Development and Analysis of A Novel Nanotherapeutic for the Treatment of Glioblastoma Multiforme

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Abstract

The most daunting obstacle in the treatment of Glioblastoma Multiforme (GBM) lies with the impenetrability of the Blood Brain Barrier. This project aimed to design and engineer nanomedicines that have the aptitude to overcome this complication. The method with which these nanomedicines were manufactured, allowed for a variety of chemotherapeutics to be encapsulated. Once the nanomedicines were proven to be stable, cytotoxicity assays were conducted. IC_{50} values of the nanomedicines were statistically lower than their free drug counterpart; however, GBM specific drugs such as tyrosine kinase inhibitors were not as effective as broad-spectrum chemotherapeutics, even when in combination with known pro-apoptotic factors. Additionally, in vitro uptake was observed and various tissue-penetrating peptides were researched and analyzed for future development.

Introduction

Glioblastoma Multiform

Arguably one of the most aggressive forms of cancers, Glioblastoma Multiforme (GBM), has remained obstinate to medical advances. GBM accounts for approximately 54% of diagnosed brain tumors (Agnihotri,S., Burrell, KE., Wolf, A., Jalali, S., Hawkins, C., Rutka, JT., Zadeh, G., 2013). Furthermore, those diagnosed generally have grim prognosis. Like other cancers, there are a great number of challenges that inhibit treatment such as systemic toxicity of high dose cytotoxins and multidrug resistance. Additionally, since GBM tumors grow exclusively in the brain, treatment must cross the Blood Brain Barrier (BBB). This barrier along with other physiological and molecular differences between the brain and the rest of body contributes to the difficulty of treating brain tumors. While investigating this problem, information was compiled to help produce a review paper on a variety of ways to combat this barrier (Saltzman 2001).

GBM is, in fact, the most aggressive form of astrocytoma. In general, astrocytoma is an invasive tumor with limited mitotic structure and abnormal nuclear constitution. Astrocytoma accounts for 76% of brain and related Central Nervous System (CNS) tumors (Agnihotri et al., 2013). It also accounts for about 2% of cancer-related deaths in North America and Europe (Agnihotri et al., 2013). These tumors are classified by the World Health Organization (WHO) as Grade I to Grade IV, depending on severity.

Formally, GBM is a Grade IV astrocytoma. The other grades of astrocytoma are Pilocytic Astrocytoma (Grade I), Low-Grade Diffuse Astrocytoma (LGA, Grade II),

and Grade III Astrocytoma. Pilocytic Astrocytoma is the mildest form and is often benign. The other grades, including GBM, form diffuse tumors of medium density (Agnihotri et al., 2013; Bai, R. Y., Staedtke, V., Riggins, G. J., 2011).

In addition to the grade classification level assigned by the WHO, GBM is split into primary and secondary subtypes. The difference between these subtypes is extreme, generally meaning the difference between life and death. However, the phenotypic features of these tumors are indistinguishable from one another. A key difference between the two, which identifies the particular subtype, is the age of the patient. The average age for primary GBM patients is 64, compared to secondary GBM, which usually occurs in patients under 45 (Agnihotri et al., 2013; Bai et al., 2011). Of diagnosed grade IV astrocytomas, 95% of patients are identified to have primary GBM (Agnihotri et al., 2013; Bai et al., 2011). Additionally, those diagnosed with secondary GBM have a much better prognosis that often leads to remission. Primary tumors present themselves de novo while secondary gliomas form as low-grade astrocytomas and develop into worsening stages. Consequently, secondary GBM tumors display a much more mild progression of GBM and therefore, have a higher survival rate.

Secondary GBM

The specific molecular mutations of secondary GBM are also far less severe than those of the primary subtype. One of the key mutations in secondary GBM involves P53. This protein helps to stop the cell cycle in the event of DNA damage (Agnihotri et al., 2013; Bai et al., 2011; Baldewpersad T., Burgers, N. M., Dawood, Y., den Boon, H. C., den Brok, M. G., Klunder, J. H, … Atai, N.A., 2013). The mutation of

this gene allows the damaged cell to continue to proliferate with the damaged DNA and pass on more harmful mutations. Additionally, the tumor over expresses Platelet Growth Factors to increase the amount of blood vessels to the tumor, supplying more nutrients. Furthermore, these tumors typically have metabolic mutations such as isocitrate dehydrogenase mutations (IDH1) (Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2002; Bai et al., 2011; Baldewpersad Tewarie et al., 2013).

Primary GBM

Primary GBM are much more complicated to classify. Physicians have further quantized primary GBM into four distinct groups: proneural, neural, classical, and mesenchymal (Agnihotri et al., 2013). Similar to the differences between primary and secondary GBM, the disparities among these 4 groups are largely genotypic and are differentiated by the levels of expression in cell signal pathways such as Epidermal Growth Factor Receptor (EGFR) and Platelets Derived Growth Factor Receptor (PDGFR) (Agnihotri et al., 2013; Bai et al., 2011).

These subtypes have few molecular quirks that distinguish one another. To begin, the proneural subtype is generally associated with younger patients, and is very similar to secondary GBM. The main mutation, which associates the proneural subtype with youth, is a mutation in the IDH1. Additionally, this subtype has increased CDK6 and CDK4 expression, which are important for cell cycle regulation (Alberts et al., 2002). Another key mutation is the activation of HIF1a, which is a pathway that helps the cell survive in oxygen poor environments (Agnihotri et al., 2013; Alberts et al., 2002). The neural subtype of primary GBM is still enigmatic and

few molecular differences are identified as of now. These tumors are difficult to excise due to their similarity in appearance to healthy brain tissue. Neural tumors seem to over express EGFR and Human Epidermal Growth Factor Receptor 2 (HER2), two receptors that are responsible for regulating the cell cycle (Agnihotri et al., 2013; Alberts et al., 2002). The classical subtype is the broadest distinction of primary GBM. They generally have EGFR amplification as well as genomic deletion of exon 2-7 (Agnihotri et al., 2013; Alberts et al., 2002). Finally, the mesenchymal subtype is associated with the poorest prognosis. These tumors have lost function of P53, over-express MET (a proto-oncogene), and contain other drastic mutations and deletions to vital cell signaling pathways (Agnihotri et al., 2013; Alberts et al., 2002). Currently, patients are not classified or treated according to their subtype of GBM (Stupp, R., Mason, W. P., van den Bent, M. J., Weller, M., 2005).

Current Treatment

The current treatment for any glioma is extremely limited. If the tumor is on the periphery of the brain, then the tumor may be removed surgically. For most patients, treatment begins and ends with a limited selection of chemotherapy or radiation. Another issue is the gradual multidrug resistance and genotypic mutation that leads to ineffective therapy. Due to these issues, current survival for most patients is only a few months (Agnihotri et al., 2013).

Temozolomide

However, there are clinical trials showing the benefits of combination therapies. One such clinical study, conducted by doctor Roger Stupp et al. in 2005, investigated the benefits of combining Temozolomide (TMZ)(Figure 1), radiation,

and surgery against surgery and radiation alone. Radiation and surgery increased mean survival rate by 10.4% (compared to surgery alone). Adding TMZ to the therapy increased survival rate by 16.1% (up by 26.5% when compared to surgery alone). This study showed that the addition of TMZ leads to an additional year of survival compared to surgery alone. Since the publication of this data, other studies have corroborated the findings, and most patients today receive TMZ as part of their standard therapy (Stupp et al., 2005).

The effectiveness of TMZ lies in its mechanism of attack. It is an alkylating agent that selectively alkylates the base, Guanine, in DNA. The location of alkylation varies, but is most efficient at prompting apoptosis if alkylating occurs at the 6^{th} position (an Oxygen). However, TMZ also methylates the 3^{rd} and 7^{th} positions, (both Nitrogens) inhibiting cell function as well (Newlands, E. S., Stevens, M. F. G., Wedge, S. R. I., Wheelhouse, R. T., Brock, C., 1997).

Molecularly, TMZ is quite a unique molecule. First synthesized in the 70's, TMZ is the fusion of two alkylating agents, MTIC and Mitozolomide (Newlands et al., 1997). TMZ is generally considered as safe, and produces little toxic effects compared to other chemotherapeutics (Stupp et al., 2005). TMZ activation is auto catalyzed in neutral or basic environments and decomposes into CO_2, N_2, a harmless metabolite, and the methyl radical. In acidic environments, the molecule breaks down and does not release the methyl group. Mechanistically, this is ideal for treating gliomas, which are generally more basic than the blood or other cells in the body (Newlands et al., 1997). Currently, TMZ is administered as an oral tablet, but this is not an efficient delivery system as only small fractions of the drug actually

cross the Blood Brain Barrier (Newlands et al., 1997; Stupp et al., 2005). Over time, tumors become resistant to TMZ treatment and consequently, the cell eventually starts over expressing Alkyl-Guanine Transferase (AGT), a suicidal enzyme that transfers the problematic methyl group from the oxygen, reversing the effects of TMZ. The methylated AGT then triggers other expression systems to increase transcription of the AGT gene, further enhancing resistance to TMZ (Newlands et al., 1997).

Tyrosine Kinase Inhibitors

Alkylating agents, such as TMZ, are not the only option for therapy. Many labs have recognized the potential of Tyrosine Kinsase Inhibitors (TKI), and have designed several to target EGFR. Since EGFR is over expressed in the majority of subgroups of GBM (Agnihotri et al., 2013), it becomes an ideal target for broad-spectrum therapy. Specifically, Erlotinib and Gefitinib are two TKI being investigated for front-line therapy (Figure 2). Currently, clinical trials are investigating the potential of Erlotinib to treat GBM, but the effectiveness of Gefitinib is questionable due to its recent performance in stage three clinical trials in which it did not improve the quality of life to a significant degree, and is therefore not currently used for treatment ("Postmarket Drug Safety Information for Patients and Providers - Gefitinib (marketed as Iressa) Information," 2013).

Clinical trials comparing alkylating agents such as TMZ and Carmustine against Erlotinib as a primary therapy option are ongoing. Both groups received radiation followed by surgical resection. The study concluded that the overall survival of the two therapies were comparable with mean survival for the Erlotinib

against TMZ or Carmustine and were 7.7 and 7.3 months, respectively. However, 1-year survival for those who received the alkylating agent was 26.7% compared to 21.9%. Though small, this decrease shows the progression of multidrug resistance of Erlotinib (Van den Bent, M.J, Brandes, A. A., Rampling, R., Kouwenhoven, M.C, Kros, J.M, Carpentier, A.F, … Gorlia, T., 2009). Continual treatment with Erlotinib puts selective pressure on the cells. This creates point mutations within the Tyrosine Kinases, causing extreme decreases in affinity of the drug for the receptors. If one was to observe the 5 year survival rate for TMZ and Erlotinib, this difference would be much more apparent.

The short-term efficacy of TKI lies with their mechanism of action. EGFR is a transmembrane receptor with a ligand binding site on the extracellular matrix of the cell and a tyrosine kinase domain on the inner membrane. Erlotinib and Gefitinib bind to the tyrosine kinase domain and compete with ATP for binding. Without ATP binding, the EGFR signal is not propagated (Bareschino, M. A., Schettino, C., Troiani, T., Martinelli, E., Morgillo, F., Ciardiello F., 2007). The theory is that many cancers become dependant on a particular pathway to express a gene or to maintain growth; therefore, without continuous signals from the pathway, the cell starts its apoptotic process (Figure 3b). This theory is referred to as oncogene addiction (Bronte, G., Rolfo, C., Giovannetti, E., Cicero, G., Pauwels, P., Passiglia, F., … Russo, A., 2013). This is why TKI are supposedly so effective. Since the tumor depends on these pathways, blocking the receptor puts an extraordinary amount of stress on the cell to adapt, and adapt it does. Usually, a single amino acid point mutation kills the inhibitors binding efficiency (Figure 3a). One of the most common mutations is T790M (point

mutation at residue 790 from Threonine to Methionine) (Bronte et al., 2013). Due to the increased steric hindrance, this mutation is quite effective at reducing Erlotinib's binding constant but does not completely blocks the inhibitor. Interestingly, this mutation does not seem to inhibit Gefitinib's binding. This provides for an interesting therapeutic option that can be investigated: the complimentary effects of Erlotinib and Gefitinib even though they both target tyrosine kinases

Currently, only Erlotinib is FDA approved; Gefitinib only just recently lost FDA approval. Though they are not used in first line therapy in any cancer, they are being investigated for use in lung, ovarian and testicular cancers. The liver metabolizes both drugs, with very little affecting the kidneys. Oral tablets appear to be a successful method for delivering these drugs to the cancer in the body, releasing the drug in a controlled manner so the bioavailability is near 100%, but they have proven to be suboptimal for treating GBM. These drugs have a difficult time crossing the BBB and are selectively targeted by ABC transporters and are exported from the BBB. (Faivrea, L., Gomoa, C., Mirb, O., Raieba, F., Schoemann-Thomasa, A., Ropertb, S., … Blancheta, B., 2011).

Nanomedicines

The prognosis for neurological disorders remains poor due to the inability to transport drugs across the highly impermeable BBB. Micelles, liposomes, dendrimers, and nanomedicines are some of the delivery systems currently under investigation as a solution to the problem of the BBB (McCall, R., Cacaccio, J., Wrabel, E., Schwartz, M., Coleman, T., Sirianni, R., 2014). Nanomedicines, in particular, are interesting as they have low permeability at the BBB, but can be functionalized with

a variety of specific targeting ligands to increase their uptake into the brain, such as transferrin, angio peptide, apolioprotein, rabies virus glycoprotein peptide, and phage display peptide (Figure 4a and McCall et al. 2014). These ligands target a variety of receptors such as the transferrin receptor, integrin receptor, low-density lipoprotein receptor, and bradykinin receptor (Pang, Z., Gao, H., Chen, J., Shen, S., Zhang, B., Ren, J., … Mei, H.,2012).

The nanomedicines engineered for this study are comprised of GRAS-grade flaxseed oil, which contains 57% by weight linolenic acid, Omega-3, and 17% by weight linoleic acid, which is an Omega-6. The combination of these two fatty acids helps to stabilize a wide range of small molecules in the lipid core of a therapeutic nanoemulsion. Drugs that are not soluble in water but present a clear therapeutic advantage can be loaded into these vehicles. Additionally, drugs that present a high toxicity to healthy tissue can be loaded into these nanomedicines and delivered safely and effectively to the specific site of the tumor. Additionally, these vesicles also protect sensitive drugs from degradative factors within the body. Drugs within these oil droplets are isolated from destabilizing factors in the surrounding aqueous environment such as oxidizers and acid base interactions. These fatty acid oil droplets are stabilized by a monolayer of phosphatidylcholine. To reduce the size of the particles to the nano-scale, the solution is homogenized via forcing the liquid through a small orifice at a high pressure. To prevent nanomedicine interaction with the immune system and prevent clearance from the Reticular Endothelial System (RES), the nanomedicines are coated in the “stealthing” agent polyethylene glycol (PEG) (Figure 4b).

This PEGylated coat is easily functionalized to have a variety of targeting ligands appropriated to the nanomedicine. Targeted nanomedicines are an effective drug delivery system as they have the potential to slip through some tight junctions and can participate in receptor mediated transport. The targeted therapy also allows a higher drug payload to be delivered to the target within the brain itself.

Nicotinic Acetylcholine Receptor Targeting

The Nicotinic Acetylcholine Receptor (nAChR) presents an advantageous route to cross the BBB. Ligands that bind to these receptors have easy access to the CNS via receptor-mediated trancytosis (Ganta, S., Deshpande, D., Korde, A., Amiji, M., 2010). There are two types of nAChR, neuronal and muscular, and they have different tasks within the body. Neuronal nAChRs play an important role in the synaptic transmissions of autonomic ganglia. There are two different subclasses of the nAChR: neuronal and muscular (Figure 5a). More is known about the muscle types than the neuronal types. The muscle subtype, "mediates all fast synaptic excitation on voluntary muscles" and is responsible for the transmission of nerve impulses given off by motor neurons to muscle fibers (Colquhoun, D., Unwin, N., Shelley, C., Hatton, C., Sivilotti, L.,2003). Every nAChR is an oligomer and is comprised of five subunits encompassing a central pathway of varying diameters for which the ions pass through. The five subunits that compose the muscle receptor in denervated muscle are α_1, α_2, β, γ, and δ, but the γ subunit is replaced by an ε subunit in the adult. Interestingly, the nAChR found in the brain are not evenly distributed. There are distinct areas where a particular receptor composition can be found. The $\alpha 7$ is the only one that is found universally throughout the brain (Figure

5b). The subunits are all similar in their sequences and topology and they have two competitive binding sites (Colquhoun et al., 2003). These sites are located on the α subunits at the interface of the adjacent δ or γ subunit (Osipov, A. V., Rucktooa, P., Kasheverov, I. E., Filkin, S. Y., Starkov, V.G., Andreeva, T. V., … Tsetlin, V. I., 2012) (Figure 5c).

In nature, snakes have evolved to target these receptors to deliver their deadly toxins to the brain to inhibit pan CNS activity. These toxins are classified as Three Finger Toxins (TFT) and are comprised of two major domains, the globular region and the fingers. The fingers consist of three adjacent loops stabilized by four disulfide bridges. Specifically, the second loop interacts with the binding site of the nAChR. The globular domain is a mostly hydrophobic mass that interacts with the membrane around the nAChR. During Loop 2 interaction with the receptor, the globular region aids the channel in opening, thereby allowing ions to pass through (Kini, R., Doley, R., 2010; Lesovoy, D. M., Bocharov, E.V., Lyukmanova, E. N., Kosinsky, Y. A., Shulepko, M. A., Dolgikh, D.A., … Arseniev, A.S., 2009; Osipov et al., 2012). Studies have shown that isolating this second loop, and attaching it to nanomedicines, can lead to better BBB penetration (Zhan, C., Yan, Z., Xie, C., Lu, W., 2010). Many of these TFTs have been isolated from a variety of venomous snakes. Each of these unique toxins display different affinities for nAChR but remarkably there are few variations in the amino acid sequences. This allows for a greater number of possibilities when selecting for possible nanomedicine targeting ligands.

Hypothesis and Specific Aims

By encapsulating active pharmaceutical ingredients (API), this study aims to increase therapeutic payload to GBM. It is our hypothesis that these encapsulated drugs will have a lower IC_{50} value because more of the drug is getting into the cells instead of relying on passive transport. A problem that GBM faces in particular is the BBB and current delivery methods encounter the problem of diluting effects that occurs during the transport of the drug to the tumor. The encapsulation technique used to engineer these nanomedicines prevents this effect by protecting the enclosed drugs from metabolic deactivation and nonspecific drug interactions. Success will be attained if the nanomedicines can be proven to be stable for a given amount of time, at least 7 weeks. Stability is defined as no significant change in size, zeta potential (dictated by the size of the hydration shells), and drug payload during that period of time.

Since short peptide sequences will be used to target nAChR and activate receptor-mediated transcytosis, the nanomedicines should be able to penetrate deep within the brain and reach the entirety of the target, increasing cell death, reducing tumors faster, and preventing relapse. To demonstrate the effectiveness of these peptides, uptake studies will be performed on bEnd.3 mouse brain endothelial cells utilizing immunofluorescence.

Combining multiple drugs that target a variety of pathways will create a superior apoptotic effect than if a single pathway was being targeted, potentially providing no chance for multidrug resistance to develop due to the controlled delivery of a highly toxic payload. By first identifying IC_{50} values for lone drugs,

molar ratios will be calculated for optimal combinational therapies at minimal toxicity levels.

Materials and Methods

General Manufacturing Nanomedicines

Nanomedicines are comprised of two separate phases: an aqueous phase and an oil phase. The active pharmaceutical ingredient (API) was loaded into the oil phase. The APIs selected for this project included Gefitinib, Erlotinib, and modified Temozolomide (TMZ). Docetaxel, Nigericin and Cisplatin were also tested for a comparison of IC_{50} values. Meanwhile, Polyethylene Glycol (PEG), a stealthing agent and an easily modified molecule, and Egg Lecithin, an emulsifying agent, were added to the aqueous phase along with glycerol, which is needed to maintain isotonicity of the final formulations. Once the phases are prepared, the two were combined and vortexed to create a crude emulsion, which was then processed via LV1 Microfluidizer. The final concentration of drug was usually around 2-4 mM. The size and surface charge of the nanomedicines were read on the Malvern SV90 and then stored at 4°C.

For use in uptake studies, the nanomedicines were loaded with the fluorescent red dye, rhodamine. Two different oils were used: flaxseed oil and safflower oil. These oils were chosen based on their vastly different concentrations of Omega-3 and Omega-6 fatty acids. In addition, two different emulsions were engineered, one with 0% and one with 0.3% PEG. Rhodamine loads in the oil core, and processing was handled in the same was as for drugs.

HPLC Method of Detecting API

Concentrations of the APIs were quantized via High Performance Liquid Chromatography (HPLC) (Malvern 1525 Binary HPLC Pump, Hypersil gold C18 5 µM, Size – 150x4.6 mm Thermo Scientific, Malvern 2487 Dual Absorbance Detector UV detector). The flow rate was always set to 1 ml/min. Standard curves were established at the beginning of each assay. In between samples, the system was washed with 100% Acetonitrile (ACN). In between runs with different drugs, or after a prolonged period of inactivity, the pumps and UV detector were purged with 5M nitric acid.

For modified TMZ, the mobile phase was comprised of 90% ACN with 0.1% trifloroacetic acid and 10% Water with 0.1% trifloroacetic acid. The UV detector was set to 220 nm. The retention time of the lipidated TMZ was approximately 4 minutes and the limit of detection and limit of quantification was <1 µg/ml and 3 µg/ml, respectively.

For Erlotinib and Gefitinib, the mobile phase was comprised of 60% ACN and 40% Water. The UV detector was set to 338 nm. The retention time of Erlotinib was approximately 3 minutes. The limit of detection and limit of quantification of Erlotinib were 0.1 µg/ml and 0.3 µg/ml, respectively. The retention time of Gefitinib was approximately 2-3 minutes. The limit of detection and limit of quantification of Gefitinib were 0.2 µg/ml and 0.3 µg/ml, respectively.

The remaining drugs that were used in this study were engineered and assessed by the Principal Investigators of the lab.

Quantification of API in Nanomedicines

Nanomedicine samples were diluted 1,000 fold in ACN and then vortexed for 2 minutes. Supernatant was then analyzed on HPLC using the method above.

Normalization of Fluorescence

The concentration of rhodamine was not quantized. Instead, the amount of fluorescence was measured and then normalized to the lowest sample. Samples were loaded, in triplicate, into a 96-well plate. The emulsions were serially diluted in water. The last row of the plate contained only water and was used as a negative control. Plates were loaded into a BioTex Synergy HT plate reader and shook for 20 seconds. The plate was excited at 530± 14.5 nm. Fluorescence was read at 590 ± 17.5 nm. The fluorescence level was plotted, and a linear range was found. The emulsion with the lowest fluorescence level was used to normalize the remaining samples.

Stability of Nanomedicines in Diluting Fluids

Nanomedicine samples were diluted 90% v/v with 0.9% sodium chloride solution, 5% dextrose, phosphate buffered saline (PBS, pH 7.4), distilled water, and fresh dog plasma. Following dilutions, samples were stored at 37°C in 5% CO_2. 10 µl of nanomedicines were taken for analysis after 0.25, 0.5, 1, 2, 4, 6, and 24 hours. Aliquots were diluted 1,000-fold with distilled water and analyzed using the Malvern SV90.

Cell Lines

The human glioma cell lines, U118 MG and U87 MG were obtained from the ATCC, and the human brain endothelial cell line, bEnd.3, was obtained from Dr. Rachel Sirianni at the Barrow Neurological Institute. They were continuously

cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin, in a humidified incubator at 37°C with 5% CO_2. All cell culture media and sterile equipment was purchased from Fisher Scientific unless otherwise stated.

Cytotoxicity Assay

Cell viability was quantitated and IC_{50} values were generated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Both U87 MG and U118 MG cell lines were seeded into 96-well plates at a density of 3 X 10^3 cells/well in normal growth media and allowed 24 hours incubation for proper attachment. Following incubation, media was removed and replaced with proper dosing of drug. DMEM was used as a negative control (0% cell death) and poly(ethyleneimine), was used as a positive control (100% cell death) and was prepared at a concentration of 0.25 mg/ml. After 72 hours of incubation, the MTT assay was performed following the accepted standard protocol. The absorbance was measured at 570 nm using the plate reader from above.

bEnd.3 Uptake Assay

bEnd.3 cells were seeded into 6-well plates containing sterilized cover slips at varying densities. The assay was conducted once cells reached approximately 70% confluency and had adhered to the coverslip. To begin, cells were washed with complete DMEM. Media was replaced with 1 ml of correct dilution of rhodamine florescence-tagged nanomedicines and incubated for the desired length of time. After incubation, cells were sufficiently washed with 1X PBS and 4% formalin was added to fix the cells. Cells were incubated for 25 minutes before SlowFade Gold

antifade reagent with DAPI (Life Technologies) was placed on a glass slide and cover slips were inverted over it. Slides were then left in the dark for 30 minutes at which point cells were viewed under Leica DMI3000 B florescence microscope.

Two different uptake experiments were conducted in this manner. The first was to determine which created the best dilution. For this experiment only one time point was used but with 3 different dilutions: 1:750, 1:1,000, and 1:1,500. The second experiment used the dilution of 1:750, and two time points, 15 minutes and 30 minutes. Additionally, four different nanoemulsions were used: 2 containing a flaxseed oil core and two with a safflower oil core. For each set of oils, one nanoemulsion was conjugated with 0.3% w/v PEG and the other contained no PEG. When the pictures were taken, the background fluorescence on the negative control was reduced to zero before the time points and different nanoemulsions were taken.

Results

Designing and Manufacturing Nanomedicines

The aim of this study was to engineer and characterize nanomedicines containing current chemotherapeutics. As a first step toward meeting this goal, nanomedicines were engineered using Microfluidics High Pressure Homogonizer LV1. This machine forces a crude emulsion, which is a mixture of an aqueous phase and an oil phase, through tiny porous membranes at high pressure creating pockets of low pressure surrounded by pockets of high pressure. The separate phases were forced through the machine repeatedly, which refined the size of the particles down to the nano-scale. The oil core is where the active pharmaceutical agent(s) (API) are stored. Each cycle through the machine reduces the size of the particles and

increases the uniformity of the colloidal suspension. After the particles have gone through the requisite number of cycles, the size and the zeta potential was measured on the Malvern SV90, which uses Dynamic Light Scattering Technique to calculate the average size and distribution of the particles.

Two different nanomedicines were engineered, one encapsulating Gefitinib, and one encapsulating Erlotinib. The average size of the Gefitinib-containing nanomedicine was 179.6±7.1 nm, and the average size of the Erlotinib-containing nanomedicine was 168.8±6.7nm. The zeta potential for the Erlotinib and Gefitinib-containing nanomedicines was -18±6 mV. The Principal Investigator, Dr. Srinivas Ganta, engineered a third nanomedicine containing modified TMZ.

In addition to checking the size of the particles, the solution was assayed to confirm the concentration of the drug loaded using a Waters HPLC Pump and UV detector. The mobile phase of both drugs was set to 60% Acetonitrile and 40% water, with a flow rate of 1 mL/min. A standard curve was constructed with each drug dissolved in Acetonitrile. The nanomedicines were diluted 1:1000 in Acetonitrile and vortexed for approximately 2 minutes in order to lyse the particles and free the APIs. Following this lysis, the concentrations of each drug was measured to be 0.87 mg/ml or 2.21 mM and 3.2 mM for Erlotinib and Gefitinib respectively.

Finally, after engineering the florescent particles, size and zeta potential was measured to ensure GMP. For the four nanomedicines (0.3% PEG in flaxseed oil, 0% PEG in flaxseed oil, 0.3% PEG with safflower oil, and 0% PEG with safflower oil) the sizes were 165.1 nm, 151.8 nm, 164.1 nm, and 198.6 nm respectively. The PDI for

the nanomedicines were 0.045, 0.091, 0.057, and 0.013 respectively. The zeta potential for these particles (in the same order) were -44.2 ± 9.32 mV, -37.1 ± 6.16 mV, -40.3 ± 8.8 mV, and -38.1 ± 11.8 mV respectively.

Prolonged Stability of Nanomedicines Containing Erlotinib and Gefitinib

After the particles were manufactured, the stability was monitored to ensure the integrity of the molecules for an extended period of time. The stability of the nanomedicines is dependent on a variety of factors, including the chemical properties of the drugs loaded into the particle. The hydrophobicity, aromaticity, and solubility also contribute to the stability of the particle. A common problem with these particles is drug precipitation or instability of size. The solution was stored at 32°C and for one month Gefitinib and Erlotinib nanomedicines were visually inspected and tested for size and zeta potential stability. Figure 6a displays the size stability of both Gefitinib and Erlotinib; neither showed a distinct increase of size. Figure 6b shows the Polydispersity Index (PDI) stability of the particles. The PDI display is a numeric representation of the dispersion of sizes, therefore the higher the PDI, the more variety in particle size. Similarly, there was no significant variance in PDI.

Diluted Nanomedicine Stability in Water

An additional stability factor is whether the nanomedicines retain their size when diluted and stored at a higher temperature. To model this, nanomedicines were diluted 90% v/v with water and stored at 37°C and 5% CO_2. The size of the nanomedicines was verified at determined time points. Figure 7a displays the changes in size for Erlotinib and modified TMZ, while Figure 7b displays the change

in their PDI. These results indicate stability, which is important for a variety of biological activities as well as for the efficacy of our nanomedicines.

Cytotoxicity Assay

A major component of this MQP's goal was to measure the efficiency with which these drugs are delivered. The industry standard for measuring the efficacy of drugs is the MTT assay, which measures cell survival. An IC_{50} value (the concentration of drug needed to kill 50% of a given population) can be extrapolated from the analysis. MTT assays were performed in order to determine IC_{50} values on both GBM cell lines, U87 and U118. The IC_{50} values and 95% Confidence Intervals of single drug dosing experiments were calculated using GraphPad Prism (Table 1a, b, and d). The IC_{50} curves for these values can be found in Figure 8a-f and Figure 9a-f. Co-therapeutic drug experiments were also performed and using the same program, the IC_{50} and 95% Confidence Interval values were calculated (Table 1c) and the IC_{50} curves can be found in Figure 10a-f

Some of the therapeutics and co-therapeutics were not able to successfully kill 50% of the GBM cell population: AZD in DMSO, AZD NE Gefitinib/AZD NE, Erlotinib/AZD in DMSO on U87, and lipidated TMZ in DMSO, XL in DMSO on U118. Since these experiments did not converge to 50% cell viability, their IC_{50} values are determined to be >100,000 nM for the single therapeutics and >10,000 nM for the co-therapeutics.

bEnd.3 Uptake Assay

In order to determine the native capability of the nanoemulsions to penetrate the BBB, an in vitro uptake assay was developed using bEnd.3 mouse brain

endothelial cells. These cells were treated with a variety of dilutions of nanomedicines and incubated for determined time points in order to asses the most practical dilution to use. After analyzing the pictures for the varying dilutions, it was determined that 1:750 produced the best results. When the slides of the different nanoemulsions at different time points were examined, the background was reduced so a clear picture of the uptake could be observed. For the subsequent pictures, the exposure, gain, and gamma for the blue and red filters were not altered. For the blue filter, the exposure was set at 6.52 ms, the gain was set at 2.0x, and the gamma was set to 0.97. For the red filter, the exposure was set to 218.5 ms, the gain was set at 2.0x, and the gamma was set to 0.99. Each time point for the nanomedicines was performed in duplicate and each slide was photographed twice. By comparing the nanoemulsions among time points, it is clear that there is an increase in uptake over time (Figure 11b-e). This is more pronounced when comparing the particles without PEG at the two time points (Figure 11c-e). Quantitatively, particles without PEG accumulated more fluorescence than those with PEG. However, the particles without PEG had a higher density in the lipid membrane of the cell and accumulated in clusters rather than penetrating to the cytoplasm and nucleus as the particles with PEG were able to do. Finally, there was a distinct difference between particles with a safflower oil core versus a flaxseed oil core (Figure 11b & c vs. d & e). The particles with a safflower oil core had a higher fluorescence than those with flaxseed.

Targeting Nanomedicines, Review and Design

A review paper summarizing Nicotinic Acetylcholine Receptors (nAChR) and the varied ligands associated with them was written in conjunction with this MQP (McCall et al. 2014). The paper was a joint collaboration among Nemucore Medical Innovations, Blue Ocean Biomanufacturing, Barrow Brain Tumor Research Center, Center for Transitional Cancer Nanomedicine at Northeastern University, and Foundation for the Advancement of Personalized Medicine Manufacturing. NMI's focus in the paper was the Three Finger Toxins (TFTs) found in snake venom, which associates with nAChR (Figure 12). The research performed for this paper led to the conclusion that short sections of toxins found in nature can be utilized for targeting, a concept which is referred to as biomimicry. By utilizing these short sequences, specific uptake of the nanomedicines will take place, enhancing delivery to the tumor and increasing survival. This review identified the major components of the TFTs specifically found on loop 2, (which is relevant to binding), the mechanism in which the nanomedicines are taken up, and relevant sequences that can be conjugated to the surface of a nanomedicine. In addition, the concept of biomimicry was applied to conceptualize other ligand-receptor pairs that can be used to add specificity to the nanomedicines.

This began with investigating other receptors found in the brain. Specifically, receptors were identified which were known to be either over-expressed in GBM or vital to the survival of the tumor. Several targets were identified and investigated as potential ligands for our nanomedicines. The research was presented to the collaborating labs via phone conference with representatives from TGEN, and the

Barrows Institute. Three of the various targets presented were chosen to be synthesized. This entailed designing linker sequences and identifying the binding sites on each peptide. Our nanomedicines were conjugated with these ligands and were sent for testing at the Barrows Institute. Unfortunately, these studies were not performed in time before the completion of this MQP.

Discussion

Since its discovery in the late 1800's, Glioblastoma Multiforme (GBM) has been one of the most prevalent forms of brain tumors observed by the medical community. The current 5-year survival rate for GBM is approximately 10% (Glioblastoma, *American Brain Tumor Association*). Unfortunately, the current standard of care does little to improve the quality of life of patients and is a regimen of radiotherapy and continual dosing of Temozolomide. Even though there have been several clinical studies to increase the efficacy of TMZ, the best option only increases mean survival rate by three months (Agnihotri et al. 2013). The initial difficulty with treating GBM is the Blood Brain Barrier (BBB). The BBB is comprised of endothelial cells that form a much tighter barrier than any other blood-tissue barrier in the body. This prevents passive transport through the tight junctions into the brain. Therefore, this physical barrier is one of the biggest challenges faced for treatment of GBM.

The use of nanomedicines to deliver targeted therapy has been innovating the field of chemotherapeutics. Encapsulating active pharmaceutical ingredients (APIs) in a nanomedicine, has the potential to eliminate many of the problems facing

current treatment; this includes conjugation with targeting ligands to bypass the BBB more effectively and deliver a wide range of therapeutics previously thought to be unusable due to their chemical properties or high toxicity. It is the aspiration of this MQP to help improve the quality of Glioblastoma therapy by utilizing nanomedicines loaded with a variety of drugs. The goal is to surpass the gold standard based on the current efficacy of TMZ by comparing it with the effectiveness of the engineered nanomedicines.

The first major problem arose when trying to load TMZ into the nanomedicines. The partition coefficient predicted that TMZ should have been able to be solubilized in the oils, but the specific chemical structure destabilized the particles, causing the drug to crash out of solution. Therefore, a structure-function relationship was developed. A modification for TMZ was suggested: an addition of a lipid tail to the circled Nitrogen residue shown in Figure 1a. The modified TMZ was then able to be loaded into the nanomedicines and no further issues were observed. It was concluded that the method of manufacturing was sufficient in producing adequately stable nanoemulsions that could be stored for an excess of more than one month.

In order to prove that the nanomedicines increased the efficacy of the drugs, a significant drop in the IC_{50} values was expected when compared to the free drug in solution. The IC_{50} value of Gefitinib was reduced 100 fold in U87 cells and 150 fold in U118 cells. Contrary to this, Erlotinib and modified TMZ did not show a significant decrease in IC_{50} values for U87 cells but showed a 6-fold and 50-fold decrease in U118 cells, respectively. This is particularly interesting, because

Gefitinib an Erlotinib have similar mechanisms with which they promote apoptosis. Both of these APIs are EGFR Tyrosine Kinase Inhibitors and as such have a detrimental effect on cell signaling. Gefitinib is not a current GBM treatment and these results raise the question of why? In the two GBM cells lines tested in this study, Gefitinib was the superior drug compared to Erlotinib. It is hypothesized that the specificity of Erlotinib is an obstacle because a single point mutation can drastically reduce the potency. Meanwhile the sterics of Gefitinib hinder complete interaction with the active site of a tyrosine kinase, therefore acquiring resistance is more difficult.

Combination therapies were also investigated with co-therapeutics, XL147, a Pan-P13K inhibitor, AZD8055, an mTORC inhibitor, and BEZ235, a P13K and mTORC duel inhibitor, to increase the efficacy of Erlotinib and Gefitinib. We proved that none of these co-therapeutics cause apoptosis on their own (Table 1b). However, because the pathways they target are extremely different than those targeted by Gefitinib and Erlotinib, a combination therapy seemed a viable option. This assay showed an increase in the IC_{50} values from DMSO to nanoemulsions (Table 1c), which is contrary to the hypothesis that inhibiting two different pathways would lead to an enhancement of pro-apoptotic factors. Instead, the inhibition of these two signaling pathways proved to be antagonistic of one another.

Chemotherapeutics that are used to treat other forms of cancer, such as ovarian and breast cancers were investigated. These drugs are not typically used to treat brain tumors, due to the impenetrability of the BBB. Since our nanomedicines theoretically nullify this problem, it was therefore feasible to test them against GBM.

The drugs selected were a Platinum derivative, Docetaxel, Nigericin, and Ceremide. This proved advantageous as three of the nanoemulsions (Platinum, Docetaxel, and Nigericin) had IC_{50} values in the nanomolar range, and Ceremide had an IC_{50} value in the low micromolar range (Table 1d). Their effectiveness can be attributed to their universality. The targets of these chemotherapeutics are those pathways that are vital for all cell function and are not specific to over or under expressed receptors. None of these drugs depend on the presence or absence of a particular pathway, but rather target vital cellular functions necessary for cell survival.

The uptake study showed the native capability of our nanomedicines to cross the BBB. This procedure still needs to be optimized, although the pictures acquired were able to give us conclusive results. One conclusion that was drawn was nanomedicines with a safflower oil core appeared to have better uptake kinetics than those with flaxseed oil cores. This can be attributed to the higher percentage of Omega-6 fatty acids. Specifically, the concentration of linoleic acid differs between the two oils, ranging from 70% w/v in safflower to 17% w/v in flaxseed. It was also concluded that the presence of PEG drastically changed the uptake kinetics. Nanomedicines with 0% PEG are able to fuse directly with the cell membrane but run the risk of not passing through to the cytoplasm. Additionally, the particles may lose their integrity and not pass through at all, dispersing the contents into the cell membrane instead of into the cytoplasm as desired.

Although these results might suggest that PEGylation is detrimental to cellular uptake, it is necessary to help the nanomedicines evade the immune system in vivo. Additionally, PEG can be easily functionalized with a variety of targeting

ligands to increase uptake through the BBB or even bypass it completely. One strategy investigated was targeting the nicotinic acetylcholine receptor (nAChR). The review paper focused on the Three Finger Toxins (TFT), specifically those related to Toxin B found in the venom of King Cobras. A blast was conducted on the protein sequence from Loop 2 on this toxin using the RCSB Protein Data Bank website. The literature describing these topics as well as their affinity to the nAChR receptor was reviewed in depth and jumpstarted the search for additional targets. One proposed goal would be to combine multiple targeting ligands on the surface of a nanomedicine to greatly increase the potential to cross the BBB.

An example of such a receptor is the mu opioid receptor (MOR), which is over-expressed in the brain (Gail E. et al. 1990). This is a promising target due to the low toxicity potential, as the MOR is not expressed in high concentrations throughout the body. Interestingly, there are few molecules, peptides, or small compounds, which interact exclusively with the MOR. Many of the small molecules, like Naloxone, are opioids without the pharamcophore. This would result in an interesting ligand for targeting. However, this study is only investigating peptides as potential ligands.

Another target investigated was the integrin and neuropilin system. These two receptors work synergistically to promote capillary growth in fetuses and new tissue and are vital to tumor growth. Many labs have already explored the possibility of using these systems, and have conjugated these peptides to nanomedicines (Zhan C. 2012, Saenz L. 2013, Sugahara K, 2009). These peptides have similar domains called the C End motif, and contain the amino acids Arginine,

Glycine, and Aspartic Acid (RGD). The C End motif first targets the integrin receptors and crosses the BBB. After crossing the barrier, the motif is enzymatically cleaved and gains affinity for the neuropilin receptor. This allows for deep penetration into the brain (Sugahara, K. N., et al. 2009).

Once these targeting moieties have been successfully conjugated to the surface of the nanomedicines, a more in depth study utilizing a mouse model will provide more conclusive results as to confirm or reject one of our original hypotheses that a nanomedicine could be functionalized to target GBM and cross the BBB. Additional studies exploring other combinational studies could result in a more successful therapy. The discovery of two drugs that can work in synergy with one another could lead to a therapy that is more potent but less toxic. Our results suggest that combination therapies between Platinum and Gefitinib, Docetaxal and modified TMZ, and Nigericin and modified TMZ would prove fruitful in this endeavor. These pairings are suggested due to different mechanisms that could synergistically promote apoptotic signals. Ideally, the drugs should target vastly different pathways in order to maximize their effects; for example, the combination of Platinum and modified TMZ would be ill advised because they both damage DNA. Finally, the uptake study designed for this MQP, must be optimized in order to produce more visually conclusive pictures. The pictures produced using this method are currently extremely dark and difficult to analyze. Two adjustments to the protocol would be to seed the cells at a higher density or to incubate the cells in non-complete media (lacking FBS or antibiotics). A higher density of cells would decrease the amount of empty space on the slide, giving a more complete picture of

uptake. It is hypothesized that the FBS in the incubation media is interacting with the nanomedicines during the experiment, hindering the uptake.

In conclusion, the development and characterization of these novel chemotherapeutics display the potential that nanomedicines have to further advance the treatment of Glioblastoma Multiform. Further experimentation is needed to confirm the best APIs to treat this disease without prompting multidrug resistance and severe cytotoxic side effects. The final step in this process is to synthesize targeting agents that are best able to cross the Blood Brain Barrier.

Tables and Figures

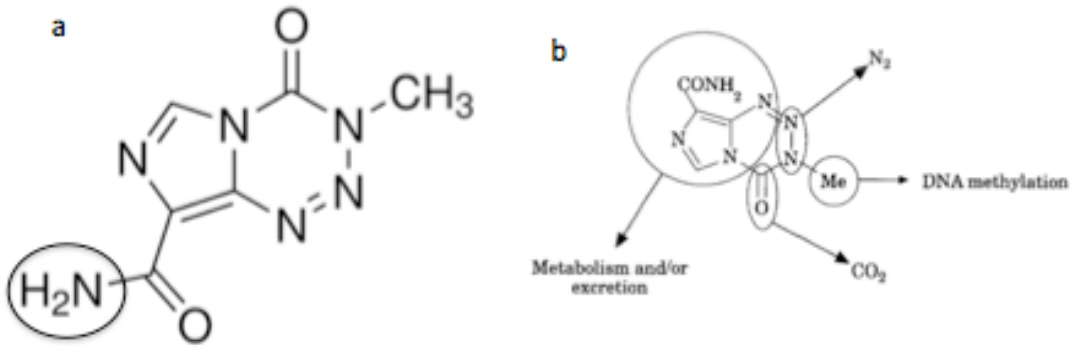
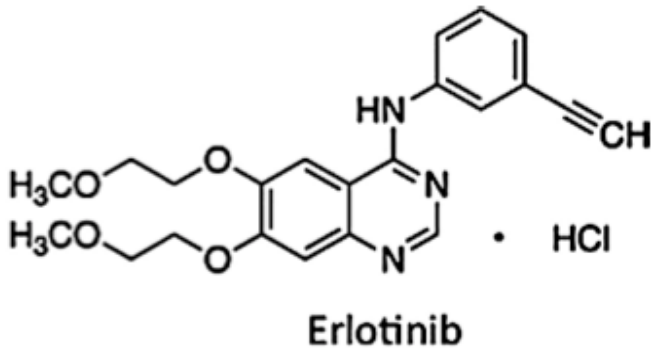


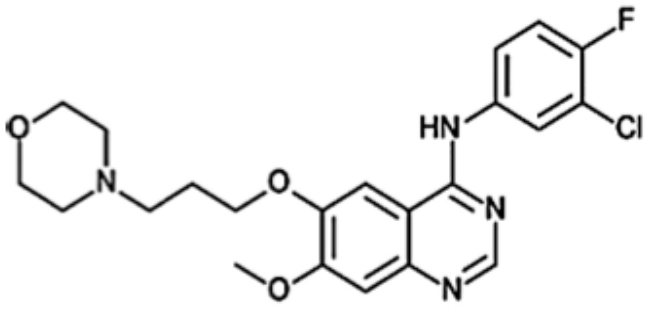
Figure 1: Molecular Structure of Temezolomide (TMZ)

TMZ (a) is an alkylating agent used to treat Glioblastoma Multiforme. TMZ is actually a prodrug, which is base catalysed to form AIC and Diazomethane. The Diazomethane then reacts with DNA to add a methyl group(b).

Source: Newlands et al. 1997



Erlotinib



Gefitinib

Figure 2: Structures of Two Tyrosine Kinase Inhibitors, Erlotinib and Gefitinib

Both Erlotinib and Gefitinib inhibit an EGFR Tyrosine Kinase Receptor on the cell membrane. Erlotinib is capable of more specific binding compared to Gefitinib and is currently approved by the FDA.

Source: Bronte et al. 2013

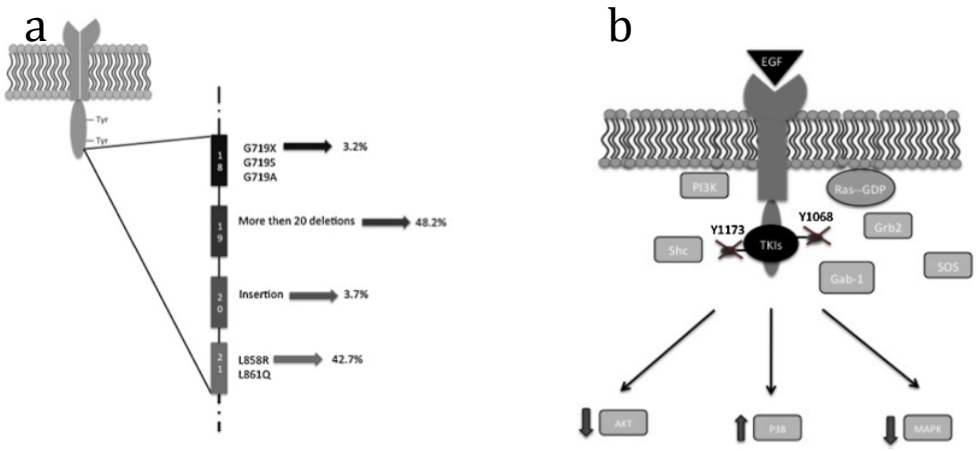


Figure 3: EGFR Tyrosine Kinase and Frequent Mutations

The EGFR Tyrosine Kinase Receptor sits on the cell membrane and is over expressed in many cancers. The problem with targeting this receptor is the frequent mutations, which occurs on the cytoplasmic side (a). Generally, single mutations completely change inhibitor-binding affinities. These inhibitors bind to the cytoplasmic side of the receptor and inhibit the phosphorylation cascade, which is intended to propagate the signal. This inhibition leads to an increase of expression of apoptosis factors (b).

Source: Bronte et al. 2013

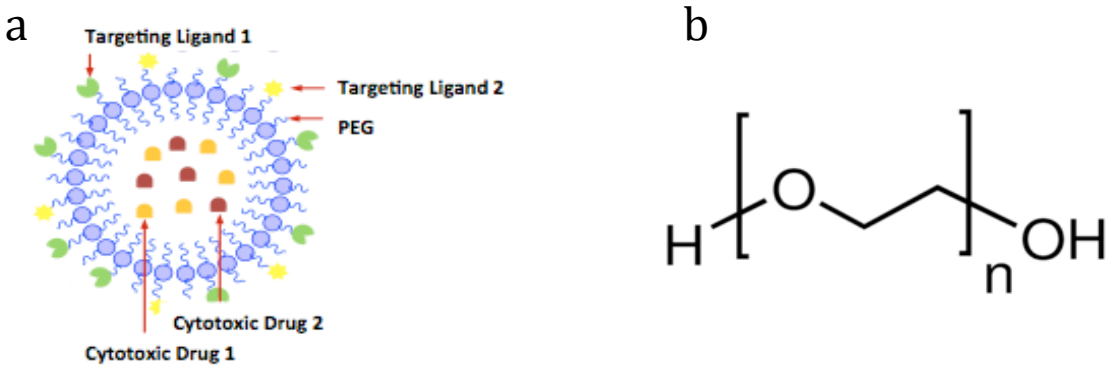


Figure 4: Diagram of Generic Nanomedicine

Nanomedicines are highly customizable drug delivery vehicles (a). Different delivery kinetics can be created by modifying the targeting ligands identity, targeting ligand density, targeting ligand combinations as well as modifying the oil core composition or the length and density of PEG (b). PEG is vital to nanomedicine engineering as it aids in evading the immune system. Additionally, utilizing the terminal hydroxyl group as a nucleophile allows for easy functionalization.

Source (b): Sigma- Aldrich, http://www.sigmaaldrich.com/catalog/product/sial/p3015?lang=en&region=US

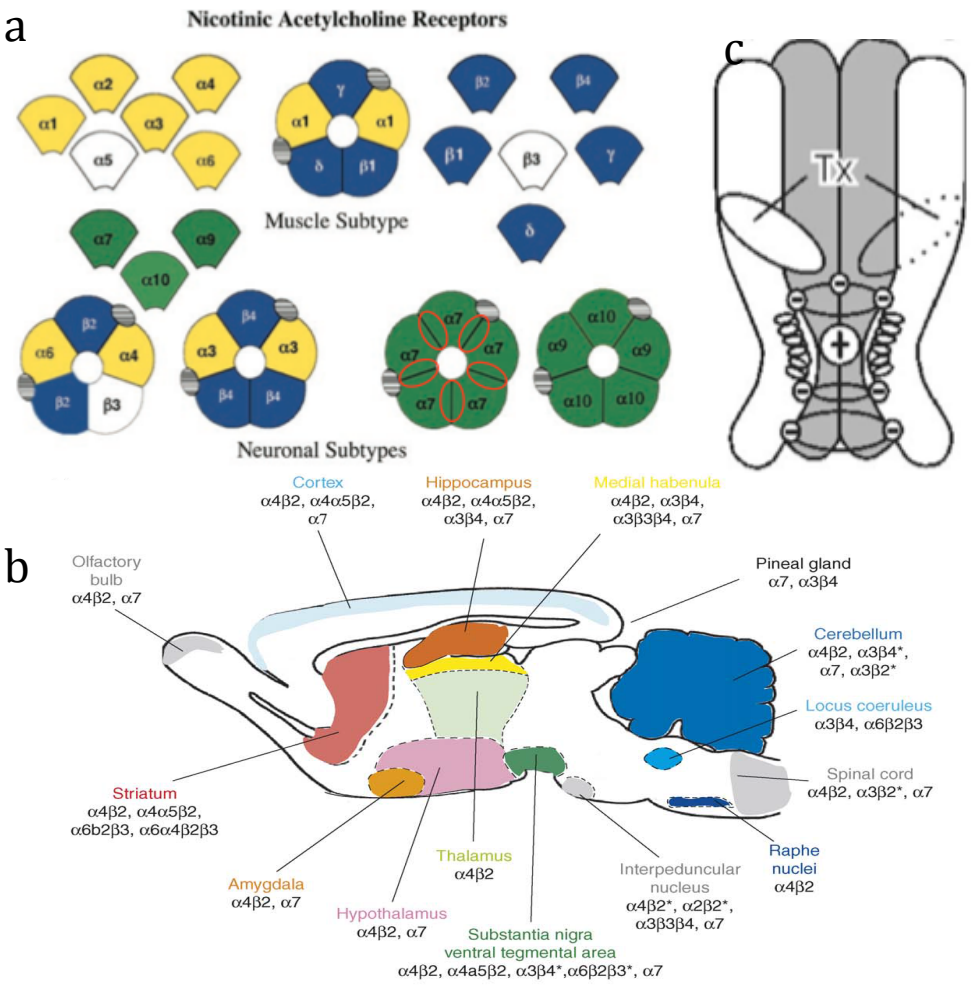


Figure 5: Nicotinic Acetyl Choline Receptor, The Gateway to The Brain

The Nicotinic Acetyl Choline Receptor (nAChR) is expressed throughout the body in two different forms (a). The nAChR is a transmembrane ion gate that allows for the passage of Ca^{2+} ions for signaling purposes. The muscular subtype is found at neuronal-muscular junctions and is comprised of a wide variety of subunits. The neuronal subtype is expressed throughout the brain and the spinal column. The nAChR structure is highly dependent on what part of the brain it is found (b). However, the α7 subunit is uniformly expressed throughout the brain. Interactions between agonists and receptors occur at the α interface.

Source:

a: Olivera, B.M., et al. 2008

b: Gotti, C. et al. 2006

c. Krabben, L., et al. 2004

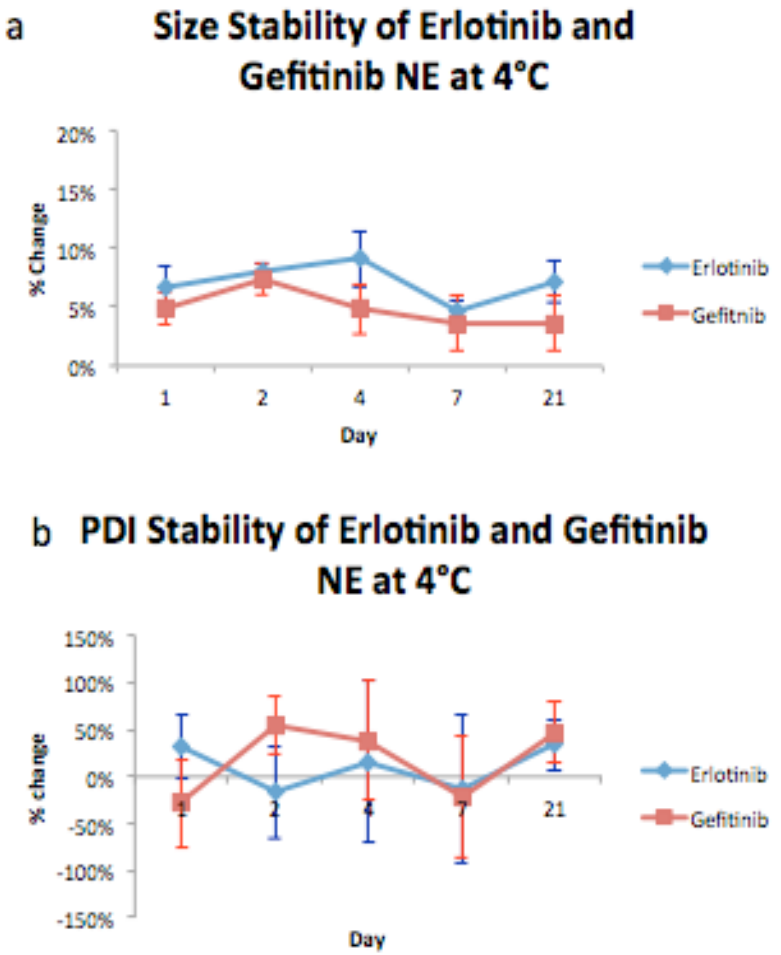


Figure 6 Stability of Erlotinib and Gefitinib Nanomedicines

Each day the size and zeta potential of the nanomedicines were checked with the Malvern SV90. Each reading was performed in triplicate. (a) Size stability in percentage change over time for both Gefitinib and Erlotinib. (b) PDI stability in percentage change over time for both Gefitinib and Erlotinib. There was no statistical difference in nanomedicine size or PDI.

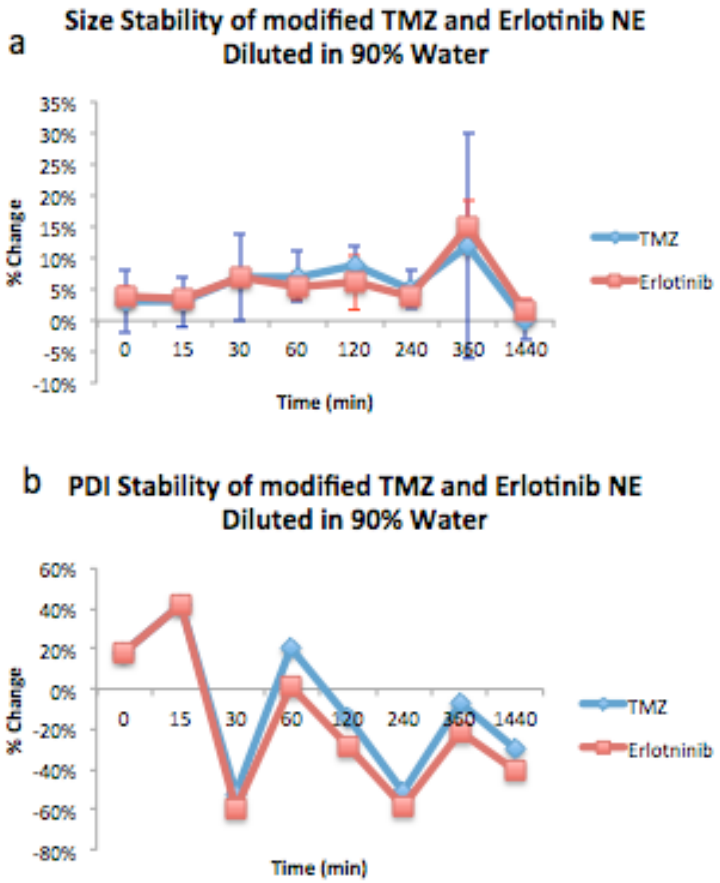


Figure 7: Size and PDI Stability of Two Nanomedicines When Diluted 90% in Water

Erlotinib and modified TMZ nanomedicines were diluted 90% in water and then incubated at 37°C. At specific time points, the samples were placed in the Zetasizer to determine the change of size diameter (a) and PDI (b).

a

Drugs	Cell Lines			
	U87		U118	
	IC50 (nM)	95% CI (nM)	IC50 (nM)	95% CI (nM)
Gefitinib in DMSO	> 100,000	404 – 3.326 e+8	> 100,000	20,150 – 6.673 e+5
Gefitinib NE	3,853	2,223 – 6,680	741.5	450.5 – 1,221
Erlotinib in DMSO	4,724	1,541 – 14,480	6,436	2,856 – 14,500
Erlotinib NE	5,814	3,349 – 10,090	1,051	770.6 – 1,433
TMZ in DMSO	6,639	24.16 – 1.824 e+6	> 100,000	Not Converged
TMZ NE	9,873	3,707 – 26,290	2,103	911.1 – 4,856

b

Drugs	Cell Lines			
	U87		U118	
	IC50 (nM)	95% CI (nM)	IC50 (nM)	95% CI (nM)
AZD in DMSO	> 100,000	Not Converged	640	~0 – 926.3
AZD NE	> 100,000	Not Converged	13,120	7,663 – 22,450
BEZ in DMSO	5,628	2,104 – 15,050	2,794	1,374 – 5,683
BEZ NE	5.616	2.726 – 11.57	6.818	4.281 – 10.858
XL in DMSO	> 100,000	38,430 – 7.85 e+5	> 100,000	Not Converged

c

Drugs	Cell Lines			
	U87		U118	
	IC50 (nM)	95% CI (nM)	IC50 (nM)	95% CI (nM)
Gefitinib and AZD in DMSO	2,209	434.1 – 11,240	506.0	62.25 – 4,112
Gefitinib and AZD NE	> 10,000	Not Converged	2,087	1,303 – 3,341
Gefitinib and BEZ in DMSO	11.73	7.087 – 19.40	11.09	4.994 – 24.60
Gefitinib and BEZ NE	1,034	537.3 – 1,992	213.8	159.6 – 286.3
Erlotinib and AZD in DMSO	> 10,000	Not Converged	64, 690	11.83 – 3.537 e+8
Erlotinib and AZD NE	1,961	1,280 – 3,004	679.2	385.9 – 1,196

d

Drugs	Cell Lines			
	U87		U118	
	IC50 (nM)	95% CI (nM)	IC50 (nM)	95% CI (nM)
Platinum NE	421.8	245.1 – 726	361.0	224.7 – 579.9
Docetaxel NE	5.505	3.524 – 8.599	8.239	5.220 – 13.004
Nigericin NE	132.9	78.15 – 225.9	61.92	37.91 – 101.14
Ceramide NE	4,377	2,550 – 7,514	1,735	1,180 – 2,550

Table 1: IC_{50} Values of Various Cytotoxic Drugs

Potency of single therapeutic drugs were tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to measure the level of respiration of a colony of cells growing in a 96 well plate. (a) The current industry standards used to treat GB In most cases the encapsulated form is more potent than the free drug form solvated in DMSO. (b) The co-therapeutics suggested t be used to increase the efficacy of the industry standards. These drugs do not kill the drugs directly, however, they also do not increase the efficacy of the industry standards to any marginal degree (c). (d) Current industry standards for treatment of other cancers are also investigated and have a significantly lower IC_{50} value than current GBM drugs.

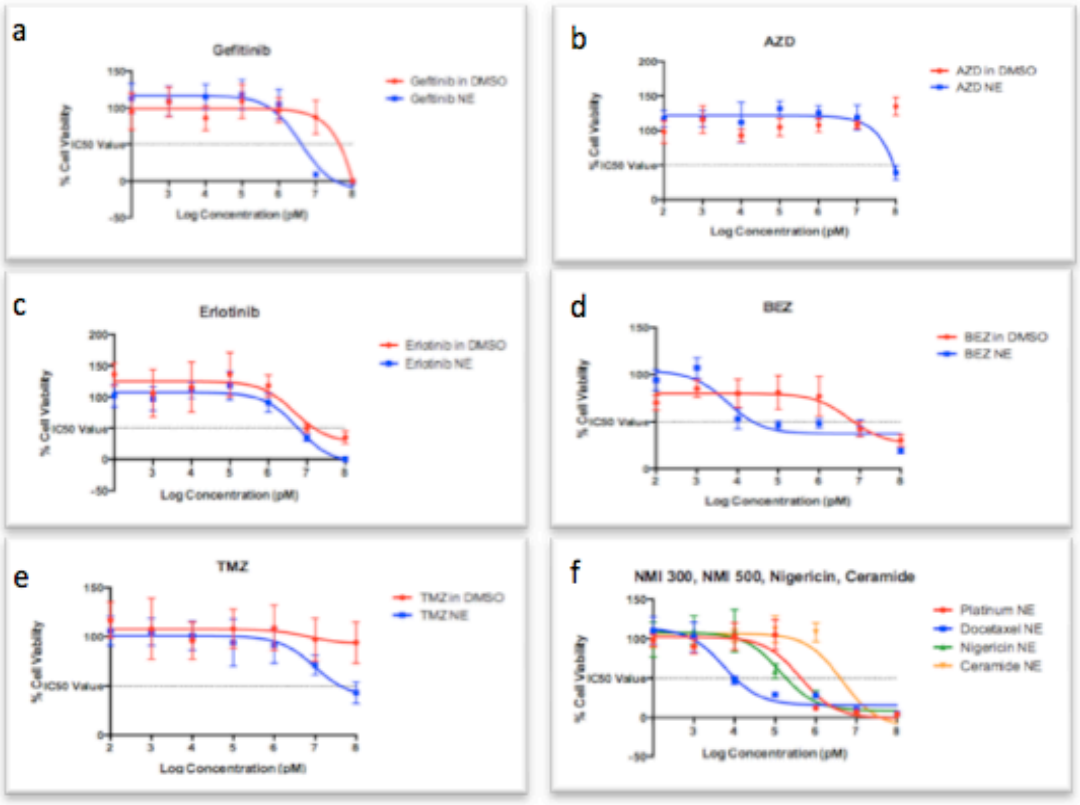


Figure 8: Kill Curves for U87 GBM Cells

Curves display the efficacy of the nanomedicines. Panels display the efficacy of single dose cytotoxic agents. Panels (a-e) are current industry standards for GBM while (f) displays cytotoxic agents used in other cancers.

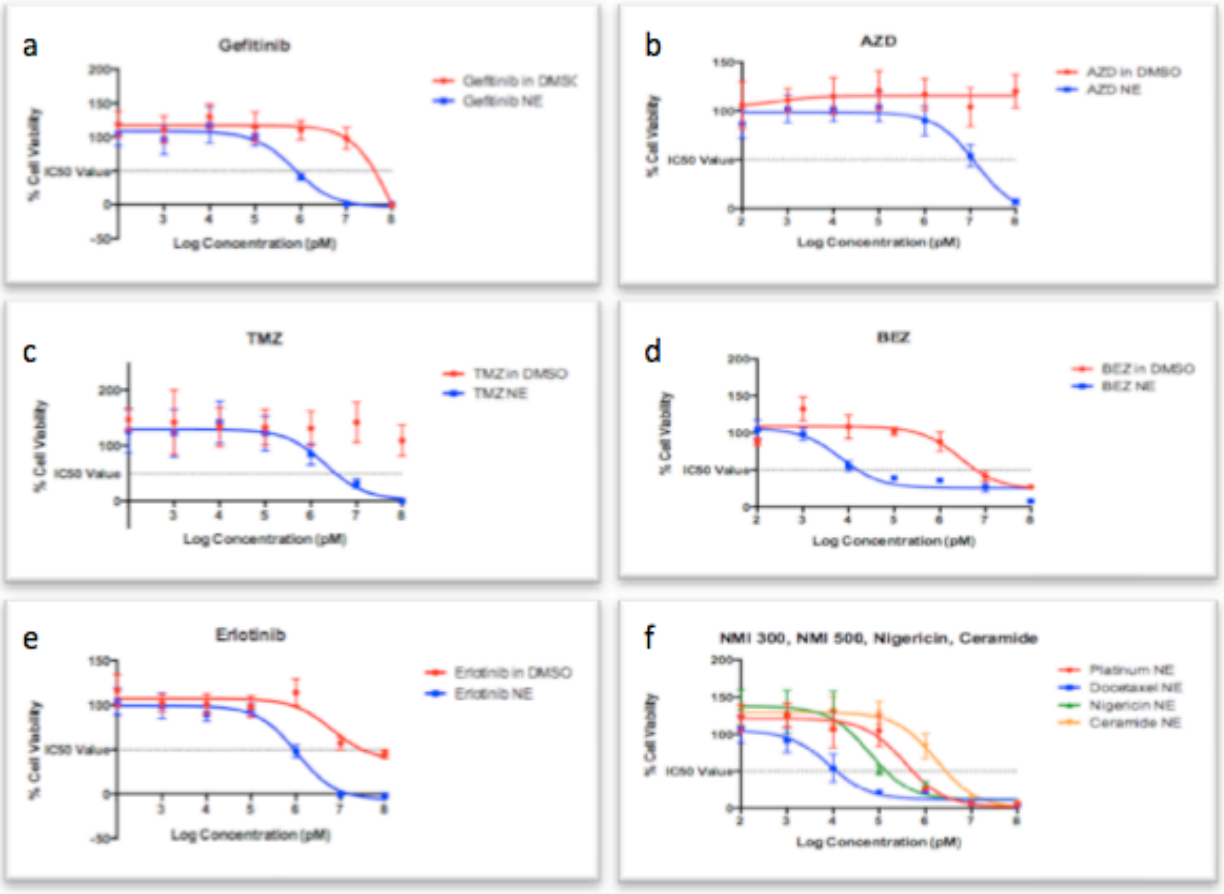


Figure 9: Kill Curves for U118 GMB Cells

Curves display the efficacy of the nanomedicines. Panels display the efficacy of single dose cytotoxic agents. Panels (a-e) are current industry standards for GBM while (f) displays cytotoxic agents used in other cancers.

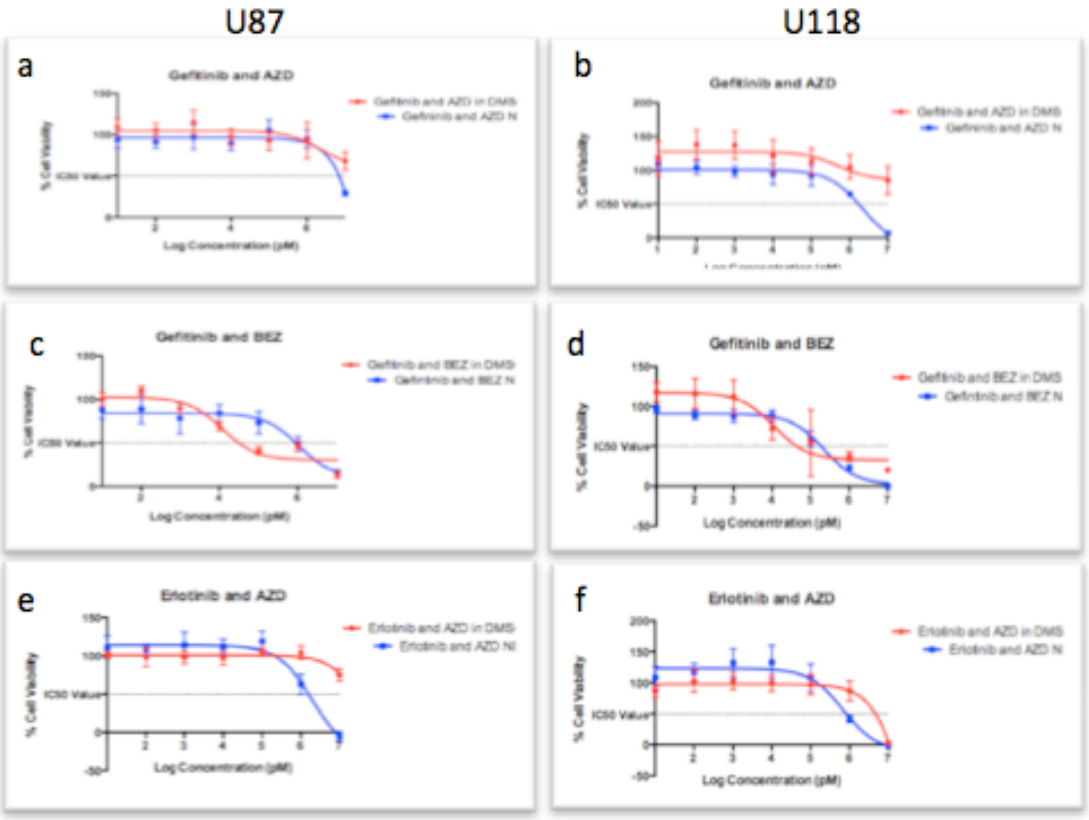


Figure 10: Kill Curves for Combination Assay

GBM standard cytotoxic agents were paired with cytostatic drugs AZD and BEZ. These drug combinations were intended t decrease the IC_{50} values of Erlotinib and Gefitinib but this was not observed in many cases.

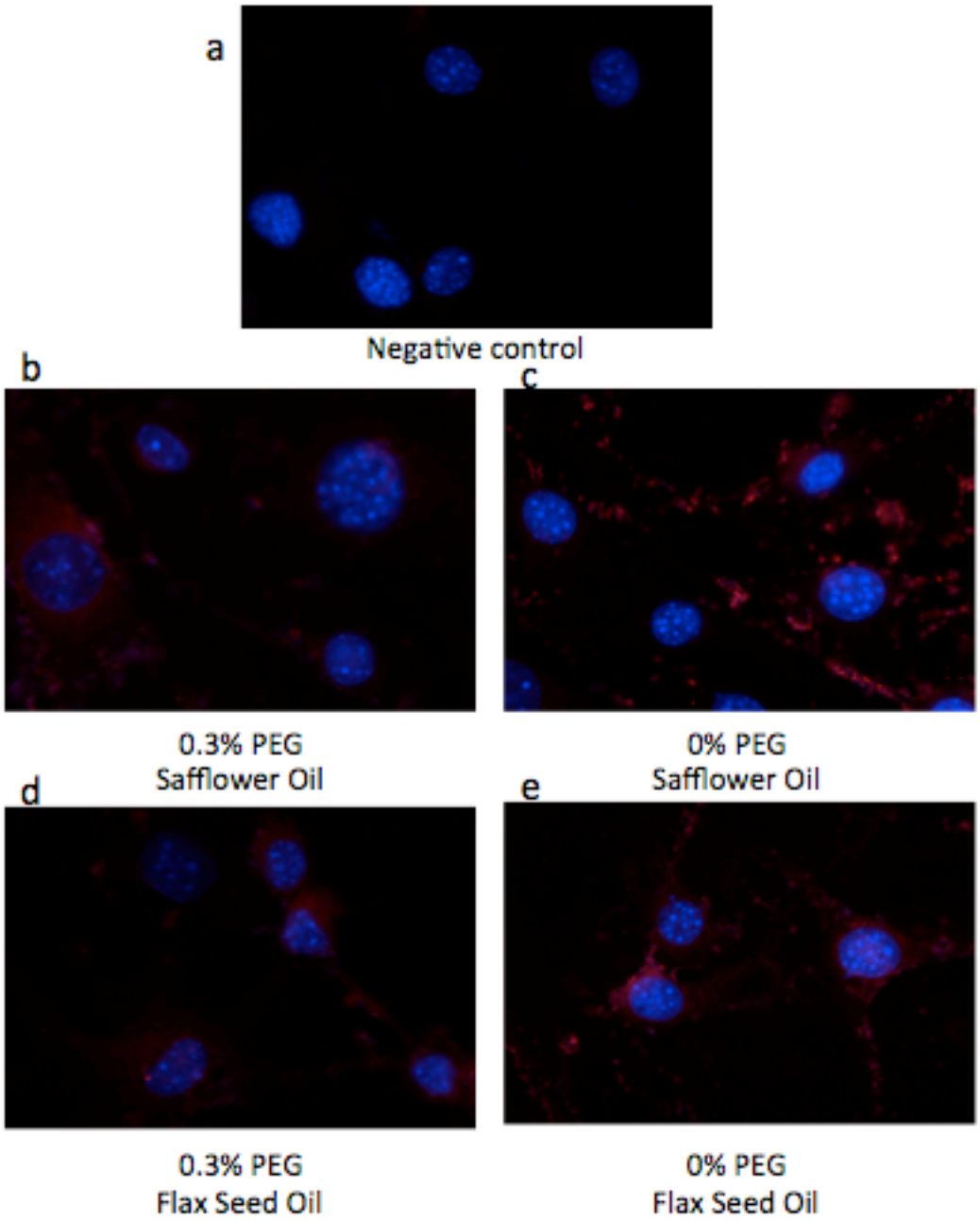


Figure 11: Uptake of Nanomedicines Across bEnd.3 Cells

The native ability of the nanomedicines to penetrate the Blood Brain Barrier was measured by developing an *in vitro* ass utilizing bEnd.3 mouse brain endothelial cells. The cells incubated with the rhodamine tagged nanomedicine for 15 min (not shown) and 30 min (above). The fluorescence background was reduced so that there was no noticeable fluorescence from the negative control (a).

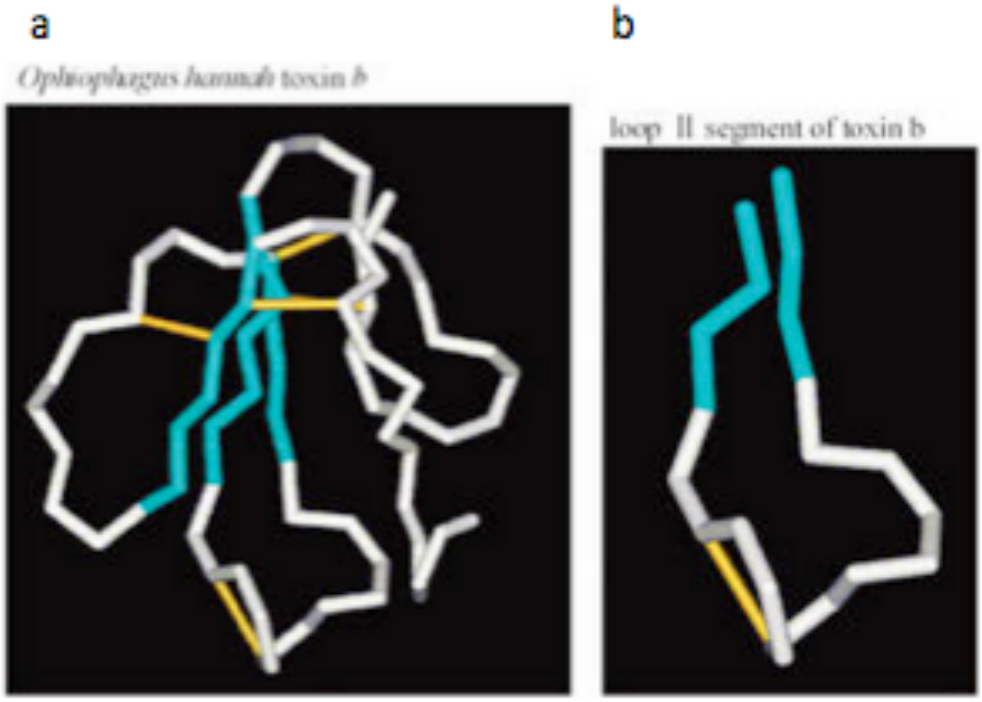


Figure 12: Three Finger Toxin

The Three Finger Toxins are peptide sequences that preferentially bind the nAChR. Their name stems from their appearance of a hand with three outstretched fingers (a). These toxins are stabilized by four disulfide bridges, which are vital for structure. (b) Only the second loop is vital for binding to the nAChR.

Appendix I: Table of Appreviations

Name	Abbreviation
(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	MTT
Acetonitrile	CAN
Active Pharmaceutical Ingredient	API
Alkyl-Guanine Transferase	AGT
Blood Brain Barrier	BBB
Central Nervous System	CNS
Dulbecco's Modified Eagle's Medium	DMEM
Epidermal Growth Factor Receptor	EGFR
Glioblastoma Multiforme	GBM
High Performance Liquid Chromatography	HPLC
Mu Opioid Receptor	MOR
Nicotinic Acetylcholine Receptor	nAChR
Phosphate buffered saline solution	PBS
Platelets Derived Growth Factor Receptor	PDGFR
Polydispersity Index	PDI
Polyethylene glycol	PEG
Reticular Endothelial System	RES
Temozolomide	TMZ
Three Finger Toxin	TFT
Tyrosine Kinase Inhibitor	TKI
World Health Organization	WHO

Appendix II: Liturature Review Publication

Title: Pathogen-inspired drug delivery to the central nervous system

Authors: Rebecca McClall, Joseph Cacaccio, Eileen Wrabel, Mary Schwartz, Timothy Coleman, Rachael Sirianni

Journal: Tissue Barriers

Abstract: For as long as the human blood-brain barrier (BBB) has been evolving to exclude bloodborne agents from the central nervous system (CNS), pathogens have adapted a multitude of strategies to bypass it. Some pathogens, notably viruses and certain bacteria, enter the CNS in whole form, achieving direct physical passage across endothelial cells to infect the brain. Other pathogens, including bacteria and multicellular eukaryotic organisms, secrete toxins that preferentially interact with neurons to exert a broad range of biological effects on peripheral and central neurons. In this review, we will discuss the directed mechanisms that viruses, bacteria, and the toxins secreted by higher order organisms use to enter the CNS. Our goal is to identify ligand-mediated strategies that could be used to improve the brain-specific delivery of engineered nanocarriers, including polymers, lipids, biologically sourced materials, and imaging agents.

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