Next Generation Intracellular Therapeutic Drug Delivery

A 3-Year Study using CRISPR/Cas9 Gene Identification and Delivery of Inhibiting Plasmids via Novel Exosomal Targeting

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ABSTRACT

This year, an estimated 19,950 people in the United States will be diagnosed with AML, the second most common leukemia. A protein called BCL2 inhibits apoptosis (programmed cell death) among cells and is often upregulated in many types of leukemia. This results in cancer cells bypassing apoptosis and multiplying rapidly. To counteract BCL2 activity, an oral therapeutic drug called Venetoclax (ABT199) was created. It inhibits BCL2 in cancer cells and is extremely effective in Chronic Lymphocytic Leukemia with a response rate of 88%. It is currently showing promise in treatment of AML as well. However, as with any drug, patients develop insensitivity (also called resistance) to these drugs. To test the genes for their relevance to ABT199 resistance, it was hypothesized that they should be expressed in leukemia patients cells and should confer resistance when inactivated individually. An analysis of over a thousand gene candidates was conducted, choosing the top 26 genes expressed in leukemia patients. sgRNA's (single guide RNA) were cloned into lentiviral vectors carrying Cas9 proteins to make lentiviruses. These viruses were used to infect Molm13 cells by means of spinoculation. Through this process, targeted genes were knocked out individually with use of CRISPR-Cas9. After 10 days, the ABT199 drug was printed on the cells at different concentrations and compared against a control cell line for survival, after 4 and 6 days. Successful clones showed more living cells at higher drug concentrations than the control. It was concluded that 11 of the candidate genes have a potential to be relevant in ABT199 resistance. These genes can now be further tested for expression in patients who show resistance to ABT199.

Realizing that there was no feasible way to delivery these plasmids to target sites in the body, next-generation intracellular delivery solutions became necessary and will provide essential roles in drug therapeutic applications as well as being critical components of genome editing approaches. Currently, advances in the fields of genome engineering push development of efficient nanocarriers. Many of these nanocarrier systems have limitations, in part because the cell rejects synthetic material regardless of whether or not it is disguised as a biological component. This project aims to use exosomes, being a biological entity, as a novel way to reach areas of the body that are resistant to nanoparticles and other bio-inspired systems. The primary problem that this project deals with is that isolation techniques have not yet been standardized. Thus it is necessary to standardize isolation and delivery techniques in order to use exosomes as efficient therapeutic nanocarriers. Utilizing the three most common isolation techniques: ultracentrifugation, ultrafiltration and an Invitrogen kit, a Multi Criteria Decision Analysis (MCDA) tool was utilized, specifically a Weighted Sum Model (WSM) to conclude that the ultracentrifugation technique was the most effective isolation technique for utilization of exosomes as nanocarriers. These exosomes were then stained with PKH26 dye and revealed to transfect HeLa cell line with great efficiency. Together, these findings demonstrate a clear way to isolate and utilize exosomes as drug delivery nanocarriers and gene identification using CRISPR/Cas9 paving the way to next generation intracellular delivery and solutions to AML.

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1. INTRODUCTION

The purpose of this project was to identify genes that have correlation to ABT199 resistance and develop a drug delivery system to send plasmid knockouts of these genes to targeted sites in the body. To do so research was divided into three sections: research on genetic identification and sequencing of ABT199 resistant genes, biological makeup and processes of exosomes (to determine their abilities to be nanocarriers) and assessment of exosome isolation techniques. This research was done in an effort to understand to what extent exosomes could be utilized as nanocarriers and how feasible it is to isolate exosomes for drug delivery. It was also conducted to identify current over-expressed genes in Acute Myeloid Leukemia (AML) patients that are thought to correlate to ABT199 resistance and utilization of the CRISPR/Cas9 system for gene identification.

1.1 Acute Myeloid Leukemia (AML) Gene Sequencing Need for ABT199

As of 2017, 19,500 new cases of this cancer are expected, of which over 10,000 will die. In AML, myeloid stem cells do not develop into mature white blood cells, rather, they freeze in an immature, abnormal state within the bone marrow. The progression of the growth of these cells in AML can become fatal in just a few months. It is for this reason that AML currently has only a 26.6% survival rate over a 5 year period. B-cell lymphoma 2 (BCL2) is a protein with a specific task of regulating apoptosis, otherwise known as programmed cell death. However in AML, the creation of BCL2 inhibits apoptosis and is often upregulated in many types of leukemia. This results in cancer cells bypassing apoptosis and multiplying rapidly. To counteract BCL2 activity, an oral therapeutic drug called ABT199 was created. It inhibits BCL2 in cancer cells and is extremely effective in Chronic Lymphocytic Leukemia (CLL) with a response rate of 88%. AML patients have developed resistance to ABT199 with response rate of only 19%.

To counteract this resistance, scientists in Dr. Jeffrey Tyner's research lab at Oregon Health and Science University (OHSU)[1] have screened the AML cell line (MOLM13). The purpose was to inactivate thousands of genes to determine possible gene candidates that are responsible for ABT199 resistance. Those conferring resistance were then extracted and sequenced to reveal their genetic makeup. In order to increase response rates in AML, genes correlated specifically with ABT199 resistance must be identified. The method used to identify genes must be accurate and reproducible over multiple trials. The solution to identifying these genes is utilizing a novel gene editing technique called CRISPR/Cas9. Through this

[1] Harding, Clifford V., John E. Heuser, and Philip D. Stahl. "Exosomes: Looking Back Three Decades and into the Future."

method genes can be accurately identified and knocked out at an efficient rate without worry of reactivation of those genes.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) proved to be easy and inexpensive, allowing accurate genome editing. The CRISPR/Cas9 system has excited the scientific community over the past two years because of its ability to be programmed to target specific stretches of genetic code and to edit DNA at precise locations[2]. CRISPR/Cas9 has the ability to permanently modify genes in living cells as well as one day correct mutations at precise locations in the human genome to treat genetic causes of disease[3]. This system is more accurate than any genome editing technique used before and for that reason was selected as the tool to be used for gene editing and modification. CRISPR/Cas9 is composed of two simple components: the sgRNA – Single guide RNA that is used to target a specific part of DNA and the Cas9 cutting enzyme which is able to make a clean cut in the DNA.

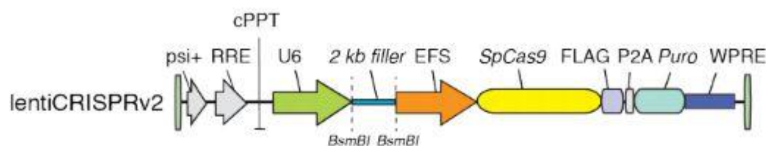


***Figure I**: To utilize CRISPR/Cas9 a specific lentiviral vector as seen with Cas9 and sgRNA customizable component.*

1.2 Exosome Biology

At the heart of most biological processes are tiny extracellular vesicles (EV's) that are responsible for intracellular communication. These vesicles have been found to have large pathological roles in the spread of widespread medical diseases such as cancer[4]. Since the discovery of EV's smaller studies of characterization of the organisms have been conducted[5]. In 1983, two papers Pan et al.[6] and Harding et al. both published that reticulocytes (immature red blood cells with no nucleus) secreted EVs at a specific time as they matured. These EV's have the characteristic of being smaller than 150nm and a few years later the term "exosome" was coined by Rose Johnstone for these specific type of entities/vesicles.

Exosomes can be described as nano-sized biological entities typically ranging from 50-120 nm in length. They are extracellular vesicles secreted by all cell types that have the ability to efficiently enter

[2] Farboud, Behnom. "Targeted Genome Editing in Caenorhabditis Elegans Using CRISPR/Cas9."
[3] Pan, B. T., and R. M. Johnstone. "Fate of the Transferrin Receptor during Maturation of Sheep Reticulocytes in Vitro: Selective Externalization of the Receptor."
[4] Wu, Yueting, Wentao Deng, and David J. Klinke. "Exosomes: Improved Methods to Characterize Their Morphology, RNA Content, and Surface Protein
Biomarkers."
[5] Tran, T. H., G. Mattheolabakis, H. Aldawsari, and M. Amiji. "Exosomes as Nanocarriers for Immunotherapy of Cancer and Inflammatory Diseases." 6
[6] Exosome and Exosomal MicroRNA: Trafficking, Sorting, and Function." Genomics, Proteomics & Bioinformatics.

other cells. Currently exosomes have been thought to be used for cell-to-cell communication, specifically between cancerous cells, as they have been shown to contain genetic material responsible for mediating intracellular communication.

Thousands of publications have revealed specific functions of exosomes and have recently started to create a new cellular pathway to next-generation delivery solutions at a micro level. Currently exosomes are being studied for possible biomarkers and early detection systems to cancers. They have also been thought to be useful in the nanomedicine field as an alternative to inefficient types of nanocarriers[7]. Exosomes may provide a more efficient alternative to such nanocarriers since they are biological entities rather than biosynthetic materials that are more likely to be rejected by the liver.

As seen in Figure II, exosomes house a variety of biological makeup, specifically soluble cell proteins, remnants of cellular membrane and microRNA (miRNA). The membrane of the exosome is created from a soluble lipid membrane taken from the cellular membrane when the exosome is excreted from the cell.

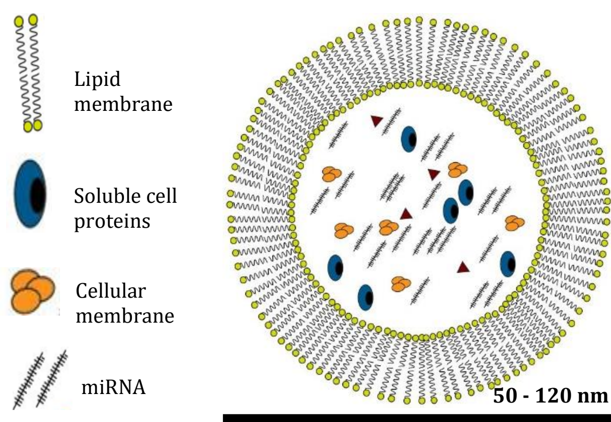


Figure II: *Exosome schematic and biological makeup*

It is important to understand the biological makeup and functions of exosomes because of their contribution to pathological diseases such as cancer and role in intracellular communication.

1.3 Exosome Isolation Techniques

Since exosomes are cell-derived vesicles, they can be isolated from a variety of biological fluids. Exosomes are primarily excreted in a cell culture medium (CCM), plasma or urine. To ensure high quality and uniformity in exosomes, this experiment will focus on techniques of isolation of exosomes from CCM isolation. Currently there are variety of CCM exosome-derived techniques both found commercially and through lab protocols. A commonly used protocol for exosome isolation is ultracentrifugation[8] which encompasses a series of centrifugal forces to pellet small vesicles eventually creating a pellet of exosomes. In recent years an easy-to-use technique is a commercially available precipitation solution called ExoQuick

[7] "Comparison of Isolation Methods of Exosomes and Exosomal RNA from Cell Culture Medium and Serum."
[8] Musante, Luca, Dorota Tataruch, Dongfeng Gu, Alberto Benito-Martin, Giulio Calzaferri, Sinead Aherne, and Harry Holthofer. "A Simplified Method to Recover Urinary Vesicles for Clinical Applications, and Sample Banking."

created by Invitrogen[9]. This procedure has many benefits for in lab in that it does not require expensive equipment and saves time. However this methodology cannot promise homogenous particles or exosomes so many other organisms can be isolated in the process.

In addition to ultracentrifugation, another technique has been applied to differential centrifugation for reliable isolation of pure fractions. Ultracentrifugation requires costly instrumentation so alternatively an ultrafiltration-based approach[10] has at times been applied. This is done through a series of sucrose gradients, extracting each gradients and testing against physical controls to see which layer contains exosomes. A benefit of this technique is that ultrafiltration has a high capacity for buffer exchange and sample concentration, allowing fractions to be easily formatted to any laboratory setting and meet requirements for any downstream analysis platform.

Current exosome isolation techniques for tissue culture have not been standardized, leading to poor concentration rates, isolation times and unclean isolations. This is a barrier for utilizing exosomes as efficient nanocarriers. Therefore it is necessary to standardize and optimize exosome isolation techniques for cell culture isolation of exosomal nanocarriers.

2. MATERIALS AND METHODS

The following objectives were identified for this project:

1. Identify gene candidates for testing
2. Construct lentiviruses with CRISPR plasmid for identified genes
3. Infect resistant cell line with lentiviral virus
4. Identify standard exosome isolation techniques for tissue culture isolation
5. Isolate exosomes with each methodology from HEKT239 cell line
6. Accurately determine which exosome isolation technique provides cleanest and most concentrated sample of exosomes through utilization of Multi Criteria Decision Making Analysis Tool (MCDA)
7. Utilize ultracentrifuged exosomes from HEKT 293T cell line
8. Create effective methodology to measure uptake of exosomes in HeLa cell line
9. Conclude that exosomes can be used as viable drug delivery nanocarriers
10. Determine uses for exosome delivery to therapeutically target cancer cells

To achieve these objectives two major parts of the project were identified.

[9] Ultrafiltration with Size-exclusion Liquid Chromatography for High Yield Isolation of Extracellular Vesicles Preserving Intact Biophysical and Functional Properties." Nanomedicine

[10] Lane, Rebecca E., Darren Korbie, Will Anderson, Ramanathan Vaidyanathan, and Matt Trau. "Analysis of Exosome Purification Methods Using a Model Liposome System and Tunable-resistive Pulse Sensing."

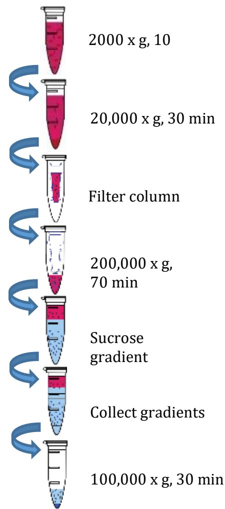


Figure III:
Ultrafiltration

2.1 ABT 199 Gene Sequencing

The top 26 sgRNA candidates identified and were cloned and individual lentiviral vectors were designed with CRISPR/Cas9 commercially. These vectors were then transfected on a 100 cell plate each cell containing its own lentiviral transfected onto an ABT199 resistant cell line. Cells were left in incubation for one week to ensure that transfection did occur.

ABT199 was then printed on top of each cell with an MTS assay (to assess concentration of alive and dead cells). Alive cells were indicators of a specific genes relevance to ABT199 resistance. Different concentrations and compared against a control cell line for survival, after 4 and 6 days. Successful clones showed more living cells at higher drug concentrations than the control.

2.2 Exosome Isolation – Ultracentrifugation, Ultrafiltration, Invitrogen Isolation Kit

The following experimental methods were utilized for a comparative analysis. The following protocols were determined by literature review for ultracentrifugation, ultrafiltration and Invitrogen Isolation Kit.

2.2.1 Ultracentrifugation Based Exosome Isolation

In this method cell media was collected from a petri dish, discarding of the cells. Media was then centrifuged in a series of spinoculations. Initially media was centrifuged for 2000g for 10 minutes. The pellet of dead cells was discarded and media was extracted and centrifuged at 20,000 g for 30 minutes. The process was once again repeated and centrifuged for 200,000g for 2 hours in ultracentrifuge. Using same tubes, same contents was spun for 300g for 30 min. Media is now discarded and the pellet was resuspended in 40um of PBS.

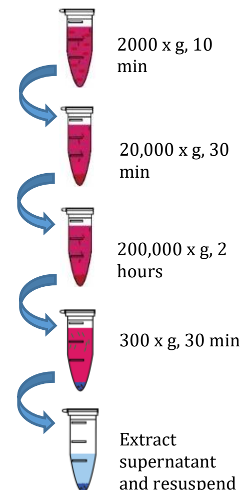


Figure IV:
Ultracentrifugation

2.2.2 Ultrafiltration Based Exosome Isolation

The primary method that was utilized was ultrafiltration and dialysis through a sucrose gradient. Cell media was extracted from petri dish, discarding of the cells. Media was then centrifuged at 2000 g for 10 minutes. After this time a pellet of dead cells forms, media was extracted

from this tube and the pellet was discarded. The media was then divided into ultracentrifuge tubes each holding 2mL of cell media and ultracentrifuged at 20,000 g for 30 minutes. The pellet was once again discarded and media was placed into a filter column >150 nm (filtering out anything greater than the size of an exosome).

Media in the filter column was ultracentrifuged at 200,000 g for 70 minutes. Filtered media was now placed in a 7 layer sucrose gradient, dividing the media based on density. After sitting in the gradients for 24 hours the layers were individually collected and ultracentrifuged at 100,000 g for 30 minutes. Each gradient was tested and gradient with particles sized for exosomes continued to be analyzed through the MCDA.

2.2.3 Invitrogen Isolation Kit

The last method utilized to compare commercial methods was an Invitrogen Exosome Isolation Kit. Cell media was collected from a petri dish, discarding of the cells. Media was centrifuged for 2000g for 10 minutes. The pellet of dead cells was discarded and media was extracted and centrifuged at 20,000g for 30 minutes. 2mL of Invitrogen Isolation solution was then added to the supernatant and spun at 300g for 30 minutes. Supernatant was discarded and exosomes (left in pellet) were resuspended in 40 µL of PBS.

3. RESULTS AND DISCUSSION

Results were isolated by the following parts of the project, the first being gene identification and confirmation, followed by analysis of exosome isolation techniques, and lastly exosome transfection for demonstration of exosomes as efficient nanocarriers via transfection on HEKT293T cell line. First raw data analysis was conducted followed by processed data analysis.

3.1 Raw Data Analysis ABT 199 Resistance

Initial resistance of clones significantly decreased as seen from 6-day resistance ABT199 MTS assay clones. Through evaluation of the line of best fit the following models were able to create that evaluates IC50, the point at which 50% of the cells have died:

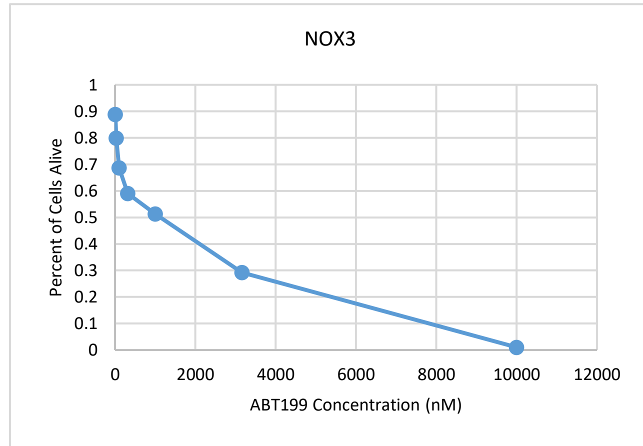
$$IC_{50} = l_{survival}/(1 + ae^{-bD_{dos}})$$

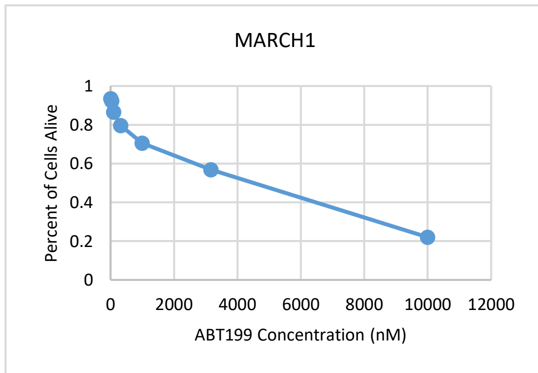
- $l_{survival}$ is the limit of alive cells at the point that there is little to no drug in the plate
- a and b are scalars and b also transforms the sigmoidal curve to best fit the data points.
- $Ddos$ is the amount of drug concentration when a cell line has reached IC50

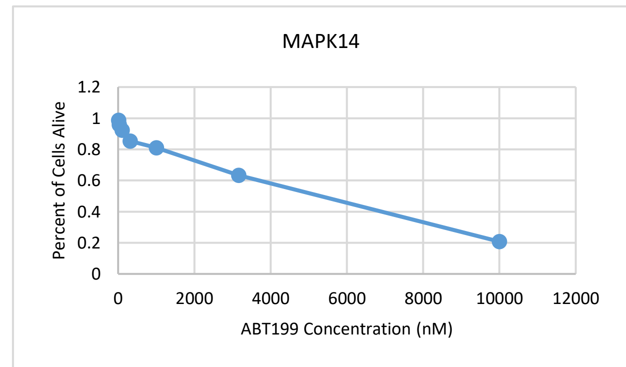
Curves were calculated from the following raw data in table.

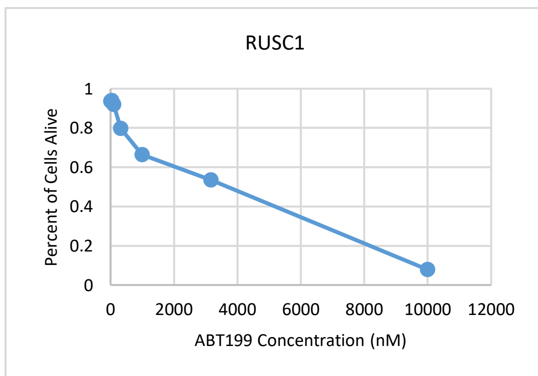
ABT199 Concentration (nM)	CRISPR Control Cell Survival	ABT199R2-1 Cell Survival	ABT199R-2 Cell Survival
10000	26.46192	80.06003	59.30462
3160	57.26695	108.3619	89.37179
1000	61.29983	99.95712	104.4251
316	68.93129	99.61407	86.80363
100	78.67225	122.2556	99.76294

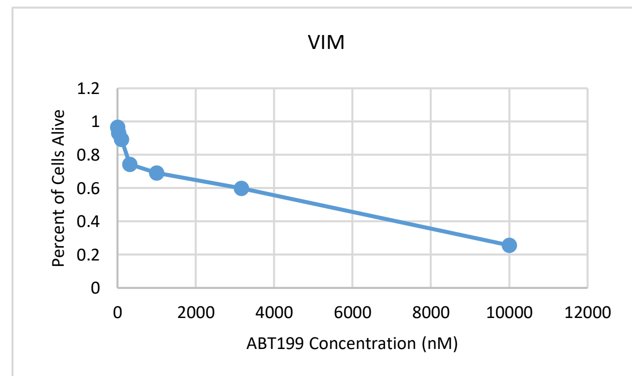
Notable survival curves are shown in the graphs below. Overall 11 overexpressed genes in Leukemia were identified for potential relevance to ABT199 resistance.

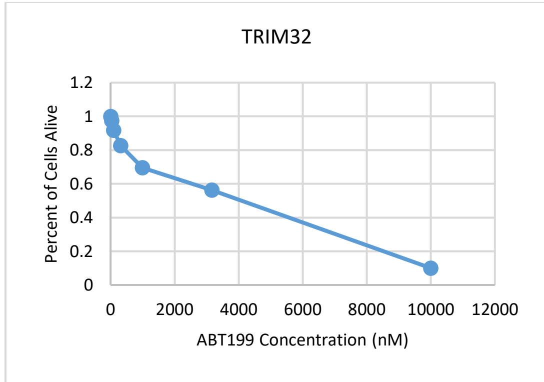


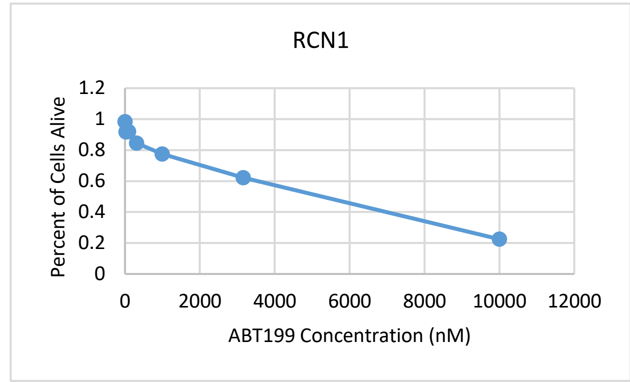


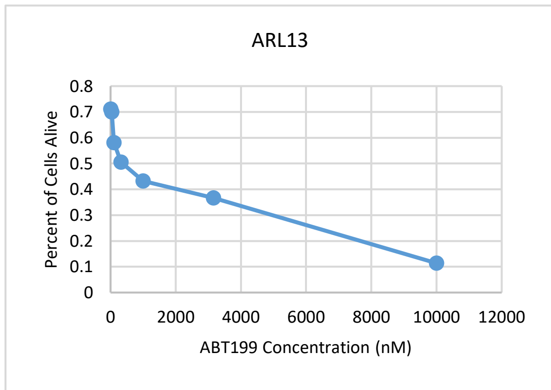




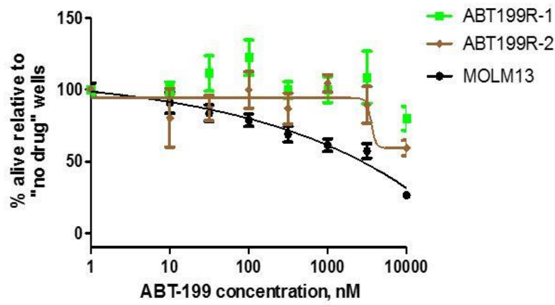








Genes that have previously not been identified with ABT199 resistance were analyzed further as seen in the able on page 9



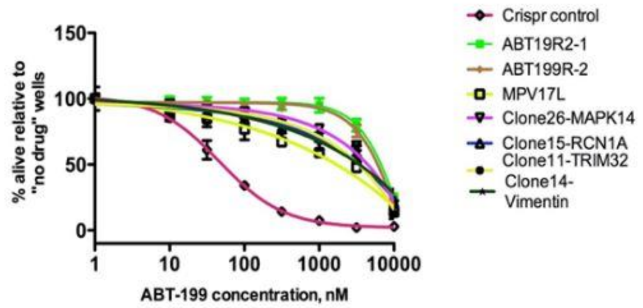


Figure V: *Overall survival curves for ABT199 resistance cells versus normal AML cell line (left) and resistant clones declining survival curves for specific knockout cell lines with ABT199*

Analysis of Identified Genes Conferring ABT199 Resistance
CXCR6 C-X-C motif chemokine receptor. Protein coding gene found in chromosome 3
NCK2 Cytoplasmic protein that belongs to a family of adaptor proteins. NCK2 is located in chromosome 2 and has been shown to bind and recruit various proteins involved in the regulation of receptor protein tyrosine kinases.
SAMD11 Sterile alpha motif domain-containing 11. Protein coding gene for a eukaryotic lineage. Found in chromosome 1
TPCN2 Two pore segment channel 2. Ion channel with 6 transmembrane segments. Associated to prostate cancer susceptibility
NRXN3 Neurexin 3. Encodes a member of a family proteins that has a function in the nervous system as receptors and cell adhesion molecules. Genetic variation has been linked to behavioral phenotypes
RCN1 Reticulocalbin 1. Calcium-binding protein located in the lumen of the ER. **Possible biochemical function besides calcium binding**. In prostate cancer cell lines this protein localizes to the plasma membrane

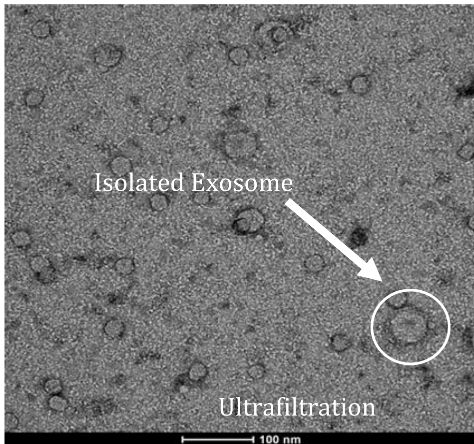
3.2 Exosome Isolation Raw Data Analysis

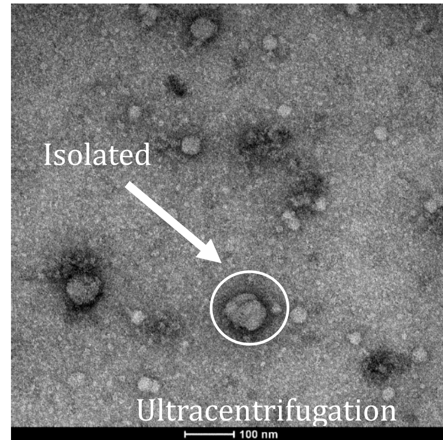
3.2.1 Visual Control Transmission Electron Microscopy (TEM)

The following visual Controls were conducted through Transmission Electron Microscopy (TEM) imaging. It was through these results that the Invitrogen isolation kit was ruled out a as a viable exosome isolation technique as isolated exosomes could be seen, and instead a large amount of aggregate was visualized.

Figure VIII: TEM Results for ultrafiltration, encircled is an example of isolated exosome

Invitrogen Isolation Technique is not a viable solution for isolation of exosome suitable for drug delivery since, there is excess matter that cannot be considered exosomes because of their shape and size. Most exosomes obtained from ultracentrifugation and ultrafiltration were close to 100 nm. In spite of utilizing these methods, there are still additional vesicles that are much smaller than 100 nm. Some of these vesicles can include proteins and pieces of cell membrane. This explains additional peaks found in PDI.

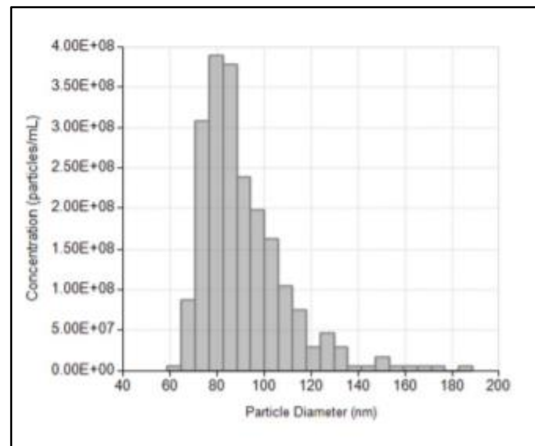




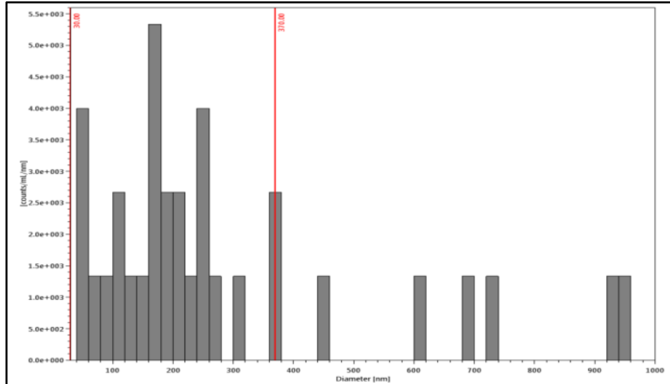
3.2.2 Physical Control

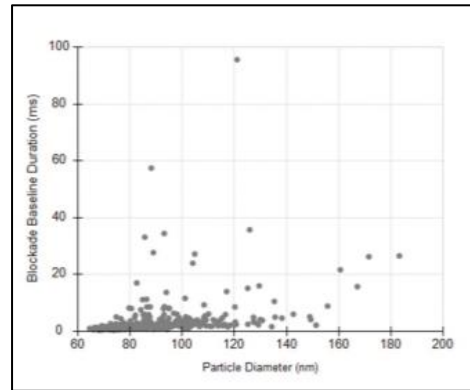
Physical control was utilized for assessment of particles fitting exosome parameters. Two dynamic Light Scattering machines were used to assess particle size, concentration and poly-dispersity index (uniformity of the particles important for nanocarriers).

Ultrafiltration provides few particles/mL, not high enough concentration to be used. Ultracentrifugation provides high quality exosomes, within range Poly Dispersity Index (PDI), meaning that exosomes are relatively uniform. Ultrafiltration PDI was too high, had a range of sizes approximating a PDI of .782. Particle size was more within range for ultracentrifugation than ultrafiltration. Both particle sizes from DLS and Manta showed that ultrafiltration sizes were too



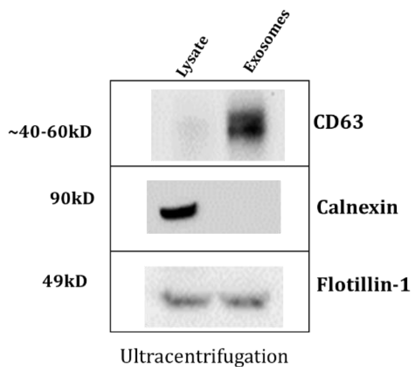
high, however the sizes were very different so systematic error should be accounted for in further data analysis of sizes for ultrafiltration.



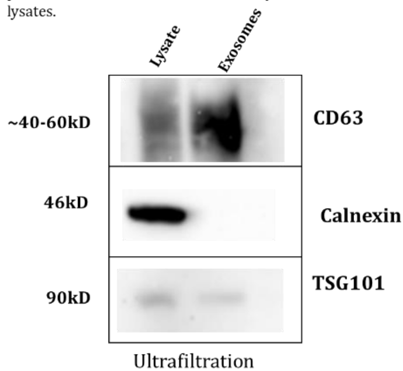


3.2.3 Biochemical Control

Exosomes isolated using ultrafiltration and ultracentrifugation appear to contain CD63, a protein that is found in exosomal membrane. Calnexin is not found in exosome sample meaning that sample does not contain other parts of cell from the ER, as Calnexin is found in the ER. Exosome +ve markers like CD63 and TSG101 are found in both whole cell lysates and exosome lysates. Exosome –ve protein Calnexin is found exclusively in the exosome lysates. Exosome +ve markers like CD63 is found in both whole cell lysates and exosome lysates. However, exosome –ve protein Calnexin is found exclusively in the exosome lysates. Full scores were given to ultrafiltration and ultracentrifugation because they both had clean bands for CD63, further trials should test CD9 as well.



Exosome +ve markers like CD63 is found in both whole cell lysates and exosome lysates. However, exosome –ve protein Calnexin is found exclusively in the exosome lysates.



Exosome +ve markers like CD63 and TSG101 are found in both whole cell lysates and exosome lysates. Exosome –ve protein Calnexin is found exclusively in the exosome lysates.

3.3 Exosome Isolation Processed Data

To process and assess each of the dependent variables a statistical analysis method was utilized for evaluating ultracentrifugation data and ultrafiltration data. Each dependent variable (criterion) was assigned a relative weight per the importance of exosome identification for each one. Primary parameters of an exosome for each criterion was identified. As seen in the following table relative weights added to 1 to then calculate in Weighted Sum Model.

Criterion	Relative Weights	Primary Parameters
Particle Size (PDI DLS)	.25	50-120nm
Particle Size (Manta)	.25	50-120nm
PDI	.10	<.3
Concentration (p/mL)	.20	<10^11 p/mL
Western Blot	.20	Scaled to 0 or 1 based on appearance of CD63 protein

To quantify the quality and success of each exosome isolation technique, each score was given a score out of 100. To calculate this score the following calculation was utilized:

$$A_i = \sum_{j=1}^{n} w_i \, a_{ij}$$

Where A_i=WSM score and a_{ij} = performance value of method

Relative weights and WSM Calculated score for each criterion

	PDI	**Particle Size (DLS)**	**Particle Size (Manta)**	**Concentration (p/mL)**	**Western Blot**
Methodologies	0.25	0.25	0.1	0.2	0.2
Ultracentrifugation	23.75	11.62375	7.8465	1.3334	20
Ultrafiltration	24.2	22.25	8.8365	2.64	20

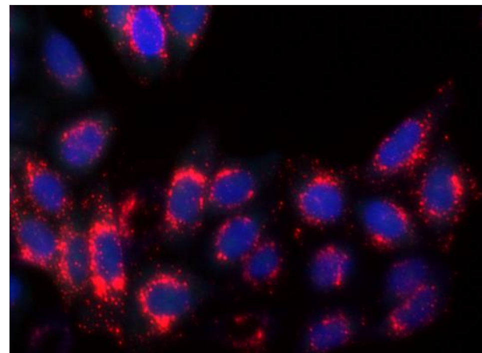
Ultrafiltration data

	PDI	Particle Size (DLS)	Particle Size (Manta)	Concentration (p/mL)	Western Blot
Weights	0.25	0.25	0.1	0.2	0.2
Ultrafiltration	0.782	307.01	243.07	666700	1
WSM Score	**64.6**				

Ultracentrifugation data

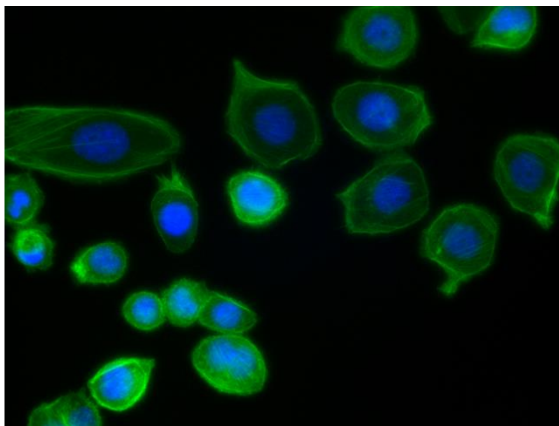
	PDI	Particle Size (DLS)	Particle Size (Manta)	Concentration (p/mL)	Western Blot
Weights	0.25	0.25	0.1	0.2	0.2
Ultracentrifugation	0.285	178	176.73	1320000	1
WSM Score	**77.9**				

3.4 HEKT 293T Exosome Transfection

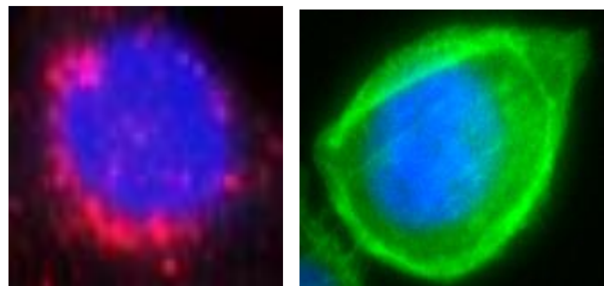


Exosomes isolated via the ultracentrifugation technique were now tested for transfection on a cell line. Particles were stained with PKH26 Staining Dye and transfected onto the HeLa cell line for 3 days. Following this time samples were sent for confocal imaging.

Observations were then noted for both positive and negative control.



Green outlines cell. Blue circles represent nuclei. Cell line without introduction of exosomes.



Observations - Positive Control
• Blue circular shapes in control represent nuclei • HeLa derived cell line • Pink nanoparticles stained with PKH26 Dye
• HeLa cell line • Benefits of utilizing HeLa cell line: HeLa cell line is cancer derived and grows rapidly
• Demonstrates that exosomes can be isolated from one cell line and introduced into a different cell line at large transfection rates
• Drug delivery systems such as mRNA need to enter cytoplasm to become effective, these images show that the nanoparticles have reached close to the nucleus thus proving effective.
• Multiple particles enter one cell at a time, ensuring that drug delivery will be efficient with exosomes
• Average amount of exosomes per cell is relatively equal, analyzing specific pattern of uptake can be beneficial to exosome drug delivery platform

4. CONCLUSIONS

Gene candidates were narrowed from 1000 genes to 11 for ABT199 resistance. I was able to use develop a methodology that is repeatable and accurate to identify these 11 genes by utilizing CRISPR/Cas9. I was able to derive a mathematical model to compare resistant cell lines to one another by calculating their IC50. The conclusion of this gene identification led to the need to find an effective drug delivery system to delivery CRISPR/Cas9 knockout plasmids. This led to an exploration in exosome isolation methods.

The investigation was successful in determining which method of exosome isolation was most successful in producing high yields of quality exosomes. Three of the most common exosome isolation procedures (ultracentrifugation, ultrafiltration and an Invitrogen isolation kit) were analyzed. It appeared that the Invitrogen Isolation kit was not able to effectively isolate exosomes as seen through the TEM. Therefore it could be concluded that the Invitrogen Isolation kit (and by proxy any other commercial isolation technique) was not a viable solution to high quality and high yield exosome isolation.

Once commercial kits were exempt from the analysis, ultrafiltration and ultracentrifugation processes were tested with Weighted Sum Model with the following criterion: particle size, PDI, concentration and western blot. For physical controls primary parameters were chosen. It was obvious through these tests that both ultracentrifugation and ultrafiltration were standardized techniques that can be used to isolate exosomes.

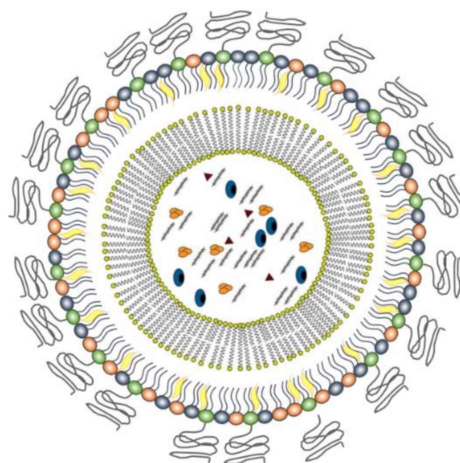
When calculated with the WSM, the ultracentrifugation technique earned a score 77.9 while ultrafiltration technique earned a score of 64.6. Even though it was concluded that ultracentrifugation and ultrafiltration are standardized techniques to isolate exosomes that provide clean samples, high concentration rates and consistent isolation times achieved by ultracentrifugation technique is most effective in isolating exosomes to be used as nanocarriers because of the particle size recovered and high concentration rate.

Now that an exosome isolation technique had been identified, transfection of exosomes had to be verified. Through study of HeLa transfection cell line, exosomes have the ability to enter cells and can be observed from confocal images that will resemble positive control. Thus it can be concluded that exosomes can be used as a novel platform for drug delivery and therapeutic targeting of cancer.

It was concluded using specific gene identification utilizing CRIPSR/Cas9, exosome isolation and demonstrated transfection that I was able to create a system for efficient identification and delivery of knockout plasmids paving the way to a future of personalized medicine and next generation intracellular drug delivery.

5. FUTURE DIRECTIONS

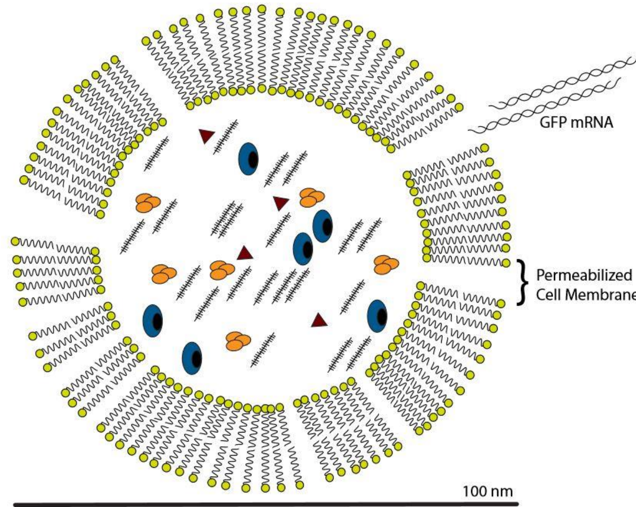
Lipid Based Nanoparticles (LNP's) are limited to their delivery to the liver. A combination of an LNP and an exosome could result in an increased uptake percentage as well as an ability to go targeted areas of the body. These combined platforms could result in an effective drug delivery technique that would be accepted by all cells. Because LNP's are larger than exosomes, this delivery system would also be able to hold larger genetic materials and technologies such as CRISPR/Cas9.



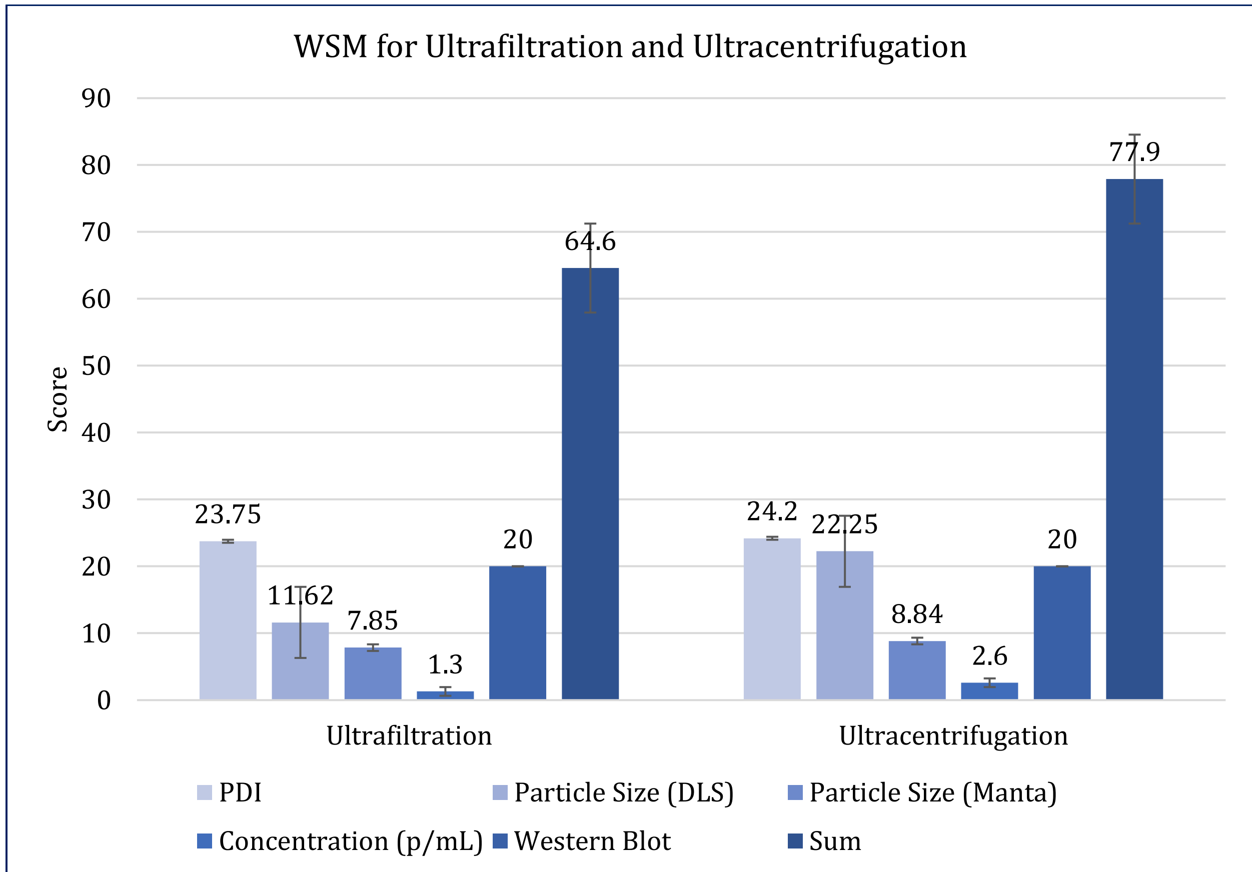
mRNA for CRISPR/Cas9 plasmid

A leading genetic technology in the field drug therapeutics is *in vitro* transcribed (IVT) mRNA. IVT mRNA has multiple benefits over conventional DNA plasmids and short interfering RNA's. IVT mRNA does not need to enter the nucleus to be functional, only the cytoplasm. Additionally, IVT mRNA is transient, meaning that it does not last more than 24 hours and it does not integrate into the genome, preventing the risk of insertional mutagenesis. Electroporation is a process through which high voltage shocks are induced in attempts to permeate a membrane. This process is often used to permeate cell membranes and show a high efficiency. Electroporation may also be able to permeate an exosome

membrane long enough for siRNA to be loaded into the exosome. This could also prove a solution for encapsulating mRNA in exosomes



7. APPENDIX



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