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Peptide-conjugated biodegradable nanoparticles as a carrier to target paclitaxel to tumor neovasculature

De-Hong Yu, Qin Lu, Jing Xie, Chao Fang*, Hong-Zhuan Chen*

Department of Pharmacology, Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai 200025, PR China

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ABSTRACT

Antiangiogenic cancer therapy can be achieved through the targeted delivery of antiangiogenic agents to the endothelial cells of tumor neovasculature. In the present study, we developed a drug delivery system (DDS), nanoparticles conjugated with K237-(HTMYYHHYQHHL) peptides for tumor neovasculature targeting drug delivery. Paclitaxel, a chemotherapeutic agent with potent antiangiogenic activity, was used as a prototype drug. We synthesized the aldehyde poly(ethylene glycol)–poly(lactide) (aldehyde-PEG–PLA) block copolymer by ring opening polymerization. The nanoparticles loading paclitaxel (PTX-NP) were fabricated using the O/W emulsion and evaporation technique. K237 ligand, a peptide that can bind to the KDR receptors predominantly expressed on the surface of tumor neovasculature endothelial cells with high affinity and specificity and inhibit the VEGF-KDR angiogenic signal pathway, was conjugated to the aldehyde group of PEG chain using the N-terminal PEGylation technique. The K237 conjugated paclitaxel-loaded nanoparticles (K237-PTX-NP) had a hydrodynamic diameter of 150 nm. The K237 density on nanoparticle surface was 474 and the mean distance between two neighboring PEG chains linked to K237 peptide was 12 nm. The K237 conjugated nanoparticles could be significantly internalized by human umbilical vein endothelial cells (HUVEC) through the K237-KDR interaction, and this facilitated uptake led to the expected enhanced antiangiogenic activity shown by HUVEC proliferation, migration and tube formation compared to cells treated with the commercial formulation Taxol® and PTX-NP. The long-circulating property and the K237 ligand of K237-PTX-NP warranted rapid, long-term, and accurate in vivo tumor neovasculature targeting, and thereafter the significant apoptosis of tumor neovasculature endothelial cells and necrosis of tumor tissues of MDA-MB-231 breast tumors implanted in female BLAB/c nude mice. This nanoparticulate DDS offers a new strategy for paclitaxel chemotherapy application and it could also be used to carry other chemotherapeutic drugs, genes, and proteins with antiangiogenic activity for antiangiogenic cancer therapy.

1. Introduction

Angiogenesis, the formation of new capillaries from existing vasculature, is a crucial event for tumor growth, evasion and metastasis [1–3]. Host endothelial cells (ECs) act as the primary building blocks of the tumor microvasculature. Thus, inhibiting tumor growth by attacking the ECs offers a major strategy for antiangiogenic intervention. There are mainly three advantages of targeting chemotherapeutic agents to the proliferating ECs in the tumor neovasculature rather than directly targeting the tumor cells. First, the therapeutic target is independent of the type of solid tumor, thus killing proliferating ECs in the tumor microenvironment can be effective against a variety of malignancies. Second, acquired drug resistance resulting from genetic and epigenetic mechanisms often reduces the effectiveness of available drugs [4–6]. Anti-angiogenic therapy targets the ECs of the tumor vasculature, which are considered genetically stable, and therefore possesses the potential to overcome drug resistance [7] and reduce the incidence of drug resistance [8,9]. Third, the fact that cancer cells depend upon ECs for survival and growth may amplify the antiangiogenic therapeutic effect, and this phenomenon is commonly referred to as 'bystander effect'. There have already been some reports on anti-angiogenic strategies mediated by nanoparticles or liposomes that targeted angiogenic ECs for cancer therapy [7,10–13].

Vascular endothelial growth factor (VEGF) is one of the most crucial mediators of tumor angiogenesis and is closely involved in tumor development and metastasis. Vascular regression occurs after initiation of anti-VEGF therapy in both preclinical models and cancer patients [14–16]. VEGF mainly binds four receptors on the surface of

* Corresponding authors. Tel./fax: +86 21 6467 4721.
E-mail addresses: fangchao_china@yahoo.com.cn (C. Fang), hongzhuan_chen@hotmail.com (H.-Z. Chen).

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the ECs of tumor neovasculature [16]. They are VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), NRP-1 and NRP-2, among which KDR/Flk-1 is identified as the primary mediator of tumor angiogenesis. Recently, a peptide designated K237-(HTMYYHHYQHHL) isolated from a phage display peptide library was found to be able to bind KDR with high affinity and specificity [17]. By interfering with the VEGF-KDR interaction, K237 peptide could inhibit proliferation of cultured primary human umbilical vein endothelial cells (HUVEC). Moreover, the peptide could significantly inhibit the growth of solid tumors implanted beneath the breasts of severe combined immunodeficient mice, as well as their metastases to the lungs. We speculate that this peptide may be used as an effective ligand for a nanoparticulate drug delivery system (DDS) which can actively target the tumor neovasculature through the binding of K237 peptide to the KDR receptor.

Paclitaxel (PTX) is a potent chemotherapeutic drug approved by the FDA against a wide spectrum of cancers including ovarian and breast cancer, non-small cell lung carcinoma, melanoma, head and neck cancer, and AIDS-related cancer. However, its short circulation half-life, poor aqueous solubility, commonly occurring drug resistance [18,19] and serious side effects due to its non-selective distribution in vivo when delivered in conventional formulations usually compromise its clinical efficacy. Recently, it was found that besides being directly cytotoxic to tumor cells, paclitaxel can inhibit endothelial cell proliferation, migration, and tube formation at a very low concentration, which is non-toxic to both endothelial and tumor cells [20–22], presenting an attractive antiangiogenic activity. In the present study, we developed a nanoparticulate DDS, K237 peptide conjugated nanoparticles for paclitaxel, aiming at strengthening the drug's antiangiogenic efficacy, reducing the dose-associated side effects, and improving the antitumor efficacy. For this purpose, we synthesized an amphiphilic copolymer, aldehyde poly(ethylene glycol)–poly(lactide) (aldehyde-PEG–PLA), to elaborate paclitaxel-loaded nanoparticles using the O/W emulsion and evaporation method. The nanoparticles were functionalized with K237 peptide by the N-terminal PEGylation technique of coupling the N-terminal amine of the peptide to the aldehyde groups of PEG protruding on the nanoparticle surface. The physicochemical characteristics, in vitro drug release, in vitro endothelial cell uptake and antiangiogenic activity, in vivo pharmacokinetics, in vivo tumor neovasculature targeting, and paclitaxel-induced apoptosis of the tumor neovasculature endothelial cells and necrosis of tumor tissues of the K237-targeted paclitaxel-loaded nanoparticle delivery system were systematically investigated.

2. Materials and methods

2.1. Materials

Hydroxy-PEG-aldehyde (SUNBRIGHT HO-050A, MW 5000) was purchased from NOF Corporation, Japan. Methoxy poly(ethylene glycol) (MPEG, MW 5000) and sodium cyanoborohydride ($NaCNBH_3$) were obtained from Sigma. D,L-Lactide (99.5% pure) was purchased from PURAC and purified by recrystallization from dried ethyl acetate twice. K237 peptide (HTMYYHHYQHHL) was synthesized by GL Biochem (Shanghai) Ltd., China. Paclitaxel (purity > 99%) was from Shanghai Jin Mao Tai Chemical Co., China. Taxol® was from Bristol–Myers Squibb Company. Coumarin-6 was from Aldrich. Acetonitrile and tert-butyl methyl ether of HPLC grade were from TEDIA. Double-distilled water was purified using a millipore simplicity system (Millipore, Bedford, USA). All other chemicals were of analytical grade and used without further purification.

2.2. Copolymer synthesis and characterization

Aldehyde-PEG–PLA and MPEG–PLA block copolymers were synthesized as described by the ring opening polymerization (Fig. 1A) [23,24]. Briefly, a predetermined amount of hydroxy-PEG-aldehyde or MPEG and D,L-lactide at the w/w ratio of 1:12 were placed in a dried round-bottomed bottle connected with a vacuum joint, and the appropriate amount of stannous octoate was added as a solution in dried toluene. The reactants were dried under reduced pressure at 70 °C for 1 h, and the reaction was allowed to proceed under vacuum at 140 °C for 6 h. The cooled product was dissolved in dichloromethane (DCM), precipitated in excess mixed solvent of ethyl ether and petroleum ether and recovered by filtration. The purified copolymers were dried in a vacuum oven at 40 °C for 24 h and then stored in a desiccator under vacuum at −20 °C.

The copolymer composition was studied by ^{1}H NMR and ^{13}C NMR spectra in $CDCl_3$ on a Mercury Plus 400 MHz spectrometer (USA). FTIR spectra (Avatar 360ESP) were obtained from a neat film cast from the chloroform copolymer solution between KBr tablets. GPC of polymers was performed in tetrahydrofuran with a Waters Associates Model ALC/GPC 244 apparatus. Molecular weight and molecular weight distribution of polymers were calculated by using polystyrene (Polysciences, Inc., Warrington, PA) as the standard.

2.3. Preparation of K237 peptide-conjugated paclitaxel-loaded PEG–PLA nanoparticles (K237-PTX-NP)

2.3.1. Preparation of paclitaxel-loaded PEG–PLA nanoparticles (PTX-NP)

The PTX–NP were prepared with a blend of aldehyde-PEG–PLA and MPEG–PLA using the O/W emulsion and evaporation technique (Fig. 1B) [23,24]. Briefly, 1.5 mg PTX was dissolved in 1 ml of a solution of 30 mg/ml of a blend of aldehyde-PEG–PLA and MPEG–PLA (1:9, w/w) in DCM. Next, 3 ml of 1% (w/v) sodium cholate solution was slowly poured into the solution and then sonicated at 160 w for 25 s (Scientz Biotechnology Co. Ltd., China). The resulted O/W emulsion was further diluted into 40 ml of a 0.5% aqueous sodium cholate solution and then gently stirred overnight at room temperature by a magnetic stirrer to evaporate the organic solvent. The resulting nanoparticles were collected by centrifugation (11,000× *g*, 30 min, 4 °C; Sigma, USA) and washed twice to remove the excessive emulsifier. The coumarin-6-labeled nanoparticles were prepared in the same way except that paclitaxel in the oil phase was replaced by 0.05% (w/v) coumarin-6.

2.3.2. Surface modification of PTX-NP by K237 peptide

The PTX-NP was incubated with K237 peptide at a 1:3 molar ratio of aldehyde to the N-terminal amine of K237. The K237 peptide conjugation reaction was processed in PBS (pH 7.4) at room temperature for 10 h in the presence of $NaCNBH_3$ as a reducing reagent (Fig. 1C) [25,26]. The unconjugated K237 peptide was removed by centrifugation (11,000× *g*, 30 min, 4 °C) and the K237 peptide-conjugated paclitaxel-loaded PEG–PLA nanoparticles (K237-PTX-NP) were collected and lyophilized.

2.4. NP characterization

2.4.1. Morphology, particle size, size distribution and zeta potential

The shape and morphology of the nanoparticles were observed using transmission electron microscopy (TEM) (H-600, Hitachi, Japan) and atomic force microscopy (AFM) (Multimode Scanning Probe Microscope, Digital Instruments, USA). The particle size and size distribution were determined with three different methods: dynamic light scattering (DLS) (Zeta Potential/Particle Sizer NICOMP 380/ZLS, PSS, Santa Barbara, USA), TEM, and AFM. In the DLS assay, the nanoparticles were diluted in double-distilled water. In TEM observation, the nanoparticle sample was negatively stained with sodium phosphotungstate solution. As for AFM detection, one drop of nanoparticle suspension was mounted on the metal slabs, air-dried and scanned by the AFM with a Nanoscope III in the tapping mode. The zeta potential was measured by DLS.

2.4.2. Encapsulation efficiency and drug loading

The drug encapsulation efficiency (EE%) was expressed as the percentage of the drug amount found in the nanoparticles to the total amount used to prepare the nanoparticles. The drug loading (DL%) was expressed as the percentage of the drug amount found in the nanoparticles. The assay of paclitaxel and coumarin-6 were determined by high performance liquid chromatography (HPLC) methods.

2.4.3. Determination of K237 conjugation efficiency and K237 density on nanoparticle surface

K237 concentration was determined using the CBQCA Protein Quantitation Kit (Molecular Probes) which provided a rapid and highly sensitive method for the quantitation of peptides and proteins [4,27]. K237 conjugation efficiency (CE%) was calculated to determine the percentage of K237 peptide conjugated on the nanoparticles surface. The calculation formula is as follows: CE% = (amount of K237 conjugated on the nanoparticle surface/total amount of K237 added) × 100%.

K237 surface density (*S*), the number of K237 molecules conjugated per particle, was calculated by dividing the number of K237 molecules by the calculated average number (*n*) of nanoparticles using the methods described by Olivier et al. [28]: $n = 6\,m/(\pi \times D^3 \times \rho)$. In this equation, m is the nanoparticle weight, *D* is the number-based mean nanoparticle diameter determined by DLS, and ρ is the nanoparticle weight per volume unit (density), estimated to be 1.1 g/cm^3 [28]. The average distance (d) between two neighboring PEG chains linked to K237 peptide can be calculated as the square root of the mean area occupied by each K237 peptide on the particle surface. The finally simplified equation is $d = D \times (\pi/S)^{1/2}$ [29].

2.4.4. X-ray photoelectron spectroscopy (XPS)

The lyophilized blank NP, K237-NP, and the mixture of aldehyde-PEG–PLA: MPEG–PLA (1:9, w/w) were analyzed to determine the surface composition of C, O and