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Lipoplex and peptide-based strategies for the delivery of steric-block oligonucleotides

Sarah Resina [a], Saïd Abes [a], John J. Turner [b], Paul Prevot [a], Adrian Travo [a], Philippe Clair [a], Michael J. Gait [b], Alain R. Thierry [a], Bernard Lebleu [a,*]

[a] *Laboratoire de Dynamique des Interactions Membranaires Normales et Pathologiques, Département de Défenses Antivirales et Antitumorales, UMR 5235-CC086, Université Montpellier II, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France*
[b] *Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK*

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Abstract

Synthetic oligonucleotides offer interesting prospects for the control of gene expression but clinical applications have been severely limited by their poor bioavailability.

Cationic lipids have been widely used for the delivery of charged oligonucleotide (ON) analogues but most of the commercial formulations are toxic and poorly stable in the presence of serum proteins. We have developed a DOGS/DOPE liposome formulation named DLS (for delivery liposomal system), that allows for the efficient nuclear delivery of negatively charged antisense ON analogues as monitored by fluorescence microscopy and by their ability to correct deficient pre-mRNA splicing, even in serum-supplemented cell culture.

Uncharged DNA mimics such as peptide nucleic acids (PNA), or phosphorodiamidate morpholino (PMO) ON are particularly interesting for their high metabolic stability and affinity for complementary RNA targets but they cannot be delivered with cationic lipids. Cell penetrating peptides (CPP), such as Tat or penetratin, have been used widely as conjugates for the delivery of various biomolecules and might be appropriate for neutral ON analogues. However, entrapment within endocytic vesicles severely limits the efficiency of PNA delivery by CPPs in the absence of endosomolytic drugs, such as chloroquine. The conjugation of new arginine-rich CPPs to PNA allows efficient nuclear delivery in the absence of chloroquine as monitored in a splicing correction assay.

Both strategies have their advantages but DLS-mediated delivery remains more efficient than CPP delivery for the nuclear targeting of splice correcting ON analogues *in vitro*.

Keywords: Antisense oligonucleotide; Splice correction; Lipoplex; Cell penetrating peptide

1. Introduction

The majority of human genes undergo alternative splicing by a complex and tightly regulated machinery involving the sequence-specific binding of a vast collection of proteins to nuclear pre-mRNA. Steric interference by transfected RNase-H incompetent oligonucleotides (ON) analogues named steric-block ON or splice switching ON has been proven efficient to re-orient the splicing machinery towards the preferential synthesis of a given mRNA species (Mercatante et al., 2001; Sazani et al., 2002b; Sazani and Kole, 2003). Clinical applications may be envisaged as for instance in the treatment of cancers through alterations of the balance between anti- and pro-apoptotic forms of bcl-X, or in the treatment of Duchenne muscular distrophy through skipping of the mutated exons in dystrophin pre-mRNA (Lu et al., 2005; Kole et al., 2004a,b).

Splice switching requires the sequence-specific binding of nuclease-resistant and high affinity RNase-H incompetent ON analogues to pre-mRNA. Both charged (such as 2′-*O*-methyl or 2′-*O*-methoxyethyl-phosphorothioate ON) or neutral (such as peptide nucleic acids or phosphorodiamidate morpholino oligomers) are well suited as splice switching ON (Abes et al., 2006a,b, 2007; DeLong et al., 1999; Kang et al., 1998; Sazani et al., 2001, 2002a; Thierry et al., 2006; Youngblood et al., 2007).

* Corresponding author. Tel.: +33 4 67 14 92 03; fax: +33 4 67 14 92 01.
E-mail address: bernard.lebleu@univ-montp2.fr (B. Lebleu).

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As for other ON-based strategies for gene regulation, efficient delivery within the appropriate cellular compartment remains a major roadblock (Thierry et al., 2003).

Negatively charged ON analogues are most generally transfected as complexes with cationic lipids. However, cytotoxicity and instability in the presence of seric proteins seriously limit their *in vivo* use. New formulations with a lower positive net charge and high particle size homogeneity as the DLS (delivery liposomal system) liposomes developed by our group have proven efficient for the delivery of nucleic acids in serum-containing medium and even *in vivo* in animal models (Lavigne et al., 2001, 2004; Thierry et al., 1997; Schmutz et al., 1999; Tavitian et al., 2002).

Lipoplexes formulations are not suitable for uncharged ON delivery. Cell penetrating peptides (CPP) have been investigated by several groups. Unfortunately, entrapment within endocytotic vesicles has been a major limitation with most CPPs (Abes et al., 2006a, 2007; Bendifallah et al., 2006; El-Andalousi et al., 2006; Koppelhus et al., 2002; Kaihatsu et al., 2004; Richard et al., 2003; Shiraishi et al., 2005; Turner et al., 2005a).

The present study aimed at comparing the splicing correction efficiency and the intracellular distribution of CPP-conjugated PNAs and lipoplexes-associated 2′-*O*-Me and MOE ON within the same system which has not been done previously. We have made use of the splicing correction assay proposed by Kang et al. (1998), in which the mutated intron 2 of the human β-globin gene has been inserted within the coding sequence of a luciferase reporter gene. The activation of a cryptic splice site in this intron prevents its complete removal unless the mutated site is masked by a steric-block ON analogue.

2. Materials and methods

2.1. Cell line

The HeLa pLuc/705 cell line was stably transfected with the pLuc/705 plasmid, in which the coding region of the luciferase sequence is interrupted by the mutated β-globin intron (Kang et al., 1998). HeLa pLuc/705 cells were grown at 37 °C, 5% CO_2 in minimal essential DMEM medium (Gibco, Invitrogen SARL, Cergy Pontoise, France) supplemented with 10% fetal calf serum (FCS), 1% sodium pyruvate, non-essential amino acids and a mix of penicillin, streptomycin and neomycin (100×, Gibco). Cells were checked routinely for the absence of mycoplasma contamination.

2.2. Antisense oligonucleotides

Antisense ON sequences are listed in Table 1. The 2′-*O*-methyl-oligoribonucleotides (2′-*O*-Me) were purchased from Eurogentec SA (Seraing, Belgium). 2′-*O*-Methoxyethyl-oligoribonucleoside-phosphorothioates (MOE) were a gift from Dr. Ryszard Kole (Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, USA). ON_{705} is the antisense sequence hybridizing to the β-globin intron mutation. SCR_{705} is the scrambled version of ON_{705}. NS_{654} is a nonsense sequence hybridizing on 654 position in the β-globin intron.

2.3. PNA and PNA-CPP conjugates

Peptide conjugates sequences are listed in Table 1.

2.3.1. Synthesis of peptides

(R-Ahx-R)$_4$C with free N-terminus and C-terminal amide was synthesized on a PerSeptive Biosystems Pioneer peptide synthesizer (100 μmol scale) using standard Fmoc/*tert*-butyl solid phase synthesis techniques as C-terminal amide peptides using NovaSyn TGR resin (Novabiochem). Deprotection of all peptides and cleavage from the solid support was achieved by treatment with trifluoroacetic acid (TFA) in the presence of triethylsilane (1%), ethane dithiol (2.5%) and water (2.5%). Purification was carried out by reversed phase HPLC as previously described (Turner et al., 2005b) and analyzed by MALDI-TOF mass spectrometry with the same matrix as for PNA.

2.3.2. Synthesis of PNA and PNA-peptides

N-terminal bromoacetyl-containing PNA_{705} was obtained from Panagene (www.panagen.com). PNA_{705}-4K and PNA_{705} were synthesized as previously described (Abes et al., 2006a).

Stably linked K_8-PNA_{705} and Tat-PNA_{705} conjugates were synthesized by continuous PNA/peptide synthesis as previously described (Turner et al., 2005a). FAM-labelled Tat-PNA_{705} was synthesized by 6-carboxyfluorescein diacetate conjugation to the N-terminus during solid phase Tat-PNA synthesis as previously described (Turner et al., 2005a). To prepare (R-Ahx-

Table 1
Antisense oligomers

Oligomers	Sequences
2′-*O*-Me ON_{705}	CCU CUU ACC UCA GUU ACA
2′-*O*-Me SCR_{705}	ACU ACC CGA UAU CUC CUC
MOE ON_{705}	CCT CTT ACC TCA GTT ACA
MOE NS_{654}	GCT ATT ACC TTA ACC CAG
PNA_{705}	H-CCT CTT ACC TCA GTT ACA-NH_2
PNA_{705}-4K	H-CCT CTT ACC TCA GTT ACA-K_4-NH_2
Tat-PNA_{705}	NH_2-GRKKRRQRRRP-(O-linker)-CCT CTT ACC TCA GTT ACA-NH_2
K_8-PNA_{705}	NH_2-K_8-CCT CTT ACC TCA GTT ACA-K-NH_2
(R-Ahx-R)$_4$-PNA_{705}	NH_2-R-Ahx-RR-Ahx-RR-Ahx-RR-Ahx-R-Ahx-R-C-thioacetyl-K-CCT CTT ACC TCA GTT ACA-KKK-NH_2

Ahx = 6-aminohexanoic acid.

R)$_4$-PNA$_{705}$, 50 nmol bromoacetyl PNA was dissolved in 45 μl formamide and 10 μl BisTris. HBr buffer (pH 7.5) and 15.6 μl C-terminal-Cys-containing peptide (8 mM, 125 nmol, 2.5 equiv.) was added. The solution was heated at 40 °C for 2 h and the product was purified by reversed phase HPLC at 45 °C using water bath heating and analyzed by MALDI-TOF mass spectrometry. MALDI-TOF mass spectrometry was carried out on a Voyager DE Pro BioSpectrometry workstation with a matrix of α-cyano-4-hydroxycinnamic acid, 10 mg/ml in acetonitrile/3% aqueous trifluoroacetic acid (1:1, v/v). The accuracy of the mass measurement in linear mode is regarded by the manufacturer as ±0.05%, but since internal calibration was not used, the determined values varied in a few cases from the calculated by ±0.1%.

2.4. Lipoplex formulation

DLS (delivery liposomal system) lipoplexes consist of small unilamellar vesicles, which can complex negatively charged ON. DLS were formed by mixing 1 mg of dioctadecylamidoglycylspermidine (DOGS, Promega France, Charbonnieres) and 1 mg of dioleoyl phosphatidylethanolamine (DOPE, Sigma–Aldrich Chimie SARL, St. Quentin Fallavier, France) as described previously (Lavigne and Thierry, 1997). ON were rapidly mixed with the liposomes in sterile dionized water at a final concentration of 45 mM NaCl. The final lipoplex preparation contained 10 μg of ON for 38 μl of DLS liposomes. Lipoplexes were stored at 4 °C until use. Complexes of ON with the Lipofectamine™ Reagent (Invitrogen SARL, Cergy Pontoise, France) were prepared according to the manufacturer's instructions.

2.5. Transfections and reporter gene assays

For CPP conjugates, 1.75×10^5 cells/well were seeded in 24-wells dishes with 10% FCS-containing medium. After overnight culture, the CPP conjugate were incubated at indicated concentrations for 4 h, in 1 ml optiMEM medium with the cells. The

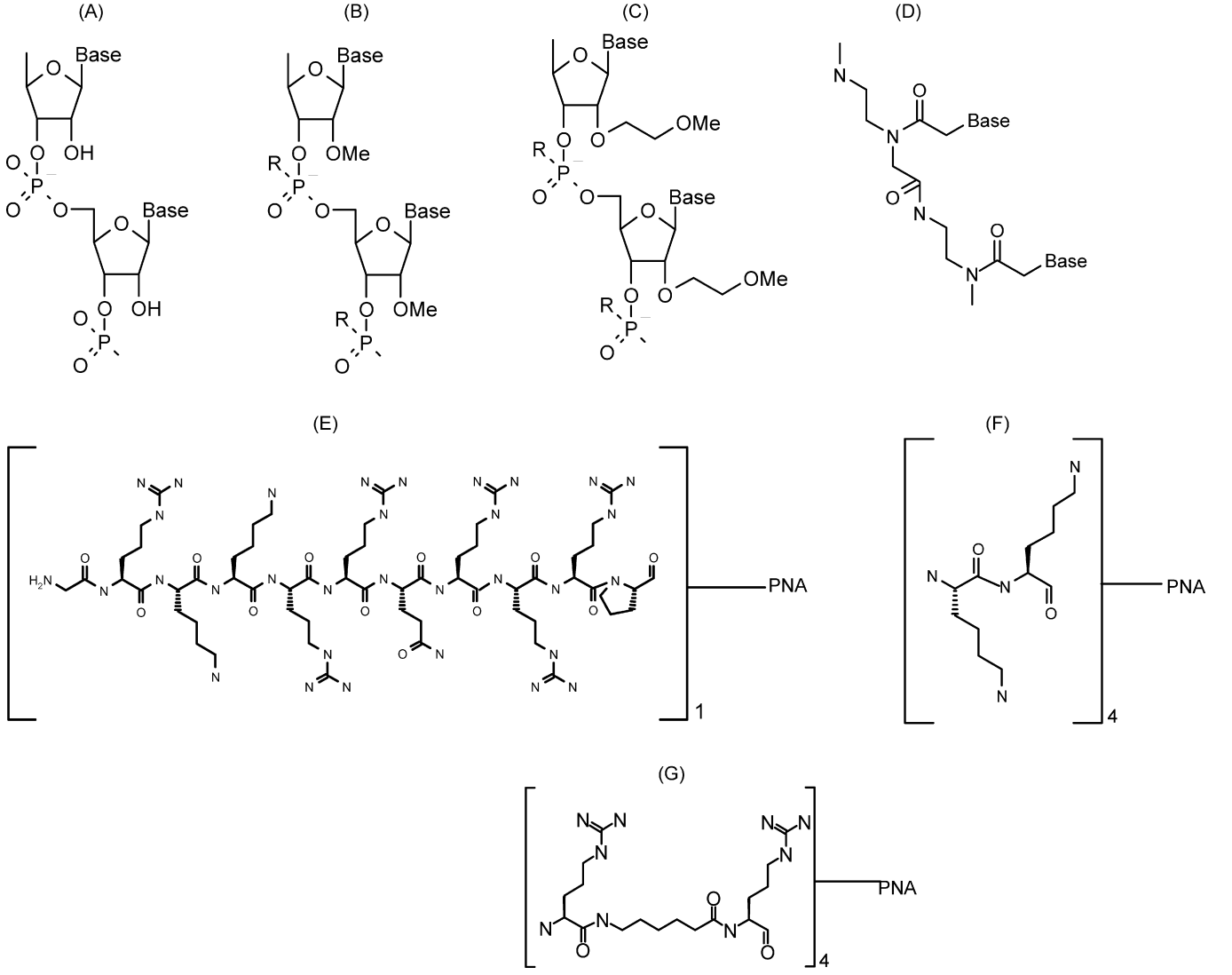

Fig. 1. Structure of oligonucleotides and peptides tested in this work. (A) RNA, (B) 2′-*O*-Me, (C) MOE, (D) PNA, (E) Tat-PNA, (F) K_8-PNA, and (G) (R-Ahx-R)$_4$-PNA, R = S (phosphorothioate) or O (phosphodiester).

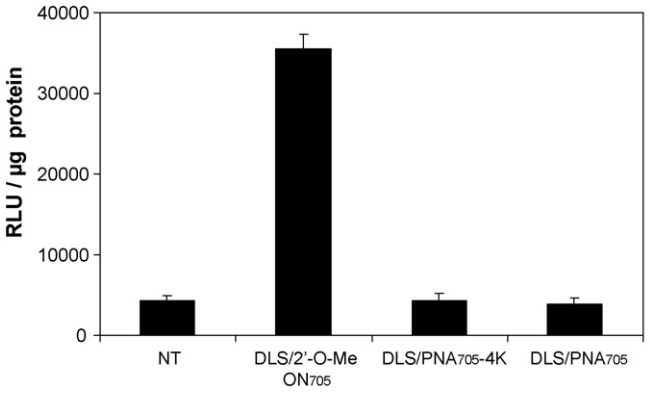

Fig. 2. Comparative study of splicing correction by DLS (for delivery liposomal system) lipoplexes complexed with various ON analogues. HeLa pLuc/705 cells were treated for 24 h in 10% serum-containing cell culture medium, with 200 nM 2′-*O*-Me ON_{705} (negatively charged), PNA_{705}-4K (positively charged) or PNA_{705} (neutral), complexed to the DLS liposomes. NT, non-treated cells. Error bars show standard deviation ($n = 3$).

CPP conjugates were then diluted with 0.5 ml complete medium (FCS-containing medium) and luciferase activity was monitored 20 h later.

For lipoplexes, 2.5–3.5 × 10^5 cells/well were seeded in 6-well dishes with 10% FCS-containing medium. After overnight culture, the cells were rinsed twice with PBS and the medium was replaced with DMEM medium-containing decomplemented serum (Invitrogen SARL, Cergy Pontoise, France). The lipoplexes were then added to the cells and luciferase activity was monitored 24 h later.

Following transfection, cells were rinsed twice with PBS and Reporter Lysis Buffer (Promega France, Charbonnieres) was added. Protein concentrations in the extracts were measured by the BCA™ Protein Assay Kit (Pierce, Perbio Science France, Brebieres) at 560 nm. Firefly luciferase activity was measured using a Luciferase Assay Kit (Promega) and was quantified in the supernatant with a Berthold Centro LB 960 luminometer (Berthold France SA, Thoiry). Luciferase activity in cultured cells was expressed as relative light units (RLU) per μg of protein. Each data point was averaged over three replicates.

2.6. Fluorescence microscopy

5 × 10^5 cells/well were plated in 35 mm single wells. Following overnight culture, pLuc/705 HeLa cells were treated with the fluorescently FAM-labelled Tat-PNA_{705} or with lipoplexes formulated with 5′-FITC-labelled 2′-*O*-Me ON_{705} (aminohexanoic linker, Eurogentec SA, Seraing, Belgium), in optiMEM medium. Cells were rinsed twice with PBS. Living cells were directly observed in the dishes, in 1.5 ml of PBS supplemented with 5% FCS, after 1–24 h of transfection. Fluorescence distribution was analyzed on a Zeiss Axiovert 200M fluorescence microscope without fixation (Carl Zeiss, Oberkochen, Germany). Images were first acquired with AxioVision software. After exportation, images were analyzed with Abobe Photoshop and ImageJ softwares.

3. Results

In order to compare CPP conjugation and lipoplex association as delivery strategies for steric-block ON, we have made use of the model initially proposed by Kang et al. (1998). This model is advantageous in providing a sensitive positive read-out over a low background when the correcting ON has been delivered in the nuclear compartment.

As already stated, the most currently used RNase-H-independent ON analogues described for splicing correction are either charged (2′-*O*-Me or MOE analogues) or neutral (PNA or PMO analogues) (Fig. 1) (DeLong et al., 1999; Kang et al., 1998; Sazani et al., 2001, 2002a).

As shown in Fig. 2, lipoplex formulation of 2′-*O*-Me ON_{705} leads to a very significant increase in luciferase activity even at low concentration (200 nM in this experiment) while uncharged PNA_{705} or positively charged PNA-$4K_{705}$ (in which a four lysine residues tail has been appended to PNA_{705}) have no effect, as expected.

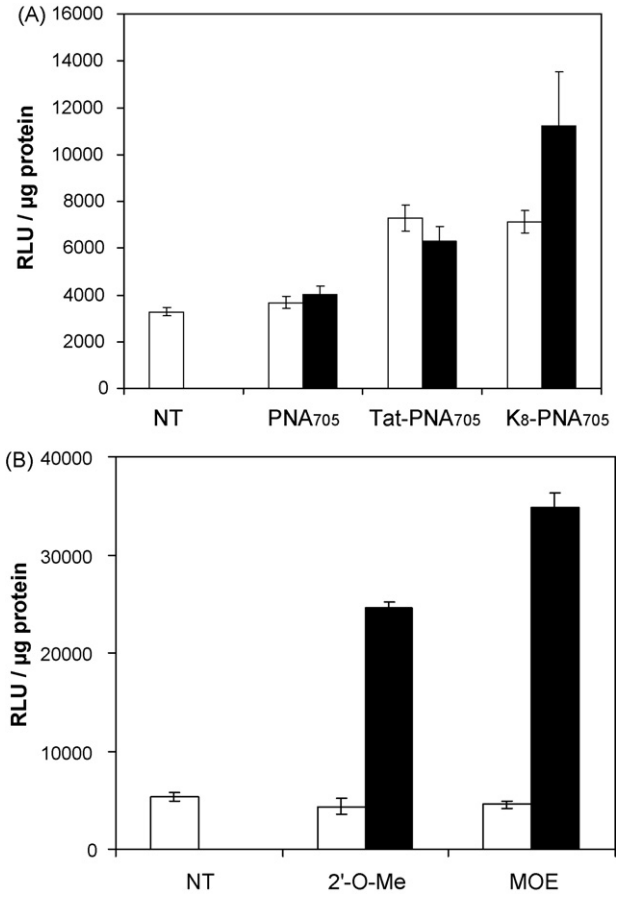

Fig. 3. Comparison of splicing correction by DLS or CPP-formulated ON analogues. (A) Splicing correction by PNA_{705}, Tat-PNA_{705} or K_8-PNA_{705} conjugates. HeLa pLuc/705 cells were incubated for 4 h in optiMEM in the absence or in the presence of 0.5 μM (white bars) or 1 μM (black bars) free PNA_{705} or CPP-PNA_{705} conjugates. Luciferase expression was quantified 20 h later. (B) Splicing correction by 2′-*O*-Me or MOE ON_{705} analogues delivered with the DLS system. HeLa pLuc/705 cells were treated for 24 h in 10% serum-containing cell culture medium, with 100 nM 2′-*O*-Me SCR_{705} or MOE NS_{654} (white bars), or with 100 nM ON_{705} (black bars). NT, non-treated cells. Error bars show standard deviation ($n = 3$).

Then, we compared in the same assay the splicing correction efficiencies of charged 2′-*O*-alkyl derivatives (2′-*O*-Me or MOE) formulated as lipoplexes and of neutral PNAs formulated as CPP conjugates (Fig. 3). In keeping with previous data from our group (Abes et al., 2006a; Richard et al., 2003), the stable conjugation of $Tat_{48–60}$ or of an oligolysine tail (K_8-PNA_{705}) to PNA_{705} only gives rise to a very modest increase over the background level, even at 1 μM concentration (Fig. 3A). On the contrary (Fig. 3B), the complexation of 2′-*O*-Me or MOE ON analogues to DLS lipoplexes leads to efficient and sequence-specific splicing correction even at the 100 nM concentration used in this experiment. At variance with most commercial cationic lipid formulations, DLS lipoplexes can be used even in the presence of serum proteins. The experiments described in Figs. 2 and 3B have indeed been performed in FCS-supplemented culture medium, e.g. in experimental conditions in which Lipofectamine™ is almost inefficient (data not shown and Thierry et al., 2006).

The intracellular fate of fluorescently tagged PNA or 2′-*O*-Me ON analogues delivered by Tat CPPs or by DLS lipoplexes, respectively, has then been followed by fluorescence microscopy of live cells. As shown in Fig. 4 (panels A–C), Tat-PNA conjugates essentially remain entrapped in cytoplasmic vesicles and little if any material can be detected in the nuclear compartment, thus explaining the poor biological response observed in the splicing correction assay. On the contrary, the DLS complexed 2′-*O*-Me ONs first appear as dotted cytoplasmic spots (panels D–F) but accumulate in nuclei upon longer incubation times (Fig. 4 and data not shown).

Entrapment within endocytic vesicles thus appears as a major roadblock for CPP-conjugated steric-block ON analogues as pointed in several studies (Abes et al., 2006a, 2007; Bendifallah et al., 2006; El-Andalousi et al., 2006; Koppelhus et al., 2002; Kaihatsu et al., 2004; Richard et al., 2003; Shiraishi et al., 2005; Turner et al., 2005a). We recently developed a new arginine-rich delivery peptide (named $(R\text{-}Ahx\text{-}R)_4$) which turned out more efficient for the delivery of PMO ON analogues (Abes et al., 2006b). As shown here, the stable chemical conjugation of $(R\text{-}Ahx\text{-}R)_4$ to PNA_{705} does allow efficient splicing correction at low concentration (0.5–1 μM) at variance with free PNA_{705} or with Tat-PNA_{705} (Fig. 5).

4. Discussion

The splicing correction assay first described by Kang et al. (1998) is now recognized as one of the most reliable to evaluate the nuclear delivery of a steric-block ON analogue and as a consequence the efficacy of a delivery vector.

As documented here with this assay, 2′-*O*-Me ON analogues delivered as complexes with cationic lipids (DLS liposomes) correct splicing more efficiently than PNA oligomers delivered as CPP conjugates. A higher level of luciferase expression is indeed achieved with 100 nM of the 2′-*O*-Me ON/DLS formu-

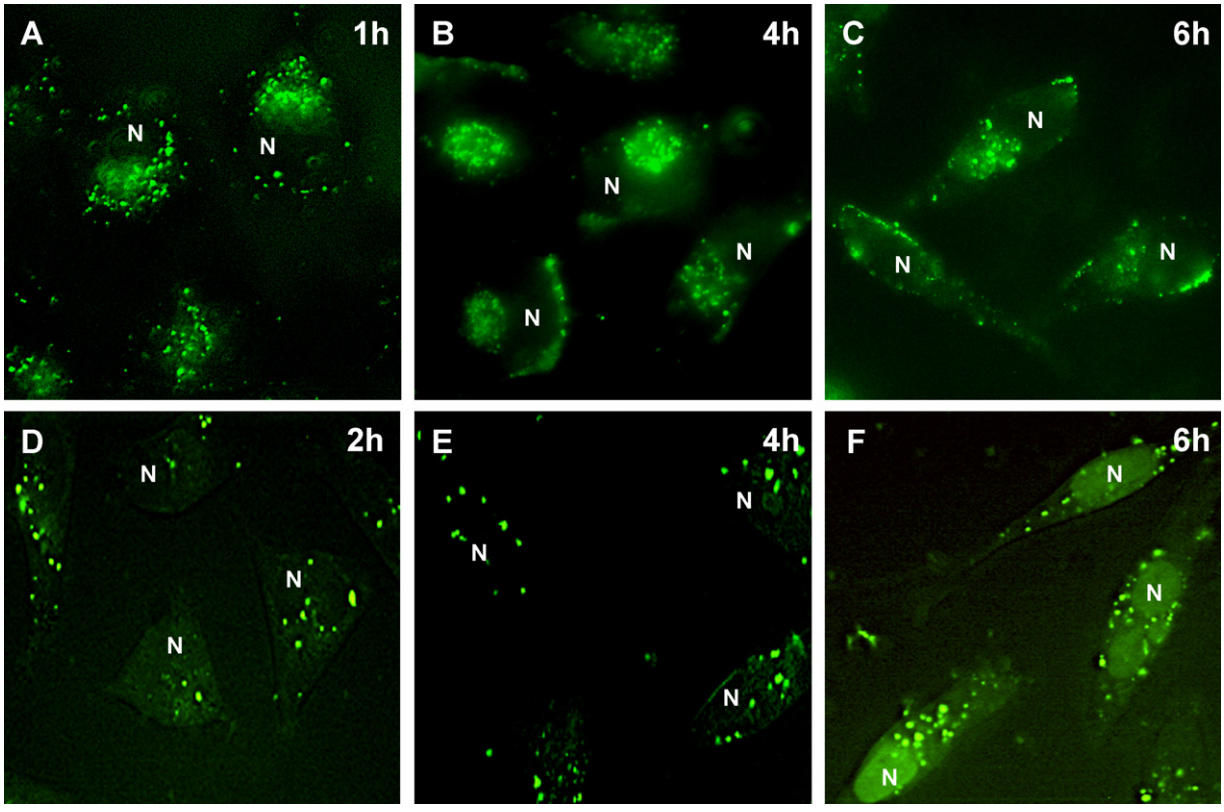

Fig. 4. Comparison of the intracellular distribution of DLS or Tat-formulated ON analogues. HeLa pLuc/705 cells were incubated with 1 μM FAM-labelled Tat-PNA_{705} conjugate (A–C) or with 200 nM FITC-labelled 2′-*O*-Me ON_{705} delivered with the DLS system (D–F). Fluorescence microscopy images in live HeLa pLuc/705 cells incubated in optiMEM in presence of oligomers (green fluorescence) at indicated times. Living cells were directly observed in optiMEM supplemented with 5% FCS. N, nucleus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

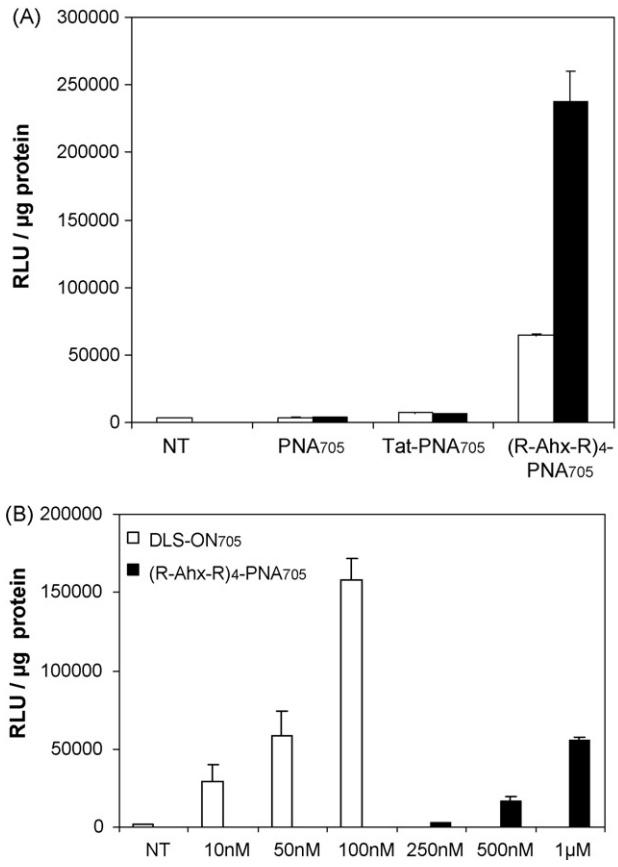

Fig. 5. Comparison of splicing correction efficiencies by naked PNA_{705}, (R-Ahx-R)$_4$-PNA_{705} and Tat-PNA_{705} at 0.5 μM (white bars) or 1 μM (black bars) (panel A) or by DLS-MOE ON_{705} (white bars) and (R-Ahx-R)$_4$-PNA_{705} (black bars) at the indicated concentrations (panel B). HeLa pLuc/705 cells were incubated for 4 h in optiMEM in the absence or presence of oligomers at the indicated concentrations. Luciferase expression was quantified 20 h later. NT, non-treated cells. Error bars show standard deviation ($n = 3$).

lation than with 1 μM of PNA-CPP conjugate (Figs. 3 and 5), in keeping with entrapment of PNA-CPP conjugates within endocytic vesicles (Fig. 4 and data not shown).

Increased splicing correction by DLS-formulated ON analogues is in line with more efficient release from endocytic vesicles and increased nuclear delivery as documented in Fig. 4. It is worth noticing here that 2′-*O*-Me and MOE ON derivatives hybridize with a lower affinity to complementary RNA and are metabolically less stable than neutral PNA and PMO analogues. Improving release of CPP-PNA or CPP-PMO conjugates for endocytic vesicles should therefore lead to very efficient splice correcting tools and is currently the object of structure–activity studies. Remarkably, these (R-Ahx-R)$_4$-PNA and PMO conjugates have very low cytotoxicity which could be advantageous for further *in vivo* applications.

Whether DLS lipoplexes or CPP conjugates will be preferable for *in vivo* applications cannot be predicted and will depend on the outcome of experiments in plan in a β-thalassemic intron transgenic mouse model for the evaluation of splicing correction.

Particulate (DLS liposomes) and soluble (CPP conjugates) delivery vectors have each advantages and problems, depending on the application. Further optimization of delivery vectors for steric-block ON will be critical for therapeutic intervention. As an example, exon skipping with mouse models of Duchenne muscular dystrophy has required very high doses of the free ON analogues (Alter et al., 2006; Fletcher et al., 2006) and commercial cationic lipid formulations have turned rather cytotoxic (Gebski et al., 2003).

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