CHEMS-LPs in endosomes. *CHOL-LPs and CHEMS-LPs mediated cellular uptake of fluorescent non-targeting #5 siRNA (red) and paclitaxel after 2 hour treatment with 50 µM liposomes. Endosomes (green) were labeled using Anti-EEA1 primary and Alexa Fluor 488-conjugated secondary antibodies. DAPI nuclear counterstain was removed to better visualize the co-localization (white), indicated by arrowheads. Co-localization of non-targeting #5 siRNA in endosomes was analyzed using the Colocalization plugin in ImageJ.*

CHAPTER 6

DISCUSSION

In this study, we identified potential gene targets in the development of drug-resistance in ovarian cancer cells. We developed two formulations of liposomes with the ability to encapsulate paclitaxel and bind siRNAs for combinatorial delivery to drug-resistant ovarian cancer cells. The concurrent delivery strategy provides the potential to knockdown genes related to resistance and re-sensitize cells to paclitaxel to improve the drug's efficacy in resistant ovarian cancer cells. Also, liposomal delivery may allow for a smaller effective drug dosage with limited systemic toxicity compared to standard intravenous chemotherapy.

Our results identified potential gene targets involved in the development of paclitaxel-resistance in ovarian cancer cells. Gene expression and microarray analysis have been useful in discovering genes which might play a role in cisplatin-resistance in ovarian cancer cells *in vitro* and in patient samples [100,101]. Similarly, paclitaxel resistance has been studied through small-scale analysis of gene expression in multidrug cross-resistance [102]. However, array analysis of gene expression in paclitaxel-resistant ovarian cancer has yet to be explored on its own. The results of the siRNA array/paclitaxel MTS assay demonstrated five different siRNAs significantly increased anticancer effects upon transfection and paclitaxel treatment compared to paclitaxel treatment alone. The identified gene targets, ABCB1, JAK2, and CFLAR, are related to different functions in the cell, confirming the complexity of drug-resistance

development and treatment. ABCB1, encodes for an efflux transporter which mediates the efflux of chemotherapeutics in cells. JAK2 is a kinase in a signaling pathway responsible for promoting cell proliferation and survival. CFLAR is a regulating protein involved in the apoptosis pathways. This experiment validated the need to target multiple pathways when treating drug-resistance in ovarian cancer. The three siRNAs with the greatest anticancer effects have been chosen as therapeutic targets and will be loaded into the liposomal delivery system in combination with paclitaxel in the future for *in vitro* cytotoxicity studies.

After determination of gene targets, liposomes were synthesized. We characterized the liposomes by evaluating size, polydispersity index, and surface charge of the different formulations. The mean particle size of both formulations was about 100 nm and the PDI ranged from 0.214-0.252, indicating uniformly sized and monodisperse particles. CHEMS-LPs possessed smaller mean particle size and PDI, likely due to the presence of interactions with protonated CHEMS and DPPC creating tightly-bound particles [103]. The zeta-potential of the liposomes ranged from +20 to +30 mV, indicating stable, cationic particles due to the incorporation of cationic lipids and protonation of CHEMS in aqueous media.

Paclitaxel loading efficiency and siRNA binding efficiency were analyzed after initial characterization studies. Paclitaxel loading efficiency was almost identical in the CHOL-LPs and CHEMS-LPs, with values of about 80%, indicating efficient drug loading and encapsulation. siRNA binding did differ between the liposome formulations, with

more siRNA binding shown in the CHOL-LPs than in the CHEMS-LPs. The difference in siRNA concentration in the liposomes could be due to quenching of the fluorescent siRNA in the CHEMS-LPs due to electrostatic interactions between CHEMS and DPPC. To ensure proper binding of siRNA in the CHEMS-LPs, we would need to control the formation of the liposomes and encourage the siRNA to bind inside, possibly by introducing the siRNA into a system in which CHEMS is not protonated. The siRNA binding could also be lower in the CHEMS-LPs, especially the disrupted samples, due to formation of self-aggregates by CHEMS. These self-aggregates could be preventing the release of siRNA during disruption, decreasing the apparent amount of siRNA. Further RNA quantification studies can be done to eliminate the interference from the lipids.

Both of these theories are also validated through the gel shift assay because the intact CHEMS-LPs sample demonstrated stained siRNA inside the well, indicating the stain was able to reach the siRNA. Also, the CHEMS-LPs disrupted with Triton X-100 showed a fainter band of stained siRNA than the CHOL-LPs and staining of siRNA near the well, showing some siRNA was still bound to lipid components even after disruption. The gel shift assay indicated siRNA protection due to the lack of free siRNA present in the intact liposomes. Further studies of siRNA binding ability in the CHEMS-LPs are needed in order to create a potential pH-sensitive alternative to the CHOL-LPs. CHEMS-containing liposomes have not commonly been used for combination delivery systems, though they are popular for single drug delivery systems. Thus, more extensive studies

on the N/P ratios and charge ratios would be beneficial to increase siRNA binding efficiency in the CHEMS-LPs.

Cellular uptake of Nile Red and fluorescent siRNA by drug-resistant ovarian cancer cells was mediated by the cholesterol-containing liposomes in 4- and 8-hour treatments. Uptake increased with increased treatment length. Immunofluorescence was utilized to analyze cellular uptake and co-localization of the CHOL-LPs and CHEMS-LPs loaded with fluorescent siRNA in endosomes after 2-hour treatments. The liposomes mediated uptake in both treatments; however, greater uptake was visualized with the CHOL-LPs than the CHEMS-LPs. The decrease in fluorescence from siRNA could be correlated to the lower binding of siRNA in CHEMS-LPs than CHOL-LPs or due to quenching and interference of lipids. Low amounts of co-localization of the fluorescent siRNA in endosomes indicates an uptake mechanism other than endocytosis. By fluorescently labeling the liposomes instead of only the loaded therapeutics, the uptake of the liposomes can be visualized with fluorescence microscopy [104].

In order to determine the potential of the liposomes and selected siRNAs as an effective therapy for drug-resistant ovarian cancer, studies examining the ability of the liposomes to mediate silencing of the targeted siRNAs need to be conducted. Quantification of gene targets in wild-type and resistant ovarian cancer cells can be analyzed with western blotting and RT-PCR to ensure upregulation of genes in resistant cells. Also, knockdown efficiency of the chosen siRNA and resulting viability can also be explored. Cytotoxicity assays, including MTT assay, wound healing assay, and flow

cytometry for live/dead analysis, can help demonstrate the therapeutic potential of the combination liposome delivery system.

CHAPTER 7

CONCLUSION

Overall, we have identified three gene targets that may be involved in the development of paclitaxel-resistance in ovarian cancer cells from a siRNA array. The three siRNA with the greatest anticancer effects will be used as therapeutics in combination with paclitaxel in our liposome delivery system. After liposome synthesis, we confirmed uniform sizing, monodisperse particles, and stable cationic liposomes through characterization studies. We demonstrated efficient loading of paclitaxel and protection of bound siRNA in both liposome formulations. In addition, liposomes mediated uptake of Nile Red and fluorescent siRNA or fluorescent siRNA alone into ovarian cancer cells. The characterization studies of the liposomes indicate their potential as a combination delivery system. However, further studies need to be done to increase siRNA binding efficiency and examine the pH-sensitive release of CHEMS-LPs. Also, *in vitro* and *in vivo* cytotoxicity studies are necessary to confirm the efficacy of the combinatorial liposome delivery system.

Liposomal combination therapies are gaining popularity as potential treatments for drug-resistant cancer. Combinatorial delivery can synergistically enhance the efficacy of therapeutics when delivered to drug-resistant cells by simultaneously re-sensitizing and treating cancer cells. The liposomes developed in this study show promise as combination delivery systems upon optimizing siRNA binding and release profiles of the

particles. We aim to create a more effective alternative to current treatments for drug-resistant ovarian cancer and increase patient survival rates.

CHAPTER 8

FUTURE DIRECTIONS

In order to further determine the therapeutic potential of the developed liposomal combination delivery systems, gene target expression, release profiles, and cytotoxicity must be analyzed for both liposomes. Expression of the selected target genes, ABCB1, JAK2, and CFLAR, must be studied in both wild-type and paclitaxel-resistant ovarian cancer cells. Western blotting will be utilized to compare the expression of the targeted genes in OVCAR3 and OVCAR3-TR ovarian cancer cells, where upregulation of the gene targets in the OVCAR3-TR cells indicates a potential involvement in paclitaxel-resistance. To further quantify the targeted genes in both cell lines, reverse-transcription polymerase chain reaction (RT-PCR) will be used to determine the RNA transcript levels.

After quantifying expression of gene targets in the OVCAR3 and OVCAR3-TR ovarian cancer cells, siRNA knockdown will be used to determine the efficiency of the siRNAs in silencing the selected genes. Western blotting and RT-PCR will be used to quantify protein and RNA levels in untreated OVCAR3-TR cells and in cells transfected with siRNAs correlating to targeted genes. The quantification of gene target expression is vital in confirming the therapeutic potential of the selected siRNAs in targeting mechanisms of paclitaxel-resistance.

To further characterize the CHOL- and CHEMS-LPs, release profile studies will be completed to analyze the pH-sensitivity of CHEMS-LPs compared to CHOL-LPs. Release

studies using release media of varying pHs will be used to determine the characteristics of pH-responsive behavior in CHEMS-LPs in environments with similar pH to tumor microenvironments. Other studies include scanning electron microscopy (SEM) and conductivity studies of the liposomes in various pHs can be used to analyze the change in shape and stability of the liposomes due to pH changes, indicating pH-sensitivity. In addition, to improve siRNA quantification in the CHEMS-LPS, it is necessary to eliminate interference or quenching due to the presence of lipids.

To quantify siRNA directly, a Take 3 microplate will be used in the Biotek Synergy plate reader. Also, a Ribogreen assay, a method using an ultra-sensitive fluorescent molecule to quantify small amounts of RNA without interference from lipids. If siRNA quantification studies demonstrate lower siRNA binding/loading efficiencies in the CHEMS-LPs, optimization of the size/charge ratio and N/P ratio of the liposomes will take place.

Immunofluorescence will be done at a later time point, 4 hours, to examine uptake mechanisms and track the localization and escape of both types of liposomes. To better visualize the liposomes, liposomes will be fluorescently labeled instead of the liposomal contents. A fluorescent tracker, like BODIPY FL or LysoTracker can then be used to examine the mechanism of uptake of the liposomes.

Additional work on the synthesis methods of the liposomes will include developing a method to improve stability of liposomes and a method of long-term storage. Currently, synthesis of the liposomes is not optimal as liposomes are

synthesized weekly, so developing a method of longer storage is needed. Synthesis of liposomes will also expand to include targeting moieties and surface modifications to increase specificity, cellular uptake, and intracellular release and accumulation.

Finally, cytotoxicity assays will be completed *in vitro* to examine the therapeutic potential of the delivered siRNAs and paclitaxel, alone and in combination. Cell viability assays, specifically MTT assays, will be used to determine viability of cells transfected with siRNAs alone, treated with paclitaxel alone, or transfected and treated with siRNAs and paclitaxel. The liposomal delivery systems will be analyzed to confirm the synergistic anticancer effects of simultaneous siRNA knockdown and paclitaxel treatment. Wound healing assays will be used to determine changes in motility of OVCAR3-TR ovarian cancer cells after liposomal treatment. Flow cytometry will be used for live/dead analysis of OVCAR3-TR cells treated with various concentrations of paclitaxel encapsulated in liposomes. Western blotting and RT-PCR will be used to confirm the knockdown of targeted genes after delivery of siRNAs by the liposomal delivery systems. Through these future steps, the therapeutic potential of the liposomal delivery systems as a combination therapy to combat paclitaxel-resistance in ovarian cancer.

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