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

Peter Järver1, Thibault Coursindel, Samir EL Andaloussi2,3, Caroline Godfrey2, Matthew JA Wood2 and Michael J Gait1

INTRODUCTION
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.

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,

Received 2 March 2012; revised 26 April 2012; accepted 26 April 2012
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)).

Current thinking is that the oligonucleotide may meet the miRNA somewhere within the endosomal pathway, 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. Therapeutic applications of anti-miRs have been recently reviewed.

Perhaps the most utilized and well-studied analogue is the PS linkage where a sulfur atom replaces one non-bridging oxygen atom in the phosphodiester 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. 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.

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).

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.

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.

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.

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. Stimulation of exon inclusion by antisense oligonucleotides is an alternative mechanism being examined for potential treatment for spinal muscular atrophy. In addition, exon skipping can be applied to wild-type genes to generate out-of-frame transcripts as a method of normal protein downregulation.

**Molecular Therapy–Nucleic Acids**
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-methoxymethyl, 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. The increased affinity allows LNA to be used in much shorter oligonucleotide than for many other analogue types (tiny LNAs). 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. 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. PNA displays high selectivity and mismatch discrimination towards its target strand and have been used effectively in many antisense applications and in vivo. 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.

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. 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 disulphide groups might enhance cellular uptake, although the reasons for this are not as yet fully understood.

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. 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. 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) 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
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

| mRNA targeting by antisense ONs | Table 1 Selection of peptide–oligonucleotide conjugates and complexes used for mRNA targeting |
|--------------------------------|---------------------------------------|
| **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/PS/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-directing 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-Penetratin              | 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 |
| MPG                        | 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 |
| PKKRRKIV                   | 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.
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

| 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.

| 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               |
| —                   | —       | LNA2′-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.
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-directing 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 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.61,62 The uptake mechanisms of peptide-complexed oligonucleotides appear to be more diverse. Some peptide complexing agents such as MPGx 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.42,63

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 hydrobicity and proteolytic resistance in serum, such as (R-Ahx-R)_n-β-Ala and (R-Ahx-R-β-Ala-R)_2-β-Ala called RXR4 and B-peptide respectively.56,63 The aminohexanoyl spacer in the R-Ahx-R motif was found to be optimal in the splicing-redirection assay among several other peptides.
spacers tried.\textsuperscript{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,\textsuperscript{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 \textit{in vivo}.\textsuperscript{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.\textsuperscript{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\textsubscript{2}-PNA conjugate also increased splice-redirecting activity, and it was suggested that the increased activity was due to micelle formation.\textsuperscript{66}

A conjugate between a 2\textsuperscript{-}OMe/PS antisense oligonucleotide and a bivalent RGD peptide, selectively targeting αvβ3 integrin, was found to enter cells \textit{via} receptor-mediated endocytosis and was used for splicing redirection in melanoma cells.\textsuperscript{67} The RGD conjugate was found to enter cells by an endocytotic pathway distinct from that of naked oligonucleotide.\textsuperscript{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 \textit{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.\textsuperscript{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 \textit{in vivo} setting. Demonstrations of quantitatively useful peptide-mediated delivery in cells and \textit{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\textsuperscript{-}OMe/PS oligonucleotide was much more effective at luciferase upregulation than stearyl-Arg\textsubscript{6} in HeLa 705 cells.\textsuperscript{70} More effort has gone into developing stearyl derivatives of the peptide Transportan 10 (TP-10) as nucleic acid delivery agents.\textsuperscript{49} In general, this class of stearylated peptide delivery agent seems to utilize the endocytotic pathway.\textsuperscript{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\textsuperscript{-}OMe/PS oligonucleotides both in the HeLa 705 cell assay as well as in exon skipping in mouse \textit{mdx} muscle cells.\textsuperscript{72}

CPP-aided splicing redirection is also a promising mechanism for \textit{in vivo} and therapeutic applications. The \textit{mdx} mouse model of Duchenne muscular dystrophy (DMD), bearing a nonsense mutation in exon 23 of the dystrophin gene, is an important \textit{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 \textit{mdx} mouse.\textsuperscript{27,63,73} PMO conjugated to a chimeric CPP comprising the B-peptide and a short muscle-specific peptide, known as B-MSF, showed higher activity in skeletal tissue in \textit{mdx} mice than B-PMO.\textsuperscript{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.\textsuperscript{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 \textit{in vivo} antisense studies targeting DMD,\textsuperscript{76,77} 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.\textsuperscript{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 \textit{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.\textsuperscript{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 a 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 E\textsubscript{μ} 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.

\textbf{Gene targeting PNA}

Three recent examples of antigen 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.\textsuperscript{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 Lys6 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.35 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 endocytosis but also can efficiently block miRNA activity without need for any enhancement of transfection by peptide or other vectors.19,20,25,52,83 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. A longer peptide is not required for activity 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.84,86 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,90 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). An amphiphilic 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.89 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.96,97 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 transactivation responsive element are nontoxic in mice at high (300 mg/kg) doses,27 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 log10 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 antiviral 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 conjugates104 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 rpmO, 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 polyethylenimine (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 PEylation 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. 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 PEI, which, when complexed with 2'-OMe oligomers and injected intramuscularly into mdx mice, improved exon skipping of dystrophin pre-mRNA.

Several successful studies on siRNA delivery have been reported. Schiffelers et al. utilized the integrin-binding peptide RGD attached to PEI for delivery of siRNA targeting VEGF-R in tumor-bearing mice. Similarly, Wang et al. conjugated a bombesin peptide via a PEG spacer to a free thiol group in an amphiphilic polymer N-[(1-aminooethyl)iminobis(N-(oleyl-cysteinyl-histidinyl-1-aminooethyl)propionamide) (EHCO) to target bombesin receptors overexpressed on various cancer cells. 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. 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.

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-DMA-melittin conjugates were mixed with siRNAs to form polyplexes that efficiently induced an RNA interference response in cell culture. 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.

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. 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.

Liposomal nanovehicles 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. 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. 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. 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. 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.

A further example of rational liposome design is the multifunctional envelope-type device (MEND) system. 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. Very recently, an even more sophisticated MEND system was efficiently utilized for systemic, targeted delivery of siRNA to tumors.

Similar liposomal delivery systems have been developed for drug delivery by Torchilin and colleagues, where the surface has been decorated with the CPP Tat. 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. 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.

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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{150,151} Of particular interest is their ability to deliver miRNAs into recipient cells to induce gene silencing.\textsuperscript{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.\textsuperscript{154} Immature dendritic cells were chosen as source for exosomes, because they lack T-cell activators and are thus immunologically inert.\textsuperscript{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),\textsuperscript{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 OLGONUCLEOTIDE 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.\textsuperscript{75} Fluorescent molecules or radioactive isotopes have been used successfully to label and follow the biodistribution of peptide conjugates of bio-cargoes such as PNA.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{98} 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\textsuperscript{152} 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.\textsuperscript{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.\textsuperscript{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).\textsuperscript{58} 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.\textsuperscript{166}

In another useful study, Wanczucicz 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.\textsuperscript{77} Similarly a PNA conjugated to an amphipathic peptide containing Lys, Leu, and Ala residues showed severe nephrotoxicity, 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.\textsuperscript{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.\textsuperscript{144} 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\textsuperscript{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.\textsuperscript{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.\textsuperscript{75} However, evidence of induced renal toxicity in monkeys for the earlier B-PMO lead\textsuperscript{80} 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.

**Acknowledgments.** This work was supported by research grants to M.J.A.W. from the Muscular Dystrophy Campaign, Duchenne Ireland, and the Medical Research Council (G0900887), and joint research grants to M.J.A.W. and M.J.G. from Association Française contre les Myopathies (programme number 14784) and from the Wellcome Trust/Health Innovation Challenge Fund (HICF-1009-025). Work in the laboratory of M.J.G. was also supported by the Medical Research Council (MRC programme no. U105178803). S.E.L.A. is supported by a research fellowship from Swedish Society of Medical Research (SSMF). The authors declared no conflict of interest.

1. Kole, R, Krainer, AR and Altman, S (2012). RNA therapeutics: beyond RNA interference and antisense oligonucleotides. Nat Rev Drug Discov 11: 125–140.
2. Kurreck, J (2008). Therapeutic Oligonucleotides. Royal Society of Chemistry: Cambridge.
3. Bljäderbøsch, MK, Manoharan, M, Rump, ET, De Vruhe, RL, van Veghel, R, Tivel, KL et al. (1997). In vivo fate of phosphorothioate antisense oligodeoxynucleotides: predominant uptake by scavenger receptors on endothelial liver cells. Nucleic Acids Res 25: 3290–3296.
4. Kaneda, Y (2010). Update on non-viral delivery methods for cancer therapy; possibilities of a drug delivery system with anticancer activities beyond delivery as a new therapeutic tool. Expert Opin Drug Deliv 7: 1079–1093.
5. Xu, L and Anchordoguy, T. (2011). Drug delivery trends in clinical trials and translational medicine: challenges and opportunities in the delivery of nucleic acid-based therapeutics. J Pharm Sci 100: 39–52.
6. Derossi, D, Joliot, AH, Chassaing, G and Prochiantz, A (1994). The third helix of the Antennapedia homeodomain translocates through biological membranes. J Biol Chem 269: 10444–10450.
7. Said Hassane, F, Saleh, AF, Abes, R, Gait, MJ and Lebleu, B (2010). Cell penetrating peptides: overview and applications to the delivery of oligonucleotides. Cell Mol Life Sci 67: 715–726.
8. Margus, H, Padari, K and Pooga, M (2012). Cell-penetrating peptides as versatile vehicles for oligonucleotide delivery. Mol Ther 20: 525–533.
Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2a dose-ranging study. Lancet 378 (2011): 159–164.

Staton, AA and Giraldez, AJ (2011). Use of target protector morpholinos to analyze the gene expression and drug development. Targeting microRNAs involved in human diseases: a novel approach for modification of antisense and antigene oligodeoxynucleotides and small interfering RNA. Nucleic Acids Res 39: 3393–3402.

Matsuura, S, Ohashi, W, Suzuki, T, Niwa, M, Tanaka, S, Ueda, K et al. (2011). Stearoylated arginine-rich oligonucleotide: a new class of transfection systems. Biochem Biophys Res Commun 398: 1005–1011.

Møse, M, Eri Andaloussi, S, Lundin, P, Oksikov, N, Johansson, HJ, Gutermstam, P et al. (2011). Stearoylated oligonucleotide ODN for delivery of splice correcting oligonucleotides using a non-covalent inoculation strategy. J Control Release 153: 221–227.

Allinguard, B, Hantraye, P, Mailleux, P, Moya, K, Boulo, C and Prochiantz, A (1995). Downregulation of amyo1 precursor protein inhibits neurite outgrowth in vitro. J Cell Biol 129: 919–927.

Borgström, JP, Aubertin, AM, Milhaud, PG and Lebeau, B (1994). Improved biological activity of antisense oligonucleotides conjugated to a fusogenic peptide. Nucleic Acids Res 22: 4681–4688.

Turner, Y, Wallakat, G, Sääläk, P, Wiesner, B, Pritz, S and Oehlke, H (2010). Cellular uptake and biological activity of peptide nucleic acids conjugated with peptides and without cell-penetrating ability. J Peptide Sci 16: 43–57.

Futaki, S, Takahara, S, Santanata, S, Masi, M, Kawamura, E, Mizukami, D et al. (2012). Peptide-conjugated antisense oligonucleotides for targeted inhibition of a transcriptional regulator in vivo. Biochem Biophys Res Commun 419: 29–34.

Morris, MC, Gros, E, Adrian-Herrada, G, Chrob, M, Archdeacon, J, Hetz, F et al. (2007). A non-covalent peptide-based carrier for in vivo delivery of DNA mimics. Nucleic Acids Res 35: 4230–4239.

Kang, SH, Cho, M and Kole, R. (1998). Upregulation of luciferase gene expression with antisense oligonucleotides: implications and applications in functional assay development. Biochemistry 37: 6235–6239.

Szaran, P, Kang, SH, Maser, MA, We, C, Dillman, J, Summerton, J et al. (2001). Nuclear antisense effects of neutral, anionic and cationic oligodeoxyribonucleotide analogues. Nucleic Acids Res 29: 3965–3974.

Wu, RP, Youngblood, DS, Hassinger, JD, Lovejoy, CE, Nelson, MH, Iversen, PL et al. (2007).Cell-penetrating peptides as transporters for morpholino oligomers: effects of amino acid composition on intracellular delivery and cytotoxicity. Nucleic Acids Res 35: 5185–5191.

Abes, S, Turner, JJ, Ivanova, GD, Owen, D, Molony, K et al. (2008). Delivery of steric block morpholino oligomers by (R)-X-RR4 peptides: structure-activity studies. Nucleic Acids Res 36: 6343–6354.

Abes, S, Turner, JJ, Ivanova, GD, Owen, D, Williams, D, Arzumanov, AA, Abes, S et al. (2005).Cell-penetrating peptide conjugates of peptide nucleic acids (PNA) as inhibitors of HIV-1 Tat-dependent trans-activation in cells. Nucleic Acids Res 33: 8837–8848.

Henke, E, Park, J, Vider, J, de Candia, P, Chyn, Y, Solt, DB et al. (2008).Peptide-conjugated antisense oligonucleotides for targeted inhibition of a transcriptional regulator in vivo. Biochem Biophys Res Commun 362: 91–100.

Morris, MC, Gros, E, Adrain-Herrada, G, Chrob, M, Archdeacon, J, Hetz, F et al. (2007). A non-covalent peptide-based carrier for in vivo delivery of DNA mimics. Nucleic Acids Res 35: 4230–4239.

Kang, SH, Cho, M and Kole, R. (1998). Upregulation of luciferase gene expression with antisense oligonucleotides: implications and applications in functional assay development. Biochemistry 37: 6235–6239.

Szaran, P, Kang, SH, Maser, MA, We, C, Dillman, J, Summerton, J et al. (2001). Nuclear antisense effects of neutral, anionic and cationic oligodeoxyribonucleotide analogues. Nucleic Acids Res 29: 3965–3974.

Wu, RP, Youngblood, DS, Hassinger, JD, Lovejoy, CE, Nelson, MH, Iversen, PL et al. (2007).Cell-penetrating peptides as transporters for morpholino oligomers: effects of amino acid composition on intracellular delivery and cytotoxicity. Nucleic Acids Res 35: 5185–5191.

Abes, S, Turner, JJ, Ivanova, GD, Owen, D, Molony, K et al. (2008). Delivery of steric block morpholino oligomers by (R)-X-RR4 peptides: structure-activity studies. Nucleic Acids Res 36: 6343–6354.

Abes, S, Turner, JJ, Ivanova, GD, Owen, D, Williams, D, Arzumanov, AA, Abes, S et al. (2005).Cell-penetrating peptide conjugates of peptide nucleic acids (PNA) as inhibitors of HIV-1 Tat-dependent trans-activation in cells. Nucleic Acids Res 33: 8837–8848.

Henke, E, Park, J, Vider, J, de Candia, P, Chyn, Y, Solt, DB et al. (2008).Peptide-conjugated antisense oligonucleotides for targeted inhibition of a transcriptional regulator in vivo. Biochem Biophys Res Commun 362: 91–100.

Morris, MC, Gros, E, Adrain-Herrada, G, Chrob, M, Archdeacon, J, Hetz, F et al. (2007). A non-covalent peptide-based carrier for in vivo delivery of DNA mimics. Nucleic Acids Res 35: 4230–4239.
65. Koppelman, U, Shiraiishi, T, Zachar, V, Pankratova, S and Nielsen, PE (2008). Improved cellular activity of antisense peptide nucleic acid conjugates by conjugation to a cationic peptide-lipid (CatLip) domain. Bioconjug Chem 19: 1526–1534.

66. Shen, G, Fang, H, Song, Y, Bielska, AW, Wang, Z and Taylor, JS (2009). Phospholipid-conjugated intracellular delivery of peptide nucleic acids. Bioconjug Chem 20: 1729–1736.

67. Alam, MR, Dixit, V, Kang, H, Li, ZB, Chen, X, Trejo, J et al. (2008). Intracellular delivery of an anionic antisense oligonucleotide via receptor-mediated endocytosis. Nucleic Acids Res 36: 2764–2776.

68. Alam, MR, Ling, X, Dixit, V, Fisher, M, Chen, X and Juliano, RL (2010). The biological effect of an antisense oligonucleotide depends on its route of endocytosis and trafficking. Oligonucleotides 20: 103–109.

69. Ming, X, Alam, MR, Fisher, M, Yan, Y, Chen, X and Juliano, RL (2010). Intracellular delivery of an antisense oligonucleotide via endocytosis of a G protein-coupled receptor. Mol Ther 18: 670–679.

70. Lehto, T, Abes, R, Oskolok, N, Suhrutserenko, J, Copolovic, DM, Mayer, I et al. (2009). Delivery of nucleic acids with a streptavidin (ReRi) peptide using a non-covalent co-incubation strategy. J Control Release 141: 42–51.

71. Hassane, FS, Abes, R, El Andrabiussi, S, Lehto, T, Sillard, R, Langel, U et al. (2011). Intraprothelial delivery into the cellular trafficking of soluble oligonucleotides conjugated with chemically modified cell-penetrating peptides. J Control Release 153: 163–172.

72. Ezzat, K, Andraliussi, SE, Zaghoul, EM, Lehto, T, Lindberg, S, Moreno, PM et al. (2011). PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation. Nucleic Acids Res 39: 5284–5296.

73. Wood, MJ (2010). Woodruff’s an oligonucleotide therapy for Duchenne muscular dystrophy: a complex development challenge. Sci Trans Med 2: 25es15.

74. Yin, H, Mouton, HM, Betts, C, Seow, Y, Boutillier, J, Iverson, PL et al. (2009). A fusion peptide directs enhanced systemic dystrophy exon skip and functional restoration in dystrophy-deficient mdx mice. Mol Hum Genet 10: 4405–4414.

75. Yin, H, Saieen, J, Cameron, P, Seow, Y, Boutillier, J, Iverson, PL et al. (2011). Piperidine transduction peptides direct high efficiency oligonucleotide-mediated dystrophy exon skipping in heart and phenotypic correction in mdx mice. Mol Ther 19: 1295–1303.

76. Yin, H, Lu, Q and Wood, M (2008). Effective exon skipping and restoration of dystrophy expression by peptide nucleic acid conjugates in mXc mice. Mol Ther 16: 38–45.

77. Wancewicz, EV, Masar, MA, Siwek, AW, Abborsthor, K, Winger, TM, Benedix, A et al. (2010). Peptide nucleic acids conjugated to short basic peptides show improved pharmacokinetics and antisense activity in mouse tissue. J Med Chem 53: 3919–3926.

78. Botla, CC, Rutron, G, Citi, M, Matis, S, Damonti, G, Manati, MR et al. (2007). Inhibition of Bcl-2 and Bcl-xL expression in SCD mice by a PNA specific for a regulatory sequence of the translated c-myc. Cancer Gene Ther 14: 5284–5298.

79. Hu, J and Corey, DR (2007). Antisense effects in Escherichia coli mediated microRNA silencing in non-human primates. J Control Release 102: 163–172.

80. Deas, TS, Bennett, CJ, Jones, SA, Tilmiger, M, Ren, P, Behr, MJ et al. (2007). In vitro competition and in vivo efficacy of peptide nucleic acid oligomers against West Nile virus. Antimicrob Agents Chemoter 51: 2470–2482.

81. Sill, K and Neundorf, I (2011). Antisense nucleic acid-mediated delivery of synthetic gene silencing reagents by peptide nucleic acids (PNA). Chem Rev 111: 1511–1529.

82. Good, L and Nielsen, PE (1999). Peptide nucleic acid (PNA) antisense effects in Escherichia coli. J Antimicrob Chemother 43: 7581–7589.

83. Oh, SY, Ju, Y, Kim, S and Park, H (2010). PNA-based antisense oligonucleotides for miRNA-122 in primates with chronic hepatitis C virus infection in humanized non-human primates. Antimicrob Agents Chemother 54: 443–450.

84. Deas, TS, Bennett, CJ, Jones, SA, Tilmiger, M, Ren, P, Behr, MJ et al. (2007). In vitro competition and in vivo efficacy of peptide nucleic acid oligomers against West Nile virus. Antimicrob Agents Chemoter 51: 2470–2482.

85. Sill, K and Neundorf, I (2011). Antisense nucleic acid-mediated delivery of synthetic gene silencing reagents by peptide nucleic acids (PNA). Chem Rev 111: 1511–1529.

86. Good, L and Nielsen, PE (1999). Peptide nucleic acid (PNA) antisense effects in Escherichia coli. J Antimicrob Chemother 43: 7581–7589.
Liposomes modified with polyarginine effectively silence the targeted gene. Delivery of siRNA to tumors using a lipid nanoparticle containing a tumor-specific cleavable PEG-lipid. Multifunctional CPP polymer system for tumor-targeted delivery of siRNA. Plasmid-lipid particles containing PEG-diacylglycerols exhibit extended circulation lifetimes. Antisense oligodeoxynucleotide. Gene silencing. Functional delivery of viral miRNAs via exosomes. Induction of apoptosis in murine neuroblastoma by systemic delivery of transferrin-shielded siRNA delivery. Multifunctional siRNA delivery systems for aerosol gene penetrative investigations of the complex structure and stability during air-jet and ultrasonic nebulization. PEGylated delivery of siRNA to tumors using a lipid nanoparticle containing a tumor-specific cleavable PEG-lipid. Systemic administration of PRO051 in Duchenne's muscular dystrophy. Membrane-derived microvesicles: important and underappreciated mediators of genetic exchange between cells.