Development of poly(lactic acid)-poly(ethylene glycol) nanoparticles for the delivery of non-toxic drug in combination therapy for the treatment of glioblastoma and neuroblastoma

Olivia Grace DeCroes
*Clemson University, odecroe@clemson.edu*

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DEVELOPMENT OF POLY(LACTIC ACID)-POLY(ETHYLENE GLYCOL) NANOPARTICLES FOR THE DELIVERY OF NON-TOXIC DRUG IN COMBINATION THERAPY FOR THE TREATMENT OF GLIOBLASTOMA AND NEUROBLASTOMA

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Presented to
the Graduate School of
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In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Olivia Grace DeCroes
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Accepted by:
Dr. Frank Alexis, Committee Chair
Dr. Daniel Whitehead
Dr. Jacqueline Kraveka
ABSTRACT

Glioblastoma and neuroblastoma are both solid-form tumors. Glioblastomas primarily reside in the brain, while neuroblastomas are found in the sympathetic nervous system. Both glioblastoma and neuroblastoma re classified as orphan diseases, which affect less than 200,000 individuals a year. Over the course of 5 years, 40% of patients diagnosed with neuroblastomas and only 6% of patients diagnosed will glioblastomas will be living.\(^{73,74}\). Glioblastoma and neuroblastoma tumors possess a gene known as MGMT. MGMT is used by tumors as a DNA repair mechanism, which increases the likelihood these tumors will become resistant to traditional drug therapies.\(^{28,37}\) Other pathways, such as the BER pathway, also contribute to drug resistance in tumors and cause them to be especially difficult to treat.\(^{65}\)

The traditional treatment options for patients with glioblastomas or neuroblastomas are chemotherapy, radiation or surgery resecting the tumor.\(^{5,18}\) These treatment options are ineffective due to the tumors’ ability to resist drug treatments\(^{28}\) and due to the complicated nature of the resection surgery.\(^{2,15}\) Combinations of traditional treatments are also becoming more popular and now patients are being treated with surgery and then with alkylating agents like TMZ for the remaining cells left behind. The focus of this research is to treat tumor cells with TMZ and increase it’s effectiveness through combination drug therapy.

The goal of this research was to formulate a polymeric nanoparticle delivery system that could deliver a non-toxic drug that will aid in the increased effectiveness of Temozolomide. Two drugs were encapsulated in poly(lactic acid)-poly(ethylene glycol)
nanoparticles. The first drug, O\textsuperscript{6}-Benzylguanine methylates the O\textsuperscript{6} loci on MGMT, which inhibits the gene. The second drug, methyl methanesulfonate, which inhibits the ALKb protein that also aids in DNA repair.\textsuperscript{75} The polymer was tested with NMR to ensure that the functional groups were all present and particle sizing ensured that drug could be loaded within the particles. Nanoparticle uptake studies were also performed to demonstrate the particle’s ability to enter the tumor cells. Nanoparticle toxicity studies, free drug studies, and drug loaded combination studies were also performed to determine the efficacy of the treatment.
DEDICATION

I would like to dedicate this work to my parents, Kim and George DeCroes, my sister Victoria DeCroes, and my grandmother, Twyla Starnes. Without their guidance and support I would not have been able to further my academic career. I also would not have been able to complete this work without the aid of my lab family, so thank you to the Alexis Lab as well.
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CHAPTER ONE

GENERAL INTRODUCTION

The research conducted in this thesis focuses on a novel approach for treating glioblastomas and neuroblastomas. Glioblastomas and neuroblastomas are both types of cancers that are not only aggressive, but also develop resistance to traditional treatments, such as chemotherapeutics. Glioblastomas are cranial tumors that affect the supportive tissue in the brain, whereas neuroblastomas are extracranial tumors that affect neurons in the sympathetic nervous system. Ultimately, the ineffectiveness of the standard medical treatment results in thousands of patient deaths annually.

An epidemiology overview of each cancer was provided to describe the demand for an innovative treatment. An outline of the physiological characteristics of each cancer treatment was also provided to discuss other viable options for treatments. Although glioblastomas and neuroblastomas both attack different structures in the nervous system, both cancers possess MGMT, which contributes to the drug resistance of both. A literature review on current treatments of both glioblastomas and neuroblastomas was also provided.

The research conducted focuses on the use of polymeric PLA-PEG nanoparticles conjugated to a peptide, GE11, a known ligand for the epidermal growth factor receptor (EGFR) expressed on glioblastomas and on some neuroblastomas. The loaded nanoparticle has both a non-toxic drug combined with a free toxic drug in a novel approach to improve the efficacy of treatment, which will be discussed later. Theoretically, the nanoparticles with GE11 conjugated PLA-PEG nanoparticles should result in increased uptake by two glioblastoma cell
lines and EGFR positive neuroblastoma cell lines. Cell lines tested contained either MGMT and EGFR or MGMT and no EGFR. Hypothetically, the targeted particles should treat cell lines with MGMT and EGFR and have enhanced therapeutic efficacy compared to non-functionalized nanoparticles. In contrast, the cell lines with only MGMT and no EGFR should see no difference treatment efficacy when using targeted nanoparticles compared to the non-functionalized nanoparticles. The increased uptake displayed allows the particles to be more effective in delivering the drug, and with fewer side effects than non-targeting particles that circulate throughout the body.

Ultimately, the goal of this research is to provide an approach that will improve the uptake of nanoparticle into the cancer cells, overcome drug resistance, reduce treatment side effects, and ultimately reducing the viability of the cancer cells.
CHAPTER TWO

EPIDEMIOLOGY AND GENERAL PHYSIOLOGY OF GLIOBLASTOMA AND NEUROBLASTOMA

2.1 Epidemiology of Glioblastomas and Neuroblastomas

Glioblastomas and neuroblastomas are classified as orphan diseases, where less than 200,000 United States Citizens are affected annually.\(^3\) Approximately 17\% of primary brain tumors are classified as glioblastomas, which makes it the most common form of solid brain tumor. Glioblastomas are especially prevalent in patients between the ages of 40 and 70.\(^3\) Between 12,000-14,000 United States citizens are diagnosed with glioblastomas annually, with less than 10\% of patients surviving more than 5 years. The older a patient was at the time of diagnosis of a glioblastoma, the less likely that patient was to survive.\(^26\) Glioblastomas have an increased concurrence with age, while only affecting 3\% of childhood brain cancers.\(^1\) The survival rate is higher in children than in adults with an average of 24 months post-diagnosis.\(^43\).

In contrast, neuroblastomas are the leading type of brain cancer in small children with prevalence in children between infancy and two years old. An astounding 90\% of neuroblastomas are diagnosed before the age of 5. Approximately 6\% of childhood cancers are classified as neuroblastomas, for which 700 new children are diagnosed annually.\(^32\) These rare types of cancer have few successful treatment options due to their aggressive nature and are in need of a new treatment approach. Prognosis for children under the age of one with neuroblastomas is fair, but patients over one year old have a very poor prognosis with extensive
metastatic disease. As a result, the long-term survival rate for children diagnosed with neuroblastomas is only 15%.

**General Physiology of Glioblastoma and Neuroblastoma**

2.2.1 Glioblastoma General Physiology

Glioblastomas, or glial cells, are highly aggressive brain tumors that attack this support tissue. Glial cells are a type of astrocyte that supports the neurons in the brain. Glioblastomas are a stage IV astrocytoma that contains calcium deposits, cystic material, and mixed cells. Microvascular proliferation is a type of angiogenesis that is typical of glioblastomas. The vascular proliferation can be attributed to loss of tumor suppression genes and the activation of oncogenes, one of which being the EGFR. There are four subtypes of glioblastomas, which are divided based on their genetic markers and characteristics. These subtypes are defined as Proneural, Neural, Classical and Mesenchymal. Of the four subtypes of glioblastomas, proneural, mesenchymal, and classical glioblastomas typically possess higher expression of the EGFR. In a recent study, 200 glioblastoma samples were selected. The samples had to have less than 40% necrosis and be within standards for microarray quality control. Microarray experiments were performed, which provided an accurate estimate of gene expression. The results based on subset did show pronounced gene expression of EGFR and the classical subset of glioblastomas displayed up to 97% of tumors with an overexpression of EGFR. EGFR overexpression is especially common in patients of increased age. It is a common biomarker for patients with glioblastomas in addition to EGFR, the RTK pathway, PI3K pathway, and IDH mutations also have been used as biomarkers for glioblastomas. The EGFR is located on is amplified in approximately 50% of the cases of glioblastomas. In adults, patients with the
overexpression of EGFR is linked with a poorer prognosis than patients without. Patients with glioblastomas were studied to determine if the tumors were EGFR positive. Tissue samples were taken from 111 patients and underwent genetic analysis. The results indicated that 41% of patients had tumors that possessed EGFR amplification.

Children, although uncommon, can also be diagnosed with glioblastomas. The outcome is more favorable for children, but is not promising for long-term. Children diagnosed with glioblastomas also have overexpressed EGFR. In a study of 27 children with glioblastomas, sections of the glial tissue were obtained and immunohistochemically stained and processed to determine if the overexpression of EGFR was present. In 22 of the 27 tumors examined, there was an overexpression of EGFR. The study graded the expression of EGFR as present in single cells (0-5%), present in minority of cells (5-50%), present in majority of cells (50-90%) or present in almost all cells (>90%). Overexpression was defined as the last two grades, either being present in the majority of cells or almost all cells. The patients with overexpression of EGFR showed no significant differences in survival rates in children, which is different than the results obtained previously in adult studies. It does, however, display that EGFR is a significant biomarker for glioblastomas.45

Another defining feature of glioblastomas are their leaky vasculature. Glial tumors often release high amounts of permeability factors, specifically vascular endothelial growth factor, or VEGF. These leaky capillaries also allow for a release of many other chemical factors, which lead to a partial disruption of the blood brain barrier in glioblastomas.38 This defect in the blood brain barrier allows for drugs to pass through the blood brain barrier to some extent. The leaky vasculature gives glioblastomas the ability to migrate to other areas of the brain. Glioblastomas are stage IV astrocytomas, which are the deadliest form. Typically,
these tumors are found in the cerebral hemispheres, the frontal lobe, and the temporal lobes. Glioblastomas arise from many cells such as astrocytes or oligodendrocytes, but affect the glial cells that support the neurons. The enhanced permeability effect (EPR) has become a characteristic associated malignant tumors. Tumor cells have increased leaky vasculature due to increased amounts of bradykinin, nitrous oxide, and other vasculature permeability factors. The increased leaky vasculature has displayed evidence of increased particle concentration within the tumor. The accumulation of particles over 60 kDa in tumor cells with leaky vasculature is evident in this study where radioactive lipidiol particles were injected in the hepatic vein. After 7 days, there was approximately 33.7 dpm/gx10^3, where in the liver and other organs it was less than 4.1 dpm/gx10^3. This demonstrates the increased retention of particles in tumor cells compared to normal cells.

Current treatment options for glioblastomas are risky and ineffective. Surgery is an option, but transecting the tumor can be elusive due to the branching nature of the tissue. In addition there is chemotherapy and radiation, which may reduce the tumor size temporarily and many glioblastomas are resistant to these treatments. In a study investigating glioblastoma response to radiation, tumors with high EGFR expression were less responsive to radiation treatment than tumors without EGFR. Neuropathologists scored the samples taken from patients tumors and stained them with antibodies to determine if EGFR was present. After treatment, the tumors were imaged using a CT scan. In patients with no overexpression of EGFR, 33% had a good response to radiation. Patients with intermediate positive staining of EGFR, 18% of patients had a positive response. Patients that strongly expressed EGFR, however, only had a good response to treatment in 9% of cases. The most effective mode of
treatment to date is using a combination of surgery, radiation, and an alkylating agent known as TMZ.

2.2.2 Neuroblastoma General Physiology

Neuroblastomas are tumors that affect the sympathetic nervous system. Developmental cells from the neural crest mutate and become neuroblasts. Histologically, neuroblastomas can be classified as undifferentiated and composed of neuroblasts or partially differentiated known as ganglioneuroblastoma. Advanced neuroblastomas can be depicted by large masses or metastases to distant organ sites. Clinically, cancer cells that have differentiated correlate with a better prognosis than undifferentiated cancer cells.

Neuroblastomas are classified into subsets based on their clinical behavior. Similarly to glioblastomas, neuroblastomas also have MGMT, which has high correlation with drug resistance and difficulty to treat with conventional therapies.

Neuroblastomas also have abnormal, overexpressed genes associated with it. This type of childhood cancer has neutorophin receptors that aid in the malignant transoformation. Specifically, three main tyrosine kinase receptors, TrkA, TrkB, and TrkC, are all overexpressed in neuroblastoma. The concentration of receptors in neuroblastoma also can be a predictor of patient outcome. Patients with a high density of TrkA receptors in the tumor cells are likely to survive, where patients with a high level of TrkB receptors are more susceptible to drug resistance and ultimately death. Neuroblastomas are, like glioblastomas, solid form tumors. Solid form tumors, as was previously mentioned, under go the enhanced permeability and retention effect and have leaky vessels and poor lymphatic drainage.

Like glioblastomas, some neuroblastomas also possess overexpressed EGFR. A study conducted showed that in 10 cells lines the western blots of 8 cell lines indicated EGFR
expression. 48 A study was conducted using cetumixlab, which is an FDA confirmed inhibitor of EGFR, to inhibit epidermal growth factor receptor in the tumors. The results indicated that inhibited EGFR lowered cell proliferation rates in the tumor. 49 An additional study investigated 4 different neuroblastoma cell lines (UKF-NB-3, IMR-32, NLF, and SH-SY5Y) and compared the same cells lines with developed resistance to cisplatin (UKF-NB-3\textsuperscript{CDDP}\textsuperscript{1000}, IMR-32\textsuperscript{CDDP}\textsuperscript{1000}, NLF\textsuperscript{CDDP}\textsuperscript{1000}, and SH-SY5Y\textsuperscript{CDDP}\textsuperscript{500}). An epidermoid carcinoma cell line A431 was used as a positive control in this experiment to test the neuroblastoma cell lines for EGFR expression. Further investigation confirmed that the neuroblastoma cell lines not resistant to cisplatin possessed similar EGFR expression to the positive control. The resistant cell lines, however, showed an increased EGFR expression 1.5-2.5 higher than the non-resistant cell lines. 67 The relationship between drug resistance and EGFR demonstrates that higher EGFR may lead to more resistance in neuroblastomas.
3.1 Traditional Treatments and Role of MGMT

3.1.1 Traditional Treatment of Glioblastoma

As previously mentioned, the aggressiveness of glioblastomas and their physiological shape makes them especially difficult to treat. Surgery is a popular option, but a complete resection of the tumor makes is nearly impossible due to the extensive invasion of tumor cells. In fact, a successful total resection of the glioblastomas only occurs in approximately 20% of patients. This further displays the difficulty with locating and resecting the tumor due to its invasive nature. Recently, patients have been given a combination of radiation therapy and a alkylating agent called temozolomide, also known as TMZ to treat the cells that could not safely be removed by surgery. Glioblastomas are known for reoccurrence and typically this happens locally where radiation was previously given. The radiotherapy and TMZ combination was given everyday for up to 49 days. Patients with TMZ combination were given 75 mg per square meter per day plus the normal dose of radiation. Despite this reoccurrence, when TMZ is in combination with radiation, patients tend to live about two months longer than with radiation therapy alone.

3.1.2 Traditional Treatment of Neuroblastoma

Traditionally, children with neuroblastomas undergo extreme chemotherapy, surgical recession, radiation, and hemapoietic transplantation. Although successful with stage I or stage II neuroblastomas, stage III and IV are rarely effective. Even with conventional treatment, there is a high likelihood of reoccurrence among patients.
Chemotherapy itself can lead to disastrous results in patients with progressive forms of neuroblastomas. In a study of patients with a severe form of neuroblastoma, of the 323 patients that died, 22 of them died as a direct result of chemotherapy treatment. Up to 6% of deaths in patients with neuroblastomas are directly related to chemotherapy treatment alone. Patient deaths are due mostly to infections caused by weakened immune systems due to an extremely low white blood cell count.

Another treatment option that is rising in popularity is treatment with retinoic-acid derivatives. These derivatives are shown to induce differentiation in neuroblastomas, which will deter cell growth in cell culture. Immunotherapy is also increasing in popularity in clinical trials. Immunotherapy makes use of antibodies that mark the tumors. Neuroblastomas are not highly immunogenic, so the body has a hard time recognizing the tumor as abnormal. The treatment with antibodies helps to stimulate the body’s immune system to recognize the abnormal tumor growth and destroy the cancer cells.

Immunotherapies for glioblastomas patients have been investigated as a new treatment mechanism. The immunosuppressant nature of glioblastomas attributes to its ability to spread at a rapid pace. Unfortunately, there has been little to no breakthrough using this method of treatment. Many of the lymphocytes bypass the brain and are only functional in the liver. Many different immune targets have been investigated, but very few seem to provide any promising results for glioblastomas.

3.1.3 DNA Repair Mechanisms MGMT

O-methylguanine-DNA methyltransferase is a gene is a DNA-repair protein that removes methyl groups on DNA. This gene is located on chromosome 10q26. Many cancers with high levels of MGMT become resistant to drugs, specifically alkylating agents. MGMT
removes an alkyl group from the O\textsuperscript{6} position of guanine, which causes increased resistance due to the importance of guanine in DNA alkylation.\textsuperscript{37} Chemotherapy is effective when it causes lesions, specifically in O\textsuperscript{6}-methylguanine, that allow for the cell to undergo apoptosis.\textsuperscript{37} Unfortunately, cells with high levels of MGMT are able to overcome the treatment by repairing the damaged cell. High levels of MGMT are higher in glioblastomas, but are still present in neuroblastomas.\textsuperscript{37}

MGMT has been thoroughly investigated in glioblastomas and other brain cancers.\textsuperscript{28} It is evident that glioblastomas do possess a high amount of MGMT, but recent research suggests that neuroblastomas also possess higher levels of MGMT. High amounts of MGMT has been defined as detection of the MGMT protein in at least 20\% of tumors cells in a sample.\textsuperscript{28} Glioblastomas and neuroblastomas that had high expression of MGMT also had a high instance of drug resistance to treatments.\textsuperscript{28,69,70}

MSH6 is another DNA repair mechanisms that affect glioblastoma progression. This mutation actually causes the alkylating agents used to become promotors of progression. This mutation was not present in any of the forty tumors that were pretreated, but it was found in 3 of the 14 recurrent cases of glioblastomas after treatment with TMZ. In addition, normal MSH6 was present in each glioblastomas examined, but after treatment with TMZ, expression was lost in 41\%. The mutation and loss of MSH6 is correlated to higher rates of tumor progression and drug resistance.\textsuperscript{50} Although this mutation and deletion does causes some increased resistance, the MGMT overexpression is the main mechanism of DNA repair.

3.1.4 BER

The BER is a repair system that eliminates damaged base residues and may be another important mechanism for overcoming glioblastomas and neuroblastomas resistance to
Specifically, Temozolomide resistance is caused by the Poly(ADP-ribosyl)ation protein modification in mammalian cells or PARP, which is a component of the BER. When the DNA in a cell is damaged PARP moves to the site of the lesion and becomes activated, signaling other DNA repair proteins to aid in mending the lesion. The PARP inhibition can lead to the cytotoxic of TMZ from O\(^6\) to N\(^7\)-meG and N\(^3\) meA. Therefore, inhibiting the BER pathway may be an effective treatment option for TMZ resistance glioblastomas and neuroblastoma. BER is investigated as thoroughly as MGMT, but over 80% of the DNA lesions that are induced by Temozolomide are N-methylated bases that are recognized by the BER pathway. Therefore, BER would be an excellent DNA repair mechanism to investigate since it is capable of potentially causing drug resistance in glioblastoma and neuroblastoma. Methyl Methanesulfonate (MMS) is one drug that has been known to act on interrupting the BER.

3.2 Treatments with Alkylating Agents and MGMT Inhibitors

3.2.1 Temozolomide Treatment

Temozolomide is an FDA approved drug for the treatment of glioblastomas and other astrocytomas. TMZ is an oral alkylating agent and an approved method for the treatment of glioblastomas. In combination studies of Temozolomide, or TMZ, results indicated that patients with glioblastomas survived almost twice as long when treated with TMZ in combination with radiation than compared to radiation alone.

According to Rubie et al. it was shown that TMZ could also be a potentially useful drug in the treatment of neuroblastomas. Similar to other studies, Rubie et al. determined the dosage of TMZ for patients to be about 200 mg and administered once a day for five days. The dosage was in combination with chemotherapy and this regiment continued over the course of 12
months. Initially, five patients showed very good partial response to TMZ, which was defined as a decrease in between 50%-90% of the sum of the products of the largest perpendicular diameter. However, overall improved clinical outcome was seen in 10 out of the 25 patients investigated (40%) over the course of 6 months. The study also showed that there was some cellular response in 21 of the 25 patients after two courses. Although there was some improvement in treatment, ultimately within 8 months each individual showed disease progression and died as a result of the neuroblastoma.61

As mentioned previously, MGMT is a leading cause of resistance for traditional treatments in glioblastomas and neuroblastomas. DNA-methylation is an important mechanism for gene silencing. Although the combination therapy with radiation and TMZ effectively treated glioblastomas in many patients, only patients with methylated MGMT promoters showed a significant benefit from this treatment. Of the 206 patients selected, 92 had methylated MGMT promoters while 114 had unmethylated MGMT promoters. The combination therapy of radiation and TMZ for patients with methylated MGMT promoters showed a median survival of 21.7 months and 15.3 months with radiation therapy alone. In contrast, patients with unmethylated MGMT promoters had a median survival rate of 12.7 months with combination therapy and 11.8 months with radiation therapy alone. Patients that were treated with the same combination of TMZ and radiation, but had unmethylated MGMT promoters had a very small, if any, increase in survival time.37

The greatest limitation for the treatment of glioblastomas and neuroblastomas with alkylating agents like Temozolomide is the dose-related acute systemic toxicity.6 Pharmokinetic studies indicate that TMZ is more effective when taken several times a day in smaller doses rather than once a day. In a study to determine the dosage of TMZ, data showed
that 200-mg level was the highest-level with patient’s glioblastomas could tolerate before undergoing hematologic toxicity. Additional studies displayed similar results, claiming that TMZ had toxic results with over 200mg dosage administered to the patient of the course of 5 days for glioblastomas and neuroblastomas.

3.2.2 O\textsuperscript{6} Benzylguanine MGMT Inhibitor

The resistance of glioblastomas and neuroblastoma to chemotherapy and alkylating agents due to MGMT leads to very little treatment options for patients with these deadly cancers. Increasing the dose of alkylating would create systemic toxicity and repeated exposure could also cause the development of secondary leukemias. However, O\textsuperscript{6} Benzylguanine inhibits MGMT, which allows alkylating agents such as TMZ to effectively increase the cytotoxicity of the cancer cells.

In addition to being effective in inhibiting MGMT, O\textsuperscript{6} Benzylguanine has shown non-toxic effects in gliomas. 30 patients were enrolled and given doses ranging from 40 mg/mL to 100 mg/mL 18 hours before a craniotomy. The results indicated that O\textsuperscript{6} Benzylguanine was non-toxic in 11 out of 11 patients treated with 100 mg/mL, which was the highest dosage given to patients in this study. An additional study also provided results that indicated O\textsuperscript{6} Benzylguanine is also non-toxic in preclinical studies for neuroblastoma cells. The maximum concentrations found for cells in a 96 well plate were 20ng/mL.

3.2.3 Chloroquine

Chloroquine, known for it’s antimalarial properties, may be an important drug to overcome resistance to TMZ. Chloroquine has antimutagenic effects that are neither cytotoxic nor are they antimitotic. Chloroquine causes intercalation of chloroquine in DNA tumor
molecules, making them more susceptible to cytotoxic events. When patients were treated with 150-300 mg of Chloroquine in combination with radiation and chemotherapy. The control group were treated with a placebo and radiation and chemotherapy. Although ultimately all subjects died as a result of glioblastomas, there was improvement when using chloroquine in combination with traditional treatments. Another study found that the life expectancy average was approximately 25 months from 41 patients treated with chloroquine, while the placebo group of 81 patients had an average lifespan of 11.4 months. Zheng et al. 2009 found treatment of colon cancer cells (CT26) with Chloroquine induces apoptosis and has found it may have a significant impact on future therapies of colon cancers.

3.2.4 Methyl Methanesulfonate

Methyl Methanesulfonate is a drug that acts on alkylating the BER pathway, which causes TMZ resistance in tumor cells. One study found that treating gliomas cells with minimally toxic dose of MMS in combination with Temozolomide showed enhanced toxic effects of TMZ.
CHAPTER 4

DRUG DELIVERY AND NANOCARRIERS

4.1 Drug Delivery

4.1.1 Conventional Drug Delivery

Chemotherapy and other traditional treatments of cancers are delivered by many different mechanisms. Typically, chemotherapy is delivered through intravenous injection, an IV drip, or a pump and can also be orally administered. Injections are instantaneous delivery of the chemotherapeutic agent, while the IV drip is more prolonged, and the pump is even more sustained and is considered outpatient treatment. Although this mode of drug delivery is effective in many patients, it is not specific. Anticancer drugs will disperse throughout the entire body and can cause significant side effects. The largest side effects most patients undergoing anticancer treatment includes fatigue, irritability, nausea, and hair loss. and that include weight loss, low white blood cell count, rashes, vomiting, and cardiotoxicity. A study conducted in mice showed that the use of hydrogel nanoparticles as drug carriers reduced side effects. The mice lost less than 10% of their body weight and at the end of 90 days, 50% of the mice were still alive. In contrast, the free drug displayed more adverse side effects and only 25% of the mice were still living after a 90-day period.

Drugs are able to diffuse through tumor cells due to their leaky blood vessels, which are due to rapid and ineffective angiogenesis. Non-targeted systemically circulating drugs can then diffuse into the tumor cells through the EPR effect. The conventional delivery systems ensure that the anticancer agents are spread systemically throughout the body in order to kill the tumor cells, but unfortunately this process also simultaneously kills healthy normal cells. This result of this is systemic toxicity to the body. Patients therefore must choose between a
near lethal dose of toxic drug or a lower dose that could potentially be below the therapeutic window once it has reached the tumor. A study investigated TMZ freely administered in the body and TMZ encapsulated in PLGA nanoparticle and compared the cellular uptake and also the half life of TMZ in gliomas. The study found the half-life of TMZ to be 1.8 hours, while TMZ had an initial release of 30% in the particle, but then underwent sustained release for 120 hours. 200 ug/mL of TMZ was tested in free and encapsulated nanoparticles. The results indicated that with a prolonged half-life that the PLGA nanoparticles provide, that the drug will decrease systemic toxicity while exposing the tumor cells to TMZ over a sustained amount of time. This demonstrates how the nanoparticles can provide sustained release and do not require continuous administration of drug.

4.2 Nanoparticles

4.2.1 General Properties of Nanoparticles

Nanomedicine is an emerging field and currently 20 nanoparticle therapeutics are approved by the FDA for clinical use. The typical nanoparticle used in therapeutic application ranges from 1-100nm. There are many important properties that make nanoparticles effective drug delivery systems.

Nanoparticles carry therapeutic agents throughout the body and protect them from degradation. The amount of therapeutic agent, or payload, encapsulated by the nanoparticle is referred to as the payload density. The payload density within the particle does not affect the surface properties of the nanoparticle. The nanoparticle will protect the drug from degradation and the healthy cells from the toxic effects of the drug. Anticancer drugs delivered freely within the systemic system are given at a higher dose because the body will naturally degrade the drug before it arrives at the cancer target. By encapsulating the therapeutics; the drug is not
exposed to the body and therefore is not released until it reaches the target. Nanoparticles have a much higher therapeutic half-life, which allows for extended release and less loading of the toxic drug into the particles.\textsuperscript{10}

The uptake of non-targeted nanoparticles by cancer cells is due to the enhanced permeability and retention effect (EPR). The leaky vessels are due to cell proliferation of cells, which cause large pores in the tumor vasculature that are large enough for nanoparticles to diffuse into the tumor.\textsuperscript{13,29} The lymphatic system is also impaired, which causes less clearance of the nanoparticles from the tumor cells.\textsuperscript{29} The combination of the increased diffusion rate and decreased clearance allows a higher concentration of nanoparticle within the cell.\textsuperscript{13,29}

Although the EPR allows for uptake of nanoparticles into the cells, surface modification can also implemented to ensure even more efficient uptake into the tumor. PEG is often added to PLA nanoparticles because it reduces aggregation and limits electrostatic interactions. With a highly charged surface, there is an increased likelihood that monocytes will remove the particle from circulation.\textsuperscript{10,20,23} In addition to charge modification, nanoparticles can be modified in order to target specific cells. The EPR effect will help the non-targeted nanoparticles passively diffuse into the tumor cells, and the defects in the lymphatic draining systems allow them to accumulate. This will allow the nanoparticle to release the drug in closer vicinity to the tumor when compared to a drug freely circulating. Peptides, antibodies, and molecules can all be attached to nanoparticles to increase uptake affinity. A specific drug with low binding affinity can be encapsulated within a targeted nanoparticle and be delivered with much higher efficacy than it would be if it were freely circulating.\textsuperscript{10}

The majority of fatal cancers are drug resistant and metastatic.\textsuperscript{9} Not only do nanoparticles have the potential to overcome systemic toxicity in cancer treatments, but also the
ability to lower the instances of cancer becoming drug resistant. Many cytotoxic treatments have dose-limiting toxicities, but by using nanoparticles to carry these drugs, there is less exposure of drug to healthy cells and more targeted treatment. Many drug resistant cancers involve surface protein pumps, but nanoparticles will enter endocytosis and bypass this potential type of resistance. Encapsulating drug in nanoparticles will lessen systemic toxicity and bypass surface protein pumps associated with drug resistance. A study using mice injected with prostate tumor cells were investigated to see the effects of free drug, drug loaded nanoparticles, and targeted drug loaded nanoparticles. The cells were treated with free docetaxel as well as docetaxel encapsulated in targeted and non-targeted polymeric nanoparticles. The mice were treated every four days and had the exact dose of docetaxel given for each drug delivery system. The targeted nanoparticle out performed the non-targeted nanoparticle and the free drug. The tumor reduced in size for the targeted nanoparticles by 26%, while the tumor size actually increased by 75% for non-targeted nanoparticles and 100% for free drug. The efficacy of polymeric targeted nanoparticles is higher than nanoparticles alone and free drug. This illustrates the capability of targeted nanoparticles for treating not only prostate cancer, but also their potential to treat other deadly cancers such as glioblastoma and neuroblastoma.

Nanoparticles are effective modes of drug delivery. Biocompatibility, low cost, longer half-lives and circulation of drug, and lower therapeutic toxicity are all attractive properties of nanoparticles. These properties of nanoparticles allow for a treatment option that is low in cost, can treat drug resistant cancers, and can deliver doses of cytotoxic agents with little exposure to the systemic environment.

4.2.1 Polymeric Nanoparticles
The use of polymeric nanoparticles is an appealing therapeutic strategy. The manufacturing of these particles is cost-effective. Polymeric particles are biocompatible and stable in the blood, they are non-toxic and non-immunogenic, and can undergo surface modifications which allow them to be specific from patient to patient. Not only do polymeric nanoparticles have the ability to be modified, they can also do so without the loss of the desired physical, chemical, and biological properties.

Although microparticles have also been investigated for drug delivery, nanoparticles are proven advantageous over microparticles because of their size. Nanoparticles can be intravenously injected and aggregate into tumor cells, whereas microparticles cannot because they are larger than the smallest capillaries. Intravenous injections must be less than 6um in diameter, otherwise they may aggregate in tissues and not make it to the targeted site. In contrast to gold or other metal nanoparticles that may be biocompatible, they are not degradable. Although polymeric nanoparticles may settle in the lungs, liver, and kidneys, they also will eventually degrade into biologically compatible components.

The capabilities of polymeric nanoparticles are dependent on the particles size, surface charge, surface modification, and hydrophobicity. Nanoparticles with slightly positively charged surfaces are more likely to undergo internalization due to increased cell interaction. There are also several modifications that polymeric nanoparticles can undergo in order to increase the circulation time. In addition to an increased circulation time, the higher the molecular weight the polymer to create the nanoparticle is, the longer the release time will be. Characterizing the nanoparticles with targeting ligands is another surface modification polymeric particles can undergo to increase efficacy. Nanoparticles can therefore be specifically designed based on a patients specific needs.
Nanoparticles can be created based on the intended treatment. Many different materials such as dendrimers, micelles, liposomes, and nanoscale ceramics, along with polymeric nanoparticles, can be used based on the anticipated drug therapy. The size of nanoparticles ranges from 1 nm to 300 nm and carries a therapeutic agent. Polymeric nanoparticles are also capable of a high payload and is an efficient way to encapsulate cytotoxic drugs. However, effective nanoparticles for cancer therapeutics range from 10-100 nm. Polymeric nanoparticles possess many beneficial properties, such as released control, stability, high loading capabilities, and can easily undergo surface modification, therefore polymers are one of the best materials to use for nanoparticles in the treatment of cancer.

Polymeric Nanoparticles can be synthesized from many different biomaterials. Typically, polymeric nanoparticles are created with two block-copolymers with different hydrophobicity, which is what gives the particle their spherical appearance when submerged in an aqueous solution. The choice of material is dependent upon the therapeutic agent that will be loaded within the particle. The more common materials used to treat central nervous system diseases are poly(ethylenimines), poly(alkyanoacrylates), poly(methylidene malonates), and polyesters. Proteins, polysaccharides, and other natural polymers can be used, but each varies in purity and requires crosslinking. This crosslinking may damage the encapsulated drug, so it is typically avoided. Polymeric nanoparticles can easily be made with different sizes, charges, and surface coatings to increase efficacy based on tumor location.

4.2.2 Poly(lactic acid) Nanoparticles

Poly(lactic acid) nanoparticles, or PLA, were first studied because of its biocompatibility and known safety in humans. PLA particles can be degraded into monomeric units of lactic acid that does not induce an immune response and is therefore highly biocompatible.
biocompatible. Polymeric nanoparticles also show increased uptake in tumor cells when compared to other nanocarriers such as liposomal carriers. Studies with central nervous system diseases show that not only is PLA a good safe nanocarrier, but also can pass through the blood brain barrier. Lower grade astrocytomas typically have the blood brain barrier intact, while glioblastomas have some disruption. Regardless, PLA’s ability to cross the blood brain will increase the efficacy of drug delivery because it can cross the disrupted BBB quicker than conventional systemic treatments.

PLA nanoparticles are typically injected intravenously. The PLA coating also provides a barrier against monocyte uptake, allowing particles to circulate for up to one week.

Release characteristics for PLA nanoparticles can be modified by a variety of methods. Larger particles have a longer release time than smaller particles because of a smaller initial burst release. Other polymers can be added to PLA nanoparticles to increase the amount of drug released. Poly(ethylene glycol), or PEG, is often added to the surface of PLA nanoparticles to decrease protein adsorption to the surface of the nanoparticles. PLA-PEG nanoparticles therefore have an increased release time, deterred degradation, and greater amount of drug released within the tumor when compared to PLA alone.

Targeted polymer nanoparticles are of increased interest in the treatment of glioblastomas and neuroblastoma. Targeted nanoparticles typically have a ligand conjugated to the surface to target a specifically overexpressed receptor. The cell line U87 in glioblastomas has an overexpressed receptor called LRP. A ligand, angiopep, was conjugated to a PEG nanoparticle. Images of the PEG nanoparticles alone compared to the targeted PEG particles confirmed that there was more uptake from the targeted nanoparticles.
CHAPTER FIVE
FORMULATION OF METHOXY-POLYLACTIC ACID PEGYLATED NANOPARTICLES

5.1 Introduction

The goal of this research was to develop a methoxy-poly(lactic acid) nanoparticle to deliver a non-toxic drug for the treatment of glioblastomas and neuroblastoma. The surface of the nanoparticle was functionalized with poly(ethylene glycol) (PEG) in order to mask the nanoparticle from the immune system in order to improve systemic circulation time. The finalized nanoparticle formulation was a block copolymer of mPLA-PEG. The nanoparticles with non-toxic drug encapsulated within were tested for toxicity and determined to be non-toxic. Another nanoparticle was also synthesized with the same mPLA-PEG ratios, but was tagged with Alexafluor647 to prove that the nanoparticles could be uptaken by the cells.

5.2 Materials

DL lactide (C_6H_8O_4) was supplied by Purac Biomaterials. Sodium sulfate (Na_2SO_4, >99%), magnesium sulfate (MgSO_4, >99.5%), Chloroform (CHCl_3, >99.8%), methanol (CH_3OH, >99.9%), O_6-Benzyl Guanine (O_6 BG), and Methyl methanesulfonate (MMS) were supplied by Sigma-Aldrich. Methoxy-poly(ethylene glycol) was supplied by JenKem Technology USA (M-PEG-OH). Acetonitrile (C_2H_3N, 99.9%) was supplied by Fisher Scientific. Temozolomide (TMZ) was provided by Molekula. PrestoBlue Cell Viability Reagent and Alexa Fluor 647 were supplied by Life Technologies. The black bottom tissue treated sterile 96 well-plates were supplied by Greiner bio-one.

5.3 Methods

5.3.1 Statistical Analysis
All statistical analysis was performed using a two-tailed t-test with at least three repeats each. Statistical significance was set at p<0.05. Error bars on graphs represent the standard deviation from the mean.

5.3.2 Cell Culture

U138-MG glioblastoma cells (American Type Culture Collection, ATCC), D283 neuroblastoma cells (ATCC), and DAOY neuroblastoma cells (ATCC) were all used for studies. All cells were of human origin and were grown in 2-D cell cultures at 37°C and 5% of CO₂. All cell lines were cultured using Eagle’s Minimum Essential Media (ATCC) supplemented with 10% fetal bovine serum (Atlanta Biologics) and 1% penicillin-streptomycin-amphotericin (MediaTech Inc.). The cell densities used for toxicity studies were 50,000 cells per well. Additionally, uptake studies were performed using T-75 flasks and seeding cell lines at 100,000 cells/flask.

5.3.3 Nanoparticle Synthesis & Characterization

Poly(lactide)-poly(ethylene glycol) block copolymers were synthesized by method of ring-opening polymerization. D,L-lactide (17.4 mmol), methoxy-PEG-OH (.133 mmol), and Sodium Sulfate (2.19 mmol) were all dried in a vacuum overnight in a round bottom flask prior to polymer synthesis. After the components were thoroughly dried, the round bottom flask containing the reagents was attached to a condenser outlet and toluene (10 mL) was added to the reaction vessel. The flask was placed over an oil bath that had reached a temperature of 120°C and stirred for 30 minutes until the D,L-lactide and methoxy-PEG-OH were dissolved. Tin(II) 2-ethylhexanoate (5.07 uL) was added to the vessel after the reagents had dissolved and then the vessel was purged with nitrogen and the stopper was replaced and stirred for 12 hours. After the duration of 12 hours, the reaction vessel was removed and cooled to room
temperature. The reaction products were washed with chloroform and water. The products were then allowed to undergo phase separation via a separation funnel. Magnesium sulfate (300 mg) was added to a beaker where the organic phase of the solution was collected. This solution was then vacuum filtered through a PTFE filter and concentrated using a rotary evaporator. The product was then precipitated in -80°C methanol overnight. The polymer was collected after centrifugation and lyophilization. The polymer was characterized by Nuclear Magnetic Resonance.

The polymeric nanoparticles were assembled by solvent evaporation, where m-PLA-PEG block copolymer was dissolved in acetonitrile at a concentration of 5 mg/mL. The polymer-acetonitrile solution was then rotated on a rotisserie for one hour. The polymer solution was then added to sterile HyPure H2O in a 1:2 ratio and stirred under the hood for 2 hours. The 1:2 solution was then removed and washed in a 100 kD centrifugal filter unit at 3500 rpm for 7 minutes. The nanoparticle solution was washed twice with water and once in phosphate buffered saline (PBS). The nanoparticles were then collected and resuspended in EMEM cell culture media at the desired concentrations.

The fluorescently tagged nanoparticles used in the uptake studies had PLA attached to AlexaFluor 647 cadaverine by EDC chemistry. PLA was first dissolved in dimethylformamide (DMF) and mixed with 10-fold excess EDC. The solution was mixed and 10-fold excess of AlexaFluor 647 was added and stirred with PLA overnight. Again, the PLA was concentrated with a rotary evaporator, dissolved in chloroform, and precipitated in the cold methanol overnight. The product was then centrifuged and collected. Then, the fluorescently labeled PLA was lyophilized overnight. To synthesize the fluorescently labeled nanoparticles, the Fluorescent PLA (F-PLA) was combined with m-PLA-PEG in a 4:6 ratio respectively. These
nanoparticles underwent the exact same protocol as the nanoparticles previously described using solvent evaporation techniques.

Both the O6-Benzyl-Guanine loaded nanoparticles and the methyl methanesulfonate nanoparticles underwent the same assembly. First, the drug was dissolved as acetonitrile at 2 mg/mL. The m-PLA-PEG was then added to the drug-containing ACN solution at 5 mg/mL. This polymer-drug solution was then rotated for one hour on the rotisserie before stirring for 2 hours in HyPure water at a 1:2 ratio respectively. The nanoparticles were then washed as previously described with centrifugal filter units twice with water and once with PBS before being resuspended in cell culture media at the desired nanoparticle concentration.

5.3.4 Nanoparticle Uptake Studies

The nanoparticle uptake studies were measured by using the fluorescently tagged PLA mixed with the m-PLA-PEG in a 4:6 ratio as previously described. The efficacy of the nanoparticle uptake by the cells was measured in U-138, D283, and DAOY cells. The cells were seeded in 96-well plates at a concentration of 10,000 cells/well. The fluorescently labeled nanoparticles, which were created as previously described, were added to the cells at a concentration of 2 mg/mL and incubated for up to 72 hours. To analyze the results, the media with nanoparticles was slowly removed and the cell wells were carefully washed 3 times with 100 µL of PBS. The cells were then trypsinized, collected, and then spun down in a microcentrifuge tube. The 150 µL of the supernatant was then slowly removed and just the cell pellet remained. The cell pellet was then resuspended in 200 PBS and samples were transferred to new 96-well plate. Results were read by a plate reader with excitation and emissions values of 645/680.

5.3.5 Nanoparticle Toxicity Studies
The toxicity of nanoparticles in U-138 glioblastoma, D283 neuroblastoma, and DAOY neuroblastoma were investigated by seeding 96-well plates at a concentration of 10,000 cells/well. The nanoparticles were then loaded with either O\(^6\)-BG or MMS at concentrations ranging from .5 mg/mL to 2 mg/mL and resuspended in media. The free drug toxicities studies dissolved the drug concentration in media, which was then added to the wells. The loaded nanoparticle studies and the free drug studies over a range of concentrations were then incubated with cells for 24, 48, and 72 hour durations. The cell viability at each time-point was then quantified using a Presto Blue cell viability assay. The combination toxicity studies used both drug loaded nanoparticles and free TMZ in the well plates. TMZ was added to wells at 256 uM, while the nanoparticles were added to the plates at 2 mg/mL. The preparation of the free drug and nanoparticle solutions is as previously described.

5.4 Experimental Results and Discussion

5.4.1 Synthesis of PLA-PEG(OCH\(_3\))

Synthesis of polymeric nanoparticles of PLA-PEG(OCH\(_3\)) was successful and yielded nanoparticles that were biodegradable, biocompatible, and able to uptake into the tumor cells. The PLA is considered the hydrophobic portion of the copolymer and is in the interior of the particle. PLA is biodegradable, but it can be targeted by the immune system due to its hydrophobicity. In order to overcome the degradation of particles due to immune system filtration the particles were PEGylated in order to increase biocompatibility. The PEG portion of the copolymer is hydrophilic and shields the particles from immune cells. Figure 1 shows the particles ability to load drug. The different sizes in particles show that the polymeric nanoparticles are capable of loading different types of drugs and are not limited to just one specific drug.
Blank nanoparticles were formed and particle sizing was performed. The results indicated that blank nanoparticles had an approximate diameter of 121.9 nm, which is an acceptable size for nanoparticles as previously discussed.

**Blank OCH3 (Combined)**
- Effective Diameter: 150.2 nm
- Polydispersity: 0.148
- Avg. Count Rate: 29.4 kcps
- Baseline Index: 8.5/93.30%
- Elapsed Time: 00:15:00

![Particle Size of Blank PLA-PEG (OCH3) Nanoparticles](image)

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**Figure 1 | Particle Size of Blank PLA-PEG (OCH3) Nanoparticles.** The diameter of unloaded nanoparticles was measured to be approximately 150.90 nm in size.

O6-Benzylguanine loaded nanoparticles were also sized using the same equipment. The results indicate that the nanoparticles are approximately 160 nm in diameter. This diameter is larger than the blank counterparts and is small enough to be considered an effective drug delivery mechanism.
5.4.2 Nanoparticle Uptake Results

Nanoparticles that were tagged with AlexaFluor 647 showed an increase in uptake into cells over a 72-hour period in U-138 cells. The graph below depicts the normalized fluorescent readings and indicates that the nanoparticles are able to be uptaken by the cells.
Figure 3 | Fluorescent Readings of U-138 cells with Fluorescently Tagged Nanoparticles. The cells show that the cells can effectively uptake the nanoparticles.

Nanoparticles that were tagged with AlexaFluor 647 showed an increase in uptake into cells over 24 hour period in D-283 cells. The graph below depicts the normalized fluorescent readings and indicates that the nanoparticles are able to be uptaken by the cells approximately 15-fold from the 1-hour time point.
Figure 4 | Fluorescent Readings of D-283 cells with Fluorescently Tagged Nanoparticles. The cells show that the cells can effectively uptake the nanoparticles.

Nanoparticles that were tagged with AlexaFluor 647 showed an increase in uptake into cells over 24 hour period in DAOY cells. The graph below depicts the normalized fluorescent readings and indicates that the nanoparticles are able to be uptaken by the cells approximately 17-fold from the 1 hours time point.
NP Uptake into Daoy Cells

**Figure 5 | Fluorescent Readings of DAOY cells with Fluorescently Tagged Nanoparticles.**
The cells show that the cells can effectively uptake the nanoparticles.

5.4.3 Free TMZ Toxicity Results

The presto blue results for D-283 indicate that TMZ does provide a semi-toxic effect in U-138 cells. U-138 cells do have the presence of the MGMT gene that results in greater resistance to TMZ and other alkylating agents. Based on the results, 256 µM is a concentration that would provide a toxic enough effect without causing systemic toxicity and is the concentration we will use in combination drug studies.
Figure 6 | Toxicity of Free Temozolomide in U-138 Cells. The results indicate that at 256µM TMZ is toxic with fewer than 80% cell viability and also would not provide systemic toxicity, which makes it an ideal concentration to use in drug combination studies.

The presto blue results for D-283 indicate that TMZ does provide a semi-toxic effect in D-283 cells. D-283 cells do have the presence of the MGMT gene that results in greater resistance to TMZ and other alkylating agents. Based on the results, 256 µM is a concentration that would provide a toxic enough effect without causing systemic toxicity and is the concentration we will use in combination drug studies.
Figure 7 | Toxicity of Free Temozolomide in D-283 Cells. The results indicate that at 256µM TMZ is toxic at fewer than 80% cell viability and also would not provide systemic toxicity, which makes it an ideal concentration to use in drug combination studies. Study performed by Jhilmil Dhulaker.

The presto blue results for DAOY indicate that TMZ does provide a semi-toxic effect in DAOY cells. DAOY cells do have the presence of the MGMT gene that results in greater resistance to TMZ and other alkylating agents. Based on the results, 256 µM is a concentration that would provide a toxic enough effect without systemic toxicity and is the concentration we will use in combination drug studies.
Figure 8 | Toxicity of Free Temozolomide in DAOY Cells. The results indicate that at 256µM TMZ is toxic at fewer than 80% cell viability and also would not provide systemic toxicity, which makes it an ideal concentration to use in drug combination studies. Study performed by Jhilmil Dhulaker.

5.4.4 MMS Loaded Nanoparticle Toxicity Results

According to the presto blue results, the MMS loaded nanoparticles resulted in an approximate cell viability above 80% in U-138 cells when the loaded nanoparticle concentrations were less than 2 mg/mL. Based on the results, it is reasonable to use a MMS loaded nanoparticle concentration of 2 mg/mL for the combination drug therapy.
Figure 9 | Toxicity of Methyl Methanesulfonate loaded PLA-PEG(OCH₃) in U-138. Cell viability around 80% for elevated nanoparticle concentration indicates acceptable toxicity.

According to the presto blue results, the MMS loaded nanoparticles resulted in approximate cell viability above 80% in D-283 cells when the loaded nanoparticle concentrations were less than 2 mg/mL. Based on the results, it is reasonable to use a MMS loaded nanoparticle concentration of 2 mg/mL for the combination drug therapy.
Figure 10 | Toxicity of Methyl Methanesulfonate loaded PLA-PEG(OCH₃) in D-283 Cell viability around 80% for elevated nanoparticle concentration indicates acceptable toxicity.

According to the presto blue results, the MMS loaded nanoparticles resulted in an approximate cell viability above 80% in DAOY cells when the loaded nanoparticle concentrations were less than 2 mg/mL. Based on the results, it is reasonable to use a MMS loaded nanoparticle concentration of 2 mg/mL for the combination drug therapy.
5.4.5 Combination Therapy MMS and TMZ Toxicity Results

The presto blue assay of the first combination therapy indicates that the MMS loaded nanoparticles are non-toxic and shows that the free TMZ does have some toxic effect on U-138 cells. The concentrations of MMS and TMZ were chosen based on previous results that showed the optimal amounts of drug that would be non-toxic in the case of MMS and a concentration of TMZ that would be considered an effective amount. Finally, the combination of the MMS loaded nanoparticles with TMZ shows a greater toxic effect on the tumor cells with cell viability below 50%.
Study indicates MMS NP had a cell viability above 80%, the TMZ free drug therapy showed cell viability around 70%, and the combination study with MMS NP and free TMZ with a reduction in cell viability in U-138 cells to approximately 40% 72 hours.

The presto blue assay of the first combination therapy indicates that the MMS loaded nanoparticles are non-toxic and shows that the free TMZ does have some toxic effect on D283 cells. The concentrations of MMS and TMZ were chosen based on previous results that showed the optimal amounts of drug that would be non-toxic in the case of MMS and a concentration of TMZ that would be considered an effective amount. Finally, the combination of the MMS loaded nanoparticles with TMZ shows a greater toxic effect on the tumor cells with cell viability below 50%.
Figure 13 | Toxicity of MMS Loaded NP, Free TMZ, and Combination Therapy in D-283
Toxicity of MMS Loaded NP, Free TMZ, and Combination Therapy in D-283. Study indicates MMS NP had a cell viability above 80%, the TMZ free drug therapy showed cell viability around 70%, and the combination study with MMS NP and free TMZ indicated reduction in cell viability in U138 cells to approximately 60%-40% over a 72 hours.

The presto blue assay of the first combination therapy indicates that the MMS loaded nanoparticles are non-toxic and shows that the free TMZ does have some toxic effect on DAOY cells. The concentrations of MMS and TMZ were chosen based on previous results that showed the optimal amounts of drug that would be non-toxic in the case of MMS and a concentration of TMZ that would be considered an effective amount. Finally, the combination of the MMS loaded nanoparticles with TMZ shows a greater toxic effect on the tumor cells with cell viability below 60%.
Figure 14 | Toxicity of MMS Loaded NP, Free TMZ, and Combination Therapy in DAOY. Study indicates MMS NP had a cell viability above 80%, the TMZ free drug therapy showed cell viability around 70%, and the combination study with MMS NP and free TMZ indicated reduction in cell viability in U138 cells to approximately 60%-55% over a 72 hours.
5.4.6 Toxicity of Chloroquine

The toxicity of free chloroquine in U-138 cells was non-toxic up to a 12.5 uM concentration. The toxicity of chloroquine for concentrations less than 12.5 uM was around or above 80%. The combination study using chloroquine used a 12.5 uM concentration based on the results below.

**Presto Blue Assay of U-138 cells with Free Chloroquine**

![Graph showing cell viability of chloroquine concentrations in U-138 cells.](image)

**Figure 15** | **Toxicity of Free Chloroquine in U-138 Cells.** Cell viability for free drug concentrations around 80% indicates an acceptable toxicity.

The presto blue assay of DAOY neuroblastoma cells indicates that concentrations up to 25 uM had approximate cell viability greater than 80%. According to the results displayed below, the combination therapy could use a chloroquine concentration of 25 uM for the combination studies.
Figure 16| Toxicity of Free Chloroquine in DAOY Cells. Cell viability for free drug concentrations around 80% indicates an acceptable toxicity. The toxicity of free chloroquine in D-283 cells shows approximate cell viability greater than 80% according to the presto blue results. Therefore it is reasonable to use a free drug chloroquine concentration of 25 uM in the combination drug therapy studies for D-283 cells.

Figure 17| Toxicity of Free Chloroquine in D-283 Cells. Cell viability for free drug concentrations around 80% indicates an acceptable toxicity.
5.4.7 Toxicity of O6 Benzylguanine Loaded Nanoparticles

The toxicity of O6-Benzylguanine encapsulated within our polymeric nanoparticles showed a non-toxic effect on the U-138 cell line. The results demonstrate that up to an elevated nanoparticle concentration of 2 mg/mL that there is no toxic effect on the cells. Cells showed an approximate toxicity of around 80% at concentrations of 2 mg/mL or lower. Therefore, 2 mg/mL is the concentration that was used in the nanoparticle combination drug therapy study.

![Presto Blue Assay of U-138 Cells with O6-BG Loaded NP](image)

Figure 18 | Toxicity of O6-Benzyl Guanine loaded PLA-PEG(OCH₃) in U-138. Cell viability around 80% for elevated nanoparticle concentration indicates acceptable toxicity.

The toxicity study of the unloaded nanoparticles in comparison with the drug-loaded nanoparticles indicates a slight difference in toxicity in D-283 cells. At 72 hours, the results demonstrates that both unloaded and loaded drugs show approximate cell viability of over 80% and are non-toxic at concentrations less than 2.5 mg/mL.
Figure 19 | Toxicity of O6-Benzyl Guanine loaded PLA-PEG(OCH₃) in D-283. Cell viability around 80% for elevated nanoparticle concentration indicates acceptable toxicity.

The toxicity study of the unloaded nanoparticles in comparison with the drug-loaded nanoparticles indicates a slight difference in toxicity in DAOY cells. At 72 hours, the results demonstrate that both unloaded and loaded drugs show approximate cell viability of over 80% and are non-toxic at concentrations less than 2.5 mg/mL.
Presto Blue Assay of DAOY Cells with O6-BG and Unloaded NP at 72 hr

Figure 20| Toxicity of O6-Benzyl Guanine loaded PLA-PEG(OCH3) in DAOY. Cell viability around 80% for elevated nanoparticle concentration indicates acceptable toxicity. Study performed by Jhilmil Dhulaker.
5.4.4 Toxicity of Chloroquine, TMZ, and Combination Studies

The presto blue assay of U-138 cells with combination of non-toxic chloroquine, non-toxic O\(^6\)-BG loaded nanoparticles, and toxic free TMZ. The study shows the TMZ is effective alone, but when it is combined with O\(^6\)-BG loaded nanoparticles it is more effective. The final treatment with O\(^6\)-loaded NP, free TMZ, and free non-toxic chloroquine yields even lower cell viability after a 72-hour treatment.

### Presto Blue Assay of U-138 Cells with Combination Drug Therapy

![Graph showing cell viability over time for different treatment groups.](image)

**Figure 21** Toxicity of O\(^6\) Loaded NP, Free TMZ, Free Chloroquine and Combination Therapy in U-138. The study indicates free chloroquine had a cell viability above 80%, the TMZ free drug therapy showed cell viability around 75%, and the combination study with O\(^6\) NP and free TMZ indicated reduction in cell viability to about 60-75% cell viability and the chloroquine, O\(^6\) NP, and TMZ to have a cell viability of about 50-60% viability in U-138 cells.

The presto blue assay of D283 cells with combination of non-toxic chloroquine, non-toxic O\(^6\)-BG loaded nanoparticles, and toxic free TMZ. The study shows the TMZ is effective alone, but when it is combined with O\(^6\)-BG loaded nanoparticles it is more effective. The final treatment with O\(^6\)-loaded NP, free TMZ, and free non-toxic chloroquine yields even lower cell viability after a 72-hour treatment.
Figure 22| Toxicity of O6 Loaded NP, Free TMZ, Free Chloroquine and Combination Therapy in D-283. The study indicates free chloroquine had a cell viability above 80%, the TMZ free drug therapy showed cell viability around 60%, and the combination study with O6 NP and free TMZ indicated reduction in cell viability to about 40-50% cell viability and the chloroquine, O6 NP, and TMZ to have a cell viability of about 40% viability in D283 cells.

The presto blue assay of DAOY cells with combination of non-toxic chloroquine, non-toxic O6-BG loaded nanoparticles, and toxic free TMZ. The study shows the TMZ is effective alone, and when it is combined with O6-BG loaded nanoparticles it is more effective. The final treatment with O6-loaded NP, free TMZ, and free non-toxic chloroquine yields even lower cell viability after a 72-hour treatment.
Figure 12| Toxicity of O6 Loaded NP, Free TMZ, Free Chloroquine and Combination Therapy in DAOY. The study indicates free chloroquine had a cell viability above 80%, the TMZ free drug therapy showed cell viability around 75%, and the combination study with O6 NP and free TMZ indicated reduction in cell viability to about 40% cell viability and the chloroquine, O6 NP, and TMZ to have a cell viability of about 25-30% viability in DAOY cells.
CHAPTER SIX

CONCLUSIONS

Glioblastomas and neuroblastomas affect thousands of patients each year. The grim prognosis of both tumors is motivation for designing new treatment options for individuals. Using nanoparticles loaded with drug in combination with TMZ may be a promising option for many affected by these tumors. The nanoparticles would provide a sustained release, increased specificity to the tumor via the EPR effect, and lower tumor resistance to TMZ. The 2D in vitro studies show that combination therapy does decrease cell viability. Less TMZ would need to be used to achieve localized toxic results with the addition of the nanoparticles with either O⁶-Benzylguanine or Methyl Methanesulfonate according to these studies. Temozolomide is known to be an FDA approved drug for the treatment of glioblastoma, but in combination with the loaded nanoparticles, the efficacy of the drug is increased.

Both treatments demonstrated better results than using TMZ alone. Using a cocktail of non-toxic drugs that act on several different resistance mechanisms of glioblastoma and neuroblastoma. Specifically, the O⁶-benzylguanine inhibits MGMT, while the methyl methanesulfonate inhibits the BER. Chloroquine was also added to the O⁶-benzylguanine and TMZ therapy to reduce mutations due to its anti-mutagenic properties. This aided in reducing cell viability in glioblastoma and neuroblastoma.
FUTURE WORK

Non-targeted nanoparticles many times can effectively carry and deliver drugs to the cancer cells, but can also lack the ability to efficiently diffuse into the cell. The cancer cells may be exposed to the drug, but if not effectively uptaken, can develop multiple-drug resistance.\textsuperscript{23} Multiple-drug resistance is especially prevalent in cancer cells because they have the ability to expel the drug due to overly expressed transport proteins. This leads to exposure at low levels and can ultimately lead to resistance.\textsuperscript{23}

Nanoparticles can be highly specific and targeted carriers of drug. Cancer cells often contain overexpressed and unique receptors. Functionalizing the surface of the nanoparticle with ligands allow for a more efficient delivery system.\textsuperscript{22} Ligands of known overexpressed receptors of specific cancers can be conjugated to the surface of nanoparticles, allowing them to be highly specialized drug delivery systems.\textsuperscript{23} Ligands on the surface of nanoparticles will interact with the corresponding receptors within the cancer and allow for greater uptake into the cell than non-targeted nanoparticles. The drug will therefore not be released until the particle has been internalized.\textsuperscript{5,23}

There are almost 1200 biomarker candidates for cancer treatment, but the FDA has only approves a handful. The approved protein biomarkers include alpha-fetoprotein, human chorionic gonadotrophin-beta, CA125, CEA, EGFR, KIT, Thyroglobulin, PSA, CA1503, CA27-29, Cytokeratins, Osestrogen receptor and progesterone receptor, HER2, NMP22, Fibrin/FDP, BTA, and HMWCEA and Mucin.\textsuperscript{55} A variety of antigens have been used in targeting, mostly antibodies for the receptor proteins.

As previously mentioned, EGFR is an FDA approved biomarker for colon cancer. In a relative study, PLGA nanoparticles were conjugated to an EGFR antibody with 6-courmrin
encapsulated within it. The study illustrated that targeted nanoparticles allowed for 13 times more uptake than non-targeted and 50 times more efficient at delivering the drug than the 6-couramin alone. According to the Cancer Genome Atlas, EGFR was recently used to define the molecular class of glioblastomas and can be used as a biomarker based on its over expression in this type of cancer. This illustrates how EGFR is not only a biomarker for cancer, but also can be used for targeted nanoparticle therapy.

Targeting by using a conjugated peptide will greatly increase the efficiency of the particle uptake, however, there are some limitations. The ligand should be for a unique receptor to cancer cells, so it will not bind to cells that are healthy. Targeting ligands are more important for internalization and not necessarily than increasing accumulation close to the location of the tumor. Cell mediated endocytosis is the major mechanism of targeted nanoparticle internalization. The ligand that is used should therefore be of a known receptor that will facilitate internalization, not just allow for attachment to surface receptors of the tumor.

Strong binding affinities correlate to more efficient drug delivery. However, if the ligand-receptor affinity is too strong, the nanoparticle will not penetrate the cell. This is known as the binding site barrier. Nanoparticles can also be conjugated to numerous targeting ligands that will provide multivalent binding to cancer cells with high receptor density. The epidermal growth factor receptor (EGFR) is an autocrine pathway that contributes a number of factors to the progression of cancer. EGFR is highly expressed in many cancers, specifically head and neck cancers. EGFR in cancer promotes cell proliferation, inhibits apoptosis, and stimulates angiogenesis. The EGFR has three major functional domains, which are important for binding: ligand binding domain, hydrophobic transmembrane domain, and a cytoplasmic
tyrosine kinase domain. When an appropriate ligand comes into contact with the receptor it will form dimer formation and allows for internalization into the cell. There are many mechanisms to which the EGFR autocrine pathway that is present in cancer can be activated, but one distinct mechanism is by these cancer cells having overexpressed EGFR. This is especially typical in head and neck cancers where the majority of tumors are EGFR positive.

As mentioned previously, both glioblastomas and neuroblastomas are EGFR positive. EGFR can be utilized for targeting capabilities because of this overexpression. This would be a novel approach for ensuring increase efficacy for nanoparticle drug delivery. One study conjugated GE11 on the surface of a liposomal nanoparticle. GE11 was synthesized in the lab and conjugated to the Mal-PEG nanoparticle with a 5:1 molar ratio before being used for testing. The particles were then inserted into mice that lung tumors with high expression of EGFR. The study used fluorescent imaging to show the targeted lipid nanoparticle biodistribution compared to the non-targeted lipid particle biodistribution. The results concluded that GE11 lipid nanoparticles were effective in targeting the overexpressed EGFR. This indicates the targeting capabilities of EGFR for nanomedicine.

Based on the discussion above, the future work of this project should entail targeting nanoparticles. Although nanoparticles are efficient, highly resistant tumors are known to respond well to targeted nanoparticles. Based on the literature, adding a GE11 targeting ligand to the surface of our already successful nanoparticle would be the next phase of work.
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