Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1758 (2006) 364 – 374
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Review

Comparison of basic peptides- and lipid-based strategies for the delivery of splice correcting oligonucleotides

Alain R. Thierry, Said Abes, Sarah Resina, Adrian Travo, Jean Philippe Richard [1], Paul Prevot, Bernard Lebleu *

UMR 5124 CNRS, CC 086, Université Montpellier 2, Place Eugène Bataillon, 34095 Montpellier, France

Received 14 September 2005; received in revised form 24 October 2005; accepted 24 October 2005
Available online 18 November 2005

Abstract

Expression of alternatively spliced mRNA variants at specific stages of development or in specific cells and tissues contributes to the functional diversity of the human genome. Aberrations in alternative splicing were found as a cause or a contributing factor to the development, progression, or maintenance of numerous diseases. The use of antisense oligonucleotides (ON) to modify aberrant expression patterns of alternatively spliced mRNAs is a novel means of potentially controlling such diseases. Oligonucleotides can be designed to repair genetic mutations, to modify genomic sequences in order to compensate for gene deletions, or to modify RNA processing in order to improve the effects of the underlying gene mutation. Steric block ON approach have proven to be effective in experimental model for various diseases. Here, we describe our experience in investigating two strategies for ON delivery: ON conjugation with basic peptides and lipid-based particulate system (lipoplex). Basic peptides or Cell Penetrating Peptides (CPP) such as the TAT-derived peptide appear to circumvent many problems associated with ON and drug delivery. This strategy may represent the next paradigm in our ability to modulate cell function and offers a unique avenue for the treatment of disease. Lipoplexes result from the intimate interaction of ON with cationic lipids leading to ON carrying particles able to be taken up by cells and to release ON in the cytoplasm. We have used as an experimental model the correction of a splicing alteration of the mutated β-globin intron causing thalassemia. Data on cell penetration and efficacy of correction of specific steric block ON delivered either by basic peptides or lipoplex are described. A comparison of the properties of both delivery systems is made respective to the use of this new class of therapeutic molecules.

Keywords: Antisense oligonucleotide; PNA; Delivery; Cell penetrating peptide; Liposome; Splicing correction

Contents

* Corresponding author. Tel.: +33 4 67 16 33 03; fax: +33 4 67 16 33 01.
Email-address: blebleu@univ-montp2.fr (B. Lebleu).
[1] Present address: NIH, Bldg. 10/Rm. 10D04, 10 Center Drive, Bethesda, MD 20892-1855, USA.

0005-2736/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2005.10.010

1. Steric-block oligonucleotides as tools for splicing modulation and for aberrant splicing correction

Conservative estimates suggest that over 75% of all pre-mRNAs are spliced to yield alternative variants, the translated products of which may have very different functions (review in [1]). How the cell decides which alternative splice site to utilize is not fully understood. However, it is generally believed that splice site selection is controlled by the binding of trans-acting protein factors to cis-acting sequences within pre mRNA during spliceosome assembly. Aberrant splicing can result in deleterious consequences for the organism. In fact, approximately 15% of all mutations that cause genetic diseases result in defective splicing of pre-mRNA. For example, aberrant pre-mRNA splicing occurs in genetic diseases such as B-thalassemia, cystic fibrosis, neurofibromatosis type 1, ataxia-telangiectasia, and congenital lipoid adrenal hyperplasia, Alzheimer disease, myotonic dystrophy, and several cancers [2].

The potential of sequence-specific recognition between complementary nucleic acid sequences (Watson–Crick base pairing) to control gene expression was first exploited over two decades ago and has since been used in basic (e.g., as a tool to assess gene function) and applied (e.g., to downregulate the expression of genes causing human diseases) research. The paradigm of the antisense strategy relies upon the hybridization of a small single-stranded DNA fragment to a complementary mRNA (or viral genomic RNA) target sequence, leading to the inactivation of the latter by steric blockade or by RNase H activation. Antisense approaches have been widely applied for target validation or therapeutic purposes, and approximately 25 phase II/III clinical trials using these approaches are now in progress (review in [3]).Catalytic RNA (ribozymes) and DNA (DNAzymes) are also being considered as tools for the specific degradation of an RNA target [4]. More recently, small interfering RNAs (siRNAs) have shown great potential to specifically trigger the destruction of an RNA target (review in [5]). Finally, synthetic DNA or RNA fragments can be used to compete for DNA- (transcription factors) or RNA-binding proteins, respectively, in the decoy approach [6]. New and increased attention has recently focused on antisense compounds as a tool for correcting mutation leading to splicing alteration [2].

To control pre-mRNA splicing patterns, antisense ON must hybridize with specific splice sites and block spliceosome assembly at the targeted splice site. This, because of the flexibility of splicing machinery, does not result in total inhibition of splicing, but in a shift of the spliceosome to another splice site. As a consequence, an alternative splicing pattern is established or a normally used alternative splice site is used with increased efficiency. It has been shown in this and other laboratories that antisense ON modify the splicing patterns of pre-mRNAs that harbor aberrant splice sites or aberrant sequence elements involved in splicing [2]. For example, antisense ON have been used to restore correct pre-mRNA splicing patterns of β-globin in β-thalassemia and cystic fibrosis transmembrane receptor (CFTR) in cystic fibrosis [2]. Antisense ON have also been used to induce an alternative splicing pattern in a dystrophin pre-mRNA in Duchenne's muscular dystrophy and to affect the level of expression of Bcl-x splice variants in cancer cells (reviewed in [7]).

It therefore seems that manipulating the splicing pattern of pre-mRNAs can be effective as a means to control a variety of diseases.

2. Requirements for efficient splicing correction by steric-block oligonucleotides

Implementation of antisense strategies has faced a number of problems (some unforeseen) such as nuclease sensitivity, target accessibility, target affinity, toxicity and delivery. Numerous ON analogs have been designed in order to improve target affinity and metabolic stability in a biological environment. Another frequently encountered drawback is the difficulty of distinguishing an authentic antisense effect from non-specific or toxic effects [8]. Steric block antisense for splicing correction and siRNA are new classes of ON with higher specificity, stability and consequently with higher potential compared with the conventional antisense molecules.

As described in Section 1, splicing correction by the hybridization of steric-block ON analogues (also called splicing-switching ON) to cryptic splice sites has been convincingly documented in several cases. Basic requirements and problems experienced with all nucleic acids-based strategies hold through in this case and will not been reviewed here. To be effective the correcting ON should hybridize the pre-mRNA in a sequence-specific fashion without activating a nuclease. The use of phosphorothioate ON derivatives (whose binding to complementary RNA recruits RNase H) or of siRNA (which trigger the degradation of the target RNA through a RISC complex-associated nuclease) is therefore precluded. RNase H-incompetent ON analogues as charged 2′Oalkyl ON derivatives, or neutral PMO (Phosphorodiamidate Morpholino Oligonucleotides) and PNA (Peptide Nucleic Acids) analogues have therefore been used in this aim [3]. These analogues have favourable pharmacologic properties in terms of nuclease resistance and affinity for the targeted RNA sequence. High affinity is particularly important since RNaseH incompetent ON analogues will at best act in stoichiometric amounts. Interestingly, RNaseH incompetent ON might be acting more specifically than RNaseH activators. Indeed RNaseH activation by antisense ON hybridized on a few consecutive positions only to the target RNA sequence has

been proposed as a source of non-specific effects. Along the same lines unmethylated DNA stretches and phosphorothioate ON derivatives bind to Toll-like receptors on denditic cells thereby triggering inflammatory responses in vivo. This has not been reported to our knowledge for PMO and PNA analogues.

Since pre-mRNA maturation takes place in nuclei, the correcting ON should be delivered in this cellular compartment. ON delivery is achievable in cell culture experiments with the physical (scrape loading, electroporation or microinjection) or chemical (formation of complex to commercial cationic lipids or to polyethylene-imine (PEI)) transfection agents, which are commonly used for plasmid DNA delivery (reviewed in [9,10]). Unfortunately, uncharged ON analogues such as PMO or PNA cannot be transfected with cationic lipids or PEI, and unassisted cellular uptake is very poor. Moreover, most commercial cationic lipids formulations are not adapted to systemic in vivo administration [9,11]. New lipoplex formulations [12–14] and peptide-based delivery vehicles (reviewed in [15,16]) with promising properties have been described by our group as detailed in Section 4.

An alternative potential strategy for splicing correction could capitalize on the fact that cis-elements governing alternative splicing often work at a suboptimal level and strictly require recognition by enhancer SR proteins [17]. Downregulation of these trans-acting elements can therefore be envisaged by an siRNA strategy [9]. Again, however, the implementation of in vivo siRNA strategies requires adequate siRNA modification (to improve metabolic stability) and more importantly suitable delivery vectors.

3. Present status with the delivery of steric block oligonucleotides

Several approaches were explored in the way in developing steric block ON for correction of splice alterations. In order to demonstrate the proof of principle of this strategy various mechanical methods were used (i) Concentrating PMO (45 μM) with cells by repetitive passages through syringe with a 25-gauge needle [18]; (ii), electroporation with 2′-*O*-methoxyethyl modified ON [19]; (iii), or percutaneous coronary intervention with PMO [20]. Interestingly, PMO delivered by streptolysin O permeabilization (at 20 μM in serum-free medium) induced missplicing of c-*myc* mRNA [21].

Polymers forming polyplexes with nucleic acids were used in a few studies. PANAM dendrimers are of interest as they form stable particles in the presence of serum. However, micromolar concentrations were needed and the heterogeneity of particle size will probably preclude systemic injection. The use of triblock copolymer F127 for the intramuscular administration of 2′-*O*-methyl phosphorothioate antisense ON was described to efficiently restore dystrophic expression in skeletal muscles [22]. Recently, antisense ON have also been used to restore the disrupted reading frame of dystrophin mRNAs in Duchenne muscular dystrophy (DMD). DMD patients suffer from severe muscle degeneration due to frame-disrupting mutations in the DMD gene that prematurely abort the synthesis of the dystrophin protein [22]. ON-induced restoration of the DMD reading frame is based on inducing the skipping of specific exons. This was successfully applied in cultured muscle cells from DMD patients and in the mdx mouse model [22,23]. High exon skipping levels of up to 90% were achieved, allowing the synthesis of significant levels of dystrophins in over 75% of treated cells. Stable long term expression of antisense ON via AAV administration into skeletal muscle generated sustained therapeutic levels of rescued dystrophin in entire group of muscles [24].

Conjugation with basic peptides was reported to improve significantly the activity of steric block ON. Sazani et al. [25] reported that PNA-4K oligomers (PNA carrying 4 lysine residues) lead to in vitro (30–300 nM) or in vivo (daily i.p. injection of 50 mg/kg in mice) steric block activity in the β-globin Kole's assay (Fig. 1). Abes et al. (in press and section 5) demonstrated that the same PNA-4K was able (at submicromolar concentration) to restore correct β-globin intron splicing in cultured cells but only when endosome-disrupting agents were concurrently administered to cells. Moulton et al. [26] investigated the ability of the Tat-derived CPP (pTat) to deliver PMO into cultured cells. pTAT-PMO conjugate targeted to c-*myc* mRNA downregulated c-*myc* reporter gene expression with an IC50 of 25 μM. pTat appeared to be 7, 5, and 6 times more efficient than three other CPP: pep-1, KFC, and NLS, respectively [26].

Lipoplexes are to date the most employed tools to deliver steric block ON. PMO delivered with the Lipofectin system (Invitrogen Corp, Carlsbad, CA) were active at 100 nM in cultured muscle cells and in vivo following intramuscular administration of 50 μg/kg in *mdx* mice [27]. Likewise, splicing correction was achieved with 2′-*O*-methyl phosphorothioate ON and PMO delivered with the Lipofectamine reagent (Invitrogen Corp., Carlsbad, CA) [18,25]. Those commercially available delivery systems suffer of instability; and serum-free medium is therefore recommended for transfection. In vivo use of lipoplex in delivering steric block ON is, consequently, poorly documented in the literature.

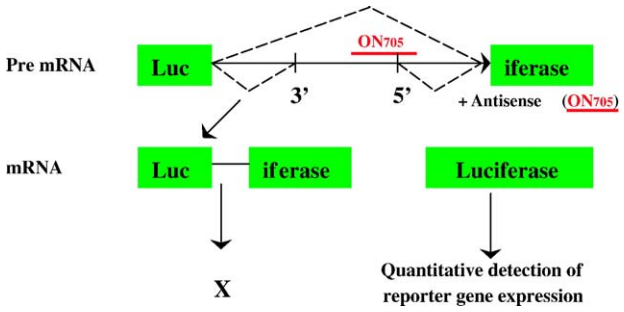

Fig. 1. Splicing correction assay: HeLa pLuc 705 cells were stably transfected with a construction in which the coding sequence of the luciferase gene is interrupted by a mutated intron 2 of the human β-globin gene. This mutation creates a 5′ splice site and activates a 3′ splice site, leading to the production of an aberrant non-functional protein (**X**). Masking of the 5′ splice site by an RNase H-incompetent antisense ON_{705} restores the production of functional luciferase mRNA and protein [2]. Adapted from Kang et al. [50].

4. Our experience with delivery strategies

Delivery within the appropriate intracellular compartments remains a limiting factor for all nucleic acids-based strategies used for the control of gene expression. Endocytosis of free material into most cell types is an inefficient process. Instability in a biological environment and poor cellular uptake are limiting factors in the therapeutic development of ON [28–30]. Appropriate delivery or chemical modification of synthetic ON should, in principle, improve their pharmacological properties through protection from nucleases and by easing their passage through biological barriers. Basically, three strategies are under examination: (i) ON chemical modification, (ii) association with a particulate delivery system, and (iii) conjugation with a hetero-component. We describe below our experience regarding the use of lipoplex-formulated ON and ON-conjugated CPPs.

4.1. ON delivery via Tat peptide conjugation

Much excitement has been generated by the serendipitous discovery by Prochiantz et al. [31] of the transmembrane passage of the *Drosophila* (Antp) protein. Membrane translocation and nuclear delivery has rapidly been assigned to a relatively small (16-mer) basic amino acid-rich peptide belonging to the DNA binding domain of Antp transcription factor [31]. Along the same lines, and to the surprise of virologists, an HIV-1 promoter could be transactivated by incubation of intact cells with the purified Tat protein. An 11-amino acid arginine-rich (GRKKRRQRRRC) peptide belonging to the RNA binding domain of Tat has been defined by our group and shown to be able to cross membranes [32]. Other peptides or peptide-mimics have since been engineered for drug delivery under the generic name of cell penetrating peptides, or CPP. CPPs can be internalized in most cell types and, more importantly, allow the cellular delivery of conjugated (or fused) biomolecules. A wide range of biomolecules such as antigenic peptides, PNA, antisense ON, full-length proteins, or even nanoparticles and liposomes have been delivered this way [15,16]. Most peptide- and nucleic acid-based drugs are poorly taken up in cells, and this is considered a major limitation in their development as therapeutic agents [32]. Conjugation of therapeutic agents to CPPs could thus become a strategy of choice to improve their pharmacological properties.

Langel et al. have provided the key initial data in the field through conjugation of a galanin receptor-specific PNA to transportan, a synthetic CPP [33]. The PNA-transportan complex was delivered within intact neurons in culture and was shown to downregulate the galanin receptor. Most impressively, these same conjugates inhibited galanin receptor expression after injection into mice. PNA delivery into neurons within the brain implied that transportan–PNA conjugates crossed the blood–brain barrier. This may have wide applications in neurobiology and in the treatment of central nervous system-associated diseases [34].

Similarly, a PS-antisense ON conjugated via a disulfide bridge to Antp or Tat peptide has been used to downregulate the expression of the MDR1 gene in cultured cells [15,35]. Although biological responses could be achieved at submicromolar concentrations, cationic lipid-mediated delivery is more efficient than the CPP-driven delivery in this assay. Interestingly, the biological response was enhanced in cells cultured in serum-rich medium in contrast to data obtained with cationic lipid-mediated delivery. The low toxicity of CPPs and their effectiveness in the presence of serum appear advantageous for in vivo applications [16]. CPP were initially thought to be internalized by energy-independent translocation through the plasma membrane. We re-evaluated the mechanisms of cellular uptake of TAT 48–60 and $(Arg)_9$. Fluorescence microscopy on live unfixed cells shows characteristic endosomal distribution of peptides [36]. Peptide uptake is inhibited by incubation at low temperature, by cellular ATP pool depletion and by inhibitors of endocytosis [37]. Similar data were obtained for Tat-conjugated PNA. These data are consistent with the involvement of endocytosis in the cellular internalization of CPP and their conjugates to PNA. Although much remains to be done to delineate the CPP mechanism of uptake, entrapment within endocytic vesicles may be limiting in this strategy as indicated in Section 5.

Numerous methods have been reported for the synthesis of CPP–ON conjugates. The few previously claimed successes for cell delivery and biological activity of CPP–ON conjugates have all used disulfide linkages between oligonucleotide and peptide moieties [38]. We have defined a simplified method for oligonucleotide activation leading to high yield synthesis of peptide-ON chimera through a disulfide bridge [39].

An example of the intracellular trafficking of ON when delivered with Tat conjugation is given in Fig. 2. We carried out fluorescence microscopy analysis of endothelial HUVEC and HeLa live cells treated with Tat–PNA. Alexa labeled Tat–PNA conjugates (green fluorescence) rapidly enter cells and essentially distribute in a punctuate pattern in the cytoplasm (Fig. 2A). This localization corresponds to endosomic vesicles as attested by co-localization with the transferrin-alexa 546 (red fluorescence, Fig. 2B) endosomes marker (yellow staining) (Fig. 2C). This distribution pattern is not modified and fades upon a longer incubation period. Intracellular fluorescence is not detected when free PNA is used suggesting no (or poor) PNA cell uptake (data not showed). However, there is no detectable release of PNA into the cytosol, and consequently, no apparent distribution in the nuclear compartment.

While investigating the role of arginine rich motifs in gene delivery, it was demonstrated that oligomers of TAT CPP compacted plasmid DNA to nanometric particles and stabilized DNA toward nuclease degradation. Such TAT peptide complexes were superior to PEI in terms of transfection efficiency. In addition, gene transfer was enhanced due to TAT nuclear localization sequence. TAT CPP interacted with plasmid DNA electrostatically and the resulting complexes were transferred to mammalian cells by an endocytosis-mediated pathway [37,40,41].

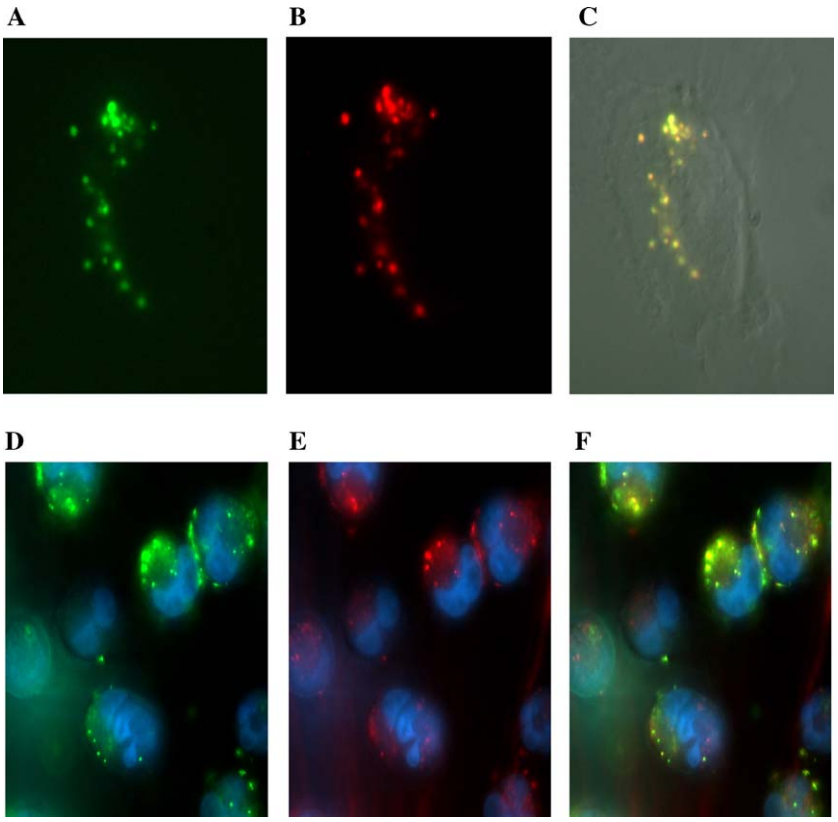

Fig. 2. Fluorescence microscopy images in unfixed HUVEC (panels A–C) or HeLa pLuc 705 (panels D–F) cells. HUVEC cells incubated with 1 μM of Tat–PNA (Alexa 488) (green color) for 30 min at 37 °C (panel A) and thereafter with 25 μg/ml transferrine-alexa 546 (red fluorescence) (panel B) for 5 min. HeLa pLuc 705 cells incubated with 2 μM Fam tagged (green fluorescence (Lys)8–PNA_{705}–Lys (panel D) for 4 h and thereafter with Alexa 546-tagged (red fluorescence) transferrin (panel E) for 5 min. Nuclei were stained with Hoechst (blue fluorescence) for 5 min. Co-localization was revealed in panels C and F (yellow staining). TAT–PNA conjugates were synthetized and characterized as described in [36].

4.2. Lipoplexes

As previously stated, the association of antisense ONs (and more recently of siRNA) to several commercially available cationic lipid formulations is, for the time being, the most reliable and easy strategy to transfect these ON into cells in vitro, despite occasional side effects and resistance of several primary cells. Careful optimization should however be completed for each application as the efficacy (and importantly the toxicity) of different formulations vary depending on the cell type and ON analog utilized. Complex formation of DNA with cationic lipids [42] leads to the respective condensation of both entities by electrostatic interactions. As a consequence, control of the thermodynamic parameters of complex formation is crucial to obtain homogeneous and reproducible particles. In addition, lipoplexes global net charge is cationic leading to unstability in the presence of serum and toxicity [9–11]. Positively charged lipoplexes indeed bind to anionic serum proteins. This leads to inactivation and toxicity (for instance to red blood cells) and first pass effect in lung [10]. This is the reason why most successful in vivo data were obtained with using local administration of nucleic acids. Pharmacologically efficient synthetic vectors are therefore still lacking for most in vivo applications and clinical developments, especially for systemic administration.

Our initial goal was to obtain pharmaceutically suitable vectors regarding stability and reproducibility. We have designed and developed an efficient lipidic vector termed as DLS [11,12]. We have set and defined the optimal thermodynamic conditions either for the lipids or the nucleic acid presentation. Lipids were presented in a liposome forming specific Small Unilamellar Vesicles (SUV) composed of a poly-cationic lipid (DOGS) and a neutral lipid (DOPE) [11–13]. DLS SUVs interacted with DNA to form highly stable (up to 3 months at 4 °C) and homogeneous (120 nm±20%; polydispersity=20) complex particles [11,43]. Uptake and intracellular distribution were studied in various cell culture models with various ON types (modified or unmodified) [29,43,44]. Our observations suggested that complete DNA release from the endocytic vesicles can be achieved and support the notion of the complete or partial release of the DNA from the lipidic carrier [28,29,44].

We have evaluated the specificity and activity of antisense ON against HIV infection with regard to dose–response range, number and choice of experimental controls, ON backbone modifications, type of cell infection, length of assays and delivery approach by the DLS lipoplex system [8]. The highest potency (IC_{50} level at picomolar range) was observed in a short-term chronic infection model with DLS-delivered ON where the DLS delivery improved the ON activity up to 10^6 times compared to free chemically modified ONs [8,43]. Likewise, we recently reported the use of antisense ON directed against VEGF RNA for treating AIDS Kaposi's

Sarcoma in vitro and in vivo setting [29]. Up to 93% of sequence specific inhibition of tumorigenesis was observed with using a clonogenic assay. Significant activity (39% inhibition) was observed at nanomolar range dose (10 nM). Daily intra-tumoral administration of VEGF-ON conduces to a marked change in tumor growth, in cell proliferation and in the number of mitotic figures as observed in thin tissue sections [29].

Biophysical examination of Neutraplex and DLS revealed a specific ultrastructure consisting of liquid crystals composed of lamellar or hexagonal phases [46]. This specific ultrastructure may be responsible for the high reproducibility and homogeneity of these lipoplex particles. Remarkably, the concentric and lamellar structure with different packing regimes was also observed with linear double-stranded DNA, single-stranded DNA and circular double-stranded plasmid DNA [46].

Systemic administration of plasmid DNA delivered with the DLS system led to widespread and long-lasting reporter gene expression [12]. We further demonstrated increased DNA plasma half-life and efficient uptake in blood cells following intravenous administration in mice [13]. The DLS system was applied in various in vivo models for therapeutic gene transfer such as human MDR1 expression in mouse bone marrow progenitor cells [47], and glucocerobrosidase gene transfer [48].

Despite their very low positive charge ratio (1.7 compared to 4.0–8.0 for commercially available formulations), DLS still suffer from drawbacks inherent to lipoplex vectors for in vivo application, especially when considering systemic administration of nucleic acids [10 11]. Whereas DLS mediated gene transfer is not inhibited by serum [11,13,47,48], DLS-ON activity is slightly inhibited in serum containing medium. However, high level ON activity was observed at concentrations in serum-containing medium to which no effect could be detected with commercially available cationic lipids such as Lipofectamine.

We have therefore developed for the first time an anionic lipoplex delivery system named Neutraplex™ [14]. These particles are composed of a poly-cationic lipid (DOGS), a neutral lipid (DOPE), and an anionic phospholipid (cardiolipin) that interacted with DNA [46]. The pharmacokinetics and bioavailability following intravenous administration in baboons of an ON delivered either in a free form, or using a cationic or the Neutraplex carrier system were compared [14]. Whole body distributions and metabolism was monitored using Positron Emission Tomography and an enzyme-based competitive hybridization assay. Free phosphodiester ON showed typical pharmacokinetics, e.g., high liver and kidney concentration, rapid plasmatic degradation and elimination from the body. The cationic vector slightly protected ON against degradation and enhanced uptake by the reticulo-endothelial system but not by other organs. In contrast, the anionic vector dramatically enhanced the uptake in several organs, including the lungs, spleen and brain, with a prolonged accumulation of radioactivity in the brain. Using this vector, intact ON were detected in the plasma for up to 2 h after injection, and the T1/2β (ON half life) and distribution volume increased by 4- and 7- fold, respectively [14]. No evidence of toxicity was found upon administration of 100 μg/kg every week over a 4-week period. The anionic vector thus improved significantly the bioavailability and the pharmacokinetics profile, and appears as a promising delivery system for in vivo administration of therapeutic ON.

As delivery to cells is not enough to ensure ON activity, ON bioavailability in cellular compartments was investigated [29,44,45]. Our long experience in studying nucleic acids intracellular distribution proved to us the need of observing labeled ON solely in live cells as fixing agents modify integrity of intracellular membranes and modify intracellular trafficking [13,29,36,44]. An example of uptake in live cells with lipoplex systems is presented in Fig. 3. Epifluorescence images of unfixed HeLa ovarian cancer cells incubated with 2′-*O*-methyl modified ON delivered with the DLS system are presented. Following 2-h incubation FITC (green fluorescence) labeled ON distribute nearly exclusively in small cytoplasmic vesicles in the cytoplasm (Fig. 3A). This vesicular localization corresponds to endosomes since Transferrin-alexa 546 (red fluorescence) nearly completely co-localizes with ON (yellow fluorescence) (Fig. 3B and C). Following 12-h incubation, the green fluorescence mainly distribute in the nucleus whereas punctuate Transferrin red fluorescence remains in cytoplasmic vesicles (Fig. 3D and E). This suggests a rapid escape of ON from the endosomes, release in the cytosol and apparent complete diffusion of ON into the nucleus as previously described [13,29,44]. It was shown that ON have a "natural tropism" to the nucleus since ON microinjected in cytoplasm rapidly localize in the nucleus [49]. Optimal nuclear penetration was observed after a 12-h incubation time period, the overall fluorescence intensity being markingly decreased following 24 h incubation (data not shown). Whereas free ON poorly penetrate in cells and mainly distribute in endocytic organelles [28,29], DLS lipoplexes thus facilitate ON delivery in the nuclear compartment.

5. Basic peptides and lipoplexes for the delivery of splice correcting oligonucleotides

The efficiency with which CPP and lipoplexes allow for the delivery of splice correcting ON has been assessed in the elegant model proceeded by Kole et al. [2]. In this assay, a mutated intron carrying an aberrant splice site has been inserted within a luciferase reporter gene, thus preventing the production of a functional mRNA. The hybridization of a RNaseH incompetent ON analogue to this cryptic splice site restores correct splicing and consequently the production of luciferase (Fig. 1). This assay is now considered as the most valuable to assess in vitro or in vivo nuclear delivery of an antisense ON since it provides a positive readout. In this assay, we use 18 mer 2′-*O*-methyl ON (ON_{705}) or PNA (PNA_{705})as designed by Kole and co-workers [50].

5.1. Use of DLS lipoplex for delivering steric block ON in Kole's model

Luciferase activity in DLS-Antisense sequence (DLS-ON_{705}) treated cells was 22- and 28-fold higher than that of

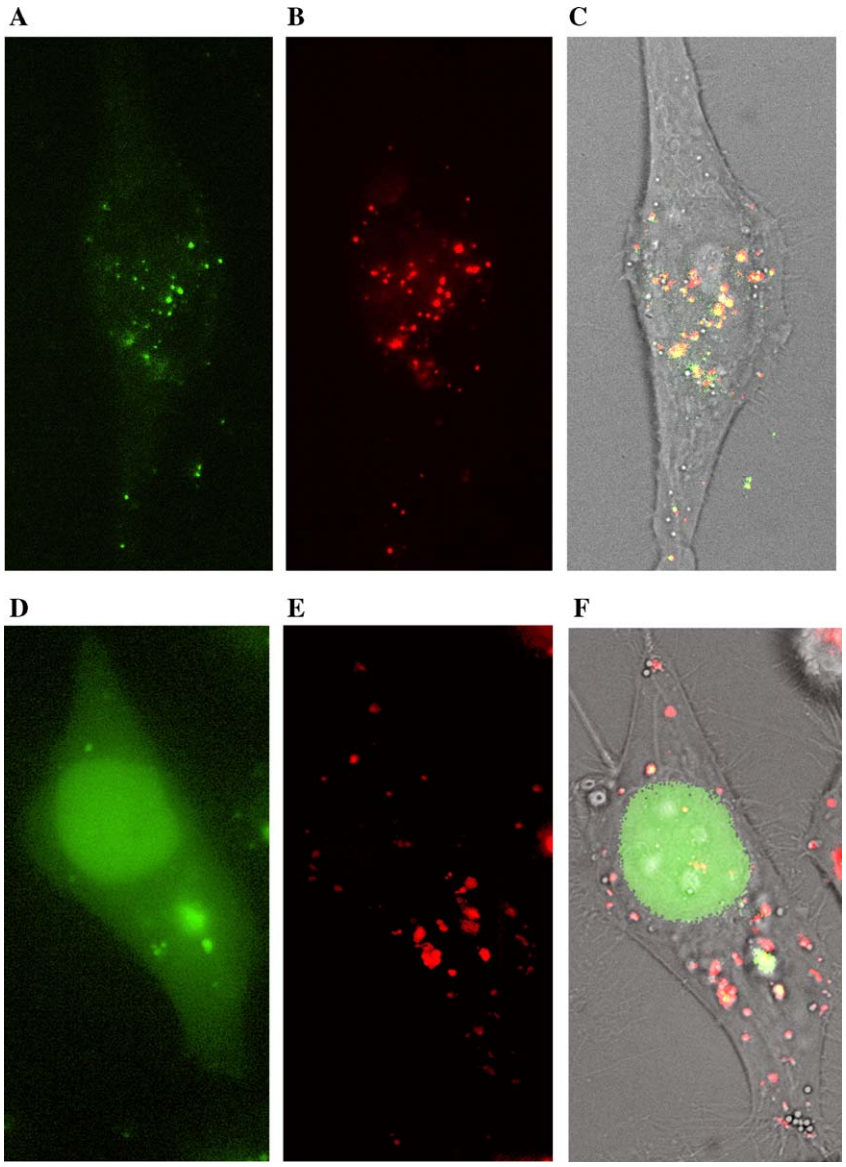

Fig. 3. Fluorescence microscopy images of live HeLa pLuc 705 cells. Cells were incubated with 200 nM FITC ON_{705} (green color) (panels A and D), using the DLS delivery system, in optiMEM medium for 2 h (panels A–C) or 12 h (panels D–F) or with 25 μg/ml transferrin-alexa 546 (red fluorescence) (panels B and C). Co-localization was revealed in panels C and F (yellow staining). DLS lipoplexes were prepared as described in [43].

cells treated with the scrambled control sequence or untreated cells, respectively (Fig. 4A). No detectable activity was observed with free antisense ON (data not shown). Kinetic study enabled us to determine an optimal treatment time between 12 and 24 h. Dose response experiments showed an optimal sequence specific activity at 100 nM with no detectable toxicity up to 300 nM. A statistically significant difference (Student's t-test, $P \leq 0.05$) between the DLS-ON_{705} and the DLS-ON_{sc} was observed at ON doses as low as 1 nM (Fig. 4B). Moreover, the DLS-ON_{705} was approximately 2.5 times more effective than lipofectamine-ON_{705} (LFA) in optiMEM medium (Fig. 4A). High level luciferase expression was observed in cells incubated with DLS-ON_{705} in serum-containing medium while no activity was found in cells treated with Lipofectamine (LFA)-delivered ON at a concentration of 100 nM (data not shown). The marked increase of luciferase activity in cells incubated with a scrambled control ON (ON_{sc}) delivered with LFA compared with that of untreated cells illustrates toxic effect or metabolic changes arising from the use of the LFA lipids. Correction of splicing alterations in thalassemia transgenic mouse models will be evaluated with these lipoplex formulations.

5.2. Use of basic peptides for delivering steric blocking ODN in Kole's model

As mentioned in section 3, the conjugation of a small oligolysine tail to the C-terminus of an uncharged steric block PNA was able to mask a splice site and to re-orient pre-mRNA maturation (PNA_{705}). This elegant and simple strategy could provide an alternative to lipoplexes or CPPs delivery.

As shown in Fig. 4, free PNA 705-Lys did not increase luciferase activity in keeping with the inefficient cellular uptake reported in previous studies [50,51]. Surprisingly however, splicing correction by the $(Lys)_8$–PNA_{705}–Lys conjugate was

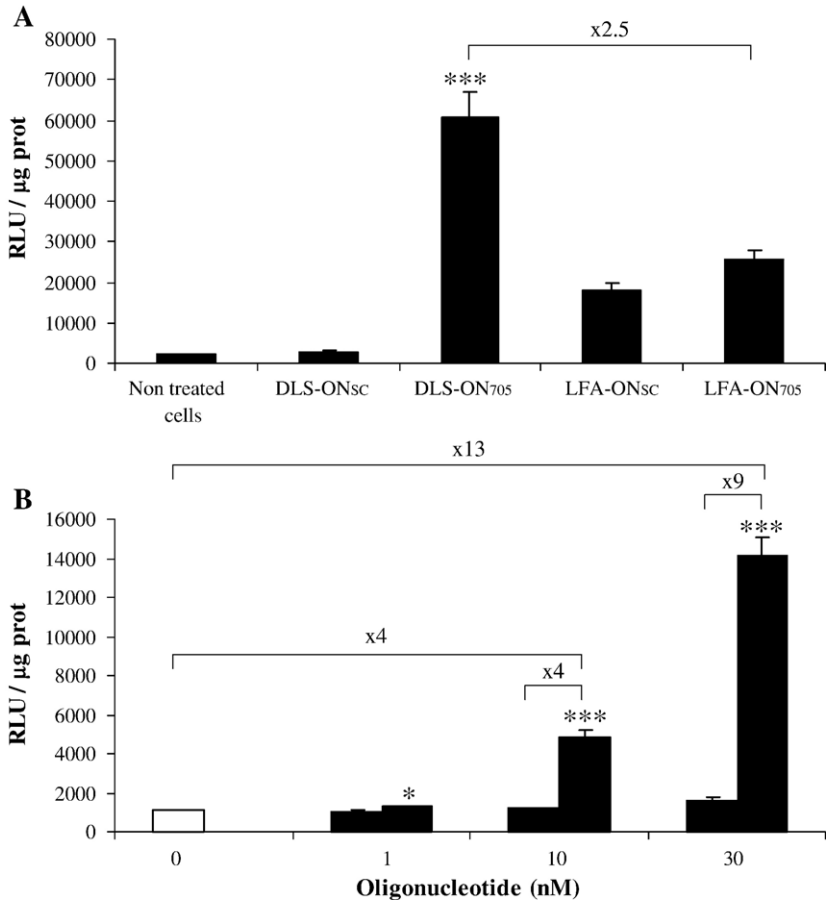

Fig. 4. Luciferase activity in pLuc705 HeLa cells transiently transfected during 24 h with antisense ON_{705} or scrambled ON (ON_{sc}), using the DLS delivery system, in optiMEM medium. (A) Comparison of transfection efficiency between the DLS delivery system and lipofectamine (LFA), at a concentration of 30 nM ON. (B) Effect of ON doses in DLS. ON_{705} (black columns) and scrambled ON (shaded) are compared. The experiment corresponding to non-treated cells is showed (white column). Values are means (±S.D.) of triplicate Luciferase determinations from three independent experiments. Bar between two columns represents multiplication factor. The activity of luciferase was normalized in total cellular protein and is presented in relative luminescence units (RLU) per µg of protein. Statistical differences between RLU/µg values from scrambled and antisense oligonucleotides treated cells were evaluated: $*P \leq 0.05$; $***P \leq 0.001$. Luciferase expression was monitored as described in [50].

barely significant as compared to luciferase expression achieved with lipoplex-delivered 2′OMet ON analogues. Likewise, a $(Lys)_8$–PNA_{705}–Lys construct of appropriate sequence was ineffective in a TAT/TAR transactivation inhibition assay in a parallel study [60].

Since FACS analysis established that $(Lys)_8$–PNA_{705}–Lys conjugates were internalized efficiently by an energy-dependent mechanism, the low biological activity in the splicing correction assay could result from sequestration in endocytic compartments and/or from degradation by lysosomal enzymes (although the modified backbone of PNAs renders them rather resistant to proteases and nucleases).

Accordingly, fluorescence microscopy analysis of live unfixed HeLa pLuc 705 cells incubated with $(Lys)_8$–PNA_{705}–Lys (Fam) revealed a characteristic cytoplasmic punctate distribution. Little if any $(Lys)_8$–PNA_{705}–Lys (Fam) staining could be detected in cell nuclei (Fig. 2D–F).

The most commonly used pharmacological agent to promote increased delivery of drugs sequestered in endocytic compartments is chloroquine, a lysosomotropic amine acting as a buffering agent and as a consequence preventing endosome acidification [51]. It has, in particular, been used to improve the functional delivery of plasmid DNA by non-viral vectors [53,54]. Cells were co-incubated with the $(Lys)_8$–PNA_{705}–Lys conjugate and 100 µM chloroquine for 4 h, a protocol in which no significant chloroquine-induced cytotoxicity was observed (as monitored by the absence of propidium iodide labeling). As shown in Fig. 5A, chloroquine co-treatment very significantly increased splicing correction by $(Lys)_8$–PNA_{705}–Lys conjugates and correction reached comparable levels observed with 2′OMet ON delivered with DLS lipoplexes. Chloroquine did not increase the efficiency of free PNA as expected since neutral PNAs are very poorly internalized [55]. The sequence-specificity of splicing correction was verified with a scrambled version of the $(Lys)_8$–PNA_{705}–Lys construct (Fig. 5). Relatively high concentrations (500 nM or more) of the conjugate were nevertheless required to achieve a significant increase in luciferase activity even in the presence of chloroquine. Although it is difficult to compare steric block agents which differ in their chemistry, 2′OMet ON were active in the low nanomolar range when delivered with DLS lipoplexes (Fig. 4). On the other hand, correction by $(Lys)_8$–PNA_{705}–Lys con-

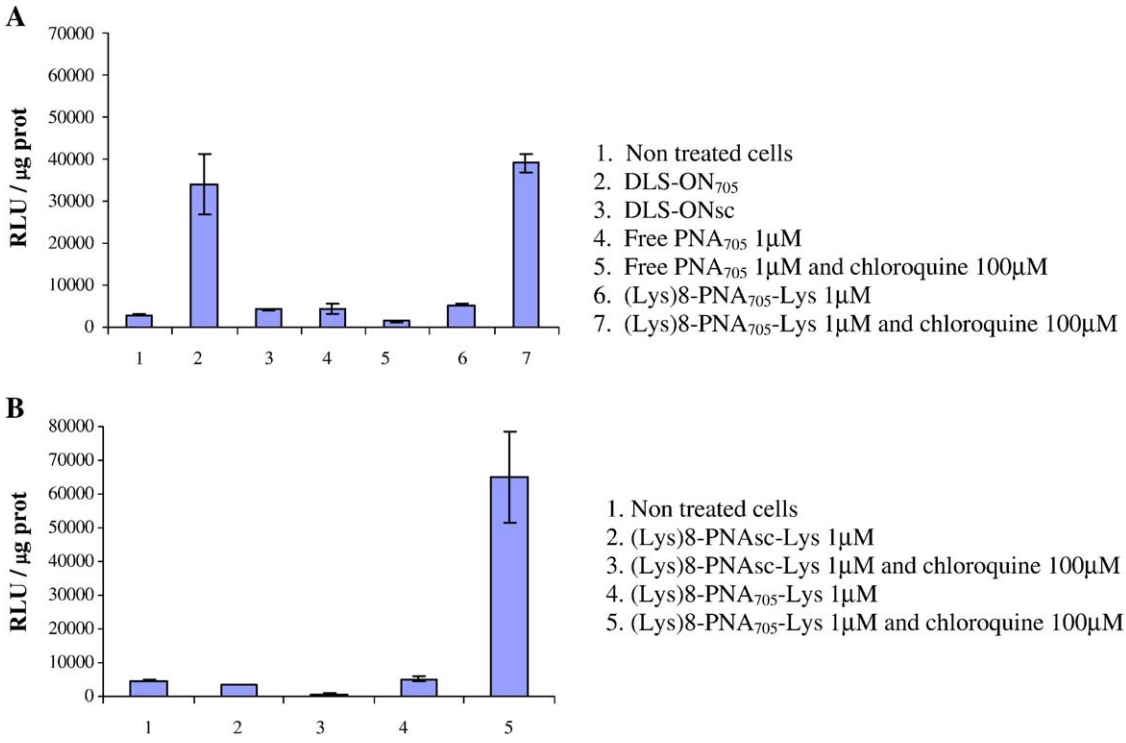

Fig. 5. Effect of chloroquine on splicing correction by (Lys)8–PNA_{705}–Lys conjugate. (A) HeLa pLuc 705 were incubated in the absence of correcting ON (1), in the presence of 30 nM ON_{705} (2) and ON_{sc}(3) delivered with DLS lipoplex, 1 µM free splice correcting PNA_{705} without (4) or with (5) chloroquine (100 µM), or in the presence of 1 µM (Lys)8–PNA_{705}–Lys without (6) or with (7) chloroquine (100 µM). Data are expressed in RLU/µg protein. Experiments have been made in triplicate and averaged. Error bars are indicated. (B) Specificity of splicing correction. Cells were incubated in presence of 1 µM (Lys)8–PNA_{705} scrambled-Lys without (2) or with (3) chloroquine (100 µM), or in presence of (Lys)8–PNA_{705}–Lys 1 µM without (4) or with (5) chloroquine (100 µM). Control in with no addition (1). Data are expressed as in panel A.

jugates was as efficient when the entire experiment was carried out in serum-containing culture medium while lipoplex delivery was partially inhibited by serum proteins in keeping with previous studies with cationic lipid formulations [30].

Likewise, cell incubation in the presence of high sucrose concentration is known to promote endosome swelling and to increase release of endosome-sequestered material [52]. Co-incubation with 0.5 M sucrose largely increased luciferase expression in $(Lys)_8$–PNA_{705}–Lys treated cells but not in cells treated with the scrambled version of the conjugate (Abes et al, in press). These experiments clearly establish that sucrose or chloroquine treatment very significantly increases splicing correction by $(Lys)_8$–PNA_{705}–Lys in a sequence-specific manner, in line with the well-established endosome-destabilizing activity of these two drugs. Likewise, endosome destabilization by lysosomotropic agents or by fusogenic peptides improved the functional delivery of Cre recombinase–TAT fusion proteins [56,57]. Moreover, chloroquine treatment significantly enhanced the inhibition of HIV-1 Tat-dependent trans-activation by PNA 16-mer either, stably polyether linked, or disulfide linked to several cell-penetrating peptides [60].

6. Future directions

In optimal experimental conditions, basic peptides or lipoplexes efficiently deliver splice correcting ON in nuclei. The apparent instability of most lipoplex formulations in serum containing medium appears as a drawback for in vivo applications. The development of negatively charged [9,14] or sterically stabilized lipoplexes will be required for systemic administration. However, cationic lipoplexes could potentially be useful when local administration is appropriate to achieve disease treatment. Second, more has to be done to reduce the toxicity of cationic constituents for red blood cells, for instance.

Basic peptides or CPP are advantageous vector system compared to particulate systems regarding synthesis, cost, and pharmaceutical development, in particular for quality control and scalable process production. In contrast to lipoplexes, basic peptides make possible delivery of uncharged PNA or PMO. One of the basic peptides drawback appears to be the need of the concurrent treatment of endosomes releasing agents in order to concentrate ON in the nucleus where splice correction occurs. Although lysosomotropic agents such as chloroquine are pharmacologically well known, the presence of a third constituent, in addition to the steric block ON and the basic peptide heteroconjugate, might decrease the tolerated clinical doses. Multifunctional basic peptides containing for instance an hydrophobic part or a nuclear localization signal [58] might be a solution to improve intracellular trafficking and their efficacy [59]. Basic peptides hetero-conjugation for ON delivery seems as well to partially protect ON towards degradation and activity requires higher doses compared to lipoplex (10- to 100-fold). All those parameters should be taken into consideration in

order to evaluate their use in therapy. Despite severely complicating formulation process, combining both approaches might improve delivery parameters, and this is under investigation in our laboratory.

Acknowledgments

This work was supported by IFCPAR (contract 3205-A) and by EC framework 5 (contract QLK3-CT-2002-01989). Said Abes is the recipient of a pre-doctoral fellowship from the Ligue contre le Cancer.

References

[1] M.A. Garcia-Blanco, A.P. Baraniak, E.L. Lasda, Alternative splicing in disease and therapy, Nat. Biotechnol. 22 (2004) 535–546.
[2] P. Sazani, R. Kole, Therapeutic potential of antisense oligonucleotides as modulators of alternative splicing, J. Clin. Invest. 112 (2003) 481–486.
[3] J. Kurreck, Antisense technologies. Improvement through novel chemical modifications, Eur. J. Biochem. 270 (2003) 1628–1644.
[4] J.B. Opalinska, A.M. Gewirtz, Nucleic acid therapeutics: a work in progress, Curr. Opin. Investig. Drugs 3 (2002) 928–933.
[5] T. Tuschl, RNA interference and small interfering RNAs, ChemBioChem 2 (2001) 239–245.
[6] M.J. Mann, V.J. Dzau, Therapeutic applications of transcription factor decoy oligonucleotides, J. Clin. Invest. 106 (2000) 1071–1075.
[7] T.A. Rando, Oligonucleotide-mediated gene therapy for muscular dystrophies, Neuromuscul. Disord. 12 (Suppl. 1) (2002) S55–S60.
[8] C. Lavigne, J. Yelle, G. Sauve, A.R. Thierry, Is antisense an appropriate nomenclature or design for oligodeoxynucleotides aimed at the inhibition of HIV-1 replication? AAPS PharmSciTech 4 (2002) E9.
[9] A.R. Thierry, A.R., L.C. Mahan, Therapeutic applications of lipid-based gene delivery systems, in: A. Rolland (Ed.), Advanced Gene Delivery, 1998, pp. 123–134.
[10] A.R. Thierry, E. Vives, J.P. Richard, P. Prevot, C. Martinand-Mari, I. Robbins, B. Lebleu, Cellular uptake and intracellular fate of antisense oligonucleotides, Curr. Opin. Mol. Ther. 5 (2003) 133–138.
[11] A.R. Thierry, Optimization of lipoplex formulations for intravenous gene delivery, J. Liposome Res. 7 (1997) 143–160.
[12] A.R. Thierry, Y. Lunardi-Iskandar, J.L. Bryant, P. Rabinovich, R.C. Gallo, L.C. Mahan, Systemic gene therapy: biodistribution and long-term expression of a transgene in mice, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 9742–9746.
[13] A.R. Thierry, P. Rabinovich, B. Peng, L.C. Mahan, J.L. Bryant, R.C. Gallo, Characterization of liposome-mediated gene delivery: expression, stability and pharmacokinetics of plasmid DNA, Gene. Ther. 4 (1997) 226–237.
[14] B. Tavitian, S. Marzabal, V. Boutet, B. Kuhnast, S. Terrazzino, M. Moynier, F. Dolle, J.R. Deverre, A.R. Thierry, Characterization of a synthetic anionic vector for oligonucleotide delivery using in vivo whole body dynamic imaging, Pharm. Res. 19 (2002) 367–376.
[15] H. Brooks, B. Lebleu, E. Vives, Tat peptide-mediated cellular delivery: back to basics, Adv. Drug Delivery Rev. 57 (2005) 559–577.
[16] J.S. Wadia, S.F. Dowdy, Transmembrane delivery of protein and peptide drugs by TAT-mediated transduction in the treatment of cancer, Adv. Drug. Delivery Rev. 57 (2005) 579–596.
[17] D.R. Mercatante, P. Sazani, R. Kole, Modification of alternative splicing by antisense oligonucleotides as a potential chemotherapy for cancer and other diseases, Curr. Cancer Drug Targets 1 (2001) 211–230.
[18] G. Lacerra, H. Sierakowska, C. Carestia, S. Fucharoen, J. Summerton, D. Weller, R. Kole, Restoration of hemoglobin A synthesis in erythroid cells from peripheral blood of thalassemic patients, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 9591–9596.
[19] J.G. Karras, R.A. McKay, N.M. Dean, B.P. Monia, Deletion of individual exons and induction of soluble murine interleukin-5 receptor-alpha chain expression through antisense oligonucleotide-mediated redirection of pre-mRNA splicing, Mol. Pharmacol. 58 (2000) 380–387.
[20] N. Kipshidze, M. Tsapenko, P. Iversen, D. Burger, Antisense therapy for restenosis following percutaneous coronary intervention, Expert Opin. Biol. Ther. 5 (2005) 79–89.
[21] R.V. Giles, D.G. Spiller, R.E. Clark, D.M. Tidd, Antisense morpholino oligonucleotide analog induces missplicing of C-myc mRNA, Antisense Nucleic Acid Drug Dev. 9 (1999) 213–220.
[22] Q.L. Lu, C.J. Mann, F. Lou, G. Bou-Gharios, G.E. Morris, S.A. Xue, S. Fletcher, T.A. Partridge, S.D. Wilton, Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse, Nat. Med. 9 (2003) 1009–1014.
[23] S. Bogdanovich, K.J. Perkins, T.O. Krag, T.S. Khurana, Therapeutics for Duchenne muscular dystrophy: current approaches and future directions, J. Mol. Med. 82 (2004) 102–115.
[24] A. Goyenvalle, A. Vulin, F. Fougerousse, F. Leturcq, J.C. Kaplan, L. Garcia, O. Danos, Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping, Science 306 (2004) 1796–1799.
[25] P. Sazani, F. Gemignani, S.H. Kang, M.A. Maier, M. Manoharan, M. Persmark, D. Bortner, R. Kole, Systemically delivered antisense oligomers upregulate gene expression in mouse tissues, Nat. Biotechnol. 20 (2002) 1228–1233.
[26] H.M. Moulton, M.C. Hase, K.M. Smith, P.L. Iversen, HIV Tat peptide enhances cellular delivery of antisense morpholino oligomers, Antisense Nucleic Acid Drug Dev. 13 (2003) 31–43.
[27] B.L. Gebski, C.J. Mann, S. Fletcher, S.D. Wilton, Morpholino antisense oligonucleotide induced dystrophin exon 23 skipping in mdx mouse muscle, Hum. Mol. Genet. 12 (2003) 1801–1811.
[28] A.R. Thierry, A. Dritschilo, Intracellular availability of unmodified, phosphorothioated and liposomally encapsulated oligodeoxynucleotides for antisense activity, Nucleic Acids Res. 20 (1992) 5691–5698.
[29] C. Lavigne, Y. Lunardi-Iskandar, B. Lebleu, A.R. Thierry, Cationic liposomes/lipids for oligonucleotide delivery: application to the inhibition of tumorigenicity of Kaposi's sarcoma by vascular endothelial growth factor antisense oligodeoxynucleotides, Methods Enzymol. 387 (2004) 189–210.
[30] O. Zelphati, C. Nguyen, M. Ferrari, J. Felgner, Y. Tsai, P.L. Felgner, Stable and monodisperse lipoplex formulations for gene delivery, Gene Ther. 5 (1998) 1272–1282.
[31] D. Derossi, A.H. Joliot, G. Chassaing, A. Prochiantz, The third helix of the Antennapedia homeodomain translocates through biological membranes, J. Biol. Chem. 269 (1994) 10444–10450.
[32] E. Vives, P. Brodin, B. Lebleu, A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus, J. Biol. Chem. 272 (1997) 16010–16017.
[33] M. Pooga, A. Jureus, K. Razaei, H. Hasanvan, K. Saar, K. Kask, P. Kjellen, T. Land, J. Halonen, U. Maeorg, A. Uri, S. Solyom, T. Bartfai, U. Langel, Novel galanin receptor ligands, J. Pept. Res. 51 (1998) 65–74.
[34] M. Zorko, U. Langel, Cell-penetrating peptides: mechanism and kinetics of cargo delivery, Adv. Drug Delivery Rev. 57 (2005) 529–545.
[35] A. Astriab-Fisher, D. Sergueev, M. Fisher, B.R. Shaw, R.L. Juliano, Conjugates of antisense oligonucleotides with the Tat and antennapedia cell-penetrating peptides: effects on cellular uptake, binding to target sequences, and biologic actions, Pharm. Res. 19 (2002) 744–754.
[36] J.P. Richard, K. Melikov, E. Vives, C. Ramos, B. Verbeure, M.J. Gait, L.V. Chernomordik, B. Lebleu, Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake, J. Biol. Chem. 278 (2003) 585–590.
[37] J.P. Richard, K. Melikov, H. Brooks, P. Prevot, B. Lebleu, L.V. Chernomordik, Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors, J. Biol. Chem. 280 (2005) 15300–15306.
[38] J.J. Turner, A.A. Arzumanov, M.J. Gait, Synthesis, cellular uptake and HIV-1 Tat-dependent trans-activation inhibition activity of oligonucleotide analogues disulphide-conjugated to cell-penetrating peptides, Nucleic Acids Res. 33 (2005) 27–42.
[39] F. Maurel, F. Debard, F. Clavelier, A.R. Thierry, B Lebleu, J.J. Vasseur, E. Vives, Toward high yield synthesis of peptide-oligonucleotide chimera

through a disulfide bridge : A simplified method for oligonucleotide activation, Bioorganic Med. Chem. Let. 15 (2005) 5084–5087.
[40] M. Silhol, M. Tyagi, M. Giacca, B. Lebleu, E. Vives, Different mechanisms for cellular internalization of the HIV-1 Tat-derived cell penetrating peptide and recombinant proteins fused to Tat, Eur. J. Biochem. 269 (2002) 494–501.
[41] B. Gupta, T.S. Levchenko, V.P. Torchilin, Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides, Adv. Drug Delivery Rev. 57 (2005) 637–651.
[42] P.L. Felgner, Y. Barenholz, J.P. Behr, S.H. Cheng, P. Cullis, L. Huang, J.A. Jessee, L. Seymour, F. Szoka, A.R. Thierry, E. Wagner, G. Wu, Nomenclature for synthetic gene delivery systems, Hum. Gene. Ther. 8 (1997) 511–512.
[43] C. Lavigne, A.R. Thierry, Enhanced antisense inhibition of human immunodeficiency virus type 1 in cell cultures by DLS delivery system, Biochem. Biophys. Res. Commun. 237 (1997) 566–571.
[44] J. Lisziewicz, D. Sun, F.F. Weichold, A.R. Thierry, P. Lusso, J. Tang, R.C. Gallo, S. Agrawal, Antisense oligodeoxynucleotide phosphorothioate complementary to Gag mRNA blocks replication of human immunodeficiency virus type 1 in human peripheral blood cells, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 7942–7946.
[45] G.B. Takle, A.R. Thierry, S.M. Flynn, B. Peng, L. White, W. Devonish, R.A. Galbraith, A.R. Goldberg, S.T. George, Delivery of oligoribonucleotides to human hepatoma cells using cationic lipid particles conjugated to ferric protoporphyrin IX (heme), Antisense Nucleic Acid Drug Dev. 7 (1997) 177–185.
[46] M. Schmutz, D. Durand, A. Debin, Y. Palvadeau, A. Etienne, A.R. Thierry, DNA packing in stable lipid complexes designed for gene transfer imitates DNA compaction in bacteriophage, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 12293–12298.
[47] I. Aksentijevich, I. Pastan, Y. Lunardi-Iskandar, R.C. Gallo, M.M. Gottesman, A.R. Thierry, In vitro and in vivo liposome-mediated gene transfer leads to human MDR1 expression in mouse bone marrow progenitor cells, Hum. Gene. Ther. 7 (1996) 1111–1122.
[48] M. Baudard, T.R. Flotte, J.M. Aran, A.R. Thierry, I. Pastan, M.G. Pang, W.G. Kearns, M.M. Gottesman, Expression of the human multidrug resistance and glucocerebrosidase cDNAs from adeno-associated vectors: efficient promoter activity of AAV sequences and in vivo delivery via liposomes, Hum. Gene. Ther. 7 (1996) 1309–1322.
[49] J.P. Leonetti, N. Mechti, G. Degols, C. Gagnor, B. Lebleu, Intracellular distribution of microinjected antisense oligonucleotides, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 2702–2706.
[50] S.H. Kang, M.J. Cho, R. Kole, Up-regulation of luciferase gene expression with antisense oligonucleotides: implications and applications in functional assay development, Biochemistry 37 (1998) 6235–6239.
[51] A.M. Siwkowski, L. Malik, C.C. Esau, M. Maier, E.V. Wancewicz, K. Albertshofer, B.P. Monia, C.F. Bennett, A.B. Eldrup, Identification and functional validation of PNAs that inhibit murine CD40 expression by redirection of splicing, Nucleic Acids Res. 32 (9) (2004) 2695–2706.
[52] V. Ferrari, D.J. Cutler, Kinetics and thermodynamics of chloroquine and hydroxychloroquine transport across the human erythrocyte membrane, Biochem. Pharmacol. 41 (1991) 23–30.
[53] K. Ciftci, R.J. Levy, Enhanced plasmid DNA transfection with Lysosomotropic agents in cultured fibroblasts, Int. J. Pharm. 218 (2001) 81–92.
[54] D.S. Manickam, H.S. Bisht, L. Wan, G. Mao, D. Oupicky, Influence of TAT–peptide polymerization on properties and transfection activity of TAT/DNA polyplexes, J. Control. Release 102 (2005) 293–306.
[55] U. Koppelhus, S.K. Awasthi, V. Zachar, H.U. Holst, P. Ebbeson, P.E. Nielsen, Cell-dependant differential cellular uptake of PNA, peptides and PNA–peptide conjugates, Antisense Nucleic Acid Drug Dev. 12 (2002) 51–63.
[56] N.J. Caron, S.P. Quenneville, J.P. Tremblay, Endosome disruption enhances the functional nuclear delivery of Tat–Fusion proteins, Biochem. Biophys. Res. Commun. 319 (2004) 12–20.
[57] J.S. Wadia, R.V. Stan, S.F. Dowdy, Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis, Nat. Med. 10 (3) (2004) 310–315.
[58] M.C. Morris, J. Depollier, J. Mery, F. Heitz, G. Divita, A peptide carrier for the delivery of biologically active proteins into mammalian cells, Nat. Biotechnol. 19 (2001) 1173–1176.
[59] S. Deshayes, A. Heitz, M.C. Morris, P. Charnet, G. Divita, F. Heitz, Insight into the mechanism of internalization of the cell-penetrating carrier peptide Pep-1 through conformational analysis, Biochemistry 43 (2004) 1449–1457.
[60] J. Turner, G.D. Ivanova, B. Verbeure, D. Williams, A.A. Arzumanov, S. Abes, B. Lebleu, M.J. Gait. Cell-penetrating peptide conjugates of peptide nucleic acids (PNA) as inhibitors of HIV-1 Tat-dependent trans-activation in cells, Nucl. Acids Res. (in press).