Citation: Molecular Therapy–Nucleic Acids (2012) 1, e7; doi:10.1038/mtna.2011.10

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Defined Folate-PEG-siRNA Conjugates for Receptor-specific Gene Silencing

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Gene silencing mediated by small interfering RNA (siRNA) is a novel approach in the development of new cancer therapeutics. Polycations used for nucleic acid delivery still remain heterogeneous compounds, despite continuous progress in polymer synthetic technologies. Here we report the development of a structural defined folic acid polyethylene glycol (PEG) siRNA conjugate accessible *via* click chemistry yielding a monodisperse ligand-PEG-siRNA conjugate. The folic acid targeting ligand was synthesized by solid phase supported peptide chemistry. The conjugate was shown to be specifically internalized into folic acid receptor expressing cells. When combined with a structurally defined polycation, again synthesized with the precision of solid phase chemistry, efficient receptor specific gene silencing is achieved.
Molecular Therapy–Nucleic Acids (2012) **1,** e7; doi:10.1038/mtna.2011.10; advance online publication 31 January 2012

Introduction

Finding new therapeutic modalities for the treatment of cancer remains one of the most relevant tasks in medicinal chemistry. Post-transcriptional downregulation of genes by a natural process called RNA interference (RNAi) is currently explored as a novel medical treatment option.[1] RNAi is triggered by double-stranded RNA.[2] Most frequently, short interfering RNA (siRNA) consisting of 21–23 nucleotides are used to modulate gene expression. Although it has been shown very clearly by experiments with cells in culture[3] that siRNA mediated gene silencing is very specific and efficient once the nucleic acid has entered the cytosol, one challenge for the development of efficient siRNA therapeutics remains: the delivery process.[4] In intravenous administration of siRNA therapeutics, besides insufficient cellular uptake and unspecific delivery, degradation in the bloodstream as well as rapid renal clearance are major limitations. Polycationic compounds have shown to act as efficient carrier systems for different classes of nucleic acids. Ionic interactions between those polymers and the negatively charged phosphate backbone of nucleic acids result in the formation of nanoparticles, called polyplexes. These structures can efficiently protect siRNA during circulation in blood and enable interactions with cell surfaces. Improved carriers incorporate polyethylene glycol (PEG) to shield the positive surface charge of polyplexes, reducing unspecific interaction with blood compounds and non-target cells. For specific target cell recognition, ligands can be covalently attached to the polyplex structure allowing for interactions with highly expressed target cell surface receptors. In a different approach PEG and/or targeting ligands have been covalently attached to the siRNA backbone.[5–8] Both concepts have shown to result in a significant increase in transfection efficiency.[9] Due to high polydispersity of the used polymers (polycation and PEG) and limited options in chemical ligation and purification, such systems often lack precise structural definition. Thus, a clear evaluation of structure activity relationships is hampered. Moreover, reproducible production of materials for clinical trials can prove difficult due to significant batch to batch variations and lack of accurate analytics.

Results

The current work aims at the design of a carrier system exclusively composed of precisely defined, pure compounds allowing for a clear structure activity relationship. Consequently, we combined a covalent siRNA-PEG-ligand conjugate with a precise monodisperse three-armed polycation to form a polyplex. As targeting ligand, folic acid was chosen, due to its small size, high-binding affinity to its receptor (10^{-10} mol/l) and the upregulation of the folic acid receptor in many tumor types.[10–12] The synthesis of the PEGylated targeting ligand was performed by solid phase supported peptide chemistry (**Figure 1a**). For the introduction of PEG, a commercially available monodisperse PEG consisting of 24 ethylene glycol units was used. **Supplementary Figure S1** and **S2** demonstrate identity and purity by mass spectrum and high performance liquid chromatography, respectively. The targeting ligand contains an azide linker to enable the covalent attachment of siRNA by Cu(I) catalyzed 1,3-dipolar cycloaddition, since the siRNA is equipped with an alkyne linker at the 5′-end of its sense strand (**Figure 1a**). After purification by ion exchange chromatography, the desired structure was obtained in high purity, as demonstrated by gel shift assay (**Figure 1b, c**) and mass spectrum (**Supplementary Figure S3**). Flow cytometry (**Figure 2**) and fluorescence microscopy using 5′-labeled siRNA (Cy5 fluorophore at 5′-end of the antisense strand, **Figure 3**) revealed that the attachment

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Received 15 November 2011; revised 22 December 2011; accepted 27 December 2011

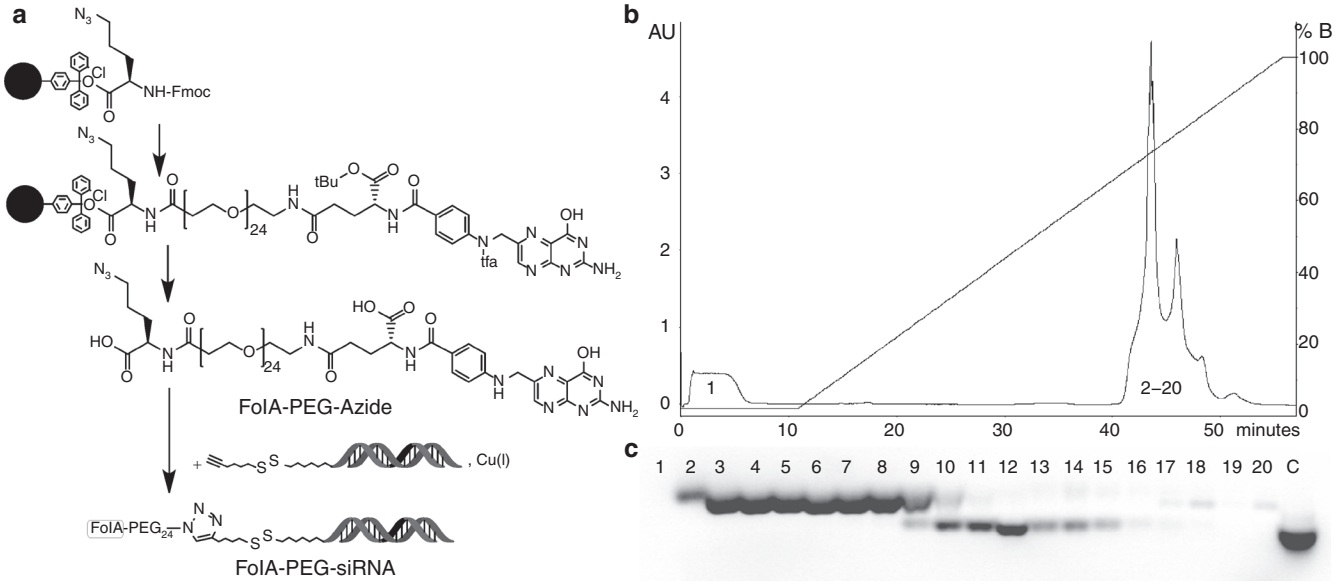

Figure 1 Synthesis of the defined FolA-PEG-siRNA conjugate. (**a**) Synthesis of folic acid targeted PEG-ligand by solid phase supported peptide synthesis and its conjugation to siRNA via Cu(I) catalyzed 1,3-dipolar cycloaddition. (**b**) Chromatogram of the ion exchange chromatographic purification of a FolA-PEG-siRNA conjugate. The purification was carried out on a Resource Q column (1 ml; GE Healthcare, Freiburg, Germany) connected to an Äkta Basic system (detection wavelength: 260 nm). After loading the column using 20 mmol/l HEPES, pH 6.5, 20 mmol/l NaCl, 30% acetonitrile, elution was achieved employing a sodium chloride gradient of 10 mmol/l NaCl/minute and a flow rate of 1 ml/minute. (**c**) Agarose gel shift assay of the fractions collected during ion exchange chromatographic purification of a FolA-PEG-siRNA conjugate. Fractions were collected and analyzed using a 2.5% agarose gel (100 V, 100 minutes). C: Unconjugated input siRNA, numbers above indicate the individual fractions. Fraction size 0.5 ml. PEG, polyethylene glycol; siRNA, small interfering RNA.

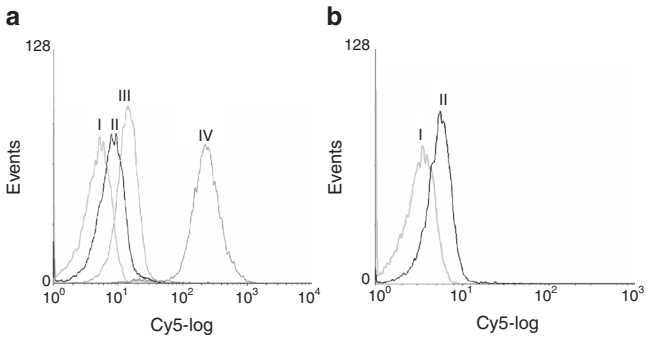

Figure 2 Histogramm of receptor mediated uptake of FolA-PEG-siRNA analyzed by flow cytometry. (**a**) KB cells, I: untransfected cells, II: FolA-PEG-siRNA with folic acid competition, III: PEG-siRNA, IV: FolA-PEG-siRNA; (**b**) folic acid receptor negative Neuro2A cells, I: untransfected cells, II: FolA-PEG-siRNA. siRNA, small interfering RNA.

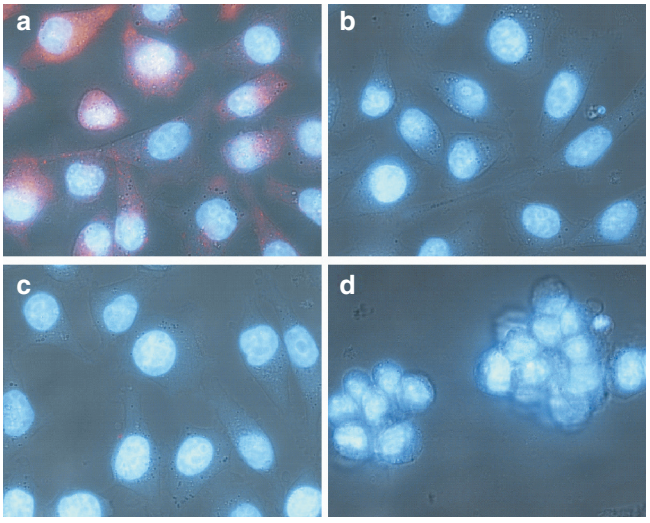

Figure 3 Fluorescence microscopic images of receptor-specific cellular uptake. (**a**–**c**) Fluorescence microscope images of the cellular uptake of Cy5-labeled siRNA into folic acid receptor positive KB cells or (**d**) Neuro2A control cells with low folic acid receptor level. (**a**,**d**) Transfection with FolA-PEG-siRNA; (**b**) Transfection with FolA-PEG-siRNA after preincubation with free folic acid; (**c**) Transfection with PEG-siRNA lacking the FolA ligand. siRNA, small interfering RNA.

of folic acid to the siRNA enabled specific and very efficient receptor mediated uptake into folic acid receptor expressing KB cells without the need of any additional carrier. Specificity was demonstrated by preincubation of cells with free folic acid inhibiting cellular uptake of the siRNA conjugate. Furthermore, Neuro2A cells with a low folic acid receptor level remained untransfected. In addition, control conjugates such as PEGylated or unconjugated siRNA were not able to enter the target cell. As the targeted siRNA conjugate was shown to be taken up by receptor-mediated endocytosis, gene silencing efficiency of the modified siRNA was tested. At first we checked whether the covalent siRNA modifications (FolA-PEG-siGFP, PEG-siGFP) are compatible with the RNAi machinery. For this we performed a receptor-independent transfection assay in Neuro2A cells (stably expressing the enhanced green fluorescent protein (eGFP)-luciferase fusion gene) using cationic lipopolymer **230** as potent but nonspecific transfection agent (**Supplementary Figure S4**).[13,14] Irrespective of the siRNA

construct employed, silencing activity was similar compared to unconjugated siRNA. Thus, the siRNA was not negatively affected by the modification. Next we tested folate receptor-targeted transfer. Although it was proven that FolA-PEG-siRNA is taken up intracellularly into folic acid receptor positive KB cells and the PEG polymer conjugation does not negatively affect the siRNA silencing potential, the use of plain FolA-PEG-siGFP conjugate did not result in reporter gene silencing (data not shown). This finding is consistent with data in the literature where siRNA conjugated to folic acid was found to be taken up by endocytosis, but did not result in significant gene silencing.[7] This compound was found trapped in intracellular vesicles and thus, lacking endosomal escape functionality, does not have access to the RNA induced silencing complex in the cell's cytosol, which is necessary for a RNAi effect. To combine our selective targeting siRNA molecule with an agent enabling efficient endosomal escape, it was complexed with the recently described monodisperse polycationic carrier **386**. This nontoxic molecule[13] is also based on solid phase supported carrier design (**Figure 4**).[13–15] The carrier is composed of three arms, each built out of three succinoyl-tetraethylenpentamine (Stp) units, connected *via* a branching lysine, and terminated by cysteines at the termini which are essential for polyplex stabilization by disulfide formation after particle formation (**Figure 4**). In the current work it was used to build polyplexes with the targeted siRNA conjugate, to protect it in the extracellular environment and to mediate endosomal escape by the proton sponge ability of its polyethylenimine-like 1,2-diaminoethane units.[16,17] By gel retardation (**Supplementary Figure S5**) stable particle formation was confirmed. PEGylated siRNA reduced the zeta potential of these polyplexes from 20 mV to 11–14 mV (~30–45% reduction), which indicates that the PEG spacer and thus also the targeting ligand is presented on the polyplex surface (**Supplementary Table S1**). Targeting specificity was retained as proven by flow cytometry experiments using folic acid receptor positive KB cells (**Supplementary Figure S6**). Transfecting KB eGFP-luc cells with FolA-PEG-siGFP bearing polyplexes resulted in significant reporter gene silencing (**Figure 4**). Even mixtures of conjugated and unconjugated siRNA in one polyplex formulation (indicated as % targeted siRNA) lead to significant knockdown. A minimum of 1% FolA-PEG conjugated siRNA was necessary to observe a significant targeting ligand effect, 5% conjugated siRNA was sufficient for a maximal knockdown. In contrast, polyplexes formed with PEGylated siRNA lacking the targeting ligand or unconjugated siRNA did not result in reporter gene silencing at any tested ratio of mixture. With the targeted polyplex formulation, potent gene silencing was observed at 25–200 nmol/l siRNA, but significant downregulation of the target gene of more than 50% was still observed at the further reduced 12.5 nmol/l siRNA concentration (**Supplementary Figure S7**).

Discussion

We report targeted siRNA polyplexes consisting of two molecularly precise components: a covalent siRNA conjugate equipped with a folic acid receptor targeting ligand and PEG shielding function, together with a defined three-arm polycation for siRNA packaging and nanoparticle formation. The synthesis of the single components by solid phase

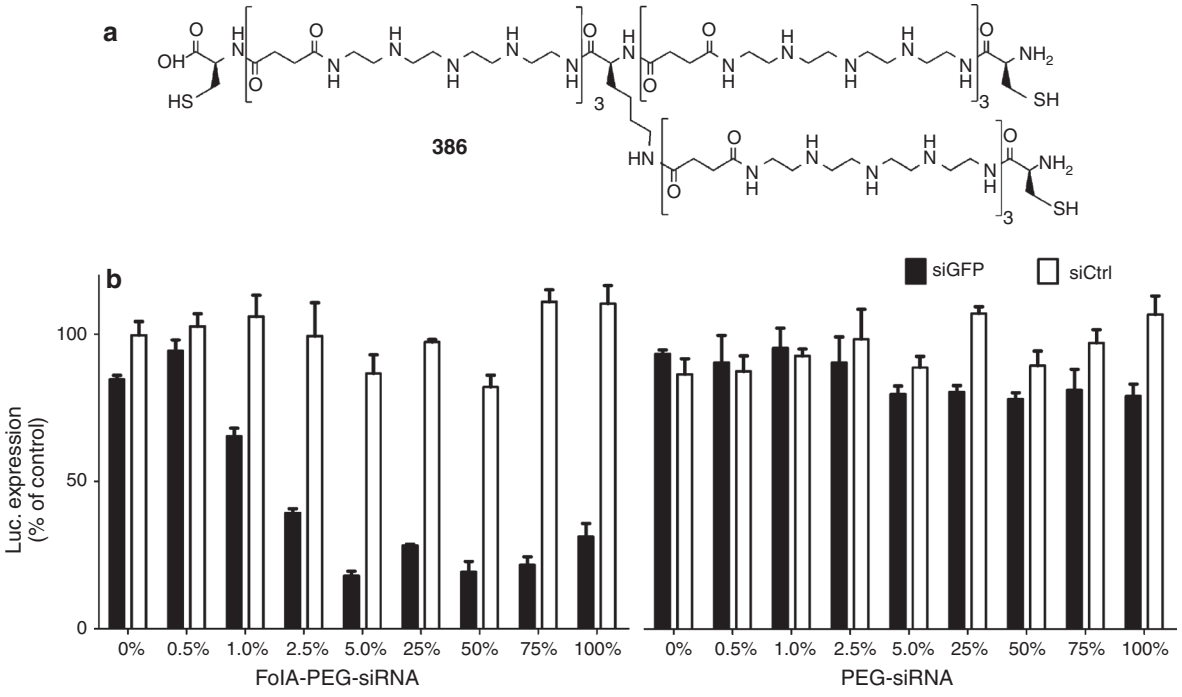

Figure 4 Receptor-specific reporter gene silencing of FolA-PEG-siRNA in combination with the defined polycation 386. (**a**) Defined polycationic carrier **386** synthesized by solid phase chemistry and used for polyplex formation. (**b**) siRNA mediated silencing of the reporter fusion gene eGFP-luciferase using polyplexes with different mixtures of targeted and nontargeted siRNA (% of targeted siRNA in total siRNA) using polycation **386** (at N/P of 6). Final siRNA concentration: 200 nmol/l. Data are presented as mean ± SD. eGFP, enhanced green fluorescent protein; N/P, nitrogen/phosphate; siRNA, small interfering RNA.

chemistry resulted in a defined targeting macromolecule. The use of Cu(I) catalyzed 1,3-dipolar cycloaddition allowed the construction of a very pure and defined siRNA conjugate, its purification and analysis. Combining this structure with the defined polycationic carrier molecule **386**, which contains polyethylenimine-like 1,2-diaminoethane units for endosomal buffering and release, leads to efficient and specific reporter gene silencing in folic acid receptor positive KB cells. The presented data make this carrier system a promising candidate for further *in vivo* gene silencing applications.

Materials and methods

Nucleic acids. Oligoribonucleotides were synthesized on solid phase according to standard phosphoramidite oligomerization methodology. Commercially available 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl) phosphoramidite monomers of uridine (U), 4-*N*-acetylcytidine (C^{Ac}), 6-*N*-benzoyladenosine (A^{bz}), and 2-*N*-isobutyrlguanosine (G^{iBu}) with 2'-*O*-*t*-butyldimethylsilyl protected phosphoramidites were used. 2′-*O*-methyl modifications were introduced employing the corresponding phosphoramidites carrying the same protecting groups as the regular building blocks; 5-ethyl thiotetrazole (500 mmol/l in acetonitrile) was used as activator solution for phosphoramidite couplings. In order to introduce phosphorothioate linkages a 50 mmol/l solution of 3-((dimethylamino-methylidene)amino)-3*H*-1,2,4-dithiazole-3-thione (obtained from AM Chemicals, Oceanside, CA) in anhydrous acetonitrile/pyridine (1:1 vol/vol) was employed. The linker on the 5′-end of the sense strand was generated without any modification of the synthesis cycle using 1-*O*-dimethoxytrityl-hexyl-disulfide,1'-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite followed by coupling of 6-hexyn-1-yl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite to the 5′-terminus. Both linker amidiates were obtained from Glen Research (Sterling, VA). After cleavage and de-protection,[18] RNA oligonucleotides were purified by anion-exchange high performance liquid chromatography and characterized by MALDI or ES mass spectrometry. To generate siRNAs from RNA single strands, equimolar amounts of complementary sense and antisense strands were mixed and annealed in a 20 mM NaCl, 4 mM sodium phosphate pH 6.8 buffer. siRNAs were further characterized by capillary gel electrophoresis and were stored frozen until use.

For cell culture experiments a siRNA directed against eGFP (siGFP, sense sequence: Hexynyl-C_6-ss-C_6-5'-AuAucAuGGc cGAcAAGcAdTsdT-3'; antisense sequence: 5'-UGCUUGU CGGCcAUGAuAUdTsdT-3'; small letters: 2'-methoxy-RNA; s: phosphorothioate) or an unrelated control siRNA (siCtrl, sense sequence: Hexynyl-C_6-ss-C_6-5'-AuGuAuuGGccuGuAu uAGdTsdT-3'; antisense sequence: 5'-CuAAuAcAGGCcA AuAcAUdTsdT-3') was used. As additional unconjugated control siRNA, the same sequences without modification at the 5'-end of the corresponding sense strands were used. Uptake studies by fluorescence-activated cell sorting and fluorescence microscopy were performed using a Cy5 labeled siRNA directed against the house keeping gene AHA1 (siAHA1, sense sequence: Hexynyl-C_6-ss-C_6-5′-GGAu GAAGuGGAGAuuAGudTsdT-3′; antisense sequence: (Cy7-NH-C_6-5′-ACuAAUCUCcACUUcAUCCdTsdT-3').

Loading of a 2-chlorotrityl chloride resin. All synthesis steps on solid support were carried out using a 5 ml syringe reactor connected to a vacuum pump.

For loading, the 2-chlorotrityl chloride (2-CTC) resin (1.6 mmol chloride/g resin; Iris Biotech, Marktredwitz, Germany) was swollen in anhydrous dichloromethane (DCM) for 10 minutes; 0.45 equivalents (150% of the desired loading) of Fmoc protected (*S*)-5-azido-2-(amino)pentanoic acid (Sigma-Aldrich, Steinheim, Germany) and 0.9 equivalent N,N-diisopropylethylamine (DIPEA) per gram resin were dissolved in anhydrous DCM, added to the swollen resin and incubated for 1 hour under constant shaking. To cap residual reactive chloride groups a mixture of DCM/MeOH/DIPEA (80/15/5, vol/vol/vol) was added twice for 30 minutes. After washing the resin five times with DCM the loading of the resin was determined by Fmoc removal and measuring the absorbance at 301 nm. For Fmoc deprotection the resin was incubated four times for 10 minutes in 20% piperidine in N,N-dimethylformamide (DMF). For short time storage, the resin was washed three times with DMF, DCM, and *n*-hexane and dried under vacuo.

Synthesis of FolA-PEG-azide. After swelling (*S*)-5-azido-2-(amino)pentanoic acid loaded 2-CTC resin (loading: 0.3 mmol/g) in DCM for 30 minutes, HO-dPEG$_{24}$-Fmoc (Quanta Biodesign, Powell, OH), tBu-Glu(OH)-Fmoc and N^{10}-(trifluoroacetyl)pteroic acid (Clausson & Kaas, Farum, Denmark) were attached consecutively using amino acid/HoBt/PyBop/DIPEA (4/4/4/8) in 1 ml DCM/DMF (1/1; vol/vol) and an incubation time of 1 hour. To cleave the Fmoc groups the resin was washed three times with DCM and three times with DMF and subsequently the resin was incubated four times with 20% piperidine in DMF for 10 minutes each. Coupling of the amino acid as well as cleavage of the protective group was checked by Kaiser test.[19] After coupling of N^{10}-(trifluoroacetyl)pteroic acid the resin was washed five times with DMF, five times with DCM, and incubated four times for 30 minutes with 1 mol/l ammonium hydroxide solution/DMF (1:1, vol/vol) to cleave the trifluoroacetyl (TFA) protecting group. After completion of the reaction the resin was washed three times with DCM and three times with *n*-hexane and finally dried in vacuo.

The desired targeting ligand was cleaved of the resin using a cocktail comprising of TFA/H_2O/Triisopropylsilane (TIS) (95.0/2.5/2.5; vol/vol/vol). After 2 hours the solution was collected by filtration and the resin was washed twice with TFA and once with DCM. After reducing the volume by evaporation, the solution was dropped slowly into a 1:1 mixture (40 ml) of ice-cold Methyl-tert-butyl-ether (MTBE) and *n*-hexane. The resulting precipitate was centrifuged at 4 °C for 10 minutes (2,000–3,000 rpm). The solvents were decanted and the pellet was washed twice with ice-cold MTBE. The pellet was dissolved in 50% acetonitrile and lyophilized.

The structural analogue dPEG$_{24}$-azide was synthesized the same way, stopping the solid phase synthesis after coupling of HO-PEG$_{24}$-Fmoc and removing its protection group.

All structures were analyzed for purity by reverse phase-high performance liquid chromatography and identity was confirmed by mass spectrometry.

Synthesis and purification of FolA-PEG-siRNA. 74 nmol siRNA (modified with a hexynyl-ss-C_6-linker at the 5′ end of its sense strand) in 100 µl H_2O were mixed with 296 nmol FolA-PEG_{24}-Azide in 300 µl dimethyl sulfoxide (DMSO)/*t*-butanol (3/1; (vol/vol)) and 60 µl Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] (TBTA)/CuBr solution (0.1 mol/l TBTA/0.1 mol/l CuBr 2/1 (vol/vol)) each in DMSO/*t*-butanol (3/1 (vol/vol)). After 3 hours at 37 °C under constant shaking, the mixture was diluted with 1.5 ml buffer A (20 mmol/l HEPES, pH 6.5, 30% acetonitrile) and centrifuged for 5 minutes at 13,000 rpm. The siRNA conjugate in the supernatant was purified using a 1 ml Resource Q column (GE Healthcare, Freiburg, Germany) connected to an Äkta basic system. For that purpose the supernatant was loaded onto the column using buffer A containing 20 mmol/l NaCl. After unbound material was washed away, the product was eluted setting a gradient of 10 mmol/l NaCl/minute and a flow rate of 1 ml/minute. Fractions showing absorption at 260 nm were analyzed by a gel shift assay. For this the samples were loaded in a 2.5% agarose gel; 100 V were applied for 100 minutes. Samples showing a retarded migration on the gel compared to unconjugated input siRNA were pooled.

Synthesis of polycationic carriers. Synthesis of the polycationic carriers **230** and **386** was performed as described elsewhere.[13]

Mass spectrometry

Peptides. Peptidic compounds (1 mg) were dissolved in 1 ml H_2O containing 0.1%TFA; 4 µl of this solution were spotted on a 4 µl matrix droplet consisting of a saturated solution of 2,5-dihydroxybenzoic acid in 50% acetonitrile containing 0.1%TFA. Samples were analyzed using an Autoflex II mass spectrometer (Bruker Daltonics, Bremen, Germany). Fifty to 100 spectra of respective probes were averaged for one sample spectrum.

Nucleic acid constructs. Samples were used as collected after ion exchange purification. To minimize additional salt peaks, 5 µl of the samples were desalted by drop dialysis using an ultrafiltration membrane (Millipore, Billerica, MA) on a petri dish filled with H_2O for 2 hours; 4 µl of this solution was spotted on a 4 µl matrix droplet consisting of a saturated solution of 3-hydroxy picolinic acid in 50% acetonitrile. Samples were analyzed using an Autoflex II mass spectrometer (Bruker Daltonics). Fifty to 100 spectra of respective probes were averaged for one sample spectrum.

Analytical reverse phase-high performance liquid chromatography. Analysis of the peptide synthesis products was performed on a Sunfire C18 column (5 µm, 4.6 × 150 mm) connected to a Waters high performance liquid chromatography system (Waters, Milford, MA). In a standard procedure the product was diluted in water containing 0.1% (vol/vol) TFA to a concentration of 1 mg/ml; 30 µl of this solution was loaded onto the column using a water/acetonitrile (0.1% TFA) mixture of 95:5 and a flow rate of 1 ml/minute for 5 minutes. The product was eluted using a water/acetonitrile gradient from 95:5 to 0:100 in 30 minutes using a detection wavelength of 220 nm.

Polyplex formation. Polyplexes for transfections and gel shift experiments were prepared as follows, unless otherwise indicated: 270 ng siRNA (final concentration: 200 nmol/l) and the calculated amount of polymer at indicated protonable nitrogen/siRNA phosphate ratios (N/P of 6, 12, or 20) were diluted in separate Eppendorf tubes, containing 10 µl of 20 mmol/l HEPES buffered 5% glucose pH 7.4 (HBG) each. Polycation solution was added to the siRNA solution, rapidly mixed by pipetting up and down (at least five times) and incubated for 40 minutes at room temperature in order to form polyplexes.

Agarose gel shift assay. A 2% agarose gel was prepared by dissolving agarose in TBE buffer (trizma base 10.8 g, boric acid 5.5 g, disodium EDTA 0.75 g, and 1 l of water). GelRed was added for the detection of nucleic acids. Polyplexes in 20 µl HBG and loading buffer (prepared from 6 ml of glycerine, 1.2 ml of 0.5 mol/l EDTA, 2.8 ml of H_2O, 0.02 g xylene cyanol) were filled into the sample pockets. Electrophoresis was performed at 80 V for 40 minutes.

Measurement of particle size and zeta potential. Particle size of siRNA polyplexes was measured by laser-light scattering using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Polyplexes, containing 10 µg siRNA, were prepared in 370 µl HEPES (20 mmol/l, pH 7.4) and filled up with HEPES (20 mmol/l, pH 7.4) to 1 ml before measurement.

Cell culture. Human KB/eGFP-luc cells, stably transfected with an eGFP-luciferase fusion gene, were grown in folate free RPMI-1640 medium, supplemented with 10% fetal calf serum (FCS), 4 mmol/l stable glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Murine Neuro2A/eGFP-luc cells, stably transfected with a eGFP-luciferase fusion gene, were grown in Dulbecco's modified Eagle medium, supplemented with 10% FCS, 4 mmol/l stable glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Transfection. Transfection was performed in 96-well plates with 5×10^3 cells per well in triplicate. Cells were seeded 24 hours before transfection and then medium was replaced with 80 µl fresh growth medium containing 10% FCS. siRNA polyplexes at indicated N/P ratios in 20 µl of HBG were added to each well and incubated at 37 °C. After 30 minutes medium change was performed. 48 hours after transfection cells were treated with 100 µl cell lysis buffer (25 mmol/l Tris, pH 7.8, 2 mmol/l EDTA, 2 mmol/l 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). Relative light units were presented as percentage of the luciferase gene expression of buffer treated control cells.

Flow cytometric assay. KB/eGFPLuc or Neuro2A/eGFPLuc cells were seeded into 24-well plates at a density of 5 × 10^4 cells/well. After 24 hours, culture medium was replaced with 400 µl fresh growth medium, containing 10% FCS. Plain Cy5-labeled siRNA (2.5 µg in 100 µl HBG) or transfection complexes (N/P ratio of 6 and 12 in 100 µl HBG, containing 2.5 µg Cy5-labeled siRNA) were added to each well and incubated at 37 °C for 30 minutes. Subsequently, cells were washed twice with 500 µl phosphate-buffered saline. Cells were detached with trypsin/EDTA, taken up in phosphate-buffered saline with 10% FCS and flow cytometry was performed using a Cyan ADP flow cytometer (Dako, Hamburg, Germany). Cellular uptake was assayed by excitation of Cy5 at 635 nm and detection of emission at 665 nm. To discriminate between viable and dead cells as well as for exclusion of doublets, cells were appropriately gated by forward/sideward scatter and pulse width, counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and 1 × 10^4 gated events per sample were collected. Data were recorded by Summit^T software (Summit, Jamesville, NY) and evaluated using FlowJo^T software.

Fluorescence microscopy. KB/eGFP-Luc or Neuro2A/eGFP-Luc cells were seeded into 8 well Labtek chamber slides at a density of 2 × 10^4 cells/well. After 24 hours, culture medium was replaced with 240 µl fresh growth medium, containing 10% FCS. Cy5-labeled siRNA (1.5 µg in 60 µl HBG) was added to each well and incubated at 37 °C for 30 minutes. Subsequently, cells were washed twice with 500 µl phosphate-buffered saline and cell nuclei were stained with Hoechst 33342 dye. Cellular uptake was assayed by excitation of Cy5 at 635 nm and detection of emission at 665 nm. An Axiovert 200 fluorescence microscope was used to collect the images and data were analyzed and processed by AxioVision LE software (Zeiss, Jena, Germany).

Acknowledgments. This work was supported by the DFG Cluster "Nanosystems Initiative Munich", a grant from Roche Kulmbach GmbH, and the Biotech Cluster m4 T12. We thank Arzu Cengizeroglu for providing stably transfected eGFP-Luc KB cells and Naresh Badgujar for providing lipopolymer **230**. The authors declared no conflict of interest.

Supplementary material

Figure S1. Mass spectrum of peptidic FolA-PEG-azide.
Figure S2. Chromatogram of the RP-HPLC analysis of FolA-PEG-azide.
Figure S3. Mass spectrum of purified FolA-PEG-siCtrl.
Figure S4. Gene silencing activity of conjugated siRNA in comparison to unconjugated siRNA directed against eGFP (siGFP).
Figure S5. Agarose gel shift assay polyplexes of siRNA conjugates with polymer **386**.
Figure S6. FACS analysis of cellular uptake of polyplexes formed with Cy5-labeled siRNA conjugates and polymer **386**.
Figure S7. Gene silencing activity using different siRNA concentrations.
Table S1. Size and zeta potential of siRNA/polymer **386** polyplexes.

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Supplementary Information accompanies this paper on the Molecular Therapy: Nucleic Acids website (http://www.nature.com/mtna)