Supplementary Information

Delivery of siRNA by tailored cell-penetrating urea-based foldamers

Mégane Bornerie,[a] Anaïs Brion[b], Gilles Guichard,[a] Antoine Kichler[b], and Céline Douat,[ac]

[a] Univ. of Bordeaux, CNRS, Bordeaux INP, CBMN, UMR 5248, Institut Européen de Chimie et Biologie, 2 rue Robert Escarpit, F-33607 Pessac, France. E-mail: g.guichard@iecb.u-bordeaux.fr
[b] Equipe 3Bio, CAMB 7199 CNRS−Univ. Strasbourg, Faculté de Pharmacie, 74 route du Rhin, F-67401 Illkirch cedex, France. E-mail: kichler@unistra.fr
[c] Department Pharmazie, Ludwig-Maximilians-Universität, Butenandtstraße 5-13, D-81377 München, Germany. E-mail: celine.douat@cup.lmu.de

Table of Contents

1. Chemistry 2
Materials and Methods 2
General procedure for the solid phase synthesis of oligoureas 1-3 3
General procedure for the synthesis of homodimers 3 and 4 8
Chemical Structure of hDim used in the initial investigations for siRNA delivery 10
Circular dichroism analysis on oligoureas 1, 2 and 5 in aqueous media 10
2. Biological evaluation 12
Material and Methods 12
siRNA 12
Cell culture 12
Delivery of the siRNA targeting luciferase 12
Silencing of Polo-like kinase 1 13
Cell viability assay 13
Size analysis by dynamic light scattering (DLS) 13
Flow cytometric quantification of cell binding/endocytosis 14
Confocal microscopy 14
Annexin V/Propidium iodide assay 14
Transfection assays 15

1. Chemistry

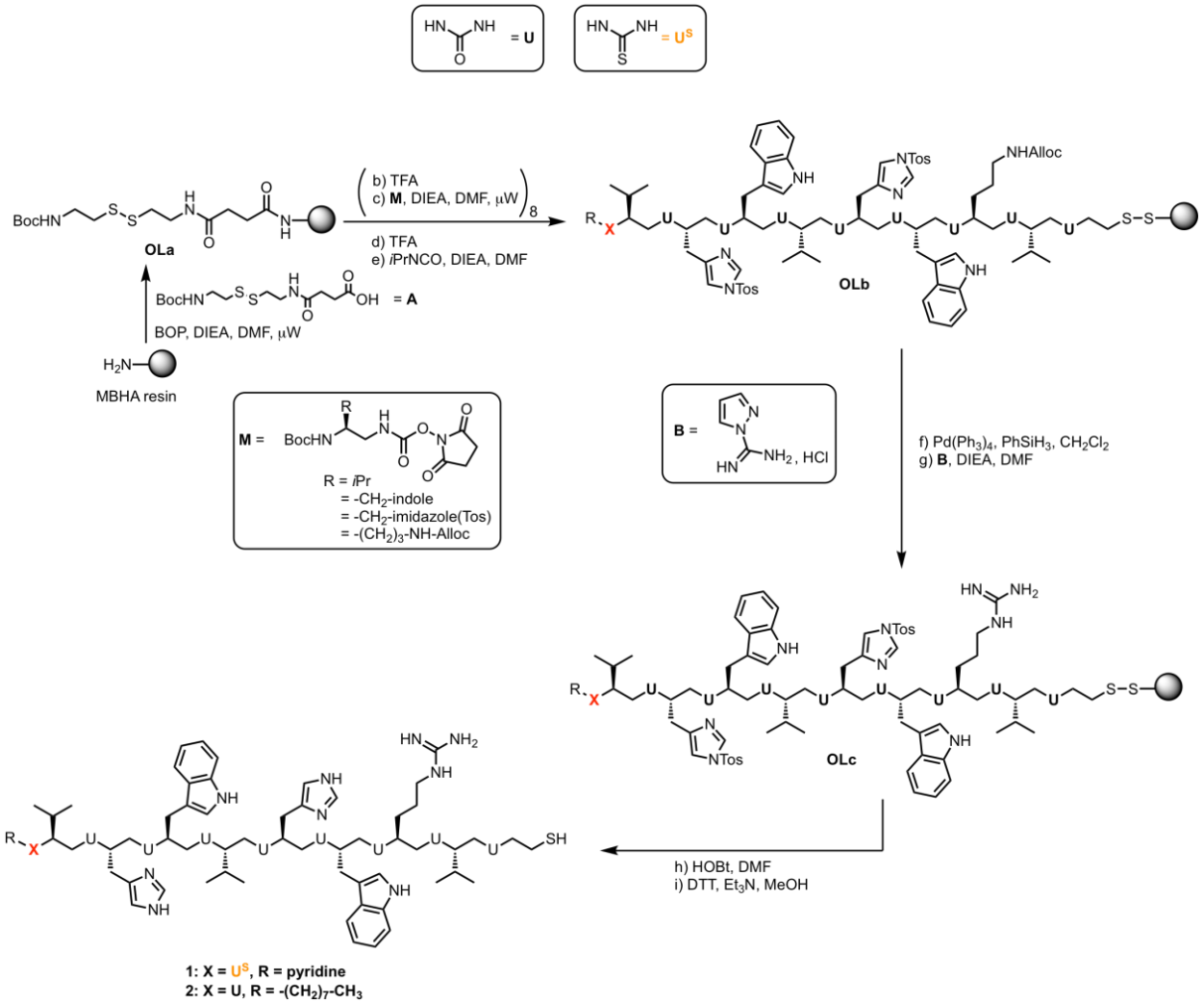
Materials and Methods

Commercially available reagents were used throughout without purification. MBHA resin (loading 1.00 mmol/g) and benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexa-fluorophosphate (BOP) reagent were purchased from PolyPeptide Laboratories, France. Solid phase peptide synthesis grade organic solvents (DMF, DCM) were used for solid phase synthesis and were purchased from Carlo Erba. RP-HPLC-quality acetonitrile (CH_3CN) and MilliQ water were used for RP-HPLC analyses and purification. Most of the activated monomers used for solid phase synthesis were prepared using previously reported procedures[1,2,3,4]. Oligourea synthesis was performed manually under microwave irradiation on a CEM discover® apparatus (*vide infra*). Thin layer chromatography (TLC) was performed on silica gel 60 F254 (Merck) with detection by UV light and charring with 1% ninhydrin in ethanol followed by heating. Flash column chromatography was carried out on silica gel (40-63 μm, Merck). RP-HPLC analyses were performed on a Dionex U3000SD using a Macherey-Nagel Nucleodur column (4.6 × 100 mm, 3 μm) at a flow rate of 1 $mL.min^{-1}$. The mobile phase was composed of 0.1% (v/v) TFA-H_2O (Solvent A) and 0.1% TFA-CH_3CN (Solvent B). Semi-preparative purifications of oligoureas were performed on a Gilson GX-281 system using a Macherey-Nagel Nucleodur column (20 × 250 mm, 5 μm) at a flow rate of 20 $mL.min^{-1}$ or on Dionex U3000SD using a Macherey-Nagel Nucleodur column (20 × 100 mm, 5 μm) at a flow rate of 4 $mL.min^{-1}$. The mobile phase was similar as for the analytic system, unless otherwise notified. Column eluent was monitored by UV detection at 214 and 254 nm. ESI-MS analyses were carried out on a ThermoElectron LCQ Advantage spectrometer equipped with an ion trap mass analyzer and coupled with a ThermoElectron Surveyor HPLC system or on an LC/MS system consisting of an Agilent 1290 Infinity liquid chromatograph coupled to an Agilent 6230 Time-of-Flight LC-MS equipped with an electrospray ion source. CD spectra were recorded on a Jasco J-815 Circular Dichroism spectrometer using quartz cells of 2 mm optical path length. The CD data represent an average of three scans.

1. A. Violette, S. Fournel, K. Lamour, O. Chaloin, B. Frisch, J.-P. Briand, H. Monteil, G. Guichard, *Chem. Biol.* **2006,** *13*, 531.
2. N. Yella-Reddy; C. Douat-Casassus, P. Claudon, B. Kauffmann, C. Didierjean, G. Guichard, *Tetrahedron* **2012**,68, 4492-4500.
3. C. Douat, C. Aisenbrey, S. Antunes, M. Decossas, O. Lambert, B. Bechinger, A. Kichler, G. Guichard, *Angew. Chem. Int. Ed.* **2015**, 54, 11133-11137
4. C. Douat, M. Bornerie, S. Antunes, G. Guichard and A. Kichler, *Bioconjugate Chem.* **2019**, 30, 1133-1139

General procedure for the solid phase synthesis of oligoureas 1-3

The synthesis of linker **A** was realized according to a previously reported procedure.[4]



Oligomers **1-3** were synthesized manually using Boc chemistry on a 50-100 µmol scale starting from MBHA resin and under microwave irradiation. The first coupling step, consisted in the installation of linker **A** and was performed with a solution of **A** (3 equiv.) in DMF (3 mL), with BOP (5 equiv.) and DIEA (7 equiv.) as coupling reagents and the resin was shaken for 15 min at 70°C with a power of 25W. This step was performed twice and completion of the coupling was monitored by Kaiser's test. For each following coupling step, a solution of succinimidyl carbamate **M** (1.5 equiv.) and DIEA (3 equiv.) in DMF (3 mL) was added to the resin, heating was maintained for 15 min at 70 °C with a power of 25W. A double coupling was performed systematically. The coupling solution was then filtered off and the resin was washed with DMF (5 × 2 mL). Completion of couplings was monitored by performing a chloranil test. Boc protecting group was removed using TFA (2 × 5 min). TFA was removed by filtration and the resin was washed with DCM (3 × 2 mL) and with DMF (3 × 2 mL). Finally, the resin was washed several times with DCM and dried under high vacuum.

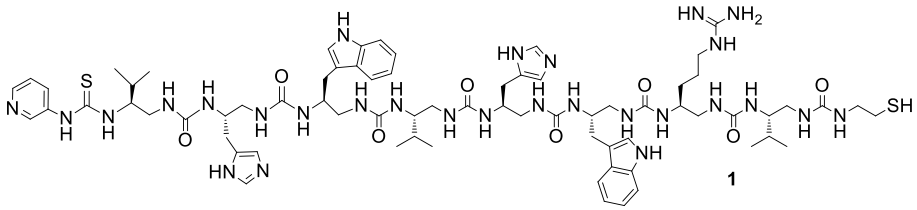
The Alloc deprotection on Orn^{U} side chain was performed on solid support by adding to the resin-bound oligourea (**OLb**) a solution of $Pd(Ph_3)_4$ (0.1 equiv.) in dry DCM (2 mL) under positive Argon atmosphere and phenylsilane (20 equiv.). The resin was mechanically shaken

for 30 min. After resin filtration and washing with dry DCM (3 × 2 mL) this deprotection was repeated once in the same conditions. The resin was then filtered off washed several times with dry DCM (5 × 2 mL) and a Kaiser's test revealed a positive purple coloration of beads thus indicating efficient Alloc removal.

Guanidinylation of Orn^{u} side-chain (OLc) was carried out on resin-bound oligourea in the presence of 1H-pyrazole-1-carboxamidine, HCl (**B**) (5 equiv.) and DIPEA (5 equiv.) in dry DMF (3 mL). The resin was mechanically shaken overnight and completion of the reaction was monitored with the Kaiser's test.

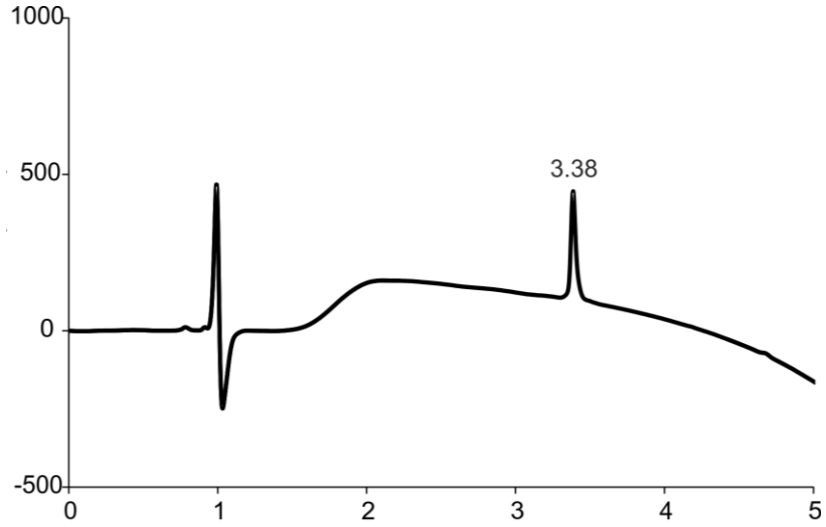
Removal of τ-Tosyl protection on imidazole side chains was performed prior cleavage by using twice a solution of hydroxybenzotriazole (HOBt, 10 equiv.) in DMF for 15 min.

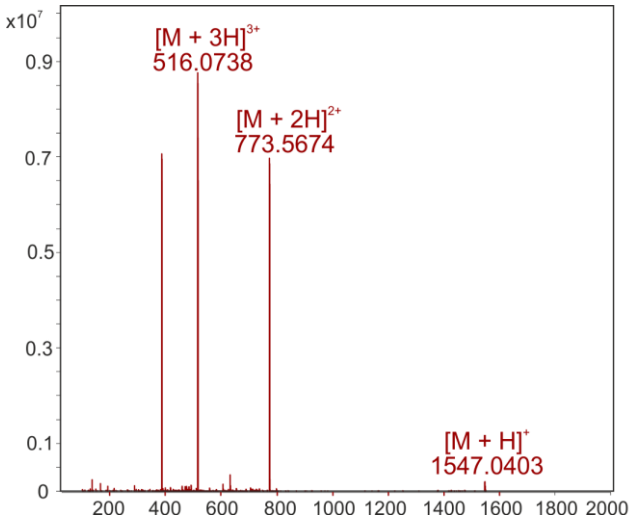
Oligourea release by disulfide bridge reduction was performed in the presence of 50 mM dithiothreitol solution (3 mL) in MeOH-Et_3N solvent mixture (11:1, v/v). The resin was mechanically shaken overnight at room temperature. The resin was then filtered off, washed several times with MeOH and the supernatant was evaporated. The crude was precipitated at 0°C with Et_2O and the recovered precipitate was solubilized in water/ACN solvent mixture to be freeze-dried. The crude oligourea was finally purified by preparative RP-HPLC using the appropriate gradient to a final purity >95%.

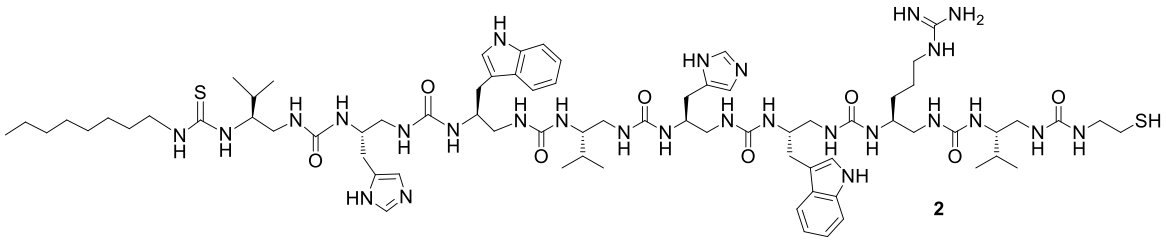


1

Oligomer 1. Yield after purification 14 mg, 14 %; white powder; RP-UPLC t_R 3.38 min (linear gradient, 10-100% B, 5 min, λ = 214 nm); HRMS calcd. for $C_{71}H_{108}N_{28}O_8S_2$ $[M+H]^+$ 1546.83; found 1547.04, $[M+2H]^{2+}$ 773.46, found 773.56.

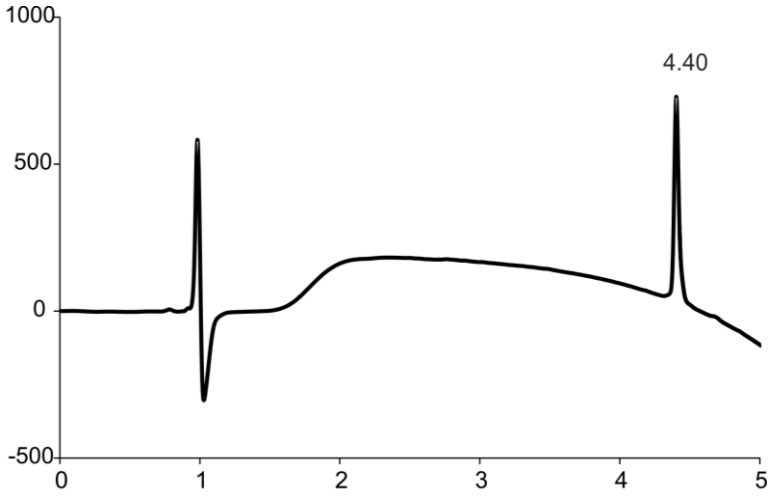


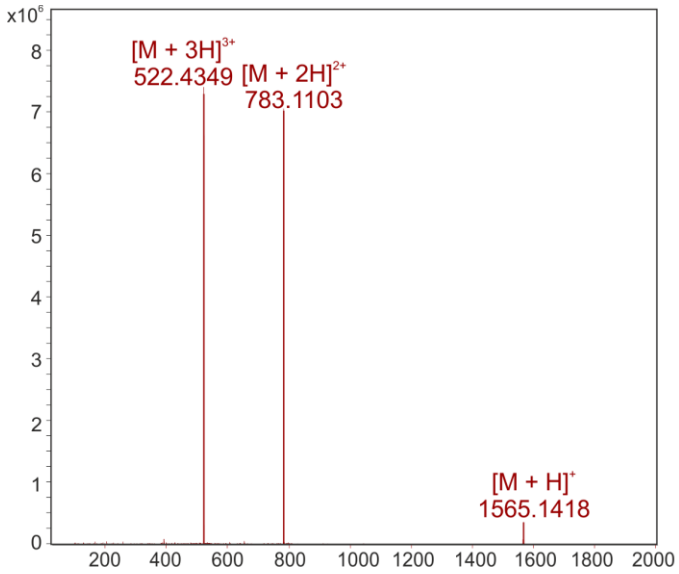


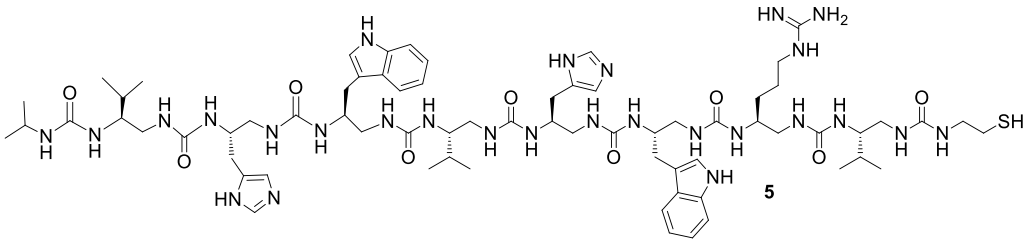


2

Oligomer 2. Yield after purification 6 mg, 6 %; white powder; RP-UPLC t_R 4.40 min (linear gradient, 10-100% B, 5 min, λ = 214 nm); HRMS calcd. for $C_{74}H_{121}N_{27}O_9S$ $[M+H]^+$ 1564.96; found 1565.14, $[M+2H]^{2+}$ 782.99, found 783.11.

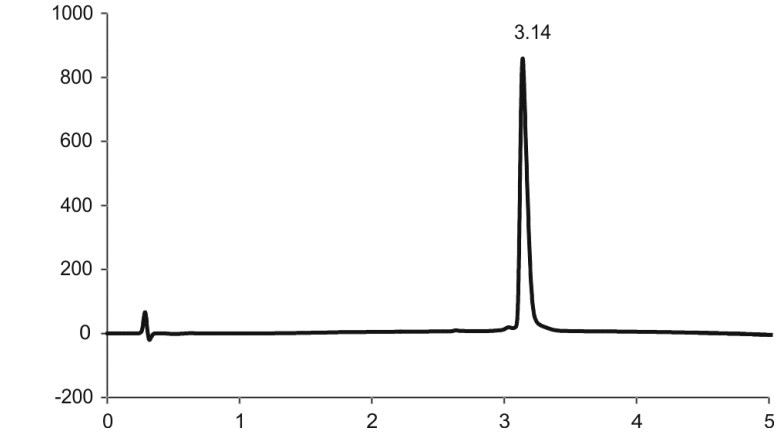


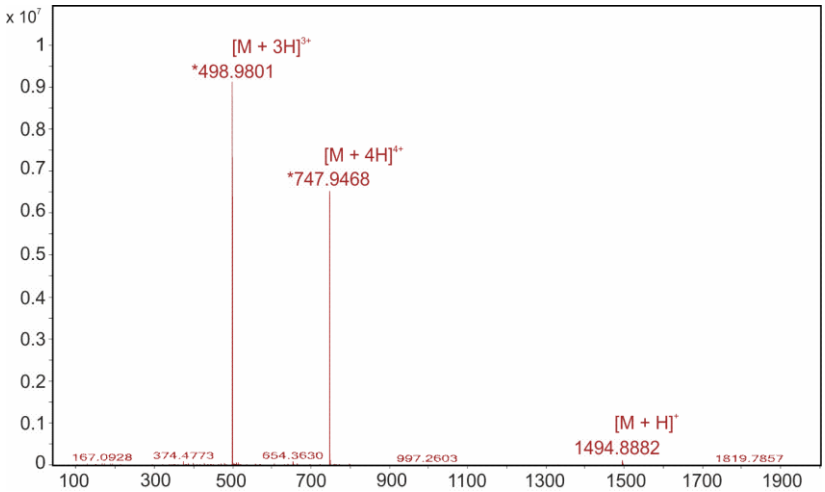




5

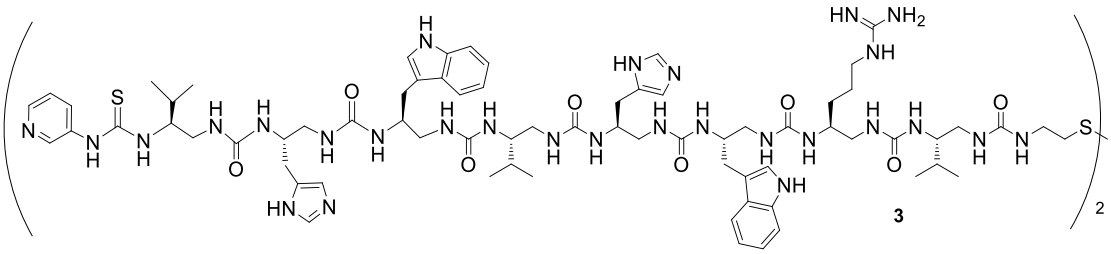
Oligomer 5. Yield after purification 18.5 mg, 13 %; white powder; RP-UPLC t_R 3.14 min (linear gradient, 10-100% B, 5 min, λ = 214 nm); HRMS calcd. for $C_{69}H_{112}N_{27}O_9S$ $[M+H]^+$ 1494.8857; found 1494.8882.



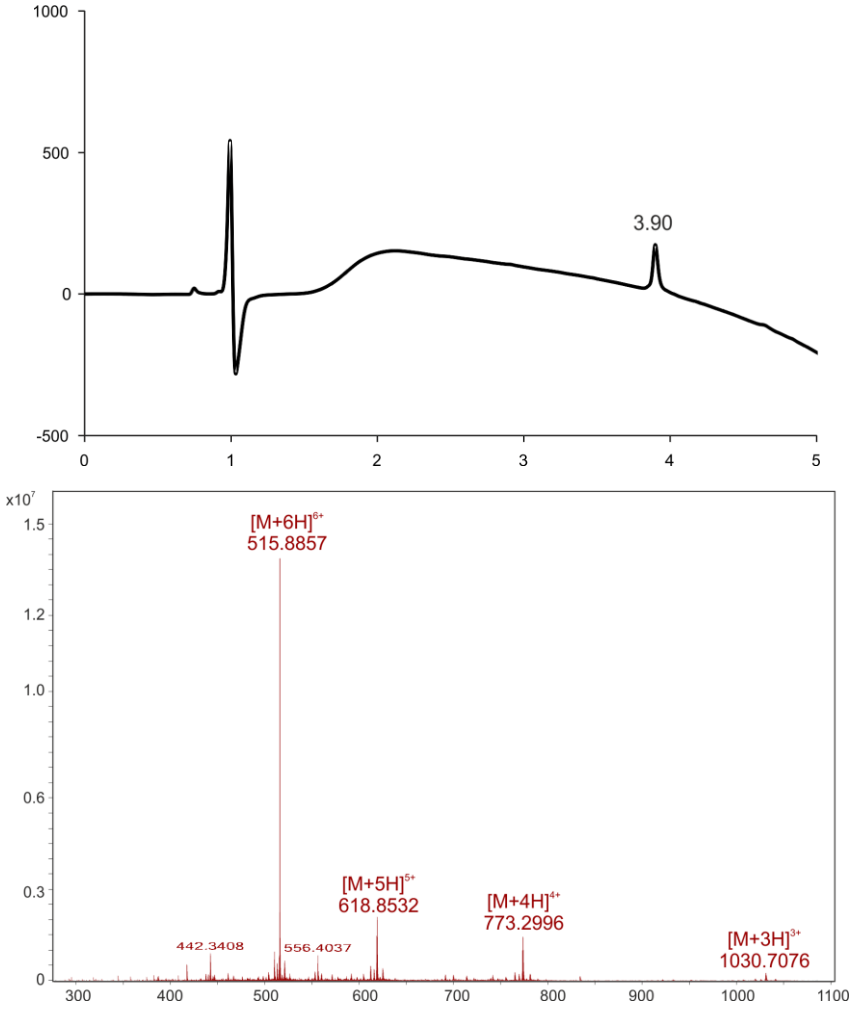


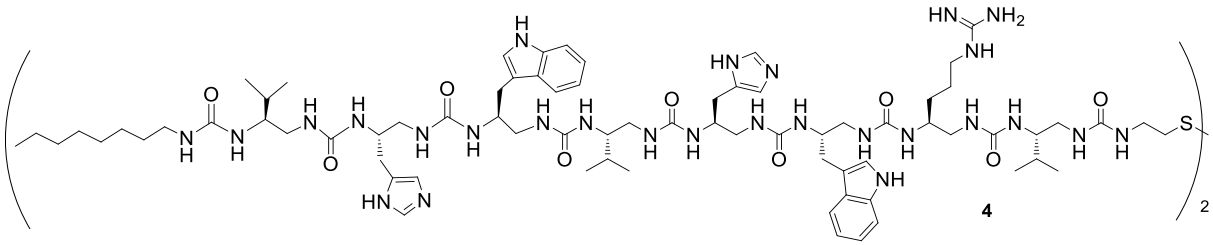
General procedure for the synthesis of homodimers 3 and 4

Oligomer **1** (1.0 equiv., 2.5 mg) was dissolved in 0.5 mL of 0.15 M ammonium acetate buffer pH 8.5 and 0.170 mL of DMSO was added. The reaction was left for two days after which RP-HPLC monitoring showed complete conversion of monomer **1** into dimer **3**. The crude was then neutralized with 0.1% TFA in water solution and directly purified on semi-prep HPLC.



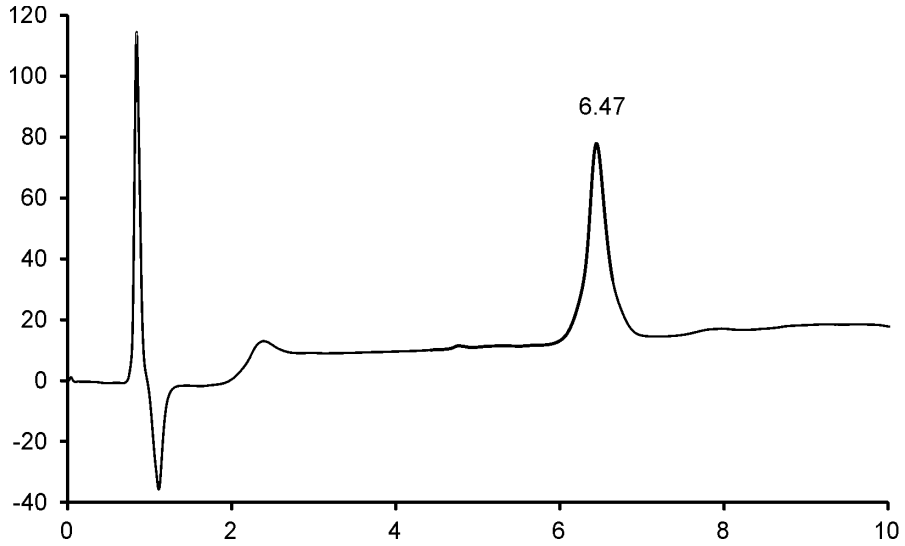
Oligomer **3**. Yield after purification 1 mg, 42 %; white powder; RP-HPLC t_R 3.90 min (linear gradient, 10-100% B, 10 min, $\lambda = 214$ nm); HRMS calcd. for $C_{142}H_{214}N_{56}O_{16}S_4$ $[M+3H]^{3+}$ 1030.21; found 1030.71, $[M+4H]^{4+}$ 772.91 found 773.39.

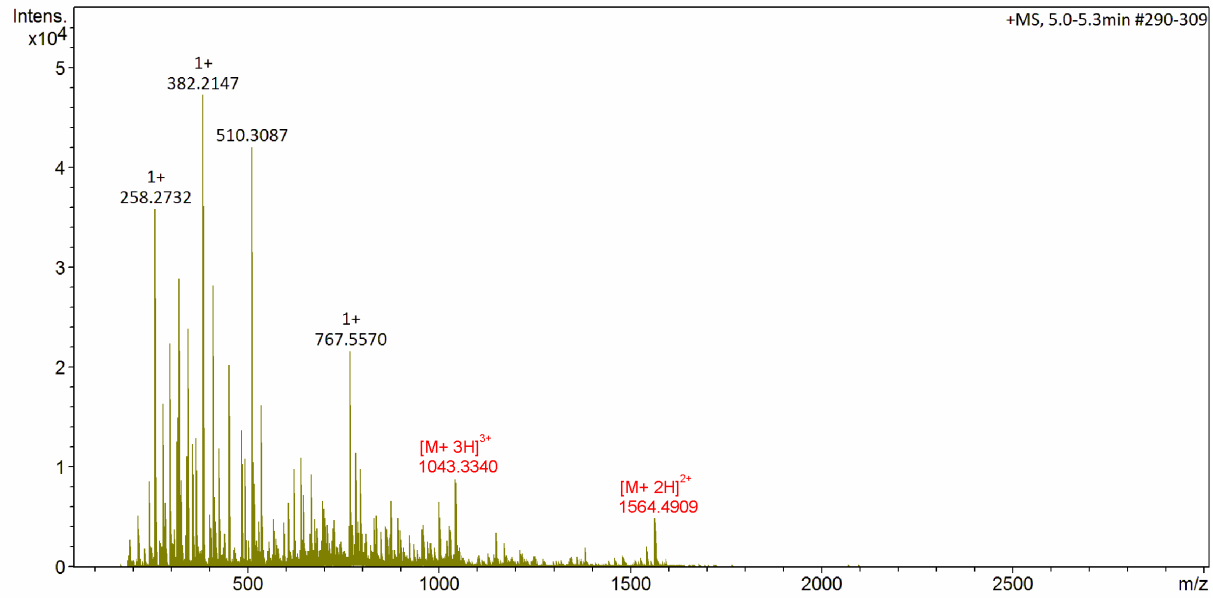




4

Oligomer **4**. Same procedure to the one used for **3**. Yield after purification 0.66 mg, 73%; white powder; RP-HPLC t_R 6.47 min (linear gradient, 10-100% B, 10 min, $\lambda = 214$ nm); HRMS calcd. for $C_{148}H_{240}N_{54}O_{18}S_2$ $[M+2H]^{2+}$ 1564.49; found 1564.4865, $[M+3H]^{3+}$ 1042.99 found 1043.33.





Chemical Structure of hDim used in the initial investigations for siRNA delivery

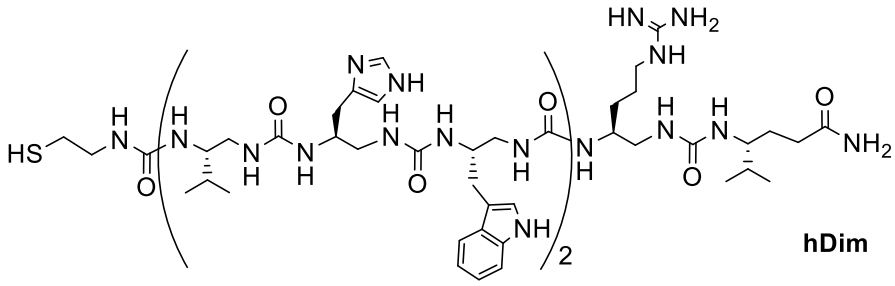


Figure S1. Chemical structure of **hDim**.

Circular dichroism analysis on oligoureas 1, 2 and 5 in aqueous media

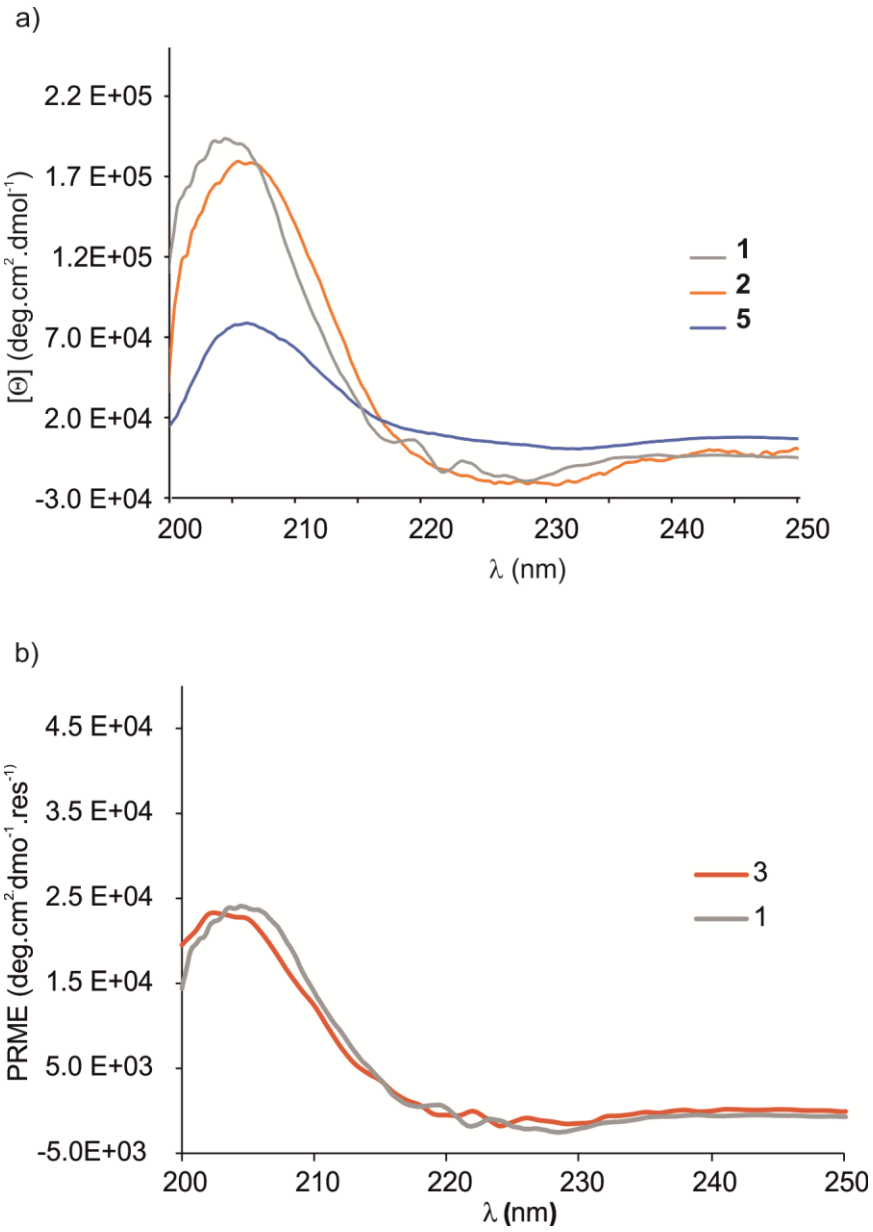


Figure S2. Comparison of far-UV CD spectra of a) **1**, **2** and **5** recorded in NaCl (150 mM) at 20 μM concentration and b) **1** and **3** reported as per residue molar ellipticity (PRME) in NaCl (150 mM).

2. Biological evaluation

Material and Methods

siRNA

The siRNAs, provided in annealed form, were synthetized by Eurogentec. Sequences are as follows (with ***TT*** = DNA bases):
siLuc: sense strand = 5'-CUU ACG CUG AGU ACU UCG A ***TT***-3'; antisense strand = 5'-U CGA AGU ACU CAG CGU AAG ***TT***-3'
siPLK1: sense strand = 5'-AGA **U**CA CCC **U**CC U**U**A AA**U** A UU-3'; antisense strand = 5'-UAU UUA A**G**G AGG GUG A**U**C UUU-3' (in bold blue: 2'-OMethylated modified nucleotides).
The control siRNA was obtained from Qiagen (AllStars Negative Control siRNA).

Cell culture

A549-Luc cells that stably express the reporter gene luciferase were established by Dr. J.-L. Coll's group (Université de Grenoble), by transfecting the human lung carcinoma cells A549 (CCL-185; American Type Culture Collection) with the pGL3-Luc plasmid (Clontech). The cells are grown in RPMI 1640 medium supplemented with 10% FBS, 1% antibiotic solution (penicillin-streptomycin, Gibco) and maintained under 800 µg/mL of G418 selection antibiotic (Promega) at 37°C in a 5% CO_2 humidified atmosphere. The human colon cancer cells HCT116 were cultured using RPMI 1640 medium supplemented with 10% FBS and 1% antibiotic solution (penicillin-streptomycin).

Delivery of the siRNA targeting luciferase

Twenty-four hours prior to transfection, 2.5×10^4 A549-Luc cells/well were seeded in 48-well culture plates. The desired amounts of siRNA duplexes (targeting luciferase (siLuc) or control (siCtrl)) and foldamer were each diluted in 20 µL of NaCl 150 mM. The samples were then mixed and incubated 20-30 min at room temperature to favor association between siRNA and the oligourea. The mixture was finally diluted with serum-free medium to a final volume of 0.5 mL (for duplicates).

For transfection, culture media was removed and replaced by 0.25 mL/well of serum-free RPMI containing the siRNA complexes. After incubation for 2.5 h at 37°C the medium was replaced with fresh one containing serum and luciferase activity in the cell lysate was measured 2 days after transfection. Each experiment was carried out several times; within a series experiments were done in duplicates. The foldamer/DOPE/siRNA complexes were generated by mixing the desired amounts of oligourea and DOPE prior to dilution with the siRNA solution. Transfection experiments in the presence of serum were realized using the same protocol as described above except that the complexes were diluted in culture medium containing the desired percent of serum before addition to the cells.

The luciferase activity was measured as previously described[5] Luciferase background was

[5] A. Kichler, C. Leborgne, P.B. Savage, O. Danos, J. Control. Release **2005**, 174-182.

subtracted from each value and the protein content of the transfected cells was measured by Bradford dye-binding using the BioRad protein assay. The transfection efficiency is expressed as light units/s/mg protein and the values are the means of the duplicates. Error bars represent the standard deviation of the mean.

Silencing of Polo-like kinase 1

The experiments with siPLK1 were done in triplicates with a final concentration of siRNA of 50 nM/well. The siRNA and the foldamer were each diluted in 25 µL of NaCl 150 mM. The samples were then mixed and incubated 20-30 min at room temperature. The mixture was diluted with culture medium containing 10% of serum to a final volume of 0.75 mL (for triplicates). For transfection, culture media was removed and replaced by 0.25 mL/well of RPMI containing the siRNA complexes. After incubation for 2.5 h at 37°C the culture medium was replaced with fresh one. After 2 days, the cell viability in each well was determined using a MTS assay.

Cell viability assay

The cell viability was determined on A549-Luc cells by performing the MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) (CellTiter 96® Aqueous One Solution Cell Proliferation Assay from Promega). This assay was performed using biological triplicates (same amount of foldamer/siRNA were added to 3 different wells). After 48h of incubation, the culture medium was removed and replaced with 300 µL/well of RPMI 10% serum containing the MTS reagent (50 µL/well). After incubation at 37°C for 1h10, 50 µL of medium were withdrawn, then 150 µL of culture medium 10% serum were added to these samples and the absorbance at 490 nm (and 680 nm for background) was measured. Untreated cells were used as control (= 100% of cell viability).

Size analysis by dynamic light scattering (DLS)

The mean size of the 7.5 µg **3**/2.5 µg DOPE/0.33 µg siRNA formulation in 15 mM NaCl was determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments).

Flow cytometric quantification of cell binding/endocytosis

75,000 CHO-K1 and pgsA745 cells/well and 40,000 A745-Luc cells/well were plated into 48-well plates. The day after, the assay was performed using 50 nM of the fluorescent siRNA (siLuc labelled with cyanine 5). The siRNA was diluted in NaCl 150 mM and mixed or not with the **3**/DOPE formulation. After 20 minutes of incubation at room temperature, culture medium was added and the mixture was put onto the cells. After 4 h of incubation at 37 °C, cells were washed and analyzed by flow cytometry (Becton Dickinson FACSCalibur flow cytometer). Non-treated cells were used as control.

Confocal microscopy

30,000 A745-Luc cells were plated into a 8-well chamber Lab-Tek slide. The next day, cells were transfected using 3.75 μg **3**/1.25 μg DOPE/165 ng siRNA-cyanine5/well. After 2h30 of incubation in serum-free medium, transfection medium was removed and replaced with complete medium. After another 1h30, cells were washed with PBS, fixed with 4% paraformaldehyde and treated with DAPI (4',6-diamidino-2-phenylindole) for nuclear staining. The cells were then imaged using a Leica DMI 4000 microscope and a X63 objective.

Annexin V/Propidium iodide assay

Cell death and apoptosis were investigated by double staining with Annexin V, a protein which binds to phosphatidylserine, an inner leaflet membrane phospholipid which is externalized during apoptosis, and propidium iodide (PI), a membrane impermeable DNA intercalating agent that enters cells which have lost membrane integrity, and fluoresces only when bound to nucleic acids. Following treatment, the cells were recovered via trypsinisation, and washed in Annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.3- 7.4). Triplicates were pooled and stained with APC-conjugated Annexin V (eBioscience) diluted 1/100 with prepared Annexin V binding buffer which was incubated for 15 min at room temperature sheltered from light. Cells were washed with Annexin V binding buffer and transferred to tubes containing 15 μM propidium iodide (Sigma-Aldrich), which were analyzed immediately via flow cytometry.

Transfection assays

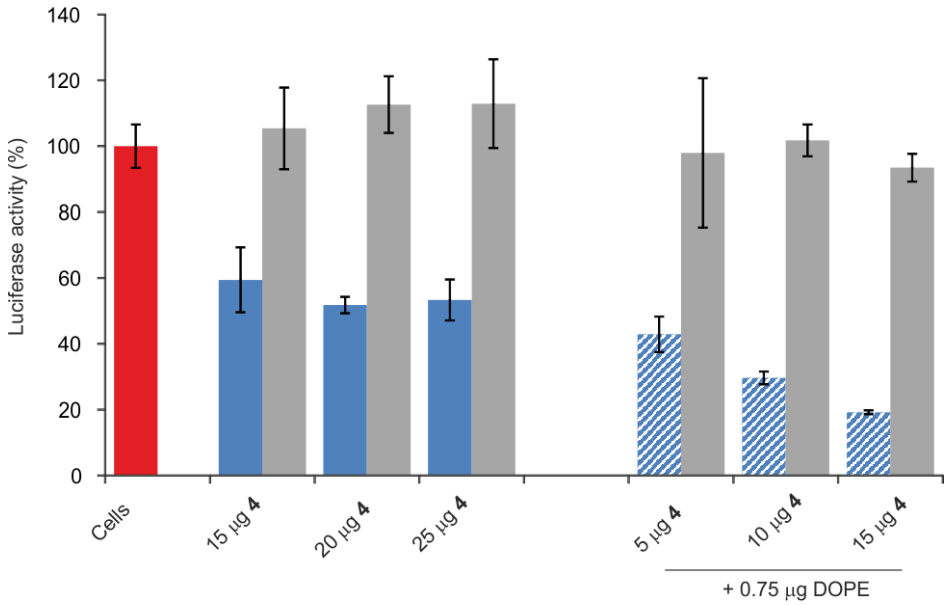


Figure S3. Evaluation of the siRNA delivery properties of the homodimer 4. A549-Luc cells were transfected with dimer **4** mixed or not with helper lipid DOPE using siRNAs at 50 nM. Luciferase activity was measured after 48h and reported relative to untreated cells. Data represent means and standard deviation (SD) of duplicate. Luciferase reporter gene expression after delivery of siLuc (dark blue bars) is compared to untreated cells (orange bar) and cells transfected with control siRNA (light grey bars). The reported mass (in µg) of oligourea and lipid DOPE are the amounts/duplicate.

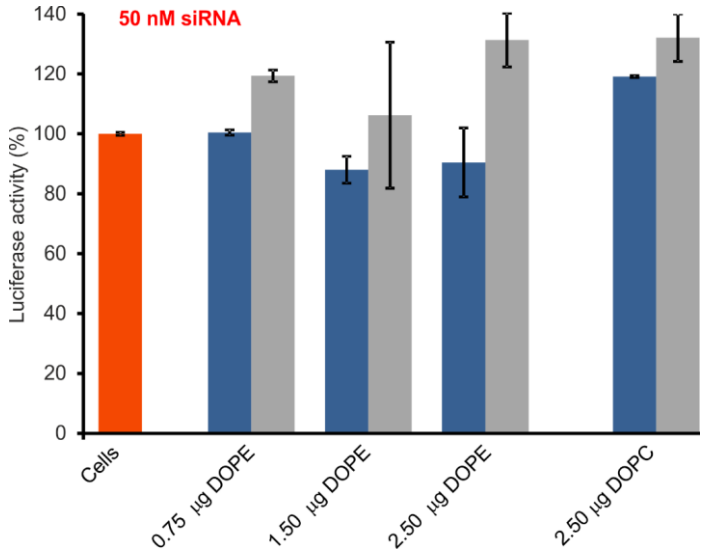


Figure S4. Evaluation of the siRNA delivery capabilities of the zwitterionic lipids DOPE and DOPC in the absence of oligourea. A549-Luc cells were transfected with siRNAs used at 50 nM and luciferase activity was measured after 48h and reported relative to untreated cells. Data represent means and standard deviation (SD) of duplicate. Luciferase reporter gene expression after delivery of siLuc (dark blue bars) is compared to untreated cells (orange bar) and cells transfected with control siRNA (light grey bars). The indicated mass (in µg) of lipid used in the assay is the amount/duplicate.

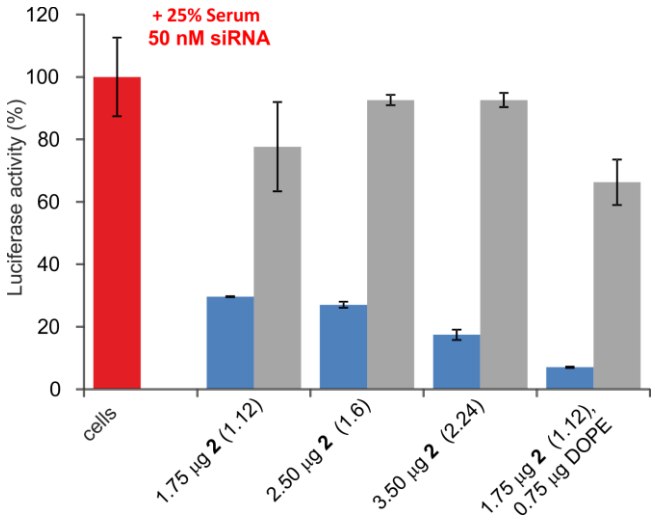


Figure S5. **Influence of 25% serum on the transfection activity of oligourea 2.** A549-Luc cells were transfected in the presence of 25% of serum with compound **2** mixed or not with helper lipid DOPE using siRNAs at 50 nM. Luciferase activity was measured after 48h and reported relative to untreated cells. Data represent means and standard deviation (SD) of duplicate. Luciferase reporter gene expression after delivery of siLuc (dark blue bars) is compared to untreated cells (orange bar) and cells transfected with control siRNA (light grey bars). The reported mass (in µg) of oligourea and lipid DOPE are the amounts/duplicate.

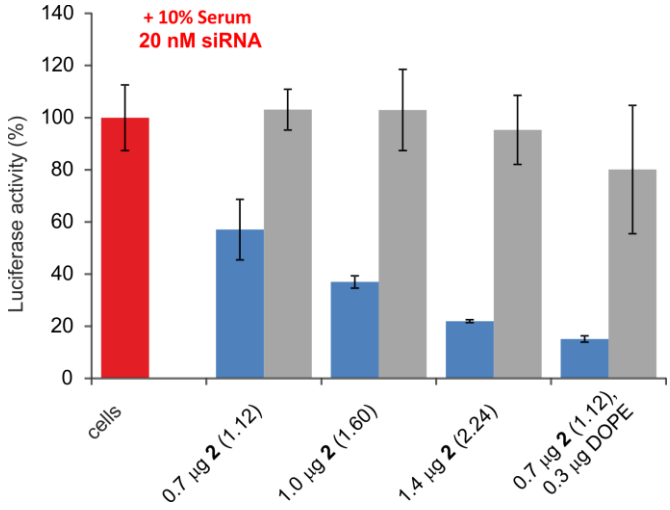


Figure S6. Evaluation of the transfection activity of oligourea 2 using reduced amounts of siRNAs. A549-Luc cells were transfected in the presence of 10% of serum with compound **2** mixed or not with helper lipid DOPE using siRNAs at 20 nM. Luciferase activity was measured after 48h and reported relative to untreated cells. Data represent means and standard deviation (SD) of duplicate. Luciferase reporter gene expression after delivery of siLuc (dark blue bars) is compared to untreated cells (orange bar) and cells transfected with control siRNA (light grey bars). The reported mass (in µg) of oligourea and lipid DOPE are the amounts/duplicate.

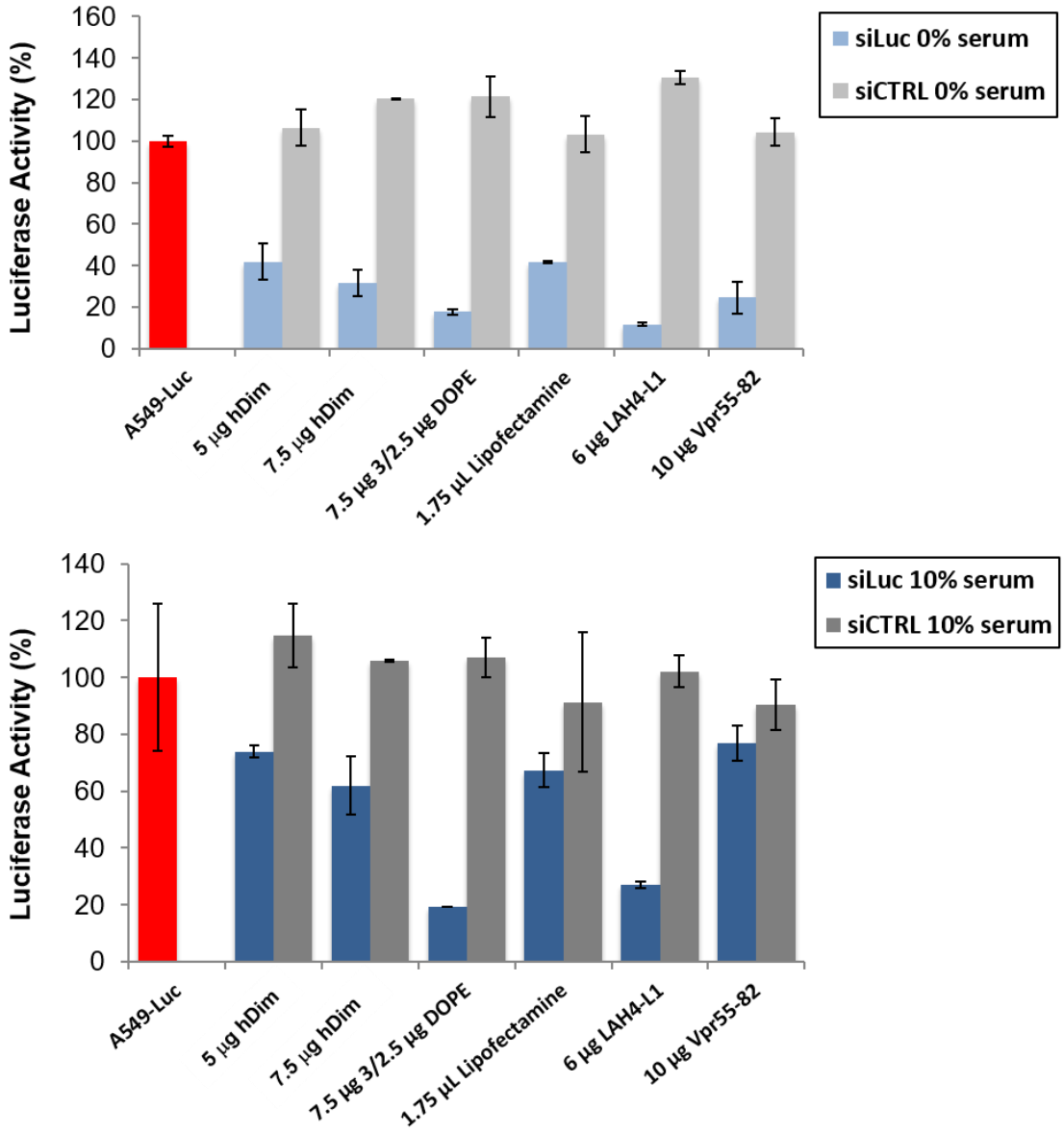


Figure S7. Comparison of the transfection efficiency of formulation **3**/DOPE with other delivery systems. Luciferase gene silencing was determined using A549-Luc cells plated in 48-well plates. The final siRNA concentration was 50 nM (=330 ng siRNA/duplicate) and luciferase activity was measured after 48h and reported relative to untreated cells. Data represent means and standard deviation (SD) of the duplicates. Luciferase reporter gene expression after delivery of siLuc (blue bars) is compared to untreated cells (orange bar) and cells transfected with control siRNA (grey bars). The amounts of vector that were used are given for a duplicate.

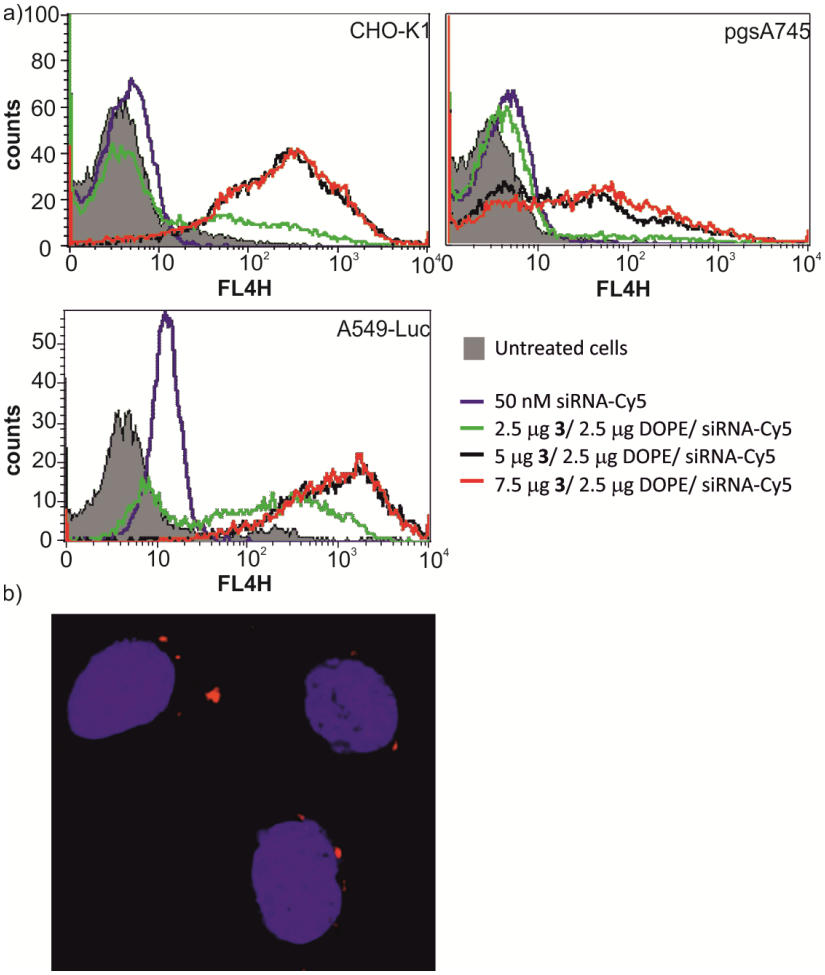


Figure S8. a) Flow cytometry analysis of CHO-K1, pgsA745 and A549-Luc cells treated with a fluorescent siRNA (siRNA-Cy5). The assay was performed using a final concentration of 50 nM siRNA (= 330 ng siRNA per duplicate; 48-well plates). The siRNA-Cy5 was used alone or mixed with different **3**/DOPE formulations. After 4h of incubation at 37°C, cells were washed once with cold PBS, detached with a Trypsin/PBS mixture, and subsequently analysed by flow cytometry. Non-treated cells were used as control (grey area). b) Confocal microscopy of A549-Luc cells transfected for 4 hours with 3.75 µg 3/1.25 µg DOPE/165 ng siRNA-Cy5/well. DAPI (blue stain) was used for nuclear staining. Images were taken using a Leica DMI 4000 microscope with a × 63 objective.

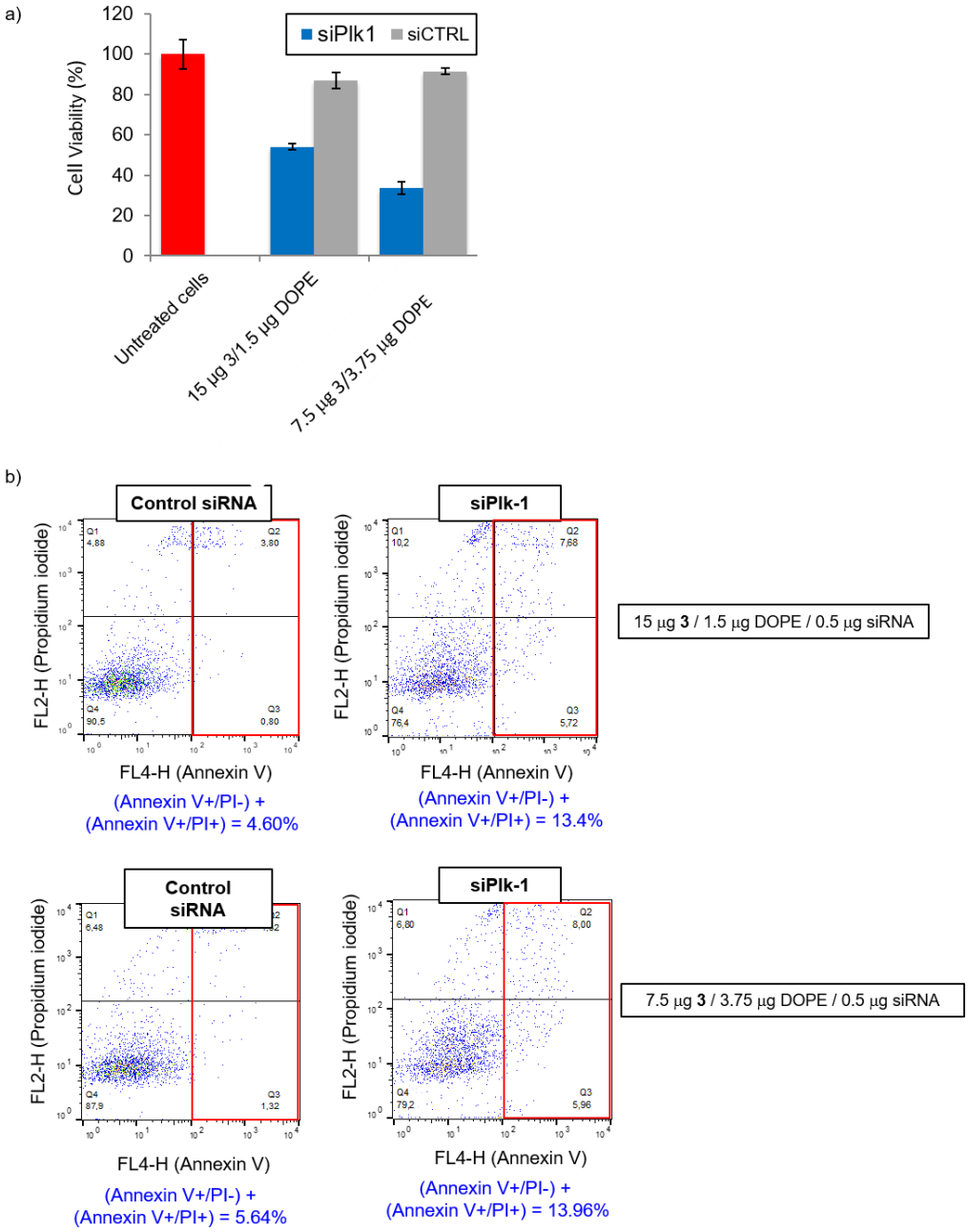


Figure S9. Two 48-well plates with A549-Luc cells were transfected in culture medium containing 10% serum with CPF **3**/DOPE using a final siRNA concentration of 50 nM (=500 ng siRNA for a triplicate ; 48-well plates). After 2h40, transfection medium was replaced with fresh one. After 72h, one plate was used to determine cell viability with the MTS assay (a) while the second one was used to determine apoptosis (b). Therefore, annexin V (AnV)/Propidium iodide (PI) staining was quantified using flow cytometry. These plots can be divided in four regions corresponding to: 1) viable cells which are negative to both probes (Annexin V/PI -/-; Q4); 2) apoptotic cells which are PI negative and Annexin positive (Q3); 3) late apoptotic cells which are PI and Annexin positive (Q2); 4) necrotic cells which are PI positive and Annexin negative (Q1).

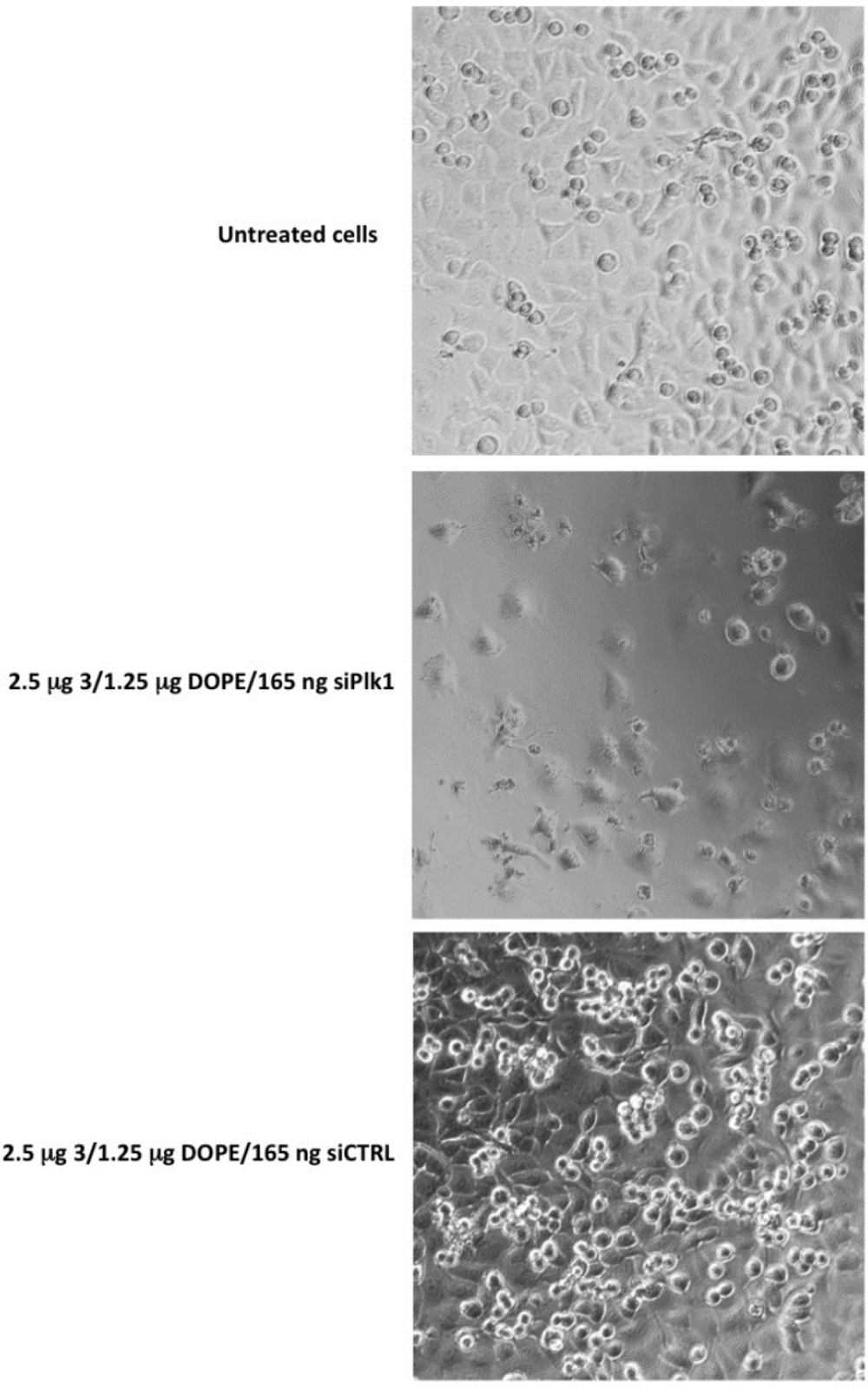


Figure S10. Microscopy pictures of untreated A549-Luc cells and cells incubated with CPF **3** complexed with 50 nM of either siPlk1 or control siRNA. The experiment was done in 96-well plates using triplicates. After 2h15 of incubation, the transfection medium was replaced with fresh one and the experiment was allowed to proceed for 72 hours at 37°C. Photographs were taken using a ZEISS Axio VERT.A1 microscope with a x10 objective.

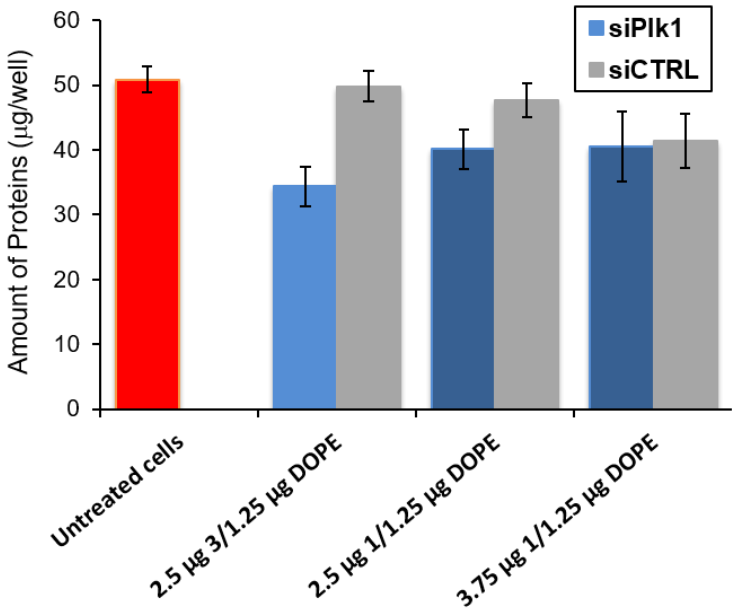


Figure S11. Transfection of A549-Luc cells with CPF **1** and **3** using 50 nM of either siPlk1 or siCTRL (=165 ng siRNA for a triplicate ; 96-well plates). The cells were transfected in serum-free medium for 2h15 and then medium was replaced with fresh one containing serum. The cells were incubated for 72 hours at 37°C and then the MTS assay was done (see figure 4b). Next, medium was removed and cells were lysed using the luciferase lysis buffer. The protein content of the transfected cells was measured by Bradford dye-binding using the BioRad protein assay. Data represent means and standard deviation (SD) of triplicate.

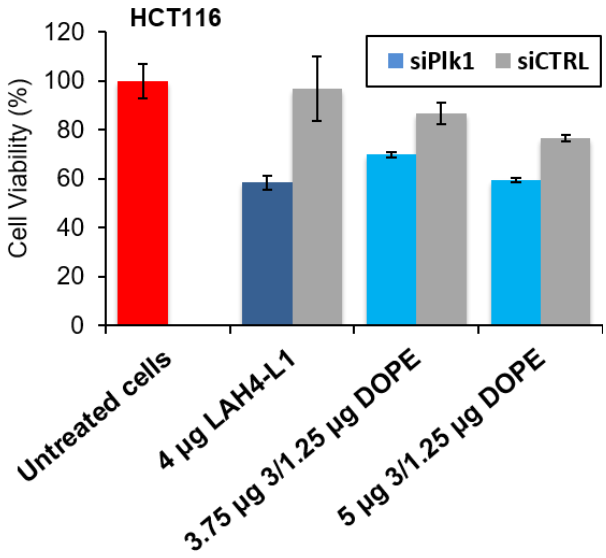


Figure S12. Transfection of HCT116 cells with CPF **3** and the cationic peptide LAH4-L1 using 50 nM of either siPlk1 or siCTRL (=165 ng siRNA for a triplicate ; 96-well plates). The cells were transfected in serum free medium for 2h30 and then medium was replaced with fresh one containing serum. The cells were incubated for 72 hours at 37°C and then the MTS assay was done. Data represent means and standard deviation (SD) of triplicates.