Journal of Controlled Release 257 (2017) 10–20

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Inhibition of CD44v3 and CD44v6 function blocks tumor invasion and metastatic colonization

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ARTICLE INFO

Article history:
Received 29 September 2016
Accepted 12 January 2017
Available online 16 January 2017

Keywords:
CD44
Migration
Polymer-drug conjugates
Targeted cancer therapy

ABSTRACT

The prevention of cancer cell dissemination and secondary tumor formation are major goals of cancer therapy. Here, we report on the development of a new CD44-targeted copolymer carrying multiple copies of the A5G27 peptide, known for its ability to bind specifically to CD44v3 and CD44v6 on cancer cells and inhibit tumor cell migration, invasion, and angiogenesis. We hypothesized that conjugation of A5G27 to *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer would enhance tumor tissue accumulation, promote selective binding to cancer cells, with concomitant increased inhibition of cancer cell invasiveness and migration. Fluorescein-5-isothiocyanate or the near-infrared fluorophore IR783 were attached to the copolymer backbone through a non-cleavable linkage to assess *in vitro* binding to cancer cells and biodistribution of the polymer in 4T1 murine mammary adenocarcinoma-bearing mice, respectively. The anti-migratory activity was evaluated both *in vitro* and *in vivo*. The binding of the targeted copolymer to cancer cells correlated well with the level of CD44 expression, with the polymer being internalized more efficiently by cancer cells. Pre-treatment of mice with polymer-bound A5G27 significantly inhibited lung colonization of migrating 4T1 cells *in vivo*, with the targeted copolymer accumulating preferentially in subcutaneous 4T1 tumors, when compared to a non-targeted system. As such, the HPMA copolymer-A5G27 conjugate is a promising candidate for inhibiting cancer cell migration and can also be used as a drug or imaging probe carrier for detection and treatment of cancer.

1. Introduction

Tumor targeted therapies developed over past decades have significantly changed the medical treatment of cancer. Targeted therapies, which include monoclonal antibodies and small molecule inhibitors, are now a component of treatment for many types of cancer, including breast, colorectal, lung, and pancreatic cancers, as well as lymphoma, leukemia, and multiple myeloma [1]. Yet, a major limitation for targeted therapies is that cancer cells can become resistant to treatment. Moreover, currently available therapies are ineffective once metastases has been established, such that there are currently no effective means to block or control metastatic dissemination of cancer cells [2].

Adhesion molecules normally associated with cell migrations that occur during embryogenesis and inflammation are often up-regulated in highly aggressive carcinomas [3]. For example, CD44, a multifunctional transmembrane glycoprotein involved in both physiological and pathological processes, including cell adhesion, angiogenesis, inflammation, tumor growth and metastasis [4–7], is up-regulated in many invasive carcinoma cells [6]. CD44 is the primary receptor for hyaluronic acid (HA), osteopontin, collagen, laminin, fibronectin and cell surface receptors like E-selectin and L-selectin [8–11]. High CD44 expression has been identified in several solid tumors, including lung cancer [12], breast cancer [13], prostate cancer [14], gastric cancer [15], colon cancer [16], malignant glioma [17], and ovarian cancer [18]. Moreover, high CD44 expression was correlated with the phenotypes of cancer stem cells [19] and epithelial–mesenchymal transition (EMT) [20], thereby contributing to tumor invasion, metastasis, recurrence, and chemo-resistance [21]. Recent studies showed that specific targeted knockdown of CD44 attenuated cancer progression [22]. This suggests that CD44 may be a promising target for cancer treatment, and that blockade of CD44-mediated activity can attenuate the malignant phenotype, slow cancer progression, and invasiveness.

Abbreviations: AIBN, 2,2′azobis(isobutyronitrile); BME, Basement membrane extract; EPR, Enhanced permeability and retention; FITC, Fluorescein-5-isothiocyanate; FPLC, Fast protein liquid chromatography; GG, Glycylglycine; HPMA, *N*-(2-hydroxypropyl) methacrylamide; HA, hyaluronan, hyaluronic acid; HPLC, High-performance liquid chromatography; MA-FITC, 5-[3-(Methacryloylaminopropyl)thioureidyl]fluorescein; MAP-Boc, Methacryloyl-aminopropyl tert-butyloxycarbonyl; MA-GG-OH, Methacryloyl-glycylglycine; MA-GG-ONp, Methacryloyl-glycylglycine *p*-nitrophenyl ester; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolinium bromide; Mw, Molecular weight; NIRF, Near-infrared fluorescence; ONp, *O*-nitrophenyl; SEC, Size-exclusion chromatography.

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http://dx.doi.org/10.1016/j.jconrel.2017.01.021
0168-3659/

Numerous nano-sized medicines (nanomedicines) have been developed in recent decades as actively targeted drug carriers for cancer therapy [23–26]. Accordingly, HA-based nanocarriers potentially offer more effective treatment with significantly reduced adverse effects through passive accumulation in solid tumors owing to the enhanced permeability and retention (EPR) effect, and the specific interactions with CD44-overexpressing cancer cells in the tumor microenvironment [27]. With this in mind, we previously designed *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer-paclitaxel conjugates with multiple copies of low molecular weight (Mw) HA fragments (P-(HA)-PTX) for targeting CD44-over-expressing cancer cells [28]. P-(HA)-PTX exhibited 50-fold higher cytotoxicity towards CD44-over-expressing cells than did control, non-targeted copolymer, indicating the selective binding, enhanced uptake, and superior efficacy of the copolymer conjugates against CD44-over-expressing cells. Yet, HA can interact with CD44 on non-cancerous cells and thus promote adverse effects. In addition, due to the poor solubility of HA and of low Mw HA fragments in aqueous solutions and most organic solvents, the conjugation of HA fragments to polymeric carriers was often found to be very challenging. Thus, despite significant promise, only few HA-based nanocarriers are currently in clinical evaluation [27].

The laminins, a family of glycoproteins assembled from alpha, beta, and gamma chain subunits, are components of epithelial cell basement membranes that interact with CD44 and regulate cell adhesion, migration, and angiogenesis [29]. The laminin-alpha 5 chain-derived synthetic peptide A5G27 (primary sequence RLVSYNGIIFFLK), was previously reported to bind specifically to CD44v3 and CD44v6 versions of CD44 that are expressed in various types of malignancies but are not found in non-metastatic tumors and the corresponding benign tissues [30–32]. A5G27 binds to the glycosaminoglycan (GAG) side chains of CD44v3 and CD44v6 and was shown to inhibit tumor cell migration, invasion, and angiogenesis *via* blocking fibroblast growth factor 2 (FGF2) binding to the GAG side chains of CD44 [33–35]. When encapsulated in a gel culture system with cancer cells, A5G27 abolished breast cancer tumorsphere formation *in vitro* and *in vivo* [36].

Previously, we demonstrated that poly(ethylene glycol)-*block*-polyethylenimine (PEG-*b*-PEI)-based polyion complexes bearing an *N*-terminally truncated version of the peptide (A5G27F) as targeting ligand could deliver small interfering RNA (siRNA) molecules directly to CD44-over-expressing tumors *in vivo*, thereby inhibiting tumor growth and cancer cell migration [37]. Now, we describe the design of water-soluble HPMA copolymer-A5G27 conjugate (a "drug-free" copolymer, *i.e.*, not carrying any drug cargo) for blocking breast cancer cell migration, invasion and metastasis. We hypothesized that the "drug-free" copolymer would passively accumulate in solid tumors, owing to its macromolecular size, and impair tumor cell migration and invasion by blocking CD44-mediated adhesive interactions. Two types of HPMA copolymers bearing the A5G27 sequence were prepared. Fluorescein-5-isothiocyanate (FITC)-labeled copolymer, P-(A5G27)-FITC (where P designates the HPMA copolymer backbone) (Fig. 1A), was designed for tracking the binding and intracellular fate of the copolymer *in vitro*, while the near-infrared fluorescent (NIRF)-labeled HPMA copolymer, P-(A5G27)-IR783 was synthesized for testing the biodistribution and tumor accumulation in 4T1 murine model of mammary adenocarcinoma (Fig. 1B). The anti-metastatic activity of the "drug-free" polymer conjugate P-(A5G27)-FITC was evaluated in both *in vitro* and *in vivo* settings. Our data demonstrate that the HPMA-copolymer A5G27 conjugate was able to inhibit metastatic dissemination of migrating prostate and breast cancer cells.

2. Materials and methods

2.1. Materials

All chemicals were reagent grade and obtained from Sigma-Aldrich (Rehovot, Israel) unless stated otherwise. N-terminal *N*-acetylated lysine-harboring A5G27 (Ac-K- RLVSYNGIIFFLR) and the control scrambled A5G27 sequence A5G27scrm (Ac-K-VLFGFLRIYSRIN) were purchased from GL Biochem (Shanghai, China). The C-terminal lysine in the original sequence [34] was replaced with arginine to avoid reactions with reactive ester groups on the polymer precursor. Mouse/human-anti CD44 pan-specific antibodies were purchased from Biolegend (San Diego, CA). Alexa-488-conjugated anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). DAPI-Fluoromount-G was from SouthernBiotech (Birmingham, AL). A Cultrex Cell Migration Assay kit was purchased from Trevigen (Gaithersburg, MD). Luciferin was purchased from Perkin (Hopkinton, MA). LysoTracker Red DND-99 was purchased from Invitrogen (Waltham, MA). The coupling reagent *N*,*N*-diisopropylethylammonium (DIPEA) was purchased from Novabiochem-Merck (Darmstadt, Germany). Paclitaxel was purchased from LC Laboratories (Woburn, MA). Methacryloyl-aminopropyl (MAP) and methacryloyl-aminopropyl tert-butyloxycarbonyl (MAP-Boc) were purchased from Polysciences (Warrington, PA). The monomers methacryloyl-glycylglycine *p*-nitrophenyl ester (MA-GG-ONp) [38] methacryloyl-aminopropyl fluorescein-5-isothiocyanate (MAP-FITC) [39] and HPMA [40] were synthesized as described previously and their structures are shown in Fig. 1A and B.

2.2. Cell lines

The murine mammary gland adenocarcinoma breast cancer (4T1), the human prostate cancer derived from bone metastasis (PC3) and the human prostate cancer derived from metastatic site left supraclavicular lymph node (LNCaP) cell lines were purchased from the American Type Culture Collection (Manassas, VA). 4T1-lutciferase cells were kindly provided by Prof. Roni N. Apte (Ben-Gurion University of the Negev, Israel). 4T1 and LNCaP cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, 100 IU/ml penicillin, and 100 μg/ml streptomycin. PC3 were grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (all from Biological Industries, Beit Haemek, Israel). The cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Mice

Female BALB/c mice were obtained from Harlan Biotech (Rehovot, Israel). Mice at 6–8 weeks of age were housed in accordance with approved institutional guidelines. All experimental procedures were approved and performed in compliance with the standards of the Ben-Gurion University of the Negev Institutional Animal Care and Use Committee.

2.4. Synthesis of the FITC-labeled CD44-targeted copolymer

The FITC-labeled HPMA precursor copolymer bearing reactive ester groups (*O*-nitrophenyl, ONp) for peptide attachment (designated as P-(GG-ONp)-FITC, where P represents the HPMA copolymer backbone) (precursor (1) in Table 1) was synthesized by radical precipitation copolymerization of HPMA, MA-GG-ONp, and MAP–FITC (92:6:2 mol%, respectively) in an acetone/dimethyl sulfoxide (DMSO) mixture in a sealed vial at 55 °C for 24 h using AIBN as the initiator, as described previously [41,42]. The polymer was precipitated into cold diethyl ether and desiccated. The polymer was then purified on a LH-20 column, using methanol as eluent. The weight average Mw of P-(GG-ONp)-FITC was estimated by size-exclusion chromatography (SEC) using a Sephacryl S-400 column on an AKTA FPLC system (GE Healthcare), calibrated with fractions of HPMA homopolymers of known molecular weight. For *in vitro* migration assays, a precursor (2) (*i.e.*, without FITC) was synthesized similarly, using HPMA and MA-GG-ONp