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REVIEW

Peptide-mediated Cell and *In Vivo* Delivery of Antisense Oligonucleotides and siRNA

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Antisense oligonucleotides and short interfering RNAs (siRNAs) are nucleic acids-targeting reagents for gene expression modulation that are being developed as drugs for many applications. A number of useful synthetic nucleic acids analogues have been introduced recently to greatly improve their properties for use as therapeutics. However, their full effectiveness in cells and *in vivo* has often only been realized through development of suitable nonviral delivery systems. Among these, a range of natural and synthetic peptides have been found useful for enhancing cellular uptake and/or cell targeting of oligonucleotide analogues and siRNA. Such peptides are synthetically conjugated, used as noncovalent complexes, or used in combination with polymer, liposomal or exosome formulation techniques. This review begins by describing the modes of action of antisense reagents and siRNA and goes on to focus on recent advances in their peptide-mediated cell and *in vivo* delivery and how peptide use has influenced drug development. The review discusses the challenges associated with understanding the physiological and toxicological aspects of peptide-mediated delivery. Developments towards clinical use are also highlighted, with particular emphasis on peptide conjugates of oligonucleotide analogues used for treatment of neuromuscular diseases.

INTRODUCTION

Antisense oligonucleotides are used widely to interfere with biological processes in cells and are being developed in some cases as drugs.[1,2] There are numerous types of oligonucleotide analogue of varying chemistries, as well as short interfering RNAs (siRNAs), and they encompass a range of intracellular targets and modes of action. Common to all oligonucleotide types is the need to be delivered into cells and tissues efficiently in order to carry out their targeting functions. Some types are able to enter certain cell classes *in vivo* (such as hepatocytes or kidney cells) reasonably efficiently in naked form (e.g., those containing both phosphorothioate (PS) linkages and certain sugar modifications),[3] but bio-availability at the right cellular location may still be limited by poor cell trafficking and endosomal entrapment. In other cell types, cell targeting and entry is found to be poor for many naked oligonucleotide types, or their ability to reach the desired tissue is limited. Accordingly, there have been massive efforts to search for new delivery systems that may enhance tissue penetration, improve cell targeting and cell entry, as well as enhance intracellular bioavailability at the desired biological target. The most prevalent delivery systems for oligonucleotides and siRNA are cationic liposomes and other nanoparticle delivery systems, based initially on cell culture studies and later translated into sophisticated multicomponent *in vivo* vectors.[4,5]

Among the simpler methods that have been utilized to enhance cell delivery of oligonucleotides and siRNA are cationic peptide vectors, often referred to as cell-penetrating peptides (CPPs) or peptide transduction domains (PTDs). The first CPP was introduced in 1994[6] and since then there has been a continuous stream of new peptide delivery vectors where increased delivery and better pharmacological properties are claimed and a variety of applications demonstrated.[7] The cellular entry and intracellular trafficking mechanisms behind peptide-mediated transduction are still not fully elucidated but are reviewed thoroughly elsewhere.[8–11] In addition to CPPs, there are peptides designed to target to specific cells or tissues (homing peptides).[12] This review concentrates on recent progress (since 2007) in all of the various peptide-mediated strategies for delivery of antisense oligonucleotides and siRNA into cells in culture and also in *in vivo* applications, and points to prospects for clinical development.

MODES OF ACTION OF OLIGONUCLEOTIDE AND siRNA THERAPEUTICS

There are several ways that short oligonucleotides and siRNA can affect cellular processes, schematically illustrated in **Figure 1**. The most obvious approach, originally proposed by Zamecnik and Stevenson,[13] is antisense binding through Watson-Crick base pairing to messenger RNA (mRNA) to block translational initiation or elongation and thus decrease the levels of the corresponding protein (**Figure 1a**). Steric blocking of ribosomal elongation is known to be hard to achieve,

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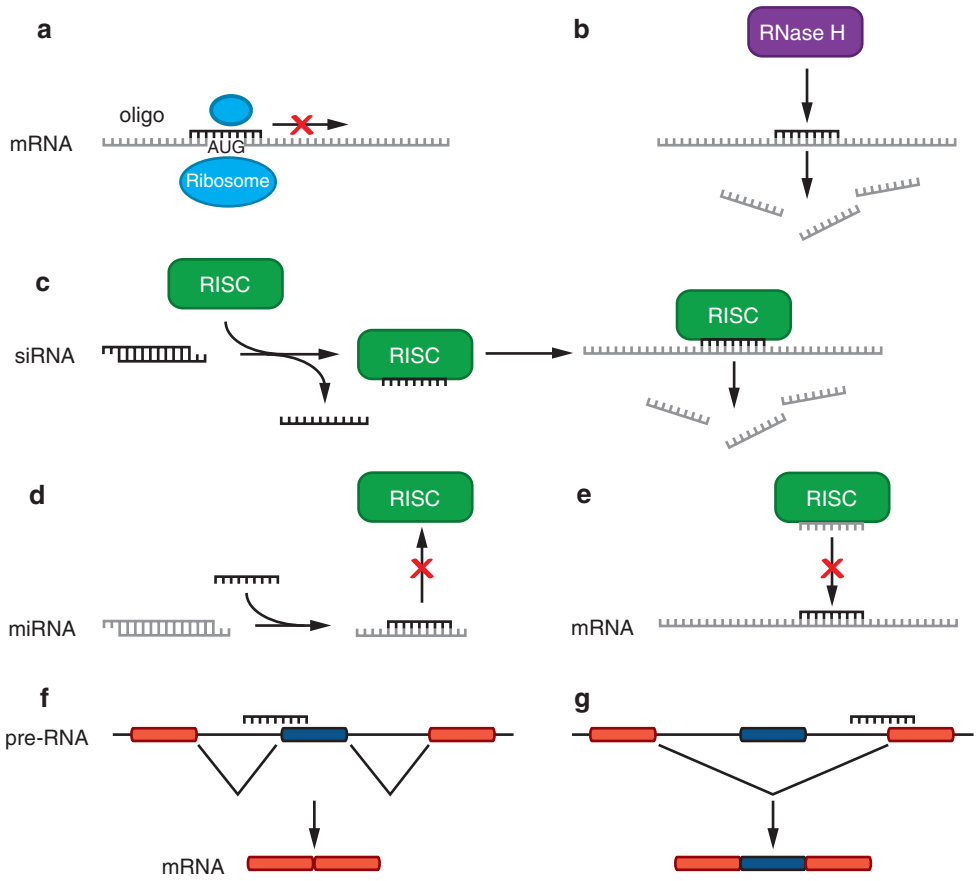

Figure 1 Schematic illustration of how synthetic oligonucleotides affect cellular processes. (**a**) Complementary oligonucleotide binds to mRNA and sterically blocks translation initiation or elongation. (**b**) Complementary oligonucleotide binds to mRNA and recruits RNase H. (**c**) siRNA cell internalization leads to one strand binding to mRNA and triggering RISC-dependent RNA cleavage. (**d**) A complementary oligonucleotide binds to the sense strand of a miRNA and blocks RISC activation. (**e**) An oligonucleotide masks the miRNA binding site on the mRNA and prevents RISC-mediated mRNA degradation or translation inhibition. (**f**) A splice site is masked on the mRNA, which results in exon exclusion. (**g**) A splice site is masked on the mRNA, which results in exon inclusion. mRNA, messenger RNA; miRNA, microRNA; RISC, RNA-induced silencing complex; siRNA, short interfering RNA.

but blocking of ribosomal initiation can be efficient by targeting the initiator AUG region, the 5′-cap region, or, where appropriate, an internal ribosome entry site. It is also possible to block polyadenylation.[14] Such targeting requires delivery of oligonucleotides in stoichiometric amount to that of the targeted mRNA present in the cell at a given time. Steric blocking also requires the use of strongly binding oligonucleotide analogues to ensure good competition for the mRNA with the proteins involved in translation or other cellular processes.

Similarly to steric-blocking mRNA binding, some oligonucleotides are also capable of binding in triplex mode to certain specific DNA sequences and inhibiting transcription in the nucleus, leading to a decrease in mRNA production,[15] but difficulties in obtaining sufficient potency when targeting chromosomal DNA selectively make gene expression inhibition through triplex formation currently less attractive as a therapeutic intervention mechanism. However, sequence-specific gene modification *via* triplex formation is a new subject that has recently shown great potential.[16]

A second mechanism involves antisense binding to RNA followed by recruitment and activation of RNase-H, which consequently leads to degradation of the mRNA target (**Figure 1b**). This mode of action is limited to those oligonucleotides containing a minimum stretch of 6–10 unmodified DNA or PS DNA units (known as gapmers), but outer nucleotides can be more heavily modified to enhance complementary RNA binding. In principle, catalytic turnover allows a lower concentration of oligonucleotides to be used compared to steric-blocking oligonucleotides. RNase-H–dependent antisense has been used very successfully to reduce expression levels of specific proteins, or to inhibit viral replication.[17,18]

In the case of RNA interference, one strand of an siRNA duplex (antisense or guide strand) becomes targeted to the mRNA as a result of recruitment by the RNA-induced silencing complex (RISC) cellular machinery and leads subsequently to RNA cleavage (**Figure 1c**). The siRNA can be targeted to any accessible part of the mRNA, similar to the RNase-H–dependent oligonucleotide targeting method. The sense (passenger) strand may include chemically modified nucleotides in any position, whereas the antisense strand only tolerates a limited range of modified nucleotides in specific locations.

Single-stranded oligonucleotide analogues have shown great promise as inhibitors of micro-RNA (miRNA) action by

preventing miRNA binding to mRNA that would otherwise trigger the RNA interference machinery and result in mRNA downregulation or degradation (**Figure 1d**). Interestingly, good levels of intracellular activity have been achieved without requiring CPP-assisted delivery both in cell culture and *in vivo* (e.g., for peptide nucleic acid (PNA) and locked nucleic acid (LNA)),[19,20] in contrast to naked oligonucleotides targeting mRNA, where high concentrations and extended periods are required in cell culture for RNase-H–dependent activity when delivered gymnotically (from Greek: *gymnos*, meaning naked).[21] Oligonucleotides targeting miRNAs have been shown to rapidly enter cells and interfere with miRNA activity at submicromolar concentrations.[19,22] Current thinking is that the oligonucleotide may meet the miRNA somewhere within the endosomal pathway,[20] and therefore CPP-enhanced endosomal release may be unnecessary, although the generality of this conclusion for all cell types and miRNAs is yet to be established. Inhibition of miRNA is thought to occur by miRNA sequestration rather than by degradation.[20] Therapeutic applications of anti-miRs have been recently reviewed.[23] An alternative mechanism has also proven successful, where a short oligonucleotide binds the mRNA to cover the miRNA-binding site to again prevent RISC binding and subsequent mRNA downregulation (**Figure 1e**).[24]

Another important mode of action of antisense oligonucleotides involves splicing redirection of pre-mRNA in the cell nucleus. An oligonucleotide directed to regions at, or close to, a splice site can mask normal or aberrant splicing events leading either to exon exclusion (**Figure 1f**) or exon inclusion (**Figure 1g**). Splicing redirection model systems have been used very successfully to monitor peptide-mediated oligonucleotide delivery.[25,26] In addition, the methodology is already being assessed in the clinic for treatment of neuromuscular and neurodegenerative diseases where targeting of specific exons in mutated dystrophin pre-mRNA with antisense phosphorodiamidate morpholino oligonucleotides (PMO) allows redirection of the splicing pattern to restore a correct reading frame (exon skipping), resulting in the regeneration of functional dystrophin protein expression.[27] Stimulation of exon inclusion by antisense oligonucleotides is an alternative mechanism being examined for potential treatment for spinal muscular atrophy.[28] In addition, exon skipping can be applied to wild-type genes to generate out-of-frame transcripts as a method of normal protein downregulation.[29]

TYPES OF OLIGONUCLEOTIDE ANALOGUE

In addition to low bioavailability, the poor stabilities of unmodified DNA and RNA and rapid clearance from the bloodstream have been major drawbacks for use as therapeutics. Thus, many different oligonucleotide analogues have been developed in order to increase stability, RNA binding, and other therapeutically useful properties. A selection of important oligonucleotide analogues that have been involved with peptide-mediated delivery is presented in **Figure 2**. Each analogue type has particular properties that make them suitable for different applications.[2,30]

Perhaps the most utilized and well-studied analogue is the PS linkage where a sulfur atom replaces one non-bridging oxygen atom in the phosphate backbone (**Figure 2a**). The PS linkage can be included in any DNA- or RNA-type oligonucleotide and increases nuclease resistance, whereas in an otherwise unmodified DNA it permits RNase-H activation. Introduction of the PS linkage reduces RNA-target affinity somewhat but leads to enhanced interaction with plasma proteins, thereby decreasing renal clearance rates.[31] Despite some concerns as to possible toxic side effects at higher concentrations, PS linkages are included in all FDA-approved oligonucleotides to date, which unfortunately are currently very few.[32,33]

The 2′-*O*-methyl nucleotide (2′-OMe), in which a methyl group replaces a hydrogen atom in the 2′-hydroxyl group in the ribose ring of RNA (**Figure 2b**), also imparts nuclease resistance, but does not permit RNase-H activation. Such non-RNase-H inducing oligonucleotides are particularly valuable in certain applications where the target RNA needs to remain intact (e.g., splicing redirection).

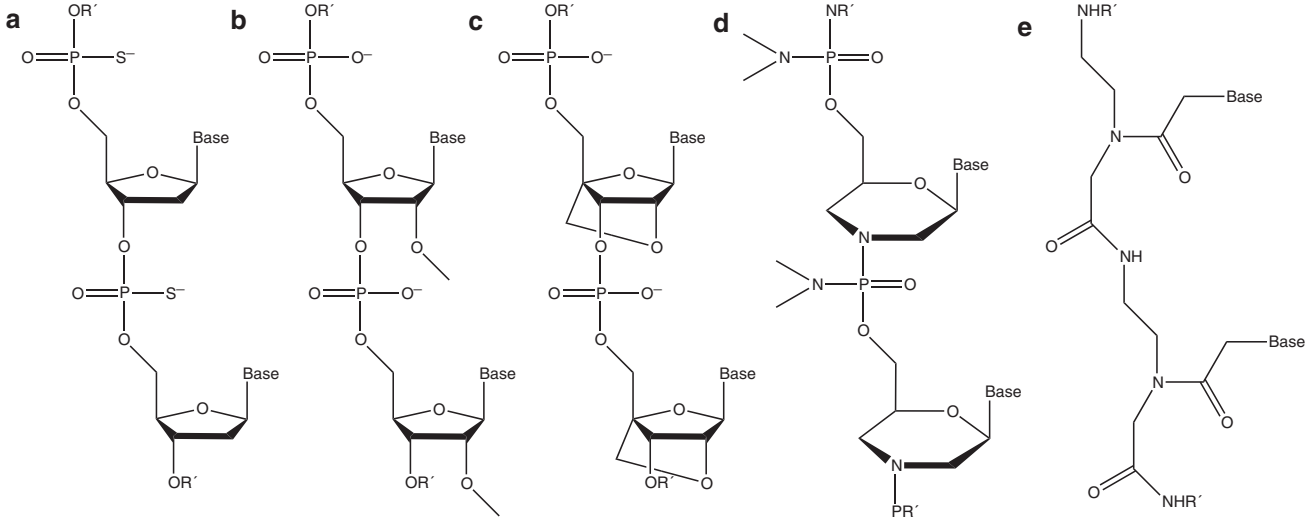

Figure 2 A selection of common oligonucleotide analogues delivered by peptide-mediated means. (**a**) Phosphorothioate (PS) linkages, (**b**) 2′-O-Methyl oligonucleotide (2′-OMe), (**c**) locked nucleic acid (LNA), (**d**) Phosphorodiamidate morpholino oligonucleotide (PMO), and (**e**) peptide nucleic acid (PNA).

Although the 2′-OMe modification is insensitive to endonucleases, it is still partially susceptible to exonuclease degradation. By combining PS linkages and 2′-OMe nucleotides (PS-2′-OMe), much greater *in vivo* stability has been achieved resulting in several successful applications. Further enhancements of therapeutic activity have been achieved in some cases by including other 2′-*O*-alkylated nucleotides (such as 2′-*O*-methoxyethyl, MOE) or 2′-fluoro-2′-deoxynucleotides, resulting in "mixmers". All these analogues are also used in the wings of gapmers, as well as in particular positions of siRNA.

A highly successful nucleotide analogue is LNA, which is a constrained RNA analogue having a methylene bridge between the 2′ and 4′ positions in the ribose ring (**Figure 2c**). LNA is also unable to activate RNase-H, but due to the constrained backbone LNA has a very high affinity for single-stranded DNA/RNA compared to other analogues. In addition to high affinity, LNAs display high *in vivo* stability and slower renal clearance, although in rare cases hepatotoxicity has been observed.[34] The increased affinity allows LNA to be used in much shorter oligonucleotide than for many other analogue types (tiny LNAs,).[35] However, nonspecific binding of longer sequences of LNAs, which could result in off-target effects, is alleviated by using LNA in combination with unmodified DNA or with other analogues, such as 2′-OMe, in steric-blocking applications. LNA is also used in RNase-H–dependent applications in the wings of all PS gapmers where the central section is PS-DNA.

A separate class of analogues are charge neutral. In PMO, the ribose is replaced by a morpholino moiety and the natural phosphodiester linkage is replaced by an uncharged phosphorodiamidate backbone (**Figure 2d**). The constrained structure and the lack of electrostatic repulsion between the oligonucleotide and its target both contribute to make PMO–RNA interactions more stable compared to DNA–RNA interactions. PMO does not activate RNase-H, but it has been used successfully for steric block antisense purposes, in many *in vivo* applications and in the clinic.[36,37] Like PMO, PNA is an uncharged oligonucleotide analogue, where the sugar-phosphate backbone is replaced by repeating *N*-(2-aminoethyl)-glycine units linked together by amide bonds (**Figure 2e**). PNA has very high affinity for DNA/RNA that, like PMO, is due partly to the lack of charge repulsion of the peptide-like backbone. PNAs display high selectivity and mismatch discrimination towards its target strand and have been used effectively in many antisense applications and *in vivo*.[16,38] Further, PNA containing a few additional cationic residues (e.g., lysine) have been used successfully to block miRNA activity without the use of a transfection agent or delivery peptide.[19,20]

The need for improved oligonucleotide therapies has accelerated advances in chemistry and many newer analogues have emerged recently. Unfortunately very few have been able to attain high stability, affinity, and selectivity, while maintaining low toxicity and off-target effects and which are available to researchers in sufficient quantities and reliability for *in vivo* studies. The analogues selected for mention in this review are those that not only have most of these above attributes but also have been most utilized in peptide-mediated delivery.

STRATEGIES FOR PEPTIDE-MEDIATED DELIVERY

There are two main ways of utilizing peptide vectors for the delivery of antisense oligonucleotides and siRNA. The first method involves chemical synthesis of peptides that are modified to enable covalent conjugation to oligonucleotides or other cargo types (**Figure 3a**). Such conjugation reactions may be carried out either on solid support or in solution, and often result in high yields. An advantage of covalent conjugation is that this results in a well-defined single entity that simplifies drug development. Linker types may be chemically stable, which ensures that the peptide–oligonucleotide conjugate remains intact throughout *in vivo* administration and the subsequent delivery process and that degradation now reflects only the proteolytic susceptibility of the particular peptide. There are numerous conjugation strategies, including thioether, thiol-maleimide, ester formation, and "click" chemistry (reviewed in ref. 39,40).

Alternatively, a labile linker cleavable within the cell, such as a disulphide linkage, has proven very successful in studies of peptide-mediated delivery, both in cells and *in vivo*.[41,42] Such disulphide bridges may remain sufficiently stable, if administration is rapid, but may be cleaved later when the conjugate reaches the reducing environment of the endosome/cytoplasm. This approach was initially favored in therapeutic design, because cleavage diminishes the risk of any detrimental effect of the peptide on the interaction of the oligonucleotide with its target. However, recent *in vivo* applications using stable linkages suggest that peptide-cargo cleavage is not essential. Recent studies have suggested also that thiol or disulfide groups might enhance cellular uptake, although the reasons for this are not as yet fully understood.[43,44]

Although covalent conjugation is very suitable for charge-neutral oligonucleotide analogues, there are technical difficulties in conjugation and purification of conjugates of highly cationic peptides with negatively charged oligonucleotides that have limited the type of peptide that can be conjugated.[45] Sadly, meaningful comparative data on the effects of different peptide-oligonucleotide linkages in biological antisense assays are mostly lacking.

The second method of peptide-mediated oligonucleotide delivery exploits the complexing properties of CPPs and their derivatives (**Figure 3b**). For example, cationic CPPs can form complexes efficiently with negatively charged oligonucleotides.[8] Further, some peptide vectors also contain hydrophobic elements (hydrophobic amino acids or addition of hydrophobic amino acids and/or other moieties such as fatty acids, lipids or cholesterol), which are designed for complex formation with both negatively charged and charge-neutral analogue types. Such complexing peptides form nanosized particles together with the oligonucleotides that are better able to translocate across the plasma membrane (in some cases perhaps avoiding the endosomal uptake system altogether)[46] and can deliver the oligonucleotide cargo to the target with greater efficiency.

It is common to optimize empirically the ratio of the peptide over cargo to obtain the best cellular delivery. The noncovalent strategy does not require the oligonucleotide to be end-modified for conjugation, and thus complex formation is often achieved by simple mixing. Complex formation also results

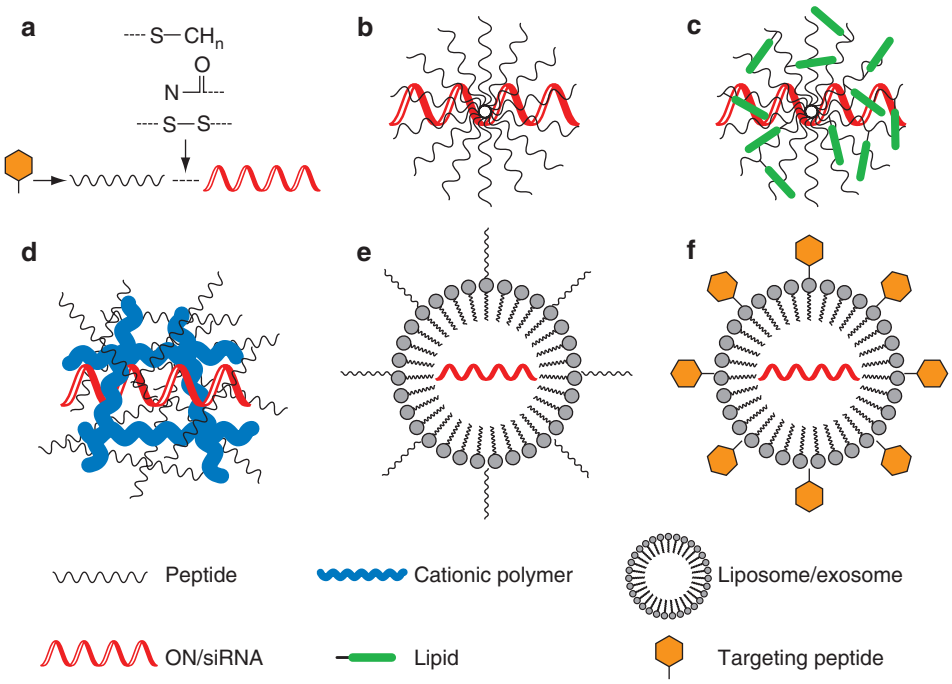

Figure 3 Schematic illustration of formulation strategies for peptide-mediated oligonucleotide delivery. (**a**) Covalent conjugation between peptide vector and oligonucleotide via a stable or cleavable linker. (**b**) Complex formation between peptide and oligonucleotides through electrostatic and/or hydrophobic interactions. (**c**) Complex formation between lipid-conjugated peptide and oligonucleotides through electrostatic and/or hydrophobic interactions. (**d**) Oligonucleotide condensation by peptide-functionalized cationic polymers. (**e**) Lipid vesicle or exosome loaded with oligonucleotides and functionalized with a CPP. (**f**) Lipid vesicle or exosome loaded with oligonucleotides and functionalized with a targeting/homing peptide. CPP, cell-penetrating peptide; ON, oligonucleotide; siRNA, short interfering RNA.

in protection of the oligonucleotide or siRNA from nuclease degradation. On the other hand, structures of varying and undefined size may be generated, which complicates drug characterization and subsequent *in vivo* use. In the cases of uncharged PMO or PNA, it has been common to first hybridize to a complementary DNA strand (leash) before complex formation with cationic peptide vectors.[47]

Recently, a new generation of chemically modified peptide vectors for noncovalent conjugation has been introduced (**Figure 3c**), based on the original work of Futaki *et al.* that involved stearylation of cationic poly-arginine peptides as delivery vectors for plasmid DNA.[48] For example, a stearylated peptide showed noncovalent complex formation and enhanced cell delivery of a 2′-OMe splice-redirecting oligonucleotide.[49] It seems that the peptide sequence needs to be carefully tuned to the type of cargo as well as to the application, since for example a stearylated poly-arginine peptide was found to be ineffective in cellular delivery of a splice-correcting oligonucleotide, despite being effective with a larger plasmid.

Other nanovectors involve peptides associated with cationic polymers (**Figure 3d**) or peptides complexed/cell surface displayed on liposomes or exosomes (**Figure 3e**). In addition to CPPs designed for general enhancement of cell entry, peptides can also be used to target specific cell surface receptors or tissues (homing peptides). These can be conjugated to an oligonucleotide by themselves (**Figure 3a**) or incorporated as a chimera with a CPP, or embedded on the surface of a nanovector (**Figure 3f**). This last concept combines the cell-penetrating or cell-targeting properties of a peptide with oligonucleotide or siRNA encapsulation technology (e.g., liposomes or exosomes) and this has recently opened up new possibilities for cell delivery.

Just as for any type of delivery agent, the advantages gained over a naked oligonucleotide or siRNA in use of a peptide vector (whether conjugate or complex) in enhancing cell and/or *in vivo* delivery must outweigh the disadvantages of the additional size of the molecule and the increased potential cost per gram. In the case of siRNA, for all except kidney and topical applications, it is clear that a complexation vector of some sort is required to obtain any significant *in vivo* activity. This is partly because the vector protects the siRNA from rapid degradation as well as helps the siRNA to passage into the cell and be released from endosomes. A simple peptide-based complexation vector is attractive compared to the better known but more complex lipid-based or polymeric nanoparticle vectors in terms of simplicity and cost of components. Here the question is more as to whether a peptide vector can be found to offer sufficient *in vivo* delivery power or tissue targeting compared to the other more sophisticated types of delivery vectors.

By contrast in the case of oligonucleotides, the advantages of using a peptide vector are fully dependent on the organ type and the application. For liver *in vivo* applications for example, there is already good delivery of naked PS oligonucleotides and a peptide vector seems unlikely to add more than a marginal benefit at best. However, delivery to muscle for example with naked oligonucleotide analogues, including PMO and PNA, requires very high dosages in animals to obtain any activity (see for example the muscular dystrophy applications

described later) and here the substantial improvement in *in vivo* activity afforded by covalent peptide conjugation easily outweighs the added cost and effort of conjugation. In the examples that follow, readers should bear in mind that there are still relatively few peptide vectors currently being considered for therapeutic use and few if any publications have addressed toxicology, potential immune recognition, or demonstrated cost/benefit analysis. Most of the vectors describing enhanced cell delivery represent therefore research in progress.

PEPTIDE CONJUGATES AND COMPLEXES FOR ANTISENSE OLIGONUCLEOTIDE AND siRNA APPLICATIONS

Peptide-mediated delivery of RNase-H–dependent antisense oligonucleotides into cells in culture was first shown in 1994 through covalent CPP conjugation.[50,51] The covalent CPP attachment concept was later used for steric-blocking PNA in downregulation of the galanin receptor *in vivo*.[41] Since then many demonstrations of antisense and siRNA cell delivery by CPPs have been published. **Tables 1–4** show some recent examples. The choice of antisense mechanism or siRNA is dependent on the particular application and this in turn influences the type of peptide delivery system available. In cases where the particular RNA target permits a choice of targeting methods available, the generally lower potency of steric-blocking oligonucleotides has to be balanced against the greater possibility of off-target effects in RNase-H–dependent or RISC-dependent activities. These effects may arise for example from binding of an oligonucleotide to incorrect target sequences that result in unwanted RNA cleavages, to miRNA-like effects of siRNA passenger strands, or to other

Table 1 Selection of peptide–oligonucleotide conjugates and complexes used for mRNA targeting

mRNA targeting by antisense ONs						
Peptide	***Conjugation strategy***	***ON/ON analogue***	***mRNA target***	***Mechanism***	**In vivo/*in cells***	***Reference***
KLA	Disulphide bond	PNA	Nociceptin/orphanin FQ receptor	Ribosome initiation block	In cells	52
F-3	Thiol-maleimide linker	2′-OMe/DNA/PS gapmer	Transcription factor Id1	RNase H-dependent mRNA degradation	*In vivo*	54
Pep-3	Complex formation	PNA	Cyclin B1	Ribosome initiation block	*In vivo*	55
Splice-redirecting ONs						
Peptide	***Conjugation strategy***	***ON/ON analogue***	***pre-mRNA target***	***Mechanism***	**In vivo/*in cells***	***Reference***
Variants of RX and Rβ	Amide bond	PMO	β-globin interrupted Luciferase	Splice correction	In cells	58
Penetratin, Tat, TP10, etc	Disulphide bond	PNA	β-globin interrupted Luciferase	Splice correction	In cells	42
R6-Peneratin	Amide bond, thioacetyl linker	PNA	β-globin interrupted Luciferase	Splice correction	In cells	60
(RXR)4	Amide bond	PMO	β-globin interrupted Luciferase	Splice correction	In cells	59
MPGα	Complex formation	2'-OMe, LNA, PNA/DNA	β-globin interrupted Luciferase	Splice correction	In cells	61
Pip2a	Thioether	PNA	Dystrophin exon 23	Splice correction	*In vivo*	64
CatLip	Ethylene glycol linker, amide bond	PNA	β-globin interrupted Luciferase	Splice correction	In cells	65
Fatty acid-PNA-Arg9	Amide bond	PNA	β-globin interrupted Luciferase	Splice correction	In cells	66
RGD	Thiol-maleimide linker	2′-OMe/PS	β-globin interrupted Luciferase	Splice correction	In cells	67
Bombesin	Thiol-maleimide linker	2′-OMe/PS	β-globin interrupted Luciferase	Splice correction	In cells	69
Stearyl-(RXR)4	Complex formation	2′-OMe/PS	β-globin interrupted Luciferase	Splice correction	In cells	70
Stearyl-TP10	Complex formation	2′-OMe/PS	β-globin interrupted Luciferase	Splice correction	In cells	49
PepFect14	Complex formation	2′-OMe/PS	Dystrophin exon 23	Splice correction	*In vivo*	72
(RXR)4, B-peptide	Amide bond	PMO	Dystrophin exon 23	Splice correction	*In vivo*	76
B-MSP	Amide bond	PMO	Dystrophin exon 23	Splice correction	*In vivo*	74
Pip5e	Thioether	PMO	Dystrophin exon 23	Splice correction	*In vivo*	75
Lys-n	Amide bond	PNA	Phosphatase and tensin homologue exons	Splice correction	In cells	77
PKKKRKV	Amide bond	PNA	Intron in Eμ enhancer	Eμ-dependent c-myc downregulation	*In vivo*	78

LNA, locked nucleic acid; mRNA, messenger RNA; ON, oligonucleotide; PMO, phosphorodiamidate morpholino oligonucleotide; PNA, peptide nucleic acid.

Table 2. Selection of peptide–-oligonucleotide conjugates and complexes used for anti gene targeting.

Gene targeting antisense ONs						
Peptide	***Conjugation strategy***	***ON/ON analogue***	***Gene target***	***Mechanism***	**In vivo/*in cells***	***Reference***
Poly Lys, Poly Arg	Amide bond	PNA	Progesterone receptor	DNA promoter binding	In cells	79
Lys8	Amide bond	PNA	Huntingtin CAG repeat	CAG repeat binding	In cells	80
Penetratin	Amide bond	PNA	supFG1 reporter gene	Targeted supFG1 mutagenesis	In vivo	16

ON, oligonucleotide; PNA, peptide nucleic acid.

Table 3 Selection of oligonucleotides and peptide–oligonucleotide conjugates used for miRNA blocking

miRNA-targeting ONs						
Peptide	***Conjugation strategy***	***ON/ON analogue***	***miRNA target***	***Mechanism***	**In vivo/*in cells***	***Reference***
—	—	LNA	miR-122	miRNA binding	*In vivo*	82
—	—	LNA/2′-OMe mixmer	miR-122	miRNA binding	In cells	19
—	—	LNA	miR-21	miRNA binding	*In vivo*	35
Lys4	Amide bond	PNA	miR-155	miRNA binding	*In vivo*	22
Lys4	Amide bond	PNA	miR-122	miRNA binding	*In vivo*	20
Tat	Amide bond	PNA	Various miR sequences	miRNA binding	In cells	84
Peptide-mediated delivery of siRNAs						
Peptide	***Conjugation strategy***	***ON/ON analogue***	***mRNA target***	***Mechanism***	**In vivo/*in cells***	***Reference***
Tat, penetratin	Disulphide bond	siRNA	P38α MAP kinase	RISC activation	In cells	86
SPACE	Amide bond	siRNA	Interleukin-10, GAPDH	RISC activation	*In vivo*	87
RGD	Thiol-maleimide linker	siRNA	Luciferase	RISC activation	In cells	88
Cholesterol-MPG-8	Complex formation	siRNA	Cyclin B1	RISC activation	*In vivo*	90
CADY	Complex formation	siRNA	Cyclin B1	RISC activation	*In vivo*	91
Tat-LK15	Complex formation	siRNA, shRNA	Oncoprotein BCR-ABL	RISC activation	In cells	93
PepFect6	Complex formation	siRNA	HPRT1	RISC activation	*In vivo*	94
Tat-DRBD fusion protein	DRBD binding	siRNA	EGFR, Akt20	RISC activation	*In vivo*	96

DRBD, double-stranded RNA binding domain; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LNA, locked nucleic acid; MAP, mitogen-activated protein; mRNA, messenger RNA; miRNA, microRNA; ON, oligonucleotide; PNA, peptide nucleic acid; RISC, RNA-induced silencing complex; shRNA; short hairpin RNA; siRNA, short interfering RNA.

cellular effects such as immune activation. Peptide-mediated delivery often results in raised levels of cellular activity but it could also lead to increases in off-target effects (compare use of cationic liposomes to enhance delivery which have suffered from the same problems). Whereas potency increases have been frequently demonstrated, off-target effects have rarely been measured systematically where peptide vectors have been used, and it is common to find only simple cell toxicity assays carried out. This limits the ability to compare methods effectively.

mRNA targeting by antisense oligonucleotides

There are only a few recent examples of peptide conjugates of oligonucleotides used for targeting mRNA (**Table 1**). A variety of CPPs of cationic or amphipathic origin disulfide-conjugated to a 12-mer PNA targeting the nociceptin/orphanin FQ receptor mRNA were studied for receptor downregulation in cardiomyocytes.[52] Among these CPPs, a repeating KLA amphipathic peptide was found to have the highest activity as a PNA conjugate in the absence of transfection agent, but no comparison was made with Arg-rich peptides. Interestingly, there was found no correlation of bioactivity with the level of fluorescence uptake of labeled conjugates, confirming previous studies with peptide-PNAs.[53] Further, receptor downregulation was improved through CPP conjugation by a relatively modest amount. The significant phenotypic activity for naked PNA and lack of clear quantitative link between phenotypic and genotypic antisense activity calls into question as to the extent mRNA antisense behavior is truly observed in this model.

Perhaps the most promising example of mRNA targeting involves an RNase-H–dependent gapmer 2'-OMe/DNA/PS oligonucleotide that targets the transcription factor Id1 which was coupled to the F-3 31-mer peptide, a fragment of the high mobility group protein N2 which homes to neovessels in xenograft tumors.[54] The conjugate showed substantial inhibition of tumor growth and metastasis in a mouse tumor xenograft model. The peptide has been shown to bind to nucleolin, which is overexpressed on the surface of cancer cells and is subsequently transported into the nucleus. It is likely that the cationic nature of the peptides may result in aggregation of this conjugate to form nanoparticles in solution that leads to partial protection against degradation in serum and which perhaps improves cell and *in vivo* activity. Although delivery of the oligonucleotide conjugate by the homing peptide into the tumor endothelium and dose-dependent Id1

Table 4 Selection of peptide–oligonucleotide conjugates and complexes with antiviral and antibacterial activity.

Viral RNA targeting

Peptide	*Conjugation strategy*	*ON/ON analogue*	*mRNA target*	*Mechanism*	**In vivo/*in cells***	*Reference*
B-peptide	Amide bond	PMO	Protease TMPRSS2 pre mRNA	Splice blocking	In cells	98
(RXR)4	Amide bond	PMO	L gene	Ribosome initiation block	*In vivo*	99
(RXR)4	Amide bond	PMO	5′-termini of viral genome	Replication inhibition	*In vivo*	100
(RXR)4	Amide bond, thioether	PMO	5′-termini of viral genome	Replication inhibition	*In vivo*	101
(RXR)4	Piperazine linker	PMO	5′-, 3′-termini of viral genome	Replication inhibition	In cells	102

Antibacterial activity of antisense ONs

Peptide	*Conjugation strategy*	*ON/ON analogue*	*Target*	*Mechanism*	**In vivo/*in cells***	*Reference*
(RFF)3RXB	Amide bond	PMO	acpP mRNA	Ribosome initiation block	*In vivo*	106
(RXR)4	Amide bond	PMO	acpP mRNA	Ribosome initiation block	*In vivo*	107
T-cell–derived CPP	Amide bond	PMO	gyrase A mRNA	RNase P activation	In cells	108
(RXR)4	Amide bond	PMO	acpP mRNA	Ribosome initiation block	*In vivo*	109
(KFF)3K	Amide bond	PNA	acpP, ftsZ, murA, fabI mRNA	Ribosome initiation block	In cells	110
(RXR)4, (KFF)3K	Amide bond	PNA	rpoD mRNA	Ribosome initiation block	In cells	111

CPP, cell-penetrating peptide; mRNA, messenger RNA; ON, oligonucleotide; PMO, phosphorodiamidate morpholino oligonucleotide; PNA, peptide nucleic acid.

protein knockdown was observed, both in cells and *in vivo*, surprisingly sequence- and dose-dependent downregulation of the corresponding Id1 mRNA was not reported, and thus there remains the question as to whether the biological activity observed was fully due to a sequence-specific antisense effect in this study. Nevertheless the strong *in vivo* activity observed is undeniable.

Among peptide-complex delivery strategies, a PEGylated amphipathic peptide Pep-3 was shown to mediate *in vivo* delivery of a PNA analogue oligonucleotide targeting cyclin B1 mRNA following intravenous injection through the formation of nanoparticles, and this resulted in reduction in rate of PC3 tumor growth.[55] Pep-3 is a short 15-mer peptide that combines lysine/arginine-rich hydrophilic domains and tryptophan/phenylalanine hydrophobic residues to form stable complexes with the antisense oligonucleotide. PEGylation was necessary for *in vivo* activity. In this case the effect is steric block, targeting close to the AUG start sequences, and sequence- and dose-dependence was well validated. A 20-fold excess of Pep-3 over oligomer was needed for optimal cell delivery, whereas *in vivo* a layer of PEGylated peptide was added after initial complexation with unmodified peptide to enhance delivery. Further Pep derivatives have been developed for siRNA (see below).

Splice-redirecting oligonucleotides

Splicing redirection has been used extensively for measuring the efficacy of peptide-mediated cell delivery of oligonucleotides. This is due to a very convenient readout system where HeLa cells are stably transfected with a luciferase gene that is interrupted by a mutated β-globin intron.[56] The mutation causes aberrant splicing of luciferase pre-mRNA, thus preventing translation. Oligonucleotides that target particular sequences at or close to the aberrant splice site (e.g., the "705" site) induce correct splicing and thus restore luciferase activity. A similar construct involves use of green fluorescent protein (GFP) in place of luciferase.[57] The reliable positive readout and large dynamic range of the method allows the efficacy of different peptide vectors, conjugation strategies, and analogue types to be compared.[58] The assay is well validated for many analogue types and delivery methods and sequence-specificity is not in doubt for such studies.

Further, the HeLa 705 assay has served as a model system to help elucidate cellular uptake mechanisms.[42] In general, CPP conjugates are delivered through endocytosis and the CPP is believed to help the release from endosomes.[7,59,60] The uptake mechanisms of peptide-complexed oligonucleotides appear to be more diverse. Some peptide complexing agents such as MPGα appear to utilize endocytosis,[61] whereas for other similar peptides a non-energy–dependent membrane-crossing pathway is proposed.[62] To obtain splicing redirection, the oligonucleotide must be delivered into the cell nucleus. Debate continues as to whether endocytosis or energy-independent mechanisms of delivery are optimal in such applications in terms of bioavailability, which is known to be poorly correlated with cell uptake.[42,61]

In recent studies of conjugates of charge-neutral antisense PNA and PMO, earlier Penetratin and Tat transduction domains have now been replaced by Arg-rich CPPs (**Table 1**). In the paradigm peptides, Arg residues are spaced by aminohexanoyl (Ahx or X) and/or β-alanyl (β) moieties to enhance hydrophobicity and proteolytic resistance in serum, such as $(R\text{-}Ahx\text{-}R)_4\text{-}Ahx\text{-}\beta Ala$ and $(R\text{-}Ahx\text{-}R\text{-}R\text{-}\beta Ala\text{-}R)_2\text{-}Ahx\text{-}\beta Ala$ called RXR4 and B-peptide respectively.[36,63] The aminohexanoyl spacer in the R-Ahx-R motif was found to be optimal in the splicing-redirection assay among several other

spacers tried.[59] Based on an initial discovery, that the addition of six Arg residues to the Penetratin peptide (R6Pen) resulted in significantly enhanced splicing redirection of PNA in HeLa 705 cells,[60] a series of peptides known as Pip (PNA/PMO internalizing peptides) was developed that include a hydrophobic core flanked by two Arg-rich regions, which when attached to PNA maintain good splicing redirection at submicromolar concentrations, yet are more serum stable and therefore usable *in vivo*.[64]

Another recent idea is to add a second chemical moiety such as a fatty acid to the end of an Arg-rich peptide to enhance delivery of the PNA conjugate.[65] It was found that a decanoic acid was the optimal chain length to balance increased splicing redirection obtained with concurrent increases in cell toxicity as the chain length was extended. In a separate study, the addition of a C14 palmitoyl lipid chain to the free terminus of the PNA in a R_9-PNA conjugate also increased splice-redirecting activity, and it was suggested that the increased activity was due to micelle formation.[66]

A conjugate between a 2′-OMe/PS antisense oligonucleotide and a bivalent RGD peptide, selectively targeting $\alpha v\beta 3$ integrin, was found to enter cells *via* receptor-mediated endocytosis and was used for splicing redirection in melanoma cells.[67] The RGD conjugate was found to enter cells by an endocytotic pathway distinct from that of naked oligonucleotide.[68] Recently, a splice-redirecting antisense oligonucleotide was conjugated to bombesin peptide and its intracellular delivery was studied in gastrin-releasing peptide receptor expressing PC3 cells, which was transfected with a luciferase gene interrupted by an abnormally spliced intron. The results suggest that the conjugate enters cells *via* a process of gastrin-releasing peptide receptor-mediated endocytosis followed by trafficking to deep endomembrane compartments, leading to significantly higher luciferase expression compared to unmodified oligonucleotides.[69]

The principle of using a peptide targeted to a cell surface receptor, particularly an overexpressed marker on cancer cells, is an excellent one. The issue lies in whether there is sufficient molecular recognition of a peptide conjugated to an individual oligonucleotide to deliver enough through a specific receptor-mediated endocytotic pathway to provide a large boost in splicing redirection that has relevance in an *in vivo* setting. Demonstrations of quantitatively useful peptide-mediated delivery in cells and *in vivo* are awaited eagerly.

Various types of stearylated peptide have been used to aid redirection of splicing using the noncovalent strategy. Stearyl-RXR4 peptide at 5:1 ratio compared to 2′-OMe/PS oligonucleotide was much more effective at luciferase upregulation than stearyl-Arg_9 in HeLa 705 cells.[70] More effort has gone into developing stearyl derivatives of the peptide Transportan 10 (TP-10) as nucleic acid delivery agents.[49] In general, this class of stearylated peptide delivery agent seems to utilize the endocytotic pathway.[71] PF14, a peptide derivative of TP-10 containing pairs of ornithine as well as pairs of leucine residues, showed particularly good splicing redirection for 2′-OMe/PS oligonucleotides both in the HeLa 705 cell assay as well as in exon skipping in mouse *mdx* muscle cells.[72]

CPP-aided splicing redirection is also a promising mechanism for *in vivo* and therapeutic applications. The *mdx* mouse model of Duchenne muscular dystrophy (DMD), bearing a nonsense mutation in exon 23 of the dystrophin gene, is an important *in vivo* system for evaluation of peptide-delivery methods. Exon skipping with oligonucleotide analogues targeting exon 23 restores the transcript reading frame and hence production of a shorter but active dystrophin protein. RXR4-PMO and B-PMO conjugates showed dramatically improved dystrophin production, compared to naked PMO, following intravenous injection in the *mdx* mouse.[27,63,73] PMO conjugated to a chimeric CPP comprising the B-peptide and a short muscle-specific peptide, known as B-MSP, showed higher activity in skeletal tissue in *mdx* mice than B-PMO.[74] An improved Pip series Arg-rich peptide (Pip5e) conjugated to PMO showed enhanced dystrophin production in both skeletal muscles and heart using a single 25 mg/kg injection.[75] This is a significant development, because, together with respiratory difficulties, cardiac deterioration is a major cause of death in DMD patients.

Peptide-PNA conjugates have also been used for *in vivo* antisense studies targeting DMD,[76] as well as other targets. For example, it was reported recently that an amphipathic peptide covalently conjugated to a PNA was able to redirect splicing of the murine PTEN primary transcript in adipose tissue of male Balb/c mice after a low-dose intraperitoneal injection of 2.5 mg/kg.[77] The study showed a clear dependence of the levels of PNA antisense activity in adipose and other tissues depending on the peptide carrier sequence.

Enhancement of splicing redirection by PNA or PMO is clearly a well-suited application of covalently attached CPPs and potentially other peptide types, and further peptide vector development here is likely. For negatively charged splice-directing oligonucleotides, good cell delivery enhancement has been observed in several cases by complexing with peptide vectors, but this has yet to translate into a sufficient advantage *in vivo* over naked oligonucleotides.

A cautionary tale serves as a reminder that not all intron-targeting leads to clear interpretation of observed biological activity. For example, a PNA targeted to an intronic sequence in a severe combined immunodeficiency mouse model of Burkitt's lymphoma conjugated to a Lys-rich nuclear localization signal peptide was shown to be effective in blocking tumor growth through multiple intravenous injections.[78] This was a surprising result, because the Lys-rich peptide is very likely to be degraded rapidly in the circulation and thus insufficiently intact to act as an nuclear localization signal. Such peptide conjugation may instead alter the bioavailability of the PNA. No other peptide conjugates were examined. Further, the mechanism of action of the PNA in this case is obscure. The PNA was targeted to the Eμ enhancer sequence, which regulates c-myc overexpression. PNA binding may perhaps alter RNA secondary structure to downregulate c-myc expression, rather than altering splicing patterns.

Gene targeting PNA

Three recent examples of antigene PNA (AgPNA) targeting are described (**Table 2**). AgPNAs conjugated to Lys-rich or Arg-rich short peptides have shown some effectiveness in targeting the progesterone receptor promoter DNA in breast cancer cells resulting in downregulation of receptor protein production, but to achieve this activity, cells needed to be treated with μmol/l concentrations of AgPNA for several days.[79]

Further, it is not clear whether targeting was truly obtained at the DNA level or instead on short RNAs derived from transcripts at the promoter site. A perhaps more promising target for PNA conjugated to the peptide Lys_8 is the repeated CAG sequences in the mRNA of huntingtin to block mutant expression selectively in cells, which would be a potential approach to treat Huntington's disease.[80] However, there are alternative promising methods of targeting announced from the same laboratory involving use of other oligonucleotide analogues, siRNAs and miRNA mimics that may turn out to have higher allele-selectivity,[81] and *in vivo* studies are awaited eagerly.

An interesting new application is the use of triplex-forming PNA conjugated with the classic CPP Penetratin to direct gene modification in the hematopoietic stem cells of mice, retaining unmodified differentiation capabilities.[16] Sequence-specific gene modification was also observed in multiple somatic tissues following systemic administration, and also preserved in bone marrow and spleen of recipient mice following transplantation of bone marrow from Penetratin-PNA–treated mice. This suggests that peptide–PNA conjugates might have good potential application for treatment of monogenic hematological diseases such as thalassemia and sickle cell anemia.

miRNA-targeting oligonucleotides (anti-miRs)

Most of the anti-miR oligonucleotide types utilize 2′-OMe, LNA or 2′-fluoro analogues usually as mixmers of more than one analogue type or with DNA (**Table 3**). *In vivo* applications all utilize PS linkages. Currently, there are no papers describing peptide delivery of such anti-miRs. As discussed above, it seems that some naked oligonucleotide analogues may have the ability not only to enter cells through endocystosis but also can efficiently block miRNA activity without need for any enhancement of transfection by peptide or other vectors.[19,20,35,82,83] Thus it seems unlikely that peptide-mediated delivery will be of value for such oligonucleotide types.

In the case of PNA anti-miRs, just a few added Lys residues enhances cell uptake significantly,[19,22] and a terminal Cys residue also adds to the uptake potential and anti-miR activity.[20] A longer peptide is not required for activity[19] but conjugation of an Arg-rich Tat peptide to PNA anti-mIRs has also shown efficient cell delivery while maintaining miRNA regulation activity in cell culture.[84,85] In addition to CPPs, it seems likely that CPPs would be worth investigating here for the future development of PNA anti-miRs targeted to specific tissue types or to cancer cells.

Peptide-mediated delivery of siRNAs

Covalent conjugation of siRNA with a CPP has met with limited success (**Table 3**). For example, reduction in mRNA expression was achieved only at high concentration of CPPs Tat or Penetratin conjugated to the 3′-end of the sense strand of siRNA,[86] which is the most appropriate position for conjugation. An interesting peptide conjugated recently in this way is the cell and skin penetrating peptide called SPACE, identified by a phage display technique, which was evaluated for delivery of siRNA *in vivo* to two different targets, interleukin-10 and GAPDH mRNAs. Conjugation of siRNA with the SPACE peptide led to enhanced skin-absorptive properties and knockdown of corresponding protein targets.[87] It is hoped to use such conjugates to treat atopic dermatitis. Unfortunately, there was no experiment described showing knockdown at the mRNA level, which is required to be sure that the effects observed in cells and mice on protein reduction are entirely due to an siRNA-directed RNA cleavage.

Cyclic RGD peptides have been investigated as tri- and tetra-valent conjugates of siRNA and shown to bring about dose-dependent reduction of reporter luciferase expression in human melanoma cells, but not the monovalent or divalent conjugates.[88] This result demonstrates that, as with oligonucleotides, multivalency is likely to be necessary for conjugates of siRNA for sufficient effectiveness as a cell-targeting approach.

In general, noncovalent complex formation has been a more fruitful strategy to siRNA delivery (**Table 3**).[89] An amphipathic cholesterol-functionalized peptide carrier, Chol-MPG-8, was shown capable of complexing siRNA-targeting cyclin B1 and to lead to tumor growth reduction after intravenous injection in a PC-3 cancer model.[90] The high stability of Chol-MPG-8–based nanoparticles and the slow release of the siRNA within cells allowed the use of low concentrations of siRNA. A peptide named CADY was also reported by the same research group to bring about inhibition of PC3 tumor growth when complexed with siRNA.[91] CADY adopts a helical conformation within cell membranes, where charged amino acids are exposed on one side of the helix and tryptophan residues, found essential to good cellular uptake, are on the other side. Importantly, CADY–siRNA complexes were shown to bind to phospholipids on biological membranes *via* electrostatic interactions and enter cells by energy-independent translocation thereby bypassing the endosomal pathway.[46,62,92] A fusion peptide of Tat (49-57) and a membrane lytic peptide LK15 was shown to form complexes with both short hairpin RNA and siRNA targeting BCR-ABL oncoprotein mRNA, but the short hairpin RNA induced a more stable silencing of the gene target in K562 chronic myeloid leukemia cells compared to siRNA complexes.[93]

Stearylated peptide vectors have also been used successfully to deliver siRNA. A TP10-derived lipopeptide (PF6) was designed to aid endosomal release through attachment of four pH titratable trifluoromethylquinoline moieties to a lysine side chain of TP10. PF6 was shown to form nanoparticles with siRNA and knockdown HPRT1 mRNA production in a range of cell types as well as in kidney, lung, and liver of mice upon tail vein infusion at 1 mg/kg.[94]

An alternative peptide–siRNA complexation approach utilizes a recombinant fusion of the HIV Tat protein PTD with a double-stranded RNA binding domain (DRBD) that binds to siRNA and neutralizes its negative charges. The PTD-DRBD peptide vector has shown excellent cellular delivery of siRNA into various primary and transformed cells.[95] PTD-DRBD was used to package two siRNAs simultaneously (against EGFR and Akt20) to induce tumor-specific apoptosis in a glioblastoma model after intracerebral injection, and to also substantially increase mouse survival.[96] The PTD-DRBD peptide is now being developed industrially, but its very large size makes it unsuitable for chemical synthesis. Further the general applicability for siRNA delivery *in vivo*, especially for systemic use, is in doubt in the absence of further examples of efficacy.

Viral RNA targeting

Although there are no examples of peptides being used for delivery of negatively charged oligonucleotides as antiviral agents, peptide conjugates of charge-neutral PNA and PMO have been investigated for some years. Peptide-PMO antiviral applications up to 2007 include targeting of Dengue virus, Cocksackievirus, Ebola, and Marburg viruses for example and are reviewed in reference 36. Despite reassuring results that PNA-peptides targeted to the HIV-1 *trans*-activation responsive element are nontoxic in mice at high (300 mg/kg) doses,[97] antiviral development seems recently to have ceased for PNA peptides. By contrast, RXR4 and B-peptide conjugates of PMO oligonucleotides have recently shown high antiviral activities both in cell culture and *in vivo* in various systems (**Table 4**). For example, P-PMO conjugates interfered with splicing of hemagglutinin-activating protease TMPRSS2 pre-mRNA and suppressed viral titers by two to three $\log_{10}$ units in Influenza A cultures.[98] Similarly, RXR4-PMOs blocked viral replication of human respiratory syncytial viruses both in cells and in BALB/c mice.[99] Here, the PMO sequence was targeted to the start of the coding region. Interestingly, there was also reduction in lung viral titers even with treatment of mice 3 hours after infection, but not 8 hours after infection. This suggests the PMO needs to be present in the cell soon after infection if it is to have an impact on virus production.

Particularly promising results have been obtained *in vivo* in pre- and post-infection P-PMO treatment of pigs infected with porcine reproductive and respiratory syndrome virus both in reducing viremia and interstitial pneumonia.[100] RXR4–PMO conjugates were also seen to substantially improve antisense activity *in vivo* over naked PMO in protection of mice upon infection with West Nile virus or coronavirus by targeting to specific viral RNA sites involved in translation or replication.[101,102]

Overall, the P-PMO approach is very promising, but it also must compete with other antiviral approaches including use of PMO analogues with cationic groups within the PMO as currently being exploited by AVI Biopharma (Bothell, WA).

Antibacterial activity of antisense PNA and PMO

Increasing multidrug resistance in bacteria and the difficulties in discovery of new small molecule antibiotics have prompted consideration of new strategies such as gene targeting by PNA and PMO for treating bacterial diseases. However, the delivery of PNAs into bacterial cells has proved to be problematic. Although some CPPs may themselves have intrinsic antimicrobial activity,[103] peptide-mediated delivery of antisense PNA and PMO targeting specific essential bacterial genes was suggested more than 10 years using peptide–PNA conjugates[104] and has shown recent promise as an antibacterial strategy (**Table 4**). In particular, Arg-rich peptides conjugated to PMO are able to penetrate the cell outer membrane and display gene-specific antibiotic activity in gram-negative bacteria.[105] The peptide–PMO conjugates are able to reduce bacterial blood titers and increase survival in *Escherichia coli* infected mice.[106,107] Very recently, a novel 22-residue Arg-rich peptide derived from human T cells, when conjugated to PMO targeting *GyrA* was able to induce broad range bacterial cell killing at concentrations much lower than required for activity by other Arg-rich peptide-PMOs.[108] The higher than perhaps anticipated activity is attributed to non-gene–specific RNA targeting of the P-PMO in addition to a gene specific effect, suggesting partial bacteriocidal activity plays an important role here. Interestingly, PMO containing positive charges in the PMO backbone also have antisense activity but did not achieve the same efficiency as peptide-conjugated PMO.[109]

PNAs conjugated to a Lys-rich peptide have also been used successfully to validate essential gene targets in *Escherichia coli* by studying the relationship between decrease in mRNA expression and growth rate decline.[110] Further, peptide-conjugated PNA targeting *rpoD*, a gene essential for bacterial growth, showed broad inhibition in multidrug-resistant *Escherichia coli, Salmonella enterica, Klebsiella pneumoniae,* and *Shigella flexneri* both in cells and *in vivo*.[111]

The variety of recent promising results shows that peptide-mediated delivery of PMO and PNA holds promise for further drug development against multidrug-resistant bacteria.

POLYMER, LIPOSOMAL, AND EXOSOME DELIVERY METHODS INVOLVING PEPTIDES

Peptide-conjugated polymers

Synthetic and naturally occurring cationic polymers represent a large group of carriers for nucleic acids. Although initially developed for the purpose of gene delivery, numerous reports have affirmed their utility in oligonucleotide delivery. These linear or branched polymers range from the first discovered poly-L-lysine (PLL), to the most frequently used polyethyleneimine (PEI).[112,113] They share the properties of condensing oligonucleotides into small particles (polyplexes) that facilitate cellular uptake *via* endocytosis. However, their delivery efficiencies and toxicities vary significantly. PEI has been produced in particular in an assortment of structural variants.[114]

A major drawback with use of PEI and many other cationic polymers is their non-biodegradable nature, which raises toxicity concerns.[115] Furthermore, their efficiencies have been poor *in vivo* in many cases, due to unwanted interactions with serum components.[115,116] Polyethylene glycol (PEG) linkers of different sizes have been included in PEI polymers in order to prolong the circulation time in blood (reviewed in ref. 117). Although such PEGylation significantly stabilizes polyplexes, it unfortunately often results in lower potency, due to inadequate cellular uptake and poor endosomal escape.[117,118] Another strategy for stabilization of polyplexes involves use of hydrophobic modifications. For example, partial modifications of amines in PEI with tyrosine stabilize polyplexes significantly increases transfection of both siRNAs and splice-redirecting 2′-OMe oligonucleotides.[119,120] Analogously, modification of polyamines with hydrophobic acrylates results in stabilization of cores for highly efficient siRNA and 2′-OMe anti-miR delivery *in vivo*.[121,122]

Peptide modifications of cationic polymers have been used successfully to either improve tissue targeting or overall delivery efficacy and to decrease the required dose. Pioneering work from the laboratory of Ernst Wagner showed that PEI-mediated gene transfer can be improved dramatically by incorporation of receptor-targeting peptide ligands (reviewed in ref. 117,123,124).

Many other groups have capitalized on this peptide/cationic polymer strategy to improve gene delivery profoundly.[125,126] Surprisingly, examples of use of targeting peptides and CPPs to enhance polyplex delivery of oligonucleotides have been sparse. In one study. Tat peptide was conjugated to PEGylated PEI which, when complexed with 2′-OMe oligomers and injected intramuscularly into *mdx* mice, improved exon skipping of dystrophin pre-mRNA.[127]

Several successful studies on siRNA delivery have been reported. Schiffelers *et al.* utilized the integrin-binding peptide RGD attached to PEGylated PEI for delivery of siRNA targeting VEGF-R in tumor-bearing mice.[128] Similarly, Wang *et al.* conjugated a bombesin peptide *via* a PEG spacer to a free thiol group in an amphiphilic polymer N-(1-aminoethyl)iminobis[N-(oleicyl-cysteinyl-histidinyl-1-aminoethyl)propionamide] (EHCO) to target bombesin receptors overexpressed on various cancer cells.[129] The siRNA/functionalized polymer complex was shown to efficiently promote RNA interference responses in cells and systemic administration of anti-HIF-1α siRNA with bombesin-containing EHCO displayed significant advantage over nontargeted polymer in inducing target reduction in a xenograft model of human glioma.

PEGylated transferrin peptide combined with oligoethyleneimine was able to deliver siRNA highly efficiently into neuroblastoma-bearing mice.[130] Further, an elegant demonstration of receptor-specific uptake of defined folate-PEG-siRNA conjugates together with a structurally defined polycation was shown in use of such complexes for specific gene silencing.[131]

Instead of targeting peptides, the Wagner group has obtained improved endosomal escape by inclusion of fusogenic peptides. In the initial study, PEGylated polylysine/PEI was covalently linked to the endosomolytic peptide, melittin. Since melittin possesses lytic properties at both neutral and low pH, amines within the peptide were masked by reaction with dimethylmaleic anhydride (DMMA). Dimethylmaleic groups are removed at low pH within endosomes, thus exposing the lytic peptide. Polycation-PEG-DMMA-melittin conjugates were mixed with siRNAs to form polyplexes that efficiently induced an RNA interferene response in cell culture.[132] In a further study with the same components, a cysteine residue was incorporated into the PLL to allow disulfide formation with an siRNA containing a 5′-thiolated sense strand. Such conjugates were almost as efficient as polyplexes and displayed reduced cytotoxicity.[133]

Another important example of peptide/polymer combinations is a cyclodextrin containing polymer (CDP)-based system, which so far is the only platform reported for RNA interference delivery in humans.[134] CDP is a short polycation that assembles with nucleic acids (e.g., siRNA) *via* electrostatic interactions. In order to facilitate endosomal escape, imidazole groups were incorporated on the ends of the polymer to act as proton sponges. For systemic delivery, the polymer was stabilized by inclusion of surface-functionalized PEG moieties. In contrast to other polymeric delivery systems, the CDP approach relies on noncovalent incorporation of PEG *via* conjugation to adamantane, a small molecule that forms a high affinity inclusion complex with cyclodextrin. Finally, in order to confer tissue targeting to solid tumors, the surface of CDP was coated with adamantane conjugated to the transferrin ligand for targeting of transferrin receptors that are expressed abundantly on the surface of tumors.[135]

Liposomal nanovectors with peptide ligands

Many common cationic liposomal vectors used for nucleic acid delivery in cell culture, such as lipofectin or lipofectamine, are not suited for *in vivo* use, because of their sensitivity to serum proteins and their toxicity.[136,137] Surface functionalization with inert hydrophilic polymers such as PEG has improved *in vivo* use of such vectors (reviewed in ref. 138), but PEGylation decreases cell delivery due to poor membrane interactions and poor endosomal escape.[118,139] New lipid formulations that include peptides have broadened the repertoire of liposomes used for oligonucleotide delivery. In particular, the inclusion of Arg-rich CPPs on such liposomes has proven as a potent strategy to enhance transfections.[10,138] In a recent example of peptide-mediated siRNA delivery, liposomal nanoparticles that are octaarginine-modified was used to encapsulate siRNA and to silence an endogenous gene in liver cells as well as *in vivo* at low concentrations of siRNA (25 μg/mouse) using a single dose, without evidence for liver toxicity.[140] In another example, cationic liposomes were modified by a chimeric peptide containing 16 lysine residues and a short multicell targeting peptide Y and used to deliver siRNA against luciferase and GAPDH targets.[141]

A further example of rational liposome design is the multifunctional envelope-type device (MEND) system.[10] In the prototype MEND, oligonucleotides are condensed with polycations (protamine) and wrapped by a lipid envelope containing cationic, anionic, and helper lipids, such as dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) that improves endosomal escape. In addition to surface modification with PEG, peptide delivery vectors have been incorporated on to the surface of such liposomes using a stearylated peptide that spontaneously inserts into the lipid membrane. Novel versions of MEND contain cleavable PEG linkers as well as targeting peptides for specific receptors or fusogenic peptides, in order to confer targeting of specific tissues and controlled release of encapsulated cargoes (reviewed in ref. 118). MEND has been efficiently exploited for delivery of unmodified single-stranded antisense oligonucleotides in cells as well as siRNAs both in cells and *ex vivo* in dendritic cells.[142–144] Very recently, an even more sophisticated MEND system was efficiently utilized for systemic, targeted delivery of siRNA to tumors.[145]

Similar liposomal delivery systems have been developed for drug delivery by Torchillin and colleagues, where the surface has been decorated with the CPP Tat.[138] To incorporate the Tat peptide in a controlled manner, it was covalently linked to the anionic lipid phosphatidic acid that spontaneously inserts into liposomes. Such liposomes have been used primarily for targeted delivery of plasmid DNA to tumors, but have also been shown to be effective for siRNA delivery.[146] CPPs have also been used in conjunction with conventional liposome-based transfection agents and synergistic effects reported for the delivery of splice redirecting 2′-OMe oligomers.[147]

Peptide-functionalized exosomes

Practically all nanoparticle vectors for oligonucleotide delivery have been developed for targeting liver or tumors. Development of efficient carriers for crossing of other biological barriers, such as the blood-brain barrier, is in its infancy. In addition to synthetic nonviral vectors, which still retain inherent risks of raising an immune response or causing systemic

toxicity *in vivo*, an alternative biologically derived vector has recently been proposed that makes use of naturally occurring membrane vesicles secreted by most cells and found in all body fluids, which are thought to be key mediators of information transmission between cells in the body.[148]

A subgroup of such vesicles are termed exosomes, which have been shown recently to contain various RNA species. They exert their biological effects either by directly activating cell surface receptors on target cells or by transferring proteins from the cell of origin to the recipient.[149] Furthermore, numerous studies suggest that exosomes, which are derived from the endolysosomal pathway and have a diameter of 40–120 nm, play a crucial role in the horizontal transfer of RNA to neighboring or distant recipient cells.[150,151] Of particular interest is their ability to deliver miRNAs into recipient cells to induce gene silencing.[152,153] Such properties indicate that they would be highly suitable candidates for oligonucleotide and siRNA delivery.

Wood *et al*. recently provided the first proof-of-concept study for delivery of exogenous siRNA using such exosomes.[154] Immature dendritic cells were chosen as source for exosomes, because they lack T-cell activators and are thus immunologically inert.[155] In order to confer targeting of exosomes to the brain, dendritic cells were transfected with a plasmid expressing an exosomal protein Lamp2b fused to a brain-specific peptide, rabies virus glycoprotein-derived peptide (RVG),[156] which becomes presented at the surface of exosomes. RVG-targeted exosomes were subsequently purified from cell cultures and loaded with exogenous siRNA by electroporation. Systemic delivery of GAPDH targeting siRNA with RVG-exosomes resulted in specific knockdown of GAPDH in several regions of mouse brain such as cortex and striatum. The potency of RVG-exosomes was tested by delivery of BACE-1 siRNA (targeting the enzyme β-secretase), which resulted in significant and specific target knockdown (60%) in the mouse brain cortex. This knockdown led to a reduction in the extracellular accumulation of Aβ1-42, the toxic amyloid species produced by BACE-1 that is implicated as a cause of Alzheimer's disease. No toxicity or immunogenicity was observed, even after repeated administration, a prerequisite for many disease applications.

PHYSIOLOGICAL ASPECTS OF PEPTIDE-MEDIATED OLIGONUCLEOTIDE AND siRNA *IN VIVO* DELIVERY

Crucial to the clinical application of peptide-based oligonucleotide and siRNA therapies is the tailoring of such drugs for whole-body distribution, in order to maximize specific tissue targeting while minimizing induced toxic or immune responses. *In vivo* biodistribution data can be derived either by the assessment of an induced biological affect, by the detection of a directly labeled bio-cargo, or by use of a transgenic animal reporter system, yet each method has caveats in their use. Measurement of an induced biological affect is restricted to those tissues where the transcript targeted by the oligonucleotide or siRNA is expressed, for example, using dystrophin exon skipping and protein restoration as a read-out in the *mdx* mouse model.[75] Fluorescent molecules or radioactive isotopes have been used successfully to label and follow the biodistribution of peptide conjugates of biocargoes such as PNA.[157] However it is important to note that where the peptide part only is labeled, label detection may not always correlate with the location of the bioactive cargo. For example, a recent report of an N-terminally FITC-labeled B-PMO having crossed the blood-brain barrier and reaching Purkinje cells in wild-type mice should be viewed with caution until confirmed by other methods.[158] An EGFP-654 transgenic mouse model that ubiquitously expresses an aberrantly spliced EGFP-654 pre-mRNA reporter gene has been used to assess functional biodistribution of B-PMO.[159] However, such distribution assessments might not reflect the potential of a peptide-PMO to influence a disease pathology, whereas in an animal model of a disease such as DMD this becomes less of an issue. Nevertheless the use of all three of these techniques together can provide valuable information in building a view of how a peptide affects biodistribution of an oligonucleotide cargo.

An important parameter in systemic delivery is the route of administration (*i.e.*, intravenous, intraperitoneal, subcutaneous, oral or nasal) in order to find the best biodistribution pattern for the particular application, but also to obtain optimal safety profiles.[160] The efficacies of the delivery routes depend on the specific peptide ligand as well as the cell-targeting requirements of the studied disease, and must be balanced against the toxicology profile of the peptide-modified cargo. Very few direct comparisons of delivery routes have been published, but for B-PMO conjugates for example, higher efficiencies have been achieved *via* the intravenous delivery route compared to the subcutaneous route.[159]

Toxicity and unwanted immune activation by peptide conjugates of oligonucleotides and siRNA need to be carefully assessed in each case. For example, Penetratin–siRNA conjugates showed innate immune activation following intratracheal administration.[161] By contrast, Penetratin–PNA conjugates did not induce an immunogenic response after intraperitoneal delivery and were well tolerated in mice at 100 mg/kg.[97] Although CPPs do have potential to stimulate unwanted immunological responses, their small size and the use of analogues has reduced this risk significantly. For example, no immune response has been seen to date for B peptide-PMO[162] and there are no reports of any peptide-PMO or peptide–PNA conjugate to date inducing a significant response.

The nature and levels of *in vivo* toxicity are not currently well understood for peptide conjugates of oligonucleotides or for peptide-based vector delivery routes, and there are very few published *in vivo* studies, probably because of commercial sensitivity of such data. However, peptide-PMO acts as a good case study. Here the PMO cargo has an amazingly good safety profile in animals and in many clinical trials.[37,163,164] Thus the safety profile of a P-PMO conjugate will depend almost entirely upon the specific peptide sequence and is assessed in the context of the therapeutic dose required in a particular clinical setting. Amantana *et al.* investigated the safety profile in rats of an anti-c-*myc* PMO conjugated to the RXR4 CPP. Although lower doses of the CPP-PMO were well tolerated (15 mg/kg), adverse events such as lethargy, weight loss, and elevated blood urea nitrogen and serum creatinine levels were reported at much higher doses (150 mg/kg), with

a lethal dose reported as 400 mg/kg.[165] Toxicity has also been reported in non-human primates following a preclinical study with the B-PMO peptide conjugate developed by AVI BioPharma. Dose-dependent toxicity was observed in healthy Cynomolgus monkeys with four weekly intravenous injections of 9 mg/kg, causing tubular degeneration in the kidneys (AVI BioPharma website www.aviobio.com).[63] No toxic effect was observed at higher doses in *mdx* mice, thus highlighting that the P-PMO toxicity threshold varies between species. Although lower toxic thresholds have been reported elsewhere, comparisons between studies assessing the use of CPPs to deliver non-oligomers are problematic, because the effect of the cargo cannot be distinguished from that of the CPP.[166]

In another useful study, Wancewicz *et al.* have reported acute toxicity in mice for a PNA containing nine additional Lys residues when dosed at 10.5 µmol/kg at three times a week for 2 weeks.[77] Similarly a PNA conjugated to an amphipathic peptide containing Lys, Leu, and Ala residues showed severe nephrotoxocity, with profound proximal tubule necrosis at 40 mg/kg dose, whereas a PNA conjugate with mostly Arg and homoArg residues did not show such toxicity at this dose. Interestingly, the tissue distributions of these two peptide-PNAs differed widely, with the latter Arg-rich conjugate showing good splicing redirection in adipose tissue (the targeted organ) even at a low dose of 2.5 mg/kg.[77]

Thus, although immunogenicity seems unlikely to be an issue for most small peptides as oligonucleotide conjugates, toxicity profiles need to be evaluated carefully on a case by case basis to establish an effective yet safe dosing regimen in humans. There is clearly an important need to determine the exact nature of peptide-induced toxicity patterns, alongside the development of further peptide modifications, if such peptide applications are to reach the clinic.

CLINICAL PERSPECTIVES OF PEPTIDE-MEDIATED DELIVERY OF NUCLEIC ACIDS

Efforts to translate synthetic peptides conjugates or complexes of oligonucleotides and siRNA to treat human disease have recently stepped up. Many of the polymeric, liposomal, and other nanovectors involving peptides have shown great promise for delivery in cell culture and in animal models. However, there are no examples yet of any of these reaching clinical trials apart from the application involving the human transferrin protein used in siRNA particle formation which was recently reported in the clinic.[134] The application of short peptides in siRNA delivery applications needs to overcome remaining obstacles of reproducible and validated manufacture of the overall vectors. In addition, there needs to be better control of off-target effects that lead to toxicities. Peptide-based ligands displayed on such vectors are bound to feature more in clinical development in the near future and these are already helping to gain greater tissue and targeting specificity. It is hoped that further preclinical development studies here will be published soon to help move such therapies to the clinic as rapidly as possible.

Therapeutic approaches for neuromuscular disorders currently lead the way in clinical development of peptide-mediated delivery. Repeat systemic dose-escalation studies of naked PMO and 2′-OMe/PS oligonucleotides having been completed recently in DMD patients and further dose-escalations studies planned[37,167] (current trials reviews in ref. 168), while recruitment for a clinical trial to treat spinal muscular atrophy is underway (clinicaltrials.gov identifier: NCT01494701), which is another neuromuscular disease where peptide-conjugated PMO is likely to be applied.

Recently there has been a concerted effort to evaluate the potential of CPPs and other peptides in some multi-systemic neuromuscular disorders. For example, our laboratories are currently involved in multi-center development of a peptide-PMO to treat DMD, based on initial evaluations in the *mdx* mouse as a model, and further preclinical therapeutic studies are underway to supplement data already published.[75,159,165] Here, a lead Pip peptide conjugated to PMO has shown excellent dystrophin production in cardiac muscle, a tissue previously shown to take up naked antisense oligomers poorly, in addition to in skeletal muscles.[75] However, evidence of induced renal toxicity in monkeys for the earlier B-PMO lead[63] has highlighted the needs for caution and for obtaining a deeper understanding regarding possible toxic side effects of cationic CPPs.

It seems likely that the simplicity of the peptide conjugation approach and the ability to apply the principle of a steric-blocking approach across a broad range of neuromuscular and neurodegenerative diseases will likely drive the clinical development of peptide conjugates of oligonucleotides and their analogues forward for several years to come. New examples of preclinical development are expected soon in several of such diseases and there is great hope that one or more of these conjugates will make it to the clinic in the near future.

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