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Suppression of colorectal cancer subcutaneous xenograft and experimental lung metastasis using nanoparticle-mediated drug delivery to tumor neovasculature

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ABSTRACT

Antiangiogenic therapy is a validated approach for colorectal cancer (CRC) treatment. However, diverse adverse effects inevitably appear due to the off-target effect of the approved antiangiogenic inhibitors on the physiological functions and homeostasis. This study was to investigate a new tumor vessel targeting nanoparticulate drug delivery system, F56 peptide conjugated nanoparticles loading vincristine (F56-VCR-NP), for the effective treatment of CRC subcutaneous xenograft and experimental lung metastasis model. The controlled release behavior and *in vivo* pharmacokinetic profile of F56-VCR-NP were characterized. The tumor vessel targeting and antiangiogenic activity of F56-VCR-NP was evaluated in human umbilical vein endothelial cells (HUVEC, a classical cell model mimicking tumor vascular EC), subcutaneous human HCT-15 xenograft in immunodeficient nude mice, and experimental CT-26 lung metastasis model in immunocompetent mice. The therapeutic efficacy (animal survival and toxicity) was further investigated in the model of CT-26 lung metastasis in mice. F56-VCR-NP could achieve 30-day controlled drug release in PBS (pH 7.4) and exhibited favorable long-circulating feature *in vivo*. F56-VCR-NP could accurately target the CRC neovasculature and elicit nanoparticle internalization in the tumor vascular EC, where the antiangiogenic VCR-induced dramatic EC apoptosis and necrosis of CRC tissue. F56-VCR-NP significantly prolonged the mouse survival with no obvious toxicity (weight loss and anepithymia) in the CT-26 lung metastasis mice model, and this pronounced antitumor effect was closely related with the decreased microvessel density in the metastases. The present nanoparticle-based targeted antiangiogenic therapy may provide a new promising approach for the therapy of CRC and lung metastasis, which deserves further translational research.

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in men and the second in women [1]. Approximately one-quarter of CRC patients have metastases at diagnosis in organs or lymph nodes adjacent to primary tumor, or even distant secondary sites (lung, liver, peritoneum, etc.) [2]. Surgical resection is only suitable for less than 10% of patients, and the systemic FOLFOX chemotherapy (oxaliplatin, fluorouracil, and leucovorin) is often used to treat patients with metastatic CRC (mCRC) [2,3]; however the clinical gains always come at the cost of considerable and inevitable toxicities [3]. Two monoclonal antibody therapies, cetuximab (Erbitux) and panitumumab (Vectibix) have been proved by FDA to block the EGFR signaling pathway of CRC [4,5]; unfortunately, the clinical benefits are greatly compromised by the emergence of KRAS mutations [4–6].

As a featured hallmark of cancer, tumor neovascularization has been revealed to be a critical factor in stimulating CRC progression and metastasis, and increased angiogenesis has been proved to be closely associated with poor prognosis and relapse of the disease [7]. This understanding of the pathology and molecular biology of CRC leads to the successful translation of three antiangiogenic inhibitors, bevacizumab (Avastin) [4,5,8], Ziv-aflibercept (Zaltrap) [9],

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and Regorafenib (Stivarga) [10] into the clinics for mCRC treatment. However, diverse adverse effects always inevitably appear which is closely associated with the off-target effect of these molecular-targeted agents on the physiological functions and homeostasis. For an instance, bevacizumab may cause, even serious and life-threatening, adverse effects such as hypertension, arterial thrombosis, cardiomyopathy, proteinuria and renal adverse effects, wound complications, gastrointestinal perforation, and fistula formation [11].

To reduce off-target associated adverse effects and achieve targeted drug delivery in cancer therapy, nanomedicine is emerging as a promising strategy. Doxorubicin in PEG-liposomes (Doxil and Caelyx) [12], polymeric micelle-based preparation of paclitaxel (Genexol-PM) [12], and paclitaxel albumin-stabilized nanoparticle formulation (Abraxane) [13] have been translated into clinical reality. The underlying mechanism for their successful performance is that the nanomedicine circulating in the blood compartment can accumulate in a large and well-vascularized tumor through the enhanced permeation and retention (EPR) effect [13–15]. However, such EPR effect has limitations: for the less-vascularized tumor lesions and particularly the multiple small metastases (<100 mm^3 in size) such as mCRC, tumor targeting via the EPR effect is usually largely compromised [16]. It is noted that the tumor vascular endothelial cells (EC) are directly exposed to the blood circulation, thus much more easily to be reached than the tumor cells especially in the deeper tumor parenchyma with high interstitial pressure [17]. Therefore, the nanoparticle-based tumor vascular EC-targeted antiangiogenic therapy may be a feasible strategy for the treatment of CRC and its metastasis.

In this study, a new nanoparticulate drug delivery system (DDS), F56 peptide conjugated biodegradable nanoparticles for vincristine (F56-VCR-NP), was developed. F56 is a peptide isolated from a phage display peptide library; it can bind to the highly expressed Flt-1 receptor (VEGFR-1) of tumor vascular EC with high affinity and specificity, and thereafter elicit internalization [18,19], which is necessary for targeted intracellular drug delivery. The model drug vincristine is a microtubule-binding anticancer agent with validated potent antiangiogenic activity of inhibiting EC proliferation, migration, and tube formation even at non-toxic concentration to both endothelial and tumor cells [20–22]. The aim of this study is to explore the efficacy of tumor vessel targeted nano-DDS in the antiangiogenic therapy of CRC subcutaneous xenograft in immunodeficient nude mice and experimental lung metastasis in immunocompetent mice. This strategy may offer a new choice for the treatment of CRC other than the present clinical methods, which have shown shortcomings in serious toxicities and/or intractable drug resistance.

2. Materials and methods

2.1. Materials, cell culture, 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 [23]. Vincristine (VCR, free base) was purchased from Pure-one Bio Technology Co., Ltd. (Shanghai, China). F56 peptide (WHSDMEWWYLLG) [18] was synthesized by GL Biochem (Shanghai, China). Coumarin 6, filipin, cytochalasin D, nystatin, nocodazole, and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were from Sigma–Aldrich (St. Louis, MO). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethyl indotricarbocyanine iodide (DiR) was obtained from Life Technologies (Carlsbad, CA). 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 M200 medium with LSGS were obtained from Life Technologies (Carlsbad, CA). The cells at 3–5 passages were used in the experiments. HCT-15 and CT-26 cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics (100 μg/ml of streptomycin and 100 U/ml of penicillin) at 37 °C in a humidified incubator with 5% CO_2.

Male Sprague–Dawley (SD) rats (190–210 g), female BALB/c nude mice (~20 g), and female BALB/c mice 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 F56-VCR-NP

F56-VCR-NP were engineered by emulsion and solvent evaporation method with a following surface functionalization. Briefly, 2 mg VCR was dissolved in 1 ml solution of 30 mg blend of aldehyde-PEG–PLA and MPEG–PLA (1:9, w/w) in dichloromethane and acetone (2:1, v/v). Next, 3 ml of 1% (w/v) sodium cholate was slowly poured into the solution and then the mixture was sonicated at 200 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 VCR-loaded nanoparticles (VCR-NP) were collected by centrifugation (11,000× *g*, 30 min, 4 °C; Sigma 3K18, Germany) and washed twice to remove the excessive emulsifier. Then, VCR-NP was incubated with F56 at a 1:3 molar ratio of aldehyde to the N-terminal amine of F56. 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 F56 was removed by centrifugation (11,000× *g*, 30 min, 4 °C) and F56-VCR-NP was collected. The coumarin 6 or DiR-labeled nanoparticles were prepared in the same way except that in the oil phase VCR was replaced or 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 stained with 2% (w/v) sodium phosphotungstate and visualized using H-600 transmission electron microscopy (TEM) (Hitachi, Japan). 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, and drug loading (DL %) was expressed as the percentage of the drug amount found in the nanoparticles. The assay of vincristine and coumarin 6 was determined by high performance liquid chromatography (HPLC) methods. The peptide conjugation efficiency (CE%), surface density (*S*, the number of F56 molecules per particle), and the average distance (*d*) between two neighboring PEG chains linked to F56 peptide were determined using the method as previously described [24]. The X-ray photoelectron spectroscopy (XPS) was used to confirm the conjugation of F56 on the nanoparticle surface [23].

2.3. Vincristine release from F56-VCR-NP

In vitro release experiments of VCR from the nanoparticles were performed in 0.01 M PBS (pH 7.4). Briefly, 20 mg of F56-VCR-NP or VCR-NP were put in a centrifuge tube and suspended in 10 ml PBS containing 0.1% Tween-80 to maintain the sink condition. The tubes were placed in the gas bath at 37 °C shaking at 100 rpm. At specific intervals, the nanoparticles were centrifuged (11,000× *g*, 30 min, 4 °C) and 1 ml release medium containing the free drug was transferred out and another 1 ml of fresh PBS was added to the test tubes to re-suspend the particles for continuous release studies. The supernatant containing the drug was extracted with DCM and analyzed by HPLC. Each measurement was performed in triplicate.

2.4. Pharmacokinetic study of F56-VCR-NP in SD rats

Male Sprague–Dawley (SD) rats (190–210 g) were randomly divided into three groups (6 in each group) and fasted overnight with free access to water before drug administration. Rats in control group were intravenously administered through the caudal vein with free VCR of 1.2 mg/kg. The other two groups were injected with VCR-NP and F56-VCR-NP formulations in saline solution (0.9%, w/v) at the same dose as the control group. After administration, 200 μl blood samples was collected from the retro-orbital plexus at 0.083, 0.25, 0.5, 1, 2, 4, 8, 12, 24 and 48 h, respectively, and were placed into heparinized micro-centrifuge tubes. The blood sample was centrifuged at 4000 g for 5 min and the separated plasma (100 μl) was stored at −80 °C until HPLC analysis. In brief, 10 μl of the vinblastine sulfate solution was added to the above plasma before the addition of acetonitrile (500 μl) to precipitate protein and the residue was reconstituted in 100 μl of the mobile phase. The chromatography system used was composed of a Shimadzu LC-20AT chromatographic system (Shimadzu, Kyoto, Japan) with a LC-20AT binary pump and a SPD-20A UV–Vis spectrophotometry at 276 nm. Analysis was carried out on a Dikma Dimonsil C18 column (4.6 mm × 200 mm, 5 μm, Dikma Technologies, Beijing, China). The mobile phase was composed of 0.02 M sodium dihydrogen phosphate–methanol (36:64, v/v, pH 4.7), and the flow rate was 1 ml/min. The column temperature was maintained at 25 °C, and the injection volume was 20 μl. The pharmacokinetic parameters were calculated with the WinNonlin software (Version 6.1 Pharsight, Mountain View, CA) according to non-compartmental model.