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Multifunctional liposomes loaded with paclitaxel and artemether for treatment of invasive brain glioma

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ABSTRACT

Invasive brain glioma is the most lethal type of cancer and is highly infiltrating. This leads to an extremely poor prognosis and makes complete surgical removal of the tumor virtually impossible. Non-penetration of therapeutic drugs across the blood–brain barrier (BBB), brain cancer stem cells (CSCs), and brain cancer vasculogenic mimicry (VM) results in relapse after surgical and radio therapy. We developed a functional targeting chemotherapy for transporting drugs across the BBB, destroying VM channels, and eliminating CSCs and cancer cells in the brain. The studies were undertaken on brain glioma cells *in vitro* and in brain glioma-bearing rats. Using paclitaxel as the anticancer drug and artemether as the regulator of apoptosis and inhibitor of VM channels, a kind of functional targeting paclitaxel plus artemether liposomes was developed by modifying two new functional materials: a mannose-vitamin E derivative conjugate (MAN-$TPGS_{1000}$) and a dequalinium-lipid derivative conjugate (DQA-PEG_{2000}-DSPE). The transport mechanism across the BBB was associated with receptor-mediated endocytosis by MAN-$TPGS_{1000}$ conjugate *via* glucose transporters and adsorptive-mediated endocytosis by DQA-PEG_{2000}-DSPE conjugate *via* electric charge-based interactions. The efficacy was related to the destruction of VM channels by regulating VM indicators, as well as the induction of apoptosis in brain cancer cells and CSCs by activating apoptotic enzymes and pro-apoptotic proteins and inhibiting anti-apoptotic proteins. These data suggest that the chemotherapy using functional targeting paclitaxel plus artemether liposomes could provide a new strategy for treating invasive brain glioma.

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1. Introduction

Brain glioma is the most lethal type of cancer. Patients with gliomas have an extremely poor prognosis owing to the highly infiltrating nature of gliomas [1,2]: the median survival time is <16 months [3]. Invasive glioma cells are far from cancer sites. They are even in the contralateral hemisphere, hide in the areas of the brain, and are protected by the blood–brain barrier (BBB) [4]. Hence, complete removal of gliomas by surgery is virtually impossible. Conventional surgical methods and/or radiotherapy alone cannot completely eliminate brain cancer cells [5], and relapse is inevitable.

Therefore, a crucial challenge is to deliver therapeutic agents effectively to the core of cancer cells as well as migratory cells in the infiltration zone [4]. Chemotherapy for gliomas is difficult because of two main obstacles. One obstacle is the BBB in the brain cancer, which separates blood from cerebral tissue, and prevents the penetration of drugs into the CNS [6]. The other obstacle is the heterogeneity of brain cancer, such as the existence of cancer stem cells (CSCs) and vasculogenic mimicry (VM). VM describes functional vascular channels composed only of channels of tumor cells.

A small number of CSCs in brain cancer have been characterized. Such cells have the capability of self-renewal, proliferation and high tumorigenicity, show strong resistance to drugs, and are responsible for cancer recurrence [7]. VM-capable cancer cells are responsible for the invasion and refractoriness of brain cancer, demonstrating high resistance to chemotherapy, and rarely being removed by conventional drug treatment [8]. VM-capable cancer cells could be genotype-transformed cancer cells induced by hypoxia during the early growth of brain cancer or during the regeneration of residual cancer cells after chemotherapy. Unlike cancer angiogenesis, VM channels could be constituted primarily by

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genotype-transformed cancer cells (rather than endothelial cells) to provide nutrients for the regeneration of cancer tissue.

A liposome is an artificially prepared vesicle comprising a lipid bilayer. A liposome can be used as a vehicle for the administration of nutrients or pharmaceutical agents. In the present study, we designed a functional targeting liposomal drugs system for offering a comprehensive strategy to transport drugs across the BBB. We also wanted to eradicate brain cancer cells, resistant brain cancer VM channels, and resistant brain CSCs. In the functional targeting liposomal drugs system, paclitaxel and artemether were incorporated into liposome vesicles as an anticancer agent and modulating agent, respectively.

A newly synthesized mannose-vitamin E derivative (P-aminophenyl-α-D-manno-pyranoside-D-α-tocopheryl polyethylene glycol 1000 succinate, MAN-$TPGS_{1000}$) conjugate was used a targeting molecule for transporting drugs across the BBB. A dequalinium-lipid derivative (dequalinium-polyethylene glycol 2000-distearoyl phosphatidyl ethanolamine, DQA-PEG_{2000}-DSPE) conjugate was used as a functional molecule for targeting cancer cells, CSCs, and genotype-transformed cancer cells. Dequalinium (DQA) is a quaternary ammonium cation commonly available as a dichloride salt, as well as an amphiphile with delocalized cationic charge centers. DQA can accumulate selectively in mitochondria [9], hence is used as a molecule targeting mitochondria. P-aminophenyl-α-D-manno-pyranoside (MAN) is a type of mannose analog. It can penetrate the brain efficiently *via* facilitative glucose transporters (GLUTs) because the BBB over-expresses GLUTs [10]. Accordingly, MAN is used as a ligand targeting the BBB.

Paclitaxel is a microtubule-targeting anticancer agent isolated from the bark of *Taxus brevifolia*. It shows antitumor activities against various solid tumors, such as ovarian cancer, lung cancer and gliomas [11]. However, the efficacy of commercial paclitaxel formulations against gliomas is far from satisfactory because it cannot penetrate the BBB [12].

Artemether is a potent and rapid agent for the treatment of severe resistant malaria, including multiple drug-resistant falciparum malaria and cerebral malaria [13]. In addition, artemether also demonstrates potent cytotoxic activities against tumor cells [14]. Mechanism studies show that artemether exhibits potential for down-regulating the expression of matrix metalloproteinase (MMP)-9, hypoxia-inducible factor (HIF)-1α, VEGF and other related proteins [15], and is able to inhibit the growth of embryonic stem cells [16].

The objectives of the present study were to develop a kind of functional targeting paclitaxel plus artemether liposomes, and to study the mechanism and efficacy for treatment of invasive brain gliomas by transporting across the BBB, followed by eliminating brain cancer cells, brain CSCs and VM channels.

2. Materials and methods

2.1. Animal experimentation

Male Sprague–Dawley rats (initially weighing 190–210 g) and ICR mice (initially weighing 19–21 g) were purchased from the Health Science Center of Peking University. All procedures involving the care and handing of animals were carried out with approval of the Authorities for Laboratory Animal Care of Peking University (Beijing, China).

2.2. Cell lines and reagents

Glioma C6 cells were maintained in serum-containing medium comprising Ham's F10 medium (Macgene Biotech Co. Ltd., Beijing, China) supplemented with 5% fetal bovine serum (FBS) and 15% horse serum (Gibco, Billings, MT, USA). Glioma C6 CSCs were grown in serum-free Dulbecco's modified Eagle's medium (DMEM)-F12 (Macgene Biotech) supplemented with 2% B27 (Gibco), 20 ng/mL basic fibroblast growth factor and 20 ng/mL epidermal growth factor (Macgene Biotech) as reported previously [17]. Murine brain microvascular endothelial cells (BMVECs) (Institute of Clinical Medical Sciences, China–Japan Friendship Hospital, Beijing, China) were passaged in the endothelial cell culture medium (DMEM, 20% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, 100 μg/mL endothelial cell growth factor, 40 U/mL heparin).

MAN, DCC, DMAP, D-α-tocopheryl polyethylene glycol 1000 succinate ($TPGS_{1000}$), sulforhodamine-B, and Sephadex G-50 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Paclitaxel, artemether, NHS, EDC and glutaric acid were obtained from J&K Scientific Ltd. (Beijing, China). Polyethylene glycol-distearoyl phosphatidyl ethanolamine (PEG_{2000}-DSPE) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). DMSO was purchased from Amresco (Solon, OH, USA). Other reagents were from Beijing Chemical Reagents (Beijing, China).

2.3. Synthesis of targeting molecule conjugates

DQA-PEG_{2000}-DSPE was synthesized and characterized according to the methods employed in our earlier publication [18]. MAN-$TPGS_{1000}$ conjugate was synthesized as the following procedures. Glutaric acid (0.2 mmol), DMAP (0.1 mmol) and DCC (0.24 mmol) were dissolved in DMSO (2 ml). The mixture was stirred using a magnetic stirrer at room temperature under nitrogen gas protection for 2 h. After adding $TPGS_{1000}$ (0.04 mmol), the reaction mixture was stirred using a magnetic stirrer at room temperature for 24 h. The crude product was then transferred to a regenerated cellulose dialysis tubing (molecular weight cut-off point, 1500) and dialyzed against deionized water for 48 h to remove uncoupled glutaric acid, DMAP, DCC, N, N-dicyclohexylurea, and DMSO. The resultant $TPGS_{1000}$-COOH was obtained by freeze-drying. Afterwards, $TPGS_{1000}$-COOH (10 μmol), EDC (40 μmol) and NHS (70 μmol) were dissolved in pyridine-DMSO (1:1, 2 ml). The mixture was stirred for 30 min, followed by adding MAN (10 μmol), and stirring for 12 h. The crude product was dialyzed against deionized water in the regenerated cellulose dialysis tubing for 48 h to remove uncoupled MAN, EDC, NHS, DMSO and pyridine. The resultant product was obtained by freeze-drying. The production mixture was identified and characterized using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS instrument, Shimadzu, Japan) and nuclear magnetic resonance spectroscopy (400 MHz ^{1}H NMR, Bruker AVANCE III 400).

The standard addition method was used to quantify the yield of $TPGS_{1000}$-MAN [18]. Briefly, serial known aliquots of MAN were added into the sample, yielding a linear increase in the signal from the aromatic hydrogen ($\sim$ 7.0 ppm). The slope was then used to determine the mass of $TPGS_{1000}$-MAN. As the aromatic hydrogen on MAN remained on the $TPGS_{1000}$-MAN conjugate, the signal peak was used to semi-quantify the $TPGS_{1000}$-MAN content based on calculations.

2.4. Functional targeting paclitaxel plus artemether liposomes

The functional targeting paclitaxel plus artemether liposomes were prepared as our previous report [18]. The constituents included egg phosphatidylcholine, cholesterol, $TPGS_{1000}$-MAN, DQA-PEG_{2000}-DSPE (88:3.5:3:3, molar ratio), and drugs (paclitaxel: artemether = 1:3, molar ratio). DQA-targeting paclitaxel plus artemether liposomes were prepared by replacing $TPGS_{1000}$-MAN with $TPGS_{1000}$. MAN-targeting paclitaxel plus artemether liposomes were prepared by replacing DQA-PEG_{2000}-DSPE with PEG_{2000}-DSPE. Paclitaxel plus artemether liposomes were prepared using PEG_{2000}-DSPE and $TPGS_{1000}$ to substitute for DQA-PEG_{2000}-DSPE and $TPGS_{1000}$-MAN. Paclitaxel liposomes were prepared by using the same procedures as above but excluding the addition of artemether. The morphology of paclitaxel plus artemether liposomes was observed by an atomic force microscope (NSK Ltd., Tokyo, Japan). The particle sizes and zeta potential values were measured using a Nano Series Zen 4003 Zetasizer (Malvern Instruments Ltd, Malvern, UK). *In vitro* release of paclitaxel or artemether from the liposomes was performed by dialysis against the release medium containing serum protein (pH 7.4 PBS containing 10% FBS).

2.5. Cytotoxic effects on brain cancer cells and brain CSCs

Glioma C6 cells were seeded at 5×10^3 cells/well and grown in serum-containing culture medium in an incubator at 37 °C for 24 h under an atmosphere of 5% CO_2. The medium was then replaced with fresh culture media containing varying concentrations of free paclitaxel, free artemether, free paclitaxel plus a fixed concentration of free artemether, free paclitaxel plus free artemether, paclitaxel liposomes, paclitaxel plus artemether liposomes, MAN-targeting paclitaxel plus artemether liposomes, DQA-targeting paclitaxel plus artemether liposomes, or the functional targeting paclitaxel plus artemether liposomes. The final concentration of paclitaxel was 0–5 μM. Blank culture medium was used as a blank control. At 48 h, cell viability was determined by a sulforhodamine-B staining assay based on measurement of absorbance at 540 nm using a microplate reader (Infinite F50; Tecan Group Ltd., Shanghai, China). Survival was calculated using the following formula: Survival (%) = ($A_{540\ nm}$ for treated cells/$A_{540\ nm}$ for control cells) $\times$ 100%. where $A_{540\ nm}$ is the absorbance at 540 nm. Dose–effect curves were plotted from the data of triplicate assays.

Brain CSCs were identified using a specific procedure. Briefly, after continuous culture of glioma C6 cells in a serum-free medium under 5% CO_2 at 37 °C for 3 weeks, brain CSC mammospheres were formed and collected for identification of the phenotype and purity of CSCs. That is, the mammospheres were collected, dissociated by enzymatic means, and washed in PBS using gentle agitation. After fixation with 4% paraformaldehyde and permeabilization with 0.2% Triton-X100, immunostaining was undertaken by incubation with monoclonal anti-mouse/rat nestin-

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