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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*}

Cell-penetrating foldamers (CPFs) have recently shown promises as efficient and safe nucleic acid delivery systems. However applications of CPFs to siRNA transport remain scarce. Here, we report helical CPFs tailored with specific end-groups (pyridylthio- or *n*-octyl-ureas) as effective molecular systems in combination with helper lipids to intracellularly deliver biologically-relevant siRNA.

Since its joint discovery by A. Fire and C. C. Mello, RNA interference (RNAi) has generated a tremendous interest for curing various diseases including cancer.¹ RNAi mechanism relies on non-coding double-stranded small-interfering RNAs (siRNAs) composed of 21–25 nucleotides that once incorporated into RISC (RNA-induced silencing complex) specifically assemble with a complementary messenger RNA (mRNA) sequence, this in turn leads to mRNA degradation and subsequently to the silencing of the targeted gene.² To be effective this mechanism requires the successful delivery of siRNA molecules into the cytosol. Remarkably, just twenty years after this discovery, several siRNA therapeutics are now in clinical trials with one siRNA-based strategy recently approved by the FDA.³

However, due to their chemical nature siRNAs are highly sensitive to circulating serum RNases and cannot spontaneously diffuse through the cell membrane - although modifications of siRNA backbone can extend their pharmacological activity.⁴ Accordingly, there is still an urgent need to develop efficient and chemically-defined transfection agents to safely deliver these molecules within the cells. Over the last decades, a broad variety of non-viral gene delivery agents has been developed ranging from lipids⁵ to cationic polymers⁶ and dendrimers^{7,8}, cell-penetrating peptides (CPPs)⁹, foldamers¹⁰ and physical methods.¹¹

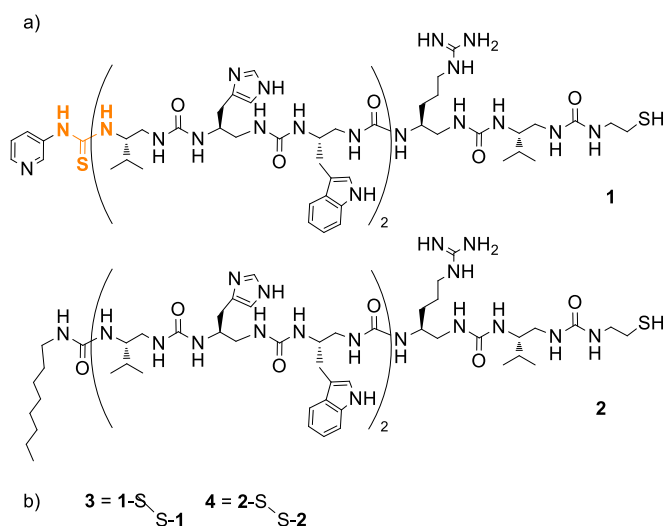


Fig. 1 Urea-based CPFs terminated either a) a pyridylthiourea moiety (1) or octyl urea (2) and b) related dimers 3 and 4.

Unanticipated, it was early demonstrated that a molecular transporter originally designed for gene transfection purposes could not be systematically employed to transport siRNAs and some showed only modest delivery capabilities. As an example, even though the branched 25 kDa-polyethylenimine (PEI) is one of the most efficient DNA transfection agents¹², it is far less efficient for siRNA delivery.¹³ One reason lies in the fact that siRNA molecules adopt a stiffer structure (rod-like structure) as compared to DNA¹⁴ which restrains the capacity of the synthetic carrier to assemble with the siRNAs and vehicle them to the cytoplasm.

Recently, we have designed pH-responsive medium-size cell-penetrating foldamers (CPFs) and related hybrids exhibiting high DNA delivery capabilities.¹⁵⁻¹⁷ Preliminary siRNA transfection investigations performed with model CPF homo dimer (**hDim**, Fig. S1) showed encouraging results in terms of gene silencing. However, when moving to more physiological conditions, *i.e.* in the presence of serum, the siRNA transfection efficiency of **hDim** considerably dropped down (Fig. S7). These first results are consistent with our recent report on **hDim** stability in serum-rich medium.¹⁶ Consequently, we sought to develop new CPFs specialized for siRNA transfection by tailoring the termini of oligourea helical foldamers. We assumed that by modifying the positive end of the helix oligourea macrodipole we could strengthen siRNA compaction and assembly properties, and by doing so, reinforce their stability in biological fluids. It is indeed now established that the positive summit of helix oligourea readily binds anions^{18,19} and therefore could be a site for siRNA binding. To this end, we considered the grafting of a pyridylthiourea (π) group, previously implemented on PEIs to enhance siRNA/PEI polyplex stability.^{20,21} The pyridylthiourea moiety may serve as a

binding recognition moiety of phosphate anions in water.²² Comparatively, the second approach concerned the attachment of a hydrocarbon tail as it has been well-validated on CPPs for siRNA delivery.²³⁻²⁸

Herein, we thus report amphiphilic oligourea foldamer modified with either a N-pyridylthiourea or an octylurea moiety at its N-terminus (compounds **1** and **2** respectively, Fig. 1a) and show that they display siRNA transfection. Additionally, a cysteamine terminus was introduced at the opposite extremity of oligoureas **1** and **2** as a chemical tool to study the consequence of disulfide homo-dimerization (compound **3** and **4** in Fig. 1b) on siRNA transport efficiency. Inclusion of a fusogenic lipid to the siRNA/CPF foldaplexes was also evaluated as a mean to further enhance the delivery properties.

We previously developed a general and robust solid phase synthesis approach to access cysteamine-terminated oligoureas starting from a cystamine-type resin and using Boc chemistry under microwave assistance.¹⁶ These conditions were applied to the synthesis of compounds **1** and **2** which were recovered in high purity and relatively good yield after semi-preparative RP-HPLC purification (see ESI for details). Their 2.5-helical conformation was next assessed by performing far-UV circular dichroism (CD) in aqueous conditions. As expected, compounds **1** and **2** showed the characteristic CD spectra attributed to helically folded oligoureas with a positive maximum of molar ellipticity ($[\Theta]$) around 203 nm. To note, these two compounds exhibited a higher 2.5-helical content in water environment than reference CPF **5** (see ESI for details)¹⁵ with an increase of $[\Theta]_{203}$ of 2.4- and 2.3-fold for **1** and **2** respectively (see Fig. S2 for details). These results indicate that both terminations, introduced at the positive end of the helix dipole, actively participate to the overall 2.5-helix stability in water.

We next investigated the capacity of CPF **1** and **2** to specifically transport siRNA within the cytoplasm and silence protein expression. A549 cell line (human lung carcinoma), which stably expresses the firefly pGL3 luciferase gene, was selected as a model and silencing by siRNA was quantified by a standard luciferase expression assay. Treatment with a control siRNA (siCtrl) which should maintain luciferase expression close to 100% unless toxicity of the CPF/siCtrl assembly occurred. The foldaplexes were generated by mixing increasing amounts of CPF with a constant amount of siRNA.

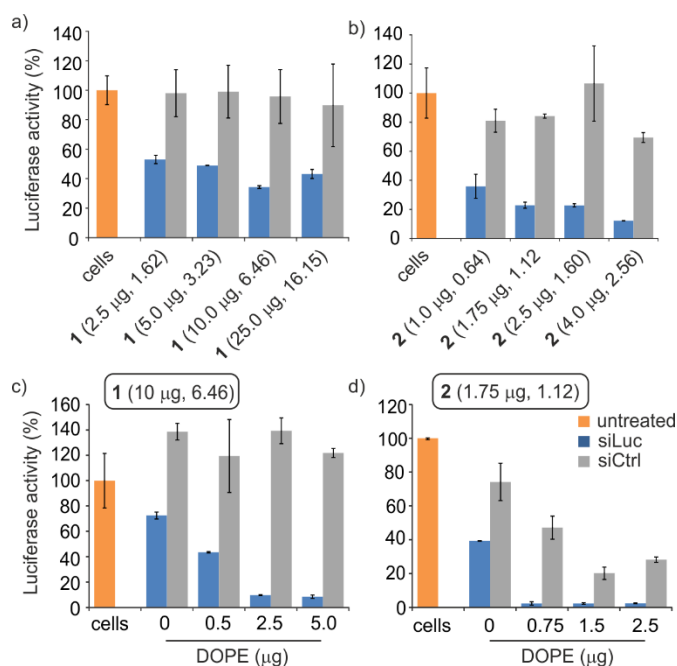


Fig. 2. siRNA delivery activity: Luciferase gene silencing by siRNA/CPF **1** and **2** foldaplexes in A549 cell line. The final siRNA concentration was 50 nM and luciferase activity was measured after 48h and reported relative to untreated cells. Data represent means and standard deviation (SD) of duplicate. Luciferase reporter gene expression after delivery of siLuc (blue bars) is compared to untreated cells (orange bar) and cells transfected with control siRNA (light grey bars). In brackets are successively reported the mass (μ g for a duplicate) of a) CPF **1** and b) **2** and the oligomer/siRNA charge ratio (N/P) used in the assay. c) and d) The luciferase activity was recorded 48 hours after incubation of the cells with siRNA/CPF formulations containing increasing amounts of the fusogenic lipid DOPE (μ g/duplicate).

As depicted in Fig. 2a, CPF **1** exhibited a good siRNA transfection efficiency with a silencing of luciferase expression up to 60% when **1** was complexed with siRNA at a charge ratio (N/P) of ca. 6.46. Above this N/P value some cytotoxicity was observed. Comparatively, CPF **2** equipped with an octyl termination showed a better siRNA delivery capacity with a loss of luciferase activity greater than 85% at N/P = 2.56 (Fig. 2b). However, the siRNA transfection activity of **2** was always accompanied with substantial cytotoxicity observable from N/P = 0.64 with a loss of \approx 20% of cell viability in the presence of the control siRNA. Consequently, we decided to seek for conditions that could not only improve the transfection properties of **1** and **2** but reduce as much as possible the observed cytotoxicity of the resulting CPF/siRNA assemblies.

In the context of gene delivery we and others have previously reported the suitability of the fusogenic lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) to enhance the transfection efficiency of DNA/vector assemblies.^{24,29,30} We thus tested

siRNA foldplexes either composed of **1** or **2** formulated with increasing amounts of DOPE. As depicted in Fig. 2c, increasing addition of the lipid to CPF **1**/siRNA formulation at a constant N/P ratio of 6.46, resulted in drastic decrease of luciferase activity (*i.e.* below 10% when 2.50 μg of DOPE) without apparent sign of cell toxicity. In contrast, the presence of DOPE did not alleviate the apparent cytotoxicity of the **2**/siCtrl complex even though the N/P ratio for this formulation was only a fifth of that of **1**/siCtrl.

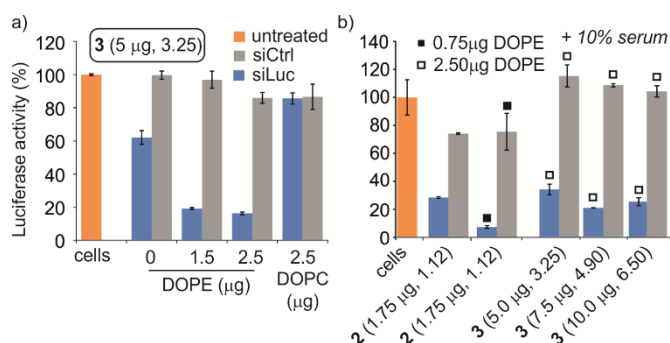


Fig. 3 a) The efficiency of dimer **3** (5 μg /duplicate) to deliver siRNA (50 nM final concentration) within A549 cells was monitored by measuring the luciferase activity with siCtrl (light grey) or siLuc (blue). Dimer **3** was associated with 0, 1.5 or 2.5 μg of DOPE, respectively or with 2.5 μg of DOPC. **b)** The siRNA delivery activity of CPF **2** and **3** was recorded in 10% serum-enriched media with either 0.75 (filled black square) or 2.5 μg (unfilled black star) of DOPE. The same conditions to those described in Fig. 2 were applied. In brackets are reported the mass (μg for a duplicate) of CPF and the N/P used in the assay, respectively.

In a second round of investigation, we applied the same strategy previously reported for DNA transfection¹⁵ by carrying out the dimerization of **1** and **2** via thiol oxidation and studying the consequence of doubling their size on gene silencing. Dimer **3** and **4** were thus prepared (Fig. 1 and see ESI for details) and studied alone and with DOPE. As additional control experiments we measured the level of luciferase expression of the DOPE/siRNA formulation and we also varied the nature of the lipid component by preparing a formulation with the non-fusogenic neutral lipid 1,2-dioleoyl-sn-glycero-3-phospho-choline (DOPC). As reported in the Fig. 3a, the addition of 1.50 μg of DOPE to the **3**/siRNA formulation (N/P ratio of 3.25) led to a pronounced reduction (ca 80%) of the level of luciferase expression. Dimer **4** also displayed a silencing activity although relatively high amounts of vector were required with respects to monomer **2**. The efficiency of **4** was further increased in combination with DOPE (Fig. S3). Comparatively, the substitution of DOPE by DOPC had a negative contribution on the cytosolic transport of siRNA by **3**. Whereas the use of **3**/siRNA alone leads to a modest reduction of luciferase activity (60%), formulation in the presence of DOPC completely abrogated the effect (86% luciferase activity remaining). This result clearly shows that it is the fusogenic nature of the associated lipid (*i.e.* DOPE) that matters for increasing the efficiency of the foldplexes.^{29,30} Of note, DOPE alone was unable to deliver siLuc within the cells thus emphasizing the synergistic effect played by this lipid during the transfection of siRNA by CPF **3** and **1** (Fig. S4). At this stage we decided to concentrate our efforts on dimer **3** as it appeared to give the most promising results.

We next tested the efficiency of the CPF/siRNA foldplexes in serum-containing media. In addition to dimer **3**, CPF **2** was included in this assay to evaluate whether the cytotoxicity of **2**/siCtrl assemblies could not partly result from some CPF **2** remaining free in solution after complexation. Hence, one could expect the cytotoxicity of **2** to decrease upon that interaction of the free form of **2** with serum proteins. As depicted in Fig. 3b, foldplexes formulated with DOPE retained high siRNA delivery activity in the presence of 10% serum in the culture media although slightly higher N/P ratio were generally necessary. At an N/P ratio of 3.25, the activity **3**/siRNA foldplexes was reduced (two-fold increase in luciferase expression) by the addition of serum but the luciferase silencing activity reached 80% when N/P = 4.90. Concurrently, when **2** was used with DOPE in a serum-enriched medium, the cell viability was in part restored (75% of luciferase production in the presence of siCtrl) with a silencing of 92% for an N/P ratio of 1.12 (Fig. 3b). This trend was confirmed by (1) using a culture medium containing up to 25% of serum (Fig. S5) and (2) reducing the final concentration of siRNA from 50 to 20nM (Fig. S6).

Additionally, the siRNA delivery capability of **3** was compared to three other formulations. The CPF-based formulation displayed comparable or even higher siRNA transfection capacity than Lipofectamine and was as efficient as the CPPs LAH4-L1³¹⁻³² and Vpr55-82^{33,34}. In serum enriched medium, among the three transfection agents tested, only LAH4-L1 could perform as well as **3**/DOPE (Fig. S7).

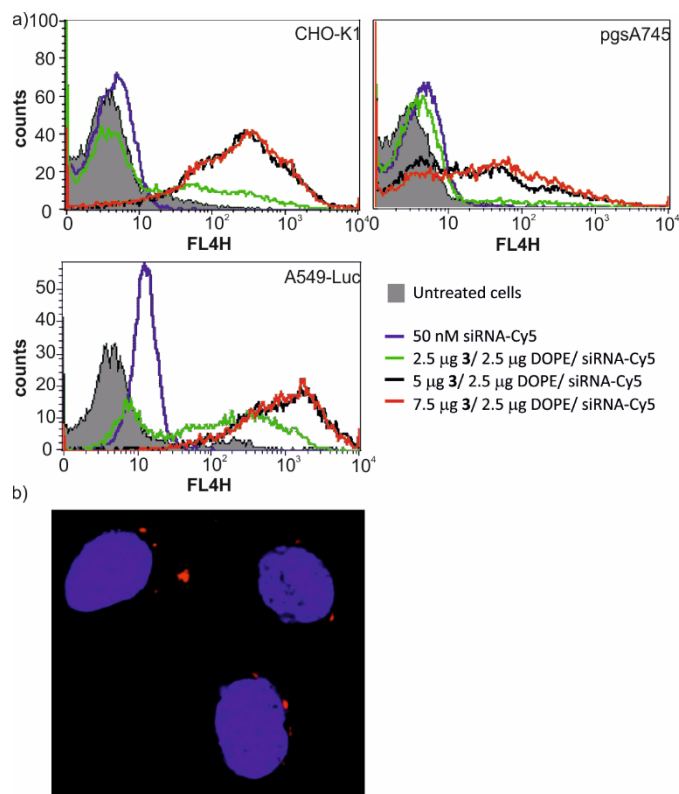


Fig. 4. a) Flow cytometry analysis of CHO-K1, pgsA745 and A549-Luc cells treated with a fluorescent siRNA (siRNA-Cy5). The assay was performed using a final concentration of 50 nM siRNA (= 330 ng siRNA per duplicate; 48-well plates). The siRNA-Cy5 was used alone or mixed with different **3**/DOPE formulations. After 4h of incubation at 37°C, cells were washed once with cold PBS, detached with a Trypsin/PBS mixture, and subsequently analyzed 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 $\times 63$ objective.

To gain additional information about these tri-partner complexes, we measured the size of **3**/DOPE/siRNA (7.5, 2.5, 0.33; $\mu\text{g}/\mu\text{g}/\mu\text{g}$) and found a diameter of 608 ± 83.5 nm (Fig. S8). Subsequently, the cell binding/uptake of these foldamer/lipid/siRNA assemblies was monitored by using a fluorescent siRNA (siRNA-Cy5). As cell surface proteoglycans (PGs) have been shown to promote binding/uptake of polycation/DNA complexes,³⁵ we asked whether it is also the case for siRNA-Cy5 foldplexes. Therefore, we made use of the xylosyltransferase-deficient Chinese hamster ovary (CHO) cell line pgsA745³⁶ which does neither produce chondroitin nor heparan-sulfated PGs. Binding/endocytosis was compared to wild-type CHO K1 cells. The results showed that: *i*) the cell surface binding is dose-dependent; *ii*) the binding reaches saturation; *iii*) the binding of **3**/DOPE/siRNA-Cy5 to the proteoglycan-deficient A745 cells is less efficient with respect to wild-type CHO cells (Fig. 4a). This result unveils that the interaction of the CPF/lipid/siRNA formulation to the cell surface occurs, in part, through binding to the PGs. We also measured binding/endocytosis to A549-Luc cells and found that it was very efficient (Fig. 4a). The cell uptake and trafficking of siRNA mediated by CPF **3** was also investigated on A549-Luc cells using confocal microscopy. The internalized fluorescence was shown to localize near the nucleus, probably in vesicular compartments in particular endosomes/lysosomes (Fig. 4b). These results are consistent with previous studies showing that only a very minor fraction of siRNAs is released from endosomes and this siRNA fraction remains undetectable by standard fluorescence microscopy methods.³⁷

To further examine the gene silencing property of our urea-based CPFs, we envisioned the cell delivery of a therapeutically-relevant siRNA. Polo-like kinase 1 (Plk1) plays a critical regulatory role in cell cycle progression, and is overexpressed in a broad range of tumors.³⁸ Its biological function consists in the inhibition of the pro-apoptotic tumor suppressor P53.³⁹ Silencing of Plk1 was shown to induce apoptosis in tumor cells.⁴⁰

We hence used a siRNA anti-plk1 (siPlk1) and monitored its activity after intracellular delivery by measuring the cell viability (MTS-based cell proliferation assay) on A549-luc cell line (see ESI for details and legend of Fig. 5). The same conditions of CPF/siRNA formulation were applied in 10% serum-containing culture medium with a final concentration of siPlk1 of 50 nM. The propensity of our CPFs to deliver siPlk1 was first correlated to the cell viability after 48 hours of transfection and compared to that recorded with the siCtrl. Fig. 5a shows that when formulated with DOPE, dimer **3** managed to effectively deliver siPlk1 with a cell death of $\approx 50\%$ at a charge ratio of $N/P = 4.87$ and this without any apparent cytotoxicity (cell viability of almost 100% with the siCtrl). This tumor cell-killing efficiency is comparable to the one recently reported by Ripoll *et al.* using polydiacetylenic micelles containing a histidine headgroup at a siPlk1 concentration of 100 nM.⁴¹

Comparatively, the formulation composed of octyl-oligourea **2**/siPlk1 showed higher efficacy (up to 68% cell death in the presence of DOPE) but with a slight unspecific toxicity (Fig. 5a) whilst monomer **1** displayed significantly lower activity with respect to **3** (Fig. 5b).

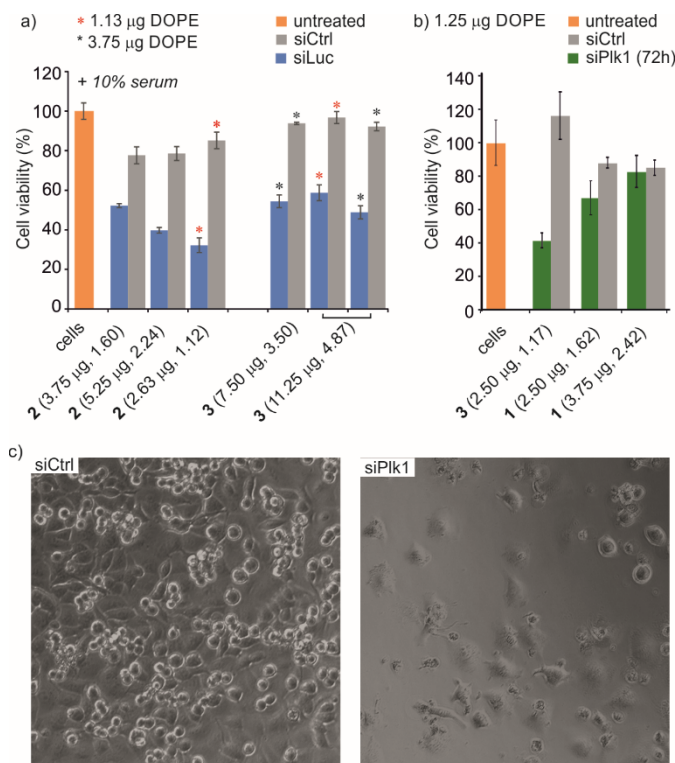


Fig 5. a) Cell viability after anti Plk1 siRNA delivery performed on A549 cells using formulations of **3** with DOPE at a final siRNA concentration of 50 nM. Data represent means and standard deviation (SD) of triplicate. b) Transfection of A549-Luc cells with CPF **1** and **3** using 50 nM of either 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. Data represent means and standard deviation (SD) of triplicate. c) Before the MTS assay described in b) was realized, photographs using optical microscopy of A549-Luc cells incubated with CPF **3** complexed with either siPlk1 or siCTRL (2.5 µg 3/1.25 µg DOPE) were taken (ZEISS Axio VERT.A1, X-Cite 120LED ProgRes C5 camera module, x10 objective).

Interestingly, when the MTS cell viability assay was done 3 days after transfection instead of 48h, the percentage of remaining cells was further decreased compared to untreated cells, independently whether serum was present or not (cell viability \leq 40%, Fig. 5b and S9a). Concurrently, we observed the cell proliferation and cell morphology using optical microscopy. In good agreement with the cell viability assay, we noticed a diminished cell proliferation and a change in cell shape when siPlk1 was formulated with **3** while both parameters remained unchanged after incubation with siCtrl when compared to untreated cells (Fig. 5c and Fig. S10). Moreover, the quantification of the amount of proteins per well at the end of the transfection experiment reflects well the cell viability assay results (Fig. S11). In parallel, to investigate the mechanism of cell death induced by the siPlk1 complexes, we repeated an MTS assay using CPF **3** (Figure S9a) and we then measured whether the cells entered apoptosis following 72h treatment by quantifying annexin V/propidium iodide staining. The results show that suppression of PIK1 induced more apoptosis than the formulation containing the control siRNA (Figure S9b). Lastly, we compared the silencing efficiency of CPF **3** to that of peptide LAH4-L1 when using siPlk1 and siCTRL on the human tumor cell line HCT116 (colorectal carcinoma). While CPF **3** was able to reduce tumor cell growth, it was less efficient than the LAH4-L1 peptide (Fig. S12).

Conclusions

We have developed effective urea-based CPFs for siRNA transfection. Our approach is based on the installation of specific chemical moieties at the N-terminus of a medium-size (8-mer) amphiphilic oligourea and on optimal co-formulation. The best siRNA/CPF formulations efficiently silenced a reporter gene in the presence of serum. Encouragingly, we were able to induce significant tumour cell killing by using a siRNA targeting polo-like kinase 1. These results pave the way for the development of urea-based foldamers as vehicle for siRNAs and potentially for other therapeutically relevant nucleic acids such as exon skipping oligonucleotides or CRISPR/Cas9 gene-editing elements.

Conflicts of interest

There are no conflicts to declare.

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