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LDLR-mediated peptide-22-conjugated nanoparticles for dual-targeting therapy of brain glioma

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ABSTRACT

Chemotherapy for brain glioma has been of limited benefit due to the inability of drugs to penetrate the blood–brain barrier (BBB) and non-selective drug accumulation in the entire brain. To obviate these limitations, dual-targeting paclitaxel-loaded nanoparticles were developed by decoration with peptide-22 (PNP–PTX), a peptide with special affinity for low-density lipoprotein receptor (LDLR), to transport the drug across the BBB, and then target brain tumour cells. Enzyme-linked immune sorbent assay (ELISA) revealed that LDLR was over-expressed in C6 cells and brain capillary endothelial cells (BCECs), but low LDLR expression was observed in H92c(2-1) cells. Nanoparticle uptake demonstrated that peptide-22-decorated nanoparticles significantly increased the cellular uptake of nanoparticles by C6 cells and BCECs but not by H92c(2-1) cells, and excess free peptide-22 significantly inhibited the cellular uptake of PNP by C6 cells and BCECs. Cellular uptake mechanism experiments showed that PNP uptake by both BCECs and C6 cells was energy-dependant and caveolae- and clathrin-mediated endocytosis pathway other than macropinocytosis were involved. Dual-targeting effects in an in vitro BBB model showed that peptide-22 decoration on nanoparticles loaded with paclitaxel significantly increased the transport ratio of PTX across the BBB and induced apoptosis of C6 glioma cells below the BBB, and these effects were significantly inhibited by excess free peptide-22. Ex vivo and in vivo fluorescence imaging indicated that PNP labelled with a near-infrared dye could permeate the BBB and accumulate more in the glioma site than unmodified NP. Glioma section observed by fluorescence microscopy further demonstrated PNP distributed more extensively in both glioma bulk and infiltrative region around than unmodified NP. Pharmacodynamics results revealed that the median survival time of glioma-bearing mice administered with dual-targeting PNP–PTX was significantly prolonged compared with that of any other group. TUNEL assay and H&E staining showed that PNP–PTX treatment induced significantly more cell apoptosis and tumour necrosis compared with other treatments. Taken together, these promising results suggested that the dual-targeting drug delivery system might have great potential for glioma therapy in clinical applications.

1. Introduction

Glioblastoma multiforme (GBM), the most frequent primary central nervous system tumour, has an extremely poor prognosis due to its highly infiltrating nature [1,2]. Those invasive glioma cells are far from the primary tumour or are even in the contralateral hemisphere and hide in areas of the brain that are protected by an intact blood–brain barrier (BBB) [3,4]. Conventional surgical methods cannot completely remove the tumour cell [5,6], and an inevitable relapse always follows. Consequently, a crucial challenge is to deliver therapeutic agents effectively to the tumour core and migratory cells in the infiltration zone [4]. However, existing chemotherapy drugs fail to elicit the desired benefit and are associated with serious adverse effects, largely due to their inability to cross the BBB and untargeted accumulation in healthy tissues [7]. The key point in chemotherapy is to maintain a high concentration of therapeutic agents at the tumour site and prevent their spread

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into the surrounding normal tissues [8]. With this in mind, polymer nanoparticles functioning as a versatile targeting platform are emerging as a good option to address specific existing limitations of conventional chemotherapy [9–11]. Furthermore, dual-targeting nanoparticles targeting both the BBB and glioma tissues functioned better than those targeting each region alone because they could specially deposit in the glioma region whether or not the BBB is compromised [12], and varying degrees of success have been obtained based on the dual-targeting system for glioma therapy [10,13–15].

Low-density lipoprotein receptor (LDLR), a member of the LDLR family, is highly expressed at the BBB [16–18] and has been exploited to transport protein therapeutics across the BBB to reach the CNS [19,20]. Furthermore, LDLR is also over-expressed in a variety of tumour cells, including glioma cells [21–23] but is sub-expressed in normal brain tissues [24,25], making LDLR a potential targeted receptor for brain tumour drug delivery systems, with dual-targeting capability for both the BBB and glioma cells. Although many studies have exploited LDLR as a target for tumour diagnosis [26–28] and treatment [21,29–31], few have extensively explored LDLR as a potential receptor for dual-targeting therapy of brain glioma.

Some targeting moieties such as Apo-B [23], Apo-E [21] and peptides derived from the LDLR binding site of Apo-B [20] or Apo-E [19,32] have been used and have been proven to be effective in LDLR targeted therapy of neurodegenerative disease in CNS or neoplastic diseases. However, they are less than ideal as targeting moieties because of some inherent disadvantages such as protein instability, competition with endogenous LDL as well as the potential risk of disturbing cholesterol homoeostasis in the brain. Recently, phage display biopanning performed by Jean-Daniel Malcor generated a series of peptides, from which peptide-22 (Ac–[cMPRLRGC]c–NH_2) was optimised to show special affinity for LDLR without competition with endogenous LDL and could be efficiently and quickly transferred to the CNS [30]. Therefore, we proposed that the peptide-22–LDLR interaction could be utilised to promote drug delivery across the BBB and simultaneously target brain tumours. Compared with those LDLR-targeting moieties mentioned above, peptide-22 harbours several advantages such as a low molecular weight, good stability, easy synthesis at a relative low cost, lack of immunogenicity [29,31] and even with no competition with endogenous LDL, and may function better in targeting drug delivery [30]. Thus, in the present work, peptide-22 was utilised as a dual-targeting moiety to modify nanoparticles for brain glioma drug delivery.

Paclitaxel (PTX), a major anticancer drug isolated from the bark of *Taxus brevifolia*, showed antitumour activity against various solid tumours such as ovarian cancer, lung cancer [33,34] and glioma [13,35]. However, the therapeutic index of PTX is extremely limited due to its poor aqueous solubility, non-targeted tumouricidal effects and serious side effects associated with its solvent Cremophor EL–ethanol [36]. The therapeutic benefit of PTX against brain tumours could also be compromised by tumour drug-resistance and the inability to readily penetrate the BBB to reach the tumour cells [37,38]. Therefore, a new glioma drug delivery system encapsulating PTX is urgently needed to improve its efficacy and decrease its adverse toxicity.

The objective of the present study was to prepare a dual-targeting drug delivery system, peptide-22-decorated nanoparticles (PNPs). Strongly liposoluble fluorescent probes, courmarin-6 and DiR are used to label PNPs to trace the behaviour of PNPs for their inertia to be released from the polymer nanoparticles [10,39–41]. Dual-targeting delivery properties of PTX-loaded or fluorescence-labelled PNP were evaluated in vitro and in vivo. The in vivo anti-glioma efficacy of PNP–PTX was also investigated using an intracranial glioma mice model.

2. Materials and methods

2.1. Materials and animals

Peptide-22 (NH_2–C6–[cMPRLRGC]c–NH_2) was synthesised by the Chinese Peptide Company (China). Methoxy-poly(ethylene glycol) (MPEG, M_w 3000 Da) was supplied by NOF Corporation (Japan) and R-carboxyl-poly(ethylene glycol) (COOH-PEG, M_w 3400 Da) was obtained from Laysan Bio (AL, USA). D,L-Lactide (purity: 99.5%) was purchased from PURAC (Holland). Methoxy-poly(ethylene glycol)–poly(lactic acid) (MPEG–PLA, M_w 33,000 Da) and R-carboxyl-poly(ethylene glycol)–poly(lactic acid) (COOH-PEG–PLA, M_w 33,400 Da) block copolymers were synthesised by ring-opening polymerisation of lactide using MPEG and HOOC-PEG as the initiator as described elsewhere [42]. Sodium cholate was from Shanghai Chemical Reagent Company. Rat LDLR Elisa Kit was purchased from Shanghai Jianglai Biotechnology Co., Ltd. Courmarin-6, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) and N-hydroxy-succinimide (NHS) were purchased from Sigma (USA). 1,10-Dioctadecyl-3,3,30,30-tetramethylindotricarbocyanine iodide (DiR), a near-infrared dye, was obtained from Biotium (Invitrogen, USA). The annexin V-FITC apoptosis detection kit, micro-BCA protein assay kit, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), radio-immunoprecipitation assay (RIPA) and Hoechst 33342 were purchased from Beyotime® Biotechnology Co., Ltd. (Nantong, China). Cellulose ester membranes (dialysis bags) with a molecular weight cut-off value (MWCO) of 8000 Da (Green Bird Inc., Shanghai, China) were used in dialysis experiments. Plastic cell culture dishes and plates were purchased from Corning Incorporation (USA). Dulbecco's modified Eagle's medium (high glucose) (DMEM), foetal bovine serum (FBS), trypsin–EDTA (0.25%) and penicillin–streptomycin were purchased from Gibco (CA). Purified deionised water (Millipore, Bedford, MA) was used throughout the entire study. All other reagents and chemicals were of analytical grade and were purchased from Sinopharm Chemical Reagent (Shanghai, China). Brain capillary endothelial cells (BCECs), C6 cell lines and H9c2(2-1) cells were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). The cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Male BALB/c nude mice and BALB/c mice (20 ± 2 g) were purchased from the Shanghai SLAC Lab Animal Ltd. (Shanghai, China) and maintained under standard housing conditions. All animal experiments were performed in accordance with protocols evaluated and approved by the ethics committee of Fudan University.

2.2. Preparation of unmodified nanoparticles and PNP

Unmodified PEG–PLA nanoparticles (NPs) were prepared using an emulsion/solvent evaporation technique [11] with few modifications. In brief, MPEG–PLA (28 mg) and COOH-PEG–PLA (2 mg) were dissolved in 1 ml of dichloromethane, and then were added into 5 ml of 0.6% sodium cholate aqueous solution. The mixture was intensively emulsified by sonication (200 w, 5 s) fifteen times in ice water using a probe sonicator (Scientz Biotechnology Co., Ltd., China). After evaporating dichloromethane with a ZX-98 rotary evaporator (Shanghai Institute of Organic Chemistry, China) at 37 °C, the obtained nanoparticles were concentrated by centrifugation at 14,500 rpm for 45 min using a TJ-25 centrifuge (Beckman Counter, USA). After discarding the supernatant, the nanoparticles were resuspended in 0.5 ml of deionised water. PTX-loaded, coumarin-6- or DiR-labelled NPs were prepared using the same procedure except that 2 mg PTX, 30 μg of coumarin-6 or 200 μg of DiR were dissolved in 1 ml of dichloromethane in advance.

Peptide-22 was conjugated to the surface of NPs using an EDC/NHS technique [9]. In brief, NP was suspended in deionised water and incubated with excess EDC (200 mM) and NHS (100 mM) at room temperature for 30 min. The resulting N-hydroxysuccinimide-activated NP was then centrifuged at 14,500 rpm for 45 min to remove the residual EDC and NHS. The activated NP was allowed to react with 20 μg of peptide-22 under magnetic stirring for 4 h. Thereafter, the covalently linked PNP was concentrated by centrifugation at 14,500 rpm for 45 min to remove free peptide-22. PTX-loaded, coumarin-6- and DiR-labelled PNP were prepared using the same procedure.

2.3. Characterisation of PNP

Particle size and zeta potential of nanoparticles were determined by dynamic light scattering using a zeta plus analyser (Zeta-sizer, Malvern nano zs, U.K.). The morphology of nanoparticles was observed using a transmission electron microscope (H-600; Hitachi, Japan) after negative staining with 2% sodium phosphotungstate solution.

2.4. Determination of peptide-22 conjugation efficiency and peptide-22 density on the nanoparticle surface

The concentration of peptide-22 in the supernatant was determined using an HPLC system (Agilent 1200 series; USA) with an analytical column (150 mm × 4.6 mm; pore size 5 μm; ZORBAX 300SB-C_{18}; Agilent). The mobile phase contained a mixture of solvent A (0.1% trifluoroacetic acid in water) and solvent B