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Design of Y-shaped targeting material for liposome-based multifunctional glioblastoma-targeted drug delivery

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ABSTRACT

Since the treatment of glioma in clinic has been hindered by the blood-brain barrier (BBB) and blood-brain tumor barrier (BBTB), multifunctional glioma-targeted drug delivery systems that can circumvent both barriers have received increasing scrutiny. Despite recent research efforts have been made to develop multifunctional glioma-targeted liposomes by decorating two or more ligands, few successful trials have been achieved due to the limitation of ligand density on the surface of liposomes. In this study, we designed a Y-shaped multifunctional targeting material c(RGDyK)-pHA-PEG-DSPE, in which cyclic RGD (c(RGDyK)) and p-hydroxybenzoic acid (pHA) were linked with a short spacer. Since c(RGDyK) and pHA could respectively circumvent the BBTB and BBB, c(RGDyK)-pHA-PEG-DSPE-incorporated liposomes could achieve multifunctional glioma-targeted drug delivery with maximal density of both functional moieties. c(RGDyK)-pHA-PEG-DSPE-incorporation enhanced cellular uptake of liposomes in bEnd.3, HUVECs and U87 cells, and increased cytotoxicity of doxorubicin (DOX) loaded liposomes on glioblastoma cells. c(RGDyK)-pHA-PEG-DSPE-incorporated liposomes (c(RGDyK)-pHA-LS) could deeply penetrate the 3D glioma spheroids after crossing the BBB and BBTB models *in vitro*. Moreover, *in vivo* fluorescence imaging showed the highest tumor distribution of c(RGDyK)-pHA-LS than did plain liposomes (no ligand modification) and liposomes modified with a single ligand (either c(RGDyK) or pHA). When loaded with DOX, c(RGDyK)-pHA-LS displayed the best anti-glioma effect with a median survival time (36.5 days) significantly longer than that of DOX loaded plain liposomes (26.5 days) and liposomes modified with a single ligand (28.5 days for RGD and 30 days for pHA). These results indicated that design of Y-shaped targeting material was promising to maximize the multifunctional targeting effects of liposomes on the therapy of glioma.

1. Introduction

Glioma is one of the most aggressive and poorly treated intracranial tumors with high morbidity and mortality [1–3]. As a grade IV glioma, glioblastoma multiforme (GBM) has been considered as the most devastating and lethal form of glioma characterized by extensive infiltration into the surrounding brain parenchyma [4]. Since GBM differs from other cancers by its diffuse invasion of the surrounding normal tissue, it is impossible to make the complete removal of tumor by the conventional surgical method and tumor recurrence from residual tumors is very possible [5]. Chemotherapy remains to be indispensable for glioma treatment [6]. Unfortunately, the therapeutic effect of anticancer agents is still unsatisfied because of the existence of several limitations, for example, systemic cytotoxic effects and limited drug penetration, which is attributed to the blood-brain barrier (BBB) and the blood-brain tumor barrier (BBTB) [2,7]. In addition, most of the drugs are prevented from entering the brain tumor core due to several physiologic barriers such as high cell density and increased interstitial pressure, which also influences the therapeutic efficacy [8,9]. Therefore, a novel drug delivery system which facilitates the transport of

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drug across the BBB, BBTB and glioma targeting is extremely desirable in clinic for anti-glioma therapy.

Liposomes are the most widely used drug delivery systems; however, they are avidly taken up by reticuloendothelial system (RES) cells in the case of absence of PEG on their surface. As is well known that PEG modified liposomes exhibit a long circulation property in the blood and accumulate in tumor *via* passive targeting [10–12], increasing evidence has suggested that the selectivity of PEG modified liposomes is far from satisfaction. Thus, actively targeted liposomal systems in which many ligands have been introduced to the surface of liposomes, including small-molecule ligands, peptides and monoclonal antibodies were useful tools to achieve efficient glioma treatment. Among all kinds of drug targeting strategies, peptide-based ligands have been widely exploited to facilitate glioma-targeted drug delivery for the ease of functionalization [13–16]. Those peptide ligands interact with cell surface receptors in a multivalent manner [17]. In this respect, the ligand density is an important factor to be considered. A positive correlation between the ligand density and cellular uptake has been reported *in vitro* by Kok et al. [18].

Instead of using two kinds of targeting ligands, modification with a single ligand has been drawing great attention. Meng et al. [19] developed a dual-targeted, single peptide containing an α_v integrin specific and a neuropilin-1 specific motif. The hybrid peptide exhibited two to threefold greater cellular uptake than separate α_v integrin and neuropilin-1- specific peptides *in vitro* [19].

It is well-known that RGD containing peptides can specifically bind the integrin $\alpha_v\beta_3$ receptor, generally recognized to be a tumor and angiogenesis marker [20], and RGD-peptides that are constrained in a preferred cyclic conformation show an increased affinity for integrin interaction [21]. In this regard, the cyclic RGD peptide (cRGDyK) was chosen as a candidate ligand because this peptide could selectively target integrin $\alpha_v\beta_3$ overexpressed on the tumor neovasculature as well as glioma cells [22,23]. In addition, benzamide analogues have high affinity with D1 and D2 dopamine receptors that are prominent in most parts of central nervous system [24]. In addition, plenty of research has been focused on synthesis of substituted benzamides as ligands for visualization of dopamine D2 receptor binding in the brain by positron emission tomography [25]. These benzamide analogues all exhibited great capabilities to cross the BBB [26].

Therefore, we developed a multifunctional glioma-targeted drug delivery system based on linking two targeting moieties, in which (cRGDyK) could recognize integrin $\alpha_v\beta_3$ on the BBTB and glioma cells, and the small molecule ligand (p-Hydroxybenzoic Acid, pHA) could target the BBB. The two ligands c(RGDyK) and pHA were connected *via* a linker, leading to a formation of c(RGDyK)-pHA which was covalently conjugated to the distal end of Mal-PEG_{3400}-DSPE by the Michael addition of thiol group and maleimide. The resulting Y-shaped c(RGDyK)-pHA-PEG-DSPE was incorporated into doxorubicin (DOX) loaded liposomes. The brain targeting efficiency and anti-glioma efficacy of c(RGDyK)-pHA-PEG-DSPE-incorporated doxorubicin loaded liposomes were evaluated both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

4-tert-butoxybenzoic acid was purchased from Accela ChemBio Co. Ltd. (Shanghai, China). Fmoc-cys(trt)-2-ctc resin was supplied by Shanghai Plus Bio Sci-Tech Co. Ltd. Protected Fmoc-amino acid derivatives were obtained from GL Biochem Ltd. (Shanghai, China). Diisopropylethylamine (DIPEA) was supplied by Fluka (USA). Mal-PEG_{3400}-DSPE was obtained from Laysan Bio Co. (Arab, AL). HSPC (hydrogenated soy phosphatidylcholine) and $mPEG_{2000}$-DSPE were purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). DiR (1,1-Dioctadecyl-3,3,3,3-tetramethyl indotricarbocyanine iodide), from Invitrogen, USA. Sephadex G50 and 5-carboxyfluorescein (FAM) were purchased from Sigma (St. Louis, MO). Doxorubicin hydrochloride and Daunorubicin hydrochloride, from Dalian Meilun Biotech Co., Ltd. (Dalian, China). Rat tail collagen Type I was provided by Shengyou Biological Technology Co. (Hangzhou, China). DNase I and collagenase were purchased from Dingguo Biological Technology Co. Ltd. (Shanghai, China). Human glioblastoma cells (U87), human umbilical vascular endothelial cells (HUVECs) and brain capillary endothelial cells (bEnd.3), from Shanghai Institute of Cell Biology, cultured in special Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). Diamidino-2-phenylindole (DAPI) was obtained from Roche (Switzerland). ICR mice and BALB/c nude mice aged 4–6 weeks, purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China), were kept under SPF conditions. All animal experiments were performed in accordance with the guidelines evaluated and approved by the Ethics Committee of Fudan University.

2.2. Synthesis and characterization of Y-shaped targeting material c(RGDyK)-pHA-PEG-DSPE

2.2.1. Synthesis and characterization of c(RGDyK)-pHA

The synthetic approach of c(RGDyK)-pHA is shown in supplemental data (Fig. S1 in the Supporting information). The cys(trt)-acp-4-tert-butoxybenzoic acid was synthesized *via* Fmoc-protected solid phase peptide synthesis strategy. The reaction was traced by TLC until it was completely consumed. The c(RGDyK) synthesized *via* Fmoc-protected solid phase peptide synthesis method was grafted onto the cys(trt)-acp-4-tert-butoxybenzoic acid by first activating its side chain carboxyl groups with NHS and then coupling with the c(RGDyK). For the carboxyl group activation, 1equivalent “equiv” of cys(trt)-acp-4-tert-butoxybenzoic acid and 1.25equiv of NHS were dissolved in dried DCM and treated with 1.25equiv of EDC·HCL. For the coupling of c(RGDyK), 1equiv of cys(trt)-acp-4-tert-butoxybenzoic acid/NHS and 1.2equiv of c(RGDyK) were dissolved in 5 mL anhydrous DMF. After adding DIPEA, the reaction mixture was stirred at room temperature and it was monitored by HPLC (Agilent 1100 series). Cys(trt)-acp-4-tert-butoxybenzoic acid/c(RGDyK), was then purified by preparative HPLC (Waters, 600 E). The trifluoroacetic acid-mediated removal of protecting groups leads to the formation of thiolated c(RGDyK)-pHA. The peptide purity and molecular weight were confirmed by analytic HPLC and ESI-MS spectrometry, respectively.

2.2.2. Synthesis and characterization of (RGDyK)-pHA-PEG-DSPE

The Y-shaped targeting material c(RGDyK)-pHA-PEG-DSPE was synthesized through covalent conjugation between thiolated peptide c(RGDyK)-pHA and Mal-PEG_{3400}-DSPE [27]. In brief, Mal-PEG_{3400}-DSPE dissolved in DMF was dropped into a peptide dissolved in PBS (pH = 7.2) and the reaction was monitored by HPLC. After that, we performed dialysis (MWCO 3.5 kDa) against distilled H_2O to remove the excessive peptide and confirmed by disappearance of Mal-PEG_{3400}-DSPE in the HPLC chromatogram.

2.3. Preparation and characterization of liposomes

The thin-film hydration and extrusion method was used to prepare the liposomes loaded with DOX, FAM or DiR as described previously [27]. A mixture of HSPC, cholesterol, mPEG-DSPE, c(RGDyK)-PEG-DSPE or pHA-PEG-DSPE or c(RGDyK)-pHA-PEG-DSPE were used at the molar ratio of 55:40:3:2 for ligand-modified liposomes or 0 for unmodified liposomes. They were dissolved in the chloroform, and the organic solvent was removed by rotary evaporation. The obtained film was dried in a vacuum oven at room temperature overnight to completely remove the residual organic solvent. For preparation of FAM-loaded liposomes, the thin film was hydrated with FAM solution in 60 °C water bath for 2 h, and extruded through a series of