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In vitro and *in vivo* intracellular distribution and anti-glioblastoma effects of docetaxel-loaded nanoparticles functioned with IL-13 peptide

Huile Gao, Shuang Zhang, Zhi Yang, Shijie Cao, Xinguo Jiang, Zhiqing Pang *

Key Laboratory of Smart Drug Delivery (Fudan University), Ministry of Education, Department of Pharmaceutics Sciences, School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai 201203, China

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ABSTRACT

An active targeting delivery system helps increase intracellular drug delivery, which is promising for the treatment of glioblastoma. Interleukin 13 (IL-13) peptide which was derived from IL-13 protein could specially bind with IL-13Rα2, a receptor highly expressed on glioblastoma cells but not on normal brain cells, suggesting IL-13 peptide is an optional ligand for glioblastoma targeted therapy. In this contribution, IL-13 peptide was functionalized to nanoparticles (ILNP) to form a glioblastoma targeted drug delivery system where docetaxel was used as a model drug. The cellular uptake and intracellular delivery of ILNP indicated that IL-13 peptide facilitated cellular uptake of nanoparticles and Golgi apparatus was involved in the sorting and trafficking of ILNP rather than NP in U87 cells. Transmission electron microscopy observation revealed that ILNP mainly distributed into endosomes, cytoplasm and Golgi apparatus. *In vitro* apoptosis assay indicated docetaxel-loaded ILNP could induce polymerization of microtubules and produce the highest early and late apoptosis of U87 cells. Growth inhibition results of tumor spheroids demonstrated ILNP displayed the best growth inhibition of tumor spheroids. *In vivo* imaging suggested that ILNP accumulated significantly more in the glioma site than NP while more NP was distributed in liver, lung and spleen than ILNP. Transmission electron microscopy further demonstrated ILNP could distribute into different organelles of cells in glioma site. Thus, docetaxel loaded ILNP could induce the most apoptosis of glioma cells which was demonstrated by TUNEL. In conclusion, we presented a glioblastoma-targeting drug delivery system ILNP, which could increase the intracellular delivery of nanoparticles as well as precisely target to glioblastoma cells, and significantly increase the anti-proliferation and anti-spheroid growth effect.

1. Introduction

Brain cancer, one of the top ten causes of death by cancers, remains a serious threat and is largely incurable. Glioblastoma multiforme (GBM) is the most frequent primary malignant brain cancer (Zhan et al., 2010), with less than 5% of patients survival for five years post diagnosis (Agarwal et al., 2011). Surgery followed by chemoradiotherapy was a frequent method for this cancer. However, the unsharp boarder and proximity to critical functional regions make it hardly to completely remove tumor and recurrence is probable (Ong et al., 2009; Zhan et al., 2010). As a widely used chemotherapeutics, structurally similar to paclitaxel, docetaxel (DTX) is more effective as an inhibitor of microtubule depolymerization (Gueritte-Voegelein et al., 1991). It was demonstrated DTX could significantly inhibit brain tumor growth by local injection (Sampath et al., 2006). However, hydrophobic property restricted its use. Presently, commercial formulation was made by tween-80 and ethanol 1:3 (v/v), which should be diluted by saline or 5% dextrose solution before injection. As the main adjuvant, polysorbate 80 shares side effects like acute hypersensitivity reactions and peripheral neuropathy (Ten et al., 2003; Upadhyay et al., 2010; Zhao et al., 2010).

The development of nanotechnology presented a promising method for hydrophobic chemotherapeutics. In our previous experiments, nanoemulsion was utilized to encapsulate DTX and displayed better anti-GBM effect and lower side effects (Gao et al., 2012a). However, conventional nanoformulations could only distribute into tumors by enhanced penetration and retention (EPR) effect. Nanoformulations functioned with specific ligands could target to cancer cells and increase intracellular drug delivery

* Corresponding author at: School of Pharmacy, Department of Pharmaceutics, 615 Room, Research Building, 826, Shanghai, China. Tel.: +86 21 51980069; fax: +86 21 51980069.
E-mail address: zqpang@fudan.edu.cn (Z. Pang).

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through specific receptor- or carrier- mediated endocytosis. Interleukin 13 (IL-13) is an inflammatory cytokine. One of its receptors, IL-13Rα2 binds IL-13 with high affinity and is a tumor-specific receptor that overexpressed in GBM (Madhankumar et al., 2006,2009; Shultz et al., 2010). Several successful attempts to utilize IL-13 to enhance GBM therapy have been reported (Madhankumar et al., 2006). However, full length protein was usually related to immunogenicity and competition with endogenous ligands. More importantly, there are several receptors could bind with IL-13 protein, but only IL-13Rα2 was highly expressed on GBM cells, and full length IL-13 protein may lead to homing to untargeted cells and cause unexpected side effects. IL-13 peptide which was derived from IL-13 protein could specially bind with IL-13Rα2 (Madhankumar et al., 2004). Compared with IL-13 protein, IL-13 peptide harboring several advantages such as a low molecular weight, good stability, easy synthesis at a relatively low cost, and lack of immunogenicity may display better GBM-targeting effect than full length protein. In our previous reports, the nanoparticles modified with the IL-13 peptide could selectively increase cellular uptake, facilitate cell internalization, alter the uptake pathway and increase GBM localization in GBM-bearing mice (Gao et al., 2013a). More interestingly, IL-13 peptide could possess cell-penetrating properties that could specifically enhance the uptake by tumor cells compared with endothelial cells and it could also significantly improve the speed and rate of nanoparticles penetration from vessels to tumor cells in subcutaneous U87 tumors (Gao et al., 2013b). Thus, drug-loaded nanoparticles anchoring with IL-13 peptide may greatly improve the anti-GBM effects of chemotherapeutics which has little been shown in our previous research. Moreover, intracellular delivery and subcellular fate of IL-13 peptide-functioned nanoparticles was rarely known, which is extremely important for understanding the activity of intracellular cargos and potential toxicity of nanocarriers.

In this study, IL-13 peptide was conjugated to nanoparticles (NP) to form a GBM targeting platform (ILNP) where DTX was used as a model drug. The cell uptake and intracellular delivery of ILNP were investigated using coumarin-6 as a fluorescent probe. The pharmacokinetics, tumor and tissue distribution of ILNP were also presented by *in vivo* imaging. Subcellular fate of ILNP in U87 cells and *in vivo* internalization of ILNP in tumor site were visualized under transmission electron microscopy. Finally, *in vitro* anti-GBM effects were carried out using DTX-loaded ILNP.

2. Materials and methods

2.1. Materials

IL-13 peptide (TAMRAVDKLLLHLKKLFREGQFNRNFESIIICRDRT) was synthesized by the Sangon Biotech Co., Ltd. (Shanghai, China). Methoxy poly(ethylene glycol)-poly(ε-caprolactone) (MPEG-PCL) (Mw: 3–15k) and R-carboxyl poly(ethylene glycol)-poly(ε-caprolactone) (HOOC-PEG-PCL) (Mw: 3.4–15k) were synthesized as previously described (Pang et al., 2008). 4,6-Diamidino-2-phenylindole (DAPI) was purchased from Beyotime (Haimen, China). Coumarin-6, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), and N-hydroxy-succinimide (NHS) was purchased from Sigma (USA). DTX was purchased from Knowshine (China). Docetaxel (DTX) was purchased from Knowshine (Shanghai, China). DiR was obtained from Biotium (Hayward, CA). Super-paramagnetic iron oxide (SPIO, 15 nm) was kindly gifted by Dr. Shun Shen, Fudan University. Goat anti-tubulin was purchased from Santa Cruz (USA) and Alexa Fluor 488-conjugated donkey anti-goat IgG was obtained from Cell Signaling (USA). The U87 cell line was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Plastic cell culture dishes and plates were purchased from Wuxi NEST Biotechnology Co., Ltd. (Wuxi, China). Dulbecco's Modified Eagle Medium (high glucose) cell culture medium (DMEM) and fetal bovine serum (FBS) was purchased from Gibco (CA). All the other chemicals were analytical reagent grades, purchased from Sinopharm Chemical Reagent (China).

BALB/c nude mice (male, 4–5 weeks, 18–22 g) were obtained from the Shanghai Slac Laboratory Animal Co., Ltd. (China) and maintained under standard housing conditions. All animal experiments were carried out in accordance with protocols evaluated and approved by the ethics committee of Fudan University.

2.2. Preparation and characterization of DTX-loaded NP

NP was prepared using a blend of diblock copolymers MPEG-PCL and HOOC-PEG-PCL by a single emulsion evaporation method (Gao et al., 2011). Briefly, the solution, 1 mg of DTX, 28 mg of MPEG-PCL and 2 mg of HOOC-PEG-PCL in 1 mL of dichloromethane (DCM) were added into 5 mL of 0.6% sodium cholate hydrate solution, and then subjected to pulse sonication for 75 s at 200 W in iced water bath. After removing DCM by rotary evaporation, NP were condensed and diluted in pH 6.0 MES containing 200 mM EDC and 100 mM NHS to activate carboxyl group of NP for 0.5 h. After MES buffer was replaced with pH 7.4 PBS by passing Hitrap™ desalting column, 10 μg of IL-3 peptide were added into the NP suspension and stirred for 6 h in the dark. The free peptide was removed by sepharose CL-4B column elution. SPIO-, courmarin-6 or DiR-loaded NP and ILNP were prepared as described above except replacing 1 mg of DTX with 1 mg of SPIO, 30 μg of courmarin-6 or 500 μg of DiR.

The particle sizes and zeta potentials were determined by a Malvern Zeta Sizer (Malvern, NanoZS, UK). The ILNP morphology was examined by transmission electronic microscopy (TEM) after staining with 2% (w/v) phosphotungstic acid solution.

2.3. Drug encapsulation efficiency and loading capacity

The drug encapsulation efficiency (EE) and loading capacity (LC) of DTX-loaded NP (NP-DTX) and DTX-loaded ILNP (ILNP-DTX) were determined as previously described (Gao et al., 2012b). The amount of DTX was measured by the HPLC system (Agilent 1200, USA) equipped with an analytical column (150 mm × 4.6 mm, pore size 5 μm, Diamonsil™, Dikma). The mobile phase was a mixture of CH_3CN:H_2O (50:50, v/v) at a flow rate of 1.0 mL/min. The sample injection volume was 20 μL and the detection wavelength was 230 nm. The encapsulation efficiency (EE) was calculated as EE = Drug encapsulated in nanoparticles/Total drug added × 100% and drug loading capacity (LC) as LC = Drug encapsulated/Total materials × 100%.

2.4. In vitro *release profiles*

The *in vitro* release behaviors of DTX from NP-DTX and ILNP-DTX were evaluated by a dialysis method using PBS (0.01 M, pH 7.4 and pH 5.0) as the release medium (Gao et al., 2012a). For the experiment, 1 mL of the DTX formulation (containing 10 mg of nanoparticles) was added into a dialysis bag (MWCO 8000 Da; Green bird Inc., Shanghai, China) and immersed into 10 mL of release medium at 37 °C at the shaking speed of 120 rpm for 48 h. At each pre-set time point, 0.3 mL of aliquot was withdrawn and, immediately, an equal volume of fresh release medium was added. The amount of DTX released was determined by the same method mentioned above.

2.5. Cell uptake

U87 cells in the logarithmic growth phase were seeded into 6-well plates at a density of 2×10^4 cells in 1 mL of DMEM containing 10% FBS per well. Twenty four hours later, the culture medium was