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Paclitaxel loaded liposomes decorated with a multifunctional tandem peptide for glioma targeting

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ABSTRACT

The treatment of glioma is a great challenge because of the existence of the blood-brain barrier (BBB). In order to reduce toxicity to the normal brain tissue and achieve efficient treatment, it is also important for drugs to specifically accumulate in the glioma foci and penetrate into the tumor core after entering into the brain. In this study, a specific ligand cyclic RGD peptide was conjugated to a cell penetrating peptide R8 to develop a multifunctional peptide R8-RGD. R8-RGD increased the cellular uptake of liposomes by 2-fold and nearly 30-fold compared to separate R8 and RGD respectively, and displayed effective penetration of three-dimensional glioma spheroids and BBB model *in vitro*. *In vivo* studies showed that R8-RGD-lipo could be efficiently delivered into the brain and selectively accumulated in the glioma foci after systemic administration in C6 glioma bearing mice. When paclitaxel (PTX) was loaded in liposomes, R8-RGD-lipo could induce the strongest inhibition and apoptosis against C6 cells and finally achieved the longest survival in intracranial C6 glioma bearing mice. In conclusion, all the results indicated that the tandem peptide R8-RGD was a promising ligand possessing multi functions including BBB transporting, glioma targeting and tumor penetrating. And R8-RGD-lipo was proved to be a potential anti-glioma drug delivery system.

1. Introduction

Glioma is considered as the most aggressive primary malignant tumor of the brain. The treatment of glioma with lower side effects remains a great challenge because of the existence of several barriers. The first obstacle for a brain drug delivery system to conquer is the blood–brain barrier (BBB), which prevents nearly all large-molecule and 98% of small-molecule drugs entering the central nervous system [1–4]. Secondly, at the later stage of glioma, the blood–brain tumor barrier (BBTB) starts to form [5–7]. As most anti-tumor drugs are highly toxic to normal brain tissue, it is more important for drugs to accumulate in the glioma foci specifically after being transported across the blood–brain barrier. Finally, when drug delivery systems reach the glioma regions, 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]. Thus, researchers utilized various methods to conquer these barriers described above and achieved efficient glioma treatment. Since brain capillary endothelial cells express numerous different receptors, receptor-mediated transcytosis (RMT) is considered as one of the most common strategies among all the methods [10,11]. RMT provides a selective means for active BBB transporting and has been extensively studied for brain targeting [12–16]. However, specific ligands only have high affinity for targeted receptors and are usually not efficient enough to enhance endocytosis or solid tumor penetration [10,17,18]. On the other hand, cell penetrating peptides (CPP), a class of diverse short peptides widely used for siRNA, proteins and small molecular drugs delivery, also have the ability to carry drugs to penetrate BBB efficiently [19,20]. Nevertheless, because of their non-specific affinity to different cells, CPP-mediated brain delivery systems showed high drug distribution in the whole brain *in vivo* after systemic administration [21,22], and this property would lead to unwanted toxicity to normal brain tissues.

Thus, to overcome the impediments of both CPP and specific ligands, it is important to add a selective domain to CPP or enhance the penetrating capability of specific ligands. On this basis, a

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tandem peptide with multi functions was designed here. This kind of tandem peptide was consisted of a specific peptide in the front position and a cell penetrating peptide in the backend so that it could possess both specific targeting and high penetrating ability. Recently these multifunctional ligands have been drawing great attention [23–26]. Ren et al. [23] designed and screened a library of tandem peptides by linking LyP-1, a constant tumor penetrating domain, to a series of CPPs as vectors for siRNA delivery *in vitro*. However, current researches mainly focused on studying the structure–activity relationship of different kinds of CPP-linked tandem peptides and are lack of *in vivo* results. These tandem peptides have not been reported being used as a targeting ligand to modify nano carriers neither. In this study, cyclic RGD [c(RGDfK)] and octa-arginine (R8) were chosen as the specific and CPP domain of the tandem peptide respectively. RGD peptide is widely used as a specific ligand for integrin $\alpha_v\beta_3$ family, which is over expressed on angiogenic endothelial cells and most malignant tumor cells including glioma cells, melanoma cells, oophoroma cells and so on [27–29]. It is also reported that cyclic RGD peptide has 1000 times greater binding affinity compared to linear RGD peptide. The advantages of cyclic RGD peptide in glioma targeting drug delivery systems were already confirmed [9,30,31]. Here cyclic RGD peptide was conjugated to R8 through amide bond to obtain a tandem peptide R8-RGD [RRRRRRRR-c(RGDfK), the branch of lysine is conjugated to octa-arginine]. We used liposomes as drug carriers and paclitaxel as a model drug. Since $\alpha_v\beta_3$ integrin receptor is over expressed on both brain capillary endothelial cells and glioma cells [32], R8-RGD can be used as a promising multifunctional ligand for BBB transporting, glioma targeting and tumor penetrating. When RGD in the front position recognize $\alpha_v\beta_3$ integrin receptor on brain capillary endothelial cells, R8 in the backend can exert the high penetrating capability to mediate drug delivery systems to transport across BBB. Then R8-RGD modified liposomes can also be specifically targeted to the brain tumor region and finally delivered into the glioma core on the synergetic effect of R8 and RGD (as illustrated in Fig. 1). We evaluated the cellular uptake efficiency, the BBB penetrating and brain tumor penetrating capability of R8-RGD peptide *in vitro*, studied the brain targeting properties *in vivo* systemically, and investigated the therapeutic efficacy of paclitaxel loaded R8-RGD modified liposomes.

2. Materials and methods

2.1. Materials

Cyclic RGD peptide with a terminal cysteine [Cys-c(RGDfK), cysteine conjugated to the branch of lysine] and R8-RGD peptide with a terminal cysteine [Cys-RRRRRRRR-c(RGDfK), cysteine modified octa-arginine conjugated to the branch of lysine] were synthesized according to the standard solid phase peptide synthesis by Chinapeptides Co. Ltd. (Shanghai, China). R8 peptide with a terminal cysteine (Cys-RRRRRRRR) was synthesized according to the standard solid phase peptide synthesis by Chengdu Kai Jie Bio-pharmaceutical Co. Ltd. (Chengdu, China). Soybean phospholipids (SPC) were purchased from Shanghai Taiwei Chemical Company (Shanghai, China). Cholesterol was purchased from Chengdu Kelong Chemical Company (Chengdu, China). DSPE-PEG_{2000}, DSPE-PEG_{2000}-Mal and 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein) (CFPE) were purchased from Avanti Polar Lipids (USA). Paclitaxel (PTX) was purchased from AP Pharmaceutical Co. Ltd. (Chongqing, China). 4′-6-diamidino-2-pheylindole (DAPI) and 3-(4, 5-Dimethylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Beyotime Institute Biotechnology (Haimen, China). Rabbit anti-mouse β-actin and β_3 integrin primary antibodies were purchased from Epitomics, Abcam (California, USA). Horseradish peroxidase (HRP) -labeled goat anti-rabbit secondary antibodies was purchased from ZSGB-BIO (Beijing, China). Poly-lysine, sodium azide, amiloride, chlorpromazine and filipin were obtained from Sigma–Aldrich (St. Louis, MO, USA). Lyso-tracker™ was purchased from Invitrogen (Carlsbad, CA, USA). 1, 10-Dioctadecyl-3, 3, 30, 30-tetramethylindotricarb-ocya-nine iodide (DiR) and 1, 10-dioctadecyl-3, 3, 30, 30-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD) were purchased from Biotium (USA). Annexin V-FITC/PI apoptosis detection kit was obtained from KeyGEN Biotech (China). Millicell Hanging Cell Culture Inserts were purchased from Millipore (USA). Plastic cell culture dishes and plates were purchased from Wuxi NEST Biotechnology Co. (Wuxi, China). The other chemicals were obtained from commercial sources.

Balb/c mice were purchased from West China animal center of Sichuan University (Sichuan, China). All animal procedures for this study were approved by the Experiment Animal Administrative Committee of Sichuan University.

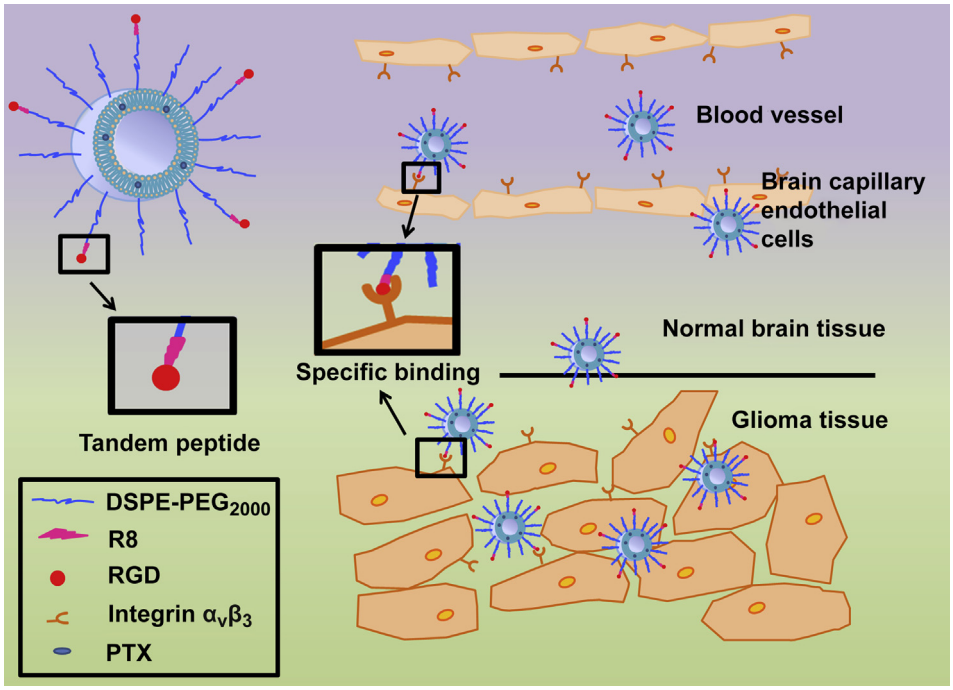

Fig. 1. Schematic illustration of PTX-loaded R8-RGD modified liposomes (PTX-R8-RGD-lipo). Liposomes could specifically bind to integrin $\alpha_v\beta_3$ receptors expressed on the brain capillary endothelial cells and transport across the BBB through a synergetic effect. Then the liposomes could accumulate in the glioma tissue selectively, penetrate into the core region of tumor and release drugs.