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The Use of Mannose-Grafted and Lipopeptide-Conjugated PE Liposomes in the Delivery of Docetaxel for the Treatment of Glioblastoma Multiforme: A Research Protocol

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Abstract

Introduction: One of the biggest obstacles in delivering anti-cancer drugs to brain tumours is the penetration of the blood-brain barrier. Docetaxel is a promising drug used for glioblastoma multiforme that works by promoting mitotic arrest and cell death of tumorous cells, yet it encounters this obstacle presented by the selectivity of the blood-brain barrier. Due to the barrier's highly selective nature and the imprecision of current cancer treatments, the use of nanoparticles in drug delivery has been an area of significant interest. To address these issues, we propose using mannose and lipopeptide-grafted phosphatidylethanolamine liposomes as a drug delivery mechanism to effectively eliminate the obstacle of penetrating the blood-brain barrier in the treatment of glioblastomas. The truncated fibroblast growth factor and GALA lipopeptides increase the precision of the chemotherapeutic agent in targeting the tumour cells. Simultaneously, the mannose allows the nanoparticle to be recognized by sugar receptors on the blood-brain barrier, enabling it to pass through. This novel drug delivery system broadens the variety and increases the effectiveness of anti-tumoral drugs used in the treatment of brain cancer.

Methods: The lipopeptides are prepared through pyridyl disulfide reactions. The phosphatidylethanolamine liposomes are prepared using standard thin-film hydration in which the lipopeptides, docetaxel, and calcein (to track the drug delivery) are incorporated into the liposomal lumen. Mannose is then grafted onto the liposomal surfaces through the covalent coupling of p-aminophenyl-D-glycosides to phosphatidylethanolamine liposomes. The synthesized liposomes would be administered intravenously alongside radiation. Statistical analyses will be conducted to measure the growth of the tumour and the accuracy of drug delivery.

Discussion: The tumour cells should display a greater level of fluorescence, indicating a more accurate administration of the drug. It is expected that the patients will respond favourably to the treatment with the tumorous tissues showing a reduced growth rate and greater bioavailability of the drug.

Conclusion: The liposomal drug delivery mechanism presents a novel method by which anti-tumoral drugs can both cross the blood-brain barrier and precisely target the tumorous mass, thereby reducing the risk of drugs getting lost within the vasculature and expanding the horizons for brain tumour prognoses.

Keywords: docetaxel; blood-brain barrier; liposomes; lipopeptide; mannose; glioblastoma multiforme; drug delivery

Introduction

Glioblastoma Multiforme (GBM) is a rare primary brain tumour originating in the astrocytes. GBMs are categorized as grade IV brain tumours due to their rapid growth and invasion of nearby tissue [1,2]. The cause of GBMs is currently unknown, but it is widely believed that the tumour starts developing when mutations cause astrocytes to no longer display their principal characteristics of being highly controlled and organized cells. There are various treatment pathways including surgery, chemotherapy, clinical trials, repeat radiation and palliative care; one of these options entails the administration of docetaxel in conjunction with radiation. Docetaxel (DTX), sold under the name Taxotere, is an anti-mitotic taxane medication administered intravenously. It is considered to be one of the most effective drugs in the treatment of brain tumours. DTX is a "plant-based alkaloid", a class of chemotherapeutic agents, which binds to the β-subunit of the tubulin protein of microtubules resulting in the hyperstabilization of microtubule assembly. The stabilization of microtubules reduces their dynamic instability which is essential to processes such as cellular reproduction. As such, DTX can promote mitotic arrest and cell death. DTX is highly lipophilic with an aqueous solubility (25°C) of 4.93 μg/mL [3,4]. Past research has indicated that cellular absorption of lipid-soluble drugs is more effective than that of hydrophilic drugs, making DTX an excellent candidate for this study [5,6].

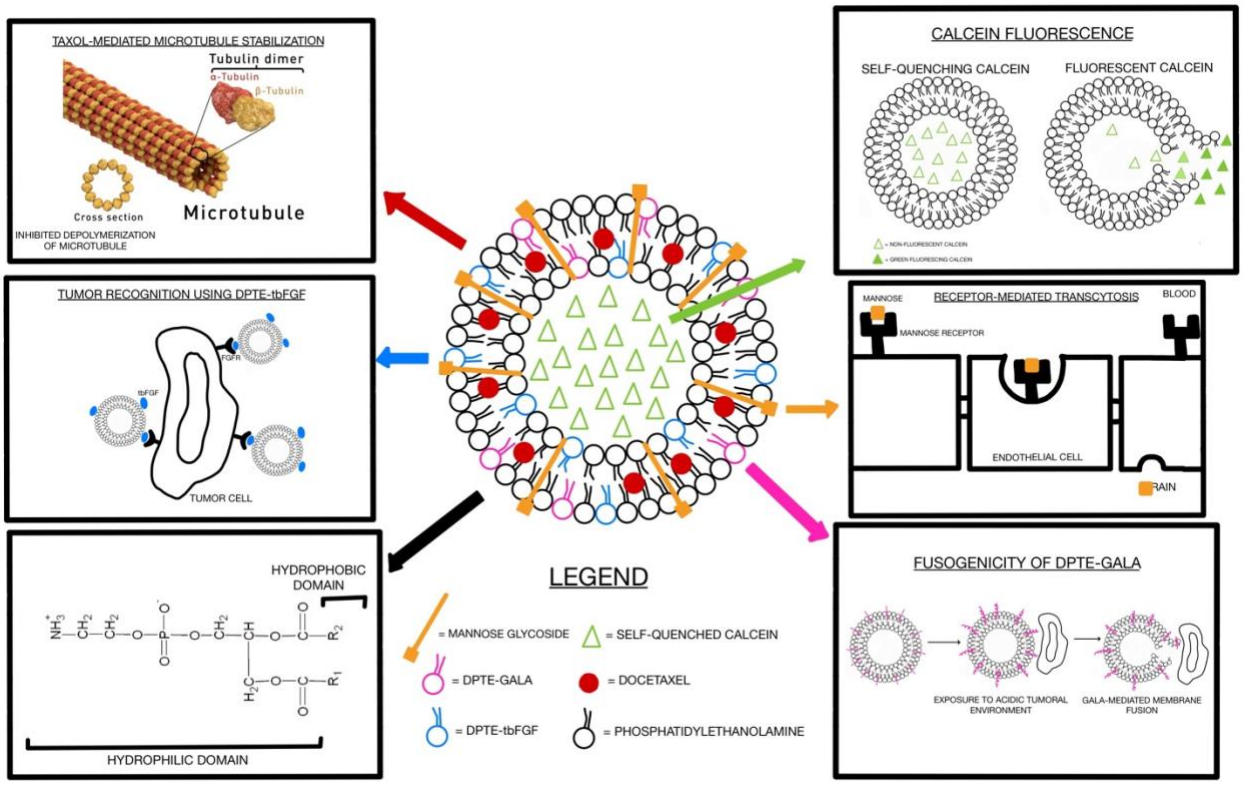

Figure 1. Diagrammatic summary of the use of mannose-grafted and lipopeptide-conjugated PE liposomes in the delivery of docetaxel. Diagram created using GoodNotes 5. "Microtubule Structure" by Thomas Splettstoesser is licensed under Creative Commons Attribution-Share Alike 4.0 International. "Phosphatidylethanolamine" by Bob Collowan is licensed under Creative Commons Attribution-Share Alike 3.0 Unported.

The blood-brain barrier (BBB) is made up of a network of continuous non-fenestrated, continuous fenestrated, and discontinuous capillaries [7]. This network is responsible for regulating the homeostasis of the central nervous system (CNS) through the formation of cellular networks that allow for the influx of essential molecules for the functioning of the CNS and the efflux of toxic cellular byproducts [8]. However, a major obstacle in the treatment of GBM is the presence of the BBB which is modified to the blood-tumour barrier (BTB) upon the development of a brain tumour. There is suboptimal delivery of drugs to brain tumours due to the heterogenous selectivity for the passage of fluid and molecules through the BTB cells. As a result, current treatments for GBM are largely palliative in nature, rather than curative, rendering the average survival rate of a patient diagnosed with a GBM to 15 months [9]. Although DTX is regarded as one of the most effective drugs against brain tumours, the highly selective nature of the BTB has long been an obstacle in the delivery of this chemotherapeutic agent [10]. DTX can cross the BTB with a meagre brain-plasma exposure of only 8%. A novel delivery system by which DTX can safely and efficiently cross the BBB to specifically target tumour cells is necessary to eliminate this obstacle.

We propose a drug delivery system consisting of mannose-coated and lipopeptide-conjugated phosphatidyl-ethanolamine (PE) liposomes that will carry DTX to the site of the tumour. Studies employ PE liposomes as they are small, biodegradable, biocompatible, amphipathic, nontoxic, and minimally immunogenic, resulting in a highly efficient avenue for cancer therapy [11]. The PE liposomes' formation will also incorporate the tbFGF and GALA peptides. The tbFGF peptide is a truncated fibroblast growth factor peptide that is nine amino acids long, with cysteine at the carboxyl end. It is commonly used as a targeting molecule due to its mitogenic, chemotactic, and angiogenic activity promoting the rapid proliferation of cells; even though it can bind to the fibroblast growth factor receptors on a tumorous mass, it will not induce cell proliferation due to its truncated nature [12]. The GALA peptide, a member of the pH-sensitive peptide family, originates from the amino-terminal segment of the H2A subunit of hemagglutinin from the influenza virus. The GALA peptide effectively penetrates and permeates cell lipid bilayers due to its activation in response to the acidic

environment of the GBM cells, allowing the liposomal escape of the encased drugs. In other words, at low pH (ie. pH=5) the GALA peptide will convert from its original coil configuration to an alpha helix configuration allowing for the perforation of the lipid bilayer [13].

Before forming the liposomal casing, the lipopeptides must be synthesized using a pyridyl disulfide reaction. Through two pyridyl disulfide exchange reactions, the thiol group of DPTE will be activated and reacted with the cysteine residue of tbFGF and GALA. The first pyridyl disulfide exchange reaction consists of lipid activation and the second reaction consists of lipid bioconjugation. These two reactions are performed for both peptides to create the lipopeptides DPTE-tbFGF and DPTE-GALA (Fig. 1) [14]. The synthesis of PE liposomes entails thin-film hydration followed by extrusion. The synthesized lipopeptides will be added to the lipid solution of PE phospholipids as they constitute the liposomal exterior of the PE vesicle. Hydration will consist of an aqueous solution containing DTX, calcein and phosphate-buffered saline being added to the dry lipid film and this solution will then be vortexed and sonicated [15]. This process is frequently followed by extrusion to efficiently create homogenous liposomes with controlled average size and minimal to no contamination. In a study conducted by Ong, et al. (2016), extrusion resulted in an average liposome size of 103.3 ± 13.5 nm with a smaller polydispersity index while other methods yielded average liposome sizes much larger accompanied by larger polydispersity indices [16]. The use of extrusion permits the formation of large unilamellar vesicles (LUVs) which fall within the range of 100 to 500 nm [17]. Due to their small size, LUVs are often excellent candidates for studies focused on intravenous drug delivery.

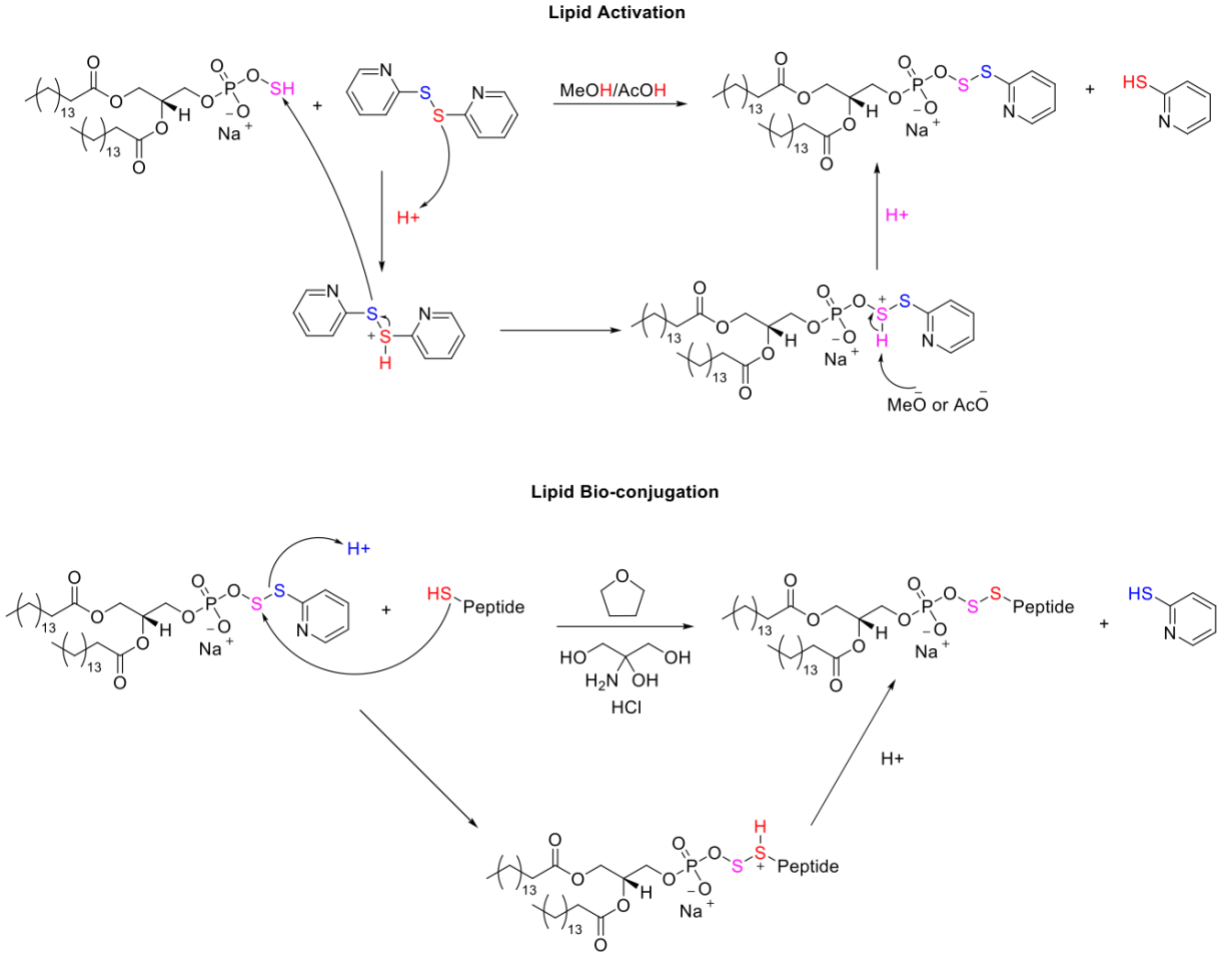

Figure 2. Two-step pyridyl disulfide reaction. The first part consists of lipid activation with mercaptopyridine, using a 16:1 (volume:volume) ratio of methanol and acetic acid creating a symmetric DPTE disulfide. The second part consists of lipid bioconjugation between DPTE disulfide and the sulfhydryl group of either the tbFGF or GALA peptide using a 2:1 (volume:volume) ratio of THF and 1M Tris HCl at a pH of 9. Lipopeptides DPTE-tbFGF and DPTE-GALA are formed. Diagram created using ChemDraw.

Furthermore, calcein is a water-soluble fluorescent dye that can be loaded into liposomes. It has been thoroughly used in past research requiring the use of fluorescent indicators [18]. As thin-film hydration involves the addition of an aqueous buffer to inflate the liposome, calcein will be added alongside the buffer. This allows it to be incorporated into the core of the liposome due to its polar nature, and prevents any disruption of the hydrophobic exterior. The release of calcein is mediated by the conformation of the lipopeptides conjugated into the bilayer of the liposome. The strength of the fluorescence signal would indicate the lysis of the liposome and the release of the drug. The calcein will serve as fluorescence markers to track the movement of the liposome, and subsequently DTX, through the body.

Additionally, past research in the field of sugar-grafting indicates that mannose improves drug targeting and mannose-grafted liposomes are more efficient in the transportation of drugs in comparison to glucose-grafted liposomes [19]. The efficiency of mannose grafting was tested by examining the efficiency of the delivery of anti-parasitic drugs to the infected spleens of 30-day infected hamster models of *Leishmania donovani*. While the percentage suppression of parasitic load in the spleen for the liposome-encapsulated drug sans mannose is a meager 55%, the incorporation of mannose increases this value to 72% [20]. The study also stated that the mannose-bearing delivery system yielded more efficient and less toxic results than the glucose-bearing systems. Toxicity studies conducted using adipose tissues indicated no apparent toxicity following the administration of the mannose-grafted liposomes and an improved result compared to glucose. As such, the uncoated liposomes will be mannose-grafted using a process detailed by Ghosh and Bacchawat (1980) in which p-aminophenyl-α-D-mannoside (pAαDM), PE liposomal suspension, and a sodium phosphate buffer containing NaCl are combined with the slow addition of glutaraldehyde [21]. Through this process mannose glycosides may be conjugated to the surface of the PE liposomes, thereby grafting the liposomes with a sugar surface to be recognized and uptook by mannose receptors [22].

DTX acts by stabilizing the microtubule cytoskeleton, thereby impairing mitosis and cell proliferation in tumours. However, several factors contribute to poor treatment response, one being the inefficiency of the drug to penetrate the BTB. The rationale of the drug delivery method proposed focuses on making this process more streamlined and accurate. First, using an organic PE liposome as the carrier of the drug allows for a natural and easily metabolizable substance to enter the body. Second, the liposome's mannose coating allows the sugar receptors on the BBB to recognize the liposome simply as mannose. Finally, the use of lipopeptides allows for accurate targeting of the tumour cells and the breaking down of the liposome to release the drug in the acidic environment surrounding a tumorous mass [23]. Combining these features could enable DTX to effectively target GBM. Both DPTE-tbFGF and DPTE-GALA have been used in the past, most notably in the delivery of cargo molecules to mouse embryonic fibroblasts [24].

It is hypothesized that the use of mannose-grafted and lipopeptide-conjugated DTX liposomes will allow for the drug to successfully penetrate the BTB and accurately target tumour cells, thereby improving treatment responses of brain tumour patients prescribed DTX. More specifically, it is predicted that patients prescribed the liposomal delivery of DTX will display a greater intensity of fluorescence near the tumour as well as a greater proportion of tumour mass fluorescing, therefore indicating a more precise delivery of the drug. As more DTX would be accurately delivered to the tumour mass, we also expect to observe a more significant decrease in the area of the tumour in the treatment group compared to the control group.

Methods

Pyridyl Disulfide Reactions for Lipid Peptide Conjugation

The conjugation of the lipopeptides through pyridyl disulfide reactions will occur in two distinct steps [24]. The first step consists of the activation of the thiol group of the lipid 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol (DPTE), and the second step entails the conjugation of the reactive disulfide DPTE with the cysteine residues of the tbFGF and the GALA peptides. The method we will use is analogous to that outlined by Fuente-Herreruela et al. in which 1-palmitoyl-2-oleoyl-sn-phosphatidylcholine (POPC) liposomes were conjugated with lipopeptides to improve fibroblast cell targeting and promote the direct delivery of cargo molecules to cancerous cells. First, 200 mg (275 μmol) of DPTE and 120 mg (550 μmol) of 2,2′-dipyridyldisulfide (DPDS) will be dissolved in methanol:acetic acid (160:1, volume: volume), for a final volume of 4 mL. This will then be incubated under stirring for 48 hours at room temperature in the dark. This will lead to lipid activation in which a disulfide exchange occurs between 2-2-pyridyl disulfide and the thiol group of DPTE in acidic conditions. Following purification of the activated DPTE (aDPTE), the disulfide bridge formed by DPTE and 2-mercaptopyridine will be substituted by DPTE and the cysteine-containing peptide. For the second reaction, 5 mg (6 μmol) of aDPTE will be incubated with 40 mg (40 μmol) of tbFGF or 120 mg (40 μmol) of GALA-Cys (1:7 mol:mol aDPTE:peptide ratio) in a mixture of tetrahydrofuran (THF) and 1 M Tris HCl pH 9 (2:1; volume:volume). This will result in a final volume of 3 mL which must then be stirred for 48 hours at 20 °C in the dark. The second reaction consists of lipid bioconjugation at pH=9. A pH of 9 is above the pKa of cysteine residues, forcing the cysteine residue of the peptides to react, resulting in a lipopeptide. In both reactions, the release of the mercaptopyridine takes place in the first 10 minutes, but the reaction is left to run for 48 hours to ensure completion. These two reactions are performed for both peptides in question to create the DPTE-GALA and DPTE-tbFGF lipopeptides.

Thin Film Hydration

The formation of PE liposomes grafted with surface DPTE-tbFGF and DPTE-GALA lipopeptides entails the use of a method known as the thin-film hydration method which will produce liposomes that are 1-5μm [25]. This procedure mimics one outlined by Xiang et al. which looked at the preparation of POPC liposomes using standard thin-film hydration followed by homogenization. First, DTX, PE lipids (manufactured by Cayman Chemicals) and cholesterol (stored at -20˚C) will be added to a round bottom flask such that there would be a 1:20 (weight:weight) drug:lipid ratio and a 2:1 (mol:mol) PE:cholesterol ratio. The contents of the flask will be dissolved in chloroform and the organic solvent will be evaporated under vacuum until the formation of a thin lipid film on the inner walls of the flask. The thin film will be blown with an inert gas (likely nitrogen or argon) for 10 minutes and the flask will be kept overnight to remove residual solvents. Next, 1 mL of a suitable aqueous phase (likely d.d H_2O) will be added to the flask containing the dry thin film, along with a suitable concentration of calcein. Finally, the dried lipids will be dispersed into the hydration fluid by shaking the flask, followed by repeated vortex agitation. At this point, we expect liposomes of 1-5 μm to be formed.

Extrusion

Extrusion is a method considered to be the most effective at creating homogenous and identically structured liposomes [17]. Extrusion will be carried out at 25°C to maintain the liposomes above the phase transition temperature. Using a mini extrusion kit, a membrane with a pore size of 100-500 nm will be applied such that LUVs may be formed. Liposomes will then be passed through a high-pressure extruder for 10 cycles to obtain uniformly structured liposomes [26].

Mannose Grafting

Mannose will be grafted onto the surface of the liposomes in the form of pAαDM following the procedure outlined in Biochemical and Biophysical Research Communications by Torchilin et al. The reaction itself entails the amino group of phosphatidylethanolamine and the amino group of pAαDM reacting with glutaraldehyde. The incorporation of glutaraldehyde between PE and mannose creates a hydrophobic domain capable of lodging itself within the bilayer such that the mannose is effectively exposed on the surface of the liposome without resulting in a significant change in liposomal size [20]. The specific measurements detailed below were determined as per a previous study conducted by Ghosh and Bachhawat (1980) in which mannose grafting of PE liposomes was successfully performed. As such, we expect similar measurements to yield promising results. A 1 mL suspension (30 mg lipid/mL) of PE liposomes will be combined in 0.025 M sodium phosphate buffer (pH of 7.2) containing 0.15 M NaCl. This will then be combined with 20 mg (contained in 2 mL) of pAαDM. Glutaraldehyde must then be slowly added to achieve a final concentration of 15 mM at which point the mixture will be incubated for 5 min at 20°C. Uncoupled glycosides and glutaraldehyde were removed by dialysis against the same buffer [21]. Since the reaction primarily involves the amino groups of pAαDM and PE, the coupling of glycosides on liposomes can be monitored through titration of the liposomal PE amino groups with trinitrobenzene sulfonic acid in which only exposed (and therefore unreacted) amino groups would be affected by the titration. As such, a rough indication of the amount of modified, hence successfully reacted PE liposomes can be obtained.

Results

We expect to observe nanoliposomes with mean diameters of approximately 150-200 nanometers. These parameters can be approximated from the findings of Fuente-Herreruela, et.al (2019) in which the formation of lipopeptide-conjugated POPC vesicles was found to exhibit sizes of approximately 150-200 nm following extrusion. Since the lipopeptides contain a negatively charged phosphate group within their structure, we expect the surface charge of the nanoliposomes to be modified following the incorporation of the lipopeptides such that a decrease in charge is observed.

Before conducting in vivo trials, an MTT cell viability assay must be performed. As such, healthy brain tissue will be exposed to liposomes that are not loaded with DTX to avoid confounding with the potential toxicity of liposomal constituents, such as calcein. The MTT viability assay will provide insight on liposomal toxicity as it measures the metabolic activity of the cells. DTX will now be incorporated in the thin-film hydration stage, resulting in mannose-grafted lipopeptide and PE liposomes encasing calcein. According to the results of related studies, it is found that DPTE-peptide conjugates do not affect cell viability and are compatible to be employed in in vivo studies [24]. The data will be analyzed with a two-way ANOVA test relating cell death to the ratios of DPTE-GALA/DPTE-tbFGF and the total concentration of transfected liposomes.

Developmental Plan

In the first trial phase, a set of tissue cultures will be prepared in the form of a cancer cell line. This primary cell line will consist of cancerous cells obtained from patients with a GBM. The objective of this trial phase is to determine the concentration of DPTE-GALA in the liposomes that will lead to the greatest intensity of calcein fluorescence, hence, the highest rate of membrane rupture. The control group will consist of a cancer cell line that is treated with fluorescence-tagged liposomes containing DTX. The experimental group will be treated with the synthesized DPTE-GALA conjugated, fluorescence-tagged liposomes. The concentrations of DPTE-GALA in the liposomes will be varied, while DPTE-tbFGF and mannose are not to be incorporated. The optimal

concentration of DPTE-GALA concentrations is analyzed using 3 experimental groups, each receiving liposomal suspensions with either 0%, 1%, or 5% coated in DPTE-GALA. The objective of this trial phase is to determine the concentration of DPTE-GALA in the liposomes that will lead to the greatest intensity of calcein fluorescence, hence, the highest rate of membrane rupture. We expect the PE liposomes lacking DPTE-GALA to lead to calcein being stuck in the endosomal pathway resulting in colocalization. However, we expect the 5% DPTE-GALA coated PE liposomes to show a significantly higher intensity of fluorescence at approximately 515-555 nm (emission wavelength of calcein) [24].

Phase two of the developmental plan will consist of experimentation and analyses using mice with GBM, each of which will be administered a DTX drug treatment in conjunction with radiation. The mice strain *Nf1;Trp53* mutants susceptible to GBM will be used for this trial [27]. The objective of this phase of the trial is twofold: (1) to test the optimal concentration of mannose that will lead to the successful penetration of the BBB and (2) to test the optimal concentration of DPTE-tbFGF for precise cancerous cell targetting. To determine the optimal concentration of mannose, a control group of mice will be administered 5% DPTE-GALA, 10% DPTE-tbFGF uncoated liposomes containing DTX and calcein. An experimental group of mice will be given the same liposomes (in regards to lipopeptide concentrations), but with varying concentrations of mannose- 0%, 3%, 6%, and 9% surface area coverage of the liposomes. We expect a greater bioavailability of the drug surrounding the tumorous tissue with mannose concentration at 9% as indicated in previous studies by Hagimori et.al. (2018) [28].

Next, to assess the efficacy of DPTE-tbFGF in cellular targeting, a measurement of its liposomal uptake must be obtained. As a result, four subdivisions of *Nf1;Trp53* mice are made, each receiving either 0%, 1%, 5% or 10% liposomal surface coverage with DPTE-tbFGF. The concentrations of DPTE-GALA, calcein, and mannose are not varied amongst these groups to minimize the possibility of confounding. The accuracy of tumour targeting can be quantified based on the intensity of fluorescence of calcein at the tumorous mass for which a greater intensity of green light is expected for the group administered nanoliposomes at a 10% DPTE-tbFGF liposomal surface coverage. Whereas the weakest fluorescence is expected in the 0% group. These findings are exemplified in prior studies conducted employing lipopeptide liposomal conjugation [24].

The success of phase two mice trials leads to the progression of phase three, human trials. In this phase, the experimental group of patients will be administered the novel liposomal drug delivery system for DTX intravenously, while a control group of patients will be administered non-liposomal DTX intravenously; both groups will receive their treatment in conjunction with radiation. At this point in the in vivo trials, we expect to see a higher efficacy of release and bioavailability of DTX surrounding the tumorous cells. A T-test will be used to identify the difference in means. We expect to see a greater decrease in the mass of the tumour as well as a significant increase in the average intensity of fluorescence of the tumour in patients given the novel drug delivery system. A large sample procedure to obtain the difference in proportions will also be conducted. We expect a greater proportion of tumour mass fluorescence in the experimental group administered the liposomal treatment.

Discussion

The current state of GBM treatment initially involves surgery to remove as much of the tumorous mass as possible. Next, patients can be administered anywhere from one to six cycles of DTX for one hour every three weeks, in conjunction with radiation. However, the treatment of GBM, and other types of brain cancer, is limited due to the poor transport of drugs across the BTB. In fact, in prior studies, DTX is shown to display no significant activity in patients with malignant recurrent gliomas. To combat this issue, we propose a novel drug delivery system of DTX using mannose and lipopeptide-coated liposomes as nanoparticle drug carriers. With the release of DTX in a sustained manner, we believe that this drug will be able to effectively cross the BTB and precisely target the GBM.

There are many important characteristics of the liposomes that allow for this novel drug delivery system of DTX to be effective and safe. First of all, the liposomes were synthesized to be 150-200 nm in diameter following extrusion. Another important characteristic of liposomes is their negative charge. Previous studies show that liposomes above a certain size may block lung capillaries and that a positively charged surface may bind circulating i2-macroglobulin, resulting in weaker coagulation processes [20]. However, when a patient was injected intravenously with 100 mg lipid with negatively charged liposomes, there was no decrease in cc2-macroglobulin levels. As a result, the liposomes used in this novel drug delivery system were specifically sized at 150-200 nm and have a negative surface charge. In addition, PE glycerophospholipids were used to create liposomes instead of POPC. PE lipids contain an amino group on the polar head which is essential for mannose grafting while POPC glycerophospholipids consist of a quaternary amine group instead which is not suitable for interactions with mannose [29]. Potential POPC liposomal toxicity was also observed in previous studies as it was found that phosphatidylcholine (PC) liposomes (the base of POPC liposomes) inhibit lymphocyte activation by attracting and thus reducing cellular cholesterol [20]. Due to the toxicity effects and the incompatibility for mannose grafting, PE liposomes are ideal over POPC liposomes.

Furthermore, an MTT cell viability assay will also be conducted before synthesizing a cancer cell line. This assay will allow us to ascertain that the nanoparticles being used have no toxic effect on cells. The cell viability assay can also

be used to test the toxicity of calcein. Calcein in high concentrations can be very toxic and this assay will allow us to determine if 100 mM (concentration of calcein being used) has any toxic effects. A successful cell viability assay will ensure cell survival during the novel drug delivery system treatment.

Developmental Plan

A primary cancer cell line will be used to measure the optimal concentration of DPTE-GALA in the liposomal composition of the liposome that will ensure effective fusogenicity. As opposed to traditional cell lines, primary cell lines are isolated directly from human tissue and have a finite lifespan. However, primary cell lines are less relevant and reflective of the in vivo environment, hence the need for the following in vivo trial phases. To extract the tumour cells and create a primary cell line from an explant, enzymatic degradation using dispase can be successful for brain tumours and it has shown great success in isolating tumorous cells from cervical, prostate and ovarian cancer when cell viability and tissue dissociation were optimal. A 3D culture will be created using the isolated tumour cells as it is well representative of the in vitro and in vivo cellular environments, by mimicking the natural characteristics of the cell. The results from these tests will be analyzed using calcein and confocal fluorescent microscopy [30]. The use of confocal fluorescent microscopy allows successful excitation of calcein. Calcein at high concentrations is notable for its ability to self-quench and therefore result in very little fluorescence. However, upon its release from the liposomal structure, a sudden spike in fluorescence levels can be observed [31]. As such, an increase in the fluorescence levels can be indicative of the lysis of the liposomal membrane which is triggered due to the mechanism of the DPTE-GALA lipopeptide.

Experimentation and analyses using *Nf1;Trp53* mice will be conducted to better understand the applicability of the liposomal drug delivery system in vivo. These mice were selected due to their predisposed susceptibility to GBM and are to be accessed through Jackson Laboratories. Using the mice, studies pertaining to the optimal concentrations and efficacy of mannose and DPTE-tbFGF will be conducted. These are to be conducted using a mouse model as opposed to a tissue culture model as both DPTE-tbFGF and mannose play significant roles in the targeting of the tumorous mass and movement within the vasculature. The liposomal suspension will be administered intravenously in conjunction with radiation, mimicking the current route of treatment with DTX. The concentration of DTX administered to each group will be maintained constant. We expect that both mannose and DPTE-tbFGF improve the responses of *Nf1;Trp53* mice administered DTX. It is expected that the optimal concentration of DPTE-tbFGF on the liposomal surface is 10% while that of mannose would be approximately 9%. The results are analyzed using calcein and two-photon electron microscopy (rather than confocal fluorescent microscopy) as this type of microscopy allows for live imaging of thick brain tissue. The remaining vital signs of the mice will be monitored throughout experimentation to track any other potential side effects of treatment. Each cycle of DTX is typically administered through IV for one hour every 3 weeks [32]. For experimentation purposes, the control and treatment groups will be subjected to five cycles of their respective DTX treatments, after which the results will be collected and compared.

Lastly, the final phase entails clinical trials on individuals diagnosed with GBM. In this stage, the real-life intended application of the drug delivery system will be tested alongside radiation. As detailed in the Results section, the proportion of fluorescent tumorous mass, relative fluorescence intensity of the tumour, and the decrease in tumour area will be compared amongst a control group – traditionally administered DTX, sans liposomes - and a treatment group - administered the novel liposomal delivery of DTX. The concentration of DTX administered will remain constant amongst the two groups to eliminate the possibility of the amount of DTX confounding the results of the experiment. The fluorescence will be monitored identically to phase two. This phase is incredibly important in concluding whether the method of DTX delivery is effective, safe, and applicable as a mode of treatment for patients with GBM. Much like the mouse trial phase, the control and treatment groups will be exposed to their respective treatments for 5 cycles total, amounting to a total time commitment of approximately 15 weeks.

Data Analysis

All statistical analyses will be performed using the statistical software R, in which two T-tests for the difference in means and a large sample procedure for the difference in proportions will be conducted ($p=0.05$). These tests allow the accurate quantification of differences amongst the results of the control and treatment groups to accurately and reliably make conclusions where appropriate. In the T-test for the difference in means, μ_1= decrease in tumour area following 5 cycles of DTX treatment in the control group and μ_2= decrease in tumour area following 5 cycles of DTX treatment in the treatment group. It is predicted that the decrease in area of the tumour in the experimental group (administered the liposomal treatment of DTX) would be more significant than that of the control group ($\mu_2-\mu_1>0$). Furthermore, the effectiveness of tumour targeting was measured using another t-test for the difference in means in which μ_1= average fluorescence intensity of the tumorous mass post-DTX therapy in the control group and μ_2= average fluorescence intensity of the tumorous mass post-DTX therapy in the treatment group. It is predicted that the liposomal delivery of DTX would increase the bioavailability of DTX, resulting in an increased level of fluorescence ($\mu_2-\mu_1>0$). Using this test, we would be able to examine whether the use of liposomal delivery truly

enhances the ability of the drug to target the tumour and act effectively. Finally, to assess the increased bioavailability of the drug surrounding the tumour mass, a large sample procedure for the difference in proportions will be conducted in which p_1= proportion of tumour mass fluorescing post-treatment in the control group and p_2= proportion of tumour mass fluorescing post-treatment in the treatment group. It is expected that there would be a greater proportion of tumour mass fluorescence in the treatment group (p_2-p_1>0).

Implications

This novel method of drug delivery ideally eliminates the obstacle of the blood-brain barrier in the treatment of not only glioblastomas but other tumours present beyond the BBB. As such, a major strength of this method would be its potential generalizability to other brain tumours entailing the use of different chemotherapeutic agents. In addition, the methods proposed have been used in countless other studies with success; we simply aim to combine these different protocols, creating a novel drug delivery system. As such, this method would be generalizable to other brain tumours entailing the use of different chemotherapeutic agents. More specifically, the structure of the liposome permits both hydrophobic and hydrophilic drugs to be encapsulated. While there is great optimism surrounding this novel method of DTX delivery, it should be noted that there may be certain limitations to keep in mind. Although the grafting of mannose was done in order to cross the BBB through the manipulation of mannose receptors, there may be similar receptors elsewhere in the body resulting in liposomal uptake in other parts of the body, thereby limiting the efficacy of delivery. One example we speculate is the epithelial tissues of the gut and lungs which may be susceptible due to their structural similarities to the BBB. Given the process of synthesizing the liposomes entails various steps, each of which requires various reagents and equipment, this delivery mechanism may be both cost-expensive and time-consuming [33]. As of right now, the protocols which constitute the method by which these liposomes are synthesized are not automated. As such, it may be difficult for this process to be industrialized and made in a grander and more efficient manner.

Conclusion

Docetaxel is a promising treatment for cancers such as glioblastoma multiforme but it is inhibited by the extreme selectivity of the blood-brain barrier. As such, we propose a novel drug delivery system for docetaxel using mannose-coated and lipopeptide-conjugated liposomal nanoparticles. The mannose coating will aid in the recognition of the liposome as mannose, leading to the penetration of the BBB. The lipopeptides DPTE-tbFGF and DPTE-GALA are crucial for tumour recognition and fusogenicity, respectively, once the BTB has been crossed. Calcein, a fluorescent molecule, will be incorporated into the liposome to track drug delivery.

When testing this novel drug delivery system in conjunction with radiation therapy, we expect the area of the tumour to have decreased significantly in the experimental group compared to the control group. We also expect both, a greater average fluorescence intensity and a greater proportion of fluorescent tumorous mass in patients of the experimental group in comparison to patients of the control group. Assuming the expected results for the above tests were obtained, we would be able to accurately conclude that the lipopeptide and mannose-grafted PE liposomal delivery of docetaxel is a minimally-toxic, highly precise, and greatly efficient method to be employed in the treatment of glioblastomas and potentially other brain tumours. Following this study, subsequent investigations using different therapeutic agents and different tumours can be conducted to examine the generalizability of the method proposed. As such, this work helps to bring to the forefront the obstacles brought on by the presence of the BBB and the intricacies of brain tumour treatment.

List of Abbreviations Used

GBM: glioblastoma multiforme
DTX: docetaxel
BBB: blood-brain barrier
BTB: blood-tumour barrier
PE: phosphatidylethanolamine
tbFGF: truncated fibroblast growth factor
LUV: large unilamellar vesicle
MLV: multilamellar vesicle
pAαDM: p-aminophenyl-α-D-mannoside
DPTE: 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol
aDPTE: activated 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol
DPDS: 2,2′-dipyridyldisulfide
THF: tetrahydrofuran
POPC: 1-palmitoyl-2-oleoyl-sn-phosphatidylcholine

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Ethics Approval and/or Participant Consent

This research protocol is based on hypothetical theories for which laboratory applications have not yet begun. As such, there is no need for ethics approval or participant consent at the moment.

Authors' Contributions

CG: made substantial contributions to the conception, design, acquisition, analysis and interpretation of data for the work, drafted and revised the work critically for important intellectual content, gave final approval of the version to be published and agreed to be accountable for all aspects of the work.
MRK: made substantial contributions to the conception, design, acquisition, analysis and interpretation of data for the work, drafted and revised the work critically for important intellectual content, gave final approval of the version to be published and agreed to be accountable for all aspects of the work.

Acknowledgements
We would like to thank Monika Snowdon for giving us ample mentorship and guidance throughout this process.

Funding
This study was not funded.

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Article Information
Managing Editor: Jeremy Y. Ng
Peer Reviewers: Monika Snowdon, Foram Vyas
Article Dates: Received April 09 22; Accepted Jul 07 22; Published Oct 19 22

Citation
Please cite this article as follows:
Gupta C, Radhamma Krishnan M. The use of mannose-grafted and lipopeptide-conjugated PE liposomes in the delivery of docetaxel for the treatment of glioblastoma multiforme: A research protocol. URNCST Journal. 2022 Oct 19: 6(10).
https://urncst.com/index.php/urncst/article/view/373
DOI Link: https://doi.org/10.26685/urncst.373