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Enhanced tumor therapy *via* drug co-delivery and *in situ* vascular-promoting strategy

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ABSTRACT

Conventional tumor starving therapy by reducing its vessel density may be effective at early treatment but potentially contributes to tumor hypoxia, drug resistance and metastasis. A new strategy through enhancing tumor angiogenesis in combination with effective chemotherapeutic drugs, has shown successful tumor growth and spread inhibition. To achieve *in situ* release of angiogenic and antitumor drugs in tumor, we designed a precise ratiometric polymeric hybrid micelle system for co-delivering nitric oxide and paclitaxel. The hybrid micelles could accumulate in tumor *via* the long blood circulation and enhanced permeability and retention (EPR) effect, promote the drug accumulation and penetration in tumor by *in situ* increased vascular permeability, blood perfusion and vessel density, achieve the synergistic antitumor effect of nitric oxide and paclitaxel through modified tumor microenvironment, overcome multidrug resistance and inhibit metastasis. This study presents a combinational therapy against tumor progression and spread, which shows great potential in cancer therapy of the future.

1. Introduction

Tumor angiogenesis was once considered as a negative factor in cancer therapy [1]. In the past decades, many efforts have been made on reducing tumor vessel density to cut off its nutrient supplement such as using vascular endothelial growth factor (VEGF) pathway inhibitor [2]. Unfortunately, this starving strategy still needs further investigation as it may raise the possibility of tumor hypoxia, drug resistance and metastasis [3–6]. An alternative strategy is to combine VEGF pathway inhibitor with some chemotherapeutic drugs, but it still keeps challenges. The inhibitor can reduce vessel density and vascular functions but the reduced blood perfusion limited the drug delivery in tumor [2,6] and thus failed to meet the therapeutic expectations. To improve the drug delivery, vascular normalization strategy by reducing tumor vessel density and improving blood perfusion was developed with good therapeutic efficacy. However, this strategy might be limited in clinical application due to the narrow therapeutic window and the resultant difficulty in operation [7]. Even so, it still manifests the essential role of blood perfusion in drug delivery for cancer treatment. As recent investigations demonstrated, when the blood flow in tumor was improved *via* an angiogenesis promoting and vessel dilation strategy, better therapeutic efficiency was observed [8–11]. In this case, drug co-administration becomes to be important to achieve expected pharmacokinetics and distribution of the drugs for synergistic efficacy. Combination therapy using co-delivery system has proven significant advantages in cancer therapy such as delivering functional drugs in a precise ratio to targeted organs together and realizing multidrug synergistic treatment [12–15]. Nano-sized carriers such as micelles, liposomes, nanogels, polymeric/inorganic nanoparticles and macromolecular prodrug have exhibited many advanced properties in co-delivery system including prolonged circulation time, reduced side effect and targeted delivery [14,16–22]. The application of nanotechnology has promoted the development of drug co-delivery system with precise ratiometric delivery and site-special, synchronous, controlled release behaviors [12,13].

To enhance tumor therapy *via* drug co-delivery and vascular-promoting strategy, a novel polymeric hybrid micelle system with precise ratiometric drug delivery property was designed for co-delivering angiogenic and antitumor drugs, *i.e.* nitric oxide (NO) and paclitaxel (PTX). PTX is widely used in cancer chemotherapy but its application is limited by the low solubility and side effects mainly caused by the adjuvant Cremophor EL. NO, a multifunctional gaseous molecule,

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regulates various pathophysiological and physiological processes. It is well known that NO has many vascular functions such as promoting the vessel dilation, increasing vessel density and enhancing vascular permeability [23,24]. Moreover, NO is capable of inhibiting tumor growth at a relatively high concentration [25,26], but the poor chemical stability and very short half-life in blood circulation makes it hard to be applied as therapeutic agent for cancer [27,28]. Co-loading these two drugs with largely different physicochemical properties in a single nano-sized carrier is a great challenge [16]. Modifying drugs onto polymers with similar structure provides a possible way to solve this as it is available to form hybrid macromolecular drug co-loading system with highly tunable drug ratio [12]. Based on our previous work, we selected D-α-tocopherol polyethylene 1000 glycol succinate (TPGS), an FDA approved pharmaceutical excipient, as the vehicle to modify and co-deliver these two synergistic drugs [29]. We previously synthesized a TPGS-based redox-sensitive PTX prodrug (TPGS-SS-PTX) which exhibited some advantages to Taxol (clinical formulation of PTX) as the extended blood circulation, enhanced antitumor efficiency, reversed multidrug resistance (MDR) and reduced side effects [21]. Another TPGS-derived NO donor (TPGS-NO_3) was synthesized to overcome the shortages of gaseous NO by improving stability, extending half-life and *in situ* releasing of NO in tumor tissue [30]. As derivatives of TPGS, it is available for TPGS-SS-PTX and TPGS-NO_3 to form hybrid micelle system (TSP-TN) in various ratios, which are able to achieve the precise ratiometric co-delivery of PTX and NO [12]. Additionally, TPGS was reported to have the ability to overcome MDR and inhibit metastasis [31–33], which may expand the application of this co-delivery system.

Herein, we developed the co-delivery system using TPGS-SS-PTX and TPGS-NO_3 to realize a stereo combinational cancer therapy *via in situ* vascular-promoting strategy and self-promoted drug accumulation (Fig. 1). TPGS-SS-PTX and TPGS-NO_3 could self-assemble to stable hybrid micelles (TSP-TN) and accumulate in tumor tissue by enhanced permeability and retention (EPR) effect [19,20]. After being endocytosed by tumor cells, the hybrid micelles can release PTX and NO quickly under the reductive environment with synergistic cytotoxicity. Furthermore, NO could diffuse outside tumor cells, and then realize its vascular functions such as vessel dilation and angiogenesis. The promoted tumor blood perfusion may make the drug delivery in benign circle. Besides, intracellular disassociated TPGS was capable of overcoming MDR *via* P-glycoprotein (P-gp) inhibition and inhibiting tumor metastasis.

2. Materials and methods

2.1. Materials

DL-Dithiothreitol (DTT) and rhodamine B (RhB) were purchased from Aladdin, China. 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Biosharp, South Korea. RPMI 1640 medium, DMEM medium, penicillin-streptomycin, fetal bovine serum (FBS) and trypsin-EDTA were purchased from Hyclone, USA. D-α-Tocopherol polyethylene 1000 glycol succinate (TPGS) was purchased from Sigma Aldrich, USA. Griess reagent, Bovine fixation solution and 3-amino-4-aminomethyl-2′,7′-difluorescein, diacetate (DAF-FM DA) probe were obtained from Beyotime Institute of Biotechnology, China. All the antibodies used in this paper were purchased from Becton Dickinson Bioscience, USA. All the solvents used were of analytical grade and were produced from Sinopharm, China.

MCF-7, A2780 and A2780/T cell lines were supplied by KeyGEN, China. MCF-7/ADR cell line was kindly donated by professor Li, Yaping (CAS, Shanghai). Mouse sarcoma tumor cell line S180 and melanoma B16F10 cell line were provided by the Shanghai Institute of Biochemistry & Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences.

Sprague–Dawley (SD) rats of weight 200 ± 20 g and C57BL/6 mice (6 weeks) were obtained from Laboratory Animal Centre of Wuhan University (certificate no. SCXK 2014-0004, Wuhan, China). Kunming (KM) mice (5 weeks) were obtained from Laboratory Animal Resources of Huazhong University of Science & Technology (certificate no. SCXK 2010-0009, Wuhan, China). BALB/c-nude mice (5 weeks) were obtained from Beijing HFK Bioscience CO, LTD (certificate no. SCXK 2014-0004, Beijing, China). All the animals were maintained under specific pathogen-free condition in the Animal Centre of Huazhong University of Science and Technology (certificate no. SYXK 2010-0057, Wuhan, China). All animals were treated according to the regulations of Chinese law and the study was approved by the local Ethical Committee Quantita.

2.2. Preparation and characterization of TSP-TN

TPGS-SS-PTX and TPGS-NO_3 were synthesized according to our previous works (the chemical structures were displayed in Fig. S1) [21,30]. TSP-TN was prepared by solvent-evaporation method. Briefly, TPGS-SS-PTX and TPGS-NO_3 were dissolved in ethanol and then added into phosphate buffer saline (PBS) dropwise under stirring. The micelle solution was stirred overnight and filtered through 0.45 μm syringe filter. Size distribution and morphology of TSP-TN were observed by dynamic light scattering (DLS) and transmission electron microscope (TEM), respectively. Moreover, TSP-TN was diluted into FBS or PBS and kept at 37 °C to evaluate the stability through the diameter changes.

In order to determine the release profiles of PTX/NO from TSP-TN, 5 mL freshly prepared micelles were dialyzed against 50 mL pH 7.4 PBS with or without DTT (10 mM) at 37 °C with constant shaking (TSP-TN1 was used in this detection and followed experiment). PBS with 10 mM DTT was commonly used to simulate the reductive microenvironment in cancer cells [34]. At the desired time intervals, the dialysis solution was partly removed and replaced with equal volume of fresh PBS. The released PTX was determined by high-performance liquid chromatography (HPLC), while the concentration of nitrite, the stable breakdown product of gaseous nitric oxide, was determined by Griess assay [30].

2.3. Cell culture

Drug-sensitive human breast cancer MCF-7 and -resistant MCF-7/ADR cells were cultured in RPMI 1640 complete medium. MCF-7/ADR cells were supplemented with 1 μg mL^{-1} doxorubicin. Drug-sensitive human ovarian cancer A2780 and -resistant A2780/T cells were cultured in RPMI 1640 complete medium. A2780/T cells were supplemented with 400 ng mL^{-1} Taxol. Murine melanoma B16F10 cells were cultured in DMEM complete medium.

2.4. Drug co-delivery and NO release in tumor cells and tumor tissue

TPGS-conjugated rhodamine B (T-RhB) labeled fluorescent micelles were used as probes to show the location of TSP-TN or TSP, while DAF-FM DA was chosen to detect NO [35]. For the intracellular uptake and NO release experiment, MCF-7/ADR cells were seeded in a 24-well plate with phenol red-free complete medium. After overnight attachment, the cells were incubated with DAF-FM DA (5 μM) for 40 min, followed by treatment with fluorescent micelles for 2 h. The cells were then washed with PBS, fixed with 4% paraformaldehyde solution, stained with DAPI and mounted on glass slides for observation by confocal laser scanning microscopy (CLSM).

The drug co-delivery and *in situ* NO release in tumor tissue was investigated in S180 tumor-bearing mice. Briefly, S180 cells (1×10^7) were subcutaneously (*s.c.*) injected in the right flank of KM mice (5 weeks, female). Then, the tumor size was measured daily and calculated as: $V = L \times W^2 \times 0.5$ (where W is width and L is length). When the tumor volume grew to 500 mm^3, all the mice were intratumorally (*i.t.*) injected with 200 μL of DAF-FM DA probe (10 μg mL^{-1}). After 45 min, mice were intravenously (*i.v.*) injected