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Vectorization of morpholino oligomers by the (R-Ahx-R)$_4$ peptide allows efficient splicing correction in the absence of endosomolytic agents☆

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Abstract

The efficient and non-toxic nuclear delivery of steric-block oligonucleotides (ON) is a prerequisite for therapeutic strategies involving splice correction or exon skipping. Cationic cell penetrating peptides (CPPs) have given rise to much interest for the intracellular delivery of biomolecules, but their efficiency in promoting cytoplasmic or nuclear delivery of oligonucleotides has been hampered by endocytic sequestration and subsequent degradation of most internalized material in endocytic compartments. In the present study, we compared the splice correction activity of three different CPPs conjugated to PMO$_{705}$, a steric-block ON targeted against the mutated splicing site of human β-globin pre-mRNA in the HeLa pLuc705 splice correction model. In contrast to Tat48–60 (Tat) and oligoarginine (R_9F_2) PMO$_{705}$ conjugates, the 6-aminohexanoic-spaced oligoarginine (R-Ahx-R)$_4$–PMO$_{705}$ conjugate was able to promote an efficient splice correction in the absence of endosomolytic agents. Our mechanistic investigations about its uptake mechanisms lead to the conclusion that these three vectors are internalized using the same endocytic route involving proteoglycans, but that the (R-Ahx-R)$_4$–PMO$_{705}$ conjugate has the unique ability to escape from lysosomial fate and to access to the nuclear compartment. This vector, which has displays an extremely low cytotoxicity, the ability to function without chloroquine adjunction and in the presence of serum proteins. It thus offers a promising lead for the development of vectors able to enhance the delivery of therapeutic steric-block ON in clinically relevant models.

Keywords: Splice correction; PMO antisens; CPPs; (R-Ahx-R) 4; Endosomolytic agents

1. Introduction

Promising oligonucleotide (ON)-based strategies leading to sequence-specific control of gene expression have been proposed but their applications are limited by poor delivery into cytoplasm and nuclear compartments [1]. The most popular tools for nucleic acids delivery are cationic vectors such as lipoplexes or PEI. However they are not devoid of cytotoxicity, and most are not effective in the presence of serum proteins [2,3] Moreover, they cannot easily be used to transfect neutral ON analogs such as peptide nucleic acids (PNAs) [4] or phosphorodiamidate morpholino oligomers (PMOs) [5].

Cell penetrating peptides (CPPs) have been of much interest as delivery vectors for biomolecules such as peptides or nucleic acids [6]. Many publications describe the use of CPPs for protein or peptide transfection [7] but, surprisingly, relatively little has been documented for the CPP-based delivery of antisense ON analogs [8]. A series of independent studies have established recently that CPPs rich in basic amino acids (oligolysine, oligoarginine or Tat) did not efficiently promote the nuclear delivery of conjugated antisense ON analogs [9–11]. This might

Abbreviations: CHO, Chinese hamster ovary; CPP, cell penetrating peptide; Fam, carboxyfluorescein; FCS, Fetal calf serum; HPLC, high pressure liquid chromatography; PI, propidium iodide; PBS, phosphate buffered saline; PMO, phosphorodiamidate morpholino oligomers; PEI, polyethylene imine; PNA, peptide-nucleic acid; ON, oligonucleotide.

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be due to the internalization mechanism of CPP–ON conjugate, which involves endocytotic pathways rather than direct membrane translocation as initially thought [12].

Most studies about ON delivery by CPPs capitalized on the splicing correction assay initially described by the group of R. Kole [13], which is considered as the most reliable to assess the nuclear delivery of an antisense ON. The coding sequence of a luciferase reporter gene is interrupted by the human β-globin thalassemic intron 2 which carries a crytic splice site. This aberrant splice site prevents total removal of the intron unless the site is masked by a steric blocking (RNase H-independent) ON analogue. The advantage of this easy to implement assay is that the nuclear delivery of the correcting ON analogue gives rise to a positive read-out over a very low background.

Using this model, it was demonstrated that CPP–ON conjugates were efficiently taken up but largely remained trapped within endocytic vesicles [9,11]. Inefficient endosome release and/or lysosome degradation were therefore proposed as the major limitation for the cytoplasm/nuclear delivery of ONs. Consistent with this hypothesis, various treatments known to promote endosome leakage or endosome disruption such as chloroquine, Ca^{2+} treatment, high sucrose concentration or photochemical activation strongly increased splice correction by CPP–PNA conjugates [9,11,14]. Similar conclusions were reached for the CPP-mediated delivery of ON analogues designed to interfere with Tat transactivation by binding to the HIV-1 TAR element [10].

Since most of these endosomolytic agents are not easy to use in an *in-vivo* setting, alternative strategies had to be searched for. Co-treatment with a CPP conjugate and an endosome-destabilizing peptide is a possibility as proposed by Wadia et al. [15], but it has the potential disadvantage of complicating the delivery vector formulation.

Screening analogs of existing CPPs for more efficient cellular uptake is an alternative strategy. An interesting structure activity relationship (SAR) study has been described by Rothbard et al. [16]. Knowing the seemingly key role of arginine's guandinium headgroups for CPP uptake, they evaluated the influence of backbone spacing on cellular uptake. One of the most efficient arginine-based CPP included a 6-aminohexanoic spacer, most probably because it provided both increased flexibility and metabolic stability to the peptide. However, this SAR study was carried out using fluorescein as the probe and no biologically functional cargos were used. As the materials trapped in endosomes can fluoresce, it is unknown whether intensity of cell fluorescence directly correlates with the amount of cargo in the cytoplasm/nucleus.

PMOs have proven to be effective steric blockers in various studies [17,18]. They have, in particular, been used successfully to promote exon skipping in skeletal muscles of the mdx dystrophic mice [19]. Although CPPs have had some success in enhancing the cellular delivery of PMOs in the virology field [20–23], it still requires much more investigation to understand SAR in terms of delivery efficiencies, internalization mechanisms and cellular toxicity of CPP–PMO conjugates.

In the present study, we compared the nuclear delivery efficiencies of three different CPPs namely the 6-aminohexanoic-spaced oligoarginine ((R-Ahx-R)$_4$), Tat48–60 (Tat), and oligoarginine (R_9F_2) in the absence of endosomolytic agents. Each peptide was conjugated to the PMO targeted to the mutated splicing site of human β-globin pre-mRNA in Kole's splice correction model. In addition, we investigated the internalization mechanisms of these CPP–PMO conjugates and hypothesized mechanisms explaining why (R-Ahx-R)$_4$–PMO is more effective in splicing correction than Tat or R_9F_2 conjugates.

2. Experimental methods

2.1. Synthesis of peptides and of morpholino-peptide conjugates

PMOs were synthesized as described elsewhere [24,25]. CPPs were synthesized using standard FMOC chemistry and purified to >95% purity at AVI BioPharma (Corvallis, OR). R_9F_2–PMO and Tat–PMO were synthesized and purified as described previously [26,27]. The synthesis and purification of (R-Ahx-R)$_4$–PMO is described below. The peptide was conjugated to the nitrogen of a piperazine ring at the 5′-terminus of the PMO. First, a C-terminally reactive peptide-benzotriazolyl ester was prepared by dissolving the peptide acid with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBT) in 1-methyl-2-pyrrolidinone (NMP). The concentration of the peptide was 50 mM. Diisopropylethylamine (DIEA) was added to the peptide solution. The molar ratios of peptide acid:HBTU:HOBt:DIEA were 1.0:1.2:1.3:1.9, respectively. Immediately after the addition of DIEA, the peptide solution was added to a DMSO solution containing either 5′-piperazine-functionalized, 3′-acetyl–PMO or 3′-fluorescein–PMO (13 mM) at 1:1 molar ratio. After stirring at 37 °C for 2 h, the reaction was stopped by adding a four-fold volumetric excess of water. The resulting solution was loaded onto a CM-sepharose (Sigma, St. Louis, MO) column. The unconjugated PMO and other reaction product were washed from the column using 10-column volumes of water. The conjugate was eluted from the column by 3-column volumes of 2 M guanidinium–HCl. The conjugate/salt solution was then loaded onto a HLB column (Waters, Milford, MA), which was subsequently washed with 10-column volumes of water to remove salt. Finally, the CPP–PMO conjugate was eluted off the HLB column with 3-column volumes of 50% CH_3CN and lyophilized. The final products were analyzed by matrix assisted laser desorption ionization time of flight mass spectrometry and HPLC. The purities of the final products were >85%.

2.2. Cells and cell culture

HeLa pLuc705 cells (a generous gift from Dr. R. Kole) were cultured as exponentially growing subconfluent monolayers in DMEM medium (Gibco, Carlsbad, CA) supplemented with 10% FCS, 1 mM Na pyruvate and nonessential amino-acids. CHO-K1, CHO-pgs745 cells were cultured as exponentially growing subconfluent monolayers in F-12K medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum and 2 mM glutamine.