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Cell penetrating peptide conjugates of steric block oligonucleotides ☆

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Abstract

Charge neutral steric block oligonucleotide analogues, such as peptide nucleic acids (PNA) or phosphorodiamidate morpholino oligomers (PMO), have promising biological and pharmacological properties for antisense applications, such as for example in mRNA splicing redirection. However, cellular uptake of free oligomers is poor and the utility of conjugates of PNA or PMO to cell penetrating peptides (CPP), such as Tat or Penetratin, is limited by endosomal sequestration. Two new families of arginine-rich CPPs named $(R\text{-}Ahx\text{-}R)_4$ AhxB and R_6Pen allow efficient nuclear delivery of splice correcting PNA and PMO at micromolar concentrations in the absence of endosomolytic agents. The *in vivo* efficacy of $(R\text{-}Ahx\text{-}R)_4$ AhxB PMO conjugates has been demonstrated in mouse models of Duchenne muscular dystrophy and in various viral infections.

Keywords: CPP; PNA; PMO; Splicing modulation; Nuclear delivery; Bioavailability

Contents

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1. Introduction

The concept of a synthetic oligonucleotide (ON) as a potential therapeutic agent was first demonstrated experimentally through oligodeoxyribonucleotide targeting of a Rous sarcoma virus RNA translation initiation site. The ON was used to form an RNA–DNA duplex that sterically blocked the RNA, resulting in inhibition of gene expression and consequently of viral replication [1]. Later it was found that a second inhibitory mechanism operates in cells. Recognition of the RNA:DNA duplex by the cellular enzyme RNase H results in subsequent RNA cleavage, and hence prevents gene expression. This second mechanism of action became the one primarily pursued and known generally as "antisense". This led to the industrial development of therapeutic DNA-based ON and their analogues for control of gene expression, but sadly so far to little clinical success [2,3].

The original concept of inhibition of protein translation through steric block continued to be studied [4,5], and in recent years ON that are not substrates for RNase H when duplexed to RNA have begun to be developed as therapeutic agents [6]. In addition to inhibition of mRNA translation initiation, ON have also been designed to sterically inhibit critically important targets in RNA processing, such as for example nuclear splicing, a series of events that involve numerous RNA–protein interactions [7]. Further, other small RNAs involved in gene control have been targeted both *in vitro* and *in vivo*, such as endogenous microRNAs [8].

The effective target region of a steric block ON for inhibiting translation is generally limited to the 5'-UTR and the start codon region of a mRNA. One important advantage of the steric block approach of modulating gene expression is its greater specificity, and thus potentially lower off-target effects, compared to conventional antisense, since binding of an ON to a partially matched, inappropriate RNA sequence is unlikely to have biological consequences. A second advantage is the ability to use a considerably wider range of synthetic ON analogues than is possible with conventional antisense, since there is no requirement for molecular recognition by cellular RNase H. Instead, the only key initial necessities for a suitable steric block ON are tight binding to the RNA target as well as good resistance to nuclease degradation. The greater flexibility to manipulate ON chemistry makes it easier also to focus on other essential requirements for therapeutic development, such as good delivery to cells and tissues, low toxicity, and other pharmacological parameters.

The first type of steric block ON to become widely established consisted of fully 2'-*O*-methylated (OMe) nucleosides [9] and also often contained phosphorothioate (PS) linkages [10–12]. PS linkages improve cellular uptake, metabolic stability and pharmacology, but also give rise to reduced RNA binding. Several other types of negatively charged, phosphate-containing analogues have better binding to RNA and higher resistance to nuclease degradation, for example 2'-*O*-methoxyethyl (MOE) (with or without PS linkages) [5] and N3'–P5'-phosphoramidates (NP) [13]. When bound to an RNA target, such analogue types adopt an A-like conformation, similar to that of an oligoribonucleotide.

Locked nucleic acids (LNA) also adopt an A-like conformation and bind very tightly to RNA. For example, LNA ONs have been used for *in vivo* tumour growth inhibition [14]. LNA is generally used in combination with another nucleotide derivative, for example with 2'-deoxyribonucleotides [15] or with OMe residues [16], to obtain optimally balanced binding and specificity characteristics. An alternating LNA/DNA 16-mer containing all PS linkages was found to give efficient splice switching in mice [17]. A luciferase reporter assay was used to show that OMe/LNA mixmers bound strongly to the *trans*-activation responsive element (TAR) of HIV-1 viral RNA to block Tat-dependent *trans*-activation in HeLa cells [18,19].

However, two types of uncharged ON analogues, peptide nucleic acids (PNA) [20] (Fig. 1) and phosphorodiamidate morpholino oligomers (PMO, also known as morpholino) [21] (Fig. 2) have come to dominate steric block applications recently. Although departing significantly from the sugar-phosphate backbone found in regular DNA, oligomers of both types retain very strong and sequence-specific RNA binding characteristics [22,23]. For example, PNAs targeting the 5'-UTR of luciferase mRNA were shown to inhibit translation of protein synthesis [24]. PMOs also have been found to be remarkable steric block ONs for inhibiting translation [25], altering pre-mRNA [26] and blocking miRNA activity [27], as demonstrated in embryos, cells and animals. Now PMOs have been taken to pre-clinical studies for treatment of cardiovascular diseases, viral diseases and genetic disorders, such as Duchenne muscular dystrophy (DMD) (see Section 5).

Despite being charge neutral, PNA and PMO do not enter cells in culture any more readily than do negatively charged ON. For example, many methods of PNA delivery have been devised, such as electroporation, microinjection, transfection in complex with a DNA ON, lipofection of acridine or other polyheteroaromate functionalized PNA, and photochemically-induced delivery. Cell transfection efficiencies are highly variable and sensitive to small variations in conditions and the type of transfection method used, with the best achievable IC_{50} values usually around 200 nM in a model splice redirection system [28]. The relatively high molar levels of these oligomers required to obtain significant steric block action, compared to ON with an RNase H-dependent mechanism of action or short interfering RNA (siRNA), are probably due to the need to deliver at least a stoichiometric amount of the ON to the RNA