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Effective targeting of gemcitabine to pancreatic cancer through PEG-cored Flt-1 antibody-conjugated dendrimers

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ABSTRACT

Tumor-targeted delivery of anticancer drugs using dendrimers has been recognized as a promising strategy to increase efficiency and reduce adverse effects of chemotherapy. Herein, we developed a dendrimer-based drug delivery system targeting Flt-1 (a receptor for vascular endothelial growth factors (VEGF)) receptor to improve therapeutic efficacy of gemcitabine in pancreatic cancer. Synthesized polyethylene glycol (PEG)-cored PAMAM dendrimers, which bear anionic carboxylic acid groups on the surface were modified with PEG chains, which were then conjugated with Flt-1 antibody. Following structural and chemical characterization studies, gemcitabine HCl-dendrimer inclusion complexes were successfully prepared. These complexes were efficiently engulfed by Flt-1 expressing pancreatic cancer cells, which enhanced the cytotoxicity of gemcitabine. Moreover, pancreatic tumors established in mice were highly targeted by PEG-cored Flt-1 antibody-conjugated dendrimers and increased accumulation of these gemcitabine-loaded complexes exhibited satisfactory *in vivo* anti-cancer efficacy. In conclusion, dendrimer-based targeted delivery of chemotherapeutics may serve as a promising approach for the treatment of malignancies such as pancreatic cancer that do not benefit from conventional chemotherapy.

1. Introduction

Compared to conventional linear polymers, highly branched macromolecules, *i.e.* dendrimers, are more advantageous due to their multi-functionality and have applications in numerous fields (Klajnert and Bryszewska, 2001). Dendrimers are precisely controlled structures that are globular in shape with low poly dispersity (Abbasi et al., 2014). Moreover, due to many surface groups, they display a large number of controllable 'peripheral' functionalities. The core, interior and surface of dendrimers can be tailored for different applications. These applications are based on their multifunctional surface, unparalleled molecular structure and internal cavities (Svenson and Tomalia, 2005).

High generation dendrimers commonly possess symmetrical shape (Lee et al., 2005). A compact globular structure can be formed as dendrimers extend out to the periphery. Therefore, these non-linear polymers possess capacities to be used in biomedical applications (Sevenson and Tomalia, 2012). Cationic dendrimers, *e.g.* amine-terminated PAMAM or poly(propylene imine) dendrimers, alter the conformation of membrane proteins and are generally hemolytic and cytotoxic (Chen et al., 2004). As the generation and concentration of cationic PAMAM dendrimers increase, their capacity to interact with cell membrane improves. Unfortunately, not only the cancer cells but also normal cells are negatively affected by this non-specific membrane affinity. Nevertheless, the functional groups of dendrimers can be modified for targeting the tumor cells more selectively (Saovapakhiran et al., 2009; Jevprasesphant et al., 2003).

PAMAM dendrimers are useful molecules for targeted delivery of anti-neoplastic drugs into the tumor microenvironment. For example, modification of dendrimers with poly(ethylene glycol) (PEG) improves their solubility and stability, increases the solubility of hydrophobic compounds in aqueous solutions, and maintains the permeability and retention (EPR) effect that augments intratumoral accumulation (Shukla et al., 2008; Wangler et al., 2008; Zhu et al., 2010; Patri et al., 2004; Kulhari et al., 2016). Moreover, incorporation of ligands (*e.g.*, folate, transferrin, dextran, galactose) (Gupta et al., 2010; He et al., 2011; Kesharwani et al., 2011) or antibodies (*e.g.*, anti-growth factor receptor-2 antibody, anti-epidermal growth factor receptor antibody) (Shukla et al.,

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2008; Wangler et al., 2008) recognizing the target molecules expressed in the tumor microenvironment. Accessorizing dendrimers with specific ligands or antibodies has been acknowledged as a promising strategy that enables the active targeting of tumors including breast, skin and prostate cancers (Shukla et al., 2008; Zhu et al., 2010; Patri et al., 2004; Kulhari et al., 2016).

Even though its incidence is not high, due to late diagnosis and poor prognosis, pancreatic cancer is currently the fourth leading cause of cancer deaths (Hariharan et al., 2008). The patients diagnosed with pancreatic cancer do not ultimately benefit from conventional anti-cancer therapeutics (Oberstein and Olive, 2013). Gemcitabine [20,20-difluoro-20-deoxycytidine (dFdC)], which is the current standard therapy for advanced pancreatic cancer, can prolong survival of patients, however the median survival duration is less than 6 months (Moore et al., 2007). This cytostatic drug is a low molecular weight molecule and acts as a deoxycytine analog inhibiting cellular DNA synthesis (Gil'deeva and Semeikin, 2009). Short half-life and rapid metabolism of gemcitabine limit tumor uptake and anti-cancer efficacy (Vandana and Sahoo, 2010). Thus, novel approaches including targeted and combinatory modalities have been proposed for treatment of pancreatic cancer. In order to obtain an effective cancer therapy regimen, a well-designed combination of multi-therapeutic agents with different modes of action is essential (Lee et al., 2008).

To maintain their malignant growth, tumors need a continuous supply of oxygen and nutrients. Through secreting various factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), tumors induce capillary growth (angiogenesis) that provide them with accessing the blood stream (Nishida et al., 2006). Therefore, anti-angiogenic therapies inhibiting VEGF and downstream signaling pathways are being employed as an adjuvant treatment (Niu and Chen, 2010). The tumor cells can also express the receptors for VEGF and establish an autocrine positive feedback loop promoting their growth and survival (Goel and Mercurio, 2013). VEGFR-1 (Flt-1) and VEGFR-2 are two cognate receptors for VEGF (Gille et al., 2001). Flt-1 expression is increased in ischemia and inflammation, and upregulated in many types of tumors including colorectal, prostate, esophageal, breast and in non-small cell lung cancer and hepatocellular carcinoma (Fischer et al., 2008). Flt-1 is associated with poor prognosis, metastasis, and recurrence in breast and lung cancers and in leukemia. Accordingly, targeting of this receptor with specific antibodies or small peptides has been regarded as a successful anti-cancer strategy (Fischer et al., 2008).

In this study, high generation PEG-cored PAMAM dendrimers were modified with PEG 2000 and then conjugated with anti-Flt-1 antibody. Enhanced ligand binding to receptor and specific uptake by pancreatic cancer cells through antibody conjugation and PEGylation were aimed. Following the loading of gemcitabine into designed multifunctional dendrimers, their capacity to target pancreatic cancer cells *in vitro* and *in vivo* was evaluated. Combination of Flt-1-targeting antibodies and gemcitabine in a dendrimer-based carrier platform displayed improved anti-cancer efficacy, and showed promising results for the treatment of pancreatic cancer.

2. Materials and methods

2.1. Conjugation of anti-human Flt-1 or isotype-matched control antibodies onto PEG-cored PAMAM type dendrimers with PEG2000 surface modification (PEGcPAMAM-PEG)

Microwave-assisted synthesis (MAS) of PAMAM-OCH_3 dendrimers was performed as described in our previous studies (Ertürk et al., 2014; Ozturk et al., 2014; Gurbuz et al., 2016). Surface modification of PAMAM dendrimers with PEG bisamine (JemKem Technology USA) and anti-Flt-1 antibody or isotype-matched control antibody was performed according to literature procedures (Clayton et al., 2011). (Benzotriazol-1-yloxy)tris(dimethylamino) phosphonium hexafluorophosphate (BOP) (Merck) (1.5 μg) in anhydrous dimethylformamide (DMF) (Sigma Aldrich) (1 mL) was added into a solution of Anti-Flt-1 antibody or isotype-matched control antibody (400 μg, 2.2 nmol) in anhydrous DMF in a 10 mL round-bottomed flask and stirred for 2 h at room temperature. A well-stirred solution of PEGcPAMAM-PEG (1 mg) in anhydrous DMF (2 mL) was added into the flask with triethylamine (TEA) (Merck) (1.2 μL). The reaction was stirred for 5 days, under nitrogen. Then, the solution containing dendrimer-antibody conjugate was dialyzed in deionized water (membrane MW cut-off, 100 kDa) in order to remove excess of PAMAM and then lyophilized overnight to obtain the final product as a pale yellow gel. Success of the antibody conjugation to the surface PEG of dendrimer was monitored by ^{1}H nuclear magnetic resonance (NMR) (Fig. 1A). Typical resonance peaks around 7–8 ppm arising from antibody was detected (Starovasnik et al., 1999).

LPR ultrafiltration membranes, Amicon 8000 Stirred Cell and dialysis membranes with molecular cut of size (MWCO) of 1–100 kDa were supplied from Millipore. NMR spectra were recorded on a Bruker Avance 500 MHz Spectrometer. A schematic demonstration of the PEG-cored Flt-1 antibody-conjugated dendrimers that were used for gemcitabine loading is given in Fig. 1B.

2.2. Quantification of gemcitabine HCl

The amount of Gemcitabine HCl (Sigma-Aldrich Chemical Company, Steinheim, Germany) in water was quantified by reversed-phase-HPLC. HPLC system (Agilent 1200) was operated in a binary mode with a photodiode array detector, SIL-10AD VP auto injector and a communication bus module. The analysis was performed at 25 °C on Kromasil C18, 250_4.0 mm; 5 μm HPLC column using a mobile phase of phosphate (60%) and methanol (40%) (Sigma-Aldrich Chemical Company, Steinheim, Germany) pumped at a flow rate of 1.0 mL/min, monitored at a wavelength of 270 nm. The total amount of Gemcitabine HCl in Gemcitabine-dendrimer inclusion complex was determined by the peak area correlated with standard curve. Stability studies conducted under pH 5.5 and 7.4 at 37 °C for 5 μg/mL concentration point. The standard curve of Gemcitabine HCl was prepared under identical conditions. This method was validated for selectivity, linearity range, sensitivity, precision, accuracy, and stability according to the International Conference on Harmonization (ICH) guideline.

As a result, retention time of gemcitabine was 3.18 min and the calibration graph was rectilinear in the concentration range of 0.1–100 μg/mL with a correlation coefficient of 0.99. The intra- and inter-day accuracy and precision was within CV of >2%. The sensitivity of the analytical method was evaluated by determining the limits of detection (LOD) and quantitation (LOQ), which were found 46.7 ng/mL and 102.4 ng/mL, respectively. For all the concentrations studied, the relative standard deviations were less than 1.97%. The chromatogram of dendrimer solution did not show any other peaks, thus, it confirmed the selectivity of the method. The drug remained stable for at least 24 h since 97.6% and 98.59% recovery were obtained at pH 5.5 and pH 7.4, respectively.

2.3. Preparation of gemcitabine-loaded dendrimer complexes and determination of encapsulation efficiency

Various formulations of synthesized dendrimers (Table 1) were loaded with gemcitabine HCl. Gemcitabine-encapsulated complexes were produced with slight modifications according to a method previously described by our group (Ozturk et al., 2014).