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Tumor-specific penetrating peptides-functionalized hyaluronic acid-D-α-tocopheryl succinate based nanoparticles for multi-task delivery to invasive cancers

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ABSTRACT

Poor site-specific delivery and incapable deep-penetration into tumor are the intrinsic limitations to successful chemotherapy. Here, the tumor-homing penetrating peptide tLyP-1-functionalized nanoparticles (tLPTS/HATS NPs), composed of two modularized amphiphilic conjugates of tLyP-1-PEG-TOS (tLPTS) and TOS-grafted hyaluronic acid (HATS), had been fabricated for tumor-targeted delivery of docetaxel (DTX). The prepared tLPTS/HATS NPs had about 110 nm in mean diameter, high drug encapsulation efficiency (93%), and sustained drug release behavior. *In vitro* studies demonstrated that the tLPTS/HATS NPs exhibited enhanced intracellular delivery and much better anti-invasion ability, cytotoxicity, and apoptosis against both invasive PC-3 and MDA-MB-231 cells as compared to the non-tLyP-1-functionalized HATS NPs. The remarkable penetrability and inhibitory effect on both PC-3 and MDA-MB-231 multicellular tumor spheroids were also identified for the tLPTS/HATS NPs. *In vivo* biodistribution imaging demonstrated the tLPTS/HATS NPs possessed much more lasting accumulation and extensive distribution throughout tumor regions than the HATS NPs. The higher *in vivo* therapeutic efficacy with lower systemic toxicity of the tLPTS/HATS NPs was also verified by the PC-3 xenograft model in athymic nude mice. These results suggested that the designed novel tLPTS/HATS NPs were endowed with tumor recognition, internalization, penetration, and anti-invasion, and thus might be a promising anticancer drug delivery vehicle for targeted cancer therapy.

1. Introduction

Efficient and site-specific delivery of anticancer drugs into tumors presents a critical challenge for the success of cancer chemotherapy [1]. Currently, several of conventional nanocarriers (e.g., liposomes, micelles, and polymeric nanoparticles, etc.) have progressed greatly and offered an available cancer-targeted treatment via the leaky tumor vasculature, termed enhanced permeability and retention (EPR) effect [2,3]. And some of them have been applied in preclinical or in clinical trials. However, an adequate treatment remains to be elusive due to their poor tumor site-specific delivery and tumor tissue penetration. The development of a multi-task delivery platform for more effectively targeted treatment is therefore still urgently required [4]. Over the past decades, a variety of different strategies have been proposed to improve the targeted delivery and intracellular internalization of nanocarriers by conjugation of ligands or antibodies. The ligand-conjugated nanocarriers directly interact with complementary receptors present on the surface of target cells [5–8]. The major benefit of the actively targeted nanocarriers over those passively targeted ones is that they can be enriched within tumors for a longer period of time because of their binding to or their uptake by cancer cells, preventing them from rapidly re-entering systemic circulation [9].

The penetration into the inner tumor tissue to deliver sufficient anticancer drugs is important to the efficacy of the actively targeted nanocarriers. However, the disordered tumor vascular system, deregulated extracellular matrix and high interstitial pressure in

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the abnormal tumor microenvironment cooperatively hinder the drug distribution into tumor region. This is a formidable barrier to cancer chemotherapy and a driving force leading to tumor recurrence, progression and multidrug resistance [10,11]. In light of this difficulty, the cell penetrating peptides (CPPs)-modified nanocarriers offers an alternative strategy to address the incapable tumor tissue penetration. Unfortunately, the majority of the known CPPs were devoid of tissue-selectivity, readily to be contacted and internalized by nearly all cell types, which in turn accelerated *in vivo* plasma clearance, and thereby lessening the therapeutic response [12,13].

The Cend Rule (CendR, (R/K)XX(R/K)) motif peptides were reported to bear tumor-specific penetration via a mechanism of ligand-receptor recognition [14,15]. The receptor for the CendR motif is neuropilin (NRP-1), which is a modular transmembrane protein overexpressed on the surface of a wide range of cancers, while a relatively low expression in normal tissues [16–18]. The tLyp-1 peptide (CGNKRTR), identified as a substrate for NRP-1 receptor with high affinity and specificity, contains both a tumor-homing motif and a cryptic CendR, which can induce tumor cell recognition and tissue penetration through NRP-dependent internalization pathway [19,20]. This promising feature makes the tLyp-1 peptide as an effective targeting moiety to mediate tumor site-specific delivery and penetration of nanocarriers into solid tumor.

Nanocarriers functionalized with biological molecules may trigger additive therapeutic effect with the delivered drugs after intracellular uptake. One typical example is D-α-tocopheryl derivatives-based nanocarriers for anticancer drug delivery [21,22]. The two D-α-tocopheryl derivatives, D-α-tocopheryl succinate (TOS) and D-α-tocopheryl polyethylene glycol-succinate (TPGS), exert highly selective antitumor effect by specifically destabilizing cancer cell mitochondria but nontoxic toward normal cells [23–26]. TOS has been grafted on a variety of polymers to allow for better encapsulation and delivery of poorly water soluble drugs. Both TOS-grafted chitosan copolymer and TOS-modified pluronic P123 copolymer demonstrated high drug loading capacity for paclitaxel, and excellent *in vivo* circulation property [27,28]. The synergic capabilities of TOS to act with the delivered chemotherapeutics have also been verified *in vitro* and *in vivo* for the TOS-based nanocarriers [26,29].

Here, an active tumor-targeted hyaluronic acid (HA) and TOS-based nanoparticle delivery system was rationally designed using tLyp-1 peptide as targeting moiety. HA bears specific binding property to cancer cells overexpressing CD44 receptor and its derivatives have been extensively utilized as nanocarriers for tumor-targeted drug delivery [30,31]. We first attempted to build the amphiphilic targeting molecule, tLyP-1-PEG-TOS (tLPTS), by chemical linking the TOS to tLyp-1 peptide via the hydrophilic polyethylene glycol (PEG) chain. This ligand-PEG-lipid conjugate was widely utilized as active-targeting components in the targeted nanocarriers design [32–35]. Besides, the amphiphilic TOS-grafted HA copolymer (HATS) was constructed by the hydrophobic TOS chemically conjugated to the backbone of HA. Thus, a multifunctional nanoparticle delivery platform (tLPTS/HATS NPs) was fabricated by simply mixing two conjugates of tLPTS and HATS (Scheme 1). The PEG and tLyP-1, located on the outer layer, was for extended blood circulation and active tumor-targeting, respectively, while the TOS was for synergistic effect with the delivered therapeutics of docetaxel (DTX). Nanocarriers functionalized with elements related to tumor cell-surface molecules are susceptible to tumor cell recognition and the ensuing transcytosis, which has been demonstrated to be an attractive choice to overcome the sequential delivery barriers [36,37]. But no research that we know has reported using biologically functional HA and CendR motif peptides tLyP-1 to develop a nanoparticle delivery system for more effectively targeted treatment of solid tumor. It was assumed that a combination of CD44 targeting and tumor lineage-homing penetrating peptides of tLyP-1 may contribute to tumor site-specific accumulation and penetration into tumor. The objectives of this study were to evaluate the potential of the developed tLPTS/HATS NPs to improve the anticancer drug delivery and therapeutic efficacy and to explore the underlying mechanisms.

2. Materials and methods

2.1. Materials

Hyaluronic acid (HA, 7.8 kDa) was obtained from Shandong Freda Biochem Co., Ltd. (Shandong, China). tLyP-1 peptide (CGNKRTR, MW 833.97) was synthesized by GL Biochem. (Shanghai, China). Maleimide-PEG_{2000}-NH_2 was purchased from JenKem Technology Co., Ltd. (Beijing, China). Docetaxel (DTX) was obtained from Norzer Pharmaceutical Co., Ltd. Taxotere® was commercially available from the local hospital of Beijing (Aventis Pharma S.A., France). D-α-tocopheryl succinate (TOS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). N,N′-dicyclohexylcarbodiimide (DCC) and N-Hydroxysuccinimide (NHS) were purchased from Sinopharm Group Co., Ltd. (Beijing, China) and Advanced Chem. Tech. Co., Ltd. (Shenzhen, China), respectively. Anhydrous formamide, anhydrous dimethylformamide (DMF) were obtained from Beijing Chemical Reagent Co., Ltd. (Beijing, China). Coumarin-6 (COU) and DiR fluorescent probes were purchased from Life Technologies (Eugene, OR, USA). Lyso-Tracker Red was obtained from Invitrogen (Carlsbad, CA, USA). Matrigel was purchased from BD Biocoat (Franklin, NJ, USA). Crystal violet was purchased from Amresco (Solon, OH, USA). Ham's F12 medium, Leibovitz's L15 medium, penicillin-streptomycin, trypsin and Hoechst 33258 were provided by Macgene Co., Ltd. (Beijing, China). Fetal bovine serum (FBS) was obtained from Invitrogen/Gibco (Grand Island, NY, USA). Annexin V-FITC apoptosis detection kit was purchased from Nanjing KeyGen Biotech. Co., Ltd (Nanjing, China). All other reagents and chemicals were of analytical grade and obtained from commercial sources.

The male BALB/c nude mice (16–18 g) were provided by the Vital Laboratory Animal Center (Beijing, China). All care of the animals were performed under specific pathogen free (SPF) conditions with free access to standard food and water, and all the animal experiments were conducted with the approval of the Ethics Committee of Peking University.

2.2. Synthesis of tLPTS and HATS conjugate

The amphiphilic targeting molecules of tLyP-1-PEG-TOS (tLPTS) were synthesized by two-step procedure as illustrated in Fig. 1A. First, the carboxyl group of TOS (0.025 mmol) was activated with EDC (0.05 mmol) and NHS (0.05 mmol) in 2 mL of DMF for 8 h, followed by the addition of Maleimide-PEG_{2000}-NH_2 (0.025 mmol). After stirring in dark for another 8 h, the reaction mixture was dialyzed against distilled water for 48 h (MWCO 7000 Da). The residue was filtrated and lyophilized to obtain the intermediate Maleimide-PEG-TOS. Then, the targeting moiety of tLyP-1 peptide was conjugated to Maleimide-PEG-TOS by sulfydryl-maleimide coupling reaction. Briefly, in the presence of triethylamine (TEA, 15 μL), the tLyP-1 peptides (0.01 mmol) were added to the Maleimide-PEG-TOS (0.01 mmol) in 2 mL of DMF. After stirring for 8 h under nitrogen gas, the reaction mixture was dialyzed against