ACCOUNTS
of chemical research

Peptide Vectors for the Nonviral Delivery of Nucleic Acids

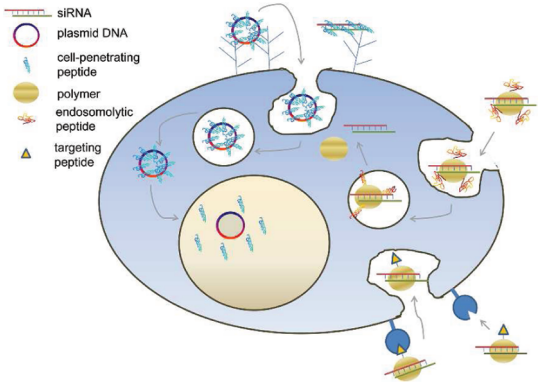
JAN HOYER[†] AND INES NEUNDORF[*,†,‡]

[†]*Translational Centre for Regenerative Medicine, University of Leipzig, Philipp-Rosenthal-Strasse 55, 04103 Leipzig, Germany, and* [‡]*Institute of Biochemistry, Department of Chemistry, University of Cologne, Zülpicher Strasse 47, 50674 Cologne, Germany*

RECEIVED ON SEPTEMBER 1, 2011

CONSPECTUS

Over the past two decades, gene therapy has garnered tremendous attention and is heralded by many as the ultimate cure to treat diseases such as cancer, viral infections, and inherited genetic disorders. However, the therapeutic applications of nucleic acids extend beyond the delivery of double-stranded DNA and subsequent expression of deficient gene products in diseased tissue. Other strategies include antisense oligonucleotides and most notably RNA interference (RNAi). Antisense strategies bear great potential for the treatment of diseases that are caused by misspliced mRNA, and RNAi is a universal and extraordinarily efficient tool to knock down the expression of virtually any gene by specific degradation of the desired target mRNA.



However, because of the hurdles associated with effective delivery of nucleic acids across a cell membrane, the initial euphoria surrounding siRNA therapy soon subsided. The ability of oligonucleotides to cross the plasma membrane is hampered by their size and highly negative charge. Viral vectors have long been the gold standard to overcome this barrier, but they are associated with severe immunogenic effects and possible tumorigenesis. Cell-penetrating peptides (CPPs), cationic peptides that can translocate through the cell membrane independent of receptors and can transport cargo including proteins, small organic molecules, nanoparticles, and oligonucleotides, represent a promising class of nonviral delivery vectors.

This Account focuses on peptide carrier systems for the cellular delivery of various types of therapeutic nucleic acids with a special emphasis on cell-penetrating peptides. We also emphasize the clinical relevance of this research through examples of promising *in vivo* studies. Although CPPs are often derived from naturally occurring protein transduction domains, they can also be artificially designed. Because CPPs typically include many positively charged amino acids, those electrostatic interactions facilitate the formation of complexes between the carriers and the oligonucleotides. One drawback of CPP-mediated delivery includes entrapment of the cargo in endosomes because uptake tends to be endocytic: coupling of fatty acids or endosome-disruptive peptides to the CPPs can overcome this problem. CPPs can also lack specificity for a single cell type, which can be addressed through the use of targeting moieties, such as peptide ligands that bind to specific receptors. Researchers have also applied these strategies to cationic carrier systems for nonviral oligonucleotide delivery, such as liposomes or polymers, but CPPs tend to be less cytotoxic than other delivery vehicles.

1. Introduction

Since the early 1990s, nucleic acids have become increasingly popular among researchers due to their tremendous potential as therapeutics for the treatment of severe and difficult to cure diseases such as genetic disorders, cancer, and viral infections. Certainly one source of enthusiasm arose from their ability to interfere with posttranscriptional processing and translation of mRNA (e.g., antisense oligonucleotides, small interfering (si) RNA). In particular, siRNA has been heralded as the ultimate cure by highly specifically regulating gene expression. Thus, virtually any target involved in signaling or metabolic pathways can be addressed, rendering siRNA also a powerful tool for the elucidation of molecular mechanisms in healthy and

Published on the Web 03/28/2012 www.pubs.acs.org/accounts
10.1021/ar2002304

TABLE 1. Cell-Penetrating and Endosomolytic Peptides

name	sequence	origin	ref
Tat(49−57)	RKKRRQRRR	HIV-1 transactivator	5
Antp	RQIKIWFQNRRMKWKK	antennapedia homeodomain	6
polyarginine	$(R)_n$	artificially designed	7
transportan 10	AGYLLGKINLKALAALAKKIL	galanin + mastoparan	8
PepFect 14	stearyl-AGYLLGKLLOOLAAAALOOLL	transportan 10	9
CADY	Ac-GLWRALWRLLRSLWRLLWRA-cya[a]	JTS-1	10
LAH4	KKALLALALHHLAHLALHLALALKKA	artificially designed	11
Endo-Porter	proprietary		12
LK15	KLLKLLLKLLLKLLK	amphipathic model peptide	5
KALA	$WEAKLA(KALA)_2KHLAKALAKALKACEA$	amphipathic model peptide	6
sHGP	RGWEVLKYWWNLLQY	HIV gp41	13
melittin	IGAVLKVLTTGLPALISWIKRKRQQ	bee venom	14

[a] Ac = acetyl group; cya = cysteamine.

diseased tissue apart from its therapeutic application. However, the euphoria associated with siRNA therapy has been somewhat dampened by the fact that delivery of nucleic acids across the cellular plasma membrane and release from endosomal compartments as well as stability and residence time in the blood circulation turned out to be problematic. Nucleic acids per se are not able to overcome the plasma membrane due to their size and negative charge. Their remarkable transfection efficiencies notwithstanding, viral delivery vectors raised safety concerns owing to their immunogenicity and risk for mutagenesis, while on the other hand synthetic vectors based on liposomes and polymers suffer from poor efficacies and considerable cytotoxicity. Other methods such as microinjection are restricted to the *in vitro* use. Electroporation has been used *in vivo* for gene delivery to, for example, skin and skeletal muscle,[1] but is of little therapeutic relevance for the delivery to internal organs due to their inaccessibility.

In order to overcome the drawbacks of nucleic acid transport, a new class of peptide carriers most commonly known as cell-penetrating peptides (CPPs) or protein transduction domains capable to transport therapeutic molecules across cellular membranes emerged. However, the application of peptides is not restricted to intracellular transport but can be extended to homing drugs to their target tissues by, among others, specific ligand−receptor interactions. The scope of this Account lies in the most recent advances in harnessing peptide vectors for the nonviral delivery of nucleic acids with particular focus on cell-penetrating peptides and promising therapeutic applications. For those interested in chemical conjugation strategies of peptides and nucleic acids, a review by Lu et al. is recommended.[2]

2. Delivery of Nucleic Acids by Cell-Penetrating Peptides

Since the landmark paper by Frankel et al.[3] in 1988 reporting that the human immunodeficiency virus-1 transactivator of transcription (Tat) protein is able to penetrate cellular membranes, a variety of CPPs have been described. They are usually short (up to 30 amino acids) and positively charged and can be either derived from naturally occurring protein transduction domains or rationally designed, an example of which is the chimeric transportan consisting of the amino terminus of the neuropeptide galanin and the wasp toxin mastoparan[4] (see Table 1).

CPPs have garnered widespread attention as delivery vehicles due to their capability of autonomous and receptor-independent intracellular translocation with comparably low cytotoxicity and risk for immune responses. In most cases, cytotoxic effects are only observed at elevated peptide concentrations that are often not needed for efficient internalization depending on what cargo is to be transported.[15] Accordingly, cell-penetrating peptides have been exploited for *in vitro* and *in vivo* delivery of an ever increasing plethora of therapeutic cargoes as diverse as small organic molecules, proteins, nanoparticles and oligonucleotides (ON). In recent years, noncovalent electrostatic interactions of cell-penetrating peptides with nucleic acids have prevailed over covalent conjugates, the synthesis of which is cumbersome and hampered by the aggregation of the positively charged peptides with the negatively charged nucleic acids. It is crucial, though, that the size of the resulting CPP/ON nanoparticles be thoroughly adjusted. Ideally, the diameter should not exceed 100−300 nm for efficient uptake. Care should also be taken with respect to fine-tuning of the stability of the complexes by varying the charge ratio. It has often been observed that dissociation of the nucleic acids from the carrier inside the cell is hindered, if not prevented, when the electrostatic interaction is too strong.

Besides their undeniable advantages, most CPPs suffer from endosomal entrapment after internalization resulting from endocytosis being their preferred route of uptake. In order to tackle this problem, lysosomotropic agents such as

chloroquine and Ca^{2+} ions[16] are often added to the transfection medium for the disruption of endosomes and release of the cargo into the cytosol. Systemic administration of chloroquine is unsuitable, though, due to its significant cytotoxicity. This issue together with the various kinds of oligonucleotides that were used in combination with cell-penetrating peptides as well as mechanistic studies will be addressed in this section.

2.1. Plasmid DNA. The concept of gene therapy is based on the delivery of DNA coding for a gene product that is deficient in the respective diseased tissue. In the past decade, intracellular transport of DNA with the help of cell-penetrating peptides has seen a substantial increase of attention, a great number of approaches being related to Tat and polyarginine peptides. Current research frequently deals with the improvement of the CPP vectors in terms of their ability to release their cargo into the cytosol. Lehto et al. reported on a stearylated $(RxR)_4$ peptide, x representing aminohexanoic acid, which was able to convey a firefly luciferase-encoding plasmid into various cell lines.[17] Luciferase-induced luminescence was many times higher compared with the nonstearylated peptide. This effect was attributed to increased endosomal escape conferred by the fatty acid moiety. However, chloroquine was still able to significantly enhance luciferase expression, indicating that a great amount of plasmid is still entrapped in endocytic vesicles. Interestingly, stearic acid-modified nonaarginine did not lead to equally pronounced luciferase expression, highlighting the role of the CPP in the effectiveness of stearylation. Conjugation of fatty acids to cell-penetrating peptides has already been described in the past[18] and is generally accepted to render a peptide more hydrophobic, thus improving membrane interaction. The stability of the ON/peptide nanoparticles is also found to be enhanced, presumably by promoting DNA condensation and shielding from degradative enzymes.[17]

Another way to impart facilitated endosomal escape on CPPs is the coupling of membrane-destabilizing peptide sequences (see Table 1). These include the pH-responsive KALA peptide[6] and LK15 consisting solely of leucine and lysine residues.[5] The effect of KALA was found to be dependent on endosomal acidification, since bafilomycin A, an inhibitor of the H^+-ATPase, suppressed gene expression.[6] This is plausible since KALA was designed to undergo a conformational change from random coil to an amphipathic α-helix at pH 5, that is, in the endocytic compartment, inspired by the endosomolytic properties of HA2, the fusogenic peptide domain of influenza virus hemagglutinin.[19]

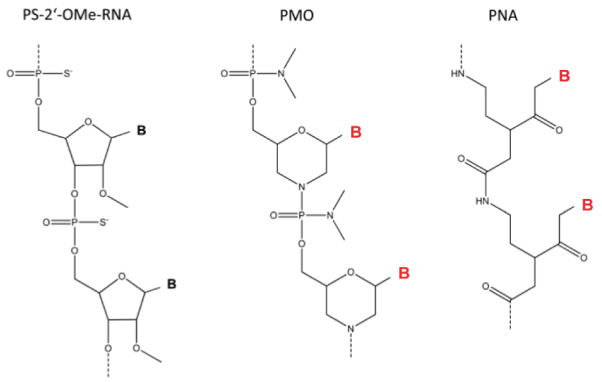


FIGURE 1. Chemical structures of various kinds of antisense oligonucleotides. PS-2′-OMe-RNA, 2′-*O*-methyl phosphorothioate RNA; PMO, phosphorodiamidate morpholino oligonucleotide; PNA, peptide nucleic acid; B, nucleobase (black, A, C, G, U; red, A, C, G, T).

A combined noncovalent strategy using polyethylenimine (PEI) and K-Antp, which is a fusion peptide of KALA and the third α-helix of the *Drosophila* Antennapedia homeodomain, resulted in further increased transfection efficiencies exceeding those of the single components. Notably, chloroquine did not augment the transfection efficiency of the PEI/K-Antp complex.[6] PEI, just like chloroquine, expedites disruption of endosomes due to the so-called proton sponge effect.

Khondee et al. followed a different approach to improve Tat-mediated transfection. They made use of calcium ions to expedite DNA condensation, resulting in compact ON/peptide complexes and enhanced gene expression. Coupling of a poly(ethylene glycol) (PEG) unit to increase serum stability reduced transfection, which was restored by addition of a receptor-targeting ligand.[20]

An elegant method for the formation of noncovalent DNA/peptide complexes other than by purely electrostatic interaction is presented by Rice et al. using an oligoarginine peptide modified with acridine units for DNA intercalation.[21]

2.2. Steric Block Oligonucleotides. Another technique for the regulation of gene expression is provided by antisense strategies, among which steric block oligonucleotides are a prominent example. Those are short single-stranded oligonucleotides that are chemically modified for improved nuclease stability and annealing ability (see Figure 1). They specifically bind to a complementary target sequence by Watson–Crick base pairing without recruitment of RNase H that would lead to target degradation. Thus, this technique is applicable to the regulation of splicing site selection, which can be used, for instance, to induce excision of exons carrying a nonsense mutation as in the case of Duchenne muscular dystrophy (DMD) caused by production of a truncated version of dystrophin.[22]

Koppelhus et al. designed a variety of fatty acid-modified CPPs covalently conjugated to neutral peptide nucleic acids that cannot be delivered by lipofection.[23] They clearly demonstrated the need for a minimum chain length for efficient splice correction but also observed that the longer the fatty acid, the higher the cytotoxicity of the construct. Recently, the dependence of the transfection result on the fatty acid chain length has also been shown by Langel et al., who synthesized acylated transportan 10 (TP10) derivatives for the noncovalent delivery of splice-correcting 2′-*O*-methyl RNA, stearyl-TP10 being the most efficient.[8] Introduction of a single phosphoryl group enhanced the splice correction efficacy even further, making it four times more efficient than Lipofectamine 2000.[24] A stearyl-TP10 derivative coined PepFect 14, where lysines and isoleucines were exchanged by ornithines and leucines, exhibited remarkable splice correction of dystrophin pre-mRNA even in the presence of serum in mdx mouse myotubes, an *in vitro* model of DMD, after delivery of 2′-*O*-methyl phosphorothioate oligonucleotides. Notably, solid formulations of the ON/peptide nanoparticles proved to be stable over extended periods of time without losing their transfection capacity, making it a promising candidate as a CPP-based therapeutic.[9]

In a different approach aimed at enhanced endosomal release, Trabulo et al. observed that a combined strategy using a chimeric cell-penetrating peptide, $SV_{13}-PV$ carrying the nuclear localization sequence of the simian virus 40 large T antigen, and cationic liposomes leads to synergistic effects on splice correction.[25]

2.3. siRNA. RNA interference (RNAi) has become widely popular due to its ability to specifically mediate knock-down of gene expression. The double-stranded siRNA is loaded into the RNA-induced silencing complex, where the guide strand binds to a complementary mRNA sequence that is later degraded by Argonaute 2.[26] A single siRNA molecule is able to cleave multiple copies of mRNA, making this technique extraordinarily efficient. The delivery of small-interfering RNA poses a challenge, which is in many aspects different from plasmid delivery. First of all, an siRNA strand, being only around 19−23 bp in length, is significantly shorter than DNA, which can be condensed and tightly packed by cationic agents. Besides, siRNA adopts an A conformation with narrow major groove and shallow minor groove as opposed to the B conformation of DNA. Thus, binding to cationic vectors may be difficult.[27] Another crucial difference lies in the fact that siRNA does not need to be delivered into the nucleus since the RNAi machinery is located in the cytosol. Taken together, just because a vector is good for DNA does not necessarily mean it is also suitable for siRNA and vice versa.

A particularly interesting method for the noncovalent binding of siRNA has been developed by Dowdy and coworkers.[28] They recombinantly expressed a Tat fusion protein bearing a double-strand RNA binding domain (DRBD) for masking the negative charges of the small-interfering RNA and thus were able to efficiently deliver it even into difficult to transfect primary cells. In another study, Ifediba et al. were able to transfect primary neurons and astrocytes with siRNA directed against a protein that is involved in stroke pathology using a myristoylated oligoarginine peptide.[7]

Langlet-Bertin et al. developed a peptide, LAH4, that is rich in pH-responsive histidine residues to tackle the problem of endosomal release.[11] Histidine-rich peptides have been shown to exert a PEI-like proton sponge effect, and accordingly, the ability of LAH4 to mediate an siRNA-induced knockdown was dependent on endosomal acidification. This has also been shown for another histidine-rich peptide, Endoporter, by Bartz et al.[12] Arthanari et al. reported on efficient delivery of siRNA directed against the BCR-ABL oncoprotein to a chronic myeloid leukemia cell line, that is, a cancer-related model. For this purpose, they used the aforementioned endosomolytic LK15 peptide conjugated to Tat.[29]

2.4. Mechanistic Aspects. Even after two decades of CPP research, there is still much debate over their mechanism of uptake, which is not yet fully understood. This issue is exacerbated by the fact that comparison of different studies may be confusing, which is due to the influence of various factors on the precise uptake mode, such as membrane composition, cargo size, peptide concentration, and of course the nature of the CPP itself, just to name a few. Besides, more than one distinct entry pathway seems likely.[30] However, there is a general consensus that most cell-penetrating peptides are taken up primarily by endocytosis, that is, an energy-dependent mechanism. It may occur via several distinct pathways, for example, clathrin- or caveolin-dependent, all of which can be distinguished using specific inhibitors[31] (see Figure 2).

According to various reports, the principal endocytic nature of the cellular uptake is not altered when CPPs form electrostatic complexes with nucleic acids.[5,32] Nevertheless, Saleh et al. hypothesized that too large a size of the CPP/ON nanoparticles might hinder uptake and shift the endocytic pathway toward macropinocytosis.[5]

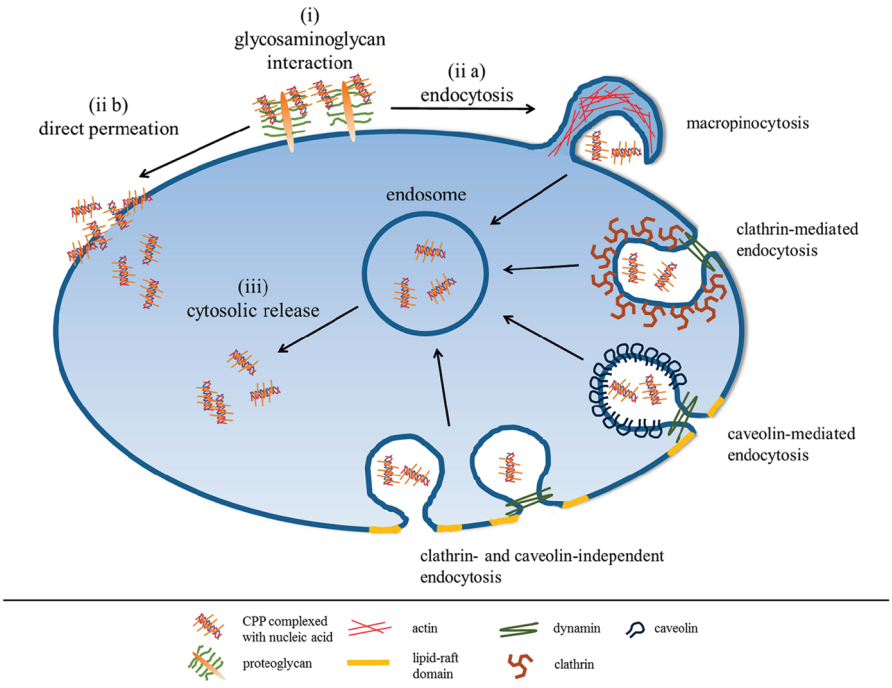


FIGURE 2. Simplified schematic presentation of the general mechanism of cell entry of cell-penetrating peptides by either direct permeation or different endocytic processes.

Generally, the initial step of the uptake of cationic cell-penetrating peptides is believed to be the interaction with negatively charged glycosaminoglycans (GAGs) of the extracellular matrix, for example, heparin. There are conflicting results, though, as to whether the glycosaminoglycans themselves actually trigger the signal leading to endocytosis or whether it might instead be mediated by insertion of the peptide into the plasma membrane.[33] Naik et al. investigated the influence of exogenous and cell surface GAGs on the DNA transfection efficiency of 16-mer polyarginine and polylysine peptides.[34] They found that cell entry of K_{16}/DNA complexes is dependent on cell surface GAGs, while this did not hold true for the respective arginine homopeptide, suggesting a different stimulus for endocytosis at least for the polyarginine peptide. Addition of exogenous GAGs decreased plasmid delivery by K_{16} due to displacement of the DNA, which was not observed for R_{16} unless very high GAG concentrations were used, probably due to the bidentate binding by the guanidino groups of arginine. Remarkably, R_{16} was only internalized endocytotically when packed with DNA, otherwise it would enter the cell via direct permeation. However, there is no mandatory requirement for endocytosis for CPP/ON conjugates since CADY, an amphipathic CPP designed by Divita and coworkers, was still able to deliver siRNA directly into the cytosol.[10]

3. Peptides in Multifunctional Delivery Systems

The nonviral delivery of nucleic acids is by far not restricted to cell-penetrating peptides. Cationic liposomes and polymers that can compact nucleic acids have also largely been used, prominent examples of the latter being polyethylenimine (PEI), chitosan, and polyamidoamine (PAMAM) dendrimers. The advantage of functionalization of these vectors with peptides is obvious, because they can confer useful extra features due to their enormous versatility. Since cationic liposomal and polymeric carriers undergo endocytosis, decorating them with endosomolytic peptides for enhanced cytosolic release may be of help. Moreover, combination with peptides endowed with the ability to target a specific tissue of interest is highly beneficial, since this allows for reduced doses and, therefore, reduced side effects following systemic administration (see, Figure 3).

Kwon et al. covalently attached a truncated endosomolytic peptide derived from the carboxy-terminus of the HIV cell entry protein gp41 to a polyethylenimine scaffold, obtaining improved gene transfection results compared with unmodified PEI.[13] A rather intricate system for siRNA delivery was presented by Meyer et al.[14] They covalently attached the nucleic acid to a polylysine carrier containing a PEG moiety for enhanced solubility and enzymatic stability via an intracellularly reducible disulfide linker.

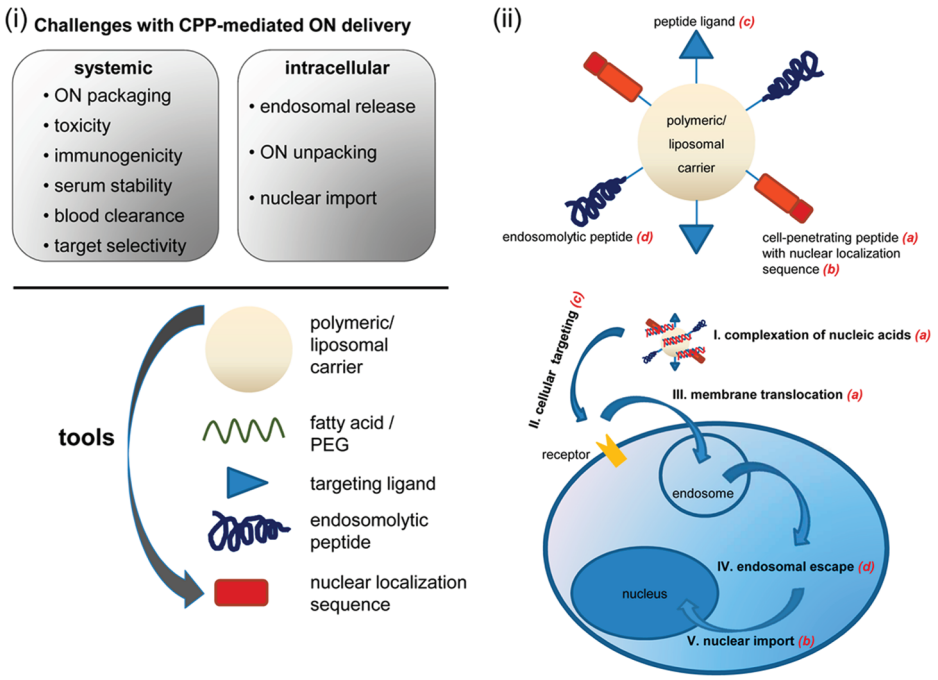


FIGURE 3. (i) Overview of the challenges associated with cell-penetrating peptide-mediated delivery of nucleic acids *in vivo* and useful modifications to address some of them. (ii) Schematic presentation of a multifunctional delivery system combining various distinct peptides and their specific role during cellular delivery of nucleic acids.

Noncovalently bound siRNA was removed by treatment with heparin. For enhanced endosomal release, the bee venom toxin melittin was covalently linked to the system. To ensure that the peptide would only exert its membranelytic effect inside the endosomes, melittin was masked by an anhydride that is cleaved in the acidic endosomal environment.

A common approach for receptor-targeting is the use of peptides with an intrinsic RGD motif binding to integrin receptors, which are overexpressed on tumor cells. Pandita et al. demonstrated that modifying PAMAM with an RGD peptide affords increased gene delivery efficiency, which was shown to be due to the specific interaction of the peptide ligand with its receptor.[35] Likewise, a self-assembling nanocomplex of liposomes and a receptor-targeting peptide was shown to markedly enhance siRNA-induced gene knockdown.[36] A different method involving homing peptides was pursued by Tsuchiya et al.[37] Their idea was to conjugate a polyacrylamide scaffold with a cationic peptide that would act both as agent for DNA condensation and substrate for specific protein kinases overexpressed in tumor cells. They could show that under certain conditions phosphorylation of the peptide facilitates dissociation of the DNA from the complex due to the negative charge of the phosphate groups, which lead to enhanced gene expression.

In an effort to combine the advantages of targeting peptide ligands and cell-penetrating peptides, Jung et al. designed quantum dots functionalized with Tat and an RGD peptide.[38] With this system at hand, they were able to selectively accumulate siRNA in brain tumor cells, while the quantum dots simultaneously act as an imaging probe suitable for diagnostics. Similarly, Kibria et al. developed PEGylated liposomes modified with stearylated octaarginine and a cyclic RGD peptide for synergistic enhancement of gene delivery.[39]

A sophisticated, yet simple solution for combined targeting and cellular internalization of a splice-correcting antisense oligonucleotide without the need for complex delivery systems was proposed by Ming et al. They linked a 2′-*O*-methyl oligonucleotide to bombesin, a peptide binding to a G protein-coupled receptor that is internalized upon ligand binding. However, it remained unclear how the cargo was actually able to get inside the nucleus.[40]

4. *In Vivo* Studies

Notwithstanding the very convincing results of *in vitro* studies, many of the delivery vectors presented in this Account may not be applicable *in vivo* due to the many times higher level of complexity in living organisms. An often encountered problem is the failure of the delivery vectors presented in this Account to trigger a significant

biological therapeutic effect in the presence of serum, for instance, due to aggregation or dissociation of the complexes, while other promising candidates induced systemic cytotoxicity.[14] Further criteria defining an ideal carrier for nucleic acid delivery are protection of its cargo from enzymatic degradation, absence of immune responses, and prolonged circulation, since naked nucleic acids are rapidly excreted by the renal system.[41] Despite these challenges, in the last years, the number of promising vectors in preclinical studies has steadily increased.

Examples of effective *in vivo* gene delivery include the aforementioned stearyl-TP10 peptide, which was shown to significantly induce luciferase expression in mice after intradermal and intramuscular injection.[42] Most importantly, immunogenicity and systemic cytotoxicity was not observed. The same was true for systemic siRNA delivery using PepFect 6, a stearyl-TP10 analog bearing covalently attached units of a chloroquine derivative to further promote endosomal escape. A pronounced knockdown of a house-keeping gene was found in various organs.[43] Sakurai et al. reported on the efficient subcutaneous administration of siRNA directed against β-actin for the inhibition of tumor growth in mice.[44] For this purpose, they designed a PEG-conjugated liposomal nanocarrier modified with stearyl-octaarginine. It was further functionalized with a truncated version of the endosomolytic peptide GALA, the progenitor of above-mentioned KALA, for decreased immunogenicity compared with the full-length peptide.

A remarkable study dealing with the therapeutic application of antisense strategies was conducted by Yin et al.[45] It was aimed at the treatment of Duchenne muscular dystrophy in mice utilizing a conjugate of an arginine-rich cell-penetrating peptide with phosphorodiamidate morpholino oligonucleotides (PMOs) to improve the delivery efficacy compared with naked PMOs. Those are neutral single-stranded DNA derivatives with a morpholine ring instead of the sugar moiety that are commonly used for splice correction. A high degree of exon skipping was observed, and eventually, more than 50% of the normal level of functional dystrophin in the heart was reached.

In another study, a reduction of tumor size in mice by the delivery of a plasmid coding for interferon-α after injection into tumor xenografts was achieved by Huang et al. using cross-linked low molecular weight PEI. It was functionalized with a peptide ligand targeting the human epidermal growth factor receptor 2, which is overexpressed in various types of cancer.[46]

Of particular interest is the number of successful *in vivo* applications involving a combination of cell-penetrating peptides with targeting ligands. Xiong et al.[47] developed polymeric micelles incorporating multiple functional modifications such as Tat for cellular internalization, an RGD peptide for integrin receptor targeting and spermine, that is, a polyamine for siRNA complexation and endosomal leakage due to the proton sponge effect. The cytostatic compound doxorubicin was attached to the spermine units via an acid-labile hydrazone linker. To overcome drug resistance, siRNA targeting P-glycoprotein mRNA was used as a second cargo, which indeed enhanced the antiproliferative effect of doxorubicin *in vitro* in resistant tumor cell lines. *In vivo* imaging studies revealed significant tumor accumulation. Ye et al. induced silencing of viral replication in mice by systemic delivery of antiviral siRNA noncovalently bound to nonaarginine, which was coupled to RVG, a peptide derived from the rabies virus glycoprotein targeting macrophages and neuronal cells.[48] RVG was also used in another study aimed at the intracerebral delivery of siRNA directed against a protein involved in the pathogenesis of Alzheimer's disease. In this case, RVG was conjugated to an all-D-nonaarginine peptide, which is characterized by improved serum stability.[49]

5. Summary and Perspectives

In this Account, the many ways in which peptides can be beneficial in the process of nucleic acid delivery were briefly outlined. On the one hand, they are able to compact nucleic acids and translocate them across the plasma membrane, as in the case of cell-penetrating peptides. Once inside the cell, endosomolytic peptides can help the oligonucleotides to escape the endocytic vesicles. Another key aspect is the import, if necessary, into the nucleus via the nuclear pore complex. This process is mediated by peptides bearing a nuclear localization sequence, which is inherent to several natural and rationally designed CPPs. On the other hand, we demonstrated how peptides can be used for homing therapeutic oligonucleotides to the tissue where they are needed by making use of specific receptor–ligand interactions. A combination of targeting ligands with cell-penetrating peptides is highly favorable to overcome an often unwanted blatant disadvantage of CPPs, namely their non-cell specific uptake. Apart from that, another elegant approach for rendering them cell-type specific includes activation after cleavage by distinct metalloproteinases.[50] For efficient systemic delivery of peptide vectors, the problem of protease degradation in the blood circulation also needs

to be addressed, which can be solved by modification with unnatural amino acids and D-amino acids or peptide cyclization without impairing functionality.[51] As mentioned above, higher proteolytic stability can also be achieved by lipidation or PEGylation in order to shield the peptide from its environment. This may also address another key issue related to the systemic delivery of CPP vectors, fast blood clearance,[52] which is mediated by the kidneys and the reticuloendothelial system (RES). RES clearance is associated with all delivery vectors carrying a high surface charge and thus also cationic lipid and polymer formulations.[53] Hence, keeping the charge ratio of the delivery vectors as close to neutral as possible is also of benefit.

A possible advantage of CPPs besides their versatility paired with easy functionalization is the absence of cytotoxicity at concentrations needed to obtain the desired biological effect in most of the above-mentioned studies, whereas cationic lipid and polymer formulations were often observed to significantly reduce cell viability at their most effective concentrations.[17,20,43] However, to date there are no long-term cytotoxicity studies *in vivo* to confirm the biocompatibility of the peptides presented here. Moreover, the therapeutic scope of cell-penetrating peptides is expanded by the fact that some of them have been shown to be able to cross the blood–brain barrier.[54]

When it comes to the nucleic acids, RNAi is becoming increasingly popular, as can be deduced from the large number of recent *in vivo* studies involving siRNA therapeutics, which is due to their enormous potential and novel chemical modifications improving serum stability and off-target effects.

However, despite the increasing number of successful preclinical trials and ongoing clinical trials that involve either cell-penetrating peptides[55] or nucleic acid therapeutics,[26] only recently have peptide vectors designated for nucleic acid delivery entered clinical trials.[56] Whether they will one day actually come into the market greatly depends on them being affordable for the patient, which in the end will, very likely, favor less complex delivery systems that can easily and reproducibly be synthesized on a large scale. Thus, purely peptidic vectors as opposed to combinations with lipids or polymers may be of benefit.

The authors are supported by funding from the German Federal Ministry of Education and Research (BMBF, PtJ-Bio, 0315338).

FOOTNOTES

*To whom correspondence should be addressed. E-mail: ines.neundorf@uni-koeln.de.
The authors declare no competing financial interest.

REFERENCES

1 Murakami, T.; Sunada, Y. Plasmid DNA Gene Therapy by Electroporation: Principles and Recent Advances. *Curr. Gene Ther.* **2011**, *11*, 447–456.
2 Lu, K.; Duan, Q. P.; Ma, L.; Zhao, D. X. Chemical Strategies for the Synthesis of Peptide-Oligonucleotide Conjugates. *Bioconjugate. Chem.* **2010**, *21*, 187–202.
3 Frankel, A. D.; Pabo, C. O. Cellular Uptake of the Tat Protein from Human Immunodeficiency Virus. *Cell* **1988**, *55*, 1189–1193.
4 Pooga, M.; Hallbrink, M.; Zorko, M.; Langel, U. Cell Penetration by Transportan. *FASEB J* **1998**, *12*, 67–77.
5 Pluen, A.; Saleh, A. F.; Aojula, H.; Arthanari, Y.; Offerman, S.; Alkotaji, M. Improved Tat-Mediated Plasmid DNA Transfer by Fusion to LK15 Peptide. *J. Controlled Release* **2010**, *143*, 233–242.
6 Yeom, Y. I.; Min, S. H.; Kim, D. M.; Kim, M. N.; Ge, J.; Lee, D. C.; Park, I. Y.; Park, K. C.; Hwang, J. S.; Cho, C. W. Gene Delivery Using a Derivative of the Protein Transduction Domain Peptide, K-Antp. *Biomaterials* **2010**, *31*, 1858–1864.
7 Ifediba, M. A.; Medarova, Z.; Ng, S. W.; Yang, J. Z.; Moore, A. siRNA Delivery to CNS Cells Using a Membrane Translocation Peptide. *Bioconjugate Chem.* **2010**, *21*, 803–806.
8 Langel, K.; Lindberg, S.; Copolovici, D.; Arukuusk, P.; Sillard, R.; Langel, Ü. Novel Fatty Acid Modifications of Transportan 10. *Int. J. Pept. Res. Ther.* **2010**, *16*, 247–255.
9 Ezzat, K.; Andaloussi, S. E.; Zaghloul, E. M.; Lehto, T.; Lindberg, S.; Moreno, P. M.; Viola, J. R.; Magdy, T.; Abdo, R.; Guterstam, P.; Sillard, R.; Hammond, S. M.; Wood, M. J.; Arzumanov, A. A.; Gait, M. J.; Smith, C. I.; Hallbrink, M.; Langel, U. PepFect 14, a Novel Cell-Penetrating Peptide for Oligonucleotide Delivery in Solution and As Solid Formulation. *Nucleic Acids Res.* **2011**, *39*, 5284–5298.
10 Divita, G.; Konate, K.; Crombez, L.; Deshayes, S.; Decaffmeyer, M.; Thomas, A.; Brasseur, R.; Aldrian, G.; Heitz, F. Insight into the Cellular Uptake Mechanism of a Secondary Amphipathic Cell-Penetrating Peptide for siRNA Delivery. *Biochemistry* **2010**, *49*, 3393–3402.
11 Kichler, A.; Langlet-Bertin, B.; Leborgne, C.; Scherman, D.; Bechinger, B.; Mason, A. J. Design and Evaluation of Histidine-Rich Amphipathic Peptides for siRNA Delivery. *Pharm. Res.* **2010**, *27*, 1426–1436.
12 Bartz, R.; Fan, H. H.; Zhang, J. T.; Innocent, N.; Cherrin, C.; Beck, S. C.; Pei, Y.; Momose, A.; Jadhav, V.; Tellers, D. M.; Meng, F. Y.; Crocker, L. S.; Sepp-Lorenzino, L.; Barnett, S. F. Effective siRNA delivery and target mRNA degradation using an amphipathic peptide to facilitate pH-dependent endosomal escape. *Biochem. J.* **2011**, *435*, 475–487.
13 Kwon, E. J.; Liong, S.; Pun, S. H. A truncated HGP peptide sequence that retains endosomolytic activity and improves gene delivery efficiencies. *Mol. Pharmaceutics* **2010**, *7*, 1260–1265.
14 Wagner, E.; Meyer, M.; Dohmen, C.; Philipp, A.; Kiener, D.; Maiwald, G.; Scheu, C.; Ogris, M. Synthesis and Biological Evaluation of a Bioresponsive and Endosomolytic siRNA-Polymer Conjugate. *Mol. Pharmaceutics* **2009**, *6*, 752–762.
15 El-Andaloussi, S.; Jarver, P.; Johansson, H. J.; Langel, U. Cargo-Dependent Cytotoxicity and Delivery Efficacy of Cell-Penetrating Peptides: A Comparative Study. *Biochem. J.* **2007**, *407*, 285–292.
16 Shiraishi, T.; Pankratova, S.; Nielsen, P. E. Calcium Ions Effectively Enhance the Effect of Antisense Peptide Nucleic Acids Conjugated to Cationic Tat and Oligoarginine Peptides. *Chem. Biol.* **2005**, *12*, 923–929.
17 Lehto, T.; Abes, R.; Oskolkov, N.; Suhorutsenko, J.; Copolovici, D. M.; Mager, I.; Viola, J. R.; Simonson, O. E.; Ezzat, K.; Guterstam, P.; Eriste, E.; Smith, C. I. E.; Lebleu, B.; El Andaloussi, S.; Langel, U. Delivery of Nucleic Acids with a Stearylated (RxR)(4) Peptide Using a Non-covalent Co-incubation Strategy. *J. Controlled Release* **2010**, *141*, 42–51.
18 Sugiura, Y.; Futaki, S.; Ohashi, W.; Suzuki, T.; Niwa, M.; Tanaka, S.; Ueda, K.; Harashima, H. Stearylated Arginine-Rich Peptides: A New Class of Transfection Systems. *Bioconjugate Chem.* **2001**, *12*, 1005–1011.
19 Li, W.; Nicol, F.; Szoka, F. C., Jr. GALA: A Designed Synthetic pH-Responsive Amphipathic Peptide with Applications in Drug and Gene Delivery. *Adv Drug Delivery Rev.* **2004**, *56*, 967–985.
20 Khondee, S.; Baoum, A.; Siahaan, T. J.; Berkland, C. Calcium Condensed LABL-TAT Complexes Effectively Target Gene Delivery to ICAM-1 Expressing Cells. *Mol. Pharmaceutics* **2011**, *8*, 788–798.
21 Rice, K. G.; Baumhover, N. J.; Anderson, K.; Fernandez, C. A. Synthesis and In Vitro Testing of New Potent Polyacridine-Melittin Gene Delivery Peptides. *Bioconjugate Chem.* **2010**, *21*, 74–83.
22 McClorey, G.; Fletcher, S.; Wilton, S. Splicing Intervention for Duchenne Muscular Dystrophy. *Curr. Opin. Pharmacol.* **2005**, *5*, 529–34.
23 Koppelhus, U.; Shiraishi, T.; Zachar, V.; Pankratova, S.; Nielsen, P. E. Improved Cellular Activity of Antisense Peptide Nucleic Acids by Conjugation to a Cationic Peptide-Lipid (CatLip) Domain. *Bioconjugate Chem.* **2008**, *19*, 1526–34.
24 Oskolkov, N.; Arukuusk, P.; Copolovici, D.; Lindberg, S.; Margus, H.; Padari, K.; Pooga, M.; Langel, Ü. NickFects, Phosphorylated Derivatives of Transportan 10 for Cellular Delivery of Oligonucleotides. *Int. J. Pept. Res. Ther.* **2011**, *17*, 147–157.

25 Trabulo, S.; Resina, S.; Simoes, S.; Lebleu, B.; de Lima, M. C. P. A Non-covalent Strategy Combining Cationic Lipids and CPPs To Enhance the Delivery of Splice Correcting Oligonucleotides. *J. Controlled Release* **2010**, *145*, 149–158.

26 Davidson, B. L.; McCray, P. B., Jr. Current Prospects for RNA Interference-Based Therapies. *Nat. Rev. Genet.* **2011**, *12*, 329–340.

27 Xu, L.; Anchordoquy, T. Drug Delivery Trends in Clinical Trials and Translational Medicine: Challenges and Opportunities in the Delivery of Nucleic Acid-Based Therapeutics. *J. Pharm. Sci.* **2011**, *100*, 38–52.

28 Eguchi, A.; Meade, B. R.; Chang, Y. C.; Fredrickson, C. T.; Willert, K.; Puri, N.; Dowdy, S. F. Efficient siRNA Delivery into Primary Cells by a Peptide Transduction Domain-dsRNA Binding Domain Fusion Protein. *Nat. Biotechnol.* **2009**, *27*, 567–571.

29 Arthanari, Y.; Pluen, A.; Rajendran, R.; Aojula, H.; Demonacos, C. Delivery of Therapeutic shRNA and siRNA by Tat Fusion Peptide Targeting bcr-abl Fusion Gene in Chronic Myeloid Leukemia Cells. *J. Controlled Release* **2010**, *145*, 272–280.

30 Madani, F.; Lindberg, S.; Langel, U.; Futaki, S.; Graslund, A. Mechanisms of Cellular Uptake of Cell-Penetrating Peptides. *J. Biophys.* **2011**, *2011*, No. 414729.

31 Lundin, P.; Johansson, H.; Guterstam, P.; Holm, T.; Hansen, M.; Langel, Ü.; Andaloussi, S. E. Distinct Uptake Routes of Cell-Penetrating Peptide Conjugates. *Bioconjugate Chem.* **2008**, *19*, 2535–2542.

32 Said Hassane, F.; Abes, R.; El Andaloussi, S.; Lehto, T.; Sillard, R.; Langel, U.; Lebleu, B. Insights into the Cellular Trafficking of Splice Redirecting Oligonucleotides Complexed with Chemically Modified Cell-Penetrating Peptides. *J. Controlled Release* **2011**, *153*, 163–172.

33 Verdurmen, W. P.; Brock, R. Biological Responses Towards Cationic Peptides and Drug Carriers. *Trends Pharmacol. Sci.* **2011**, *32*, 116–124.

34 Ganguli, M.; Naik, R. J.; Chandra, P.; Mann, A. Exogenous and Cell Surface Glycosaminoglycans Alter DNA Delivery Efficiency of Arginine and Lysine Homopeptides in Distinctly Different Ways. *J. Biol. Chem.* **2011**, *286*, 18982–18993.

35 Pandita, D.; Santos, J. L.; Rodrigues, J.; Pego, A. P.; Granja, P. L.; Tomas, H. Gene Delivery into Mesenchymal Stem Cells: A Biomimetic Approach Using RGD Nanoclusters Based on Poly(amidoamine) Dendrimers. *Biomacromolecules* **2011**, *12*, 472–481.

36 Tagalakis, A. D.; He, L.; Saraiva, L.; Gustafsson, K. T.; Hart, S. L. Receptor-Targeted Liposome-Peptide Nanocomplexes for siRNA Delivery. *Biomaterials* **2011**, *32*, 6302–6315.

37 Tsuchiya, A.; Kang, J. H.; Asai, D.; Mori, T.; Niidome, T.; Katayama, Y. Transgene Regulation System Responding to Rho Associated Coiled-Coil Kinase (ROCK) Activation. *J. Controlled Release* **2011**, *155*, 40–46.

38 Jung, J.; Solanki, A.; Memoli, K. A.; Kamei, K.; Kim, H.; Drahl, M. A.; Williams, L. J.; Tseng, H. R.; Lee, K. Selective Inhibition of Human Brain Tumor Cells through Multifunctional Quantum-Dot-Based siRNA Delivery. *Angew. Chem., Int. Ed.* **2010**, *49*, 103–107.

39 Kibria, G.; Hatakeyama, H.; Ohga, N.; Hida, K.; Harashima, H. Dual-Ligand Modification of PEGylated Liposomes Shows Better Cell Selectivity and Efficient Gene Delivery. *J. Controlled Release* **2011**, *153*, 141–148.

40 Ming, X.; Alam, M. R.; Fisher, M.; Yan, Y.; Chen, X.; Juliano, R. L. Intracellular Delivery of an Antisense Oligonucleotide via Endocytosis of a G Protein-Coupled Receptor. *Nucleic Acids Res.* **2010**, *38*, 6567–6576.

41 Shim, M. S.; Kwon, Y. J. Efficient and Targeted Delivery of siRNA in Vivo. *FEBS J.* **2010**, *277*, 4814–4827.

42 Andaloussi, S. E.; Lehto, T.; Lundin, P.; Langel, U. Application of PepFect Peptides for the Delivery of Splice-Correcting Oligonucleotides. *Methods Mol. Biol.* **2011**, *683*, 361–373.

43 Andaloussi, S. E. L.; Lehto, T.; Mager, I.; Rosenthal-Aizman, K.; Oprea, I. I.; Simonson, O. E.; Sork, H.; Ezzat, K.; Copolovici, D. M.; Kurrikoff, K.; Viola, J. R.; Zaghloul, E. M.; Sillard, R.; Johansson, H. J.; Hassane, F. S.; Guterstam, P.; Suhorutsenko, J.; Moreno, P. M. D.; Oskolkov, N.; Halldin, J.; Tedebark, U.; Metspalu, A.; Lebleu, B.; Lehtio, J.; Smith, C. I. E.; Langel, U. Design of a Peptide-Based Vector, PepFect6, for Efficient Delivery of siRNA in Cell Culture and Systemically in Vivo. *Nucleic Acids Res.* **2011**, *39*, 3972–3987.

44 Sakurai, Y.; Hatakeyama, H.; Sato, Y.; Akita, H.; Takayama, K.; Kobayashi, S.; Futaki, S.; Harashima, H. Endosomal Escape and the Knockdown Efficiency of Liposomal-siRNA by the Fusogenic Peptide shGALA. *Biomaterials* **2011**, *32*, 5733–5742.

45 Yin, H.; Saleh, A. F.; Betts, C.; Camelliti, P.; Seow, Y.; Ashraf, S.; Arzumanov, A.; Hammond, S.; Merritt, T.; Gait, M. J.; Wood, M. J. Pip5 Transduction Peptides Direct High Efficiency Oligonucleotide-mediated Dystrophin Exon Skipping in Heart and Phenotypic Correction in mdx Mice. *Mol. Ther.* **2011**, *19*, 1295–1303.

46 Huang, H.; Yu, H.; Tang, G.; Wang, Q.; Li, J. Low Molecular Weight Polyethylenimine Cross-Linked by 2-Hydroxypropyl-gamma-cyclodextrin Coupled to Peptide Targeting HER2 as a Gene Delivery Vector. *Biomaterials* **2010**, *31*, 1830–1838.

47 Xiong, X. B.; Lavasanifar, A. Traceable Multifunctional Micellar Nanocarriers for Cancer-Targeted Co-delivery of MDR-1 siRNA and Doxorubicin. *ACS Nano* **2011**, *5*, 5202–5213.

48 Ye, C. T.; Abraham, S.; Wu, H. Q.; Shankar, P.; Manjunath, N. Silencing Early Viral Replication in Macrophages and Dendritic Cells Effectively Suppresses Flavivirus Encephalitis. *PLoS ONE* **2011**, *6*, No. e17889.

49 Wood, M. J. A.; Alvarez-Erviti, L.; Seow, Y. Q.; Yin, H. F.; Betts, C.; Lakhal, S. Delivery of siRNA to the Mouse Brain by Systemic Injection of Targeted Exosomes. *Nat. Biotechnol.* **2011**, *29*, 341–345.

50 Olson, E. S.; Aguilera, T. A.; Jiang, T.; Ellies, L. G.; Nguyen, Q. T.; Wong, E. H.; Gross, L. A.; Tsien, R. Y. In Vivo Characterization of Activatable Cell Penetrating Peptides for Targeting Protease Activity in Cancer. *Integr. Biol.* **2009**, *1*, 382–393.

51 Gentilucci, L.; De Marco, R.; Cerisoli, L. Chemical Modifications Designed to Improve Peptide Stability: Incorporation of Non-natural Amino Acids, Pseudo-peptide Bonds, and Cyclization. *Curr. Pharm. Des.* **2010**, *16*, 3185–3203.

52 Sarko, D.; Beijer, B.; Garcia Boy, R.; Nothelfer, E. M.; Leotta, K.; Eisenhut, M.; Altmann, A.; Haberkorn, U.; Mier, W. The Pharmacokinetics of Cell-Penetrating Peptides. *Mol. Pharmaceutics* **2010**, *7*, 2224–2231.

53 Tong, A. W.; Jay, C. M.; Senzer, N.; Maples, P. B.; Nemunaitis, J. Systemic Therapeutic Gene Delivery for Cancer: Crafting Paris' Arrow. *Curr. Gene Ther.* **2009**, *9*, 45–60.

54 Du, L.; Kayali, R.; Bertoni, C.; Fike, F.; Hu, H.; Iversen, P. L.; Gatti, R. A. Arginine-Rich Cell-Penetrating Peptide Dramatically Enhances AMO-Mediated ATM Aberrant Splicing Correction and Enables Delivery to Brain and Cerebellum. *Hum. Mol. Genet.* **2011**, *20*, 3151–3160.

55 Johnson, R. M.; Harrison, S. D.; Maclean, D. Therapeutic Applications of Cell-Penetrating Peptides. *Methods Mol. Biol.* **2011**, *683*, 535–551.

56 Steele, J. C.; Rao, A.; Marsden, J. R.; Armstrong, C. J.; Berhane, S.; Billingham, L. J.; Graham, N.; Roberts, C.; Ryan, G.; Uppal, H.; Walker, C.; Young, L. S.; Steven, N. M. Phase I/II Trial of a Dendritic Cell Vaccine Transfected with DNA Encoding Melan A and gp100 for Patients with Metastatic Melanoma. *Gene Ther.* **2011**, *18*, 584–593.