Biomaterials xxx (2018) 1–13

Contents lists available at ScienceDirect

Biomaterials

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

Folate receptor-directed orthogonal click-functionalization of siRNA lipopolyplexes for tumor cell killing *in vivo*

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ARTICLE INFO

Article history:
Received 30 December 2017
Received in revised form
15 March 2018
Accepted 17 March 2018
Available online xxx

Keywords:
Click chemistry
Folate receptor
Gene silencing
siRNA delivery
Tumor targeting

ABSTRACT

The delivery of small interfering RNA (siRNA) and its therapeutic usage as an anti-cancer agent requires a carrier system for selective internalization into the cytosol of tumor cells. We prepared folate-bearing formulations by first complexing siRNA with the novel azido-functionalized sequence-defined cationizable lipo-oligomer *1106* (containing two cholanic acids attached to an oligoaminoamide backbone in T-shape configuration) into spherical, ~100–200 nm sized lipopolyplexes, followed by surface-functionalization with various folate-conjugated DBCO-PEG agents. Both the lipo-oligomer and the different defined shielding and targeting agents with mono- and bis-DBCO and varying PEG length were generated by solid phase supported synthesis. A bivalent DBCO surface agent with a PEG_{24} spacer was identified as the optimal formulation in terms of nanoparticle size, folate receptor (FR) targeting, cellular uptake and gene silencing *in vitro*. Notably, near-infrared fluorescence bioimaging studies showed that double-click incorporation of bivalent DBCO-PEG_{24} resulted in siRNA/*1106*/*$DBCO_2$-ss_2-PEG_{24}-FolA* lipopolyplexes with extended biodistribution and intratumoral delivery in a subcutaneous FR-positive leukemia mouse model. Intravenous administration of analogous therapeutic siRNA lipopolyplexes (directed against the kinesin spindle motor protein EG5) mediated tumoral EG5 mRNA knockdown by ~60% and, in combination with the novel antitubulin drug pretubulysin, significantly prolonged survival of aggressive leukemia bearing mice without noticeable side effects.

1. Introduction

Small interfering RNA (siRNA) enables the efficient down-regulation of sequence-specific gene expression and thereby offers new therapeutic approaches for severe diseases such as cancer [1–5]. Depending on the genetic dysregulation, tailored siRNAs can be synthesized to target driver gene mutations. For the treatment of heterogeneous and highly mutated cancers, the use of multiple therapeutic siRNAs enables to interfere with various targets simultaneously [5–9], or combination with other drugs is advisable.

Still, the delivery of siRNA into target cells remains a big challenge. Naked siRNA molecules have limited stability in biological fluids, since they are actively targeted and degraded by ubiquitously occurring nucleases. This issue can be addressed by the incorporation of chemical modifications into siRNA strands [10,11]. Crossing of cell membranes is another hurdle for siRNA, due to its highly negative charge and molecular weight [12]. The incorporation of siRNA into carriers, like liposome-based formulations, lipo-polymer micelles, and polymer-based complexes improve the intracellular cellular delivery [10,12–23]. Various polycations, cationic lipids and combinations with helper lipids were found to form polyplexes [3,16,18,19,24–26], lipoplexes or other lipid nanoparticles (LNPs) [1,13,27–32].

Precise editing of the components' chemical structure enables the step-wise optimization of siRNA carrier systems [33–36]. Recently, we developed monodisperse cationic oligomers, made of artificial oligoamino acids, natural α-amino acids and fatty acids sequentially connected together via solid phase-supported synthesis (SPS) [37–39]. Thereby we synthesized numerous structures with various functional moieties to enhance the extracellular stability, transfection efficiency and cell tolerability of the

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https://doi.org/10.1016/j.biomaterials.2018.03.031
0142-9612/

corresponding siRNA polyplexes [14,20,33–35,40–44].

One focus was the introduction of ligands and shielding domains for specific targeting of siRNA carriers to cancer cells [45]. For example, folate as a vitamin is widely used as a targeting ligand with high affinity to the folate receptor (FR)-overexpressing cancer cells [46–50]. Both the targeting and the siRNA binding elements do not only influence the pharmacological activity of a siRNA carrier system, they can also change the biophysical properties. The defined conjugation of a polyethylene glycol (PEG) shielding domain attached to a folate with a 2.5 kDa cationic backbone with cysteines for cross-linking ability [35,37], resulted in the 4 kDa targeted carrier molecule ***356*** [40]. siRNA polyplexes formed with this ***356*** carrier in combination with an endosomolytic INF7 peptide mediated FR-specific cellular uptake and gene silencing *in vitro* and *in vivo* when injected intratumorally. The oligomer was found to form monomolecular nanoplexes with a hydrodynamic diameter of only ~6 nm, whereas a non-PEGylated cationic carrier usually forms much larger multi-siRNA polyplexes [35,37,51]. As a consequence of small particle size, systemically administered ***356*** siRNA nanoplexes revealed fast renal clearance and a short circulation time of 15 min.

Combination with non-shielding, cationic oligomers did increase the particle size of formed, targeted combinatorial polyplexes (TCPs) to ~100–200 nm [52]. Alternatively, combination of ***356*** with lipo-oligomers [35,51] resulted in targeted siRNA lipoplexes (TLPs) with similar advantageous particle sizes [53]. Both targeted TCPs and TLPs demonstrated gene silencing activity *in vivo* in distant tumors after intravenous administration [52,53].

A different strategy presents the initial assembly of siRNA lipo-oligomer core nanoparticles followed by subsequent modification of the lipopolyplex surface with shielding and targeting domains. In previous work, functional groups like thiols of lipo-oligomers were used to attach PEG agents via mercapto-reactive linkers [54–57]. Despite suitable biophysical properties and specific gene silencing of target tumor cells *in vitro*, the limited *in vivo* stability of these oleic acid based lipopolyplexes remained a major impediment for further therapeutic applications.

In the current work, we precisely positioned an azido domain into a previously described sequence-defined T-shaped bis-(cholanic acid amido) oligoaminoamide [44] via SPS and used DBCO click reaction to equip the surface of lipopolyplexes with PEG shielding and folate targeting domains. The replacement of the two unsaturated C18 oleic acids, used as lipo-oligomer subunits for stabilization in previous folate-targeted formulations [53,54], by two tetracyclic C24 cholanic acids further increases stability in serum. The bio-orthogonal nature of the click reaction prevents inadvertent effects such as side reactions with amines or thiols impairing siRNA polyplex stability. By using SPS for the design of nanoparticle surface functionalizing agents as well, a library of defined click PEG agents with various features, like altering PEG lengths and mono- or bivalent attachment of DBCO (click and double-click agents) could be synthesized with precision. We investigated the impact of the different structural modifications on biophysical properties, pharmacological activity and *in vivo* stability. The best-performing agents were used for formulation of an antitumoral siRNA, and intravenous (i.v.) administration was performed to test tumoral gene silencing. Finally, without or with combination with the novel antitumoral compound pretubulysin, the therapeutic efficacy of these siRNA lipopolyplexes was evaluated in leukemia tumor-bearing mice.

2. Materials and methods

2.1. Materials

Protected Fmoc-α-amino acids, 2-chlorotrityl chloride resin, *N*,*N*-dimethylformamide (DMF), *N*,*N*-diisopropylethylamine (DIPEA) and trifluoroacetic acid (TFA) were purchased from Iris Biotech (Marktredewitz, Germany). Triisopropylsilane (TIS), 1-hydroxybenzotriazole (HOBt), 5β cholanic acid, dibenzocyclooctyne acid and folate were purchased from Sigma-Aldrich (Munich, Germany). (Benzotriazol-1-yloxy) tripyrrolidino phosphonium hexafluorophosphate (PyBOP) and microreactors were obtained from MultiSynTech (Witten, Germany). N^{10}-(Trifluoroacetyl)pteroic acid was purchased from Clauson-Kass A/S (Farum, Denmark) and Fmoc-N-amido-dPEG$_{24}$-acid from Quanta Biodesign (Powell, Ohio, USA). Cell culture media, antibiotics and fetal calf serum (FCS) were purchased from Invitrogen (Karlsruhe, Germany), HEPES from Biomol GmbH (Hamburg, Germany), glucose from Merck (Darmstadt, Germany), agarose (NEEO Ultra-quality) and ammonia solution 25% from Carl Roth GmbH (Karlsruhe, Germany), and GelRed™ and Fmoc-Glu-O-2-PhiPr from VWR (Darmstadt, Germany). Cell culture 5× lysis buffer and D-luciferin sodium salt were obtained from Promega (Mannheim, Germany). Ready-to-use siRNA duplexes were obtained from Axolabs GmbH (Kulmbach, Germany): eGFP-targeting siRNA (siGFP) (sense: 5′-AuAucAuGGccGAcAAGcAdTsdT-3′; antisense: 5′-UGCUU-GUCGGCcAUGAuAUdTsdT-3′) for silencing of eGFPLuc; EG5-targeting siRNA (siEG5) (sense: 5′-ucGAGAAucuAAAcuAAcudTsdT-3′; antisense: 5′- AGUuAGUUuAGAUUCUCGAdTsdT-3′) for silencing EG5 motor protein; control siRNA (siCtrl) (sense: 5′-AuGuAuuGGccuGuAuuAGdTsdT-3′; antisense: 5′-CuAAuA-cAGGCcAAuAcAUdTsdT-3′); Cy5-labled siRNA (Cy5-siAHA1) (sense: 5’-(Cy5)(NHC6)GGAuGAAGuGGAGAuuAGudTsdT-3’; antisense: 5′-ACuAAUCUCcACUUcAUCCdTsdT-3′); Cy7-labled siRNA (Cy7-siAHA1) (sense: 5’-(Cy7)(NHC6)GGAuGAAGuGGAGAuuAGudTsdT-3’; antisense: 5′-ACuAAUCUCcACUUcAUCCdTsdT-3′) small letters: 2′methoxy; s: phosphorothioate. All other solvents and reagents were purchased from Sigma-Aldrich (Munich, Germany), Iris Biotech (Marktredwitz, Germany), Merck (Darmstadt, Germany) or AppliChem (Darmstadt, Germany).

2.2. Methods

2.2.1. Synthesis of oligomer and DBCO agents

See supporting information for detailed description on synthesis of oligomer ***1106*** and DBCO agents.

2.2.2. siRNA polyplex formation

Nucleic acid was dissolved in 20 mM HEPES buffered 5% glucose pH 7.4 (HBG) at a concentration of 500 ng/μL. According to the indicated nitrogen/phosphate (N/P) ratio (N/P 10 unless stated otherwise), the azido-oligomer ***1106*** solution was prepared in a separate tube and the same volume of siRNA was added to the oligomer. Only protonatable nitrogens were considered in the N/P calculation. The mixture was rapidly pipetted at least 5× and incubated for 40 min at RT resulting in a polyplex solution with 250 ng of siRNA/μL.

2.2.3. Modification with DBCO reagents

For modifying siRNA polyplexes with DBCO click agents, solutions with reagents were prepared in ¼ of the volume of polyplex solutions prepared before. The concentration of the solution was calculated according to the respective equivalents (eq). Equivalents represent the molar ratio of shielding agents to oligomers in the polyplex solution. The reaction time was 4 h and the final concentration of the polyplex solution was 200 ng of siRNA/μL.

2.2.4. siRNA binding assays

A 1% agarose gel was prepared by dissolving agarose in TBE buffer (10.8 g of trizma base, 5.5 g of boric acid, 0.75 g of disodium

Please cite this article in press as: P.M. Klein, et al., Folate receptor-directed orthogonal click-functionalization of siRNA lipopolyplexes for tumor cell killing *in vivo*, Biomaterials (2018), https://doi.org/10.1016/j.biomaterials.2018.03.031