Self-Assembling Peptide Hydrogel for Local Anticancer Prodrug Delivery in the Treatment of Glioblastoma Multiforme

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ABSTRACT

Gliomas represent approximately 80% of all malignant brain tumors, and glioblastoma multiforme (GBM) accounts for nearly half of all gliomas. GBM is the most common and aggressive primary brain tumor. Despite aggressive treatment including tumor resection followed by radiation and chemotherapy, the median survival rate for GBM averages between 12 and 15 months, with a 2-year survival rate less than 25%. Therefore, new therapeutic strategies are needed to improve the survival rate of those affected by GBM. Chemotherapy with the DNA alkylating agent, Temozolomide (TMZ), is commonly used as a first-line treatment for GBM. TMZ is an orally delivered drug that is known to be stable or inactive in the acidic pH environment of the stomach and begins to convert to its active form at a neutral pH in the bloodstream. However, studies have shown that full conversion of TMZ to its active form occurs at a more basic pH. Further, after TMZ is converted to its active form, it is unable to cross the blood-brain barrier, thereby limiting availability in the brain and lowering the effectiveness of TMZ.

Recently, peptides have been utilized as delivery systems for hydrophobic anticancer drugs or agents. These peptides are advantageous due to their biocompatibility, and high loading capacity for hydrophilic and hydrophobic drugs. To facilitate the delivery of TMZ, and potentially other hydrophobic drugs, we propose an innovative local delivery strategy using hydrophilic and alternating hydrophobic and acidic amino acids to form a peptide hydrogel. We hypothesize that the hydrophobic residues of the peptide hydrogel will load TMZ, and the hydrophilic residues will convert TMZ to its active form as the hydrogel degrades. This therapeutic strategy will allow for extended release of TMZ, thus increasing the efficacy of the drug.
In this study, peptide sequences were designed, synthesized, and formed into hydrogels for the delivery of TMZ. *In vitro* experiments validated that TMZ dissolved at a higher pH resulted in significantly increased cytotoxicity in LN-18 and T98G human glioblastoma cells. We also demonstrated that at various concentrations, the proposed peptide hydrogel efficiently loads TMZ and the release profiles can be adjusted based on peptide composition. Further, studies showed that TMZ-loaded peptide hydrogels mediated greater anticancer activity in LN-18 cells compared to delivery of TMZ alone. Overall, our results demonstrate the therapeutic potential of the TMZ-loaded peptide hydrogels for local drug delivery in treating GBM.
DEDICATION

I would like to dedicate this work to my Mom, Christine Gregory. You have instilled in me strength, courage, and confidence. Thank you for inspiring me every day, and for your never-ending sacrifice, support, and love. All that I am I owe to you. I will be forever grateful.
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CHAPTER 1

INTRODUCTION & BACKGROUND

Each year an estimated over 25,000 adults and children in the United States will be diagnosed with cancerous tumors in the brain or spinal cord.\(^1\) The frequency of brain tumors increases with age, with the median age of diagnosis being 59,\(^1\) and an average incidence of 40.1 per 100,000 in those over 40.\(^1\) Brain tumors are also the most common cancer in children between the age of 0-14, and the third most common cancer-related death in people between the ages 15 and 39.\(^1\)

Of the many types of central nervous system and primary brain tumors, gliomas and meningiomas are the most common.\(^2\) Gliomas represent 24.7% of all primary brain tumors and other CNS tumors and 74.6% of malignant brain tumors.\(^2\) Gliomas are known to arise from supporting cells of the brain, such as astrocytes or oligodendrocytes, and are classified based on the malignancy grade. Grade I tumors are benign with an almost normal appearance, grade II is considered low-grade diffuse glioma, grade III is classified as malignant diffuse glioma, and grade IV is considered glioblastoma.\(^2\) The grades are assigned based on the presence/absence of necrosis, amount of proliferation, and mitotic activity.\(^2\) Glioblastoma multiforme (GBM) is the most common malignant brain tumor with an incidence of 5-8 people per 100,000, and overall accounting for 55.4% of gliomas\(^1\). With a median age of onset at 64, GBM follows the same trend as all cancerous brain tumors; the incidence rate increases with age, with the highest diagnoses rates occurring between ages 75-84 years old. The incidence of GBM is slightly higher in men than in women, and occurs more often in Caucasians compared to other ethnicities.\(^3\) The median survival rate for patients with GBM is
between 12 to 15 months, even with aggressive treatment involving surgery, chemotherapy, and radiation.

1.1 Current Treatment

The current standard of care for treating GBM includes maximal surgical resection, followed by radiation, and then chemotherapy. However, complete resection of GBM tumors is difficult due to the invasive nature of the tumors and the areas of the brain in which they are located, which are usually responsible for controlling speech and motor function. The current standard treatment following surgical resection consists of radiotherapy plus concomitant chemotherapy with temozolomide (TMZ). TMZ is a prodrug that undergoes spontaneous hydrolysis at physiological pH to the active metabolite 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC). The drug further hydrolyzes into 5-amino-imidazole-4-carboxamide (AIC), providing the active alkylating or reactive portion of the drug needed to promote DNA methylation. TMZ primarily exerts its affect by methylation of guanine DNA at the O6 position, initiating a base pair mismatch, and ultimately leading to apoptosis. TMZ was approved for treatment of glioblastoma in 1999, and has a 96-100% bioavailability when delivered orally. Due to the pharmacokinetic properties of TMZ, only 30% of the drug has been found to reach the central nervous system. Despite this, studies have shown that patients receiving radiation therapy along with concomitant and adjuvant TMZ treatment was more effective than radiation alone. In one study, patients who received radiotherapy with TMZ had a median survival time of 14.6 months with a two-year survival rate of 27.2%, compared to 12.1 months and 10.9% of patients surviving two years that received radiotherapy alone. Since GBM is a highly vascularized tumor and is characterized by increased endothelial growth factor signaling, utilizing antiangiogenic therapeutic
strategies in addition to TMZ have been explored. Bevacizumab is a monoclonal antibody antiangiogenic therapy that was approved in 2009 for treatment of GBM. Bevacizumab binding to vascular endothelial growth factor (VEGF) prevents interaction with the VEGF receptor, resulting in a reduction of vascular growth within the tumor. Clinical trials testing the combination of bevacizumab with TMZ and radiotherapy have reported improvement of progression-free survival in patients with GBM, but no gain in overall survival.

In addition, local delivery of an alkylating drug, bis-chloroethylnitrosourea (BCNU), has been explored using Gliadel wafers, a polymeric (poly carboxyphenoxy-propane/sebacic acid anhydride) substrate that allows release of BCNU for approximately 3 weeks. Locally administered gliadel wafers allow for a reduction in systemic toxicity and a higher drug concentration at the tumor resection site. Gliadel wafers have been used as an adjuvant therapy in combination with systemic delivery of temozolomide, but the adjuvant therapy effectiveness was limited due to toxicity and small survival increase.

1.2 Barriers to Current Treatment

Several barriers remain for providing effective drug treatment to patients with GBM; these include crossing the blood-brain barrier (BBB) and development of chemotherapeutic resistance.

1.2.1 Blood Brain Barrier

The blood-brain barrier (BBB) is composed of brain endothelial cells with tight junctions that allow diffusion of water and small lipophilic molecules, but prevent the
passage of large, charged, or potentially dangerous molecules.\textsuperscript{11,12} This limitation creates a challenge for drug treatment of brain tumors, like GBM. Drug delivery to the brain is most commonly performed by using either a pharmacological or neurosurgical-based approach.\textsuperscript{13} The pharmacological-based approaches use various drug conjugations or drug delivery platforms such as self-assembling peptides,\textsuperscript{14} liposomes,\textsuperscript{15} or polymeric drug carriers.\textsuperscript{15} The pharmacological-based approach uses invasive methods that involve intraventricular infusion, intracerebral delivery, and convection-enhanced diffusion.\textsuperscript{12}

A common way to improve the delivery of a brain cancer drug is through drug lyophilization or conversion of the drug into a lipophilic molecule. Prodrugs for delivery across the BBB are formed by linking nonpolar groups to the active molecule,\textsuperscript{11} allowing concealment of the polar groups in the active drug, therefore transforming a water-soluble drug into a lipid-soluble prodrug.\textsuperscript{11} The prodrug is then able to cross the BBB and is converted to the active drug. Furthermore, if the active drug is polar, exit from the brain into the systemic circulation will be hindered.\textsuperscript{16} Conjugation of drugs, by either addition of a nonpolar group or molecular alteration, to form prodrugs are popular due to their enhanced BBB permeability.

1.2.2 Chemotherapeutic Resistance

Glioblastoma has been found to have either intrinsic or acquired resistance to chemotherapy. Acquired drug resistance to chemotherapeutic prodrugs, like temozolomide, has been attributed to the (O)6-methylguanine-DNA-methyltransferase (MGMT) gene. MGMT is a DNA repair protein that functions by removing alkyl groups from the O\textsuperscript{6} position of guanine, correcting drug-induced DNA damage and preventing
apoptosis. Therefore, when TMZ and other alkylating agents are used for therapy, they encounter resistance due to upregulation of MGMT. Since alkylating agents exert therapeutic effects by generating DNA damage, the MGMT status of a patient is strongly correlated with chemotherapeutic resistance, and as a result, the efficacy of treatment when using alkylating drugs. Methylation or lower expression of MGMT has been associated with an increase in positive outcomes when treating patients with glioblastoma with TMZ.
CHAPTER 2

RELEASE APPROACHES AND ACTIVATION STRATEGIES FOR PRODRUGS

2.1 Introduction to Prodrugs

Prodrugs are used extensively in the delivery of cytotoxic compounds to cancer cells. The development of successful therapies for cancer treatment is increasingly challenging due to the barriers that diminish drug efficacy. These difficulties potentially affect the drug bioavailability, solubility, and distribution. Consequently, further significant issues may arise, such as adverse side effects, shorter drug duration, and reduced drug stability. In order to overcome these barriers, the use of prodrugs has become an effective tool in cancer therapies.

The term prodrug, introduced by Adrien Albert in 1951, refers to a reversible molecule with desirable drug properties, which does not possess any pharmacological effects until conversion to its active form. The aim behind prodrug design is to mask undesirable properties of therapeutics, such as poor water solubility or chemical instability. This mask allows prodrugs to remain in their inactive form until enzymatic or chemical transformation initiates release of the active form of the drug. The justification behind the use of prodrugs is to improve the overall bioavailability, biodistribution, metabolism, excretion, and undesirable toxic effects of the parent drug (or adsorption, distribution, metabolism, excretion and toxic properties). These properties remain muted until the prodrug reaches the targeted tumor or intended site of action.
Figure 1.1 Schematic representation of the prodrug concept. (A) Carrier-linked prodrug allow improved pharmacokinetics of the drug. The carrier-linked portion of the prodrug indicates the part of the prodrug that is pharmacologically inactive. The barrier represents the limitations of the parent drug that prevent optimal pharmacokinetic performance. (B) Carrier-linked prodrugs are activated through enzymatic or non-enzymatic cleavage of the drug from the carrier (adapted from Rautio et al. 2008).

There are two main classes of prodrugs: carrier-linked prodrugs and bioprecursor prodrugs. In carrier-linked prodrugs, the drug is linked to a moiety (or temporary carrier) through a bioreversible covalent linkage. This carrier generally improves the
pharmacokinetics of the drug due to its lipophilic nature, allowing it to overcome barriers
to optimal pharmacokinetic performance (Fig. 1.1A). To release the parent drug or
activate the prodrug, the carrier can be easily removed in vivo through enzymatic and/or
non-enzymatic cleavage\textsuperscript{22} (Fig. 1.1B). Bioprecursor prodrugs result from molecular
alteration of the active drug itself. This modification allows the prodrug to be converted
metabolically or chemically to its active compound.

Prodrugs are commonly activated in two ways: actively, via enzyme hydrolysis,
or passively, via reduced pH or hypoxia. Our aim is to present an overview of the two
prodrug activating strategies. Specifically, we will highlight the common approaches to
converting prodrugs to their active form.

\section*{2.2 Active Targeting: Enzyme responsive therapies}

Anticancer prodrugs can be designed to target certain molecules, such as enzymes and antigens, which are commonly overexpressed in tumor cells.\textsuperscript{23} Enzyme Prodrug Therapy (EPT) is widely-used as a method for converting cancer prodrugs into active drugs through hydrolysis in the body. The principal technique of EPT is to deliver a drug-activating enzyme to cancerous tissues and then systemically dispense the prodrug.\textsuperscript{23} This method allows local activation of the prodrug, which is essential in preventing systemic adverse side effects.

Activation of prodrugs in tumor tissues by enzymes can be achieved in two ways: antibody-directed enzyme prodrug therapy (ADEPT), and gene-directed enzyme prodrug therapy (GDEPT)\textsuperscript{23}. Here we discuss strategies for active transformation of prodrugs using enzymatic therapies in detail as well as their benefits and limitations.
2.2.1 Antibody directed enzyme prodrug therapy (ADEPT)

Antibody directed enzyme prodrug therapy (ADEPT) was first proposed almost
30 years ago to overcome the restrictions of early antibody directed cancer therapy. ADEPT is a strategy that uses a drug-activated enzyme attached to a tumor-related antibody. After the prodrug is systemically administered, the enzyme located on the tumor site will convert the prodrug to its active form. This results in localized cytotoxic effects to the tumor due to the targeting of the tumor-related antibody. Limitations of the ADEPT system include immunogenicity of the antibody, inefficient delivery of antibody and enzyme to all tumor cells, and difficulty of producing large amounts of active drug, resulting in low levels of cytotoxicity.

2.2.2 Gene-directed enzyme prodrug therapy (GDEPT)

Gene-directed enzyme prodrug therapy (GDEPT), also known as viral directed enzyme prodrug therapy (VDEPT) or suicide gene therapy, utilizes a viral vector to deliver a gene encoding an enzyme that will convert a prodrug into its active, lethal form within a tumor. Upon production of the desired enzyme, the systemically delivered prodrug can then be activated. Common viruses used in GDEPT are adeno-associated viruses (AAV), retroviruses, herpes simplex virus (HSV), and Epstein-Barr virus (EBV). Enzymes commonly delivered include thymidine kinase, cytosine deaminase, and cytochrome P450. Despite the high delivery efficiency, there are several limitations to using viral vectors that still remain. There is a slight risk of the virus reverting to its wild-type form, causing mutations in the genome. Also, most retroviral vectors can only integrate into dividing cells, which even in high-grade invasive tumors only represents approximately 11.2-23.4% of tumor cells. This excludes the type of retroviral vector
known as lentiviruses, which are able to infect both proliferating and non-proliferating cells. Despite this, the majority of tumor cells would not be targeted using retroviruses.

2.3 Common Enzyme/Prodrug Systems

2.3.1 Carboxypeptidase G2 (CPG2)/Nitrogen mustard

The enzyme carboxypeptidase G2 (CPG2) is derived from the *Pseudomonas* strain RS-16. CPG2 works by cleaving glutamic acid from nitrogen mustard-based drugs creating DNA-alkylating agents with the capacity to methylate DNA. The activated drug then has the ability to cause DNA cross-linking, which leads to apoptosis. CPG2 also possesses the ability to diffuse through cell membranes without the need for gap junctions, demonstrating versatility compared to other enzymes. Using CPG2 in enzyme-prodrug chemotherapy is effective, but this strategy is hindered by the enzymes' off-site targeting of the prodrug. Currently, studies are being conducted to create an inhibited version of the enzyme that can be regulated through proteolysis by a tumor-specific protease, allowing an increase in the localization effects of the prodrug and a decrease in off-site targeting. A study conducted by Yachnin et al. showed that it is possible to generate a protease-regulatory form of CPG2, which will be valuable in upcoming enzyme-prodrug therapy.

2.3.2 Herpes Simplex Virus-Thymidine Kinase (HSV-TK)/Ganciclovir

In the Herpes Simplex Virus-Thymidine Kinase (HSV-TK) system, ganciclovir (GCV) is phosphorylated by the thymidine kinase enzyme. Following phosphorylation,
the triphosphate form of GCV is produced.\textsuperscript{35} This GCV triphosphate form can then cause apoptosis in tumor cells by introducing single-stranded breaks into DNA. Unfortunately, GCV can only affect dividing cells, and it has limited transportation capacity because gap junctions are required for transport.\textsuperscript{35} There are also risks in using a viral system, such as triggering the immune system, mutations, and restricted migration.\textsuperscript{35} Recently, Hashemi et al. demonstrated in a GBM disease model that the HSV-TK system could be improved by incorporating olfactory ensheathing cells (OECs).\textsuperscript{37} These cells are removed from human olfactory mucosa and have the ability to migrate toward astrocytes.\textsuperscript{37} The cells then can deliver the HSV-TK gene to tumorigenic astrocytes, inducing cell death. Another study conducted by Wu et al. showed that transfer of GCV could be increased by improving a study that used an approach combining two GDEPT systems.\textsuperscript{38} Cell were retrovirally transduced with cytosine deaminase-uracil phosphoribosyltransferase and HSV-TK.\textsuperscript{38} The result of the combination was inhibited growth and high therapeutic efficacy in lung metastases.\textsuperscript{31} Other approaches examined the use of valacyclovir, the prodrug form of acyclovir, instead of GCV, and higher efficacy was observed.\textsuperscript{27,35}

### 2.3.3 Cytosine Deaminase (CD)/5-Fluorocytosine

In the cytosine deaminase (CD) system, 5-fluorocytosine (5-FC), an antifungal prodrug, is converted to its active form, 5-fluorouracil (5-FU), by the enzyme CD.\textsuperscript{25} After conversion, active 5-FU is effective in killing tumor cells but causes side effects, such as dermatitis, cardiac toxicity, and mucositis.\textsuperscript{35} Despite this, 5-FU has the ability to travel independently of gap junctions by diffusion, unlike HSV-TK. This feature of 5-FU allows the prodrug to induce more cell death than most systems. CD/5-FC has been utilized for
treat various types of cancer, including colon,\textsuperscript{39} liver,\textsuperscript{39} breast,\textsuperscript{40} glioma,\textsuperscript{41} and ovarian cancer.\textsuperscript{29,42} To overcome the weaknesses of CD/5-FC, groups have worked to address off-targeting, optimizing the prodrug, and optimizing delivery capacity. Takahashi et al. demonstrated that CD/5-FC increases cytotoxic effects in glioma cells in response to the radiosensitizing effect of 5-FU.\textsuperscript{43} Another study combined CD/5-FC with a nonlytic retroviral replicating vector (TOCA 511), where TOCA 511 acts as a carrier for cytosine deaminase (CD). Ostertag et al. proved that TOCA 511 was capable of effectively delivering and activating the prodrug, which resulted in tumor shrinkage and growth inhibition.\textsuperscript{44}

\textbf{2.3.4 Cytochrome P450 (CYP)/Oxazaphosphorines}

One third of all P450 enzymes are involved in activating anticancer prodrugs.\textsuperscript{30} P450 enzyme concentrations differ in various areas of the body, so the P450 enzymes may be manipulated to target select tissues or areas for drug activation.\textsuperscript{30} Anticancer prodrugs involved with P450 enzymes are oxazaphosphorines, which include Cyclophosphamide (CPA) and Ifosfamide (IFA). These alkylating prodrugs are substrates that the P450 enzyme metabolizes to yield a 4-hydroxy derivative product, the active form of the prodrugs.\textsuperscript{35} The yielded product forms DNA cross-links that ultimately lead to cell death. Similar to 5-FU, the resulting active prodrug from CPA/IFA can be transported independently of gap junctions,\textsuperscript{35} which provides the system with a higher level of potency than others. Another advantage of the CYP system is the decrease in immunogenicity due to P450 enzymes already existing in the human body.\textsuperscript{35} Lack of specificity is a major weakness of the system because P450 liver enzymes metabolize prodrugs, generating active metabolites throughout the entire body instead of
in one specific location. This causes serious side effects, including neurotoxicity, cardiotoxicity, and nephrotoxicity.\textsuperscript{35} However, a few approaches have been used to reduce systemic toxicity. In one approach, encapsulated cells overexpressing the CYP enzyme were implanted around malignant mammary tumors, and the patients were given CPA following implantation. The results showed no adverse systemic side effects.\textsuperscript{45} Despite these promising results, there are still fears about using the CYP system due to the systemic activation of CYP450 in humans.

2.3.5 Summary

There has been significant progress in the development of enzyme/prodrug systems. Developing safe vectors and enzymes to achieve local drug delivery and prevent off-target effects remains the focus of current research. Efforts are being made to move away from traditional prodrug activation methods due to their instability and other drawbacks mentioned previously. For example, Du et al. has developed metal-contained enzyme mimics (MEMs) for prodrug activation. These MEMs can copy the functions of natural enzymes and be used for prodrug activation.\textsuperscript{46} Overcoming these issues may increase the possibility of eradicating incurable and hard to treat cancers.

2.4 Passive Targeting: Environment Responsive Therapies

Unlike active targeting, which utilizes enzymes or antigens, passive targeting exploits the pathophysiological abnormalities that result from malignant tissue.\textsuperscript{47} These abnormalities include the hypoxic environment of tumor tissue and the difference in intracellular pH. These changes can be manipulated beneficially by designing prodrugs that will selectively activate in these tumor environments. Here we discuss common pH-responsive linkages and hypoxia-activated prodrugs (HAPs).
2.4.1 pH Responsive

The intracellular pH within solid tumors is maintained near a pH range of 5.7-7.6 due to the buildup of acid metabolites resulting from an imbalance of metabolic rates, glucose consumption rates, and proton transport. Therapeutic strategies have been designed to exploit tumor pH by using prodrugs which activate at low pH. pH-responsive prodrugs can be attained through drug conjugation to cleavable acid-linkages. Common pH-responsive linkages include acetals, anhydrides, and hydrazones. These linkages are bonded to the drug, rendering it inactive or in prodrug form, until the chemical bonds are cleaved at a low pH to allow drug hydrolysis. Hydrazone linkages have demonstrated fast hydrolytic cleavage at a low pH compared to a neutral physiological pH. Alendronate-monoethyl adipate-hydrazone (ALN-MA-hyd) has been used as a linker for doxorubicin (DOX) to deliver the chemotherapeutic to bone tumor tissue. The conjugate released DOX at a pH of 5, and also proved to be relatively stable at physiological pH. In another study, a hydrazone bond was used to conjugate DOX to a di-block copolymer composed of poly(L-lactic acid) (PLLA) and methoxy-poly(ethylene glycol) (mPEG). The study showed that in an acidic environment of pH 3 or 5, most of the DOX was cleaved from the hydrazone linkage, allowing for DOX to hydrolyze and mediate cell death. At a pH of 7, the amount of DOX released was less than 10%, which confirmed that the encapsulated DOX remained stable at neutral pH. The DOX-conjugated polymer allowed for increased drug uptake in lymphoblast cells at a low pH and slower release of DOX at a physiological pH.

2.4.2 Hypoxia Responsive
The environment within most tumors contains areas of hypoxia due to poor blood supply, or a state of low oxygen, which is known to promote tumor resistance to chemotherapy and radiotherapy. \(^{53}\) Oxygen is a radiosensitizer, a molecule that facilitates oxidation of free radicals in DNA during tissue irradiation. \(^{54}\) Thus, hypoxic tumor cells are resistant to radiotherapy. The hypoxic tumor cells are also resistant to most chemotherapies, due to the tumor cells being quiescent and chemotherapeutic drugs only targeting proliferating cells. \(^{53}\) Chemotherapy and radiotherapy resistance is also often due to the development of an aggressive phenotype that promotes resistance to apoptosis, inhibition of DNA repair, increased angiogenesis, metastasis, and an abundance of other effects. \(^{53}\) Thus, the therapeutic strategy of hypoxia-activated prodrugs (HAPs) was developed to target hypoxic tumors.

PR104 is a nitroaromatic compound, a water-soluble phosphate ester, which upon activation, quickly hydrolyzes in a hypoxic environment into an alcohol, PR104A. \(^{55}\) PR104A is then metabolized by oxidoreductases in the body, which results in DNA cross-linking or cell death. \(^{53,55}\) Studies have shown that PR104 could be used to eradicate acute myeloid leukemia and acute lymphoblastic leukemia. \(^{55}\) Patients with these diseases are known to exhibit high levels of hypoxia at their tumor sites. \(^{55}\) In a phase I/II study, PR104 was given to patients with refractory or relapsed acute myeloid leukemia or acute lymphoblastic leukemia. The patients were administered an intravenous infusion of PR104 every 14-28 days for up to 3 cycles. Overall, the study demonstrated PR104 effectiveness, but due to the toxicity of PR104, it is unlikely to be successful long-term. \(^{55}\)

TH-302 is a hypoxia-activated prodrug of bromo-isophosphoramide. Reduction of the nitrogen group on TH-302 causes the prodrug to release a DNA-crosslinking
alkylating agent in hypoxic regions. Studies have shown that TH-302 can be utilized for a broad range of applications, but is most commonly used to treat myeloma. In one study, TH-302 was used to target hypoxic bone marrow niches in leukemia and the results showed that leukemia cells were reduced when treated with TH-302. The study also showed that using TH-302 followed by chemotherapy increased survival in mice. Despite in vivo success, phase III clinical trials did not show effective results. This was due to side effects, such as myelosuppression. TH-302 is selective by only affecting hypoxic areas and shows potential for targeted therapy in cancer treatment.

2.4.3 Conclusion

One of the key advantages of pH-responsive prodrugs is that they are able to exploit tumor tissue pH for activation. These pH-responsive prodrugs allow efficient delivery at the tumor site and minimization of harmful off-target side effects. Despite this, pH-responsive delivery systems have not performed well in clinical studies for cancer therapy. This is due to ineffective translation of the in vitro properties to in vivo studies.

Hypoxia-activated prodrugs are useful for targeting hypoxic tumors. These tumors are known for being difficult to treat, and this particular strategy offers an advantage for treating aggressively resistant tumor types. However, the therapeutic benefit of hypoxia-activated prodrugs is currently limited. The toxicity associated with the use of the hypoxia-activated prodrugs, along with the lack of prodrug optimization, reduces the clinical potential of this strategy.

2.5 Conclusion/Final Remarks

Bioengineering strategies have been explored to improve the release or activation of prodrugs through active or passive activation methods. Active targeting
methods that involve enzyme/prodrug systems are effective but include many unsafe
delivery vectors and enzymes. Passive targeting methods, which rely on environment
activation, allow for more efficient, local delivery, and in turn a reduction of harmful side
effects. Despite this, passive targeting systems still lack effective translation to \textit{in vivo}
studies. The future of prodrug therapy for both targeting methods begins with the
development of safer delivery systems, along with prodrug optimization for effective
translation to clinical trials.
CHAPTER 3

RESEARCH AIMS

3.1 Objectives

Gliomas represent approximately 80% of all malignant brain tumors, and glioblastoma multiforme (GBM) accounts for nearly half of all gliomas.\(^1\) GBM is the most common and aggressive primary brain tumor. Despite aggressive treatment including tumor resection followed by radiation and chemotherapy, the median survival rate for GBM is 15 months.\(^2\) Therefore, new therapeutic strategies are needed to improve the survival rate of those affected by GBM.

The objective of this research is to target GBM by developing a local anticancer drug delivery system using peptides, that will self-assemble in water to form a hydrogel. Chemotherapy with the DNA alkylating agent, Temozolomide (TMZ), is used as a first-line treatment for GBM. Studies have shown that full conversion of TMZ to its active form occurs at a more basic pH (pH>7.5).\(^5\) Furthermore, after TMZ is converted to its active form, it is unable to cross the blood brain barrier, thereby limiting availability in the brain and lowering the effectiveness of TMZ. Therefore, my central hypothesis and the overall goal of this research is to develop an innovative local delivery strategy using hydrophilic and alternating hydrophobic and acidic amino acids to form a hydrogel that will encapsulate TMZ and convert the drug to its active form.

By pursuing the following aims, I will gather the data essential to develop a delivery system that will improve the efficacy of TMZ and extend the survival rate for patients with GBM. The study aims are listed and discussed below.
Aim 1: Determine whether the peptide compositions/designs will allow for loading of the anticancer prodrug. To accomplish this aim, we will design, synthesize, and test the ability of three different peptides to load TMZ. These peptides will differ in number of hydrophobic and hydrophilic sites. Based on this and the hydrophobic nature of TMZ, we hypothesize that the peptides will be able to load TMZ. We also believe that increasing the number of hydrophobic amino acids will allow for loading of more TMZ.

Aim 2: Characterize the structural properties of peptide hydrogels. We will determine the sizing, zeta potential, formation of secondary structures, and release profile of each peptide hydrogel. We hypothesize that the release of drug from each peptide will be associated with the ratio of hydrophobic to hydrophilic amino acids. We also hypothesize that sizing of each peptide hydrogel will be relatively similar, there will be formation of secondary structures, and the zeta potential will correlate with the amount of charged amino acids of each peptide.

Aim 3: Assess drug release from the loaded peptide hydrogels. To accomplish this aim, we will load a dye into the hydrogels and measure the change in fluorescence over time. We hypothesize that dye release will be slower in the peptide hydrogel that consists of more hydrophobic amino acids, and faster in the peptide hydrogels with less hydrophobic residues.

Aim 4: Determine the cell viability of human glioblastoma cells treated with the TMZ-loaded peptide hydrogel compared to TMZ delivered alone. Each peptide-hydrogel loaded with TMZ will be delivered onto LN-18 cells and assessed for an increase in cytotoxicity. We hypothesize that increased conversion of TMZ into its active form will be based on the number of basic amino acids in each peptide.
3.2 Approach

**Aim 1:**

We will first evaluate whether TMZ is more active/cytotoxic when dissolved in a basic pH environment and delivered onto human glioblastoma (LN-18 and T98G) cells. This will be performed to determine whether our peptide designs will potentially be effective in conversion of TMZ into its active form.

**Aim 2:**

Once the appropriate peptide compositions have been chosen, the peptide hydrogels will be characterized using circular dichroism (CD) for secondary structure analysis, zeta potential for structure stability, and dynamic light scattering to measure particle size. After the assessment of TMZ in a basic environment, and characterization of the peptide hydrogels, the amount of TMZ loaded will be assessed. TMZ will be loaded into the peptide hydrogels at 3 peptide concentrations (0.05, 0.1, and 0.5 mg/mL) and a TMZ concentration of 200 µM. The amount of loaded TMZ will be determined by high-performance liquid chromatography (HPLC).

**Aim 3:**

Based on the TMZ loading results, the peptide concentration that exhibited the maximum amount of loading will be selected for the release and cytotoxicity study. To study release, phenol red will be loaded into each peptide hydrogel. The peptide hydrogels will then be incubated at 37°C. The absorbance of phenol red will be taken at several time points to determine the amount released over time.

**Aim 4:**

Lastly, the cytotoxicity of the TMZ-loaded peptide hydrogels in human glioblastoma (LN-18) cells will be evaluated by MTT assay.
CHAPTER 4

IMPLANTABLE SELF-ASSEMBLING PEPTIDE-HYDROGEL DRUG DELIVERY SYSTEM FOR TREATING GLIOBLASTOMA MULTIFORME

Temozolomide (TMZ) has been used for anticancer treatment since its approval in 1999. TMZ is a slightly hydrophobic prodrug, that is able to exert its anticancer effects by methylation of purine bases of DNA, including O6-guanine, N7-guanine, and N3-adenine, initiating base pair mismatches. TMZ is delivered orally and known to remain stable in its native form in the acidic environment of the stomach. The drug is absorbed in the bloodstream and begins to convert into its active form, known as 5-(3-methyltriazel-1-yl)-imidazo-4-carboxamide (MTIC), at a neutral or physiological pH. However, studies have shown that full conversion of TMZ into its active form occurs at a more basic pH (>7.4), suggesting that the therapeutic effect of TMZ is limited. Further, TMZ is the standard-of-care chemotherapy drug for treating patients with glioblastoma, but the median survival rate remains low at 14.6 months with aggressive treatment. Development of carriers tailored to the mechanism activating TMZ have not been previously explored but may enhance the effectiveness of the drug.

Peptides that are able to self-assemble into different nanostructures are very attractive for biomedical applications such as drug delivery. These self-assembling peptides are advantageous due to their biocompatibility and high loading capacity for hydrophilic and hydrophobic drugs. These peptides are able to self-assemble due to hydrogen bonding, hydrophobic interactions, and electrostatic interactions that combine and form stable nanostructures. Among these self-assembling peptides is a class of peptides that are able to self-assemble into hydrogels when introduced to water. This is due to the alternating hydrophilic and hydrophobic nature of the amino acids. Recently,
self-assembling peptides have been utilized as local delivery systems for protein drugs and hydrophobic anticancer drugs or agents. To facilitate the delivery of TMZ, and potentially other hydrophobic prodrugs, we developed a novel peptide hydrogel delivery system.

The peptides were designed and consisted of basic and alternating hydrophobic and acidic amino acids. We demonstrated that at various concentrations, the peptide hydrogels are able to efficiently load TMZ, and the release profiles can be adjusted based on peptide composition. In vitro experiments confirmed that TMZ dissolved at a higher pH resulted in significantly increased cytotoxicity in LN-18 and T98G human glioblastoma cells. Studies also showed that TMZ-loaded peptide hydrogels exhibited greater anticancer activity in LN-18 cells compared to delivery of TMZ alone.

4.1 MATERIALS AND METHODS

4.1.1 Materials

Peptides AE12K4 (AEAEAEAEAEAEKKKKK), AE8K8 (AEAEAEAEKYYYYK), and AE4K12 (AEAEKYYYYYYYYYYYYYYYYY) were synthesized and purified (purity >95%) by GenScript USA Inc (Piscataway, NJ). The N and C terminals of the peptides were protected by acetyl and amino groups, respectively. TMZ (≥98% pure), Phenol Red, and Thiazolyl Blue Tetrazolium Bromide (TBTB) were purchased from Sigma-Aldrich (St. Louis, MO). The LN-18 and T98G human glioblastoma cell lines were obtained from ATCC (Manassas, VA). The LIVE/DEAD Viability/Cytotoxicity Kit as well as cell culture reagents, including fetal bovine serum (FBS), penicillin-streptomycin, and trypsin were acquired from ThermoFisher Scientific (Waltham, MA). Cell culture medium DMEM,
EMEM, and Methanol (≥99% pure) were obtained from VWR (Radnor, PA). pH buffers were obtained from ThermoFisher Scientific.

4.1.2 Cell Culture

The human glioblastoma cell line LN-18 was cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), and 1% antibiotic (100IU/mL penicillin/100ug/mL streptomycin). The human glioblastoma cell line T98G was cultured in Eagle’s Minimum Essential Medium (EMEM) with 10% fetal bovine serum (FBS), and 1% antibiotic (100IU/mL penicillin and 100ug/mL streptomycin). Both cell lines were grown in an incubator at 37°C with 5% CO₂.

4.1.3 Cytotoxicity of TMZ at various pH

To determine whether the anticancer activity of TMZ is pH dependent, cytotoxicity of TMZ on glioblastoma cells was examined in various pH environments. LN-18 or T98G cells were plated at 1X10⁵ or 0.9X10⁵ cells per well in 24-well plates and allowed to attach overnight. TMZ was dissolved in water at a pH of 2.5, 7.5, and 12. TMZ from each pH environment was added to LN-18 and T98G cells for a final concentration of 200 µM and allowed to incubate in media containing 10% FBS. After 24 hours, cells were washed three times with media to remove TMZ and incubated for an additional 48 hours. Cytotoxicity of TMZ was determined by MTT assay after a total of 72 hours. For the MTT assay, Thiazolyl Blue Tetrazolium Bromide (TBTB) was dissolved in PBS at 2 mg/mL and added to media on cells for a final concentration of 1 mg/ml. After a 4-hour incubation at 37°C, the media containing TBTB was aspirated from the wells, and 500 µL of DMSO was added to each well to dissolve the formazan crystals. A Biotek Synergy
plate reader (Winooski, VT) was used to measure absorbance at 540 nm. Cell viability was normalized to untreated cells and determined by using the following equation:

\[
\text{Cell viability (\%)} = \frac{\text{Absorbance}_{540(\text{sample})}}{\text{Absorbance}_{540(\text{control})}} \times 100\%.
\]

4.1.4 Preparation of TMZ-loaded Peptide Hydrogels

To prepare the peptide hydrogels, AE12K4, AE8K8, and AE4K12 peptides were dissolved in a solution of 90% ethanol and 10% Milli-Q water at concentrations of 0.05 mg/mL, 0.1 mg/mL, and 0.5 mg/mL. TMZ was dissolved in Milli-Q water and combined with each peptide solution for a final TMZ concentration of 200 µM. The final solution was dehydrated to form a transparent drug-loaded film. The film was washed 3 times with Milli-Q water to remove any unloaded or residual TMZ. Milli-Q water was then added to rehydrate the film and allowed to shake for 2 hours. The amount of TMZ loaded was determined by high-performance liquid chromatography (HPLC).

4.1.5 Characterization of Peptide Hydrogels

Zeta Potential and Dynamic Light Scattering

To determine the surface charge (zeta potential) and particle size of the peptide hydrogel assemblies, laser doppler velocimetry combined with phase analysis light scattering (M3-PALS) and dynamic light scattering, respectively, was conducted on the peptide hydrogels prepared at a concentration of 0.05 mg/mL using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The temperature of the Zetasizer was maintained at 25°C. The intensity of scattered light due to the complexes was collected at an angle of 173°, a refractive index of 1.6, and an absorbance value of 3.27. Three measurements were tested to generate an intensity-based size distribution plot.
CD Measurement

The peptide hydrogels were prepared at a concentration of 0.05 mg/mL and a CD spectrum between 190 nm and 300 nm was recorded on a Jasco J-810 CD Spectropolarimeter (Jasco, Inc., Easton, MD) at 20°C. All spectra were corrected by subtracting the baseline and the data was expressed as the mean residue of ellipticity, which was given in units of degrees per cm²/dmol. Predicted percentages of α-helices and β-sheets were determined using CAPITO, a web server-based analysis and plotting tool for CD data.

4.1.6 Determination of TMZ Loading in Peptide Hydrogels

The amount of TMZ loaded into each rehydrated peptide hydrogel was determined using an HPLC system consisting of a Waters Model 2707 Autosampler connected to a 1515 Isocratic HPLC Pump (Waters, Inc., Milford, MA). Samples were injected onto the reverse phase Zorbax Rx-C18 column (4.6 x 250 mm, 5 µm). The absorbance detector was set at 316 nm. The mobile phase consisted of 0.1% aqueous acetic acid in Milli-Q water and methanol (90:10, v/v) and was delivered at 1.0 mL/min. The amount of drug loading was determined by comparison to the given temozolomide concentration (0.03 mg/mL).

4.1.7 Release of TMZ from Peptide Hydrogels in vitro

0.05 mg/mL of AE8K8 and AE4K12 peptide hydrogels, and 0.5 mg/mL of AE12K4 peptide hydrogel was prepared as previously described in section 4.1.4. Instead of TMZ, phenol red was loaded into the peptide hydrogels at a final concentration of 200 µM. To study release kinetics, the phenol red-loaded peptide hydrogels were placed into a 96-well plate and incubated at 37°C. The absorbance of the phenol red was measured.
using a Biotek Synergy plate reader at 443 nm. The absorbance of each sample was measured at 0, 1, 2, 4, 8, 12, 24, 48, 72, 96, and 120 hours. The amount of phenol red released from the peptide hydrogel was calculated by comparison to the absorbance value taken at the initial time point. The percentage of phenol remaining was determined by using the equation below:

\[
\text{Amount Remaining (\%) = \left( \frac{\text{Absorbance}_{443(T\text{ime} \times)} / \text{Absorbance}_{443(T\text{ime} \ 0)}}{1} \times 100\% \right).}
\]

4.1.8 Cytotoxicity of TMZ-Loaded Peptide Hydrogels

To analyze the anticancer activity of the TMZ-loaded peptide hydrogels, a cytotoxicity study was performed. LN-18 (1X10⁵) cells were cultured in 24-well plates and allowed to attach overnight. AE12K4, AE8K8, and AE4K12 peptide hydrogels were formed at the concentration that exhibited the highest TMZ loading for each peptide. The TMZ-loaded peptide hydrogels were incubated with LN-18 cells at 200 µM for 72 hours in media containing 10% FBS. Cells were treated with TMZ alone, unloaded peptide hydrogel, or left untreated as controls. Cytotoxicity of TMZ was determined by MTT assay after the 72-hour incubation period and cell viability was calculated as previously described in section 4.1.3.

4.1.9 Statistical Analysis

Quantitative data were presented as mean ± SEM or standard deviation of three independent experiments. Statistical analysis was performed using either Student’s t-test or one-way ANOVA, where \( *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 \). A value of \( P<0.05 \) was considered statistically significant.
4.2 RESULTS

4.2.1 Peptide design and structure of peptide hydrogels

Three different compositions of self-assembling peptides were designed, each 16 amino acids in length, consisting of Alanine (A) as the hydrophobic domain, Glutamic acid (E) as the hydrophilic/acidic domain, and Lysine (K) as the hydrophilic/basic domain (Fig. 4.1). The peptides were synthesized and designed with alternating hydrophobic and hydrophilic amino acids to promote self-assembly into a hydrogel when water is introduced. The rationale for this design is that TMZ will load into the hydrophobic core and maintain stability due to the acidic amino acid residues (Fig. 4.2). As the hydrogel degrades, the basic amino acids will convert TMZ into its active form.

![Peptide design and structure of peptide. AE12K4 (A), AE8K8 (B), and AE4K12 (C) peptides were designed using peptide sequences consisting of alanine, glutamic acid, and lysine.](image)

*Figure 4.1. Peptide design and structure of peptide. AE12K4 (A), AE8K8 (B), and AE4K12 (C) peptides were designed using peptide sequences consisting of alanine, glutamic acid, and lysine.*
4.2.2 Cytotoxicity of TMZ versus pH

TMZ begins to convert to its active form, MTIC, at neutral pH; however, previous studies have demonstrated that TMZ only fully converts to MTIC at a more basic pH (>7.5). Though this has been shown, the effectiveness of TMZ when dissolved in different pH environments has not been previously reported in the literature. Therefore, to determine whether the anticancer activity of TMZ is pH dependent, a cytotoxicity study was performed on human glioblastoma cells with TMZ dissolved in basic, neutral, and acidic pH environments. The results showed that TMZ exhibited slight anticancer activity at a pH of 2.5, but as the pH was increased, the anticancer activity of TMZ was significantly enhanced in both LN-18 (Fig. 4.3A) and T98G (Fig. 4.3B) human glioblastoma cell lines. Viability of cells treated with neutral TMZ was 56% and 83% in LN-18 and T98G cells, respectively. Viability decreased by at least 25% when cells were treated with basic TMZ compared to neutral TMZ, with viabilities of 30% and 58% in LN-18 and T98G cells, respectively. These results demonstrate that the effectiveness of TMZ is pH dependent and TMZ has the largest anticancer activity when dissolved in a basic pH environment. These results validate the potential utility of basic pH amino acids.
residues in the design of the peptide hydrogels to increase the anticancer activity of TMZ.

Figure 4.3. pH-dependent cytotoxicity of TMZ in LN-18 and T98G cells. Cell viability of (A) LN-18 and (B) T98G human glioblastoma cells 72 hours after treatment with TMZ dissolved in water at a pH of 2.5, 7.5, and 12. Data are mean ± SEM of three independent experiments performed in triplicate, where **P<0.01 and ***P<0.001 compared to untreated cells (control) which were considered 100% viable.

4.2.3 Characterization of Peptide Hydrogels
After formulating AE4K12, AE8K8, and AE12K4 peptide hydrogels, we characterized the particle size, zeta potential, and secondary structures of each peptide hydrogel. Circular dichroism (CD) spectroscopy was used to measure the secondary structure of each peptide design. The AE12K4 peptide hydrogel displayed a typical $\alpha$-helical spectra with negative peaks at 208 nm and 222 nm (Fig. 4.4A). The AE8K8 peptide hydrogel exhibited a $\beta$-sheet peak at 215 nm as well as an $\alpha$-helix peak at 208 nm (Fig. 4.4B). CD analysis of AE4K12 peptide hydrogels revealed a standard $\beta$-sheet structure with a negative peak at 215 nm (Fig 4.4C).

**Figure 4.4. Circular Dichroism (CD) analysis of the secondary structure of peptide hydrogels.** CD examination of the secondary structure of (A) AE12K4, (B) AE8K8 and (C) AE4K12 peptide hydrogels. (D) Predicted proportions of secondary structures based on wavelength and mean residue ellipticity values taken from CD results.
The particle size and zeta potential of each peptide design was tested by DLS and M3-PALS, respectively, at a concentration of 0.05 mg/mL. The mean particle sizes determined using the Zetasizer ZS were 189.4 nm, 186.3 nm, and 248.3 nm for AE12K4, AE8K8, and AE4K12 unloaded peptide hydrogels, respectively (Fig. 4.5A-C). The zeta potential, an indicator of surface charge and stability, increased with the increasing addition of lysine residues due to the positive charge of the lysine amino acid. The mean zeta potentials were -10.6mV, -4.54mV, and 9.56mV for AE12K4, AE8K8, and AE4K12 peptide hydrogels, respectively (Fig. 4.5D).

**Figure 4.5. Size and zeta potential of peptide hydrogels.** Particle size distribution of (A) AE12K4, (B) AE8K8, and (C) AE4K12 unloaded peptides hydrogels and (D) zeta potential were characterized using dynamic light scattering. Data in table (D) are reported as mean ± standard deviation of peptide hydrogels analyzed in triplicate.
To assess whether TMZ could be loaded into AE4K12, AE8K8, and AE12K4 peptide hydrogels, we determined the amount of TMZ in drug-loaded peptide hydrogels using HPLC. Drug loading ability of each peptide hydrogel composition was examined at multiple concentrations, including 0.05, 0.1, and 0.5 mg/mL. TMZ was dissolved and loaded into each formulation at a final concentration of 200 µM. The drug loading efficiency was calculated for each peptide hydrogel. The maximum drug loading efficiency for each peptide hydrogel was at least 85%, shown in figure 4.6. Based on these findings, we selected the peptide concentration with the highest TMZ loading for each peptide composition for further experimentation.

**Figure 4.6. TMZ drug loading efficiency in peptide hydrogels.** The amount of TMZ loaded for each peptide hydrogel composition was determined at peptide concentrations of 0.05, 0.1, and 0.5 mg/mL.
4.2.5 Release Profiles of TMZ-Loaded Peptide Hydrogels

To examine the effect that peptide composition has on drug release, phenol red, a pH indicator and dye, was loaded into each peptide hydrogel. The color change or absorbance of phenol red from each peptide hydrogel was determined over time. The results in figure 8A showed the AE12K4 peptide exhibited the slowest release, the AE8K8 released faster, and the peptide with the fastest release was the AE4K12 peptide. Phenol red exhibits a color change from yellow to red as pH increases. To confirm the change in pH and rate of degradation, images of each peptide hydrogel containing phenol red were taken (Fig 4.7B). The well containing the AE4K12 peptide hydrogel shows a faster color change or release of the phenol red dye. The AE12K4 and AE8K8 peptide hydrogels start to exhibit a noticeable color change at day 3. This color change confirms the phenol red absorbance measured over time represented in Figure 4.7A.
4.2.6 Effect of TMZ-Loaded Peptide Hydrogels on Glioblastoma Cells in vitro

To evaluate the therapeutic potential of TMZ-loaded peptide hydrogels, cytotoxicity of the drug-loaded hydrogels was determined using LN-18 cells at each peptide composition. Cells were treated with TMZ alone, unloaded peptide hydrogel, or TMZ-loaded peptide hydrogels at a TMZ concentration of 200 µM. The amount of TMZ alone delivered onto cells was based on the highest amount of TMZ loaded into each peptide (Fig 4.8). The percent viability of the LN-18 cells treated with TMZ alone was
69% at the TMZ concentration of 200 µM. The percent viabilities of the LN-18 cells treated with the peptide hydrogels were 72%, 54%, and 44% for AE12K4, AE8K8, and AE4K12, respectively. These results were compared to the cytotoxicity of TMZ delivered alone. The cytotoxicity of TMZ delivered by the AE8K8 and AE4K12 peptide hydrogels were found to be statistically significant compared to delivery of TMZ alone. Overall, these results demonstrate that each peptide design has either similar efficacy or is more effective in delivering TMZ into glioblastoma cells than TMZ alone.

Figure 4.8. Peptide hydrogels enhance the anticancer effect of TMZ in vitro. Quantitative analysis of LN-18 cell viability via MTT assay following 72-hour incubation with TMZ loaded into AE12K4 peptide, AE8K8 peptide, and AE4K12. Data are mean ± SEM of three independent experiments performed in triplicate, where **P<0.01 compared to TMZ treated cells.
CHAPTER 5

DISCUSSION

In this study, we developed a delivery strategy for TMZ that uses amino acids to form a hydrogel that encapsulates the prodrug and converts it into its active form. We demonstrated the efficacy of the delivery system, confirming the potential of the TMZ-loaded peptide hydrogel as a new treatment strategy for glioblastoma, which increases TMZ anticancer activity and may ultimately result in improved therapeutic efficacy. Furthermore, the peptide hydrogel may allow for delivery of smaller dosages of TMZ, which could decrease the toxic side effects of the drug.

Our results demonstrate the pH-dependent efficacy of TMZ in human glioblastoma cells. This is particularly significant because though the pH driven activation of TMZ has been shown, to our knowledge, the pH-dependent efficacy of the drug on cancer cells has not been previously reported. Our results confirm that the anticancer activity of TMZ is significantly greater when the drug is dissolved at basic pH compared to neutral or acidic pH. This result aligns with studies demonstrating that TMZ is only partially activated at neutral pH and has implications on the current clinical strategy for delivery of TMZ. Oral delivery of TMZ relies on the conversion of the drug to its active form at physiological pH once it reaches the bloodstream. The low therapeutic efficacy of TMZ seen clinically in glioblastoma patients may in part be due to the incomplete conversion of TMZ at physiological pH, resulting in limited anticancer activity, as we have demonstrated in vitro. This experiment validated the potential of our peptide hydrogel design including acidic and basic amino acids to improve the efficacy of
TMZ. Acidic amino acids are included in the interior to provide stability since TMZ is stable as a prodrug at acidic pH,\textsuperscript{62} which we also confirmed \textit{in vitro}; TMZ dissolved in an acidic environment was only slightly cytotoxic to glioblastoma cells. Basic amino acids are included on one end of the peptide to mediate the conversion of TMZ to its active form upon release from the hydrogel.

After the peptides were designed and synthesized, we conducted characterization studies to evaluate the size, stability, and secondary structures of each peptide hydrogel. The diameter of all the peptide hydrogels ranged from 150-300 nm. Nanoparticles are ideal for systemic delivery of cancer therapeutics due to their small size,\textsuperscript{63} therefore, the TMZ-loaded peptide hydrogels may have the potential for intravenous delivery. The stability and potential cytotoxicity can be verified by the zeta potential measurements. Values between +30mV to -30mV are considered to be less stable and are more likely to coagulate or gel. The zeta potential of the peptide designs ranged from -10mV to +10mV, indicating more slightly charged or neutral hydrogels. Circular dichroism (CD) spectroscopy was used to understand the secondary structures of the peptides. The AE4K12 peptide hydrogel displayed a typical $\beta$-sheet structure with a negative peak at 215 nm. The AE8K8 peptide hydrogel displayed a $\beta$-sheet peak at 215 nm, but also an $\alpha$-helix peak at 208 nm. AE12K4 peptide hydrogels displayed a typical $\alpha$-helical structure with a negative peak at 208 nm and 222 nm. The formation of $\alpha$-helices and/or random coils are due to the number of repeated patterns of hydrophobic and charged amino acid residues. The presence of lysine amino acids can explain the formation of more $\beta$-sheet structures, which is most likely due to better crosslinking of the peptide. We found it difficult to achieve a high count number when using the zeta sizer. This could be due to the low peptide concentration chosen or the
peptide index of refraction being too similar to water. Further studies using scanning electron microscopy (SEM) need to be conducted to further confirm the sizing of each peptide hydrogel.

Following characterization, we demonstrated that the peptide hydrogels efficiently loaded TMZ, with an average loading efficiency of at least 85%. The AE12K4 peptide hydrogel was found to load the greatest amount of TMZ and also exhibited an increase in loading capacity with increasing peptide concentration. The AE8K8 and AE4K12 peptide hydrogels required higher peptide concentrations than the concentrations tested to increase loading, likely due to smaller hydrophobic regions compared to the AE12K4 peptide. These results demonstrate that hydrophobic drug loading capacity increases with increasing number of alanine amino acids. Future work should include optimizing TMZ loading into each peptide hydrogel, as well as attempting to load other alkylating prodrugs to demonstrate the versatility of the delivery system.

The release profiles of each peptide hydrogel loaded with phenol red dye was then examined. We found that the AE12K4 peptide had the slowest rate of pH change over time, and the release rate did in fact increase with the reduction of alanine amino acids. We hypothesized that including alanine residues in the peptide design would allow for an increase in release rate, due to the hydrophobic nature of alanine. Our results demonstrate the tunability of the peptide hydrogels, or the ability to adjust the rate of drug release from the delivery vehicle.

To determine the efficacy of TMZ-loaded peptide hydrogels, cytotoxicity studies were performed using LN-18 human glioblastoma cells. We found TMZ-loaded AE12K4
peptide hydrogels exhibited anticancer activity similar to delivering TMZ alone. The TMZ-loaded AE8K8 and AE4K12 peptide hydrogels increased cytotoxicity in LN-18 cells by approximately 15% and 25% respectively, compared to TMZ alone. We hypothesized that including lysine resides in the peptide design would enable conversion of TMZ into its active form. Our results demonstrate that the efficacy of TMZ increases with the number of basic amino acid residues included in the peptide design. Efficacy could also be affected by slower release of TMZ from AE12K4 and AE8K8 peptide hydrogels caused by the additional alanine amino acids. Future studies will include the T98G human glioblastoma cell line to evaluate the effectiveness of the TMZ-loaded peptide hydrogel on a more resistant cell line. A TUNEL assay that quantifies the apoptotic activity of cells, may also be attempted for future studies. Due to the mechanism in which TMZ causes cell death, the TUNEL assay may be a more suitable method in confirming the efficacy of the delivery system.
 CHAPTER 6

CONCLUSION

In conclusion, our *in vitro* results confirmed that TMZ is more effective in a basic pH environment, likely due to increased conversion of the drug to its active form, validating the design of our peptide hydrogels. We demonstrated that AE12K4, AE8K8, and AE4K12 peptide hydrogels efficiently load TMZ and their release profiles can be adjusted based on peptide composition or the hydrophobic to hydrophilic amino acid ratio. Further, the AE8K8 and AE4K12 TMZ-loaded peptide hydrogels increased anticancer activity in human glioblastoma cells compared to delivery of TMZ alone. The physical and biological results demonstrate the therapeutic potential of the TMZ-loaded peptide hydrogels for local drug delivery in treating GBM. However, further studies using *in vivo* models of GBM are necessary to further confirm the clinical potential of the TMZ-loaded peptide hydrogels.

Peptide-based drug delivery shows great promise in the treatment of cancer. Drug delivery using peptides can assist in overcoming the blood brain barrier (BBB), and in increasing the conversion of pH-activated prodrugs to their active parent drug. The peptide hydrogels in this study show potential for becoming a new treatment for GBM. With further studies, we aim to optimize this vehicle for delivery of not only TMZ, but other anticancer prodrugs. In doing this, we hope to advance therapeutic strategies using carriers that enhance drug activity and ultimately increase GBM patient survival rates and reduce disease progression.
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