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Delivery of therapeutic oligonucleotides with cell penetrating peptides[☆]



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ABSTRACT

Oligonucleotide-based drugs have received considerable attention for their capacity to modulate gene expression very specifically and as a consequence they have found applications in the treatment of many human acquired or genetic diseases. Clinical translation has been often hampered by poor biodistribution, however. Cell-penetrating peptides (CPPs) appear as a possibility to increase the cellular delivery of non-permeant biomolecules such as nucleic acids. This review focuses on CPP-delivery of several classes of oligonucleotides (ONs), namely antisense oligonucleotides, splice switching oligonucleotides (SSOs) and siRNAs. Two main strategies have been used to transport ONs with CPPs: covalent conjugation (which is more appropriate for charge-neutral ON analogues) and non-covalent complexation (which has been used for siRNA delivery essentially). Chemical synthesis, mechanisms of cellular internalization and various applications will be reviewed. A comprehensive coverage of the enormous amount of published data was not possible. Instead, emphasis has been put on strategies that have proven to be effective in animal models of important human diseases and on examples taken from the authors' own expertise.

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1. Introduction

In recent years there has been a dramatic re-evaluation of how genes are regulated and of gene expression modalities. Two major sets of discoveries centre on the key roles of non-coding RNAs, and in particular those involved in the RNA interference (RNAi) pathway, as well as the mosaic structure of eukaryotic genes.

The antisense ON strategy was proposed more than two decades ago for the artificial regulation of eukaryotic gene expression in cultured cells *via* the hybridization of short ONs to mRNA targets through the pioneering work of Stephenson and Zamecnik [1,2]. The immense potential of this strategy, which in principle only requires knowledge of the target mRNA sequence, was quickly realized within both academic and industrial laboratories. Interestingly, “Mother Nature” has also exploited this potential, as convincingly demonstrated in bacteria [3] and later on in eukaryotes [4] by their ability to capitalize on such potent biochemical and genetic tools. The best demonstration that the antisense concept is exploited came from the discovery that antisense genes are able to control transcription in bacteria (and with less experimental evidence in eukaryotic cells) and finely tune the expression of target genes. However, these natural antisense RNAs turned out to be long and highly structured, and attempts to use this knowledge in the design of synthetic antisense genes proved disappointing. Most studies so far in the antisense field have instead focussed on short single-stranded DNA mimics, the hybridization of which allows recruitment of the cellular enzyme RNase H and as a consequence leads to the destruction of the RNA target [5].

Somewhat unexpectedly, eukaryotic coding genes are transcribed as immature mRNA precursors, the splicing of which by the complex nuclear machinery leads to intron removal. It became rapidly realized that a major outcome is the possibility of exon re-assortment and that this is the case for the majority of human genes. Importantly in terms of potential clinical translation, several human diseases are associated with dysfunction of the splicing machinery (as in β -thalassemia) or with the preferential use of one splicing event rather than another. Intervention with the process of exon selection using low molecular weight drugs has turned out to be difficult and the most promising strategy was proposed instead by Ryszard Kole and colleagues as detailed in Section 2 of this issue. In contrast to the “classical” antisense ON strategy, splice switching oligonucleotides (SSOs) are designed to prevent (or promote) the insertion of exons through high affinity binding at obligatory splicing sequences in the nuclear pre-mRNA (for example donor or acceptor splice sites) and therefore RNase H-incompetent ON analogues must be used [6].

The discovery of RNA interference by Fire et al. has also revolutionized our concepts of gene expression regulation [7]. The current detailed knowledge of RNAi processing and recognition by the RNA-induced silencing complex (RISC) has led to the possibility of rational design and synthesis of artificial siRNAs able to recognize any mRNA on the sole basis of their sequences. As for RNase H-competent antisense ON, sequence-specific recognition of the RNA target leads to its degradation by the RISC-associated nuclease. Further, several hundred human genes have now been identified to code for short stem-loop structures, known as micro RNAs (miRNAs), which are processed to allow targeting of the 3'-UTRs of mRNAs and many such mRNA targets have been identified [8–10]. Astonishingly, miRNAs do not need to hybridize over their entire sequence to promote the down-regulation of an mRNA target [11]. As a consequence, a single miRNA is able to regulate the expression of a complex set of mRNAs, which are often related in terms of cellular

function. Although still incomplete, ongoing studies show that specific sets of miRNAs control most cellular functions and that dysregulation of their expression is associated with many human diseases. Regulation of the levels of certain miRNAs or interference with their binding to mRNA targets are both now heavily explored strategies.

Synthetic antisense ONs, SSOs, siRNAs and miRNAs have become routine and invaluable tools to dissect cellular functions and they can be efficiently transfected into most laboratory cell lines using commercially available reagents such as cationic lipid formulations. However, their systemic *in vivo* administration has been plagued by toxicity and by low efficiency in the presence of serum proteins. Therapeutic developments centred on the use of naked ONs have thus met with only limited successes. Indeed, more than two decades of therapeutic developments have only led to three FDA approved drugs, with two used for easier-to-manage topical ocular applications (Fomivirsen as an antisense treatment for ocular cytomegalovirus infections in immunocompromised patients [12] and Ranibizumab, an aptamer for the treatment of macular degeneration [13]). Of more promise is the recent approval in the US (but not yet in Europe) of Mipomersen [14], an antisense ON for the treatment of homozygous familial hypercholesterolemia, which capitalizes on the accumulation of phosphorothioate (PS) ONs in the liver after systemic administration. Encouraging small scale clinical data have also been reported in the treatment of Duchenne muscular dystrophy with 2'-O-methyl phosphorothioate (2'-OMePS) ONs (Drisapersen [15–17]) and phosphorodiamidate morpholino oligonucleotides (PMO, Eteplirsen [18–20]), as will be extensively discussed later in Section 4 of this chapter.

Despite these successes, it is generally agreed that degradation in biological fluids, passage across cellular barriers and intracellular trafficking are all limiting factors in nucleic acids-based therapies [21]. Extensive searches for ON chemical modifications have improved their metabolic stabilities significantly as well as their affinities for RNA targets, and have to some extent reduced off-target effects. No ON chemical modification has significantly improved cellular uptake or tissue targeting, however.

The design of efficient and non-toxic delivery vectors for ON-based drugs has therefore become a major concern in both academic and industrial laboratories. Among the many proposed tools for delivery, cell penetrating peptides (CPPs) have appeared as an easy to implement strategy and have led to encouraging data at least in murine models of human diseases, as will be reviewed later in this chapter.

This review does not pretend to be exhaustive and will be restricted (1) to the CPP delivery of nucleic acids-based drugs and mainly to SSOs and siRNAs and (2) to delivery strategies which have turned efficient in animal models of human diseases.

2. Historical background of CPPs as delivery vectors for nucleic acids and their limitations

The harnessing of cationic peptides to deliver drugs across biological membranes was first attempted by Ryser and his colleagues [22]. They demonstrated that an anticancer drug could be delivered as a poly-L-lysine (PLL) conjugate in drug resistant cells *in vitro* and *in vivo* in mouse models [23]. Likewise, the chemical conjugation of antisense ONs to PLL led to the generation of a potent antiviral activity in several *in vitro* models of viral infections [24]. Unfortunately, conjugates were poorly characterized in view of the polydispersed character of commercial PLL preparations and, more importantly, they led to acute cytotoxicity upon systemic administration in mice.

It was discovered serendipitously by virologists that much shorter stretches of cationic peptides could promote the cellular uptake of macromolecules. For example, it was found that the full size purified HIV-1 Tat protein *trans*-activates the HIV-1 LTR promoter when incubated with cells [25]. More astonishingly, this same Tat protein was able as a conjugate to promote the cellular internalization of the non-permeable protein β -galactosidase and this paved the way for the use of Tat as a delivery vector for biomolecules. Along the same lines, the purified *Antennapedia* protein from drosophila was able to exert its transcriptional activity when incubated with nerve cells and this property was ascribed to a short peptide named Penetratin [26]. Dissection of the Tat protein did also show that cell penetration was due to a small, basic amino acids-rich peptide known as the Tat peptide [27]. Initial studies of the cellular trafficking of both Penetratin and Tat suggested an unusual non-receptor dependent mechanism of direct translocation across the plasma membrane. Although challenged later on as detailed in Section 7, this mechanism and the possibility to use such so called cell-penetrating peptides (CPPs) as non-viral delivery vectors for biomolecules, fostered a very large interest. Many CPPs were rapidly discovered and proposed as vectors for the transport of various drugs across biological membranes, starting from low molecular mass drugs to very large molecular entities such as nanoparticles.

Most CPPs were designed for the transport of a chemically conjugated cargo (see Table 1). Since the chemical conjugation and purification of negatively charged ONs with the most popular cationic CPPs has turned out to be difficult, most applications have concerned charge-neutral ON analogues such as Peptide Nucleic Acids (PNAs) and PMO (see Section 3). The most advanced studies have involved the use of these conjugates for RNA splicing regulation.

Early work described the use of Penetratin for the delivery of conjugated PS ONs [55] and of Transportan (another popular CPP) for PNA transport [56]. Unexpectedly, in a well-characterized HeLa 705 cell assay with a positive read-out (Fig. 1), splicing redirection using PNA or PMO oligomers conjugated to various standard CPPs (Tat, Penetratin or oligo-arginines) was not achieved in our research groups [57].

HeLa pLuc 705 cells were stably transfected with a construction in which the coding sequence of the luciferase gene is interrupted by a

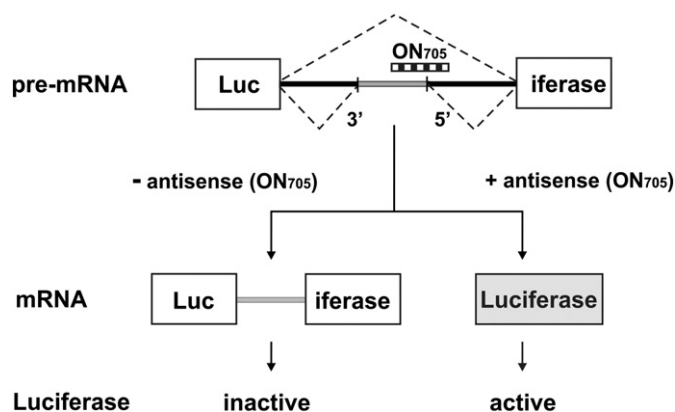


Fig. 1. Outline of the HeLa splicing redirection assay. Note that antisense ON (705) needs a delivery method for HeLa cell entry.

mutated intron 2 of the human β -globin gene. This mutation creates a 5' splice site and activates a 3' splice site. Masking of the 5' splice site by a RNase H-incompetent antisense ON (705) restores the production of functional luciferase mRNA and protein. This assay was provided by Kole and colleagues [58] and allows a reliable and easy to implement comparative evaluation of ON analogues and ON-delivery vectors. Intronic point mutations in a β -thalassemia globin gene activate cryptic splice sites leading to the aberrant splicing of this intron and as a consequence to a non-functional protein. Masking of the mutated site with a steric-block ON re-oriens the splicing machinery toward complete removal of the intron and leads to the production of a correctly spliced mRNA. This mutated intron has been introduced into the coding region of a reporter luciferase gene and the construction has been stably transfected in HeLa cells, which are available from ATCC (ATCC® CCL-2™). Luciferase expression can be easily monitored enzymatically or by PCR. This assay is advantageous in providing a positive read-out with a low background and a large dynamic range. It has been adopted by many laboratories in the field thus allowing easy comparisons.

Extensive studies of their cellular trafficking revealed that these CPP-ON conjugates were efficiently taken up by cells but remained stuck in endocytic vesicles. In keeping with this hypothesis, further incubation with chloroquine (an endosomolytic drug) or with saponin (a membrane-permeabilizing agent) allowed by-pass of this restriction [57]. Similar conclusions were reached using the same well-characterized assay by the Nielsen group [59].

Understanding the mechanisms of cell uptake using CPPs *via* comparison of literature data has proved extremely difficult, since the behaviours of CPPs seem to be strongly influenced by experimental conditions. Among relevant factors are CPP sequence, type of cargo, concentration (which seems to be crucial to foster endocytosis or direct translocation) and cell type [60].

The first data showing strong activity in the HeLa 705 splicing redirection assay were obtained with a derivative of oligo-arginines in which the spatial distribution of guanidinium side chains was optimized by the use of a non-natural amino-hexanoic acid spacer [61]. Similarly, the addition of 6 arginine residues on the N-terminus of Penetratin also resulted in strong activity in this HeLa 705 assay for PNA conjugates [57]. This led to the development of several arginine-rich peptides as PMO conjugates for use in muscle cells and *in vivo* mouse models of DMD as outlined in Section 4.

In addition to covalent conjugation, some CPPs have been designed for use as complexes, particularly for siRNA delivery (Table 1). This is because, as previously alluded to, chemical conjugation and purification of ONs and siRNAs with cationic CPPs has been difficult to achieve. For example, in the case of ONs, the Heitz and Divita group described a new class of such CPPs, with MPG as the lead compound, which can be complexed with negatively charged ONs essentially through

Table 1
Selection of oligonucleotides and peptide-oligonucleotide conjugates used *in vivo*.

Peptide	ON/ON analogue	Strategy	Reference
w/o	LNA	–	[28]
w/o	LNA	–	[11]
F-3	2'-OMe/DNA/PS gapmer	CL	[29]
Pip2a	PNA	CL	[30]
B-peptide	PMO	CL	[31]
B-MSP	PMO	CL	[32]
Pip5e	PMO	CL	[33]
PKKKRKV	PNA	CL	[34]
Penetratin	PNA	CL	[35]
Lys ₄	PNA	CL	[36,37]
SPACE	siRNA	CL	[38]
Tat-DRBD	siRNA	CL	[39]
(RXR) ₄	PMO	CL	[40–44]
(RFF) ₃ RXB	PMO	CL	[45]
(KFF) ₃ K	PNA	CL	[46]
R ₉ F ₂	PMO	CL	[47]
T-cell-derived CPP	PMO	CL	[48]
Pep-3	PNA	CF	[49]
PEGPep-3	PNA	CF	[49]
MPG-8	siRNA	CF	[50]
MPG-8-Chol	siRNA	CF	[50]
PepFect6	siRNA	CF	[51]
P5RHH	siRNA	CF	[52]
R ₁₅	siRNA	CF	[53]
Chol-R ₉	siRNA	CF	[54]

Footnotes: w/o, without CPP; LNA, locked nucleic acid; ON, oligonucleotide; PMO, phosphorodiamidate morpholino oligonucleotide; PNA, peptide nucleic acid; CL, covalently linked; CF, complex formation; Pip, PNA internalization peptide; DRBD, Domain-dsRNA Binding Domain; Chol, cholesterol.

electrostatic interactions. This and other complexation peptides are detailed in Section 6.

3. Chemical synthesis of CPP–ON conjugates

3.1. Negatively charged oligonucleotides and siRNA

Since practically all CPPs have a high cationic charge (due to Lys and Arg residues), it has proved extremely hard technically for covalent conjugation of most CPPs to standard negatively charged (phosphodiester or phosphorothioate) ONs because of aggregation or precipitation that can occur. It was possible for thiol linker-functionalised ONs (for example mixmers of 2'-O-methyl/LNA steric blocking ONs) to be conjugated with cysteine-functionalised classic CPPs such as Tat and Penetratin to give disulphide linkages, if the conjugations were carried out in the presence of a denaturing agent such as formamide [62]. In this way, it was found that fluorescent versions of such conjugates were taken up much better into endosomal compartments of model HeLa cells than unconjugated versions, but release into the nucleus to generate steric block RNA targeting activity proved not to be achievable [62]. A disulphide linkage is also not thought to be compatible with sufficient conjugate stability using systemic delivery, although lung delivery by disulphide-linked Penetratin and Tat conjugated siRNA has been attempted [63].

Thus the few rare examples of peptide-conjugated negatively charged ONs used *in vivo* have generally utilised a thiol-maleimide linkage between ON and peptide (Fig. 2). In one case the ON was 5'-functionalised during synthesis with an aminohexyl linker and the resulting amino group reacted with a bifunctional N-(gamma-maleimidobutyryloxy)-succinimide (GMBS) reagent to give a maleimide derivative. The maleimide group was subsequently reacted with a Cys-containing peptide, a cationic CPP known as F3, to furnish the desired conjugate (Fig. 2A) [29]. Surprisingly, chain aggregation was not reported in this conjugation reaction.

Alternatively and more conveniently, 7-mer non-cationic peptides discovered by a phage display technique were functionalised at the N-terminus by reaction with maleimidopropionic acid at the final stage of peptide synthesis and after purification were conjugated to a 2'-O-Me phosphorothioate ON synthesized with a 5'-thiohexyl linker

(Fig. 2B). Such conjugates were designed for enhanced uptake in muscle and heart due to use of homing peptides selected by a phage display method [64]. Non-cationic homing peptides are likely to be explored further for delivery of ONs to specific tissue types. Indeed there was a recent report of a phage-selected SPACE peptide conjugated covalently to siRNA showing enhanced skin penetration [38]. One might also expect increasing use of conjugations using the modern “Click” chemistry, such as *via* the copper-catalysed reaction between an azido functionality and an alkyne group, where each of the peptide and ON contains a clickable functionality [65] or where the ON contains a 2'-O-propargyl functionality [66].

3.2. Peptide nucleic acid (PNA)

Despite the large body of literature on the use of peptide-conjugated PNAs in cell culture [67] there are surprisingly few examples of their use *in vivo*. In the case of short peptide attachments to PNA, these can be assembled directly on the N or C-terminus of the PNA chain during solid-phase synthesis without needing any specific conjugation step. The amide couplings of protected amino acids are identical to that of protected PNA monomers as long as a compatible protecting group scheme is used. In the standard syntheses of PNA, it has been common to add a few Lys residues in any case, particularly to enhance the aqueous solubility of the PNA. Thus the first report of PNA activity involved merely use of (Lys)₄ N-terminally functionalised PNA (the Lys residues effectively acting as a CPP) in an *in vivo* splicing assay using a green fluorescent protein reporter that was up-regulated through redirection of splicing by the PNA [68].

Further, *in vivo* results were obtained using (Lys)₈ derivatives of PNA, synthesized in the same continuous way on the N-terminus of the PNA [69]. The results showed that an (Lys)₈-PNA conjugate was rapidly cleared from the circulation but distributed relatively broadly in a mouse with highest concentrations reached in liver, kidney, and spleen. Only very low amounts were detected in lungs, heart, skeletal muscle and testes, however. *In vivo* analysis was extended to an amphipathic Lys and Leu-containing D-peptide and another rich in Arg and homo-Arg as PNA conjugates, again synthesized continuously on solid phase [70]. Neither showed splicing redirection in liver and only the Lys/Leu peptide-PNA conjugate showed activity in kidney, but both

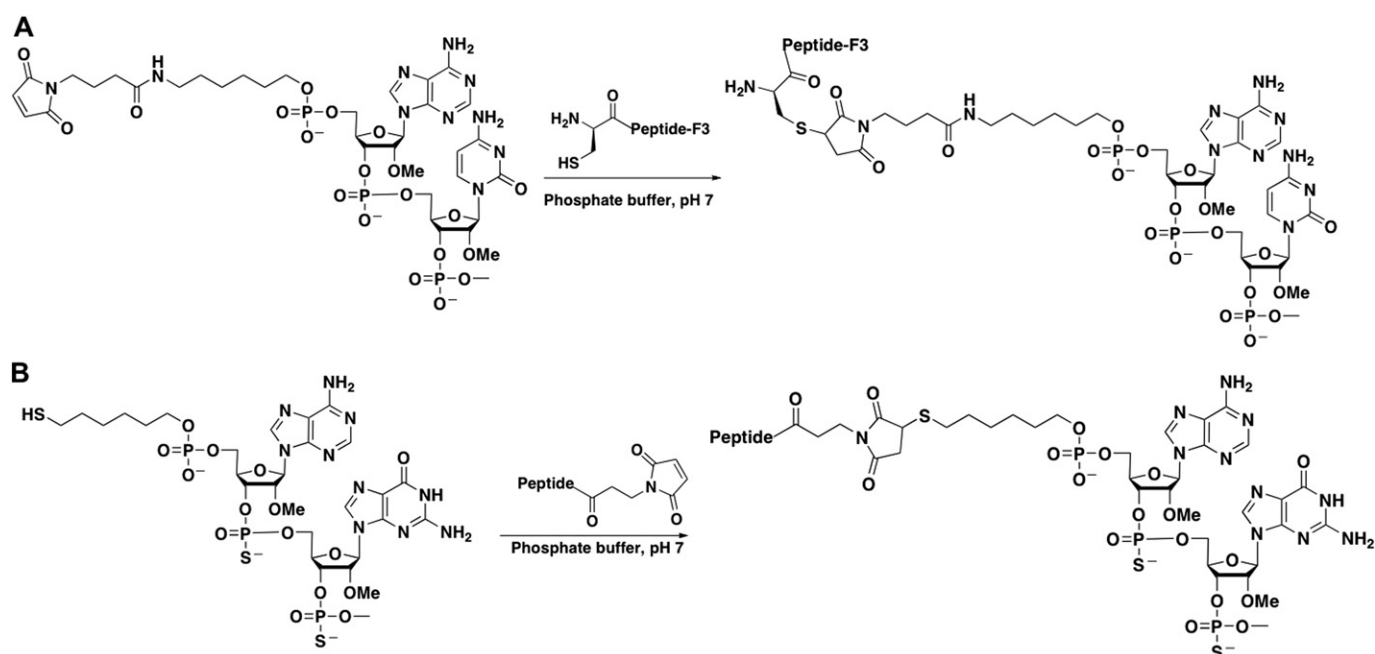


Fig. 2. Schemes showing methods of conjugation of negatively charged ONs to peptides *via* thiol-maleimide linkage (A and B).

showed significant activity in adipose tissue. However, the lack of potency compared to RNase-H activating ONs and their perceived toxicity profile at high doses led to abandonment of peptide–PNA conjugates as a drug modality for Isis Pharmaceuticals.

A continuously synthesized nuclear localization signal (NLS) peptide (PKKKRKV) attached to a PNA was also reported to be active in a severe combined immunodeficiency (SCID) mouse model in intronic targeting of c-myc in Burkitt's lymphoma-aimed therapy [34]. Further, a Penetratin peptide was synthesized continuously at the C-terminus of a triplex-forming PNA to target chromosomal DNA for genetic modification of haematopoietic progenitor cells in mice [35]. Promising microRNA knockdown results were obtained also when an antisense Lys–PNA–(Lys)₃ derivative was tested in mouse spleen for targeting microRNA-155 [36]. Very recently an anti-microRNA 155 PNA disulphide-conjugated (Fig. 3A) to a tumour-targeting pHLIP peptide has been shown to have potent activity in a mouse model of lymphoma [71]. The only other example of a peptide–PNA active *in vivo* that was not synthesized by continuous solid-phase assembly was an Arg-rich CPP Pip2a (and related Pip2b) conjugated through a thioether bridge by reaction of a C-terminal Cys residue on the peptide to an *N*-terminal bromoacetyl group on the PNA (Fig. 3B) [30]. The conjugates were active by intramuscular injection into the *mdx* mouse, a model of Duchenne muscular dystrophy (DMD), but later higher activity was found *in vivo* when using PMO rather than PNA as the ON material [33].

Despite results of mixed fortunes *in vivo* for CPP conjugates of PNA, these materials deserve further investigation especially in the case of tissues that are harder to reach with other types of ON. A new method of parallel synthesis of arrays of CPP–PNA conjugates (known as SELPEPCON) could prove useful for pre-screening of suitable drug candidates in cell assays or for example by intramuscular delivery

3.3. Phosphordiamidate morpholino oligomers (PMO)

By far the majority of successfully *in vivo* used CPP conjugates have utilised PMO as ON cargo. The application of CPP–PMO to muscular dystrophies is outlined in Section 4. Regarding Peptide–PMO (P–PMO) synthesis, suffice it to say that it has not been possible to date to use continuous synthesis methods for P–PMO conjugates, since synthesis has been restricted to commercial PMO suppliers. Methods of conjugation are therefore limited to what is available commercially regarding functionalised PMO or instead must employ un-functionalised PMO.

Practically all CPPs used with PMO to date have been Arg-rich starting from the initial observations of Moulton and colleagues [72]. Early work involved use of a bi-functional cross-linker to couple a 5'-piperazine PMO through a maleimide linkage to the peptide (Fig. 4A). Later the 5'-piperazine was directly conjugated through an amide linkage using C-terminally activated peptides (Fig. 4B) [61]. 5'-conjugation can also be carried out with commercially available amino-link functionalised PMO by direct coupling to the C-terminal carboxylic acid of the peptide through an amide link [30,33]. More generally now, the secondary amine of the 3'-morpholino group is coupled directly to the C-terminal carboxylic acid of the PMO *via* an amide link

(Fig. 4C) [73,74]. For direct amide conjugation, it is not necessary for Arg-containing peptides to be chemically protected. However, Lys-containing CPPs require a protecting group on the epsilon amino group removable after conjugation.

Direct 3'-amide conjugation of un-functionalised PMO also allows for peptides to be conjugated to the PMO containing for example alkyne functionalities to allow for azide Click coupling of a fluorescent label to the P–PMO [75]. In addition Click chemistry conjugation features in an adaptation for P–PMO of the SELPEPCON method of parallel conjugate synthesis (Fig. 4D) [76]. Combinations of compatible conjugation techniques such as use of Click chemistries are likely to dominate future P–PMO synthesis methods.

4. Development of CPP–SSO conjugates for the treatment of muscular dystrophies

The term muscular dystrophy describes a large group of hereditary diseases characterized by progressive weakness and degeneration of skeletal muscle [77]. A variety of gene therapies are being developed for this clinically and genetically heterogeneous group of disorders, including antisense ON mediated splice modulation (SSO). Recent advances have placed Duchenne muscular dystrophy (DMD) at the forefront of developments in SSO therapy. In order to overcome the remaining obstacles to the success of this approach, peptide-conjugated SSOs have been extensively investigated in animal models of DMD.

DMD is the most common subtype of muscular dystrophy in the UK [Online Mendelian Inheritance in Man (OMIM [78]) database reference: 310200] affecting one in 3500 new born boys. This severe, X-linked recessive disease results from mutations in the DMD gene [79]. The disorder is characterized by progressive muscle degeneration and wasting, along with the emergence of respiratory and cardiac complications, ultimately leading to premature death [80]. The majority of mutations underlying DMD are genomic deletions, encompassing multiple exons thereby producing a premature truncation of the open reading frame and resulting in the absence of the dystrophin protein. Dystrophin is an integral component of the dystrophin associated protein complex (DAPC), forming a crucial connection between the intracellular actin-cytoskeleton and the extracellular matrix. Exon skipping therapy utilises SSOs to target specific regions of the DMD transcript, inducing the exclusion of individual exons, leading to the restoration of aberrant reading frames and resulting in the production of an internally deleted, yet largely functional, dystrophin protein [8].

Following development in animal models, two AO chemistries have undergone proof-of-concept studies and repeat systemic dose-escalation studies, which have now been completed in the clinical trial setting [15,17–19,81]. Systemic administration of both PMO and 2' OMePS SSOs have yielded specific exon exclusion and partial restoration of dystrophin protein in peripheral muscle of DMD boys. Despite the undoubted potential of exon skipping ON therapy for DMD, the successful application of this approach is currently limited by the relatively inefficient targeting of skeletal muscle, as well as by the inadequate targeting of SSOs to other affected tissues such as the heart [82].

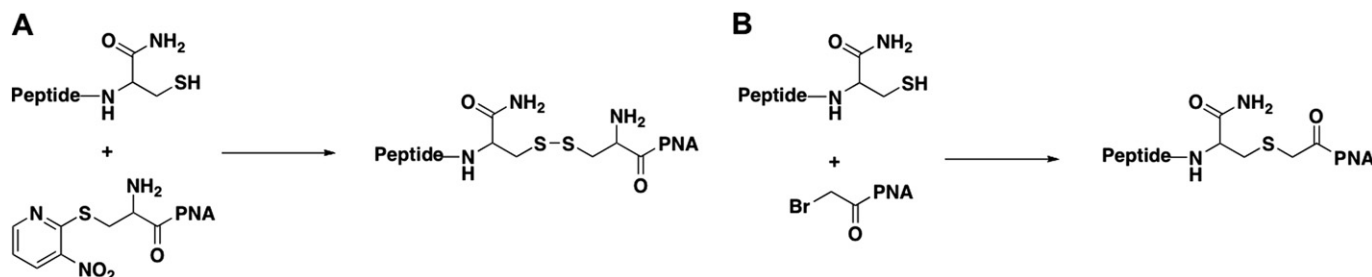


Fig. 3. Schemes showing peptide–PNA conjugation through a disulphide linkage (A) and peptide–PNA conjugation through a thioether linkage (B).

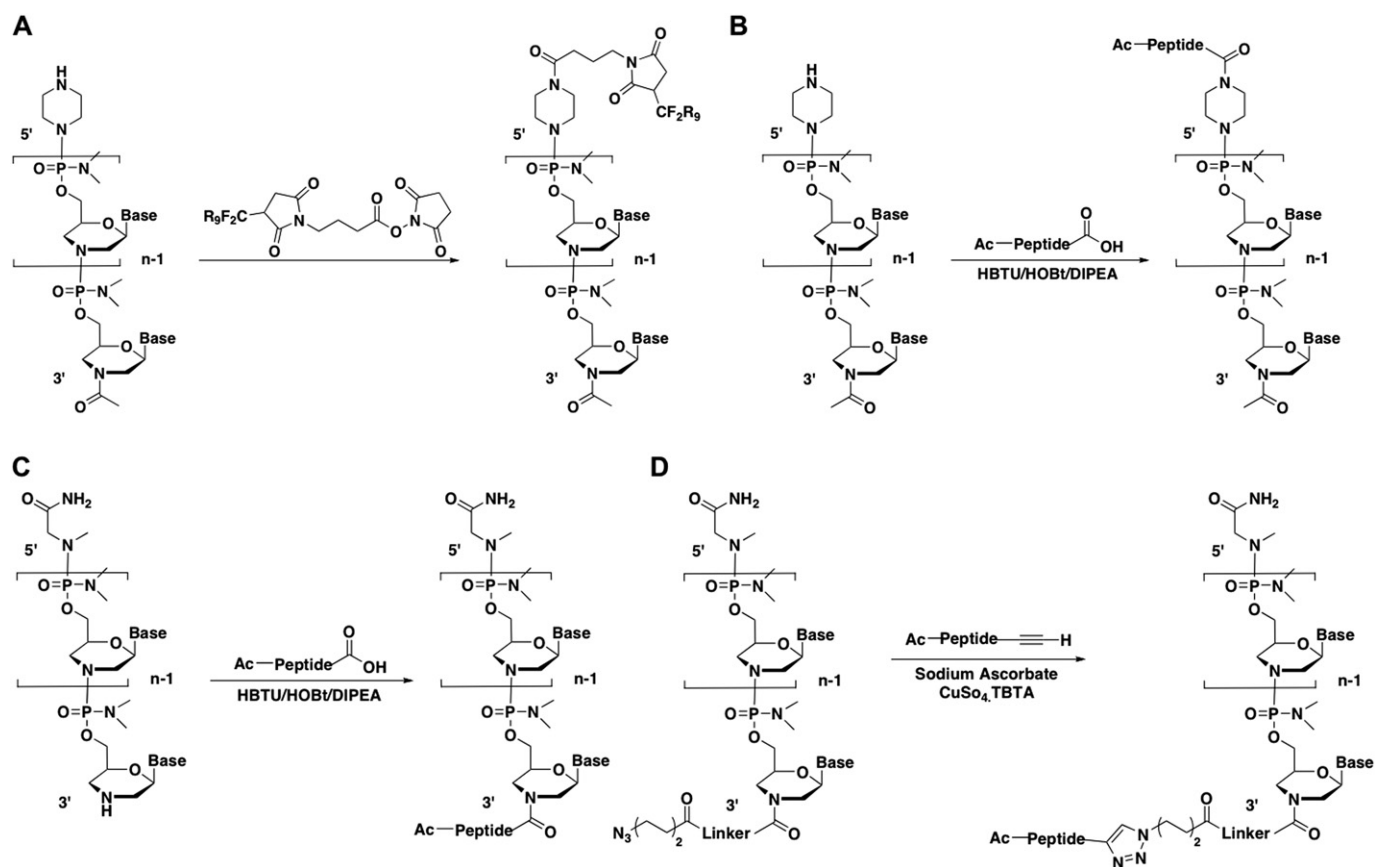


Fig. 4. Schemes showing methods of conjugation of peptides to PMO.

As such, cationic CPPs, which may be co-administered by conjugation with SSOs, have shown dramatic improvement in delivery and induce high levels of dystrophin correction in muscle at comparatively low doses. The majority of work has been performed in the *mdx* DMD mouse model, which is the most widely utilised model in the DMD field. The dystrophic murine phenotype arises as a result of a spontaneous mutation in exon 23 which encodes for a premature termination

Table 2

The derivation of Pip series peptides and their sequences.

	Name	Peptide sequence	Reference
Parent peptide	Penetratin	RQIKIWFQNRRMKWKK	[26,97]
First generation CPP	R6-Pen	RRRRRRR QIKIWFQNRRMKWKKGG	[98]
Second generation CPP	Pip1	RXRRRRXR-IKILFQN-RRMKWKK	[30]
	Pip2a	RXRRRRXR-IdKILFQN-dRRMKWHKB	
	Pip2b	RXRRRRXR-IHILFQN-dRRMKWHKB	
Third generation CPP	Pip5e	RXRRBRXR-ILFQY-RXRBRXB	[33]
	Pip5f	RXRRBRXR-ILFQY-RXRBRXB	
	Pip5h	RXRRXR-ILFQY-RXRRXR	
	Pip5j	RBRRXRBR-ILFQY-RBRBRBR	
	Pip5k	RBRRXRBR-ILFQY-RXRBRXB	
	Pip5l	RBRRXRBR-ILFQY-RXRBRXB	
	Pip5m	RBRRXRBR-ILFQY-RXRBRXB	
	Pip5n	RXRRBRXR-ILFQY-RXRBRXB	
	Pip5o	RXRRBRXR-ILFQY-RXRBRXB	
Fourth generation CPP	Pip6a	RXRRBRXR-YQFLI-RXRBRXB	[73]
	Pip6b	RXRRBRXR-IQFLI-RXRBRXB	
	Pip6c	RXRRBRXR-QFLI-RXRBRXB	
	Pip6d	RXRRBRXR-QFLI-RXRBRXB	
	Pip6e	RXRRBRXR-YRFLI-RXRBRXB	
	Pip6f	RXRRBRXR-FQILY-RXRBRXB	
	Pip6g	RXRRBRXR-YRFLI-RXRBRXB	
	Pip6h	RXRRBRXR-ILFQY-RXRBRXB	

Notes: B – beta-alanine, X – amino hexanoic acid, dK/dR – amino acid as D-isomer.

site, thus preventing the production of dystrophin protein [83,84]. The targeted exclusion of exon 23 in this mouse models the therapeutic strategy in patients by restoring the production of dystrophin protein.

The majority of CPPs utilised in the DMD field (Tables 1 and 2) may be covalently linked to PMO [31,85]. However, another class of non-covalent peptides from the Transportan family has been engineered to deliver anionic SSOs such as 2'OMePS, as detailed in Section 6 [86]. A number of modifications of stearylated TP10 [87] gave rise to CPPs capable of inducing high splice correction *in vitro* in murine H2k *mdx* myotubes (PepFect6; [51] and PepFect14; [88]) (Table 1). It should be noted that the *in vivo* efficacies of these CPPs have yet to be assessed.

The covalent class of CPPs comprises 3 subtypes, specifically oligo-arginine derivatives, phage peptides, and Penetratin derivatives. Oligo-arginines spaced by 6-amino hexanoic acid and/or β -alanine exhibit high splicing efficiency and serum stability *in vitro* [89–91]. The (RXR)₄ peptide was the first CPP conjugate to be administered in the *mdx* mouse at a range of doses, time-intervals and *via* different delivery routes. Generally, a single intravenous administration induced high dystrophin exon skipping in skeletal muscle, diaphragm and, for the first time, in heart [92]. Another arginine-rich peptide, (RXRRBR)₂ peptide (B-peptide), identified from a screen using the EGFP-654 splicing reporter mouse model [74], also gave rise to impressive exon skipping notably in the heart, when delivered using higher doses and *via* the retro-orbital route [85]. Improvements in cardiac function such as resistance to dobutamine stress testing and improvements in end systolic volume and end diastolic volume were also observed.

The identification of phage motifs from phage display-libraries allows the specific homing of a target tissue. As such, peptides with preferential binding to muscle and cardiac tissue were identified specifically for the treatment of DMD. These include muscle specific peptide (MSP) which exhibits enhanced *in vivo* muscle binding capacity [93], a 12-mer peptide (M12) which revealed better splicing efficiency over

MSP [94], and a 7-mer peptide (P4) which exhibited a moderate improvement in splicing activity over naked SSO [64]. The beneficial tissue targeting attributes of phage peptides may be further enhanced when combined with the delivery efficiency of CPPs, to develop a chimeric peptide. This was demonstrated when MSP was coupled to the B-peptide to determine the combined efficacy in *mdx* mice [32,95]. The specific orientation of these peptides was crucial to dystrophin splicing activity, and when coupled in the configuration 'B-MSP-PMO' revealed a 2–5 fold improvement in skeletal muscle restoration compared to B-PMO [96]. However, no improvement in dystrophin restoration was observed in cardiac muscle.

More recently, the PNA/PMO internalization peptide (Pip) series was derived from the parent Penetratin peptide [26,97]. Subsequent modifications including the addition of 6 arginine residues to the N-terminus (R6-Penetratin) [98], the addition of a C-terminal cysteine residue, and the utilisation of disulphide conjugation methods [30] fashioned this group of peptides into the established 'Pip' sequence conformation, consisting of a central hydrophobic core flanked on either side by arginine-rich sequences. Additional *in vivo* screening of Pip-PMO compounds was carried out to identify optimal CPPs for dystrophin splicing and correction in *mdx* mice. In the Pip5-series (Pip5e-o), the core sequence, ILFQY, was retained and there was alteration in the composition and length of the flanking regions [33]. The number of arginine residues ranged between 8 and 10, and the number and placement of 6-aminohexanoic acid (X) and β -alanine (B) spacer residues also varied in the flanking regions (Table 2). Pip5e-, Pip5j-, Pip5l-, and Pip5n-PMO resulted in the greatest number of dystrophin positive fibres following intramuscular administration (*tibialis anterior* muscle). Of these highly efficient CPPs, Pip5e-PMO induced the highest levels of exon skipping and dystrophin restoration body wide including in the

heart, following a single 25 mg/kg intravenous administration. When directly compared to B-PMO, Pip5e-PMO was shown to restore considerably greater dystrophin protein levels in the heart (intravenous administration comparison).

As B-PMO and Pip5e-PMO comprised similar arginine sequence and content, it was deduced that the core region of Pip5e- (ILFQY) was responsible for the splicing activity in heart. Therefore, the Pip6- series was designed with identical flanking regions to Pip5e and the core sequence was altered (Table 2). Pip6a-, Pip6b- and Pip6f-PMO, which maintained a 5 amino acid core, exhibited the greatest dystrophin splicing activity in heart over the previous lead candidate, Pip5e-PMO [73] (Fig. 5). Peptides such as Pip6c- and Pip6d-PMO, with a shortened core, resulted in a substantial reduction in efficacy. As cardiac and respiratory complications are the leading causes of death amongst DMD patients, the ability of Pip6-PMO to restore cardiac function is vital. These compounds demonstrated their restorative ability in a long term, low dose administration study (10 mg/kg over 3 month time-course) in which 30% dystrophin protein was restored in heart and the onset of cardiomyopathy was prevented in an exercised *mdx* mouse model (unpublished data). Liver and kidney toxicity has been assessed 2 weeks after the administration of Pip-PMO conjugates in *mdx* mice with no sign of toxicity [33,99]. Further toxicology studies are currently being pursued however have not been published yet. Of course, continuous optimisations to dose, pharmacokinetics and toxicity are underway which will facilitate the progression of this class of CPPs to clinical trial.

The development of CPP-ON conjugates for the treatment of DMD has served as a foundation for the treatment of other diseases facing similar antisense ON delivery challenges. Antisense ONs have been used to modulate RNA processing in the triplet repeat disorder myotonic dystrophy [OMIM: 160900, 602668]. This degenerative disease arises

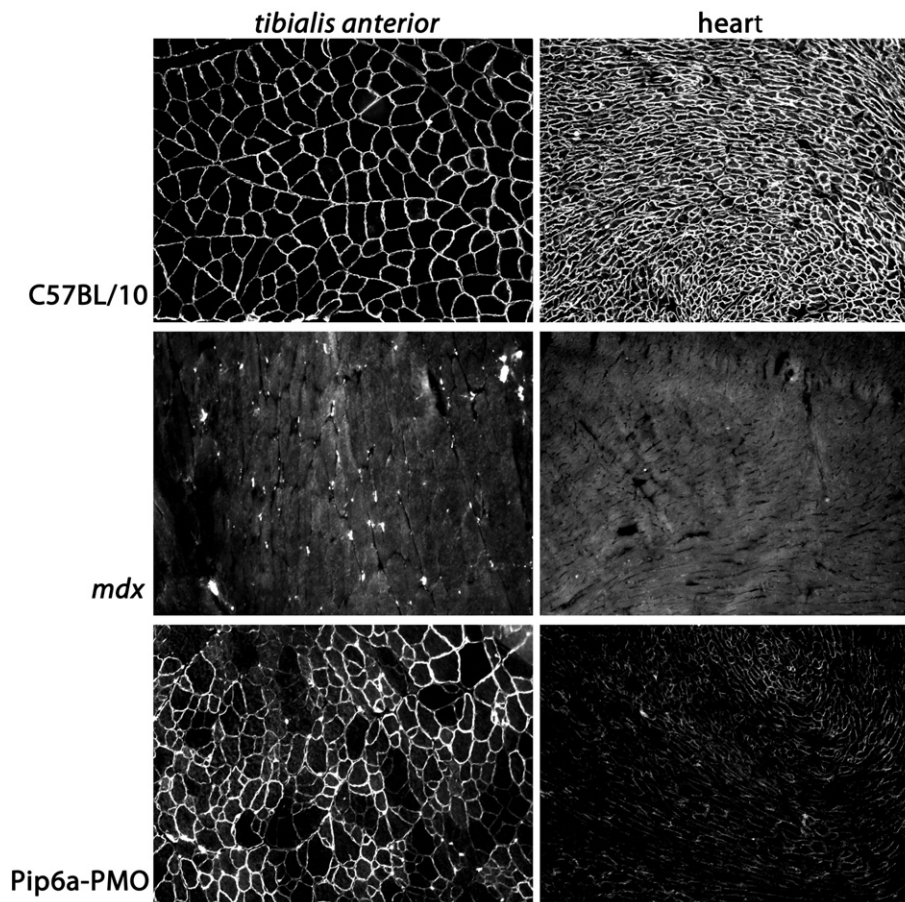


Fig. 5. Immunohistochemical staining of dystrophin following Pip6a-PMO treatment. Dystrophin staining in the *tibialis anterior* and heart of C57BL/10, untreated *mdx* and Pip6a-PMO treated *mdx* mice. The treated cohort received a single, intravenous 12.5 mg/kg dose, and tissues were harvested 2 weeks later.

from the expansion of pathogenic microsatellite repeats within noncoding regions of either the dystrophin myotonic-protein kinase (DMPK) or CCHC-type zinc finger, nucleic acid binding protein (CNBP) gene loci [99,100]. A variety of antisense ON strategies have been used to combat the effect of these toxic RNA expansions, although currently the most promising is the use of steric block ONs [101]. Using this approach, ONs were designed to bind to the DMPK repeat regions, therefore preventing the detrimental sequestering of RNA-binding proteins to within the expansion. Due to the multisystemic nature of myotonic dystrophy, a CPP conjugated antisense ON strategy has recently been tested in a mouse model to induce body wide distribution [102].

Spinal muscular atrophy [OMIM: 253300, 253400, 253550, 271150] is a neuromuscular disorder that results from the loss of motor neurons and skeletal muscle atrophy. This autosomal recessive disease is caused by mutations in the survival of motor neuron 1 (SMN1) gene. The related SMN2 gene undergoes alternative splicing due to a single nucleotide polymorphism, and therefore lacks exon 7 in the majority of transcripts, resulting in low protein expression. In order to functionally compensate for the loss of SMN1 protein in patients, SSOs have been used to promote the inclusion of exon 7 in SMN2 transcripts, and therefore increase the production of SMN2 protein [8]. Substantial headway has been made with naked SSOs in animal models of SMA [103] and clinical trials are ongoing. However, the effective delivery of antisense ONs to motor neurons in the CNS constitutes a major challenge, which may be combatted by CPP conjugation.

As described, multiple diseases may benefit from antisense ON therapy and indeed some have reached clinical trial and demonstrated significant therapeutic potential. These successes may be further amassed with the utility of CPPs, which is a rapidly evolving field of research. Whilst studies pertaining to toxicity and bio-distribution are underway, it is anticipated that CPP-ON therapies will reach clinical trial in the near future.

5. Antiviral and antibacterial applications of CPP-ONs

5.1. Antiviral applications

Amongst antiviral applications, CPP-PNAs were designed several years ago for targeting the HIV-1 trans-activation responsive element TAR [62,104]. Reassuringly, a Tat-PNA was found to be non-toxic in mice at high (300 mg/kg) doses [105]. However, no further *in vivo* antiviral studies of CPP-PNA have been published since. By contrast, CPP-PMO antiviral applications have been plentiful for a range of viruses, such as Dengue, Cocksackie, Ebola and Marburg viruses [106].

Only a few *in vivo* studies of CPP-directed antiviral delivery have been published, all of these involving Arg-rich CPPs, notably (RXR)₄XB or R₉F₂, as conjugates of PMO (Table 1). For example, (RXR)₄XB-PMO blocked viral replication of Human respiratory syncytial virus (RSV) expression in BALB/c mice, where the PMO sequence was targeted to the start of the coding region [40]. There was also a reduction in lung viral titres seen when mice were treated 3 h after infection, but not 8 h after infection. This suggested that the PMO must be present in the cell soon after infection if it is to have an impact on virus production.

Promising results were obtained in pre- and post-infection (RXR)₄XB-PMO treatment of 3-week old piglets infected with porcine reproductive and respiratory syndrome virus (PRRSV) both in reducing viremia and interstitial pneumonia [41]. An (RXR)₄XB-PMO conjugate, where the PMO was targeted to the 5'-terminus of the genomic RNA strand, was found to be substantially more active than naked PMO in reducing viral titers in livers of mice infected with murine hepatitis virus (MHV), a coronavirus, and protected mice from tissue-associated liver damage [42]. CPP-PMO treatment also prolonged survival in two lethal challenge models. However in the case of high dose viral challenge and delayed treatment, the CPP-PMO was not protective and there was some evidence of toxicity in the diseased mice. AG 129 mice treated by intra-peritoneal injection

with (RXR)₄XB-PMO targeted to the 5'-terminus or the 3'-cyclization sequence (CS) regions of the dengue 2 virus (DENV-2) pre-infection (but not post-infection) were able to increase the average survival time to up to 8 days, but viral rebound was seen eventually [107]. Similarly, dosing of (RXR)₄XB-PMO targeted either to the 5'-terminus or the 3'-CS region of West Nile Virus (WNV) RNA partially protected mice from viral challenge but higher doses caused toxicity [108]. Unconjugated PMO had no efficacy.

Although the CPP-PMO approach is very promising in principle for targeting viral RNA, an alternative antiviral approach involves use of PMO analogues with cationic piperazine groups within the PMO. Such "second generation" PMOs, known as PMOplus™, have now generally supplanted CPP-PMO in antiviral applications and these have been reviewed [109,110].

5.2. Antibacterial applications

CPP-PNA was suggested more than 10 years ago for targeting specific essential bacterial genes [111] and subsequently both CPP-PNA and CPP-PMO have been studied extensively in bacterial cell culture [112], but surprisingly few experiments showing *in vivo* efficacy have been published. The first proof of principle *in vivo* was shown in 2005 where a single intravenous injection of a (KFF)₃K-PNA targeted to acpP (an essential gene) RNA administered into *Escherichia coli* K-12-infected BALB/c mice 30 min after bacterial challenge reduced bacterial blood titres and enhanced survival of the infected mice [46]. Similar results were obtained for the Arg-rich (RFF)₃RXB-PMO conjugate targeted to the same acpP gene when dosed in mice 12 h after infection with *E. coli* W3110 [45]. The effect on potency of varying the peptide sequence was investigated in this model and (RXR)₄XB was found to be the most potent of various Arg-rich CPPs studied in mice [44].

More recently, CPP-PNA targeting rpoD, a gene that encodes an RNA polymerase primary σ 70 subunit 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*, with (RXR)₄XB CPP as a PNA conjugate having higher activity in general than (KFF)₃K CPP [113].

(RFF)₃R-PMO targeted to acpP or gyrA genes was found to be highly effective to increase mouse survival time when mice were challenged with the deadly Ames strain of *Bacillus anthracis* (the causative agent of anthrax) [114], suggesting that such treatment of anthrax-infected humans might one day become possible. However, just like in the case of pathogenic viruses, cationic backbone-containing PMO (PMOplus™) although not achieving the same efficiency as CPP-PMO [43], may prove to have a more favourable toxicity profile for therapeutic development.

6. CPP:ON non-covalent complexes and their applications

6.1. CPPs used for the non-covalent strategy

As briefly mentioned above, the non-covalent strategy is based on short peptides which are able to form complexes with cargoes without requiring any cross-linking or chemical modifications [115,116]. Most of the CPPs used in the non-covalent approach have an amphipathic character which enables a combination of electrostatic and hydrophobic interactions with the cargo. The amphipathicity may arise from either the primary structure or the secondary structure. Primary amphipathic peptides can be defined as the sequential assembly of hydrophobic residues with hydrophilic residues, whereas secondary amphipathic peptides are generated by the conformational state that allows distribution of hydrophobic and hydrophilic residues on opposite sides of the molecule [117]. Though originally based on amphipathic peptides, the non-covalent approach has been extended to peptides and peptidic analogues that are able to self-assemble with ONs to form stable CPP:ON complexes [118] and several of them have been reported to improve ON delivery into mammalian cells [116,118].

The non-covalent approach was originally developed for the delivery of large molecules and several peptides able to bind and condense DNA such as GALA, KALA, JTS1 have been used to improve gene delivery [119,120]. In 1997, the Divita and Heitz group designed the MPG peptide for the delivery of single and double stranded short ONs [86] and the strategy was then extended to proteins and peptides by the development of Pep-1 [121]. Since MPG, numerous CPP:ON complexes have been developed for the delivery of different types of ONs including AO, SSO and siRNA.

The main advantage of the non-covalent strategy over covalent conjugation lies in its simplicity and the protection that CPP:ON complexes confer to the ON from digestion by nucleases [118]. Compared to a chemical CPP–ON conjugate, the non-covalent complexes usually involve a one-step process consisting of a simple mixing of both partners, CPP and ON [115] (Fig. 6).

CPP:ON complexes do not require any chemical cleavage, prevent steric hindrance between peptide and ON, favour a better release of the ON inside the targeted cells and facilitate modifications to increase specificity for the ON and/or the target [118]. With regard to the mechanism of internalization, there is no consensus point of view for the cell entry of non-covalent complexes (see Section 7). However, as these complexes rely on electrostatic and hydrophobic interactions between positively charged CPPs and negatively charged ONs, their cellular uptake mechanism could be also controlled by structural polymorphism of peptides, particle stability and the nature of complexes/membrane interactions [122]. Interestingly, some CPP:ON non-covalent complexes were shown to enter cells through a direct translocation process [123]. Most non-covalent CPPs enable a wide range of chemical modifications and combinations and the modular nature of both peptides and resulting CPP:ONs particles also allows new developments.

6.2. Formulation of Peptide-Based Nanoparticles (PBNs)

The first developments of MPG were based on peptide/ON interactions and investigations of CPP:ON affinity by fluorescence spectrometry to measure the dissociation constant (K_d) as well as the optimal molar ratio and/or charge ratio to obtain stable complexes [86,124]. A molar ratio (MR) of 7 peptides per ON and a charged-corrected (N/P) ratio of 2 positively charged amino acids (N) for a phosphate group (P) were estimated [125]. Similar approaches were used for other non-covalent CPPs such as Pep-3 for HypNA–pPNA [49], CADY, C6 and MPEG–PCL–CH2R4H2C for siRNA condensation [126–129]. Fluorescence assays were also combined to circular dichroism (CD) analyses to monitor conformational changes that might occur in the presence of the ON and during CPP:ON complex formation [125,128–130]. Although both fluorescence and CD investigations suggest interactions and formation of CPP:ON complexes, the use of gel shift assays has been generalized to demonstrate the formation of complexes [124,131]. However, none gives a clear characterization of CPP:ON particles in terms of colloidal properties (size, charge and shape). Studies on colloidal properties of CPP:ON complexes have therefore been witnessed over recent years. Methods used for other nanomedicines were applied in order to characterize the size, surface charge and morphology of these CPP:ON complexes. In 2008, Law et al. were the first to report the hydrodynamic

diameter and the Zeta potential of R_9 :siRNA non-covalent complexes [132]. After UV–visible absorbance and CD investigations suggesting R_9 :siRNA interactions, size and Zeta potential were measured for several charge ratios (+/–) and revealed that R_9 forms particles with siRNA, with a hydrodynamic diameter of $\sim 1 \mu\text{m}$ at siRNA saturation [132]. Zeta potential increased with the hydrodynamic diameter of particles, supporting the positive contribution of the guanidinium group of R_9 in the surface charge. Size and Zeta potential have to be estimated in parallel since, according to DVLO theory for colloidal systems, low Zeta potential suggest low electrostatic repulsion which induce aggregation of particles whereas high absolute value of Zeta potential are indicative of stable particles suspension [133].

In this light, investigations of size and charge were generalized to other CPP:ON complexes, for different types of ONs (siRNA, SSO, and AS-ON). However, these parameters might be very different according to the CPP, the nature of the ON or the formulation protocol [131]. For example, Kim et al. identified small R_{15} :siRNA nanoparticles with a 224 nm diameter and a +7 mV Zeta potential for a charge-corrected N/P ratio of 3 in PBS [53] whilst CADY:siRNA nanoparticles have a size of 156 nm and surface charge of +50 mV whatever the MR (MR20 to MR80) in 5 mM NaCl [134]. CADY:siRNA nanoparticles have a polydispersity index (PDI) of 0.37, suggesting homogeneous and unimodal nanoparticles [134]. For other CPP:ON complexes, the size and the biological activity clearly vary with the MR [50]. Size and charge can also depend on the environment which is important since nanoparticles stability and homogeneity might vary according to the nature of the buffer, as described for C6M1:siRNA particles [130]. In addition physiological conditions such as serum addition can influence colloidal properties. PepFect6:siRNA complexes form homogenous unimodal nanoparticles of 70–100 nm (PDI ~ 0.1 – 0.2), which remained stable in water, whilst the presence of serum proteins induces larger particles of 125–200 nm with a wider distribution [51]. CPP:ON complexes can also be stabilized by specific excipients, such as lactose [88], 5% mannitol [135] or albumin [52].

Since the shape of nanoparticles also influences the bloodstream circulation half-life [136], the morphology of PBN was studied by electronic microscopy and atomic force microscopy (AFM) and most studies pointed to globular or spherical nano-objects [129,134,137]. *In fine* colloidal characterization suggested a redefinition of the CPP:ON complexes, and with regard to chemical modifications that were included in the design of non-covalent CPP as well as the discrepancy in the formulation of complexes, the more appropriate term of “Peptide-Based-Nanoparticles” (PBNs) was proposed [116,122]. In this light, formulation of PBN clearly became the key point to consider for further developments, especially with regard to *in vivo* administration.

As PBNs aim for *in vivo* perspectives, they have to be improved for membrane permeation and cell targeting abilities, whilst being biocompatible and biodegradable, with minimal cytotoxicity and inflammatory response (see Section 7.5).

6.3. *In vivo* applications of PBN

Few PBN have been successfully used for the *in vivo* delivery of anti-sense ONs, SSOs or siRNA [49–51,54,127,138]. With regard to siRNA, the

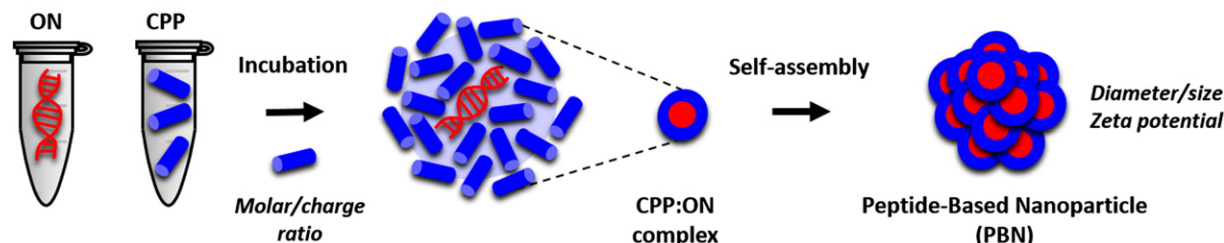


Fig. 6. CPP:ON non-covalent complexes and the peptide-based nanoparticle (PBN) strategy.

first reports on PBN-mediated delivery of siRNA *in vivo* involved MPG- Δ^{NLS} peptide and a cholesterol oligo-D-arginine (Chol- R_9) [54,138]. MPG- Δ^{NLS} was used for the *in vivo* delivery of siRNA targeting Oct-4 into mouse blastocysts. Oct-3/4 (POU5f1) is one of the earliest transcription factors expressed in the embryo and both the pluripotency and the fate of ES cells depend upon a tight control of Oct-3/4 expression. MPG- Δ^{NLS} -based nanoparticles induced a siRNA-mediated inhibition of up regulation of Oct-3/4 in ES cells which prevents their specification toward the mesoderm and their differentiation into cardiomyocytes [138]. Similarly, injection of PBN in the inner cell mass of blastocysts impairs cardiogenesis in early embryos [138]. Chol- R_9 was applied for the delivery of siRNA targeting VEGF (siVGEF) in CT-26 cells xenografted tumour. Intratumoural administration of Chol- R_9 :siVGEF complexes induced a significant inhibition of tumour growth associated with a pronounced decrease in VEGF level in the tumour, suggesting a synergistic effect between the D-amino acids of the CPP and the cholesterol moiety [54].

In a similar approach, MPG- Δ^{NLS} was modified in a shortened (MPG-8) and cholesterol-functionalized (MPG-8-Chol) peptide analogue. MPG-8-mediated delivery of siRNA targeting cyclin B1 induces a significant down regulation of both protein and mRNA levels and a systemic administration of MPG-8-based nanoparticles targeting cyclin B1 prevented tumour growth in a xenografted tumour mouse model [50]. Intravenous injections of 0.25 mg/kg reduced tumour growth for 60% by day 50, and administration of 0.5 mg/kg PBN entirely abolished tumour growth [50]. In addition formulation of a mix of MPG-8 and MPG-8-Chol (15%) in PBN revealed that cholesterol increases the biodistribution of siRNA in the tumour by maintaining siRNA in the plasma [50]. Cytokine levels in the plasma were quantified in order to assess the ability of MPG-8:siRNA and MPG-8/MPG-8-Chol:siRNA formulations to induce innate immune response *in vivo*. No increase in cytokine level was observed 6 h after injection of any formulation, suggesting the lack of immune response induction [50].

In a similar approach, PepFect6 was tested for *in vivo* RNAi silencing. The hypoxanthine phosphoribosyltransferase 1 (HPRT1) gene was targeted due to the long cellular half-life of the protein (~48 h) and the minimal impact on the viability of the transfected cell. Systemic administration of PepFect6:siRNA nanoparticles (1 mg/kg) induced a significant down regulation (>60%) of the HPRT1 mRNA level in liver, kidney, and lung, without induction of immune response [51]. In addition, PepFect6-based nanoparticles were also used for RNAi-mediated silencing of luciferase (Luc-siRNA) in mice expressing luciferase in the liver and intravenous injection of PepFect6:luc-siRNA (1 mg/kg) displayed decrease of bioluminescence for 2 weeks, reaching 75% gene silencing whilst naked Luc-siRNA was unable to induce a RNAi response [51]. Likewise, Chol- R_9 , R_{15} :siRNA nanoparticles were formulated for siRNA-mediated knockdown of human epidermal growth factor receptor 2 (HER-2) in human ovary adenocarcinoma cells (SK-OV-3). A reduction of HER-2 expression was associated with the inhibition of SK-OV-3 xenograft tumour growth [139]. Intratumoural injection of R_{15} :siRNA nanoparticles, every 3 days at a dose of 4 μ g siRNA per mouse, significantly reduced tumour growth without significant toxicity [53].

Furthermore, different strategies were investigated to stabilize the nanoparticles and to increase blood circulation such as exemplified by albumin- or polyethylene glycol (PEG)-coated nanoparticles. For example, Hou and co-workers have shown that albumin-coated formulation of p5RHH exhibits remarkable transfection efficiency attributable to pH triggered nanoparticle disassembly [52]. Furthermore, Tanaka and co-workers have demonstrated that intravenous injection of MPEG-PCL-CH₂R₄H₂C/siRNA complexes had a significantly higher anti-tumour effect in sarcoma-bearing mice [127].

Most non-covalent CPPs tested *in vivo* have been formulated for siRNA delivery. However the non-covalent strategy is also intended to deliver other types of ONs, such AS-ONs or SSOs, *in vivo*. In this light, Pep-3 was formulated with the antisense cyclin B1 HypNA-pPNA and

the resulting PBN were evaluated on PC3 xenografted mice [49]. PBN were administrated through intratumoural or intravenous injection every 3 days, and the biological effect of the antisense HyPNA-pPNA was evaluated by monitoring tumour growth during 2 weeks after injection. Intratumoural administration of PBN induced a 50% inhibition of tumour growth at a 1 mg ON dose and more than 92% at a 5 mg dose, whereas intravenous injection only reduced tumour growth by 20% at a 10 mg dose. Consequently in order to improve the *in vivo* stability of PBN, small amounts of PEGylated-Pep-3 were included in formulation [49]. Intravenous administration of 10 mg of ON with PBN containing 20% of PEGylated-Pep-3 significantly improved PBN stability and inhibited tumour growth by more than 90%, which was 4–5-fold more efficient than un-PEGylated-Pep-3 [49]. These data emphasize the potential of non-covalent CPP for *in vivo* AS-ON delivery and the importance of PEGylation on nanoparticle stability.

In addition to the physicochemical properties of PBN, some specific characteristics of formulations have to be addressed. Parameters such as affinity, molar or charge ratios, excipients, size and colloidal properties have a strong influence on biological interactions and minor changes can have a major impact on drug delivery efficacy [136]. Thus, as in the FDA draft guidance for liposomal products, the physicochemical properties and specifications should include: morphology, net charge, particle size of PBN, spectroscopic data, light scattering index, *in vitro* release, content of peptide-engaged in PBN versus free peptide, biodegradation of the PBN.

7. CPP/ON mechanisms of cellular internalization

One of the most challenging questions concerning CPPs is the mechanism by which these peptides enter cells. Early studies using fluorescent dyes linked to CPPs concluded that internalization was energy- and temperature-independent. However, this was later found to be an experimental artefact due to strong CPP adherence to the cell membrane leading to fluorescence overestimation and/or to fixation protocols using methanol allowing the CPP-dye complex to enter cells [140]. Recent studies have suggested that CPP uptake takes place by both endocytosis and energy-independent translocation, with the balance between these two pathways influenced by factors such as CPP sequence [141], temperature and CPP concentration [142–145]. It is now admitted that both biophysical methods, cell biology assays and biological end-points to assess activity need to be used to dissect the cell import mechanism.

7.1. First contacts with the cell membrane

Molecules approaching a cell first encounter a layer of oligosaccharides – the endothelial glycocalyx which is a network of membrane-bound proteoglycans and glycoproteins, covering the endothelium lumenally [146]. Proteoglycans in particular carry large O-linked oligosaccharides consisting of highly negatively charged repeating disaccharide units, the glycosaminoglycans (GAGs), such as heparan sulphates (HS). GAGs are involved in the cellular binding and uptake of several viruses [147,148] as well as cationic CPPs and polycationic nanoparticles [123,149].

Oligo-arginines bind to HS with affinities in the upper nanomolar range suggesting that GAGs may act as CPP receptors (reviewed in [150]). For the interaction of cationic CPPs with GAGs, the number of positive charges (number of arginines) was shown critical [151]. As examples, TP10-PNA and Penetratin-PNA conjugates are mainly internalized *via* macropinocytosis after initial HS interaction on the cell surface [152,153]. Beside the important role of the positive charges, Sagan and co-workers also show a strong positive correlation between the number of tryptophan residues, GAG binding and cell uptake [154].

Likewise, CADY:siRNA complexes interact with HSPGs, which probably allow the binding and accumulation of the particles at the

cell surface as demonstrated by electromobility gel shift assay and nanoparticle dissociation [128].

7.2. Internalization via direct translocation

Several models to explain a direct membrane translocation of CPPs have been proposed, such as the formation of inverted micelles, pore formation (toroidal or barrel shape), the carpet model and the sinking-raft model. These models have been initially proposed for the uptake of membrane-active peptides (reviewed in [155–157]) and then adapted for CPPs, such as oligo-arginines [158,159] or Transportan [56].

Likewise, the amphipathic MPG and CADY peptides are internalized via direct membrane translocation. On the basis of physico-chemical investigations (e.g., circular dichroism, Fourier transform infrared and electrophysiological measurements on model membranes), two very similar models have been proposed for MPG:siRNA and CADY:siRNA PBNs based on the formation of transient pore-like structures [125, 160]. A partial conformational change takes place upon MPG complexation with nucleic acids, and an increase in β -sheet content upon association with the cell membrane [125]. CADY:siRNA PBNs were characterized in more details (reviewed in [160]). CADY-mediated cellular uptake of siRNA is extremely rapid [126]. Moreover, internalization and biological activity of CADY:siRNA occurred even at 4 °C and under inhibition of mitochondrial oxidative phosphorylation revealing an energy-independent mechanism [123]. Fluorescence microscopy revealed that neither Transferrin, nor Rab5 co-localized with CADY:siRNA nanoparticles whereas co-localization with LysoTracker was observed to some extent [123]. These data further support an uptake mechanism largely independent of classical endocytosis with a degradation of the nanoparticles via the lysosomal pathway.

7.3. Internalization via endocytotic pathways

Apart for the few cases described in Section 7.2, the present consensus is that cell uptake occurs mainly by endocytosis for arginine-rich CPPs at least at low concentrations [140]. Whether clathrin-coated pit endocytosis, macropinocytosis, or another endocytic route is used is still a matter of debate and the route might differ with cell type, nature of the payload, or concentration [161,162].

The internalization mechanism was mainly characterized using the HeLa splicing redirection assay (Fig. 1) by correlating cellular uptake and biological efficiency of CPP-PNA or CPP-PMO conjugates. Several conjugates have been analysed in detail such as (Lys)₈-PNA-Lys [163], (RXR)₄-PMO [61] or Pip2b-PNA [30]. Cell uptake is energy-dependent and leads to sequestration of conjugates in cytoplasmic vesicles after an endocytic mechanism of internalization.

The limitations provided by the endosome sequestration of the payload are well illustrated by the two following sets of data.

PepFect3, a stearylated version of Transportan [87], is able to complex SSOs and these PepFect3:SSO PBNs are rather efficient in the HeLa splicing-redirection assay. However, a significant part of the complexes still remains entrapped in endosomes and can be released upon chloroquine treatment [88]. Based on these results, endosomolytic trifluoromethylquinoline (QN) moieties were grafted on the PepFect3 CPP leading to PepFect6 with largely improved cytoplasmic delivery of the transfected ON and an increased splicing redirection activity. Likewise, PepFect6 was efficient to transfect siRNAs and to promote gene silencing at low concentrations at variance to the PepFect3 formulation [51].

Along the same line and more recently, we could relate the high efficiency of Pip6a-PMO in H2k *mdx* skeletal muscles with an efficient release from endocytic vesicles after internalization via caveolae-dependent endocytosis. At variance, this same conjugate is internalized by clathrin-dependent endocytosis in primary *mdx* cardiomyocytes, is less efficiently released from endocytic vesicles and has a lower exon skipping activity [164].

7.4. Methods to analyse the mechanism of cellular internalization

Several strategies can be used to study the cellular internalization of CPP-ON conjugates or CPP:ON complexes [160,165].

1. Biophysical characterization of CPP/ON using circular dichroism, infra-red spectroscopy, dynamic light scattering or by insertion studies into phospholipid layers.
2. Inhibition of a specific endocytic pathway and assessment of its effect on CPP/ON internalization and biological activity.
3. Co-localization microscopy studies of the association of CPP/ON with fluorescently labelled endocytic markers

The association of CPPs with membranes induces the modification of several physical properties, such as the surface pressure of monolayer (Langmuir Blodgett) and the secondary structure of the peptide (FT-IR, CD, etc.). Deformation of the lipid bilayer due to hydrophobic and hydrophilic interactions between the components as well as the peptide localization in the membrane can be also assessed using NMR, X-ray diffraction, coupled plasmon waveguide resonance, EPR or FRET [166].

Assessing endosomal escape directly has turned difficult and can only be monitored using artificial models such as liposomal leakage assays. Different protocols were developed using large unilamellar vesicles (LUVs) with entrapped dyes such as calcein [167], carboxyfluorescein [168] or ANTS (fluorescent dye)/DPX (quencher) [169,170].

In cellulo, clathrin-mediated endocytosis can be predominantly characterized by the use of transferrin. Its uptake in cells can indeed be inhibited by a clathrin-specific siRNA or via cell transfection cells with a mutant form of dynamin [171]. However, dynamin is now known to regulate other endocytic and membrane trafficking pathways. Alternatively pharmacological inhibitors, such as chlorpromazine or dynasore (a dynamin inhibitor), can be used to inhibit this pathway even if these reagents cause significant cell toxicity and have to be used over short incubation times [18–20]. Potassium depletion or hypertonic medium incubation is also commonly used to investigate clathrin-mediated endocytosis [21,22].

Caveolae are invaginations of the plasma membrane that are localized in lipid rafts. A number of ligands, such as cholera toxin B, SV40 virus and albumin have been shown to be internalized more or less specifically via caveolae. The glycosphingolipid analogue lactosylceramide (LacCer), a fluorescent probe, was also shown to be internalized via this route and may represent a more selective marker for this pathway [172]. A range of pharmacological inhibitors of this pathway have been described and, in the main, they are agents that deplete cholesterol synthesis (lovastatin), agents that rapidly extract cholesterol from lipid rafts (methyl- β -cyclodextrin), and other cholesterol-interacting molecules such as the antibiotics nystatin and filipin.

Dowdy, Futaki and their colleagues have shown that Tat and other arginine-rich CPPs induce a ubiquitous form of fluid-phase endocytosis termed micropinocytosis [174,173]. For example, the uptake of octa-arginine (R8) peptide by HeLa cells was significantly suppressed by the macropinocytosis inhibitor ethylisopropylamiloride (EIPA) and the F-actin polymerization inhibitor cytochalasin D, suggesting a role for macropinocytosis in the uptake of the peptide.

7.5. Examples of CPP modifications to improve ON delivery

As mentioned above, CPPs are taken up primarily by endocytic pathways, and in order to promote endosomal escape and increase the transfection efficiency of CPPs, many different strategies have been used. We have mentioned the insertion of endosomolytic moieties in Section 7.3 and additional strategies are described below.

Since endosomes have a low pH, researchers have incorporated histidine residues or appended oligo-histidine tails in the CPP sequence, aiming at capitalizing on a “proton sponge” effect (see review [174]). Due to its protonation at pH 6.0, the imidazole ring of histidine (which is a weak base) counterbalances the accumulation of protons generated

by a specific ATPase inside acidic vesicles, neutralizes the lumen of endocytic vesicles and increases their osmolarity. As a consequence, the endosomal vesicles swell and their content is delivered into the cytosol. This has been exploited with success for the Tat-CPP [175], for microsphere coated with ornithine and histidine repeats ($O_{10}H_6$) and for self-assembling nano-constructs of amphiphilic copolymers (see review [176]).

Mason and co-workers have focused on amphipathic α -helical peptides incorporating pH sensitive residues [177]. The histidine residues in the LAH4 peptide are uncharged at neutral pH but when the pH of the endosomal lumen drops, the side chains become protonated, large numbers of peptides are released from the complex and adopt a conformation and alignment in the membrane that induces membrane disorder [178,179].

Other groups have focused on the addition of cholesterol or on fatty acid modifications. For example, Chol- R_9 improves siRNA delivery in a mouse model bearing a subcutaneous tumour [54]. Tat-PNA-mediated splice correction is increased by up to two orders of magnitude when conjugated with decanoic acid [180]. Stearylation of CPPs represents another strategy to increase endosome escape and as a consequence to increase the transfection efficiency of siRNA [181] and phosphorothioate 2'-OME RNA [182] as already detailed in Section 6.

For *in vivo* applications, the most frequently modification involves the grafting of a polyethylene glycol (PEG) moiety to prevent rapid clearance. As reported for Pep-3-mediated delivery of antisense-cyclin B1-charged-PNA which blocks tumour growth *in vivo* upon intratumoural and intravenous injection, PEGylation of Pep-3 significantly improves complex stability and efficiency [49].

In the context of clinical translation, it will be crucial to improve the tissue specificity of CPPs through their functionalization. The screening by *in vivo* phage display has enabled the identification of numerous peptides that home specifically to various organs under normal or pathological conditions [183]. For example the five residues homing peptides CREKA, which was identified by *in vivo* screening of phage-displayed peptide libraries in tumour-bearing MMTV-PyMT transgenic breast cancer mice [184], has been combined to the pVEC peptide to yield a CPP with tumour homing specificity [185].

8. Conclusions

Despite their huge potential, the clinical use of nucleic acids-based drugs has been limited by their poor biodistribution. Cell penetrating peptides have therefore been considered as a possible strategy to improve passage across biological barriers and intracellular delivery as reviewed extensively in this chapter.

Both covalent conjugates with neutral antisense ON mimics and non-covalent complexes with either charged antisense ONs or siRNAs have been engineered. Several such constructs have undergone extensive evaluation in animal models (mainly in mice) of both acquired (e.g., viral infections or cancers, in particular) and genetic (e.g., Duchenne muscular dystrophy) human diseases. CPP-delivery has been convincingly shown to increase significantly the efficiency of these nucleic acids cargoes. Clinical trials have however not yet been started to our knowledge. Extensive studies of biodistribution and of possible toxic effects have in particular to be completed.

Despite many studies, mechanisms responsible for the extravasation and for the cellular trafficking of these CPP-drug conjugates or complexes are still poorly understood. Direct translocation across cell membranes appears to be operational in some instances. In most cases however, cell internalization occurs through endocytosis and escape from endocytic vesicles limits biological efficiency. Likewise, CPP delivery does not occur with a similar efficiency in all tissues after systemic administration. Understanding better these limitations will obviously help in engineering of new CPP generations with a superior potential to target various tissues (and pathological conditions) and to deliver their payload within the appropriate cellular compartment. Efforts in

these directions have already been started as described in this chapter but much remains to be done.

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