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Delta-like ligand 4-targeted nanomedicine for antiangiogenic cancer therapy

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ABSTRACT

Tumor angiogenesis is a multistep process involved with multiple molecular events in cancer microenvironment. Several molecular-targeted agents aiming to suppress tumor angiogenesis have been successfully translated into cancer clinic. However, new strategies are still urgently desired to be excavated to overcome the poor response and resistance in some antiangiogenic therapies. Recently, Delta-like ligand 4 (Dll4) is identified to be specifically over-expressed on tumor vascular endothelial cells (EC), and the Dll4-Notch pathway serves as a critical regulator in the development and maintenance of tumor angiogenesis. The intensively up-regulated phenotype of Dll4 on the membrane of tumor vascular EC implies that Dll4 may act as a targetable address for drug delivery system (DDS) to achieve targeted antiangiogenic cancer therapy. Here, a nano-DDS, GD16 peptide (H_2N-GRCTNFHNFIYICFPD-$CONH_2$, containing a disulfide bond between Cys_3 and Cys_{13}) conjugated nanoparticles loading paclitaxel (GD16-PTX-NP), which can specifically target the angiogenic marker Dll4, was fabricated for the investigation of antiangiogenic therapeutic efficacy in human head and neck cancer FaDu (Dll4-negative) xenograft in nude mice. The results demonstrate that GD16-PTX-NP achieved controlled drug release and exhibited favorable *in vivo* long-circulating feature. GD16-PTX-NP exerted enhanced antiangiogenic activity in the inhibition of human umbilical vein endothelial cell (HUVEC) viability, motility, migration, and tube formation, and in the Matrigel plug model as well, which can be definitely ascribed to the active internalization mediated by the interaction of GD16 and the over-expressed Dll4 on EC. GD16-PTX-NP showed accurate *in vivo* tumor neovasculature targeting property in FaDu tumor, where the paclitaxel was specifically delivered into the tumor vascular EC, leading to significant apoptosis of tumor vascular EC and necrosis of tumor tissues. The antiangiogenic activity of GD16-PTX-NP significantly contributed to its *in vivo* anticancer efficacy in Fadu tumor; moreover, no overt toxicity to the mice was observed. Our research firstly presents the potency and significance of a Dll4-targeted nanomedicine in antiangiogenic cancer therapy.

1. Introduction

Angiogenesis is responsible for the growth and metastasis of most types of tumors. Numerous signaling molecules and pathways have been found to be involved in human tumor neovascularization, and some of them have been successfully used as antiangiogenic target to enhance the effect of chemotherapy in inhibiting tumor growth and extending survival [1–5]. Inhibiting tumor angiogenesis is a well validated major strategy in cancer therapy. However, most current antiangiogenic therapies focus on targeting the vascular endothelial growth factor (VEGF) pathway [3,6]; poor response in some circumstances and emerging resistance necessitate the search for new strategies to combat the tumor angiogenesis [7–10]. It is now recognized that Delta-like ligand 4 (Dll4), one ligand of the Notch receptors, is highly and selectively expressed on the surface of tumor vascular EC [11–13]. A series of studies have indicated that Dll4-Notch signaling pathway is critical to the development and maintenance of tumor angiogenesis, and the interventions that hinder Dll4-Notch signaling can produce

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vascular networks with increased ramifications but with reduced blood perfusion, ultimately leading to the inhibition of tumor growth [14–17]. Moreover, Dll4 blockade has shown potent growth inhibitory effects on some tumors that are resistant to VEGF inhibition [14,15,18].

Antiangiogenic cancer therapy based on nanoparticulate drug delivery systems (nano-DDS) is emerging as a promising new approach besides the proved molecular-targeted antiangiogenic agent (Avastin, Nexavar, Votrient, Sutent, Caprelsa, Inlyta, Zaltrap, etc.) [19]. It is reasonably speculated that the specifically high expressed Dll4 on the EC of tumor vasculature may present a potential targetable address for nano-DDS in antiangiogenic cancer therapy. However, to our knowledge there has been no report regarding the Dll4-targeted nanomedicine in antiangiogenic cancer therapy. In this study, a new nanomedicine, GD16-conjugated, Dll4-targeted nanoparticles loading paclitaxel (GD16-PTX-NP), was developed for antiangiogenic therapy of FaDu (a Dll4-negative human head and neck cancer cell line) xenograft in nude mice. Here, GD16 was a peptide (H_2N-GR**C**TNFHNFIYI**C**FPD-$CONH_2$, containing a disulfide bond between Cys_3 and Cys_{13}) recently designed to bind Dll4 over-expressed in tumor neovasculature with high affinity and specificity [20]. The model drug paclitaxel is a promoter of microtubule polymerization and exhibit potent antiangiogenic activity [21–23]. The targeting delivery and antiangiogenic ability of GD16-PTX-NP *in vitro* and *in vivo*, and its potential therapeutic efficacy in Dll4-negative FaDu xenograft were investigated.

2. Materials and methods

2.1. Materials, cell lines and animals

Aldehyde poly (ethylene glycol)-poly (lactide) (aldehyde-PEG-PLA, MW 64 kDa) and MPEG-PLA (MW 61 kDa) block copolymers were synthesized by the ring opening polymerization in our lab as previously described [24]. Paclitaxel (PTX) was purchased from Knowshine Pharmachemicals (Shanghai, China). GD16 peptide was synthesized by GL Biochem (Shanghai, China). Coumarin 6, filipin, phenylarsine oxide, cytochalasin D, nystatin, chlorpromazine, and nocodazole were from Sigma Aldrich (St. Louis, MO). Double distilled water was purified using a Millipore Simplicity System (Millipore, Bedford, MA). All other chemicals were of analytical grade and used without further purification.

Primary human umbilical vein endothelial cells (HUVEC) and VascuLife VEGF Medium Complete Kit were from Lifeline Cell Technology (Frederick, MD). The cells at 3 to 5 passages were used in the experiments. Human head and neck cancer FaDu cell line was obtained from the American Type Culture Collection (Manassas, VA) and cultured in MEM medium supplemented with 10% fetal bovine serum, 1 × non-essential amino acids (Life Technologies, Carlsbad, CA) and sodium pyruvate (0.11 g/L) at 37 °C in a humidified incubator with 5% CO_2.

Female Sprague Dawley (SD) rats (180–200 g) and female BALB/c mice (~20 g) were provided by the Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China). The animal experiment designed in this study was approved by the ethical committee of Shanghai Jiao Tong University School of Medicine.

2.2. Preparation and characterization of GD16-PTX-NP

GD16-PTX-NP were fabricated by emulsion and solvent evaporation method with a following surface functionalization. Briefly, 3 mg PTX was dissolved in 1 ml solution of 30 mg blend of aldehyde-PEG-PLA and MPEG-PLA (1:9, w/w) in dichloromethane. Then, 3 ml of 1% (w/v) sodium cholate was slowly poured into the solution and then the mixture was sonicated at 280 w for 25 s (Scientz Biotechnology, Ningbo, China). The O/W emulsion was further diluted in 40 ml of 0.5% (w/v) sodium cholate solution and the organic solvent was removed by rotary evaporation under reduced pressure. The resulting PTX-loaded nanoparticles (PTX-NP) were collected by centrifugation (11,000 × g, 30 min, 4 °C; Eppendorf AG 5810R, Germany) and washed twice to remove the excessive emulsifier. Then, PTX-NP was incubated with GD16 at a 1:3 molar ratio of aldehyde to the N-terminal amine of GD16. The conjugation reaction was processed in 0.01 M PBS (pH 7.4) at room temperature for 10 h in the presence of $NaCNBH_3$ as a reducing reagent. The unconjugated GD16 was removed by centrifugation (11,000 × g, 30 min, 4 °C) and GD16-PTX-NP were collected. The coumarin 6-labeled or DiR-labeled nanoparticles were prepared in the same way except that in the oil phase PTX was mixed with 0.05% (w/v) coumarin 6 or 0.2% (w/v) DiR, respectively.

The particle size and zeta potential were determined using a Zetasizer Nano ZS instrument (Malvern, Worcestershire, UK). The nanoparticles were negatively

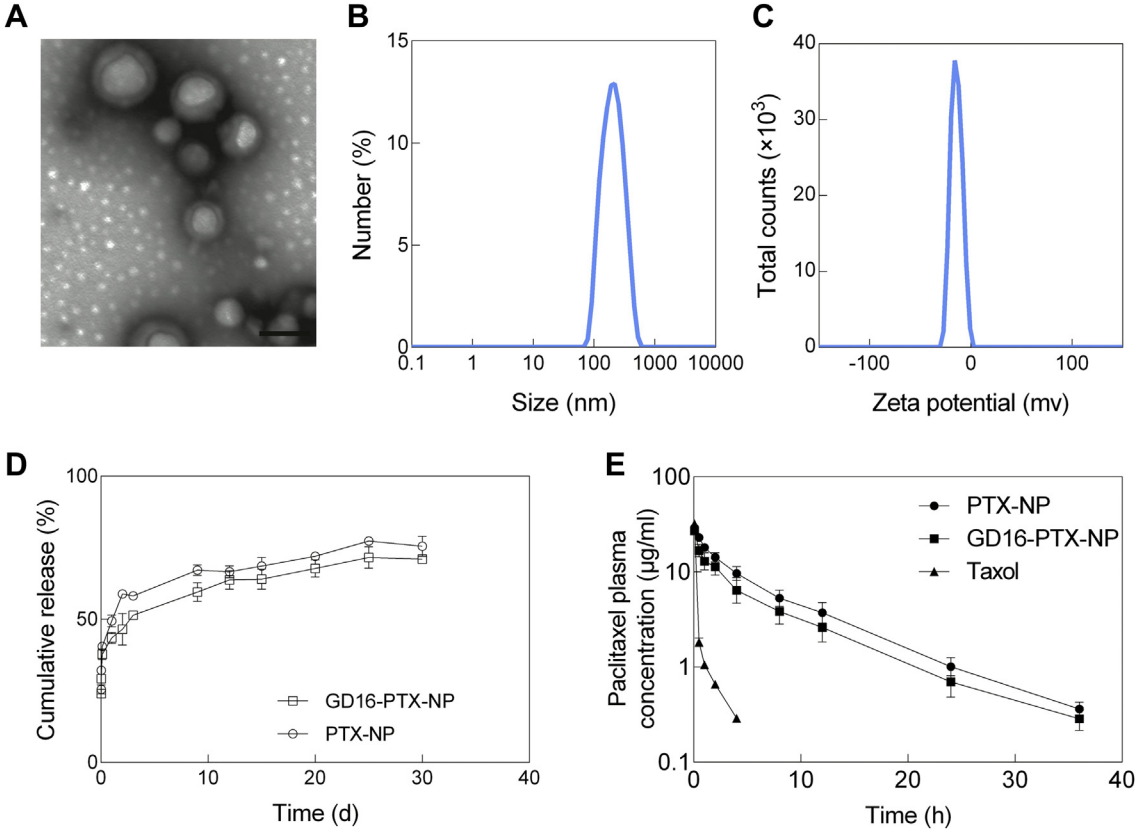

Fig. 1. Characterization of GD16-PTX-NP. A, TEM photograph of GD16-PTX-NP. Bar, 100 nm. B, Dynamic light scattering size distribution. C, Zeta potential. D, PTX release from nanoparticles in PBS (pH 7.4). E, Blood clearance kinetics in SD rats after a single intravenous injection of free drug or nanoparticles at PTX dose of 4 mg/kg body, $n = 5–6$.