Journal of Controlled Release xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Targeted delivery of platinum-taxane combination therapy in ovarian cancer

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ARTICLE INFO

Article history:
Received 16 July 2015
Received in revised form 1 September 2015
Accepted 7 September 2015
Available online xxxx

Keywords:
Combination drug delivery
Nanogels
Ovarian cancer
Cisplatin
Paclitaxel
Receptor targeting

ABSTRACT

Biodegradable polypeptide-based nanogels have been developed from amphiphilic block copolymers, poly(ethylene glycol)-b-poly(L-glutamic acid)-b-poly(L-phenylalanine), which effectively co-incorporate cisplatin and paclitaxel, the clinically used drug combination for the treatment of advanced ovarian cancer. In order to target both drugs selectively to the tumor cells, we explored the benefits of ligand-mediated drug delivery by targeting folate receptors, which are overexpressed in most ovarian cancers. Drug-loaded nanogels were surface-functionalized with folic acid (FA) with the help of a PEG spacer without affecting the ligand binding affinity and maintaining the stability of the carrier system. FA-decorated nanogels significantly suppressed the growth of intraperitoneal ovarian tumor xenografts outperforming their nontargeted counterparts without extending their cytotoxicity to the normal tissues. We also confirmed that synchronized co-delivery of the platinum-taxane drug combination via single carrier to the same targeted cells is more advantageous than a combination of targeted single drug formulations administered at the same drug ratio. Lastly, we demonstrated that the same platform can also be used for localized chemotherapy. Our data indicate that intraperitoneal administration can be more effective in the context of targeted combination therapy. Our findings suggest that multifunctional nanogels are promising drug delivery carriers for improvement of current treatment for ovarian cancer.

1. Introduction

Combination chemotherapy is preferred over treatment with single agents to combat most cancers as it targets multiple cell-survival pathways at the same time and delays the onset of resistance. This helps in achieving long-term tumor remission and increases median survival [1]. Cisplatin (CDDP)-based therapy has been the standard treatment for ovarian cancer since its discovery. After paclitaxel (PTX) was shown to be effective in ovarian cancer, multiple clinical trials studied the overall efficacy of CDDP and PTX and found significant benefit over the pre-existing treatments. Since then, this combination has been the treatment of choice for both early stage as well as advanced cases of ovarian cancer [2,3]. However, administration of two different agents comes with the inconvenience of repeated or extended duration of drug infusion in patients. Moreover, the most extensively used conventional formulation of paclitaxel, Taxol®, utilizes Cremophor EL (polyethoxylated castor oil) that has been linked to significant toxicities including allergic, hypersensitivity and anaphylactic reactions during infusion that require premedication and prolonged peripheral neuropathy. Combining such drugs in one delivery carrier is therefore a well-suited and convenient strategy for controlling the pharmacokinetics and co-delivery of the desired drug ratio in vivo, to maximize the therapeutic potency and minimize drug-associated toxicities.

Cross-linked nanogels have been found to be promising drug carriers to achieve this goal. Being mostly hydrophilic in nature, nanogels are highly biocompatible with a high loading capacity for guest molecules [4]. The nanogel structure can be readily adjusted to integrate features of different materials and, thus, offer advantages for combinatorial encapsulation of drugs with varying physicochemical properties. One of the widespread synthetic techniques for the synthesis of nanogels is the crosslinking of preformed core-shell self-assemblies such as polymer micelles that allows introducing a high degree of spatial organization into the nanogels [4,5]. Cross-linking is known to impart control over the swelling behavior of the nanogels and thus helps in achieving controlled release of the incorporated cargo, which is an added advantage over the structural integrity imparted to the carrier system upon *in vivo* administration [4,6]. The enhanced stability also makes the prolonged circulation of the nanogels possible, which in turn allows for increased drug accumulation at the target site [5–9].

We have previously described a biodegradable hybrid nanogel carrier system (NG) for the combination of CDDP and PTX for ovarian cancer therapy, which not only mitigated the toxicities associated with the use of free drugs but also improved treatment outcome [10]. However, our

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http://dx.doi.org/10.1016/j.jconrel.2015.09.007
0168-3659/

system relied solely on the enhanced permeation and retention (EPR) effect to facilitate the delivery of the drug combination to the tumor site [11]. Regardless of the importance and popularity of EPR effect-based drug delivery, this strategy has some limitations related to the inter- and intra-tumor heterogeneity, variations in the density as well as permeability of the tumor vasculature that can affect the accumulation of nanocarriers. One of the popular approaches to circumvent these problems is by surface-functionalization of the drug carrier with ligands that can target receptors with differential expression on the cancer cell surface, which helps in increasing the mean residence time of the delivery system at the tumor site and improving target cell uptake [12–15]. One such receptor of interest is the folate receptor (FR). Malignant cells, due to their high rate of cell division, have an increased requirement of folic acid (FA), since it is an essential component of cell metabolism and DNA synthesis and repair. To fulfill this higher need of FA, FR is known to be over-expressed in a large number of malignant tissues, including ovarian cancer, compared to normal tissues with the exception of the kidney and choroid plexus [16,17]. Furthermore, this receptor becomes accessible via the plasma compartment only after the cells lose their polarity owing to malignant transformation which makes it a differential target for cancer tissue that is easily accessible for intravenously administered FA-conjugated systems. Its natural ligand, FA, comes with the advantages of high binding affinity, stability and a simple chemical structure together with ease of availability, making it a suitable targeting ligand for ovarian cancer therapy. FA can thus be successfully conjugated to macromolecular systems without loss of binding affinity to its receptor [18]. Many different agents targeting the folate pathway are currently in clinical development [19]. To date, FA-targeted agents showed significant promise in phase II clinical trials but it has not been confirmed in phase III studies [20–22]. Accordingly, there is a need for further identification of new therapeutic combinations and refinement of patient selection. To this end, FA-conjugated imaging agents could be used for pre-selection of patients based on the expression of FR [23] and several methods have been already developed for this purpose [24,25].

Our group has previously demonstrated a tumor-specific delivery and improved anti-cancer effect *in vivo* of CDDP-loaded NGs decorated with FA targeting groups [26]. In the current study, we designed FA-linked NGs incorporating platinum-taxane combination, and examined whether FR-targeted concurrent delivery of synergistic combination of CDDP and PTX can lead to enhanced therapeutic efficacy compared to nontargeted NG system.

2. Materials and methods

2.1. Materials

α-Amino-ω-methoxy poly(ethylene glycol) (mPEG-NH_2, Mw = 5000 g mol^{-1}, M_w/M_n = 1.05) was purchased from Creative PEGWorks Inc., (NC, USA). Fmoc-NH-PEG-NH_2 (M_w = 7500 g mol^{-1}, Mw/Mn = 1.04) was purchased from JenKem technology (TX, USA). CDDP was purchased from Acros Organics (NJ, USA). L-Glutamic acid γ-benzyl ester (BGlu), L-phenylalanine (Phe), 1,2-ethylenediamine (ED), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), paclitaxel, folic acid and other chemicals were purchased from Sigma-Aldrich (MO, USA) and were used without further purification. Fetal bovine serum (FBS), RPMI 1640 medium, penicillin, streptomycin, Trypsin–ethylenediaminetetraacetic acid (EDTA) (0.5% trypsin, 5.3 mM EDTA tetra-sodium) and other chemicals were purchased from Invitrogen (CA, USA).

2.2. Preparation of polymeric NGs

Poly(ethylene glycol)-*b*-poly(L-glutamic acid)-*b*-poly(L-phenylalanine) block copolymer with 90:25 ratio of glutamic acid and phenylalanine units (PEG–$PGlu_{90}$–$PPhe_{25}$) was synthesized as previously described [10]. Details of the procedures used for polymer synthesis and characterization are described in the Supplementary Information. NGs were prepared by using previously described method [10] using PEG–$PGlu_{90}$–$PPhe_{25}$ micelles as templates for further cross-linking by ED and EDC ([EDC]/[ED] = 2; [COOH]/[EDC] = 5) at r.t., overnight. Byproducts of the cross-linking reaction were removed by exhaustive dialysis of the reaction mixtures against distilled water. To synthesize fluorescence (Alexa 594)-labeled NGs, solution of Alexa Fluor 594 hydrazide (0.25 μmol, equivalent to 1% of carboxylate groups in NGs) in DMF was added to aqueous dispersion of NGs and the mixture was incubated for 2 h in the dark. Unbound dye was removed by filtration on Ultracon filter units (MWCO 10,000 Da, Millipore) at 3000 rpm for 15 min (3 washes) and then additionally purified using NAP-10 column (GE healthcare). Notably, the same stock of Alexa 594-labeled NGs was used to prepare FA-decorated NGs.

2.3. Synthesis of FA-decorated NGs and drug loading

FA targeting moieties were conjugated to drug-loaded NGs via PEG spacer having Fmoc protection group (Fmoc-NH-PEG-NH_2). Firstly, Fmoc-NH-PEG-NH_2 (0.013 mmol, 0.05 eq with respect to the amount of carboxylate groups and 1.5 eq with respect to required FA) was conjugated to the free carboxyl groups (0.25 mmol) of NGs via EDC (0.023 mmol) chemistry. Fmoc group was then removed by adding 5 μL of 30% piperidine/DMF (1.1 eq piperidine, 0.014 mmol) and incubating the reaction mixture for 10 min. Resulting constructs were purified using repeated ultrafiltration (MWCO 30,000, Millipore) at 3000 rpm for 15 min (3 washes). Size exclusion chromatography (ÄKTA Fast Protein Liquid Chromatography system) was used to determine the presence of residual PEG. PEG-NG conjugates were analyzed by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) using Varian INOVA 500 NMR spectrometer (Varian, Palo Alto, CA) operating at 500 MHz (1 mg/mL, D_2O, pH 7.0, 25 °C). The dispersion of PEG-conjugated NGs was mixed with an aqueous solution of CDDP (1 mg/mL) at pH 9.0 at a 0.5 molar ratio of CDDP (0.12 mmol) to carboxylate groups (0.24 mmol) of the NGs followed by incubation at 37 °C for 48 h. Unbound CDDP was removed by Ultracon filter units (MWCO 10,000 Da, Millipore) at 3000 rpm for 15 min. FA (0.025 mmol) dissolved in water was subsequently reacted with 1.5 eq EDC (0.037 mmol) at pH 7 for 2 h. This EDC-activated FA was then conjugated to amine terminus of the PEG linker. Amount of FA was selected based on the previously reported optimal density of FA on NGs (0.1–0.2 μmol/mg of polymer) to maintain good loading, stability and cell uptake [26]. The amount of conjugated FA was determined by UV spectrometry (ϵ_{363} = 6500 M^{-1} cm^{-1}) using non-modified PEG-conjugated NGs a reference. Finally, PTX (0.01 mmol) was solubilized into the hydrophobic PPhe core of CDDP-loaded FA-modified NGs using an extraction method [27]. According to this method, a thin film of PTX (prepared by evaporation of a methanol solution of PTX) was incubated with aqueous dispersion of NGs (24 h, r.t.). Unincorporated PTX was removed by filtration with 0.8 μm syringe filters (Thermo Scientific). Similar procedures were used to prepare single drug NGs formulations. Pt content in NGs (Pt194/Pt195) was measured by the inductively coupled plasma-mass spectrometer (ICP-MS, NexION 300Q, ICP-MS spectrometer, PerkinElmer) calibrated with Pt (2–100 ng/ml) and Holmium as the internal standard. Samples were diluted in 1% HNO_3. PTX levels were determined by HPLC analysis under isocratic conditions using an Agilent 1200 HPLC system with a diode array detector set at 227 nm. As stationary phase a Nucleosil C18 column was used (250 mm × 4.6 mm), a mobile phase of acetonitrile/water mixture (55/45, v/v) was applied at a flow rate of 1 mL/min.

2.4. Physicochemical characterization of the NGs and drug release study

Intensity-mean Z-averaged particle diameter (D_{eff}) and ζ-potential of NGs were determined using a Zetasizer NanoZS (Malvern Instruments

Please cite this article as: S.S. Desale, et al., Targeted delivery of platinum-taxane combination therapy in ovarian cancer, J. Control. Release (2015), http://dx.doi.org/10.1016/j.jconrel.2015.09.007