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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-Ahx-R)₄ AhxB and R₆Pen 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-Ahx-R)₄ AhxB PMO conjugates has been demonstrated in mouse models of Duchenne muscular dystrophy and in various viral infections. © 2007 Elsevier B.V. All rights reserved.

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

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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 IC₅₀ 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

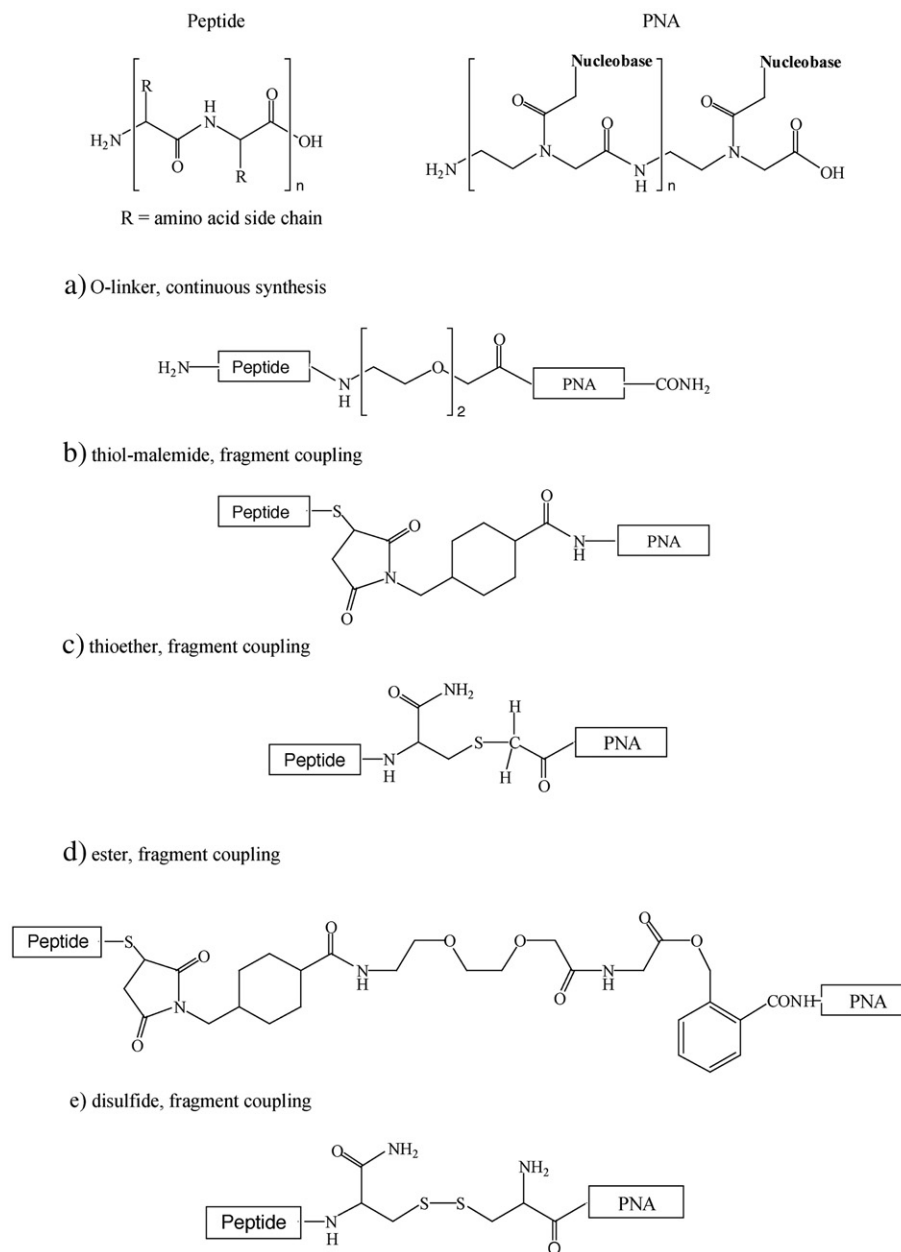


Fig. 1. Structures of peptides and PNA and various types of peptide–PNA conjugate linkage.

target into the right cell compartment to obtain the desired biological effect.

One idea to enhance cell delivery was first proposed 20 years ago, the covalent conjugation of ON to cationic peptides [29]. Later, conjugates of an antisense ON with a peptide derived from helix 3 of the *Antennapedia* homeodomain protein from *Drosophila melanogaster* (residues 43–58, Penetratin) were shown to have biological activity in neuronal cells, which was attributed to the cell penetrating properties of the peptide [30–32]. Another cell penetrating peptide (CPP) was discovered in the basic region of HIV-1 Tat protein (residues 48–60) [33]. Many CPPs (also known as “protein transduction domains”) with remarkable properties of cell penetration either on their own, or as conjugates with a range of other types of biomolecules, such as peptides, proteins, liposomes and nanoparticles have now been

characterised (reviewed in [34]). However, very few examples of strong delivery and biological activity of negatively charged ON or siRNA cargoes into cells through covalent attachment of a CPP have been published (reviewed in [35–37]), and in many cases, the conjugation of a peptide does not allow sufficient cargo delivery through the endocytotic pathway and into the cell compartments necessary for biological activity.

By contrast, CPP conjugates have shown significant promise towards improving cell delivery of charge neutral PNA and PMO. For PNA–peptides, an early example was the blocking of expression of the galanin receptor mRNA in human Bowes cells by a 21-mer PNA disulfide-coupled to the CPPs Penetratin or Transportan (a hybrid of a section of the neuropeptide galanin and the wasp venom peptide mastoparan), which constituted a step towards the modification of pain transmission [38].

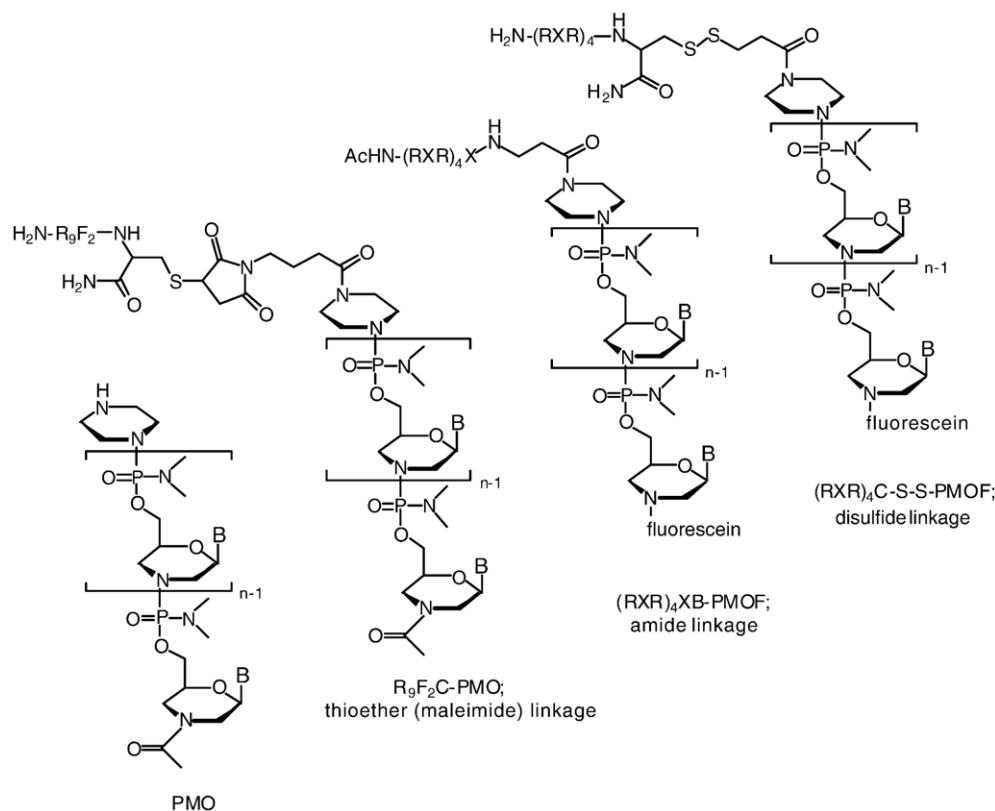


Fig. 2. Structure of PMO and three types of peptide–PMO conjugate linkages. R=arginine, Ahx=6-aminohexanoic acid, B=beta-alanine and F=phenylalanine.

Remarkably potent inhibition (down to 70–80 nM EC_{50}) in the same model was claimed with shorter PNAs targeting different regions of the mRNA and conjugated to a truncated Transportan version TP10 [39]. This is exceptional, since most cellular inhibition of protein translation by PNA has been achieved only in the μ M range. For example a 13-mer PNA disulfide-linked to Penetratin was able to inhibit telomerase activity in JR8 melanoma cells with $IC_{50} \sim 7 \mu$ M [40]. In a different model system, an amphipathic peptide, MAP, disulfide-conjugated to a PNA complementary to the nociceptin/orphanin FQ receptor mRNA was shown to impart improved cell uptake and steric block biological effects in both CHO cells and neonatal rat cardiomyocytes again in the μ M range [41]. When linked to a D (AAKK)₄ peptide, a 19-mer PNA complementary to human caveolin 1 mRNA inhibited expression in HeLa cells ($IC_{50} = 2 \mu$ M) and in primary endothelial cells at concentrations $> 15 \mu$ M [42].

CPP–PMO conjugates have been used successfully in cell culture and animal models to inhibit the replication of several viruses that are harmful to humans, including for example Coxsackievirus B3, SARS coronavirus, influenza, dengue, West Nile and Ebola viruses (see Section 6) [43–49].

In addition to the applications of CPP–PNA and CPP–PMO in translational blocking mentioned above, PMOs and PNAs delivered by CPP have been used to alter pre-mRNA splicing. 3–10 μ M of a PNA 18-mer with 4 added Lys residues (PNA-4 K) complementary to a splice site (654) within an enhanced green fluorescent protein (EGFP) reporter gene containing an aberrant human β -globin intron was enough to result in some

splice correction and up-regulation of EGFP in a HeLa cell culture assay [12]. In transgenic mice, PNA-4 K 654 showed activity in several tissues (heart, kidney lung, liver, muscles and small intestine) when delivered intraperitoneally at 50 mg/kg for up to 4 days, with up to 40% splicing correction observed [50].

CPP–PMO conjugates have also been used effectively to alter pre-mRNA splicing in DMD models. DMD is usually caused by nonsense or frame-shifting exon mutations in the dystrophin gene that result in an absence of functional protein. (R-Ahx-R)₄AhxB–PMO (R=arginine, Ahx=6-aminohexanoic acid and B=beta-alanine) conjugates have been used in several models to promote skipping of the mutated exon to produce internally shortened but functional dystrophin protein. An (R-Ahx-R)₄AhxB–PMO conjugate was much more effective to cause desired exon-skipping in primary muscle cells isolated from a golden retriever muscular dystrophy dog than the corresponding non-conjugated PMO or OMe ON. A 300 nM extracellular concentration of the conjugate caused complete exon-skipping in the muscle cells. Ten days after a single treatment, conjugate-treated cells continued to show abundant exon-skipped RNA product [51]. Another (R-Ahx-R)₄–PMO conjugate caused desired exon-skipping in both *mdx* mouse muscle explants [52] and in several muscles of the intact *mdx* mice [53] (see Section 5).

(R-Ahx-R)₄AhxB–PMO altered pre-mRNA splicing in primary murine splenic CD4⁺ T-cells. The PMOs were targeted to splice acceptor sites of the pre-mRNAs of the cell surface signalling protein OX-40 and the nuclear transcription factor Foxp3. Treatment of the T-cells with 4 μ M of the conjugates resulted in marked reduction of OX-40 and Foxp3 protein

expressions up to 24 h after removing the conjugates, and detection of the splice-altered OX-40 and Foxp3 gene products 48 h after removing the conjugates [54].

Several studies have used a model cell culture system involving splicing redirection of murine CD40 pre-mRNA to cause a down-regulation of activity. A PNA complementary to the splice site with 8 Lys residues on the N-terminus was significantly more active in cell culture than a similar PNA with 4 Lys residues, but still 3–10 μM concentrations were needed for strong down-regulation of this target in BCL₁ cells or primary murine macrophages [55]. A variety of stably linked PNA conjugates with variously spaced oligo-Lys peptides showed some small enhancements of splicing redirection activity in some cases compared to Lys₈ [56], and a series of 28 cationic peptides based on Lys or Arg that had amphipathic character showed even stronger splicing redirection activity at 1–6 μM concentrations, and without toxicity [57], but *in vivo* efficacies for these constructs have not yet been reported.

A particularly useful splice redirection model system, developed by Kole and colleagues is based on the use of a stably integrated plasmid containing a gene coding for firefly luciferase interrupted by an aberrant β -globin intron with an in-frame stop codon [58]. Up-regulation of luciferase resulted when an 18-mer PS/OMe oligonucleotide complementary to the aberrant 705 splice site is introduced into the HeLa cells to redirect splicing [12,50]. Use of this sensitive splicing redirection model allows optimization of CPP conjugate technology for delivery of PNA or PMO into the nuclear compartment.

2. Synthesis of ON–CPP conjugates

Since in our experience the conjugation of CPP to negatively charged ON did not result in a level of delivery into cells sufficient for biological activity [59], we concentrate here on PNA- and PMO–peptide conjugates which have been much more successful. General reviews describing methods of synthesis of peptide–ON conjugates have been published [60,61].

2.1. Synthesis of PNA–peptides

Peptides and PNA are synthesized through similar sequential coupling reactions of monomer units on a solid support to form amide bonds. Strategies for manual or automated PNA synthesis by 9-fluorenylmethoxycarbonyl/benzhydryloxycarbonyl (Fmoc/Bhoc) or *tert*-butyloxycarbonyl/carbobenzoyloxy (Boc/Cbz) routes are well established [62–64]. Thus peptide–PNA conjugates may be assembled by continuous synthesis with activated amino acids or activated PNA monomers being added in the coupling reactions. In general there are no restrictions in combining amino acids with PNA residues and these may be added in any order. It is common to include at least one Lys residue on the end of a PNA synthesis to aid water solubility. Fmoc/Bhoc chemistry is more often preferred for PNA assembly and we have reported protocols for PNA and PNA–peptide syntheses using a robotic Apex 396 Synthesizer [65]. A short polyether spacer (AEEA, 8-amino-3,5-dioxo-octanoic acid, also known as an O-linker) is often added between the

peptide and PNA moieties in continuous synthesis (Fig. 1a) [66,67].

Alternatively, fragment conjugation of pre-synthesized peptide and PNA components in solution is usually more convenient for structure-activity (SAR) studies and the overall yields and purities of the final conjugate are generally higher. Each fragment is synthesized separately on solid-phase and contains a specific functionality on one end. Each fragment is purified prior to the conjugation step. Usually, an excess of one component is added over the other to drive the conjugation reaction to completion and the product conjugate is purified by HPLC and characterized by mass spectrometry. No general rules have yet emerged as to optimal linkage types, since the factors affecting biological activity are often complex. For example, in some studies, use of labile disulfide linkages has resulted in higher biological activities [67,68]. By contrast, a stable thioether linkage or a disulfide linkage gave equally good results in a splicing redirection system [69].

Several types of stable linkage have been reported. For example, a thiol–maleimide linkage requires addition of a Cys residue to the CPP and preparation of a N–maleimide derivative of the PNA, which may be obtained by reaction of the terminal primary amino group with the hetero-bifunctional cross-linker succinimidyl 4-[N–maleimidomethyl]cyclohexane-1-carboxylate (Fig. 1b) [70]. A thioether linkage may be formed between a bromoacetyl ON and a Cys-containing peptide [71] and we have adapted this reaction for conjugation of a CPP containing a Cys residue at the C-terminus with N-terminally bromoacetylated PNA (Fig. 1c) [69].

An ester-containing conjugate is synthesized with a PNA bearing a glycyloxybenzoic acid residue at the N-terminus, and this linkage was designed to be labile to esterases within cells (Fig. 1d) [68]. However, the most popular labile linkage is a disulfide bond between the peptide and the CPP (Fig. 1e), which was proposed to be cleavable within the reducing environment of the cell, and which was first used for studies of Transportan–PNA and Penetratin–PNA conjugates [38]. Both CPP and PNA are functionalized with a Cys residue, and the disulfide bond is formed between the Cys thiol groups. One of the thiols is activated by reaction with either a pyridylsulfenyl or a 3-nitropyridylsulfenyl group. We have used the disulfide linkage predominantly in studies of HIV Tat-dependent *trans*-activation inhibition and in splicing redirection [67,69]. The disulfide linkage is convenient for *in vitro* and cell studies, even though it may be less suitable for *in vivo* use.

2.2. Synthesis of PMO–peptides

PMO syntheses have been described previously [21,72], while the CPPs may be synthesized by standard Fmoc chemistry. The conjugation of CPPs and PMOs through a thioether (maleimide), disulfide or amide linker have been described previously [73–75] and the structures of these conjugates are shown in Fig. 2.

The effect of linker type on the activity [74,75] and stability [76] of CPP–PMO conjugates has been determined. The nuclear antisense activities of the CPP–PMOs with the three

linkage types were similar ([74] and Moulton, H. M, unpublished results). Synthetically, the amide linkage has two advantages over disulfide and thioether linkages. First a PMO can easily be conjugated to a CPP via an amine at either end of the oligomer. Second, formation of an amide bond requires only a one-step synthetic and purification scheme as compared to a two-step strategy for other linkages. Therefore, the amide linkage leads to an overall greater yield of CPP–PMO. In addition, amide bonds appear to be more stable *in vivo*, since an unhindered disulfide-linked CPP–PMO was found to be unstable in human serum [76].

Although a CPP can be conjugated to either end of a PMO, the position of the CPP affects the activity of the attached PMO. A PMO having a CPP conjugated at the 5'-end was more functionally active than that with the CPP attached at the 3'-end [74]. Adding a bulky fluorochrome such as carboxyfluorescein at the 3'-end of PMO decreased the activity of the CPP–PMO by about 30% (unpublished data).

3. Our experience with CPP delivery of ON

As pointed out in Section 1, at variance with numerous reported successes for peptide payloads [77], successful delivery of antisense ON by CPPs has not often been documented.

Nielsen and colleagues [78] investigated the cellular uptake of several PNA–CPP constructs in live cells to avoid artefacts of redistribution caused by cell fixation. The PNA-Tat and PNA-Penetratin constructs were taken up by an energy-dependent mechanism and essentially accumulated in endocytotic vesicles but not in nuclei, in keeping with our own data [79]. Although not encouraging at first sight, these data could not predict whether biological effects would be achievable or not. For example, even though fusion constructs between Cre recombinase and Tat CPP seemed to remain localized in endocytotic vesicles, they gave rise to significant biological activity [80].

As mentioned in Section 1, many groups in the field of ON delivery are now relying on the splicing redirection model of Kole [58] to assess the nuclear delivery of steric block ON. This model is useful by virtue of being highly sensitive and sequence-specific, by providing a positive read-out over a low background level, and in giving rise to a linear response over a large range of concentrations. Luciferase expression is however highly sensitive to cell density and other variables as pointed by Bendifallah et al. [68]. RT-PCR evaluation of splicing correction is easy to implement, gives an estimation of the proportion of mis- versus correctly-spliced luciferase mRNA, and it may therefore be preferred to luciferase assays.

Tat- and Arg–PMO constructs gave rise to a sequence-specific correction of mis-spliced luciferase pre-mRNA. However high concentrations (5 μ M and higher) of the correcting steric block ON were required to achieve luciferase expression [73,75]. As a point of comparison, OMe and MOE ON rapidly accumulated in nuclei and led to splice correction at doses as low as 10 nM when transfected with cationic lipids [81]. Likewise, Tat-, Arg- and Penetratin-PNA constructs were poorly efficient in the splice redirection assay at low concentrations [69].

A series of publications reported successful splicing correction with PNA appended to short oligolysine chains, as reviewed in Section 1. Intriguingly, splicing correction was claimed to be energy-independent [12], at variance with most recent data on the mechanism of cellular uptake of basic amino acid-rich CPP-conjugated ON [82]. In our hands, (Lys)₈–PNA conjugates were efficiently taken up in cells as monitored by FACS analysis and by fluorescence microscopy, but their biological activity remained rather low, in keeping with our previous data with Tat- or Arg-rich steric block ON conjugates [83]. Interestingly, cellular uptake was clearly energy-dependent at low concentration, but became temperature-independent at high concentration, in parallel with membrane destabilization as monitored by propidium iodide uptake [83]. Endocytosis may be thus the predominant mechanism of uptake at low concentrations, while direct translocation linked to CPP-induced membrane destabilization could take place at higher concentrations. CPP-induced membrane permeabilization may itself be rather dependent on peptide, cell type and experimental conditions, providing a possible explanation for the ongoing controversy concerning CPP mechanism of cell uptake. Sequestration of internalized material in endocytotic vesicles could also explain the observed low biological activity of these various CPP conjugates. Indeed, treatment with endosomolytic agents, such as chloroquine or high sucrose, gave rise to a large increase in splice correcting activity even when added after CPP–PNA internalization [83].

Similarly, in a HeLa cell assay containing integrated luciferase reporter plasmids, cationic lipid-transfected OMe/LNA ONs targeting the HIV-1 TAR element were able to dose-dependently inhibit HIV-1 Tat-dependent *trans*-activation [18]. However, most CPP conjugates (such as Tat and Pen) of PNA targeted to TAR when incubated with the HeLa cells were unable to inhibit *trans*-activation unless chloroquine was added, and even the few CPP–PNA that did show some inhibition required 24 h incubation to show activity [67]. Endosomal sequestration appeared to be the explanation for these results.

Alternative endosome disrupting strategies have been evaluated using the splicing correction assay. Addition of Ca²⁺ at high concentration (6 mM) to the culture medium strongly increased splicing correction by Tat- or (Arg)₆ PNA constructs [84]. Evidence for a partial redistribution from endocytotic vesicles to cytoplasm was obtained but no nuclear staining was observed. Likewise chloroquine treatment improved functional activity of CPP–PNA or CPP–PMO conjugates, but we were unable to detect nuclear accumulation of conjugate or oligomer (Abes et al., unpublished observations). In any event, none of the above endosome-disrupting strategies is likely to be feasible *in vivo*. Therefore several groups have explored alternative strategies.

Co-treatment with endosome-disruptive peptides has been studied extensively to improve plasmid DNA transfection with non-viral delivery vectors. Both synthetic (mostly amphipathic) and natural (mostly viral) endosome-disruptive peptides have been described, the most popular being the N-terminal peptide of Influenza virus HA2 protein ([85] and references therein).

A HA2-Pen fusion construct only slightly increased splicing correction by Tat-, Pen- or Transportan-PNA conjugates [86]. Likewise, the addition of several natural and synthetic membrane destabilizing peptides to Tat-PNA had only a modest effect on splicing correction at non-cytotoxic doses [87].

Photochemical internalization (PCI) capitalizes on the production of reactive oxygen species, which damage endosomes upon irradiation, thus facilitating escape of endosome-entrapped drugs [88]. PCI has been exploited successfully to increase the uptake of various biomolecules from low molecular weight drugs to plasmid DNA. Bøe and Hovig [89] have documented PCI-induced gene silencing with several PNA–CPP constructs together with re-localization of the PNA–peptides from endosomes to nuclei. Shiraishi et al. provided similar data with PNA–CPP constructs aimed at splicing correction [90]. PCI thus represents an interesting strategy to overcome endosomal entrapment of CPP-ON, since it is amenable to *in vivo* development [91].

4. Design and structure-activity studies on RXR and R₆Pen conjugates

Structure-activity studies of CPPs derived from proteins (Tat, Penetratin) and synthetic CPPs have emphasized the crucial role played by basic amino acids and particularly by the guanidinium side-chain of arginine. Such basic amino acids are responsible for binding to cell surface heparan sulfate proteoglycans (HSPG) through electrostatic interactions and for subsequent cell internalization.

4.1. RXR peptides

The RXR family of synthetic CPPs was first proposed by J. Rothbard, P.A. Wender and their colleagues on the assumption that not all Arg side chains in an oligo Arg peptide would point in the same direction and would therefore be available for HSPG (or membrane lipid polar heads) binding. They accordingly designed and synthesized a large collection of (RXR)_nR, (RX)_nR, (RXX)_nR and (RXXX)_nR transport peptides in which X represents various natural or non-natural amino acids [92]. All peptides were conjugated to fluorescein and assayed for cellular uptake in a Jurkat T cell line. These studies emphasized the crucial role of Arg side chain spacing for cellular uptake, and the most efficient peptide was (R-Ahx-R)₄R in which Ahx represents a 6-atom aminohexanoic acid spacer. This strategy of interspersing Arg residues with non-natural, uncharged amino acids also appeared interesting in decreasing the number of natural peptide bonds (thus providing increased metabolic stability) and in decreasing the positive charge density often responsible for cytotoxicity. However, no studies of biological responses were reported, although the synthesis of conjugates to cyclosporin or acyclovir has been described [93]. (R-Ahx-R)₄ was chosen therefore as a lead peptide and has been stably conjugated to a PMO oligomer via a Ahx-β-Ala linker. At variance with Tat–PMO and Arg₆–PMO conjugates, (R-Ahx-R)₄AhxB–PMO leads to a dose-dependent and specific increase of luciferase expression in the absence of endosomolytic agents.

Importantly a significant increase could already be observed at sub-micromolar concentration [75].

As mentioned above, measurement of luciferase expression allows the functional comparison of various CPP constructs but does not allow the quantification of splicing redirection. We therefore made use of RT-PCR which easily discriminates correctly from aberrantly-spliced luciferase transcripts and confirmed that highly significant splicing correction could be achieved at μM concentrations of (R-Ahx-R)₄AhxB–PMO. (R-Ahx-R)₄ has also been stably conjugated to a splice correcting PNA oligomer and found to be effective in the same concentration range [87]. Interestingly for *in vivo* applications, the (R-Ahx-R)₄ constructs did not show membrane permeabilization at doses up to 50 μM (i.e. concentrations at which Tat-, (Lys)_n- or (Arg)_n- constructs lead to significant propidium iodide uptake).

We then compared the mechanism of cellular internalization of fluorochrome-labelled (R-Ahx-R)₄AhxB–PMO, Tat–PMO and (Arg)_n–PMO in a search for a mechanistic explanation for their different efficiencies. Surprisingly, the (R-Ahx-R)₄ construct was taken up less efficiently than Tat- or (Arg)_n ones [75], at variance with the data reported by Rothbard et al. [93]. An increased activity could be explained by a non-endocytotic uptake route, but studies involving energy deprivation or use of endocytosis inhibitors did not support this hypothesis [75] and further studies are needed to clarify this issue. Along the same lines, fluorescence microscopy studies in live cells revealed that the majority of (R-Ahx-R)₄AhxB–PMO distributes as dotted cytoplasmic spots, characteristic of accumulation in endocytotic vesicles [75]. Similar conclusions are reached when using a functional assay such as splicing redirection to assess the energy-dependence of the (R-Ahx-R)₄ AhxB–PMO conjugates (Fig. 3).

As expected, (R-Ahx-R)₄AhxB–PMO binds to cell surface HSPG as revealed by both genetic and biochemical tools [72]. Of possible interest, (R-Ahx-R)₄ constructs bind to Sepharose-immobilized heparin (taken as a model heparan sulfate) with a

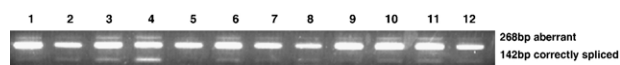


Fig. 3. (R-Ahx-R)₄AhxB–PMO conjugates are internalized by an energy-dependent mechanism. A splicing correction assay was used to assess the energy-dependence by ATP deprivation (lanes 5–8) or by low temperature incubation (lanes 9 to 12). HeLa pLuc 705 cells were pre-incubated for 1 h at 37 °C in OptiMEM, supplemented with 10 mM sodium azide and 6 mM 2-deoxy-D-glucose or at 4 °C in OptiMEM. Cells were then incubated with the PMO conjugates at the indicated concentrations for 4 h in the same conditions. Cells were then washed and incubation was continued at 37 °C for 20 h in DMEM. Total RNA was extracted and amplified by RT-PCR. PCR products from incorrectly (268 bp band) and correctly (142 bp band) spliced luciferase pre-mRNA were separated on a 2% agarose gel. Lanes 1, 5 and 9: controls in the absence of (R-Ahx-R)₄ AhxB–PMO conjugate, 2–4: cells incubated at 37 °C and treated with 0.5, 1 or 2.5 μM (R-Ahx-R)₄ AhxB–PMO, respectively, 6–8: cells incubated at 37 °C in the presence of 10 mM sodium azide and 6 mM 2-deoxy-D-glucose and treated with 0.5, 1 or 2.5 μM (R-Ahx-R)₄ AhxB–PMO, respectively, and 9–12: cells incubated at 4 °C and treated with 0.5, 1 or 2.5 μM (R-Ahx-R)₄ AhxB–PMO, respectively.

significantly lower affinity than Tat- or (Arg)_n-constructs [72]. This could lead to improved efficiency, if one postulates that HSPG-bound ligands need to dissociate after cellular internalization in order to escape from endocytotic vesicles.

SAR studies with a series of (RXR)₄AhxB-PMO constructs in which X varies in terms of length, affinity for heparin or hydrophobicity have been conducted (Abes et al., in preparation). These studies demonstrate that increased hydrophobicity or affinity for heparin improves cellular uptake, but is detrimental to the efficiency of splicing correction.

As briefly mentioned above, (R-Ahx-R)₄ has a lower number of adjacent natural amino acids than other CPPs, a factor which could increase its metabolic stability and consequently its biological efficiency. Although serum stability was indeed increased, analysis of intracellular cleavage products did not support this hypothesis [94]. Furthermore, the introduction of D-Arg residues to give (DR-Ahx-R)₄-R-PMO, for the purpose of increasing resistance to proteolysis, did not lead to increased splicing correction efficiency, thus indicating that metabolic stability did not limit functional efficacy (Abes et al., in preparation).

Altogether, no definitive explanation for the superior activity of (R-Ahx-R)₄ over other Arg-rich CPPs can be provided at present. Further SAR and endosomal escape studies will hopefully shed light on this mystery and will contribute to the design of more active steric block ON delivery vectors. We note recent results from use of fluorescent microscopy and the splice correction assay, which show that a large percentage of the (R-Ahx-R)₄AhxB-PMO that enters cells appears to localize in endocytotic vesicles, even at doses that produce nearly complete splicing correction. These results are not incompatible, since steric block ON conjugates were applied at μM concentrations, while the mis-spliced pre-mRNA target is expressed at a far lower molar concentration.

4.2. R₆Pen peptide

A second class of Arg-rich CPPs that we have been studying, known as R₆Pen, is based on the well known Penetratin peptide [95] to which six Arg residues are added at the N-terminus. First synthesized for conjugation to an OME/LNA oligonucleotide [59], R₆Pen was disulfide-conjugated to a 16-mer PNA complementary to the HIV-1 *trans*-activation responsive element TAR and was found to enter the HeLa cell nuclei and to inhibit Tat-dependent *trans*-activation in a luciferase plasmid assay system at sub-micromolar concentrations both dose-dependently and PNA sequence-dependently [67]. Activity could be achieved more readily when the R₆Pen-PNA was incubated with HeLa cells in the presence of the endosomolytic reagent chloroquine, suggesting an endocytotic mode of cell uptake [67].

Similarly to (R-Ahx-R)₄-PNA or PMO conjugates, an R₆Pen-PNA conjugate targeted to the 705 splice acceptor site showed potent and sequence-specific correction in the HeLa 705 cell assay at low concentrations in the absence of endosomolytic agents [69]. The mechanism of cell internalization of R₆Pen-PNA705 has not yet been thoroughly investigated, but it is clearly energy-dependent and likely to involve endocytosis (Abes et al., unpublished results).

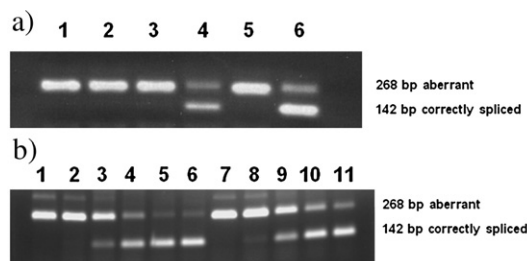


Fig. 4. RT-PCR analysis of splice correction by R₆Pen-PNA conjugates. (a) HeLa pLuc705 cells were incubated for 4 h in OptiMEM in the absence (control), in the presence of 1 μM PNA705 alone or in the presence of 1 μM CPP-PNA705 conjugates. Total RNA was extracted 20 h later and amplified by RT-PCR. PCR products from incorrectly (268 bp) and correctly (142 bp) spliced luciferase pre-mRNA were analysed on a 2% agarose gel. Lane 1: control, 2: PNA705 alone, 3: Pen-ss-PNA705, 4: R₆Pen-ss-PNA705, 5: R₆Pen-ss-scrambled PNA705, 6: R₆Pen(W-L)-ss-PNA705. (b) Dose dependencies of splice correction using 1 μg of total RNA extracted, amplified by RT-PCR and analyses as in (a). Lane 1: control of untreated cells, 2–6: cells treated with 0.25, 0.5, 1, 2 or 4 μM R₆Pen-ss-PNA705 respectively and 7–11: cells treated with 0.25, 0.5, 1, 2 or 4 μM stably linked R₆Pen-PNA705 respectively (This figure is reproduced from Fig. 7 of Abes, S. et al. (2007) *Nucleic Acids Res.*, 35, 4495–4502).

When employing the splicing redirection assay using RT-PCR to measure the levels of aberrantly and correctly-spliced RNA transcript, R₆Pen is the most active of all CPP-PNA conjugates investigated so far by our group (Fig. 4a). It is not yet clear why R₆Pen is more efficient than other CPPs, such as Penetratin. For example, an essential difference has been documented using a mutant of the R₆Pen peptide in which a Trp residue known to be essential for Pen peptide to penetrate membranes has been replaced by a Leu residue. This R₆Pen (W → L) mutant is even more active than the wild-type R₆Pen version (Fig. 4a) [66]. This suggests that the mechanism of cell entry and release into cytosol and nucleus must differ significantly between the R₆Pen-PNA conjugate and the Pen peptide. Further, the importance of the appended oligo-Arg tail is affirmed by varying the number of Arg residues. Neither shorter nor longer tails are as efficient as the original R₆Pen peptide [66]. Further SAR studies are underway aimed at understanding better the roles of various domains of the R₆Pen peptide, at enhancing conjugate activity and serum stability, and at decreasing cytotoxicity.

An often-debated issue is the nature of the linker between the CPP delivery vector and the transported payload, as mentioned in Section 1. The role of a delivery vector is obviously to transport a cargo to its intracellular site. Intracellular dissociation between the two entities would intuitively be preferred, since it would be predicted to avoid steric hindrance or other potential complications. However, the situation is more complex for steric block ON analogues, since a bound, highly charged delivery peptide should increase affinity for the targeted RNA sequence and favour invasion of secondary structures, thus leading to enhanced activity [96]. We thus compared splicing correction efficiency and the specificity of PNA sequences conjugated to R₆Pen through a stable (thioether) or a reducible (disulfide) linker. The disulfide-linked

material is only slightly more efficient than the stably linked one (Fig. 4b). All data gathered so far with stably linked (R-Ahx-R)₄ or R₆Pen conjugates indicate that the appended CPPs do not prevent target recognition and do not alter oligomer sequence specificity. Alternatively, the similar activity of the stably- and disulfide-linked material could be due to equal rates of intracellular degradation of the peptidic delivery vector of both compounds.

5. *In vivo* data on splicing modulation, bioavailability and toxicity

5.1. *In vivo* data on splicing modulation

DMD is an X-linked recessive disease causing muscle degeneration and decreased span and quality of life in affected humans. A mouse model of muscular dystrophy (*mdx*) has been used to study the effectiveness of free PMO and CPP–PMO conjugate. *Mdx* mice carry a nonsense mutation in exon 23. Excision of exon 23 in *mdx* mouse cells can restore mostly-functional dystrophin expression [26]. Unconjugated PMO targeted to a donor site of exon 23 partially restored dystrophin function in skeletal muscles of *mdx* mice at 100 mg/kg/dose intravenously [26] or at 25 mg/kg/dose intra-peritoneally [97], both with 7 consecutive weekly doses. The PMO conjugated to (R-Ahx-R)₄AhxB CPP produced high efficacy when administered intra-peritoneally at 5 mg/kg /dose once a week for 4 weeks [53]. The dystrophin level in diaphragm muscles of *mdx* mice, detected two weeks after a single intra-peritoneal injection of (R-Ahx-R)₄AhxB–PMO, was similar to the levels in normal mice. Four doses of the conjugate at 5 mg/kg/dose, administered once a week for four weeks to neonatal mice, resulted in normal levels of dystrophin in the diaphragms of 6-week-old mice. At 22 weeks post the fourth injection, dystrophin was still detectable in the diaphragm. The conjugate effect was lower in the tibialis anterior, gluteus maximus, and triceps brachialis, weaker still in the colon and stomach, and absent in the heart. Western blots detected normal levels of dystrophin in the diaphragm at six weeks, lower levels in the tibialis and gluteus, but no dystrophin was detected in cardiac muscle. Animals of one day, four weeks or one year of age were treated to test the effect of age. Whilst splice-modified dystrophin expression increased for all ages treated, muscle architecture was most improved in mice treated at younger age. Normalized pathology and central nucleation were observed for the sections taken from the 6-week-old mice treated with (R-Ahx-R)₄AhxB–PMO. The study revealed that treatment of DMD mice at a young age is more beneficial than at an older age [53].

5.2. Tissue distribution, pharmacokinetics and stability

Tissue distribution and pharmacokinetics of a PMO and its corresponding (R-Ahx-R)₄AhxB conjugate, administered intravenously, have been determined in rats [98]. Conjugation of a PMO to the (R-Ahx-R)₄AhxB peptide increased the tissue uptake and retention [98]. The CPP conjugation increased uptake of PMO in all organs assayed, except in brain, with

greater increases being seen in liver, spleen and lungs. Remarkably, a similar amount of PMO was still detected in the liver 5 days after the last dose, indicating that the conjugated PMO had a long retention time in this organ. Interestingly, the concentration of conjugated PMO in rat spleens assayed 5 days after the last dose was twice the concentration in rat spleens assayed 1 day after the last dose. These results suggest that efflux of the conjugated PMO from tissues to the vascular space is slow, which is an important consideration for its therapeutic applications. Conjugation of PMO to (R-Ahx-R)₄AhxB improved the kinetic behaviour of PMO as demonstrated by a 4-fold increased in volume of distribution, 2-fold increase in the estimated elimination half life, and an increased area under the plasma concentration versus time curve.

The stabilities of 12 CPP–PMO conjugates varying in CPP sequences, amino acid compositions and/or linkers were studied in cells and in human serum. The stability of the peptide moiety in serum ranked in the order D-CPPs=(R-Ahx)₈B=(RB)₈B>(R-Ahx-R)₄AhxB>R₉F₂C=Tat. Stabilities of peptide moieties within cells were found to be D-CPPs>(RB)₈B>(R-Ahx)₈B=(R-Ahx-R)₄AhxB=R₉F₂C=Tat. Stabilities of linkers in serum or in cells were found to be amide = maleimide > disulfide [76]. The stability of a (R-Ahx-R)₄AhxB–PMO was determined in rat serum and organ lysates. The CPP portion of the conjugate exhibited time- and tissue-dependent degradation, with biological stability ranked in the order of liver > heart = kidney > plasma. Up to 6 h, the CPP portion of CPP–PMO was partially degraded in the plasma but had no apparent degradation in tissues [98]. The PMO portion of the conjugates was completely stable in cells, serum, plasma and tissues [76,98].

5.3. Toxicity

The toxicity of (R-AhxR)₄AhxB–PMOs is caused by (R-Ahx-R)₄AhxB while the PMO portions of the conjugates are essentially non-toxic [48,98]. The degree of toxicity depends on the dose, dose frequency and route of administration. Mice tolerated (R-Ahx-R)₄AhxB–PMOs well with repeated doses at ≤ 10 mg/kg [43,48,53,98,99]. Four daily i.p. injections into mice at 3.7 mg/kg of an (R-Ahx-R)₄AhxB–PMO conjugate did not produce any observable toxicity [99]. No changes in mice behaviour, weight and serum chemistry were observed for the *mdx* mice injected intraperitoneally with the conjugate at 5 mg/kg once a week for four weeks [53]. No histopathological abnormalities were detected in heart, liver, kidney, spleen and pancreas after two i.v. doses of an (R-Ahx-R)₄AhxB–PMO conjugate administered 48 h apart at 10 and 15 mg/kg [43]. However, at higher doses and dosing frequency, animals experienced weight loss. Mice lost weight after five daily doses (i.p.) at 15 mg/kg of an (R-Ahx-R)₄AhxB–PMO conjugate despite maintaining their normal organ weights and appearances. Fibrosis was found in the livers of treated animals. These mice regained their normal weights after the treatment was stopped [48]. Five daily intravenous injections of an (R-Ahx-R)₄AhxB–PMO to rats at 15 mg/kg did not cause any detectable toxicity, as measured by behaviour, appearance, eating/drinking habits, body weight and

serum chemistry. Rats treated with a single 150 mg/kg dose appeared lethargic immediately after the injection and proceeded to lose weight, accompanied by elevated serum BUN and creatinine, indicating that kidney function was most likely affected. The LD₅₀ of an (R-Ahx-R)₄AhxB-PMO in rats was around 220–250 mg/kg [98].

6. Perspectives from *in vivo* applications

The ability of CPPs to deliver steric block PNA and PMO for treatment of viral and genetic diseases has been demonstrated in a number of publications both in cell culture and *in vivo*.

Very few studies have been carried out so far *in vivo* with CPP-PNA conjugates. For example, PNA-Lys₄ showed some activity in several tissues (heart, kidney lung, liver, muscles and small intestine) when delivered i.p. at 50 mg/kg for up to 4 days in transgenic mice with the EGFP 654 splice correction model without showing acute toxicity [50]. An 18-mer PNA complementary to the enhancer E_μ intronic sequence and stably linked to a 7-mer NLS peptide inhibited *c-myc* oncogene expression in Burkitt's Lymphoma (BL) cells [100] and showed very little toxicity in a human tumour SCID mice model [101]. When inoculated subcutaneously, the NLS-PNA showed inhibition of BL cell-induced tumour growth in SCID mice [102]. A PNA conjugated to K₈ showed broad tissue distribution in mice after i.v. injection and a relatively slow excretion level [56], whereas two Lys/Arg-rich amphipathic D-peptides stably conjugated to a PNA distributed substantially differently in mice [57].

By contrast CPP-PMO conjugates have been taken into numerous pre-clinical studies. For example, CPPs have been shown to be powerful vectors to deliver PMOs into the cytosol of cells. There have been a number of reports of CPP-PMO inhibition of virus replication in cell cultures and in mouse models. Coxsackievirus B3 is a picornavirus and a significant cause of myocarditis, especially in children and adolescents. An (R-Ahx-R)₄AhxB-PMO targeting the Internal Ribosome Entry Site (IRES) in the 5' UTR of the Coxsackievirus B3 viral genome reduced viral titer by 4 logs in virus infected cells in culture. Two 10 mg/kg doses of the IRES-targeted compound, administered intravenously once 3 h before and once 2 days after infection with virus, reduced heart tissue damage and viral titer by about 100-fold, when measured at one week post-infection [43]. The recently emerged severe acute respiratory syndrome (SARS) coronavirus raised the threat of a worldwide pandemic in 2002–2003. Experiments in cultured cells showed that antisense R₉F₂C-PMO dramatically reduced virus-induced cytopathic effects, viral titers, and cell to cell spread of the virus [44]. Recently, in mouse models with a variety of murine coronaviruses, an (R-Ahx-R)₄AhxB-PMO, administered intraperitoneally, reduced liver damage and viral titers, reflecting the improved survival observed [99].

Influenza A virus (FLUAV) causes a considerable impact on human health every year, and also poses a pandemic threat. A panel of CPP-conjugated PMO targeted against various locations in FLUAV gene segments involved in viral RNA synthesis showed that a PB1- and/or a NP- directed compound were

found to markedly inhibit the replication of all viral strains tested (including H1N1, H3N2, H7N7, H3N8 and H5N1). Both effective compounds target a sequence that is highly conserved across FLUAV strains and subtypes [45].

Dengue virus (DENV) causes 50–100 million cases of dengue fever yearly, with about 300,000 requiring hospitalization, and 15,000 deaths. About 40% of the world's population is currently exposed to potential infection. Steric block R₅F₂R₄C-PMO specifically inhibited DENV replication by over 10,000-fold in cell culture experiments [46]. Similarly (R-Ahx-R)₄AhxB-PMO potently reduced viral titers of another flavivirus, West Nile virus (WNV), in cell culture experiments, and improved survival over 50% compared to controls in a WNV mouse model [47,48]. Ebola virus causes severe hemorrhagic fever with fatality rates up to 90% in humans. In an *in vivo* mouse model, pre- or post-infection treatment with a R₉F₂C-PMO conjugate rescued mice from an otherwise-lethal Ebola virus infection [49].

We expect CPPs will continue to be a very useful research tool to deliver PMO and PNA for studying various biological processes, including splicing correction, where the leading potential therapeutic application is treatment of DMD [53]. However, CPP toxicity and endosomal entrapment appear to be limiting factors to the potential for systemic delivery of steric blocking ONs to humans. Future studies on the nature of the toxicity, endosomal entrapment and uptake mechanism of CPP-ONs will hopefully instruct the design of the next generation of CPPs having a higher therapeutic index. At the current time, CPP-ONs are more suitable for diseases amenable to either local treatments, infrequent systemic dosing, such as DMD, or perhaps a small number of systemic treatments, such as some acute viral infections. An initial Phase Ib/II clinical trial to evaluate the safety and efficacy of a CPP-PMO conjugate for the *ex-vivo* treatment of vein tissue in coronary artery bypass grafts has started in Poland and Ukraine. This clinical trial will evaluate AVI-5126, a (R-Ahx-R)₄AhxB-PMO targeted to human *c-myc*, and is designed to prevent inappropriate cell proliferation that can occur after the grafting procedure and frequently causes eventual failure of the grafted vein. Excised vein tissue will be immersed in a solution containing 10 μM AVI-5126 for 30 min and then implanted as a bypass graft (www.clinicaltrials.gov). Hopefully, further clinical trials with CPP-PMO will become possible soon, and further commercial development of CPP-PNA as well.

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