SUPPORTING INFORMATION

LIGAND-DIRECTED ACID-SENSITIVE AMIDOPHOSPHATE 5-TRIFLUOROMETHYL-2′-DEOXYURIDINE CONJUGATE AS A POTENTIAL THERANOSTIC AGENT

Tatyana S. Godovikova,[a,b,#] Vladimir A. Lisitskiy,[a,b] Natalya M. Antonova,[a,b] Tatyana V. Popova,[a,b] Olga D. Zakharova,[a] Alexey S. Chubarov,[a,b] Igor V. Koptyug,[b,c] Renad Z. Sagdeev,[c] Robert Kaptein,[b,d] Andrey E. Akulov,[e] Vassily I. Kaledin,[e] Valeriy P. Nikolin,[e] Sergei I. Baiborodin,[e] Ludmila S. Koroleva,[a,b] Vladimir N. Silnikov[a,#]

[a]Institute of Chemical Biology and Fundamental Medicine, SB RAS, 630090 Novosibirsk, Russia

[b]Novosibirsk State University, 630090 Novosibirsk, Russia

[c]International Tomography Center, SB RAS, 630090 Novosibirsk, Russia

[d]Bijvoet Center, University of Utrecht, 3584 CH Utrecht, The Netherlands

[e]Institute of Cytology and Genetics, SB RAS, 630090 Novosibirsk, Russia

[#]To whom correspondence should be addressed.

Dr. Tatyana Godovikova, Institute of Chemical Biology and Fundamental Medicine, SB RAS, 8 Lavrentiev prosp., 630090 Novosibirsk, Russia. Tel.: +7 383 363 5174; fax: +7 383 363 5153. E-mail address: godov@niboch.nsc.ru

Dr. Vladimir N. Silnikov, Institute of Chemical Biology and Fundamental Medicine, SB RAS, 8 Lavrentiev prosp., 630090 Novosibirsk, Russia. Tel.: +7 383 363 512; fax: +7 383 363 5153. E-mail address: silnik@niboch.nsc.ru

TABLE OF CONTENTS

1. Synthetic methods

Synthesis and characterization of linoleic acid-substituted polyethyleneimine (PEI-LA)

PEI-LA was synthesized by *N*-acylation of 25 kDa PEI with linoleyl chloride. Briefly, linoleyl chloride was dissolved in 1 mL of DMF and added dropwise to 100 mg of PEI in 1 mL of DMSO. The linoleyl chloride : PEI molar ratios were systematically varied during the synthesis procedure between 3 and 10. The mixture was allowed to react for 24 h at room temperature. The excess of diethyl ether was used to precipitate the polymer and to wash the pellet (10 mL × 3). After that, the polymer was dried under vacuum at ambient temperature overnight. PEI-LA was analyzed by ^{1}H NMR. The characteristic proton shifts for the PEI-LA-2 polymer (Figure S1) (D_2O) are: **PEI**: N-CH_2-CH_2-N, δ 2.40–3.60 (m, 2300 H). **LA**: δ 1.0 (m, 6H, H–d), 1.41 (m, 32H, H–b), 1.58 (m, 4H, H–a), 2.18 (m, 12H, H–c), 5.43 ppm (m, 8H, H–e). The characteristic chemical shifts for the protons of LA (δ 1.0 ppm; -CH_3, H–d) and PEI (δ 2.4–3.6 ppm) were integrated, normalized for the number of protons in each peak, and used to obtain the extent of lipid substitutions on the modified polymer. At the linoleyl chloride : PEI molar ratios of 3, 5, and 10, the extent of lipid substitutions was calculated to be 2.0, 3.5 and 6 linoleic acid residues per PEI molecule, respectively. Two of the obtained polymers were found to be soluble in pure water. The polymer conjugates containing two lipid residues per PEI molecule were used in this study.

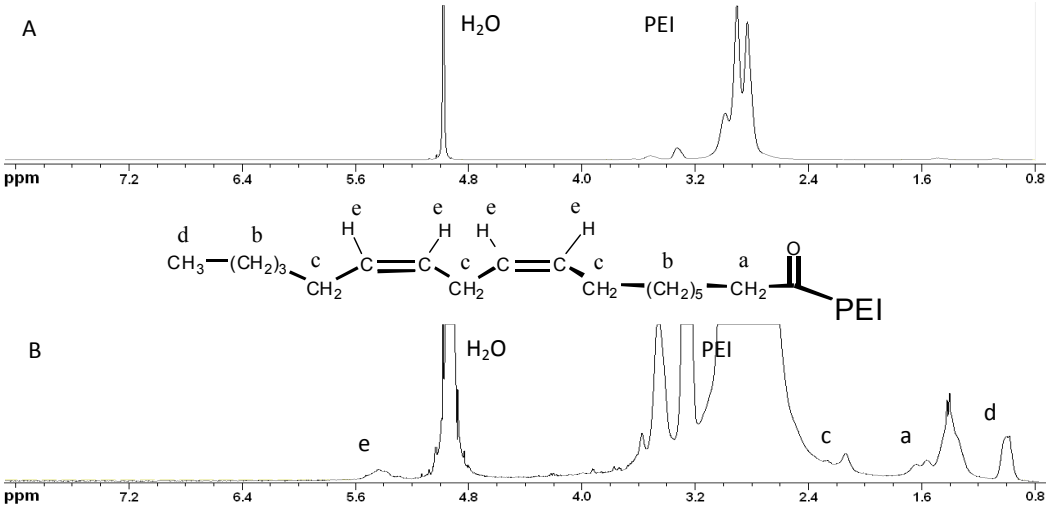


Figure S1. ^{1}H NMR spectra of PEI (A) and PEI-LA (B) in D_2O. Signals in the spectrum B have been assigned to protons (a through e) of the structure shown above the spectrum.

Synthesis of PEI-LA-UA conjugates via pentafluorophenyl ester of urocanic acid

Urocanic acid (2.45 mg, 0.018 mmol), DMAP (36.45 mg, 0.299 mmol), triphenylphosphine (36.15 mg, 0.149 mmol), pentafluorophenol (4.0 mg, 0.216 mmol) and 2,2'-dipyridyldisulfide (33.0 mg, 0.149 mmol) were dissolved in DMSO (0.1 mL, 0.2 mL, 0.15 mL, 0.05 mL and 0.1 mL, respectively). These solutions were rapidly mixed and kept at 37 ºC for 5 min. Then the mixture was immediately used for the next step of the synthesis. The aqueous solution (0.128 mL H_2O) of PEI-LA (0.45 μmol, 11.50 mg,) was added to the pentafluorophenyl urocanate (40-fold excess of urocanic acid ester). The reaction mixture was incubated at 37 ºC with stirring at 400 rpm. After 16 h, diethyl ether (1 mL) was added to the mixture, and the pellet was obtained by centrifugation, washed with diethyl ether (1 mL × 3) and

dissolved in 1 mL of DMSO. The resultant PEI-LA-UA conjugate was purified from low molecular weight compounds by centrifugal filtration using Centricon concentrators.

The modified polymer was analyzed by ^{1}H NMR. The characteristic proton shifts of the PEI-LA-UA-30 polymer (Figure S2) are: **PEI**: N-CH_2-CH_2-N, δ 2.4–3.6 (m, 2300 H). **LA**: δ 1.00 (m, 6H, H–d), 1.51 (m, 32H, H–b), 5.43 (m, H–e). **UA** (two stereoisomers): δ 6.48 (m, 25H, H–f), 7.05 (m, 5H, H–f′), 7.45 (m, 55H, H–h,g), 7.68 (m, 5H, H–g′), 7.81 (m, 1H, H–i) ppm.

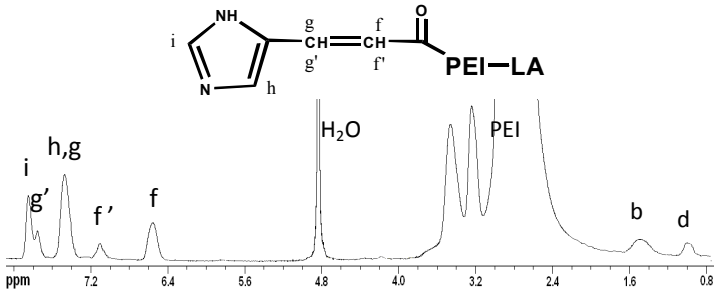


Figure S2. ^{1}H NMR spectrum of PEI-LA-UA conjugate obtained via pentafluorophenyl ester of urocanic acid in D_2O. Signals in the spectrum have been assigned to protons (f through i) of the structure shown above the spectrum. In spectrum, labels g, f correspond to *trans*-UA protons and g′,f′ – to *cis*-UA protons. Signals in the spectrum have been assigned to protons (b and d) of the structure LA shown above the spectra B (Figure S1).

Synthesis of phosphorylating derivative of pTFT (DMAP-pTFT)

The phosphorylating mononucleotide derivative was prepared by reaction of pTFT with a mixture of triphenylphosphine and 2,2′-dipyridyl disulfide in the presence of 4-(*N,N*-dimethylamino)pyridine. Briefly, the *N,N,N*-triethylammonium salt of pTFT (10.8 mg, 0.036 mmol) was dissolved in 0.2 mL of DMSO. To this solution, 4-(*N,N*-dimethylamino)pyridine (72.9 mg, 0.60 mmol) in 0.4 mL of DMSO, triphenylphosphine (78.3 mg, 0.30 mmol) in 0.3 mL of DMSO and 2,2′-dipyridylsulfide (65.6 mg, 0.30 mmol) in 0.3 mL of DMSO were sequentially added. The reaction mixture was kept at room temperature for 12 min with stirring (250 rpm). After that, diethyl ether (1 mL) was added to the reaction mixture, and the pellet was collected by centrifugation, washed with diethyl ether (1 mL × 3), dissolved in 1 mL of H_2O, and immediately used for the reaction with PEI-LA or PEI-LA-UA conjugates.

Synthesis of PEI-LA-pTFT conjugates

To introduce an anticancer nucleotide antimethabolite pTFT into PEI-LA polymer, we used 4-(*N,N*-dimethylamino)pyridine derivative of pTFT (Figure S3). DMAP-pTFT was reacted with PEI-LA (23.00 mg, 0.9 μmol) in 0.26 mL of H_2O. The PEI-LA : pTFT ratios were systematically varied during the synthesis procedure between 0.035 and 0.12, assuming one primary amine per PEI monomer unit. After 1 h of reaction, low molecular weight reactants were removed from the polymeric product by centrifugal filtration using Centricon concentrators. **PEI-LA-pTFT-70**: UV-vis (15 mM KH_2PO_4, pH 7.4): λ_{max} = 262 nm; $\varepsilon = 5.78 \times 10^5\ M^{-1}\ cm^{-1}$.

The modified polymer was analyzed by ^{1}H NMR. The characteristic proton shifts for the PEI-LA-pTFT-70 polymer (Figure S4) are: **PEI**: N-CH_2-CH_2-N, δ 2.6–3.4 (m, 2300 H). **TFT**: δ 2.49 (m, 140H, H–m), 4.05 (m, 140H, H–j), 4.25 (m, 70H, H–l), 4.60 (m, 70H, H–k), 6.40 (m, 70H, H–n), 8.25 ppm (m, 70H, H–o). ^{19}F NMR: δ 102.07 ppm (br.s). ^{31}P NMR: δ 8.63 ppm (br.s).

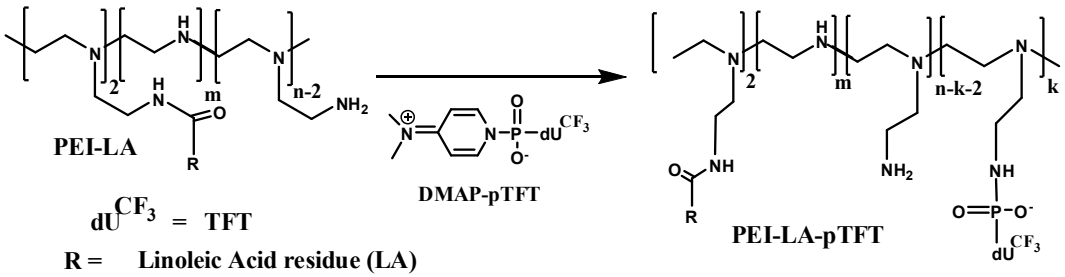


Figure S3. Scheme for the synthesis of PEI-LA-pTFT conjugate.

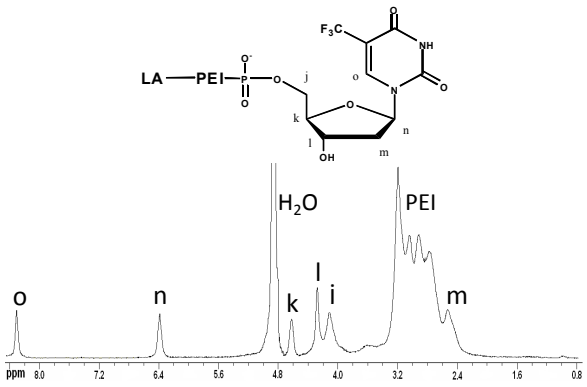


Figure S4. ^{1}H NMR spectrum of PEI-LA-pTFT conjugate in D_2O. Signals in the spectrum have been assigned to protons (j through o) of the structure shown above the spectrum.

2. Drug release properties of PEI-LA-pTFT-UA conjugates at different pH (Figure S5)

PEI-LA-pTFT-UA conjugate (1.5 mM) was incubated in acetate buffer (0.1 M) with pH values of 5.0 or 7.0 at 37 ^{o}C under constant shaking (400 rpm). At predetermined time points (0, 4, 6, 7.5, 9, 10 and 11.5 h), 0.4 mL aliquots of the sample were withdrawn, low molecular weight material was removed by centrifugal filtration using Centricon concentrators, and analyzed by UV-vis spectroscopy at 267 nm. The percentage of hydrolysis was calculated using the following equation:

$$\text{Percentage of hydrolysis (\%)} = \frac{D}{D_0} \times 100\%$$

where D_0 is an optical density of the initial conjugate and D is an optical density of the low molecular weight material. The percentage of hydrolysis for the conjugates as a function of time is shown in Figure S5.

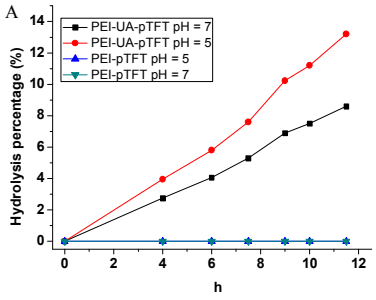


Figure S5. Acid-sensitivity of PEI-LA-pTFT and PEI-LA-UA-pTFT conjugates.

3. Stability of the PEI-LA-UA-pTFT conjugate in human plasma

The stability of PEI-LA-UA-pTFT in human plasma was studies by ^{19}F NMR spectroscopy. The substantial difference in the chemical shifts of the signals for trifluoromethyl group in PEI-LA-UA-pTFT and for free fluoride anion, and the zero-background of ^{19}F NMR signal allows real-time monitoring of the degradation of fluorinated polymer conjugate in human plasma. The levels of PEI-LA-UA-pTFT and its fluorine-containing catabolites were directly measured in human plasma incubated with 0.5 mM conjugate. The final catabolite (free fluoride anion, F^-) was detected after 3 h of TFT incubation in plasma (Figure S6). The presence of fluoride anion is explained by the conversion of 5-fluoromethyluracil, which was released from PEI-LA-UA-pTFT, into 5-carboxyuracil. Analysis of fluorinated products of TFT hydrolysis isolated from plasma by centrifugal filtration demonstrated that no more than 10% of PEI-LA-UA-pTFT was cleaved within 3 h of its incubation with human plasma. Indeed, upon incubation of PEI-LA-UA-pTFT in human plasma, the intensity of a broad ^{19}F NMR signal for TFT trifluoromethyl group ($\delta \sim 100$ ppm) decreased with the concomitant appearance of a sharp singlet at ~ 40 ppm characteristic of the degradation product, free fluoride anion (Figure S6).

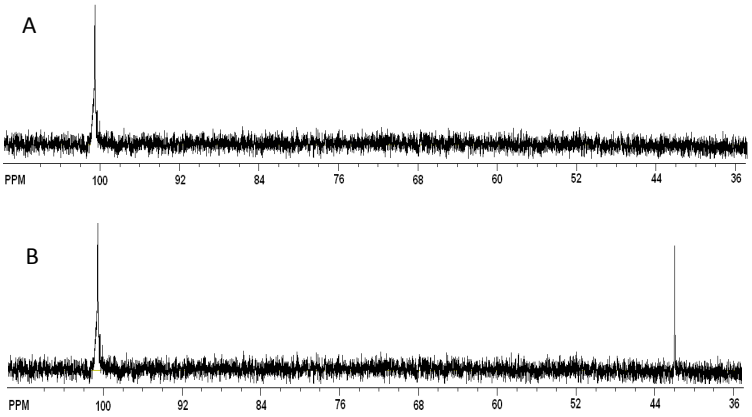


Figure S6. ^{19}F NMR spectra of PEI-LA-UA-pTFT conjugate in human plasma. The reaction mixture was incubated at 37 ºC for 0 h (A) or 3 h (B).

4. *In vitro* experiments

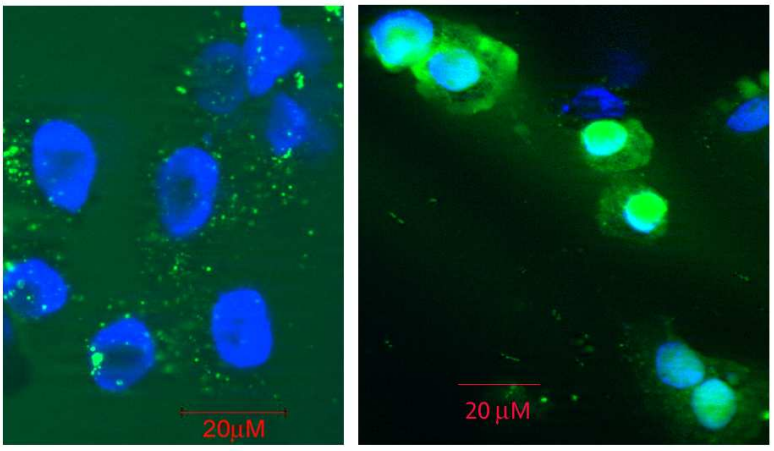
Synthesis of PEI-FITC conjugate for cellular uptake study

To determine whether the modified PEI retained the ability to bind to cancer cells and to be accumulated in them, the FITC-labeled PEI-based conjugates were synthesized. Fluorescein isothiocyanate (FITC) isomer was purchased from Sigma-Aldrich (USA). The synthetic procedure was adapted from *Lee et al.*[1] Briefly, PEI (50 mg, 2 μmol) in PBS (1 mL) was mixed with FITC isomer (1.0 mg, 2.5 μmol) dissolved in water/DMSO (1 : 1 v/v, 1 mL) and stirred at room temperature overnight while protected from light. Byproducts and unreacted FITC were removed by centrifugal filtration using Centricon concentrators. The PEI/FITC molar ratio in FITC-labeled PEI-LA-pTFT and PEI-LA-UA-pTFT conjugates was 1/1.

(1). Lee, S.-Y., Huh, M. S., Lee, S., Lee, S. J., Chung, H., Park, J. H., Oh, Y.-K., Choi, K., Kim, K., Kwon, I. C. (2010) Stability and cellular uptake of polymerized siRNA (poly-siRNA)/polyethylenimine (PEI) complexes for efficient gene silencing. *J. Control. Release 141*, 339-346.

Cell uptake

The fluorescent microscopy was utilized to investigate intracellular localization of the substances. MCF-7 cells in the exponential phase of growth were plated on the glass coverslips in 12-well plates at a density of 1×10^5 cells/well and were allowed to attach for 24 h. The FITC-labeled PEI-LA-pTFT and PEI-LA-UA-pTFT were then added to the cells to the final concentration of 1mg/mL. After 1 h of incubation the cells were washed with PBS and mounted on the object-plate in DAPI/Antifade solution (Millipore, USA). The analysis of PEI-X localization was performed using Axioscop A2 (Carl Zeiss, Germany) using optical filters BP 420–480 nm, BP 505–530 nm and LP 560 nm. Both types of FITC-labeled conjugates are characterized by a high level of accumulation in the cells (Figure S7).



A B

Figure S7. Fluorescent microscopy of MCF-7 cells incubated with FITC-labeled PEI-LA-pTFT (A) and PEI-LA-UA-pTFT (B).The conjugates were added at concentration 0.1 mg/ml and incubated for 1 h.