Biomaterials 178 (2018) 183–192

Contents lists available at ScienceDirect

Biomaterials

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

Correlating quantitative tumor accumulation and gene knockdown using SPECT/CT and bioluminescence imaging within an orthotopic ovarian cancer model

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ARTICLE INFO

Article history:
Received 29 March 2018
Received in revised form
27 May 2018
Accepted 11 June 2018
Available online 13 June 2018

Keywords:
Folate receptor alpha
Folate receptor targeting
siRNA delivery
Polymer micelleplexes
Ovarian cancer
Ovarian cancer xenograft

ABSTRACT

Using an orthotopic model of ovarian cancer, we studied the delivery of siRNA in nanoparticles of triblock copolymers consisting of hyperbranched polyethylenimine-*graft*-polycaprolactone-*block*-poly(ethylene glycol) (hyPEI-*g*-PCL-*b*-PEG) with and without a folic acid targeting ligand. A SKOV-3/LUC FRα overexpressing cell line was employed to mimic the clinical manifestations of ovarian cancer. Both targeted and non-targeted micelleplexes were able to effectively deliver siRNA to the primary tumor and its metastases, as measured by gamma scintillation counting and confocal microscopy. Stability of the micelleplexes was demonstrated with a serum albumin binding study. Regarding biodistribution, intravenous (I.V.) administration showed a slight advantage of FRα targeted over non-targeted micelleplex accumulation within the tumor. However, both formulations displayed significant liver uptake. On the other hand, intraperitoneally (I.P.) injected mice showed a modest 6% of the injected dose per gram (ID/g) uptake within the primary and most interestingly also in the metastatic lesions which subsequently resulted in a 62% knockdown of firefly luciferase expression in the tumor after a single injection. While this is, to the best of our knowledge, the first paper that correlates quantitative tumor accumulation in an orthotopic tumor model with *in vivo* gene silencing, these data demonstrate that PEI-*g*-PCL-*b*-PEG-Fol conjugates are a promising option for gene knockdown in ovarian cancer.

1. Introduction

Ovarian cancer remains one of the deadliest gynecological malignancies. With current treatment options, 65% of all women who are diagnosed will eventually succumb to the disease [1,2]. A primary reason for the low survival rates is that a majority of patients are diagnosed at an advanced stage. The disease progression of ovarian cancer produces aggressive and widespread metastatic lesions throughout the peritoneal cavity [3]. Primary treatments for these patients often include tumor de-bulking surgery along with combination chemotherapy regimens containing a platinum and taxane [1,4]. Unfortunately, with widespread metastatic lesions, resistance and reoccurrence of the disease is the usual course [5–7]. Resistance mechanisms often include an increase in anti-apoptotic proteins, increase in drug efflux pumps, or altered drug targets [8–15]. Once patients experience chemoresistance, treatment options become considerably limited.

It is estimated that over 85% of ovarian tumors have an overexpression of folate receptor alpha (FRα) [16,17]. Folate receptor is expressed in four distinct isoforms: alpha, beta, gamma, and delta [18–20]. Both alpha and beta isoforms are cell surface receptors that are glycosylphosphatidylinositol-anchored (GPI) and linked to the membrane [19]. FRα and FRβ have a very select expression profiles. FRα is expressed on the proximal side of the tubules of the kidneys, spleen, certain lug tissues, as well as a variety of cancers [16,17,21–23]. Additionally, FRβ is mainly expressed on activated macrophages [24]. Both receptors express high affinities for folic acid ($K_d = 1–10$ nM). Therefore, folate receptors have been exploited by researchers to deliver a targeted payload to specific cells of interest by linking a targeting moiety of folic acid to either a drug

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https://doi.org/10.1016/j.biomaterials.2018.06.014
0142-9612/

itself or a delivery vehicle. This approach has been implemented in treating a variety of cancers and autoimmune diseases [24–29]. Accordingly, nanomedicine researchers have utilized this approach to selectively deliver a nanoformulated payload to target cells, while decreasing off-target toxicity caused by uptake of the payload into healthy cells.

One type of payload that researchers have been utilizing in a targeted delivery approach is small interfering RNA (siRNA). siRNA has shown promising potential in treating diseases, such as cancer, by silencing genes that give rise to a diseased phenotypes [30,31]. Naked siRNA is negatively charged, hydrophilic, and easily degraded *in vivo* by nucleases [32]. Due to the properties of naked siRNA, it cannot be delivered effectively to diseased cells without a carrier to protect it and enhance its delivery. Nanoparticle delivery of siRNA has the ability to encapsulate and protect the payloads from degradation or early release, to modify the payloads bioavailability, increase circulation profiles, and modify tissue distribution profiles. However, according to a recent review by Wilhelm et al. who analyzed the tumor delivery of nanoparticles described in 232 reports between 2005 and 2015, on average, only 0.6% of the injected dose (%ID) of polymeric nanoparticles reach the tumor after systemic administration [33]. While, on the other hand, many publications describe successful gene silencing in tumor models after systemic administration of siRNA loaded nanoparticles, to the best of our knowledge, a quantitative correlation between %ID siRNA in the tumor and gene silencing effects has not yet been described. Here, we added a folic acid targeting ligand to the surface of the nanocarriers to take advantage of the FRα overexpression within the xenograft mouse model and to improve tissue distribution toward the tumor. To deliver the siRNA, triblock copolymers were utilized containing polyethyleneimine (PEI) to electrostatically condense and protect the siRNA. PEI has been documented to be an efficient carrier and transfection reagent. PEI homologues with larger molecular weight and higher degree of branching have been described to not only increase the transfection rates, but also to exhibit stronger toxic effects toward the cells [34–36]. Therefore, the polymers here were modified with a polyethylene glycol (PEG) chain to increase biocompatibility and circulation profiles, increase the stealth-like character of the nanocarriers to avoid macrophage detection, and to decrease the interaction with serum proteins [27,37]. Lastly, a polycaprolactone (PCL) block was added in the middle as a linker between PEI and PEG to increase the hydrophobic content of the polymer, drive micelle formation, and to aid in cleaving the polymer chains and releasing the siRNA once inside the cell due to its susceptibility to hydrolytic degradation [30,38]. Previous work performed with PEI-PCL-PEG, or short PPP, polymers has shown their ability to deliver siRNA *in vitro* to FRα overexpressing SKOV-3 cells, achieve a sustained protein knockdown, and display long-circulation profiles *in vivo* [5,30,34,39–41]. Here, we used modified architectures of the polymer and their block ratios in order to determine the efficacy of FRα targeted and non-targeted formulations *in vivo* and ultimately, for the first time, correlated quantitative tumor uptake results with gene silencing in a SKOV-3/LUC FRα overexpressing orthotopic murine ovarian cancer model.

2. Materials and methods

2.1. Materials

Hetero-bifunctional poly(ethylene glycol) (3.5 kDa), as well as methyl terminated monofunctional poly(ethylene glycol) (5 kDa) was purchased from JenKem Technologies (Plano, TX, USA) and chemically modified based on previously published protocols [5]. Hyperbranched polyethylenimine (*hy*-PEI, 25 kDa) was purchased from BASF (Ludwigshafen, Germany). Dicer substrate double-stranded siRNA (DsiRNA) targeting the Enhanced Green Fluorescent Protein gene (EGFP siRNA, 25/27), Firefly Luciferase (luc), and a Negative Control (scr), as well as Alexa Fluor-488 labeled siRNA were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA) [42]. Folic acid depleted Dulbecco's Modified Eagle's Medium (10×) for cell culture, phosphate buffered saline (PBS), heat-inactivated fetal bovine serum (FBS), D-(+)-glucose, and sodium bicarbonate was bought from Sigma-Aldrich (St. Louis, MO, USA). The chelator pBn-SCN-Bn-DTPA was purchased from Macrocyclics (Plano, TX, USA) while arsenazo(III) was purchased from Chem-Impex INT'L INC (Wood Dale, IL, USA), and yttrium(III) chloride was obtained from Acros Organics (Geel, Belgium).

2.2. Cell culture

The SKOV-3 human ovarian cancer cell line was obtained from ATTC (LG Promochem, Wesel, Germany). The SKOV-3/LUC cell line was engineered by stably transfecting the parental SKOV-3 cell line to stably express the reporter gene luciferase as previously reported [43]. SKOV-3 and SKOV-3/LUC ovarian cancer cells were cultured in folate free DMEM cell culture medium (Sigma-Aldrich) supplemented with 0.584 gm/L of L-glutamine, 3.7 gm/L sodium bicarbonate, 10% fetal bovine serum (Thermo Scientific Hyclone), and 1% penicillin/streptomycin at 37 °C and 5% CO_2. Cells were grown in 75 and 175 cm^2 cell culture flasks (Thermo Scientific) and passaged every 2–3 days when they had reached confluency.

2.3. Preparation of PEI-g-PCL-b-PEG-Fol micelleplexes

Each polymer was dissolved in water to yield a 1 mg/mL concentration based on the 25 kDa PEI content. Concentrations were tested with a copper assay as described before [5]. Once dissolved, samples were filtered through a 0.22 μm filter for sterilization. Subsequently, micelleplexes were prepared for both *in vitro* and *in vivo* work by mixing polymer and siRNA solutions together at N/P ratio 5 based on a previously published protocol [5].

2.4. In-111 siRNA radiolabeling and purification

To investigate *in vitro* cellular uptake and *in vivo* pharmacokinetics and biodistribution, indium-111 labeled siRNA was prepared and purified based upon a previously published protocol [44]. Briefly, siRNA modified with an amine functional group on the 5′ end was coupled with the chelator, p-SCN-Bn-DTPA. After purification, it was incubated with $^{111}InCl3$ for 30 min. Afterwards, the mixture was run though a PD-10 size exclusion column in order to separate free In-111 fractions from siRNA-DTPA-In-111 fractions. In-111 bound to siRNA was verified through gamma scintillation counting and UV absorption detection at 260 nm. If needed, peak fractions were combined for *in vivo* studies.

2.5. Cellular uptake of micelleplexes by gamma counting

In 24-well plates (Corning Incorporated, Corning, NY) 60,000 SKOV-3 cells were incubated overnight at 37 °C and 5% CO_2. In each well, freshly made micelleplexes containing 50 pmol of siRNA-DTPA-In-111 were added. Negative controls consisted of blank/untreated cells, while positive control cells were treated with siRNA containing lipoplexes made with lipofectamine (Life Technologies, Carlsbad, CA, USA) and polyplexes made with unmodified *hy*-PEI. Cells were transfected for 4 h in 37 °C and 5% CO_2. Cells were washed twice with 1X PBS + 2 mM EDTA, trypsinized and spun down at 350 g for 5 min. After centrifugation, the cells were resuspended in 1X PBS + 2 mM EDTA buffer and analyzed via