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Large amino acid transporter 1 mediated glutamate modified docetaxel-loaded liposomes for glioma targeting

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ABSTRACT

The therapeutic outcome of glioma treatment is rigorously limited by blood–brain barrier (BBB) and infiltrating growth of glioma. To tackle the dilemma, more and more attentions were focused on developing nutrient transporters-mediated dual-targeted drug delivery system, in one side for BBB penetration, another for intracranial glioma targeting. Herein, Large amino acid transporter 1 (LAT1), overexpressed both on BBB and glioma cells, was selected as a target. Docetaxel-loaded glutamate-D-α-tocopherol polyethylene glycol 1000 succinate copolymer (Glu-TPGS) functionalized LAT1-targeting liposomes (DTX-TGL) were applied to enhance the BBB penetration and glioma therapy. The *in vivo* results of the fluorescent image indicated that TGL possessed an effective BBB penetration than that of unmodified ones in mice. The LAT1 targeting effcicacy and cell cytotoxicity of TGL were investigated in C6 glioma cells. Compared with unmodified liposomes, a significant higher cellular uptake and cell cytotoxicity was found in TGL treated group. Our results indicated that LAT1-targeting docetaxel-loaded liposome paves up a new direction using LAT1 transporter as a good target in designing brain glioma-targeting nanosystems.

1. Introduction

Blood–brain barrier (BBB) and infiltrating growth of glioma severely limited the clinical treatment of glioma therapy. Surgery therapy is unsatisfied in the glioma treatment due to the infiltrating growth of solid tumor, resulting in chemotherapy as the most common method for glioma treatment [1,2]. However, BBB prevents most of small molecule drugs and almost all macromolecule drugs from entry into the brain. Many active drugs fail in the early development phase because of poor BBB penetration ability [3,4]. To address these problems, several receptors and influx nutrient transporters highly expressed on BBB and brain tumors are employed as drug targets, including transferrin receptor, glucose transporter 1 (GLUT1), large amino acid transporter 1 (LAT1) and monocarboxylate transporter 1 (MCT1), etc.

Large amino acid transporter 1 (LAT1) is abundantly expressed both on luminal and abluminal membrane side of the capillary endothelial cells comprising of the BBB for transporting large neutral amino acids, including L-leucine, L-isoleucine, and L-phenylalanine etc. [5]. LAT1 on BBB exhibits higher affinity to the substrates (K_m = 10–50 μM) than that in peripheral tissues (K_m = 1–10 mM) [6]. Recently, it is determined that LAT1 is also overexpressed in various cancer cells, including glioma and breast tumor cells. The distribution characteristics offer LAT1 a promising target in the treatment of brain tumors.

Transporter-mediated nanoparticulate drug delivery system is a prominent brain tumor therapy strategy. Facilitative glucose transporter 1 (GLUT1) was used as a dual target expressed both on BBB and glioma cells in many researches [7,8]. 2-Deoxy-D-glucose functionalized poly(ethylene glycol)-*co*-poly(trimethylene carbonate) nanoparticles could effectively penetrate across the BBB and enhance the antitumor effects in Jiang et al. studies [7]. Choline transporter was applied as another dual target of BBB and glioma cells. Choline-derivate modified nanoparticles induced more apoptosis than unmodified ones both *in vitro* and *in vivo* [9]. Vitamin C modified PEG-PE micelles or liposomes for actively targeting sodium-dependent vitamin C transporters 2 (SVCT2)

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overexpressed in glioma cells demonstrated a better tumor accumulation [10]. There was one report on exploration of LAT1 for brain active targeting nanoparticles. Phenylalanine-coupled solid lipid nanoparticles were developed to target the LAT1 on BBB and C6 glioma cells [11]. The LAT1 targeted nanoparticles could improve the delivery of drugs to brain. However, as for substrates of LAT1, free amino group and carboxyl group are the key requirements [12,13]. In the report, the α-carboxyl group of the phenylalanine was conjugated to the hydroxyl group of DSPE-PEG, which did not meet the key requirements for LAT1 substrates and had a low affinity to LAT1. Besides, the phenylalanine tends to be entrapped in the core of solid lipid nanoparticles because of the hydrophobicity of the phenylalanine.

Based on the successful applications of transporters in brain drug delivery, LAT1 is selected as a dual target expressed both on BBB and C6 glioma cells in this study. First, γ-carboxyl group of glutamate was attached to D-α-Tocopherol polyethylene glycol 1000 succinate (Glu-TPGS), leaving free α-amino and α-carboxyl groups for LAT1 recognition and ensuring the exposure of amino acid on a surface of nanovesicles. Then, docetaxel-loaded Glu-TPGS modified LAT1-targeting liposomes (TGL) were applied to investigate the LAT1-mediated BBB penetration and C6 glioma cells targeting efficacy (Fig. 1A). After LAT1-mediated BBB penetration, TGL further enhanced the cellular internalization dependent on LAT1 in C6 glioma cells (Fig. 1B).

2. Materials and methods

2.1. Materials

TPGS and cholesterol were purchased from Aladdin (Shanghai, China). Coumarin-6 and Thiazolyl blue tetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO, USA). *N*-Cbz-glutamic acid α-benzyl ester (*Z*-Glu-Obzl) was gained from GL Biochem Ltd. (Shanghai China). Soy lecithin was provided from Shanghai Taiwei Pharmaceutical Co., Ltd. (Shanghai China). Docetaxel (DTX) was obtained from Nanjing Jingzhu Bio-technology Co., Ltd. (Tianjin, China). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was gained from Shanghai Haiqu Pharm Co., Ltd. 4-Dimethylaminopyridine (DMAP) was obtained from Shanghai Medpep Co., Ltd. Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum were purchased from Gibco (BRL, MD, USA). The NIR dye (DIR) was obtained from Fanbo biochemicals (Beijing, China). All solvents applied in this study were HPLC grade.

2.2. Synthesis of glutamate-TPGS copolymer

To synthesize the targeting copolymer, *Z*-Glu-Obzl was firstly activated by EDC and DMAP in ice-bath for 1 h. Next, TPGS dissolved in dichloromethane was added to the activated solution. The reaction was carried at 37 °C under the protection of nitrogen overnight. After purified by silica column chromatography, the Cbz and Obzl group were removed by Pd/C (10%) for 6 h under H_2 at 30 °C. The structure of the glutamate-TPGS copolymer (Glu-TPGS) was finally confirmed by ^{1}H NMR (Bruker, AV-400, Switzerland) with d-DMSO as solvent.

2.3. Preparation of Glu-TPGS and TPGS decorated liposomes

Docetaxel-loaded Glu-TPGS functionalized liposomes (DTX-TGL) and TPGS decorated liposomes (DTX-TL) were prepared by the lipid film hydration with modification. In brief, soy lecithin, cholesterol, TPGS-Glu or TPGS and docetaxel (30:1:3:1, w/w) were dissolved in dichloromethane, and then dried by a rotary evaporator to obtain a uniform lipid film. Subsequently, the lipid thin film was hydrated in ddH_2O and sonicated in an ice bath. Coumarin-6 and DIR-loaded liposomes were prepared by the same method.

2.4. Characterization of DTX-TGL and DTX-TL

2.4.1. Size, zeta potential and surface morphology

The particle size and zeta potential of DTX-TGL and DTX-TL were measured by Zetasizer (Nano ZS, Malvern, Co., U.K.). All measurements were repeated in triplicate. The morphology of liposomes was observed by transmission electron microscopy (TEM) (Tecnai G20, FEI, USA). Briefly, a drop of samples was placed on a carbon-coated copper grid, stained with 1% (w/v) phosphotungstic acid, and then visualized by TEM.

2.4.2. Encapsulation efficiency (EE) and drug-loading content (DL)

The EE of DTX-TGL and DTX-TL was measured by Sephadex G50 micro column method. Briefly, DTX-TGL and DTX-TL were added to the Sephadex G50 micro column and eluted by ddH_2O respectively. The liposomes and free DTX were separated depending on the difference of the molecular weight. The collected liposomes were further destroyed by acetonitrile to dissolve the encapsulated DTX. The content of DTX was detected by UPLC-UV at 230 nm.

For determination of drug-loading content, DTX-TL and DTX-TGL were lyophilized without any lyoprotectant and weighted. Then, the lyophilized DTX-TL and DTX-TGL were dissolved in acetonitrile and centrifuged. Finally, the DTX was analyzed by UPLC-UV at 230 nm.

2.4.3. Differential scanning calorimetry (DSC)

DSC was applied to evaluate the state of DTX in liposomes. DTX, physical mixtures of DTX and lyophilized blank liposomes, lyophilized DTX-TGL and DTX-TL with trehalose as lyoprotectant were analyzed at 30–200 °C with a rate of 10 °C/min.

2.5. In vitro *colloidal stability and dilution stability*

The *in vitro* colloidal stability of DTX-TGL and DTX-TL was evaluated in the presence of 10% fetal bovine serum DMEM medium. Both liposomes were diluted in DMEM medium containing 10% FBS at 37 °C. The size changes were measured by DLS at predetermined time point. To investigate the dilution stability of the liposomes, DTX-TGL and DTX-TL were diluted by PBS at different ratio. The size changes were also measured by DLS.

2.6. In vitro *release*

In vitro DTX release from liposomes was performed with a dialysis method. 2 mL (containing 150 μg DTX) of DTX-Solution (Taxotere), DTX-TGL and DTX-TL were sealed in a dialysis bag (MW, 14000) and immersed in 30 mL of pH 7.4 PBS containing 0.5% Tween 80 in a conical flask. The release profile studies were conducted in an incubator shaker at 100 rpm/min and 37 °C for 48 h. At predetermined intervals, 2 mL samples were withdrawn and replaced with equal volume of fresh medium. The DTX content in the released medium was determined by high-performance liquid chromatography (HPLC).

2.7. Immunofluorescence analysis of LAT1 in C6 glioma cells

Rat C6 glioma cells were cultured in DMEM medium with 10% fetal bovine serum or calf serum, 80 U/mL penicillin G sodium, 100 μg/mL streptomycin sulfate at 37 °C, 5% CO_2 and 5% humidity atmosphere.

C6 glioma cells were plated at a density of 2×10^4 cells/mL in 24-well plates for 24 h culture. Thereafter, cells were washed