Novel Aminoglycoside Polymers and Combination Treatments in Triple Negative Breast Cancer Studies

by

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ABSTRACT

In the United States, 12% of women are typically diagnosed with breast cancer, where 20-30% of these cases are identified as Triple Negative Breast Cancer (TNBC). In the state of Arizona, 810 deaths occur due to breast cancer and more than 4,600 cases are diagnosed every year (American Cancer Society). The lack of estrogen, progesterone, and HER2 receptors in TNBC makes discovery of targeted therapies further challenging. To tackle this issue, a novel multi-component drug vehicle is presented. Previously, we have shown that mitoxantrone, a DNA damaging drug, can sensitize TNBC cells to TRAIL, which is a protein that can selectively kill cancer cells. In this current study, we have formulated aminoglycoside-derived nanoparticles (liposomes) loaded with mitoxantrone, PARP inhibitors, for delivery to cancer cells. PARP inhibitors are helpful in preventing cancer cells from repairing their DNA following damage with other drugs (e.g. mitoxantrone). Various treatment liposome groups, consisting of lipid-containing polymers (lipopolymers) synthesized in our laboratory, were formulated and characterized for their size, surface charge, and stability. PARP inhibitors and treatment of cells for in-vitro and in-vivo experiments with these liposomes resulted in synergistic death of cancer cells. Finally, studies to evaluate the pre-clinical efficacy of these approaches using immuno-deficient mouse models of TNBC disease have been initiated.

Dedicated to my Parents and Sister,
Murali, Uma, and Hamsini

ACKNOWLEDGEMENTS

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the support and help I had throughout my graduate education. I would like to personally thank my sister Hamsini, Kate, Srishti and Sahil for their positivity throughout my Masters and being a close pillar of support. My last two years in this program has taught me so much about my field, my personal and career aspirations, and the great impact of the research in Rege Lab. I was always passionate about working in the research field to solve healthcare problems, specifically, oncology. Having lost my grandfather and best friend to rigorous cancer battles, I wanted to work in this field as I was completing my undergraduate degree. Following my dream to be on the west coast, begin a new chapter, and learn to be independent while pursuing my career aspirations fell in place with all the support and encouragement I received from my friends and family back home. On that note, I would like to thank my second mom and dad (close family friends), Priya Auntie and Rajesh Uncle who have been instrumental since my childhood days. In addition, I would like to thank my committee members, Dr. Rege, Dr. Susan Holechek, and Dr. Nannenga. Dr. Holechek was a great pillar of support from teaching me science to help me spread the passion through teaching others. Dr. Nannenga was great to get to know and learn from as I conducted my thesis research. They were all great to work with and get to know throughout this process and am honored to have them on my committee. Lastly, I am humbled and honored to have been given this opportunity to conduct my thesis research under Dr. Rege with a great team of support both from home and here in Arizona.

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CHAPTER 1
INTRODUCTION

1.1.Overview

Cancer continues to be a deadly disease affecting men and women of various ages in the United States and all over the world. Approximately 40% of the population will face this deadly disease at some stage of their life.[1] Specifically, 12% of women in the United States are typically diagnosed with breast cancer, where 20-30% of these cases are identified as Triple Negative Breast Cancer (TNBC). Women diagnosed with TNBC have lower five-year survival rates (77%) than other types of breast cancers (93%).[2] Triple Negative Breast Cancer is known to be more aggressive as it can more easily spread tumors to other organs and also recur even after rigorous treatments. TNBC tumors are called "triple-negative" due to the lack of three hormone growth receptors: HER-2, Estrogen, and Progesterone (Figure 1.1.1) Receptor positive cells allow chemotherapeutic drugs to arrive at receptor site and block hormone attachment to kill tumor cells. However, for TNBC cells, lack of receptors makes this strategy complicated and ineffective. Therefore, conventional methods of chemotherapy, and drugs targeted to those common breast growth receptors are ineffective.[2,3] Other treatments such as local breast surgeries and mastectomy also have challenges with difficult strategies and high chances of tumor and local regional recurrence (LRR).[2, 4] As stated earlier, the lack of estrogen, progesterone, and HER2 receptors in TNBC makes discovery of targeted therapies further challenging. TNBC tumors are also closely associated with BRCA-1 and BRCA-2 mutations which are tumor suppressor genes that aid in DNA repair in cells.[5] Taking advantage of this association could be a key to identifying TNBC tumor cells and potentially treating them in the human body.

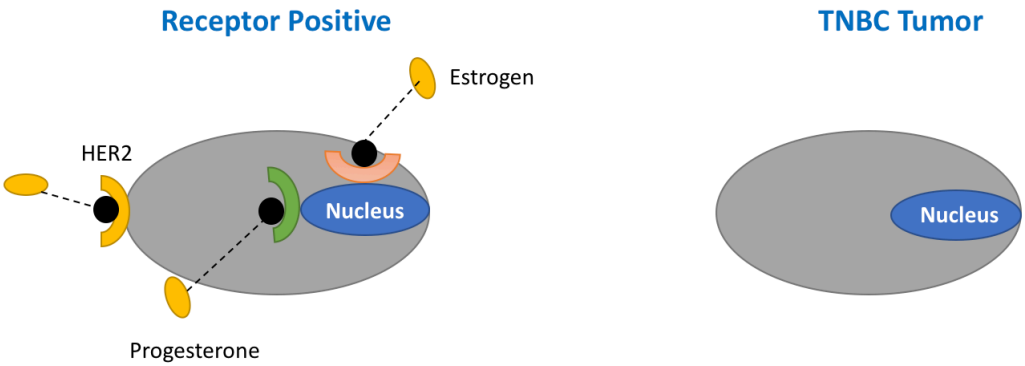


Figure 1.1.1 Triple Negative Breast Cancer Cell with three negative receptors.

Various chemotherapy agents are known widely in oncology research fields. One such agent is antineoplastic, a synthetic anthracycline derivative used in different cancer treatments, both in research and clinical settings. An anthracycline agent is a red aromatic polyketide occurring in various forms that has high anti-tumor activity. A few examples of these agents include doxorubicin, Adriamycin, and mitoxantrone. An added benefit is the potential to generate free oxygen radicals which is a strategy in damaging cell DNA via strand breaks. Anthracyclines easily diffuse through cellular membranes with its high affinity to various proteasomes in the cytoplasm which forms a complex and sent to the nucleus for binding of the anthracycline drug to DNA backbones. To tackle challenges of high toxicity, using an anthracycline derivative form is helpful to decrease tumor resistance and other potential side effects to dosage related factors. Additionally, a few other methods to avoid cardiotoxicity risks lies in delivery methods of drugs and the types of modifications associated with them(11,20).[6, 7] It is known as a common chemotherapeutic

agent in acute leukemia, and non-Hodgkin's lymphoma.[8,9,10] Being an FDA approved drug from late 80's, it became instrumental for leukemia, prostate, and breast cancer treatments. It was also FDA approved for multiple sclerosis (MS) with its rapid uptake in tissues and a longer half life of nine days. Doxorubucin, Mitoxantrone, Epirubicin, and few others are known for their mechanistic action of intercalation of DNA molecule via double strand breaks. They are called Topoisomerase II inhibitors and induce apoptosis (cell suicide) through DNA damage in human myeloid leukemia cells and observed to have potent cell death effects in other types of cancer cells as well. Topoisomerase II is an essential enzyme that aids in DNA topology modification to provide transitory double strand breaks. It utilizes ATP to pass a helix through the transitory breaks to aid in DNA topology repair and ultimate restoration of its original structure. It also is key in chromosome segregation. Using an inhibitor of this particular enzyme is influential in cycle disruption to promote apoptosis through forceful double stand breaks.

Poly (ADP-ribose) polymerase (PARP) inhibitors are also an attractive molecule in cancer therapies targeting inhibition DNA repair pathways. PARP enzymes are a group of enzymes controlled by a wide variety of genes that help in catalysis to ultimately target various proteins in the human body. They are crucial to chromatin structure modifications, as well as transcription, replication, and most importantly, DNA repair. Many cells that are deficient in homologous recombination rely on PARP enzymes mediated DNA repair for survival. When DNA undergoes any single or double strand breaks, PARP is involved in base excision repair (BER) as a part of the complex containing DNA polymerase beta, DNA ligase III, XRCC1 protein. In the PARP family, each type of enzyme is a part of

different repair pathways with NER and BER to be the instrumental ones in DNA repair with chemotherapeutic agents. The versatile properties of PARP enzymes come in to play with its involvement in the cell cycle phases (cytokensis, G_0, G_1, S, G_2, mitosis). Various studies have shown its role in G_2 cell checkpoint phase as it aids in preventing damaged DNA strands to enter in further cell phases. PARP-1 arrives at the DNA site and releases nicotinamides as a result of NAD^+ cleavage. After this cleavage, the chromatin structure is liberated to recruit proteins for scaffold modeling, allowing DNA polymerase β to attach any breaks and gaps.[11,12]

When DNA damage occurs, histones near the site are phosphorylated by ATM and ATR molecules which aids in recruitment of binding mediators, which leads to an important mediator RAP80 that can identify histones near DNA damage sites through the ubiquitin-interacting motif (UIM). RAP80 is the recruiter of BRCA1 gene complex to the specific damage site. Another key characteristic of BRCA1 is its dominance in all checkpoints of the cell cycle to assure no damaged DNA proceeds to mitosis. Especially in the G1/S checkpoint phase, when DNA damage occurs, it "arrests" cells before proceeding to replication and division.[13] The tumor suppressor gene p53 plays a large role in this phase, and as discussed earlier, p53 is an important biomarker for DNA damage and repair pathways. It prevents cyclin B and Cdc2 transcription proteins from activating to assure that these markers do not promote cell to progress towards mitosis.[14,18] This shows that interference with this cell cycle is crucial and advantageous for inhibiting certain mediators to promote cancer cell death. As mentioned earlier when SSBs occur, the PARP inhibitor blocks any activity from repair protein which promotes cell degradation and leads to DSBs.

Both cell and pre-clinical studies have gone to prove the sensitivity of PARP deficient cells (PARP $^{-/-}$) to DNA damaging and chemotherapeutic agents.[13,14,15] This case makes the use of PARP inhibitors with antineoplastic or chemotherapeutic agents a useful mechanistic tool to target multiple apoptosis pathways.

1.2. Literature Review

In literature, various drug and gene delivery were found to be common approaches to treating TNBC tumors. In one study of anti-cancer effects of a novel system against triple negative breast cancer, the agent Aminoflavone (AF) was found effective in preventing TNBC tumor growth at low doses. In addition, AF was helpful in cell cycle arrest, apoptosis, and identified as a DNA damaging agent. AF is known to have high toxicity levels in clinical and in-vivo models, therefore, this study wanted to improve drug effects to TNBC tumor sites with low toxicity levels. A multifunctional micelle was formulated and conjugated with a peptide, GE11, for safe delivery of AF agents to the tumor site. These star-polymer micelles were prepared by various dialysis and freeze-dried methods to yield studies both in-vitro and in-vivo. Results revealed that AF-T (AF loaded GE11-conjugated targeted) micelles had significant cell death when compared to the AF-NT (non-treated) micelles, showing cell death up to 85%. In-vivo results also were in accordance, with strong statistical significance ($p<.001$) for AF-T treated mice versus AF-NT mice. The results overall confirmed that GE-11 binding to micelles allowed for better targeting to tumor cells, where AF agents were unloaded, showing smaller tumor sizes in-vivo and higher cell death in in-vitro studies.[15]

In another study, researchers went to prove that sigma-2 was a significant receptor through progesterone receptor membrane component 1(PGRMC1) for TNBC tumors, to help further studies find a system to target this for aggressive treatments. Using different SMC (small mimetic compound) agents to bind to sigma-2-targeted drugs showed higher cytotoxicity than commonly used therapies for breast cancers (taxol). MDA-MB-231, had the highest sigma-2 expression of the three TNBC cell lines tested. Additionally, MDA-MB-231 also was taxol resistant along with its high sensitivity to sigma-2 receptor SMC delivery methods. Specific mechanistic studies still need to explored to understand the apoptosis pathways in relation to sigma-2 receptor targeted therapies.

Using receptor based targeted therapies, many studies have shown minimal cell survival with tailored surface properties of nanoparticles in TNBC studies. Particularly, Johnson. Et al synthesized reconstituted high density lipoproteins (rHDLs) with anthracycline anti-cancer drugs for targeting towards overexpressed scavenger receptor class B type 1 (SR-B1) receptors to perform characterization and cytotoxicity testing. These particles at 50 nm and approximately -6 to -9mV were efficient at higher half life circulation in blood systems. Testing the efficacy of these NPs (4 – 8 uM) and in comparison to free drug treatments in MDA-MB-231 (TNBC cell line), the study showed a significant (60-70%) decrease in cancer cell survival. Synthesized NPs were also tested in the H9C2 (cardiomyocyte) cell line which revealed NPs acting as a shield, increasing cell survival fourfold – which confirms minimal effect to cardiac tissues with chemotherapeutic drugs. In addition, northern blot analyses revealed a significant fluorescence shift of SR-B1 protein expression in MDA-MB-231 showing that mechanistic pathway of targeted drug delivery was instrumental. Confocal microscopy also confirmed

the higher uptake and efficacy (> 50% FU units) of encapsulated drugs in NPs versus control (free drug) treatments.[26] The targeting mechanism in this paper with the supporting results suggest the ease of transport of rHDL NPs to the cytoplasm, avoiding major challenges through extracellular membrane transport.

A recent study evaluated alternate forms of nanoparticle delivery for enhanced therapeutic effects in TNBC. Anti-Trop2 (an overexpressed glycoprotein in TNBC tumors) conjugated nanoparticles loaded with various dextran derivatives composed of bioreducible disulfide bonds. Doxorubicin, a commonly known anthracycline in chemotherapeutic studies, was also loaded and tailored to be released at tumor sites in the presence of glutathione (GSH). Average diameters of nanoparticles were between ranges of 160-190 nm, which is the appropriate size for nanocarrier methods. With various release and cytotoxicity studies, the GSH environment versus controls reduced the disulfide bonds in nanoparticles which allowed for rapid drug release (low cancer cell survival of 70-80%).[26]

1.3.Motivation

Chemotherapy and radiation are generic treatments employed for patients with cancer diseases but are not tailored specifically for cancer types. Various drug delivery methods are used in both pre-clinical and clinical settings in the healthcare field to aim for targeted and controlled release of chemotherapeutic agents. Microneedles, gene and protein delivery, cargo as nanovaccines, and nanoparticle systems such as micelles and liposomes are few of the methods known and currently being developed.[16,17,15,18] Particularly when using nanoparticles as used in this current study, various modifications to the surface

chemistry can aid in enhanced biological function and tumor activity at the molecular level. As mentioned earlier, liposomes are an efficient way to reduce the toxicity levels associated with drugs which is a real challenge in the field. Liposomes are spherical shaped amphipathic vesicles or molecules composed of a bi-layer non-toxic phospholipids.[19] They also mimic the cell membrane structure which is instrumental when these NPs come in contact with cells and release any loaded molecules with responses to pH, temperature, radiation, etc. Using these nanometer sized vesicles is advantageous to improved stability, higher compatibility within cells, reduced toxicity, and even minimal surrounding tissue to drugs released.[16, 18-20,21]

They consist of a lipid bilayer built around aqueous cores lined with polar heads on the exteriors and non-polar tails on the inside. Liposomes are known to be multi-functional vehicle system with different "pockets" to efficiently store and carry various molecules to a biological site. Once at the site, various endogenous enzymes are helpful in the lipid degradation of liposomes.[16]

Nanoparticle drug delivery facilitates ease of transport into cellular membranes and environments near targeted site. Unloading drugs at specific sites with reduced toxicity levels is important for patients with cancerous tumors for longer survival and minimal relapse rates.[21,22] Various molecules (peptides, antibodies, carbohydrates) bound to the nanocarriers are also recent progressive advances for more effective therapeutic approaches in cancer treatments. Nanoparticle and cellular membrane interactions are therefore important to gain a deeper knowledge of structural and thermodynamic properties

at a molecular level. Cell membranes are composed of a complex phospholipid bilayer barrier and act as a protective layer. Bilayers in membranes are essential for communication to external stimuli to internal areas in the cell. Fickian diffusion occurs to allow small molecules but any polar or larger molecules require protein carrier mediated transport. Particularly, these larger molecules use a bulk transport method known an endocytosis to travel between membranes and cytosol components. Nanoparticles (NPs) also participate in endocytosis when coming to contact with cellular membranes but do not necessarily need receptor mediated processes. Various studies have gathered that these phospholipid membranes are easily flexible and deformed when NPs arrive, pushing the cell to "swallow" NPs into cytosol and cytoplasm areas. For the cell to engulf these NPs, adhesion energies in the membrane must match to the energy needed to wrap around circumference of nanoparticle. Specifically, cationic molecules are utilized for drug delivery applications for their strong adhesion and "internalization" to opposite charged membranes.[23,24]

Polymer based nanoparticles (NPs) have been a rising area of focus in cancer therapy for various drug delivery systems. The use of polymers in the system allows for narrow size distribution and increased encapsulation efficiencies which are desirable features of drug loaded NPs. Drug loading into NPs are also an important point to consider when synthesizing these molecules.[13, 25] Many studies have used polyethylene glycol (PEG) coated or loaded nanoparticles for higher stability in in-vitro and in-vivo settings as it can withstand circulation challenges from the reticuloendothelial system (RES).[26] Also, when testing in cells (in vitro and in vivo), polymer nanoparticles are known to provide extra

protection against any enzymatic degradation naturally occurring in the body as well as controlled release of drugs to tumor sites.[26,27,28] Many studies have shown that use of polymer nanoparticles for combination therapies in cancer have great cytotoxicity effects both in vitro and in vivo depending on the synthesis and characterization of NPs. Obtaining appropriate sized nanoparticles to assure efficient transport into tumor cells with minimal or no damage to normal cells as well as uptake by necessary cell components is very important.

CHAPTER 2

INTRODUCTION

Recent advances in nanotechnology and drug delivery systems offer areas of improvement and novelty in cancer therapies. Use of polymer based nanoparticles for various cancer treatments is helpful in protecting encapsulated drugs in the body until appropriate release times. We employed this foundation in our study with combination of chemotherapeutic drugs in Triple Negative Breast Cancer to obtain higher and effective cell death at low concentration treatments.

FDA approved drugs that are Topoisomerase II and PARP inhibitors, mitoxantrone and olaparib, respectively were encapsulated in polymer-liposome complexes for synergistic effects on tumor cell death. The interactions between both these drugs are beneficial in TNBC therapies, due to the lack of receptors on TNBC tumor cells. Here, we report a novel aminoglycoside derived polymer liposome complex with encapsulated anticancer drugs for TNBC studies. To our knowledge, this is a novel approach of combination therapy targeting DNA damage in TNBC tumors. Our aim is to also test these novel liposomes in cancer cell lines while studying apoptotic pathways, various pre-clinical settings, to understand the implications in potential clinical oncology settings.

EXPERIMENTAL

Reagents. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), Cholesterol, PARP inhibitors (Olaparib, Veliparib), MTT reagent, dimethyl sulfoxide (DMSO) and Chloroform were purchased from Sigma-Aldrich St. Louis, USA. All reagents were used without any further purification. The anticancer drug, Mitoxantrone was purchased from Ontario chemicals, Canada.

Preparation of liposomes. PARP inhibitors (Olaparib or Veliparib), Cholesterol, and 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) in (0.25-1):1:1 molar ratios were dissolved in a mixture of chloroform in a glass vial, Mitoxantrone stock solution (1 wt % in 1:1 chloroform:methanol) in a 0.25-0.5 molar ratio was then added to this vial containing mixture of compounds. The solvent was removed using a low flow of moisture free air and the dried lipid film was then kept under high vacuum for 8 h. 1 mL of sterile 1X PBS (phosophate buffered saline) (for preparing liposomes containing 1 mM DOPC, (0.25-1) mM PAPR inhibitors and 1 mM cholesterol) was added to the vacuum dried lipid film and the mixture was allowed to swell overnight to form hydrated films. The vials were placed in the vortex mixer for 3 minutes at room temperature to yield multilamellar vesicles (MLVs). MLVs were then first bath sonicated for 5–6 min and followed up with probe sonication for 1-2 min keeping in an ice bath using a Sonifier at 100% duty cycle at 35 W output power for appropriately sized small unilamellar vesicles (SUVs). Given the liposome synthesis described above, since mitoxantrone and PARP inhibitors are highly hydrophobic, they are expected to be localized within the hydrophobic lipid bilayer of

liposomes. The liposomes were finally centrifuged for 20 min at 13000 rpm to precipitate out unencapsulated mitoxantrone. The amount of mitoxantrone entrapped in liposomes (supernatant) was quantitated by spectrophotometric absorbance measurements at λ_{max} of mitoxantrone (450 nm) by lysing the liposomal solutions with Triton X-100 (1%) and dissolving the lysed solutions in methanol. The concentrations (μg/mL) of the liposomally encapsulated mitoxantrone were then calculated from a standard calibration graph constructed by measuring absorbance of six different known concentrations of mitoxantrone of at 450 nm.

Characterization of liposomes. Hydrodynamic sizes and zeta potential values of liposomes were determined using a Zetasizer Nanosystems Nano-ZS instrument (Malvern Instruments, Mission Viejo, CA). The sizes of empty liposomes and mitoxantrone encapsulated liposomes were measured using 150 μL of corresponding liposomes. Zeta potentials or surface charges were measured using 150 μL of liposome stocks with 850 μL of nanopure water. Zeta potential and size distribution measurements were carried out in triplicate and all the liposomal solutions were incubated at room temperature. Liposomes' Size and zeta potential stability was investigated by assessing change in hydrodynamic diameter of liposomes with time (7, 30 and 60 days) using the Zetasizer Nanosystems Nano-ZS instrument. The procedure is as earlier demonstrated under this section; the results were showed on a table.

Cell Culture. MDA-MB-231 (Triple negative breast cancer), PC3-PSMA (human prostate cancer), UM-UC3 (human bladder cancer), and MDA-MB-468 (Triple Negative Breast Cancer) cells were procured from the American Type Cell Culture (Manassas, VA). All cells were cultured in DMEM media supplemented with 10% v/v fetal bovine serum (FBS)

and 1% penicillin-streptomycin (10000 units/mL) solution. All cell lines were maintained at 37 °C under an atmosphere of air (95%) and carbon dioxide (5%) in an incubator. At approximately 80% confluence, cells were trypsinized with 0.25% trypsin-EDTA and seeded at a density of 10,000 cells/well in 96-well plates (Corning, Corning, NY, USA) for all cell viability experiments.

Cell Viability studies. MDA-MB-231, PC3-PSMA, UMUC-3, and MDA-MB-468 Cells were plated in 96 well plates at a density of 10,000 cells/well and cells were incubated at 37°C and 5% CO_2 for approximately 24 hours prior to the treatment. Four different concentrations (0.625μM, 1.25μM, 2.5μM, and 5μM) were employed to the efficacy of the liposomes. The cell viability was determined using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay (described below). The empty and Mitoxantrone loaded liposomal treatments were carried out using concentration range of 0.625 μM-10 μM. The experiment was carried out for 48 hours to know the effective formulation and dosing.

Absorbance Spectroscopy. Following MTT Cytotoxicity assay to the samples, the absorbance spectra of the cells with the different treatments of liposomes were measured using a BioTek Synergy 2 plate reader. The measurements are taken at both 570 and 670 nm, which gives a good indicator of the amount of light absorbed in the samples. Samples with less tumor cells are less of a purple color, are indicated for less viable or living tumor cells, which is then indicated by lower absorbance values read by the instrument. Absorbance values at 670 nm were subtracted from 570 nm, to give the actual values, which are then analyzed accordingly.

Western Protein Analysis. For protein expression analysis, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, Waltham, MA, USA) at 4°C. Immunoblotting was performed for all samples with standard procedures. Protein assaysw were run (Thermo Scientific , Pierce^{TM} BCA Protein Assay Kit) on lysed protein in MDA-MB-231 cells to obtain corresponding protein amounts in a set volume. Briefly, a total of 135 μg of protein was resolved and run by 4%–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 200 V for 32 minutes, and then transferred on to wet nitrocellulose membranes and run at 20 V for 45 minutes. The transfer membranes were then probed with both primary (Rabbit mAb) and secondary rabbit antibodies. For this study, the primary antibodies used were: Capase-3 and TNF-alpha (Cell Signaling Technology, Danvers, MA, USA), Topoisomerase II Beta, RAD51C (Abcam, USA). β-actin (Sigma-Aldrich) was used as the loading control for all studies in dilutions of 1:250 to 1:10,000.

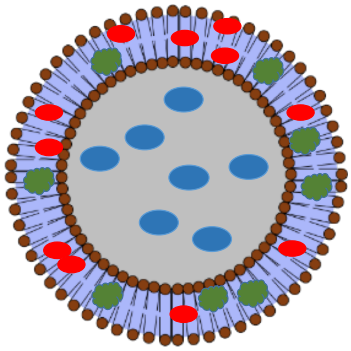
Confocal Microscopy. MDA-MB-231 cells were seeded onto glass coverslips at 10 x104 cells/ml in 24 well plates overnight. Twenty hours following plating, cells were treated with empty, single, and combination drug liposomes and kept at 37°C and 5% CO_2 for 5 hours. Following incubation, media was removed from cells and 1X PBS was washed and added to each well plate. Coverslips were then removed from well plates and inverted on to microscope glass slides containing Fluorogel (Electron Microscopy Sciences, 17985-10). Coverslips containing cells were then sealed with top coat gel and wrapped in aluminum foil to avoid light interference with samples. Cells were examined under a Leica SP5 laser scanning fluorescence confocal microscope.

RESULTS & DISCUSSION

Using a novel nanoparticle based drug system is influential in bringing alternative methods for clinical treatments for Triple Negative Breast Cancer. Synthesizing a system that can eventually become targeted for tumors with a lack of receptors can have significant implications in the oncology field. In addition, having a synergistic combination treatment might have the foundation for solving challenges such as the above in the field. In this study, we report a novel polymer based liposome that can deliver chemotherapeutic drugs (mitoxantrone and olaparib) targeting DNA helix strand damage. Aminoglycoside polymers synthesized in the lab have both hydrophilic and hydrophobic components, mitoxantrone and olaparib are hydrophobic drugs, along with neutral co-lipids that are non-polar components (Figure 2.3.1).

The ability to encapsulate and deliver chemotherapeutic drugs can have a significant impact in tumor ablation in many cancer types. Previous studies have shown that liposome encapsulated mitoxantrone significantly improved anti-tumor activity specifically in breast cancer treatments. In addition, encapsulated doxorubicin (common anthracycline derivative) in liposomes revealed lower cardiotoxicity in patients compared to free drug treatments.[29,26] Other nanoparticle vehicles with mitoxantrone and olaparib (single agent) have also been investigated for various drug delivery techniques.

(A)



(B)

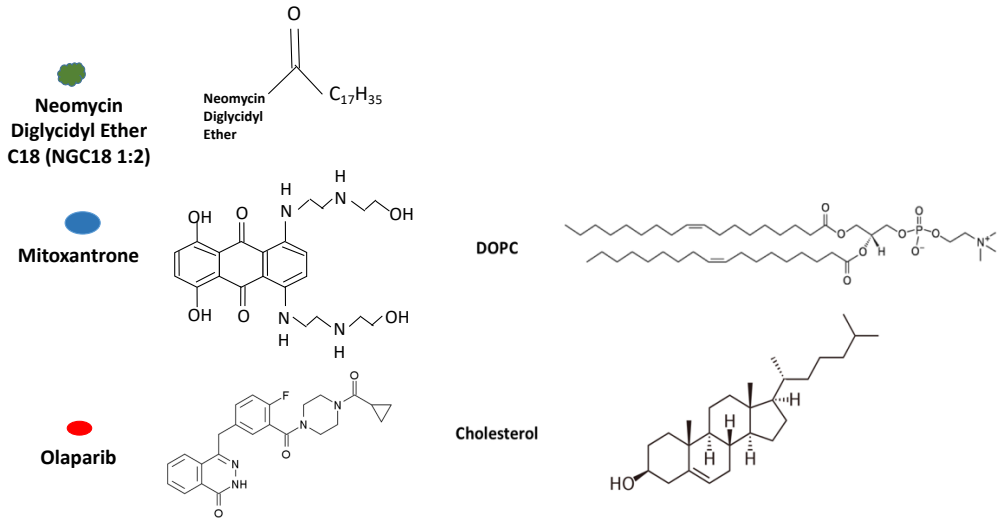


Figure 2.3.1. Schematic of final liposome synthesized with encapsulated drugs, co-lipids and novel aminoglycoside polymers for in-vitro studies. (A) General liposome visual schematic. Co-lipids constitute the liposome structure as depicted by the bi-layer of hydrophobic components. (B) chemical structures of hydrophobic and hydrophilic elements in liposomes.

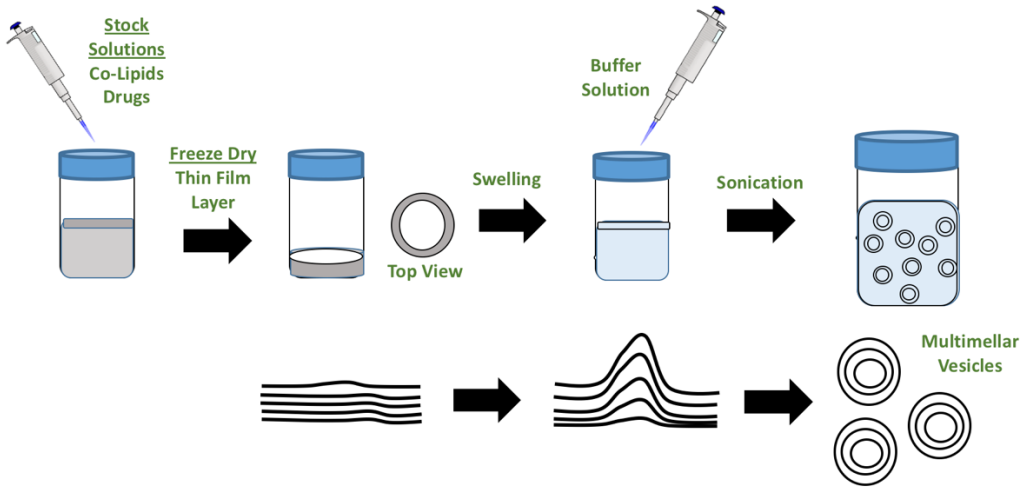


Figure 2.3.2 Schematic of Liposome Synthesis and Cell Treatment Processes. 1mM final liposome solutions are formulated in 1:1 chlorofoam and methanol and then evaporated to create a thin-film lipid layer through a freeze drying process. Then, 1X PBS is added as a buffer to help swelling of drugs and lipids. Samples are placed through a sonication process to promote the multimellar vesicular structures which then promote the final unilamellar structures.

Initially, 1 wt% stocks of various components for liposome synthesis are prepared in a 1:1 chloroform methanol solution. Preliminary experiments included various novel polymers weighed out and dissolved at 2.5wt% in the same above solution, to assure uniformity mixing. Having both non-polar and polar components in our synthesized polymer is helpful to maintain a balance of types of encapsulated molecules in the amphiphilic nanoparticle. Taking appropriate amounts of each component (including polymer) into 3mL scintillation vials, a vacuum source is applied for a thin film layer of chemicals by drying out solvents. After all amounts of solutions were added to scintillation vials, air vacuum (sufficient for small volumes) was used to create a thin-film layer. This establishes the first step of promoting the liposome structure with a lipid bi-layer.[30,31] Neutral lipids typically have a gel-liquid crystal transition temperature at 1^{o}C or less so a popular hydrating medium is usually water of 1X PBS. In this study, 1-2 mL of 1X PBS was added to scintillation vials (1mM liposome) for swelling of liposomes overnight. After sonication and vortex steps the following day, liposomes finally form the multimellar vesicular form to then be used for characterization and toxicity studies.[16, 30] In the liposomes, the hydrophilic (aqueous) core contains the neomycin group which is instrumental in its affinity of binding to Phosphatidylinositol 4,5-bisphosphate (PIP2), an important lipid component of cell membranes.[22,31] The anti-cancer drugs, mitoxantrone and olaparib are encapsulated in the hydrophobic areas of the liposome, as indicated in Figure 5. As mentioned earlier, liposomes are an attractive source for delivering both hydrophilic and hydrophobic drugs.[6,32] The hypothesis was formed on the basis of liposomal delivery of combination drug being more effective than free drug treatments. This can be explained due to potent stability of nanostructures over extended periods of times, high surface charges from

conjugation to aminoglycoside polymers, and ability to facilitate effective drug loading. This should be a tactful technique to observe in comparison to free drug treatments.

The neomycin glycerol polymer with a C18 chain (NGC18 1:2) is produced from cross-linking of neomycin monomers to glycerol diglycidyl ethers cross linkers. Using aminoglycoside polymers in drug delivery is attractive for its many unique properties as mentioned earlier.[33,37] These molecules are a major subset of antibiotics that are particularly helpful in minimizing multidrug resistance from various pathogens, making it useful in drug delivery applications. Aminoglycosides are small molecules with sugar, hydroxyl, and amine moieties with unique properties such as high hydrophilicity and versatile chemistries through slight structural modifications, making it an attractive source for various applications in the field. In addition, aminoglycosides have shown to have the capability to bind to bacterial regions in eukaryotic RNA and DNA.[24,34] Previously, aminoglycosides have been used as starting materials in polymers conjugated to DNA plasmids[35], as well as novel DNA binding ligands.[28] In this study, novel aminoglycoside derived liposomes were encapsulated with mitoxantrone and olaparib for effective drug delivery applications in various cancers, with a focus on Triple Negative Breast Cancer cells.

Liposome Characterization Studies

Four groups of liposomes were formulated for all studies carried out. Empty Liposomes (EL) contain only polymer and co-lipids cholesterol and DOPC. Single agent liposomes, mitoxantrone liposomes (LM) and olaparib liposomes (LO) have components of EL and are encapsulated with 11uL of 1 wt% stock solutions mitoxantrone or olaparib, respectively. Combination treatment liposomes (LMO) had both mitoxantrone and olaparib with co-lipids and synthesized polymers. Characterization studies further on were carried out on four primary groups of liposomes. Dynamic light scattering was used for size and zeta potential studies with diluted solutions of liposome solutions in NPW. Obtaining an appropriate nanometer size and positive surface charge was the aim of these studies in order to assure binding of amines to negative membranes in the DNA backbone. Various formulations were carried out in this study and results of sizes, surface charges, and stability obtained from DLS are outlined below (Tables 1.3.1).

Table 2.3.1 (A) DLS characterization studies of groups of various polymer conjugated mitoxantrone only liposomes. **(B**) DLS characterization studies of single drug and combination drug liposomes. Corresponding poly-disperse indexes (PDI), nanoparticle sizes, and surface charges are shown for each liposome.

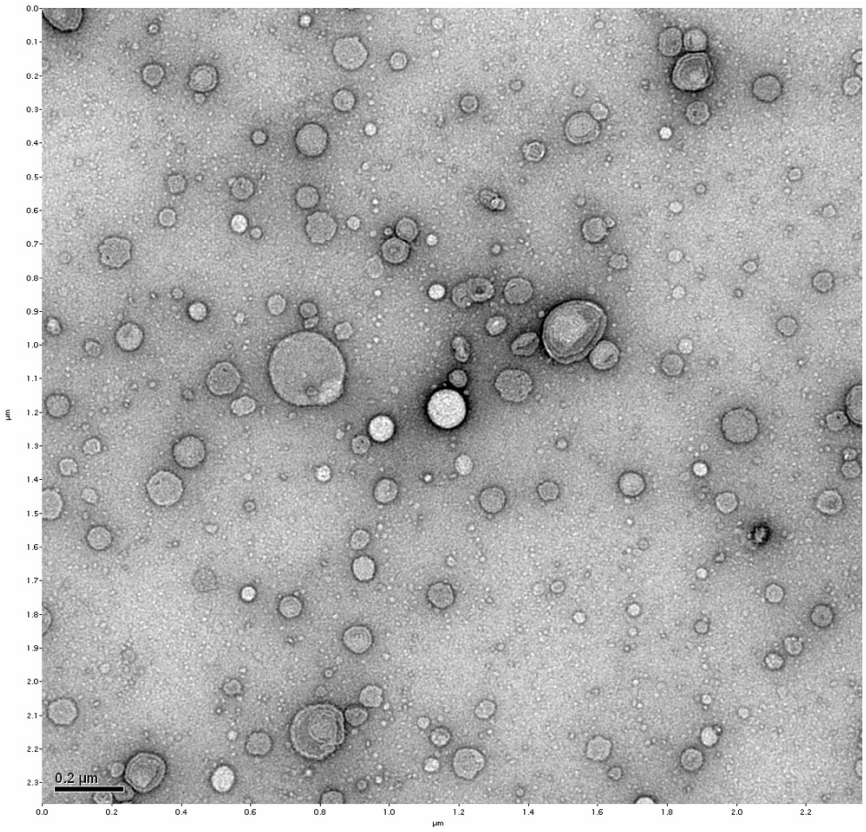
Liposome Number	Polymer	PDI	Hydrodynamic Diameter (nm)	Zeta Potential (mV)
LPSM 1	Neomycin Glycerol	0.19	113	34 ± 3.0
Control		0.43	257	24 ± 7.6
LPSM 2	Paromycin Glycerol	0.16	117	33 ± 3.3
Control		0.30	195	26 ± 6.7
LPSM 3	Paromycin Resorcinol	0.24	198	36 ± 4.3
Control		0.21	229	25 ± 4.8
LPSM 4	Aromycin Resorcinol	0.19	158	37 ± 6.8
Control		0.24	186	32 ±5.4
LPSM 5	Neomycin Glycerol	0.17	143	32± 6.6
Control		0.84	227	23 ± 6.5

The mitoxantrone encapsulated liposomes revealed lower hydrodynamic diameters and higher zeta potential values than their respective controls (without mitoxantrone), indicating their implication for drug delivery applications, both in vitro and in vivo settings. Nanoparticles at 200 nm or less are considered effective due to longer circulation time due to increased stability and uptake by EPR effects in the body. Enhanced permeability and retention (EPR) effects describe the leaking process of nanoparticles leak into tumor tissue through permeable tumor vasculature and are able to stay in the area with reduced lymphatic drainage. Therefore, our characterization studies seemed promising for drug delivery applications. Higher surface charge densities are likely due to presence of amines from neomycin or paromycin groups on outer surface of liposomes (Table 1.3.1). There was not a significant difference in zeta potential of liposomes synthesized of neomycin or paromycin. These liposomes included only encapsulated mitoxantrone with various polymers. Stability of these liposomes were tested over a time of two months. Liposomes with encapsulated mitoxantrone are stable over long periods of time (Table 2.3.2), and show their promising method when used in experiments.

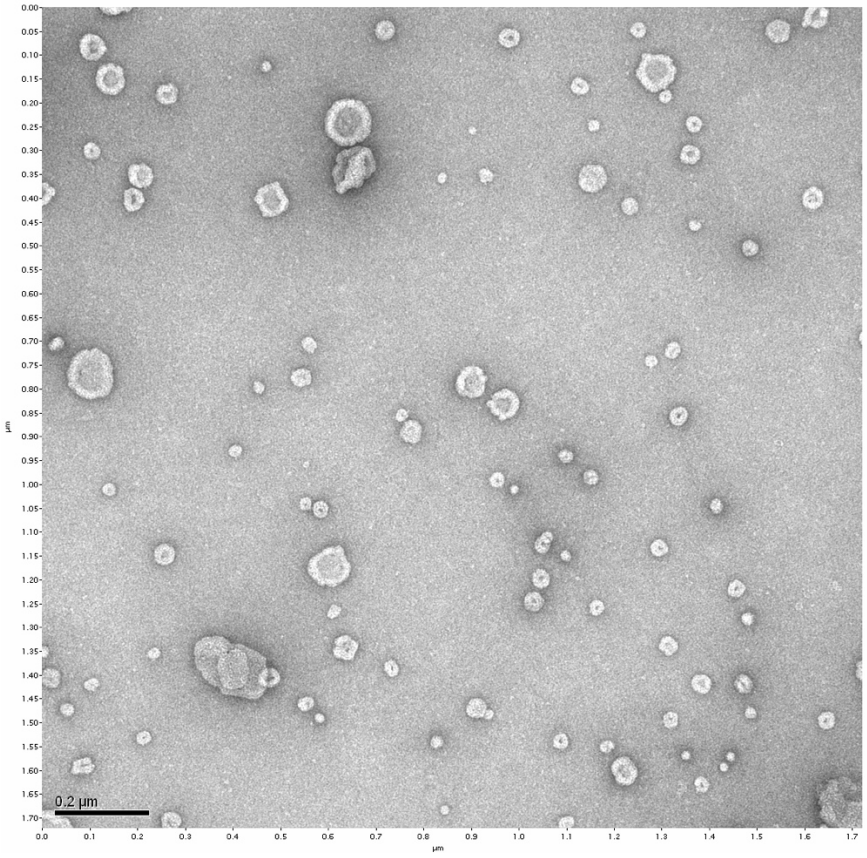
Liposome	Polymer	Hydrodynamic Diameter (nm)		Zeta Potential (mV)	
		1 month	2 month	1 month	2 month
1	NGC18 1:2	132 ± 1	132 ± 2	132 ± 3	132 ± 1
2	PGC18 1:2	127 ± 2	127 ± 2	127 ± 2	127 ± 2
3	PRC18 1:2	138 ± 2	138 ± 2	138 ± 2	138 ± 2
4	ARC18 1:2	158 ± 2	158 ± 2	158 ± 2	158 ± 2

Table 2.3.2 (A) Stability Studies on Mitoxantrone Single Drug Liposome Groups with Various Polymers.

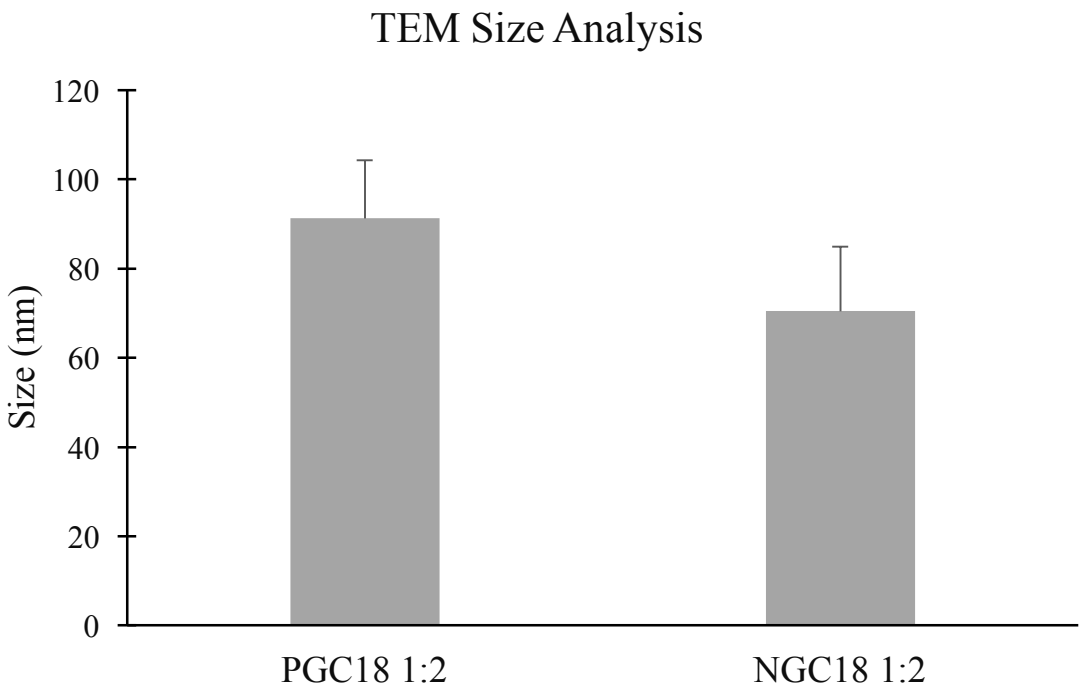
(A)



(B)



(C)



	Neomycin Glycerol Diglycidyl Ether (NGC18 1:2)	**Paromomycin Glycerol Diglycidyl Ether (NGC18 1:2)**
Average Size (nm)	91 ± 13	62 ± 14

Figure 2.3.3 Transmission Electron Microscopy (TEM) Images of Liposomes**.** Structural morphologies of **(A)** mitoxantrone encapsulated liposomes synthesized with Paromomycin glycerol diglycidyl ether and **(B)** mitoxantrone encapsulated liposomes synthesized with Neomycin glycerol diglycidyl ether. Cells with respective treatments were visualized under transmission electron microscopy (TEM), which gives images on nanoparticle sized structures. **(C)** Average sizes of liposomes with the various polymers from TEM images.

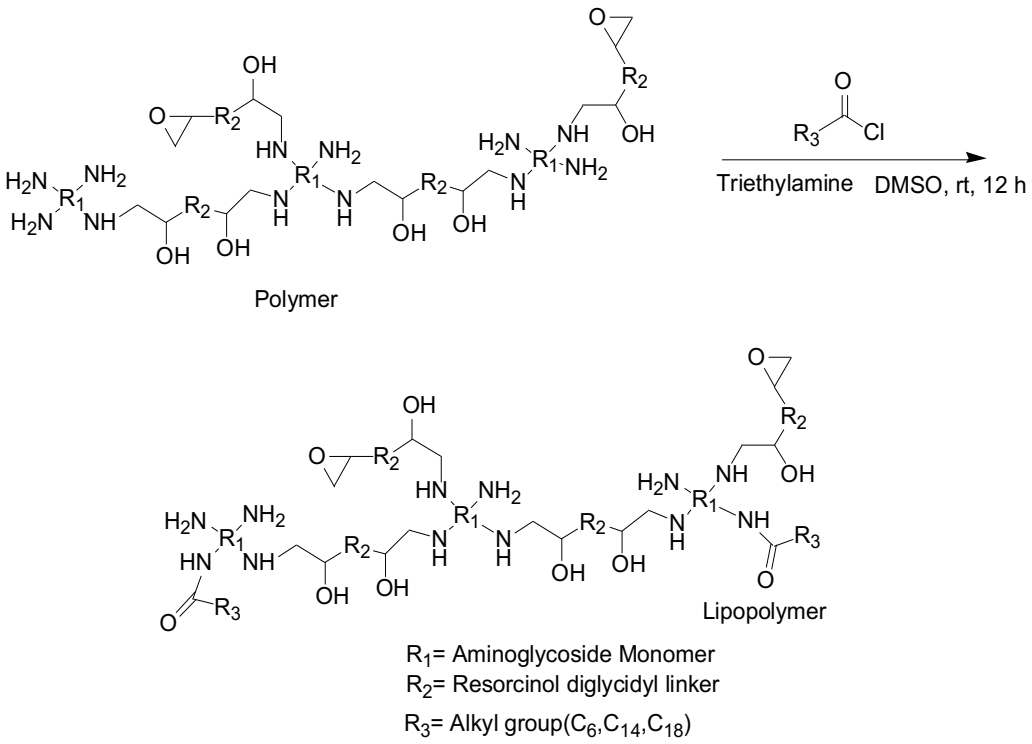
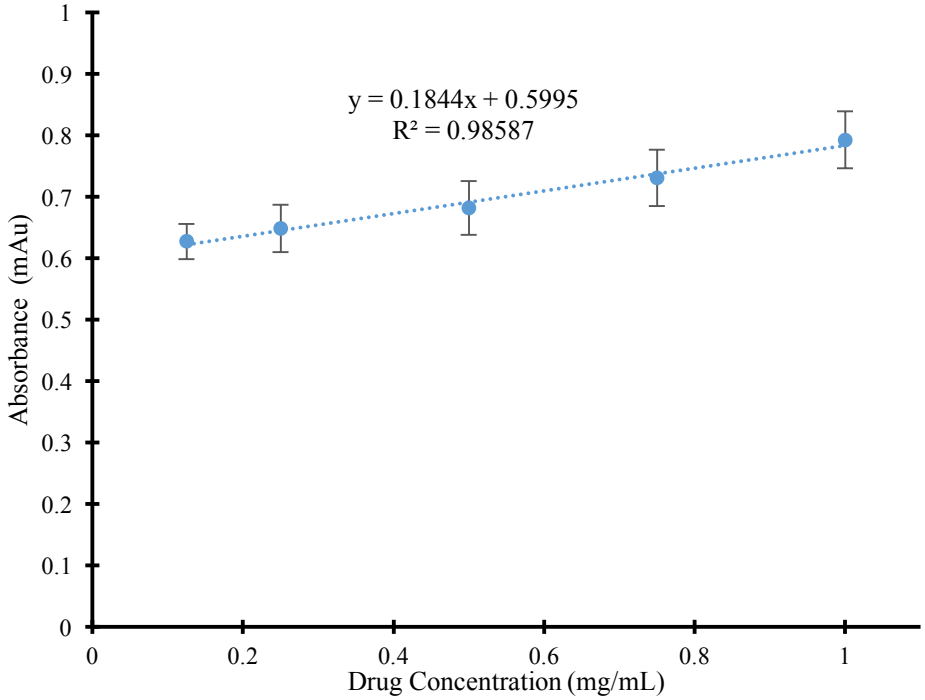


Figure 2.3.4 Aminoglycoside Parental Polymer Synthesis. Monomers were cross-linked with resorcinol diglyceryl ethers and then reacted with alkyl acid chlorides (hydrophobic chain) for parental polymer used in liposome synthesis.


y = 0.1844x + 0.5995
R² = 0.98587
Absorbance (mAu)
Drug Concentration (mg/mL)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
0.2
0.4
0.6
0.8
1

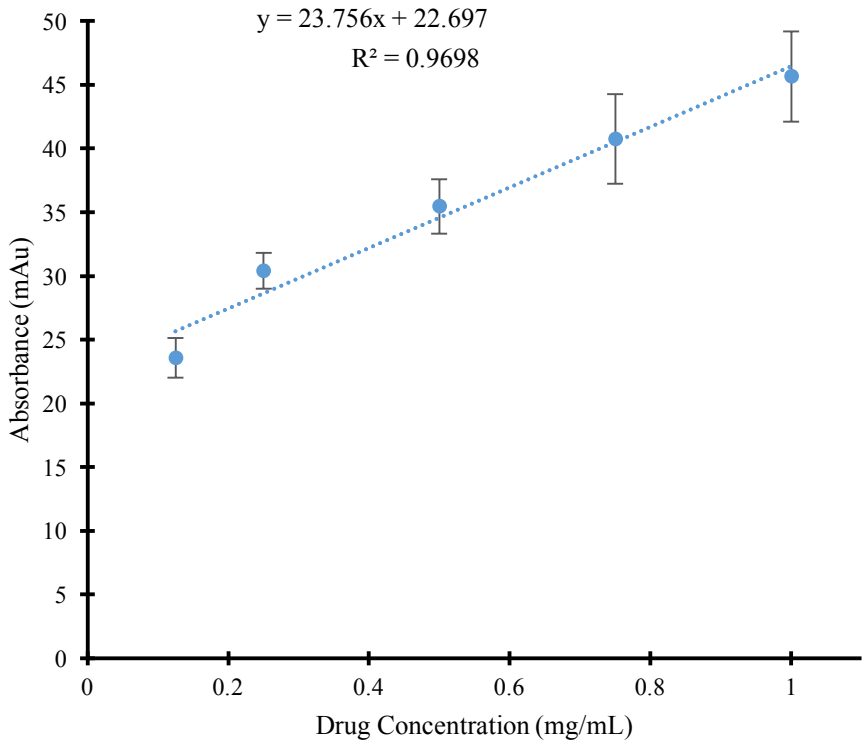


Figure 2.3.5 High Performance Liquid Chromatography (HPLC) Calibration Curves for Loading Studies. **(A)** Mitoxantrone Calibration Curve and **(B)** Olaparib Calibration Curve obtained from standards of drugs in 80:20 acetonitrile:water solutions.

Calibration studies for loading of drugs into liposomes were conducted using High Performance Liquid Chromatography (HPLC) methods. HPLC methods were run at mobile phase, at 0.25 mL/min flow rate in a C18 column with a absorbance detector, 248 nm and 625 nm, for olaparib and mitoxantrone, respectively. Concentrations of drugs were suspended in 80:20 acetonitrile: water and then run through HPLC to detect corresponding absorbances. To detect loading of mitoxantrone and olaparib, single agent and combination liposomes were also loaded into the HPLC and then compared against calibration curves. Liposomes with encapsulated drugs showed efficient drug loading of 20-23% for both mitoxantrone and olaparib. These reported values from our study are considered high and sufficient enough for treatments. The loading range is suitable for drug applications as there will be sufficient amount to be effective in cell treatments.[36]

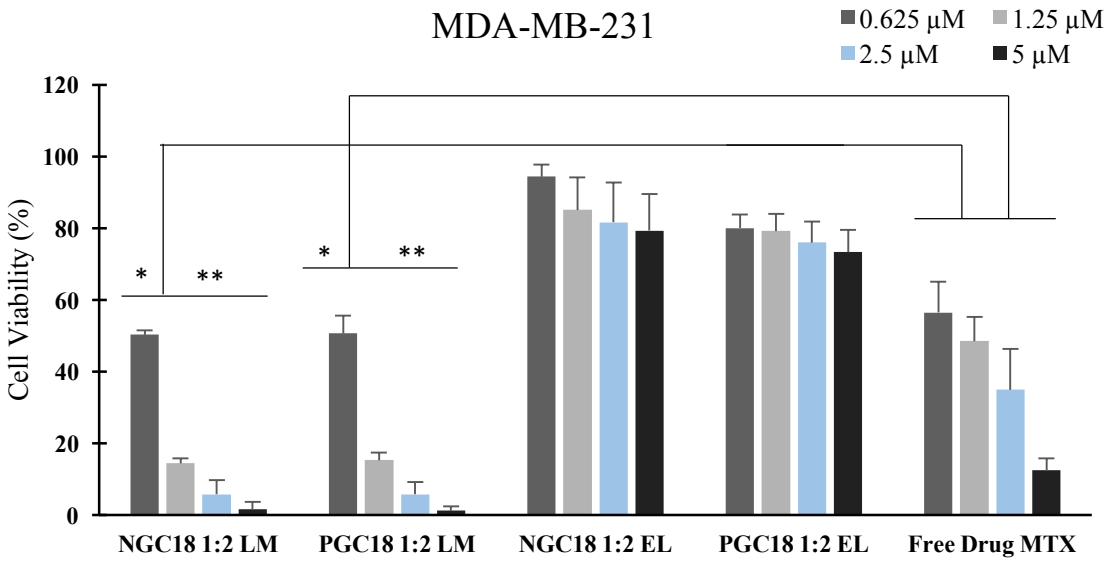
Cell Treatments

Initially, mitoxantrone only encapsulated liposomes were tested in various cancer cell lines to observe effect on cell death (Figure 4 – 6). Cell viability was measured using an MTT assay across cell lines which shows the measure of "viable" or living cells and is usually determined with various assays surrounding cell function, apoptosis, and cell proliferation. Across UMUC-3, PC3-PSMA, and MDA-MB-231 cell lines, cell death for liposomes were significantly lower ($p < 0.001$) in comparison to mitoxantrone free drug and control liposomes (empty) treatments. In MDA-MB-231 (TNBC cell line), mitoxantrone free drug treatments resulted in viabilities between 35% and 20% for 2.5 μM and 5 μM, respectively. At the same concentrations, 2.5 μM and 5 μM, liposomes encapsulating mitoxantrone had viabilities 15% and < 10%, respectively. With aminoglycoside polymers giving a higher surface charge density, the liposome is able to bind efficiently to hyaluronic molecules in

proteoglycans, an important constituent of the cell membrane. Liposomes were most likely transported across the cell membrane through an endocytosis process as the liposomes would be absorbed and form into endosomes.[37] After damaging and breaking through the cell membrane, these positively charged liposomes (endosomes) effectively bind to negative DNA molecules, to release Mitoxantrone as the endosome is cleaved by various intracellular enzymes at the nucleus site.[37,38] Even if nanoparticles are slightly bigger in size, drugs or any genetic material can still interact with nuclear DNA when mitosis occurs.[38, 39] The release of mitoxantrone from liposomes at nuclear DNA site is assumed to be significantly higher than free drug treatments after efficient transport through the cell membrane, resulting in higher cell death. This assumption will later be shown through nanoparticle uptake studies through confocal microscopy.

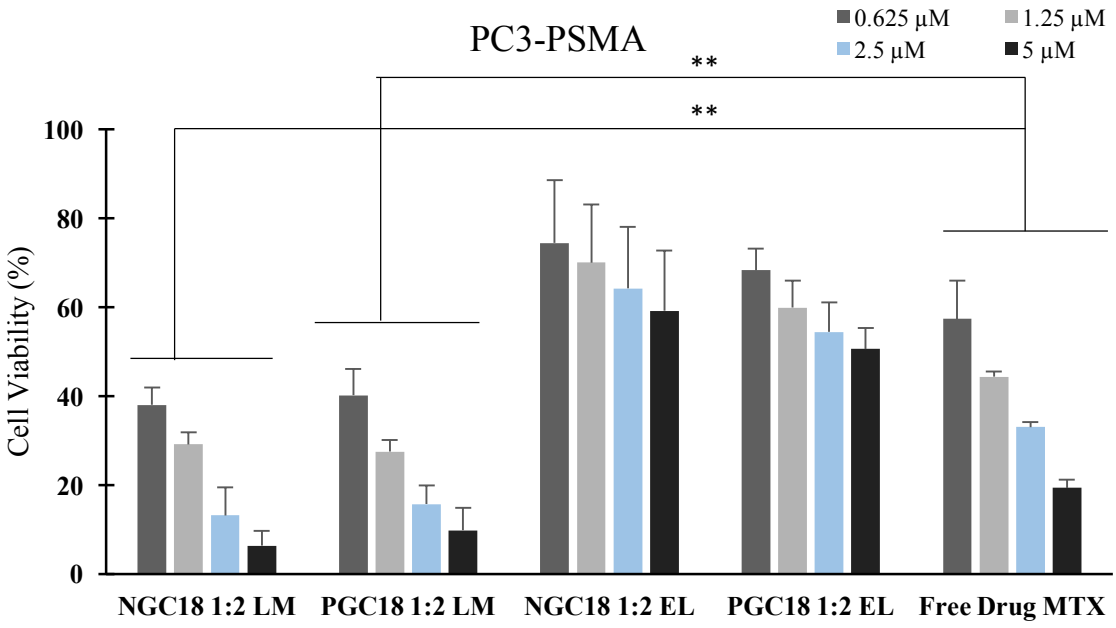
This reveals the efficacy of single agent liposomes across three cancer cell lines, with highest cancer cell death in MDA-MB-231 (Figure 4). Breast cancer cells also have a higher tendency to be more BRCCA-1 mutated, making them more sensitive to chemotherapeutic drugs.[40] With this deficiency, BRCA1 genes are not properly recruited to DNA damage sites by the specific RAP80 mediators. MDA-MB-231 (TNBC cell line) had the highest. Employing these specific drug treatments in cell lines with possible BRCA1 deficiency showed higher cell death due to higher sensitivity levels.

(A)


MDA-MB-231
0.625 µM
1.25 µM
2.5 µM
5 µM
Cell Viability (%)
120
100
80
60
40
20
0
*
**
*
**
NGC18 1:2 LM
PGC18 1:2 LM
NGC18 1:2 EL
PGC18 1:2 EL
Free Drug MTX

.

(B)


PC3-PSMA
0.625 µM
1.25 µM
2.5 µM
5 µM
**
**
Cell Viability (%)
100
80
60
40
20
0
NGC18 1:2 LM
PGC18 1:2 LM
NGC18 1:2 EL
PGC18 1:2 EL
Free Drug MTX

(C)

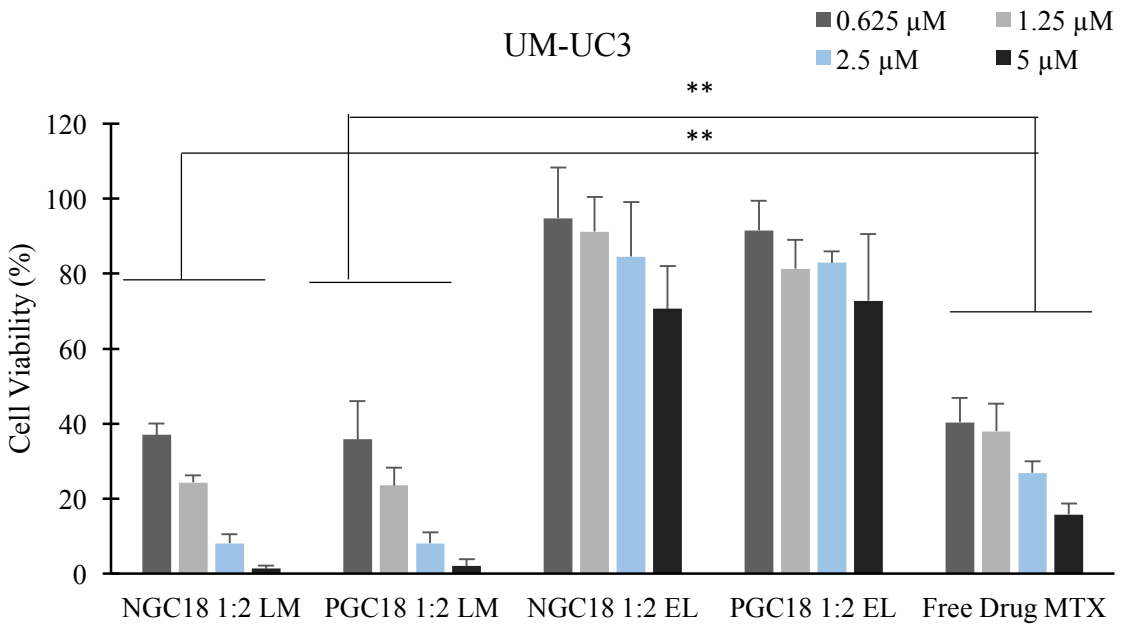
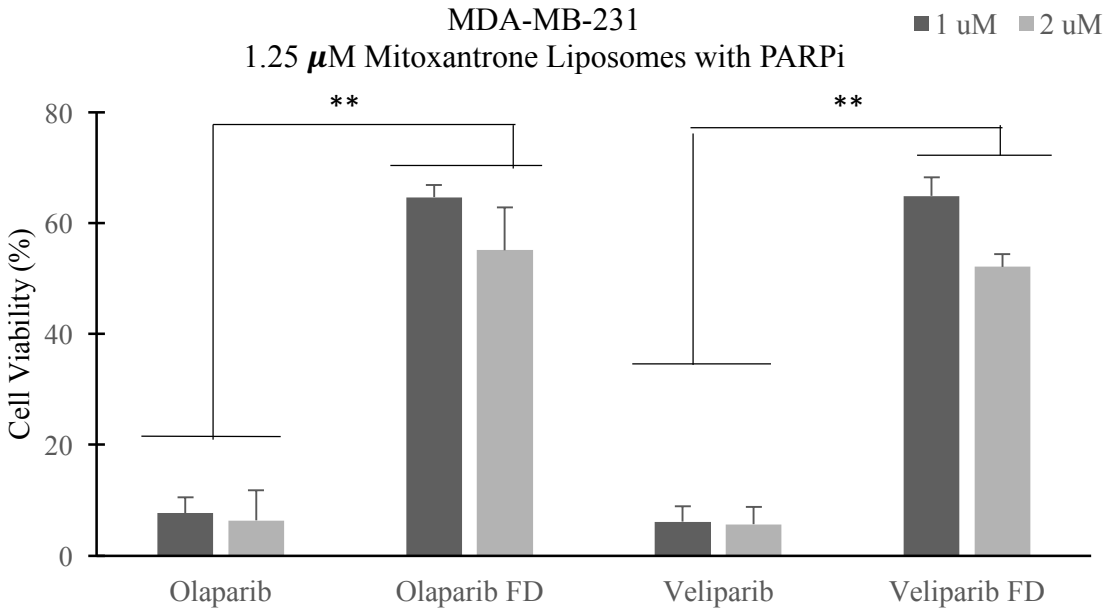


Figure 2.3.6 MTT Cell Viability of Mitoxantrone encapsulated liposomes in MDA-MB-231 (TNBC) cells. Various polymers were used with the single agent liposomes to determine efficacy of liposome mitoxantrone in comparison to free drug and unloaded liposome treatments. Results were recorded 48 hours post drug treatments. Asterisks indicate statistical significance (n=3) between olaparib free drug and combination with liposome treatments (**, indicates p-values < 0.01).

(A)


MDA-MB-231
1.25 μM Mitoxantrone Liposomes with PARPi
1 uM
2 uM
**
**
Cell Viability (%)
80
60
40
20
0
Olaparib
Olaparib FD
Veliparib
Veliparib FD

(B)

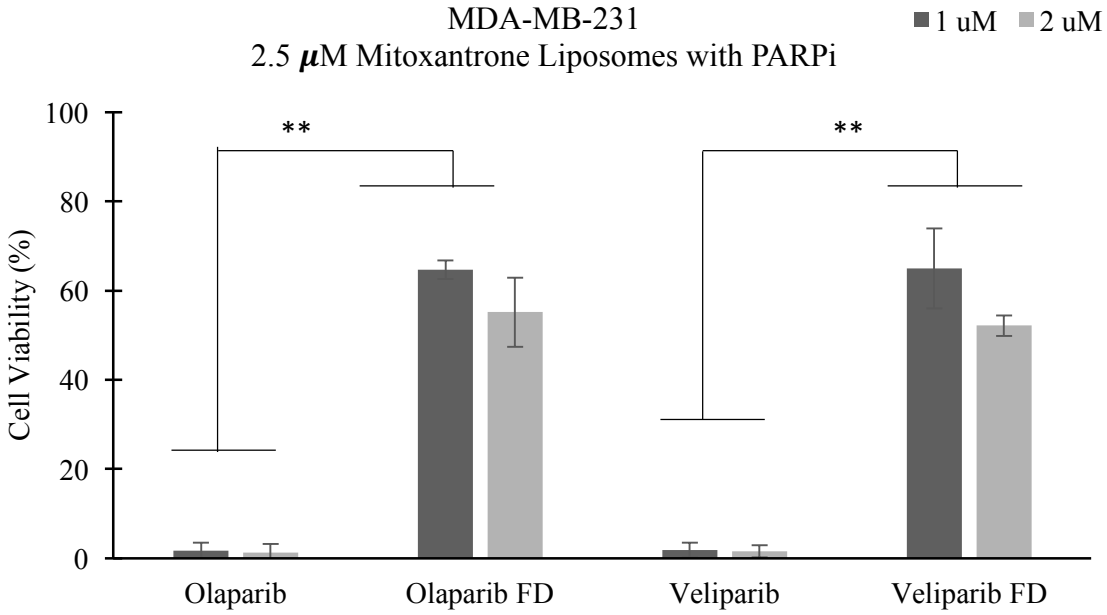


Figure 2.3.7 Mitoxantrone encapsulated liposomes with olaparib free drug treatments in MDA-MB-231 cell culture at **(A)** 1.25 ***μ***M liposomal concentrations and (B) 2.5 ***μ***M liposomal concentrations. Asterisks indicate statistical significance (n=3) between olaparib free drug and combination with liposome treatments (**, indicates p-values < 0.01).

Cytotoxicity of Combination Treatments in MDA-MB-231, PC3-PSMA, UM-UC-3 Cells

Mitoxantrone encapsulated liposomes were further tested across the same cell lines in combination with olaparib and veliparib free drug treatments (Figure 2.3.7). Both are well known PARP inhibitors that are established in phase II and III in clinical trials, with minute differences between them. MTT cell proliferation assay was conducted to obtain cell viabilities across cell lines in response to mitoxantrone encapsulated liposomes with both PARPi free drug treatments. Significant cell death was observed in mitoxantrone liposomes with olaparib and veliparib free drugs in comparison to their respective controls. Additionally, significant cell death was seen when comparing the liposome treatments to both free drug controls and liposome with only olaparib or veliparib. This proves that there is synergistic cell death that is higher than individual agents in liposomes. Higher significance (**, $p < 0.01$) of cell death in liposomes compared to PARPi free drug controls was seen in PARPi concentrations at 2 μM (liposomes constant at 1.25 μM). Additionally, mitoxantrone encapsulated with PARPi free drug treatments (Figure 2.3.6 displayed higher cell death at 1.25 μM in comparison to mitoxantrone single agent liposomes (Figure 2.3.7). It is important to note the lower cell viabilities obtained in the MDA-MB-231 cell line with the lack of 3 receptors, making this combination therapy effective. Additionally, a majority of TNBC cells are considered to be BRCA-1 deficient or defective, making them more sensitive to DNA damaging agents such as the ones in our liposomal treatments. Using a PARP inhibitor is especially useful here as it promotes damaged DNA to enter the next cell phase (mitosis), ultimately leading to apoptosis.[41, 27] All cell lines showed statistical significance, proving the efficacy of combination treatments of mitoxantrone and

PARPi agents in drug delivery applications. Negligible differences were noticed between olaparib and veliparib in performance in liposomal treatments, however, olaparib was used moving forward. Studies have shown that olaparib held higher binding affinities to the PARP complex for more effective inhibition of repair pathways, making it more prevalent in clinical practices.

Table 2.3.2 DLS characterization studies of groups of single and combination treatments of drugs (mitoxantrone and olaparib). Values from studies were taken from n=4 experiments and averaged.

Liposome	Hydrodynamic diameter (nm)	Zeta Potential (mV)
Unloaded Liposome	174 ± 6.28	23.6 ± 3.2
Liposome Olaparib (LO)	176 ± 3.89	23.1 ± 1.4
Liposome Mitoxantrone (LM)	184 ± 1.23	28.4 ± 2.6
Liposome Mitoxantrone & Olaparib (LMO) - Combination	185 ± 9.4	26.8 ± 5.8

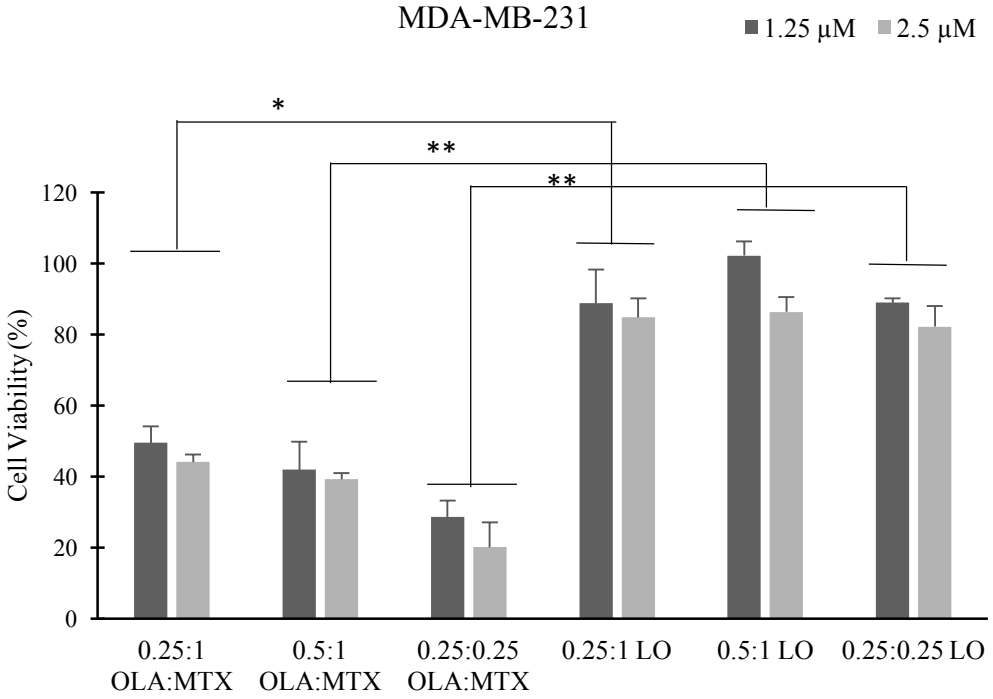


Figure 2.3.8 MTT Cell Viability Assay of Liposomes: Various Formulations of Combination Drug Liposomes in TNBC Cells. Acronyms stand as the following: OLA – Olaparib, MTX- Mitoxantrone, LO- Olaparib Liposomes, LM- Mitoxantrone Liposomes. Asterisks indicate statistical significance (n=3) between groups shown on graph combination versus single drug liposomal treatments (*, indicates p-values < 0.05; **, indicates p-values < 0.01).

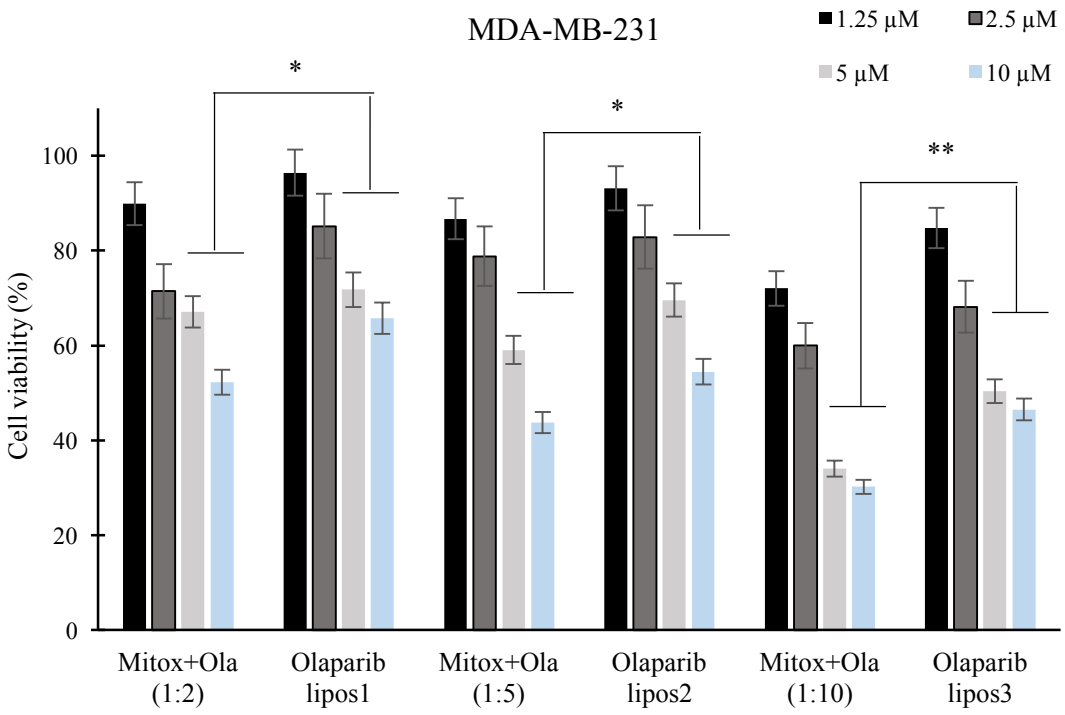


Figure 2.3.9 MTT Cell Viability Assay of Liposomes: 1:2, 1:5, 1:10 Olaparib single agent, and Combination drug liposomes in MDA-MB-231(TNBC) cells. Asterisks indicate statistical significance (n=3) between groups shown on graph combination versus single drug liposomal treatments (*, indicates p-values < 0.05; **, indicates p-values < 0.01).

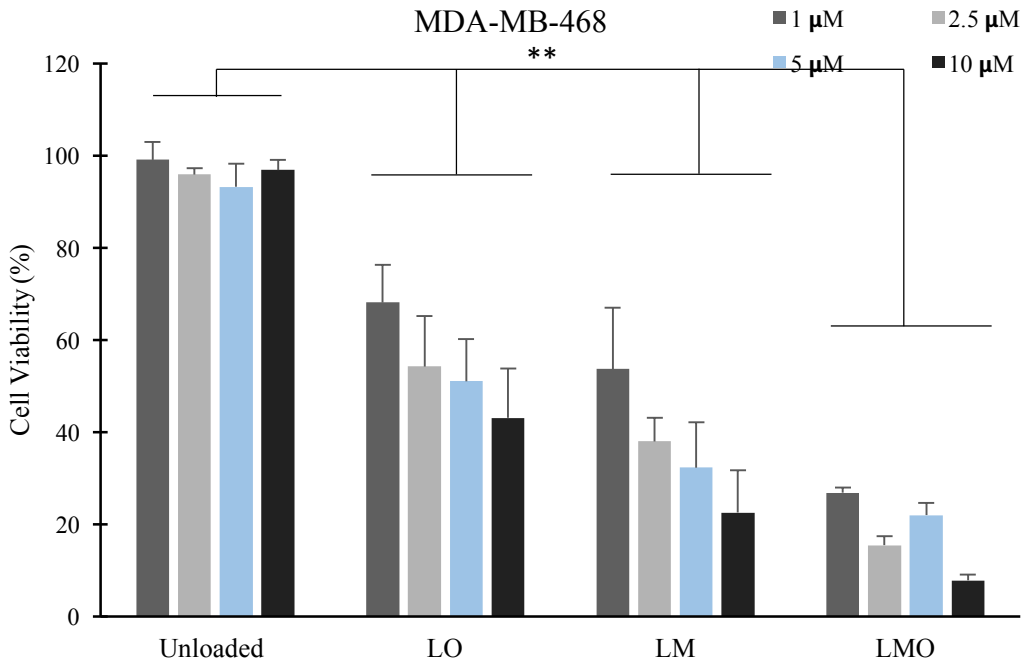


Figure 2.3.10 MTT Cell Viability Assay of Liposomes: Empty (unloaded), Mitoxantrone single agent, Olaparib single agent, and Combination drug liposomes in MDA-MB-468 (TNBC) cells. Asterisks indicate statistical significance (n=3) between groups shown on graph combination versus single drug liposomal treatments (*, indicates p-values < 0.05; **, indicates p-values < 0.01).

Characterization Studies of Encapsulated Mitoxantrone and PARPi

After observing synergistic effects of Mitoxantrone with free drug PARPi in various cell lines, the ultimate goal was to develop a liposome where both components are efficiently encapsulated and delivered at target tumor sites. For the following cell treatments, liposomes with both drugs encapsulated were synthesizes along with controls of single drug liposomes and empty or unloaded liposomes. Dynamic Light Scattering was again used to perform characterization studies of both size and zeta potential in all samples. Liposome sizes were in an appropriate range below 200 nm (Table 2.3.2), however they were bigger in diameter in comparison to Mitoxantrone (single agent) liposomes (Table 2.3.1). This could be due to the fact that the combination liposomes are bulkier with additional PARPi molecules. The zeta potential or surface charge is still high and depicts samples as cationic liposomes for ease of delivery through cellular and nuclear membranes. Having highly cationic particles is beneficial in damaging cell membrane and mitochondrial function, allowing drugs to penetrate targeted area.[29] All nanoparticles were in the range of 170-185 nm with surface charges in the range of 23 – 26 mV.

Cell Treatments of Encapsulated Mitoxantrone and PARPi

Empty (unloaded), single drug, and combination encapsulated liposomes were tested in MDA-MB-231 (TNBC) cells. Here, the synergistic effect of dual or combination drug treatments was off interest. Various molar ratios of mitoxantrone to olaparib were tested (1:0.25, 1:0.5, 0.25:0.25, respectively) with their respective controls of empty liposomes (Figure 2.3.8). Higher cell death was observed in all combination treatments with respect to controls, but highest cell death was in 0.25:0.25 (mitoxantrone : olaparib) liposomes. Equivalent molar amounts were needed in order to effectively damage DNA in tumor cells

and inhibit repair pathways, making this formulation an important one to move forward with in cell and pre-clinical settings. Encapsulating both drugs in liposomes proved to have synergistic and higher cell death than single drug treatments of both mitoxantrone and olaparib (Figure 2.3.8). A 50 to 70 % increase in cell death in dual treatments was observed at both 1μM and 2μM PARPi (olaparib) treatments when compared to single drug liposomal treatments. Liposomes were then formulated with higher ratios of PARPi in comparison to mitoxantrone (1:2, 1:5, 1:10; mitoxantrone : olaparib, respectively). This study was performed to see if there still is effective and synergistic cell death between both drugs in comparison to single agent treatments (Figure 2.3.9). We observed decreased cell death in 1:2, 1:5 mitoxantrone : olaparib in comparison to 1:10 formulated liposomes. Therefore, it can be assumed that because there are significantly lower amounts of mitoxantrone in comparison to PARPi or olaparib, a significant amount (~10 fold increase) is needed in order to obstruct PARP repair pathways, which create a scaffold for other essential repair proteins. Molar ratios of 1:2 and 1:5 are not as effective because there is not enough mitoxantrone (compared to olaparib) supplied for effective DNA damage, so an abundance of PARPi molecules are necessary to stop tumor cells from attempting repair. Empty, single, and combination drug liposomes were tested in an additional TNBC non-metastatic cell line, MDA-MB-468. Cell line variations were observed with same treatment conditions in different cells, which are bound to exist due to origins of where cell lines are extracted from. However, similar trends were seen with synergistic and significant cell death of dual drug liposomes in comparison to unloaded and single drug treatments, making our combination liposome vehicles an effective drug delivery application.

Confocal Microscopy

Mitoxantrone containing liposomes and free drug treatments were observed under Confocal Microscopy to observe nanoparticle uptake versus free drug treatments (Figure 2.3.11). These images show efficient nanoparticle uptake in both single drug and combination with PARPi liposomes as indicated by the red fluorescence shown into cells present in the sample matrix. Free drug treatments were also effective in cell uptake, with no significant differences between free drug and nanoparticle or liposome uptake by cells. However, this supports the fact that no compromise of liposome uptake by cells is made, as efficient transport is still maintained. Our liposomes are effective in treatment of TNBC cells, as seen by efficient uptake and corresponding cell death of liposomes with MTT cell viability assays. During MTT cytotoxicity testing (Figures 2.3.6 to Figures 2.3.9), liposomes were significantly effective in higher tumor cell death, which could be due to higher uptake of liposomal endosomes into nuclear membranes. Aminoglycoside polymers used in the liposomes would be instrumental here with its high binding affinity to phosphate backbones in the DNA molecules and access to any nuclei material.

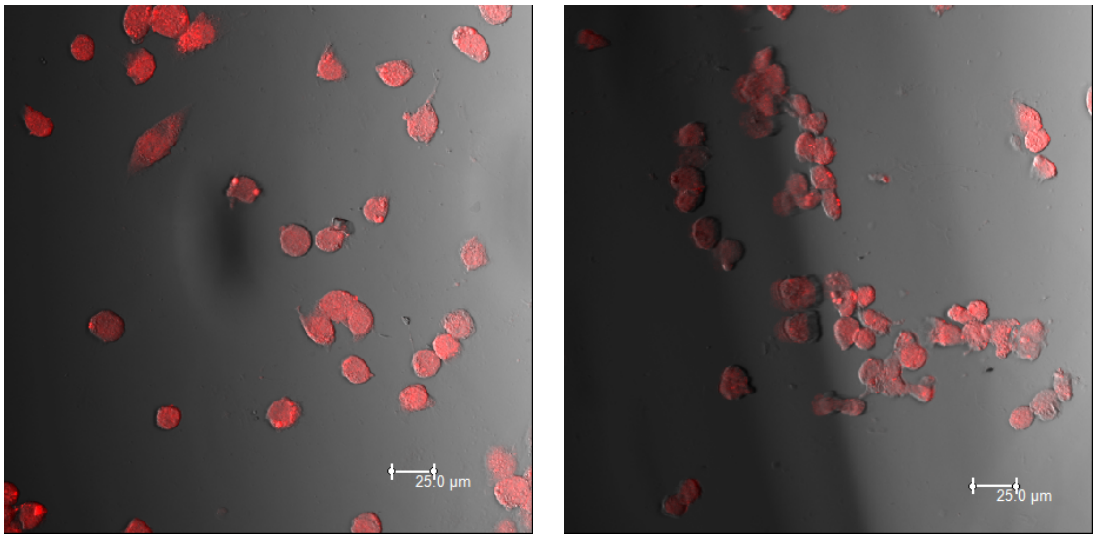


Figure 2.3.11 Confocal Microscopic Images of Liposomal and Free Drug Treatments in MDA-MB-231 cells. MDA-MB-231 cells treated at 10µM with: mitoxantrone free drug (left), combination drug (mitoxantrone and olaparib liposomes (right).

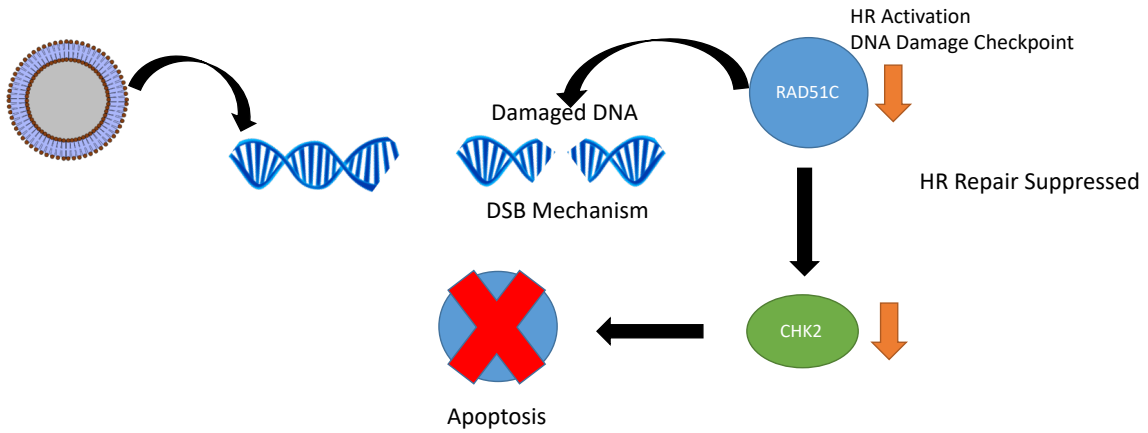


Figure 2.3.12 Hypothesis of apoptotic pathway via RAD51C expressions from liposomal treatments in MDA-MB-231 tumor cells.

(A)

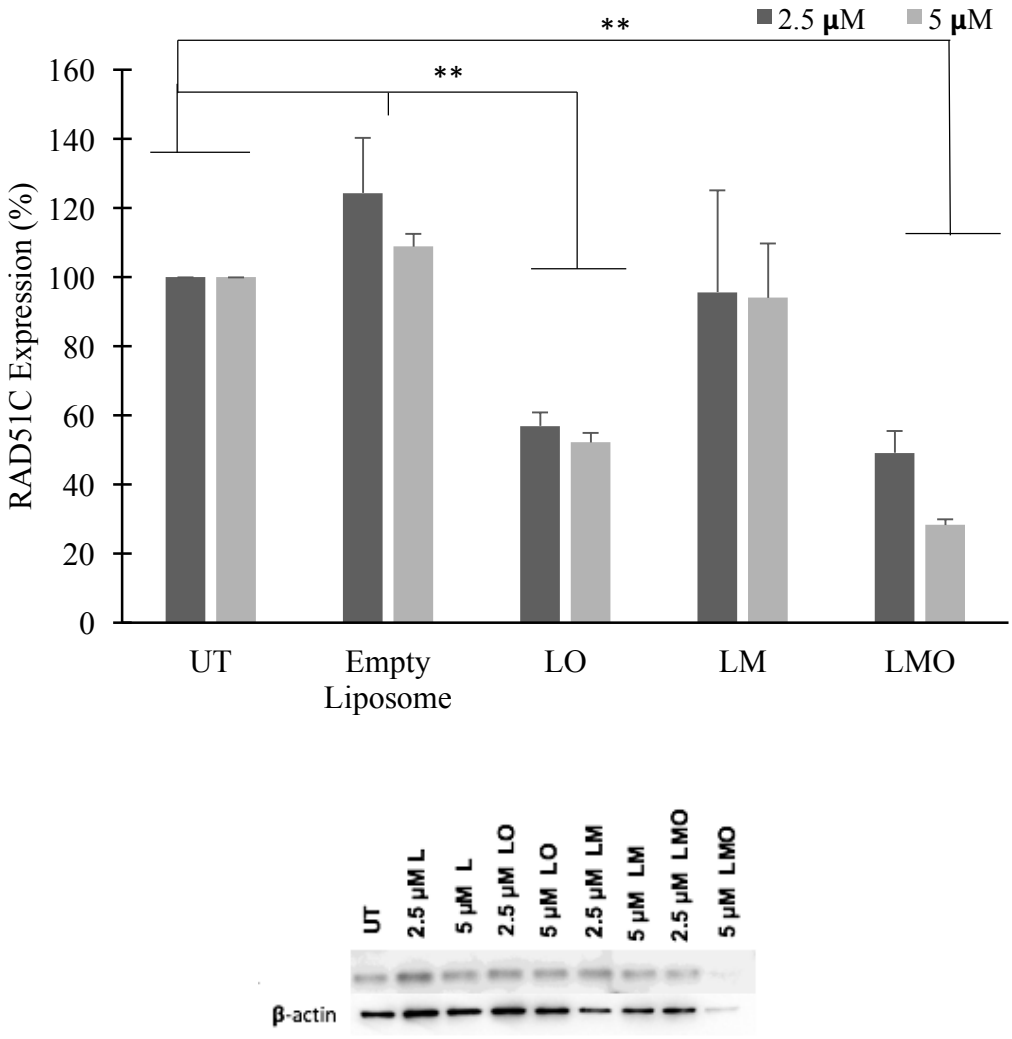


Figure 2.3.13 Densitometry measurements (n=3) of **(A)** RAD51C Expression in MDA-MB-231 Cells with Single and Combination Drug Liposomes, **(B)** RAD51C Expression Images of Liposomes and β actin controls. Images representative of n=3 conducted experiments.

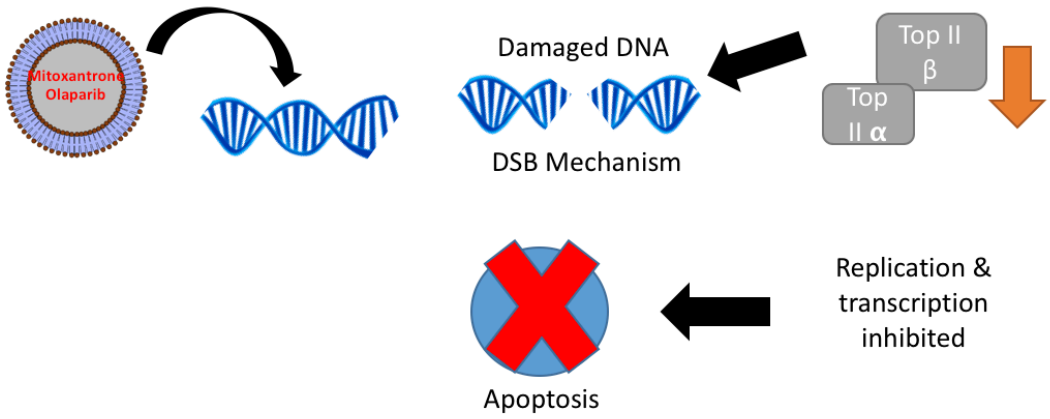


Figure 2.3.14 Hypothesis of apoptotic pathway via Top-II β expressions from liposomal treatments in MDA-MB-231 cells.

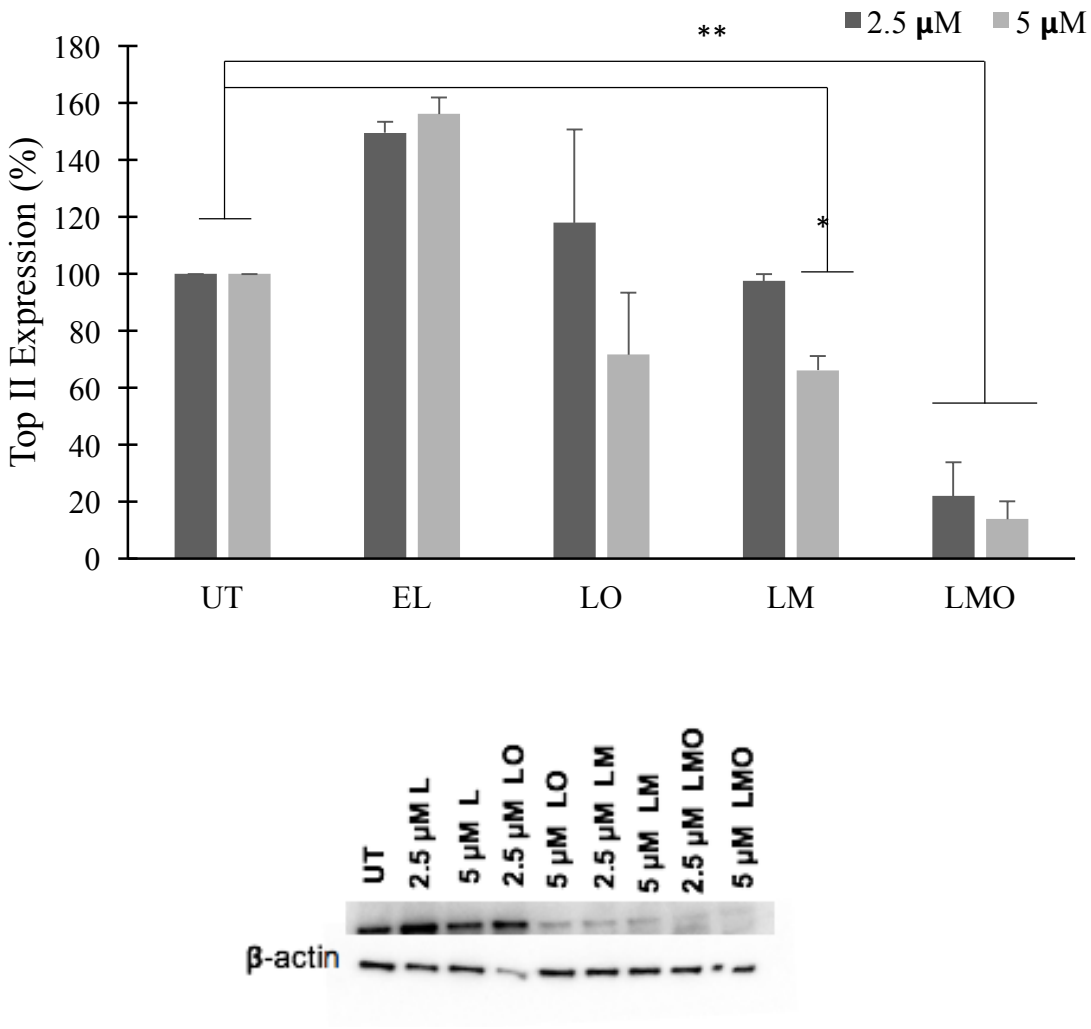


Figure 2.3.15 Densitometry measurements (n = 3) of **(A)** Topoisomerase II β Expression in MDA-MB231 (TNBC cells) in Single and Double Agent Liposomes, **(B)** Topoisomerase II β Expression Images of Liposomes and respective β actin controls. Images representative of n=3 conducted experiments.

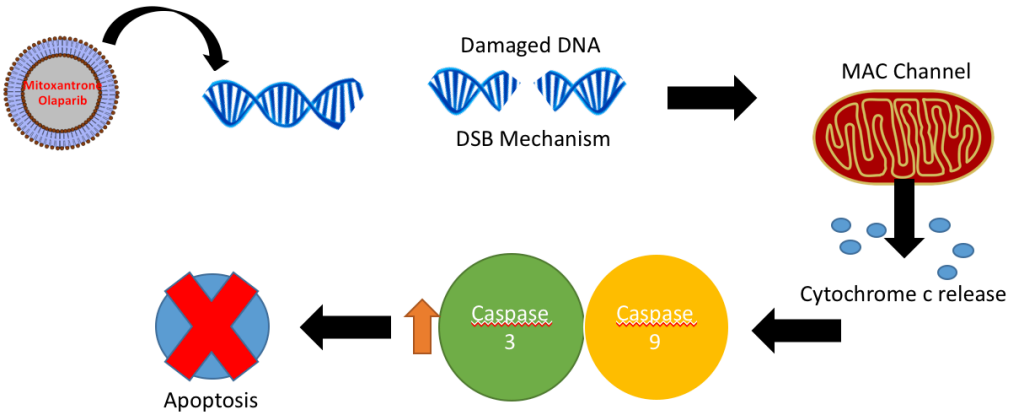


Figure 2.3.16 Hypothesis of apoptotic pathway via Caspase-3 expressions from liposomal treatments in MDA-MB-231 cells.

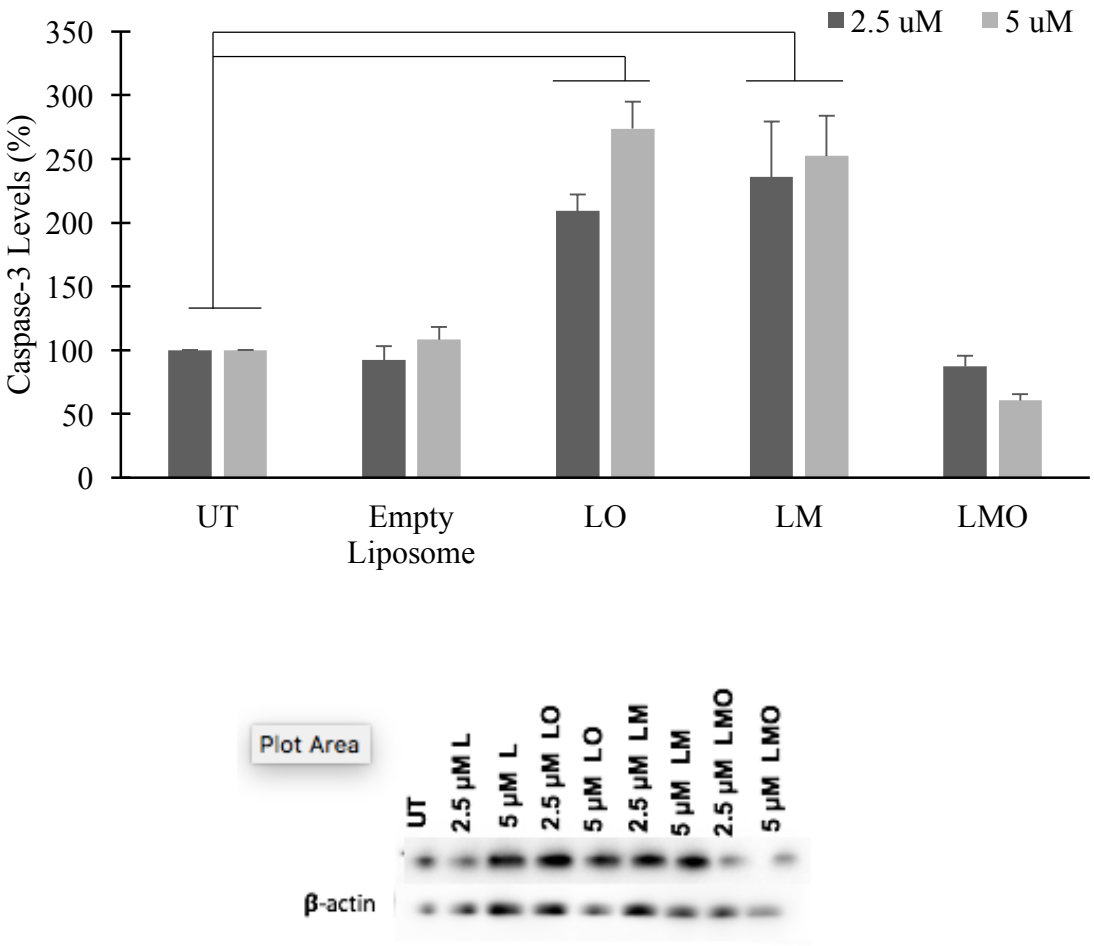


Figure 2.3.17 Densitometry measurements (n=3) of **(A)** Caspase-3 Expression in MDA-MB231 (TNBC cells) in Single and Double Agent Liposomes, **(B)** Capase-3 Expression Images of Liposomes and β actin controls.

Apoptosis Protein Analysis

Protein Analysis (Western Blots) were conducted (n=3) to understand the pathways that liposomes were involved in leading cancer cells to programmed cell death or apoptosis. RAD51C, a significant protein in PARP related activity was one of the few biomarkers evaluated in our studies. RAD51C is a participant in the family of RAD51 protein genes that helps maintain genomic stability in cells through double strand break (DSB) repairs and participates in many subsets of the family. Cells that lack any sets of the RAD51 paralogue are more sensitive to chemotherapeutic agents, radiation, and other factors.[42,43,44] Studies have shown that RAD51C is highly response to DSBs with increasing foci in response to DNA damage over various time periods. Additionally, studies have been conducted revealing that RAD51C is instrumental in delaying progress of defective cells (post DNA damage) through cell cycle (G_2-M phases).[45,44,46] With its many roles in the DNA damage repair process from control homologous recombination activation to filament assembly in RAD51 paralogues, RAD51C was of interest for its expression in response to PARP inhibitor treatments. A particular study also investigated the effects of PARP inhibitors on RAD51C deficient cells. Olaparib induced apoptosis pathways was seen in cells without RAD51C proteins.[44,46,47] Therefore, for our study, we hypothesized that with olaparib treatments, RAD51C proteins would be suppressed, leading to higher cell death via activated apoptotic pathways (Figure 2.3.12). As Olaparib is PARP inhibitor and prevents DNA damage repair pathways to activate, RAD51C and its paralogues should be restrained and not able to recruit subsequent proteins. This down regulation of RAD51C should then force the tumor cells to proceed further in the cell cycle and lead to apoptosis. The expected down regulation of RAD51C expression can be observed in response to

olaparib liposomal treatments that aid in inhibiting repair pathways recruited by RAD51 protein paralogues (Figure 2.3.13). Figure 2.3.13 (B) shows all concentrations tested for each sample corresponding with Figure 2.3.13 (A). It can be concluded that with increased PARP inhibitor sensitivity in the cells, the cell DNA repair protein, RAD51C is stopped from arriving at site to perform its functions. Also, RAD51C is an instrumental part in the 17q23 chromosomal region, a popular spot for estrogen genes, which is commonly linked to breast cancer tumors.[47, 48,49] TNBC tumors therefore will be associated with RAD51C deficiency due to the lack of all receptors (HER, progesterone, estrogen). Seeing the results from our study confirmed that with the use of PARP inhibitors such as Olaparib, RAD51C is less expressed and is not able to recruit its consequent proteins such as CHK2 to pause cells in the M phase of cell mitosis.[44,47]

Topoisomerase II- β was another biomarker studied to understand the mechanism of apoptosis in cells treated with the combination of our liposomes. Topoisomerase II is an essential enzyme instrumental for maintain topological features of DNA during cell replication and other processes to properly maintain function. The apoptosis pathway is known to be as follows: Mitoxantrone binds to Topoisomerase II, cleavages the enzyme, which leads to NF-kappa B activation to result in apoptosis.[50, 51] The covalent bond to Topoisomerase II aids in prevention of DNA strands to come together for any attempted replication stages.[52,] In the Topoisomerase II complex, there are two subunits: Top II- α and Top II- β, which have individual functions in helping with cell regulation and replication processes in DNA. Both sub units are similar in catalytic activity, however Top II- β is more commonly studied as it is the dominant subunit of the two. Studies have

showed that Top II- β levels are altered with various factors in correlation to DNA damage.[50,52] With decreased activity of Top II- β, DNA damage possibly became permanent, leading the cell to irregular function, and ultimately led to apoptosis. With our liposome treatments in cells, the idea was to induce permanent cell DNA damage in tumor cells. Cells were treated with both single and double agent liposomes (combination of mitoxantrone and olaparib) to evaluate protein expressions of Top II- β, which then in turn cannot recruit other proteins for regulating cellular replication and transcription in tumor cells. Figure 2.3.15 displays densitometry measurements of this protein with its corresponding images. Single agents of both drugs displayed less expression with DNA damage induced by mitoxantrone, a Topoisomerase II inhibitor. Combination treatments show a higher magnitude of down expression of Top II- β, making apoptosis pathways more effective. In the combination treatments, mitoxantrone inhibits Topoisomerase II activity as it forms a complex between DNA molecules and the enzyme, inhibiting any interaction between the two. Olaparib then de-activates its repair family pathways, which prohibits the cell from attempt of any repair on DNA damage. The combination treatments suppress Top II- β activity even further, to explain the mechanism behind tumor cell apoptosis in MDA-MB-231 (TNBC) cells (Figure 2.3.15). Suppressing this activity possibly led to decreased and inhibition of replication and therefore transcription processes in cells, which deems the cell "dead" due to irregular function in DNA due to disrupted ATP levels.

Caspase-3 was the final protein evaluated in our studies. Caspase-3 is a part of the caspase family that is responsible for various signaling functions in eukaryotic cells. They function

by cleaving essential proteins or enzymes needed for regular functioning, causing cells to die through apoptosis. This is a sound strategy in treatment of cancer cells as targeting the caspase family is one of the most important molecule leading to apoptosis. DNA damage in cells cause many proteins and chemical releases in cells, which in turn act as "death stimulators" and initiate caspase-8, caspase-9, and caspase-3 downstream. With our liposomes, the hypothesis was that inducing permanent DNA damage in conjunction with inhibition of repair pathways would cause up regulation of caspase-3 and other up-stream caspase molecules to lead cells to apoptosis (Figure 2.3.16). After DNA damage, death initiator molecules could activate the mitochondrial apoptotic channel (MAC), leading to release of cytochrome C, which is the main chemical to activate factors that allow caspase to activate its otherwise latent apoptotic characteristics.[53, 54]1 The cascade of this pathway was interesting for our study, and was observed in the conducted experiments. When treated with olaparib and mitoxantrone single drug liposomes, caspase-3 expression was up-regulated. Mitoxantrone only liposomes, however, had higher expression levels, possibly due to ability to cause more forceful DNA double strand breaks. Combination liposomes, also had an over expression of caspase-3 levels, leading us to believe that cytochrome C release and other upstream apoptotic factors were activated, which are instrumental in programmed cell death. The interactions between all three biomarkers studied are interesting, as they are connected via induced DNA damage to tumor cells, as well as up and down regulation of anti and pro apoptotic factors leading to cell death.

CHAPTER 3

CONCLUSIONS

Conclusion

To our knowledge, this is the first combination treatment involving DNA damage and repair pathway mechanisms for Triple Negative Breast Cancer with a novel drug carrier system. Nanoparticles (liposomes) that were synthesized with aminoglycoside polymers showed promising size (120-180 nm) and stability data by maintaining their size, charge, and structure up to 3 months. Confocal studies also revealed the significant amount and fast uptake of nanoparticles into cells when treated with combination liposomes over free drugs. In addition to breast cancer studies, the combination treatment liposomes also showed significant cell death (85-90%) in low concentrations (2.5μM and higher) in other cell lines such as bladder (UM-UC3) and prostate (PC3-PSMA) which suggests strong implications of this therapeutic strategy in many types of cancer. The two-fold strategy of damaging and inhibiting DNA damage and repair, respectively, is an attractive strategy in cancer therapy to facilitate minimal cancer relapse. Combination chemotherapeutic therapies holds significant impact and high promise in the oncology field, for both pre-clinical and clinical studies.

Future Work

Future work for these breast cancer studies include the following:

- Ongoing western analysis studies to understand additional activated apoptotic pathways
- Ongoing confocal microscopy studies to evaluate efficiency of nanoparticle uptake in comparison to free drug treatments
- Cell cycle studies as a supplemental study to western biomarker studies using flow cytometry methods
    - Provides amount of cell populations in various cell cycle phases
- Liposomes given via intravenous intra-tumor injections to TNBC tumors in mammary fat models of mice experiments
- Targeted nanoparticle studies through folate receptors overexpressed on TNBC cells

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