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Stabilizing effect of tyrosine trimers on pDNA and siRNA polyplexes

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ABSTRACT

Nine sequence-defined, polycationic oligomers were synthesized containing motifs of three consecutive tyrosines (Y3) as stabilizing components for pDNA and siRNA polyplex assembly. For pDNA, a combination of terminal oligotyrosines and cysteines was necessary and sufficient for stable polyplex formation. Stable siRNA binding required a combination of terminal cysteines and oligotyrosines, as well as a central hydrophobic modification (oligotyrosines or fatty acids). The phenolic group within the aromatic amino acids of Y3 containing oligomers further increased the endosomal buffer capacity. As a result, the new class of oligotyrosine containing oligomers was efficient in pDNA and siRNA transfection, in most cases superior to a previously established cysteine-containing, dioleic acid modified oligomer without the Y3 motif. Additionally, increased serum stability of the new oligomers with terminal Y3 motifs was demonstrated by gel shift and fluorescence correlations spectroscopy (FCS). In vivo stability and biodistribution was monitored by intravenous administration of chemically stabilized Cy7 siRNA either as free form, or complexed with the nine Y3 containing oligomers or control oligomers. Oligomer *332*, with the overall most beneficial in vitro and in vivo characteristics, was applied in RAN siRNA polyplexes for intratumoral treatment of neuroblastoma-bearing mice. This resulted in significantly reduced tumor growth compared to animal treated with control siRNA polyplexes.

1. Introduction

The intracellular localization of nucleic acid targets and the inability of siRNA and other oligonucleotides to diffuse across cellular membranes require formulation with carriers [1–5]. Several modifications of the pDNA transfection agent polyethylenimine (PEI) have been reported to convert it into a suitable siRNA carrier [6–11]. Creusat et al. modified PEI with hydrophobic natural amino acids [7] and showed that the random modification of 10–20% of PEI nitrogens with tyrosines was beneficial in regards of buffer capacity, reduced cytotoxicity, and siRNA [12] or oligonucleotide [13] delivery. Modification of PEI with tyrosines occurred at random positions and only one single tyrosine was attached per nitrogen. Tyrosine modified PEI showed favorable properties; it did not display hemolytic activity, but polyplexes nevertheless escaped endosomes due to their endosomal protonation capacity. Moreover, those polyplexes displayed an increased stability in glucose solution, favorable for in vivo application. In a different context, self-assembly of tyrosine containing biopolymers into nanostructures has been described to be facilitated through the π-π interactions of the aromatic rings of single neighboring tyrosines or tyrosine dimers [14,15].

High stability is an important requirement for in vivo administration of polyplexes, because free nucleic acids i) are degraded by DNases or RNases, ii) cannot enter the cells because of their negative charge, and iii) in case of small oligonucleotides are rapidly cleared through the urinary tract due to their size [16]. After intravenous (i.v.) injection, serum proteins or blood cells interact with the polyplexes potentially leading to aggregation or dissociation. Therefore, stable and neutral particles in a range of ~100 nm [17] are considered as ideal for i.v. injection. These nanoparticles can benefit from the so called tumor-selective enhanced permeability and retention (EPR) effect [18]. This effect leads to an accumulation of nanoparticles in the tumor because of the leaky tumor vessels and the pure lymph drainage of tumors. In contrast, different polyplex properties are required for intratumoral (i.t.) injections. Nomura et al. showed that larger and positively charged liposomes are superior for i.t. injections compared to smaller and uncharged liposomes [19]. Therefore, precise changes in the oligomer structure can affect size, charge and stability of the polyplexes

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leading to differences in biodistribution, cellular uptake and release of the nucleic acid in the cytoplasm.

In this study, we aimed at optimizing the serum stability of polyplexes based on sequence-defined carriers [20–22]. For this purpose, we evaluated the effect of three consecutive tyrosines as stabilizing components. Therefore, we synthesized various oligomers containing oligotyrosine motifs at defined positions. In addition, we evaluated their nucleic acid delivery efficiency and their toxicological profile in vitro and assessed their distribution profile in vivo in mice. We selected the oligomers *49* and *332* as siRNA carriers for in vivo therapeutic administration.

2. Materials and methods

2.1. Materials

pCMVLuc plasmid DNA (pDNA) as purchased from PlasmidFactory (Bielefeld, Germany). Ready to use Cy5-labeled siRNA, control siRNA duplexes (Mut) (sense: 5′-AuGuAuuGGccuGuAuuAGdTsdT-3′; antisense 5′-CuAAuAcAGGCcAAuAcAUdTsdT-3′, small letters: 2′methoxy-RNA; s: phosphorothioate, dT: deoxythymidine), GFP-siRNA (sense: 5′-AuAucAuGGccGAcAAGcAdTsdT-3′; antisense: 5′-UGCUU-GUCGGCcAUGAuAUdTsdT-3′) for silencing of the EGFPLuc protein, and Cy7-labeled siAHA1 (sense:5′-(Cy7) (NHC_6)-GGAuGAAGuGGAGAuuAGudTsdT-3′; antisense:5′-ACuAAUCUCcACUUcAUCCdTsdT-3′) were kindly provided by Axolabs GmbH (formerly: Roche Kulmbach, Kulmbach, Germany). RAN siRNA (antisense. 5′-AGAAGAAUCUUCAGUACUAUU-3′; sense: 5′-UAGUACUGAAGAUUCUUCUUU-3′ was purchased from Dharmacon (Thermo Fischer Scientific Inc., Lafayette, CO, USA). Cy5-labeling kit for pDNA labeling was obtained from Mirus Bio (Madison, WI, USA). Fetal bovine serum (FBS) was purchased from Invitrogen (Karlsruhe, Germany), glucose from Merck (Darmstadt, Germany), HEPES from Biomol GmbH (Hamburg, Germany) and sodium chloride from Prolabo (Haasrode, Belgium). Dilutions of siRNA and polymers were prepared in 20 mM HEPES pH 7.4 and 20 mM HEPES with 5% glucose (HBG) pH 7.4. All buffer components were solved in MilliQ water and the pH was adjusted, if necessary. Afterwards the buffers were filtered with a sterile filter with 0.2 μm pore size. Stp-based sequence-defined oligomers were synthesized as described in the Supplementary Information.

2.2. DNA and siRNA polyplex formation

Polyplex formulations for transfection and gel shift experiments were prepared as follows: 100–200 ng of pDNA or 500 ng of siRNA and the calculated amount of oligomer were diluted in separate tubes in 10 μL of 20 mM HEPES buffered 5% glucose pH 7.4 (HBG) each. The nucleic acid and the polycation solution were mixed by rapidly pipetting up and down (at least 5 times) and incubated for 30–40 min at RT in order to form the polyplexes necessary for transfection and gel shift experiments.

2.3. Cell culture

Mouse neuroblastoma cells Neuro2A and Neuro2A/EGFPLuc cells, stably expressing the CMV-EGFPLuc cassette (Clontech) encoding a fusion of enhanced green fluorescent protein and GL3 firefly luciferase under the control of the CMV promoter [11,23], were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, 4 mM stable glutamine, 100°U/mL penicillin, and 100 μg/mL streptomycin.

2.3.1. In vitro gene transfer

Neuro2A cells were seeded 24 h prior to pDNA delivery using $1*10^4$°cells/well in 96-well plates. In vitro transfection efficiency of the polymers was evaluated using 200 ng pCMVLuc per well. All experiments were performed in quintuplicates. Before transfection medium was replaced with 80 μL fresh medium containing 10% FBS. Transfection complexes formed at different protonable nitrogen/phosphate (N/P) ratios in 20 μL HBG were added to each well and incubated at 37 °C. At 24 h after transfection, cells were treated with 100 μL cell lysis buffer (25 mM Tris, pH 7.8, 2 mM EDTA, 2 mM DTT, 10% glycerol, 1% Triton X-100). Luciferase activity in the cell lysate was measured using a luciferase assay kit (100 μL Luciferase Assay buffer, Promega, Mannheim, Germany) and a Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany).

2.3.2. Gene silencing with siRNA

Gene silencing experiments were performed in stably transfected Neuro2A/EGFPLuc cells using 0.5°μg/well of either GFP-siRNA for silencing of the EGFPLuc protein, or control siRNA as a control. siRNA delivery was performed in 96-well plates with 5000 cells/well in triplicates. Cells were seeded 24 h prior to transfection and then medium was replaced with 80 μL fresh growth medium containing 10% FBS. Transfection complexes for siRNA delivery (20 μL formed in HBG) at different N/P ratios were added to each well and incubated at 37 °C. At 48 h after transfection, luciferase activity was determined as described above. The relative light units (RLU) were presented as percentage of the luciferase gene expression obtained with only buffer treated control cells.

2.4. siRNA polyplex stability in 90% FBS via gel shift assay

2.5 μg of control siRNA and the polymer at N/P 12 were diluted in separate tubes to a total volume of 12.5 μL in 20 mM HEPES pH 7.4. The nucleic acid solution was added to the diluted polycation, mixed and incubated for 30–40 min at room temperature. Afterwards fetal bovine serum (FBS) was added to the samples. All samples had a final concentration of 90% FBS. The samples were incubated either at room temperature or 37 °C for different time points. Meanwhile a 2.5% agarose gel was prepared, by dissolving agarose in TBE buffer and heating the suspension up to 100 °C. After cooling down to about 50 °C and addition of GelRed, the clear agarose gel solution was casted into an electrophoresis unit. After 0, 10, 30 and 90 min, 20 μL of the samples and 4 μL loading buffer were carefully mixed and were placed into the sample pockets. Electrophoresis was performed at 120 V for 40 min.

2.5. Fluorescent correlation spectroscopy

Unlabeled control siRNA was spiked with Cy5-labeled siRNA in formulation buffer (20 mM HEPES pH 7.4) in order to determine the size of the polyplexes with FCS. The calculated amount of polymer (N/P) diluted in formulation buffer, was mixed with siRNA solution to concentrations of 200 μg siRNA per mL. Afterwards, the polyplexes were incubated at room temperature for 30 min. For the size measurements in buffer and FBS, the samples were diluted 1:40 in buffer or fetal bovine serum. For serum measurements, FBS was added to a final concentration of 90%, after polyplex formation. Afterwards, the polyplexes were incubated at room temperature or 37 °C and measured at different time points. The minimal volume for polyplex analysis was 200 μL for all measurements with a final concentration of Cy5-labeled siRNA of 50 nM in each sample. FCS measurements were performed on an Axiovert 200 microscope with a ConfoCor 2 unit (Carl Zeiss, Jena, Germany). A HeNe laser (633 nm, average power of 50 μW at the sample) was used for excitation. The objective was a 40× (NA = 1.2) water immersion apochromat (Carls, Zeiss, Germany). Samples were measured in eight well LabTek I chamber slides (NUNC, Wiesbaden, Germany). The laser beam focused at about 200 μm above the bottom of the wells containing the samples. Autocorrelation and analysis were performed using a ConfoCor 2 software. Starting value for analysis was 3–18 μs to cut off photophysical effects. To determine the structural parameter and measurement parameters, Cy 5 dye in water was analyzed before each data acquisition. All data were evaluated with a one component fit, unless stated otherwise.

2.6. In vivo experiments

For all in vivo experiments female Rj:NMRI-nu (nu/nu) (Janvier, Le Genest-St-Isle, France) mice were chosen, housed in isolated vented cages with a 12 h day/night interval and food and water ad libitum. Animal experiments were performed according to guidelines of the German law of protection of animal life and were approved by the local animal experiments ethical committee.

2.6.1. Polyplex distribution

Different polyplexes (N/P 12) containing 50 μg siRNA including 50% Cy7-labeled siAHA1 were mixed in a total volume of 250 μL (HBG). Fluorescence imaging was performed utilizing the IVIS Lumina system with Living Image software 3.2 (Caliper Life Sciences, Hopkinton, MA, USA). After anesthetizing the mice with 3% isoflurane in oxygen, polyplexes were injected into the tail vein and the distribution was measured after 0, 0.25, 0.5, 1, 4, and 24 h with a CCD camera. Experiments were performed in triplicates and pictures were analyzed using the Living Image software.

2.6.2. N2A tumor treatment

Mice were injected subcutaneously with 5×10^6 Neuro2A-eGFPLuc cells into their left flank at day 0. Two days later the mice were separated into 6 groups ($n = 5$) based on their bioluminescence signal (Caliper Life Sciences, Hopkinton, MA, USA). Bioluminescence imaging was performed 15 min after intra peritoneal injection of 100 μL luciferin solution ($c = 60$ mg/mL), recorded by a CCD camera (IVIS Lumina™) and analyzed using Living Image software 3.2. Intratumoral treatment with polyplexes, containing oligomers *49* and *332* complexing either 50 μg RAN siRNA or control siRNA (N/P 12) in a total volume of 50 μL (HBG) was also started at day 2 and repeated at day 4, 8, 11, 14. Tumor growth was recorded via bioluminescence imaging at day 8, 11, 14, 16 and 18. Caliper measurements were performed twice every week. Mice were sacrificed after their tumors reached a size of 1500 mm^3 (length × $width^2/2$). Bioluminescence signals were analyzed with the IVIS Lumina system with Living Image software 3.2 (Caliper Life Sciences, Hopkinton, MA, USA).