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Free paclitaxel-loaded E-selectin binding peptide modified micelle self-assembled from hyaluronic acid-paclitaxel conjugate inhibit breast cancer metastasis in a murine model

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ABSTRACT

The present work seeks to construct a nanovehicle for the efficient suppression of breast cancer metastasis through targeting E-selectin on tumor vascular endothelial cells and hyaluronic acid-receptor on tumor cells. Herein, a new ligand-PEG-lipid conjugate, E-selectin binding peptide-polyethene glycol-1-octadecylamine (Esbp-PEG-OA), was used as the targeting molecule of micelle self-assembled form hyaluronic acid-paclitaxel (HA-PTX) conjugate. When loaded with free PTX, the micelles (Esbp-HA-PTX/PTX) exhibited nanoscale particle size with high drug-loading capacity (up to 31.5%). *In vitro* release study showed that the conjugated and entrapped PTX released simultaneously. Cellular uptake of micelles confirmed that Esbp-HA-PTX micelles could be specifically and efficiently internalized into E-selectin expressing human umbilical vein endothelial cells (HUVEC) and 4T1 breast cancer cells *via* receptor-meditated endocytosis. *In vitro* cytotoxicity assay further revealed that Esbp-HA-PTX/PTX micelles significantly improved the selectivity of PTX for killing the two cell types compared with PTX solution formulation. More importantly, Esbp-HA-PTX micelles raised the accumulation of payload in tumor through targeting two cell types in the tumor microenvironment simultaneously, resulting in marked *in vivo* inhibition of tumor growth, intratumoral microvessel density and metastasis, and decreased systemic toxicity over solution formulation. Overall, Esbp-HA-PTX/PTX micelle is promising in therapy of breast cancer metastasis.

1. Introduction

Over the past decades, a continuous evolution related to cancer research and treatment has been performed. However, as an important approach for cancer therapy, the efficacy of low-molecular-weight drugs is still limited by the low therapeutic efficacy, severe toxic side effects and occurrence of distant metastases (Fu et al., 2015; Li et al., 2015; Satsangi et al., 2015). Currently, active targeting nanoscale drug delivery systems (NDDSs) are widely expected to bring new hope to address these problems, due to their unique pharmacokinetics and biodistribution behavior (Altangerel et al., 2016; Wei et al., 2013; Ziemys et al., 2016). Anti-vasculature in tumors represents one of the most attractive active targeting strategies since solid tumors (beyond 1–2 mm in diameter) acquire growing number of vessels to maintain growth and form metastases (Vázquez et al., 2013). Endothelial cells are indispensable for vascular system as well as the process of new vasculature development. The specific receptors on vascular endothelial cells (*e.g.* VEGFRs, $\alpha_v\beta_3$ integrin and E-selectin, aminopeptidase-N and Eph receptors) have been reported to be accessible for anti-vasculature drug delivery (Arosio and Casagrande, 2016; Guo et al., 2015; Shamay et al., 2016; Shamay et al., 2015; Wang et al., 2012).

Among these receptors, E-selectin (CD62E) is an inducible transmembrane adhesion protein that is expressed exclusively on the activated endothelial cells in response to cytokines such as TNF-α and IL1-β, during inflammation and tumor progression (Kang et al., 2016). In tumors, E-selectin positive vessels are preferentially found in vasculature-rich areas and the expression of E-selectin was co-localized with dividing microvascular

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endothelial cells with active angiogenesis (Shamay et al., 2015; Spivak et al., 2016). Therefore, E-selectin plays a functional role in metastasis in various cancers (St Hill, 2011). Several NDDSs were previously exploited to enhance the accumulation of nanovehicles in tumor vasculature *via* E-selectin-mediated cellular uptake, using the natural ligand (*e.g.* sialyl Lewis x and sialyl Lewis a) or synthetic peptides (Jubeli et al., 2012a; Jubeli et al., 2012b; Shamay et al., 2015). In this study, E-selectin binding peptide (Esbp) with a sequence of CDKDKDITWDQLWDLMK-NH_2 was chosen as targeting motif to mediate the drug delivery to E-selectin expressing tumor vasculature, due to the high affinity with E-selectin (nanomolar range) (Martens et al., 1995).

Hyaluronic acid (HA) is a non-toxic extracellular matrix component, composed of repeating disaccharides of *N*-acetyl-D-glucosamine and D-glucuronic acid linked by β-1,3 and β-1,4 glycosidic bond (Wang et al., 2017). In recent years, HA is wildly used as the targeting and capping material for cancer therapy, because over-expression of HA binding receptors, such as CD44, RHAMM and LYVE-1, has been found on the surface of many types of tumor cells (Badwaik et al., 2016; Schledzewski et al., 2006; Tripodo et al., 2015). The easily modified structure further makes HA-based amphiphile and prodrug very attractive for tumor cell-targeted drug delivery (Li et al., 2012; Liu et al., 2016). In addition, HA was selected because of its other favorable properties, including biocompatibility, biodegradability, non-immunogenicity and hydrophilicity (Tripodo et al., 2015).

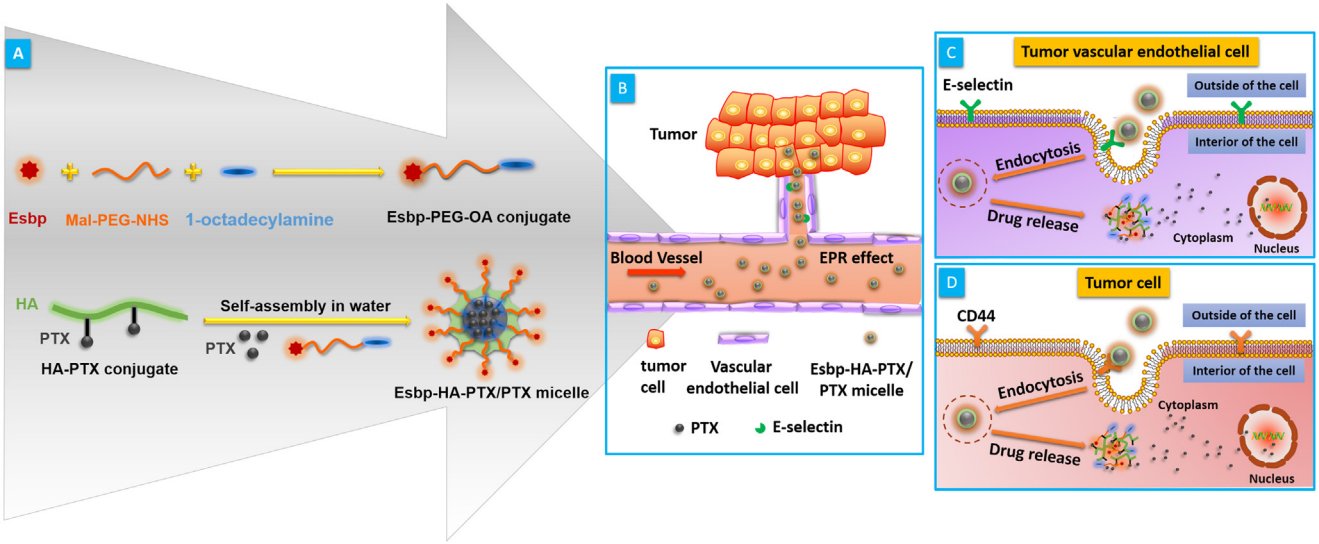
Based on the background above, a novel Esbp-modified HA-based polymeric micelle (Esbp-HA-PTX) was designed to target both tumor vascular endothelial cells and tumor cells, for therapy of breast cancer metastasis. To prepare this NDDS, an amphiphilic polymer-drug conjugate, HA-paclitaxel (HA-PTX) was firstly synthesized. The HA-PTX conjugate would not only serve as a water-soluble prodrug for PTX but also could be used as carrier of free PTX. Then, a ligand-PEG-lipid conjugate, Esbp-polyethylene glycol-1-octadecylamine (Esbp-PEG-OA), was synthesized for use as tumor vascular endothelial cell-targeting moiety of HA-PTX micelles. As shown in Scheme 1, when HA-PTX conjugates self-assembled in aqueous environment, free PTX was loaded into the hydrophobic inner-core of micelles. Meanwhile, the hydrophobic segment of Esbp-PEG-OA conjugate, 1-octadecylamine, could insert into the hydrophobic inner-core of micelles, while Esbp were modified on the surface of micelles. We hypothesize that the designed Esbp-HA-PTX/PTX micelles could accumulate in tumor site through the enhanced permeability and retention (EPR) effect in bloodstream, selectively taken up to E-selectin expressing vascular endothelial cells as well as tumor cells *via* receptors-mediated endocytosis, and then achieve therapeutic effect to suppress tumor growth and metastasis. Accordingly, the objectives of this study were to develop the targeting Esbp-HA-PTX micelles, investigate their action mechanisms, and evaluate their anti-tumor and metastasis prevention efficacy in a highly metastatic tumor model.

2. Materials and methods

2.1. Materials, cell culture and animals

Sodium hyaluronic acid (HA, molecular weights 11 kDa) was purchased from Freda Biochem Co., Ltd. (Shandong, China). Formamide and *N*,*N*-dimethyl formamide (DMF) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 1-Ethyl-3 (3-dimethylaminopropyl) carbodiimide (EDC) and *N*-Hydroxysuccinimide (NHS) were purchased from Aladdin Reagent Database Inc. (Shanghai, China). Paclitaxel (PTX) was purchased from Shanghai Zhongxi Sunve Pharmaceutical Co., Ltd. (Shanghai, China). Adipic dihydrazide, succinic anhydride and 1-octadecylamine were provided by TCI Development Co., Ltd. (Shanghai, China). Heterobifunctional PEG (Mal-PEG-NHS, molecular weights 3.5 kDa) was obtained from JenKem Technology Co., Ltd. (Beijing, China). The E-selectin binding peptide (Esbp) with primary sequence of CDKDKDITWDQLWDLMK-NH_2 was obtained from SciLight Biotechnology, LLC. (Beijing, China). Coumarin 6 was purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Human E-Selectin Antibody and NorthernLights™ Anti-mouse IgG-NL557 were provided by R&D Systems, Inc (Minneapolis, Minnesota, USA). Rabbit polyclonal to CD31 was obtained from Abcam Inc. (Cambridge, Massachusetts, USA). DAPI and BCA protein assay kit were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Water used in the experiment was doubly distilled and deionized. All other chemicals were of analytical reagent grade.

Human umbilical vein endothelial cells (HUVEC) were obtained from ScienCell Research Laboratories (California, USA). Cells were routinely grown in flasks coated with 0.5% gelatin in Endothelial



Scheme 1. Schematic illustration of the self-assembly (A), accumulation at tumor tissue through EPR effect (B) and receptor-meditated cellular internalization of Esbp-HA-PTX/PTX micelles in tumor vascular endothelial cell (C) and tumor cell (D).