The use of cell-penetrating peptides for drug delivery

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In the past decade, several peptides that can translocate cell membranes have been identified. Some of these peptides, which can be divided into different families, have short amino acid sequences (10–27 residues in length) and enter the cell by a receptor-independent mechanism. Furthermore, these peptides are capable of internalizing hydrophilic cargoes. Although the detailed mechanism by which these molecules enter cells is poorly understood, their ability to traverse the membrane into the cytoplasm has provided a new and powerful biological tool for transporting drugs across cell membranes.

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▼ Advances in genomics, biotechnology and chemistry have led to the design of a significant number of therapeutic and diagnostic agents that target intracellular molecules. Delivering these molecules at their most appropriate site (e.g. cells and tissues) is a formidable challenge that is far from being met satisfactorily. Therapeutic agents with low biomembrane permeability are generally considered to be of limited therapeutic value. Therefore, there is a growing effort to enhance the cellular uptake of these compounds. An even more difficult task is to deliver hydrophilic molecules across the blood–brain barrier (BBB), a complex biological interface that prevents transport of most drugs from the vasculature into the brain parenchyma.

Introduction to intracellular delivery

During the past decade, several cell-penetrating peptides (CPPs) that enable the intracellular delivery of polar, biologically active compounds *in vitro* and *in vivo* have been described. These peptides, such as SynB vectors, Antennapedia (Antp) and transactivator of transcription (Tat) [1–3], are heterogeneous in size (10–27 amino acids in length) and sequence. However, all these peptides possess multiple positive charges and some of them share common features, including important theoretical hydrophobicity and helical moment (reflecting the peptide amphipathicity), the ability to interact with lipid membranes and to adopt a significant secondary structure on binding to lipids. These CPPs penetrate into cells by a receptor-independent process [4,5]. Although the detailed mechanism by which these peptides enter into cells is poorly understood, their capacity to cross the membrane into the cytoplasm, even when carrying hydrophilic molecules, has afforded a new and powerful tool in biomedical research. Here, recent studies that have tested the ability of these CPPs, which penetrate by a receptor-independent mechanism, to enhance the transport of cargoes across cell membranes and the BBB are discussed.

Cell-penetrating peptides

SynB vectors

SynB vectors are a new family of vectors derived from the antimicrobial peptide protegrin-1 (PG-1), an 18 amino acid peptide originally isolated from porcine leukocytes (Table 1) [6]. The PG-1 peptide interacts with, and forms pores in, the lipid matrix of bacterial membranes [7,8]. The demonstration that the pore formation capability of PG-1 depends on its cyclization [8] has led to the design of various linear analogues of PG-1 that lack the cysteine residues. Although these linear peptides have lost their membrane-disrupting activity, they are still able to interact with the cell surface and cross the plasma membrane [5,9]. Furthermore, the internalization of these CPPs into cells does not appear to be dependent on a chiral receptor because the D-enantiomer form penetrates as efficiently as the parent peptide (L-form), and retro-inverso sequences exhibit identical penetrating activity. These linear analogues were the starting point for developing a new potent strategy for drug delivery into complex biological membranes, for example, the BBB. Further optimizations

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Table 1. Principal cell-penetrating peptides

Cell-penetrating peptide	Sequence[a]	Origin	Refs
Tat48–60	GRKKRRQRRRPPQ	HIV Tat	[17]
Penetratin	RQIKIWFQNRRMKWKK	AntpHD	[10]
SynB1	RGGRLSYSRRRFSTSTGR	Protegrin-I	[67]
SynB3	RRLSYSRRRF	Protegrin-I	[68]
Transportan	GWTLNSAGYLLKINLKALAALAKKIL	Galanin and mastoparan	[25]
Amphipathic model peptide	KLALKLALKALKAALKLA	NA	[26]
Signal sequence-based peptide	GALFLGWLGAAGSTMGAWSQPKKKRKV	gp41 fusion protein–NLS (from SV40 T antigen)	[29]
Arg	RRRRRRRRR	NA	[30]

[a]The sequence indicated in this Table corresponds to the original sequence. Various analogues were described in the literature.
Abbreviations: AntpHD, Antennapedia protein homeodomain; NA, not available; NLS, nuclear localization signal; Tat, transactivator of transcription.

have led to the development of smaller peptides (less than ten amino acids in length) that have improved properties.

Protein-derived cell-penetrating peptides

It was established that a short peptide segment, pAntp43–58 (penetratin), corresponding to the third helix of the homeodomain of the Antennapedia protein (AntpHD), is able to penetrate into various cells (Table 1) [10–12]. Analogues of penetratin corresponding to its enantio-form [all amino acids (43–58) are of the D-configuration] or retro-inverso-form (58–43) penetrated as efficiently as the parent peptide [13], which suggests that penetratin translocates through cell membranes without binding to a stereospecific receptor. The unusual cellular import of penetratin could depend on its capacity to interact with the lipid matrix of plasma membrane [14,15], although the exact mechanism has yet to be resolved.

As found for the homeoproteins, the transcription factor Tat, which is involved in the replication cycle of HIV, was demonstrated to penetrate into cells [16,17]. One of the shortest peptides containing a nuclear localization signal (NLS), Tat48–60, was defined as the minimal translocating fragment (Table 1). Since its initial description [18], many Tat-derived short peptides have been shown to translocate into the interior of different cell types. Internalization occurs within minutes and is reduced by the addition of polyanions such as heparin or dextran sulfates [19]. Although various studies claim to have shown experimentally the cellular penetration of Tat and penetratin peptides via a receptor- and endocytosis-independent mechanism, some recent studies have re-evaluated the mechanism of cellular uptake and the concept of an endocytotic mechanism has been given additional support [20–24]. Based on recent studies, the results of some previous reports need to be revisited. Although penetratin and Tat have been extensively used as carriers for relatively small cargoes (e.g. peptides and oligonucleotides), Tat has been the predominant vector used in the delivery of large molecules (e.g. proteins).

Other cell-penetrating peptides

Several other CPPs that are not derived from natural proteins but are the result of the engineering of various short peptides have been described, for example, transportan [25], the model amphipathic peptide (MAP) [26,27], various signal sequence-based peptides [28,29] and homoarginine vectors [30] (Table 1). Transportan is a 27 amino-acid chimeric peptide composed of the neuropeptide galanin and mastoparan-X linked by a central lysine. The cellular-uptake of this peptide is rapid, occurs at 4°C and is unaffected by the presence of inhibitors of endocytosis [25]. In addition, it has been reported that MAP and some of the other sequence-based peptides seemingly enter into cells via a non-endocytotic pathway [27–29]. The signal sequence-based peptides resulted from the fusion of a hydrophobic peptide (such a signal sequence or a fusion peptide) with the NLS motif, whereas the MAP peptide is a complete canonical amphipathic helix. Homoarginine peptides have also been described as entering into cells by an energy-dependent, non-endocytotic process [22,30].

Principles of the use of peptide-conjugated cargoes

The development of the peptide-conjugated cargo system is based on the premise that enhancing cellular or brain uptake of a drug will result in a therapeutic benefit. However, this development will require careful evaluation to determine if the supposed benefit gained by modifying the drug or adding a vector will not be offset by anticipated problems created by the peptide vector. Potential toxicity and immunogenicity of the peptide vectors must be considered before clinical application. Conjugation of a drug with a transport vector might often lead to a loss of biological activity, requiring the development of a linker

strategy that will enable the drug molecule to be cleaved off the drug transporter once it reaches its site of action (Figure 1). In many studies, the cargo was coupled to the vector via a chemical linker, such as a disulfide bond (Figure 2). This enables the reduction of the bond in the cell and the release of the cargo. When dealing with protein cargoes, the chemistry of linking becomes more complicated because many functional groups are present within a protein. Therefore, in most cases, the conjugates were prepared by fusion proteins and recombination (Figure 2).

CPPs have proven their ability to deliver various molecules, including oligonucleotides, small molecules, peptides and large molecules, in cultured cells. However, the ability to deliver these cargoes *in vivo* would be of tremendous benefit. Therefore, the development of vectors will not only depend on the efficiency with which these CPPs can cross the cell membrane but also on their pharmacokinetic and biodistribution profiles. Ideally, an efficient peptide vector should have the dual effects of enhancing the uptake and increasing the systemic bioavailability of the drug in plasma. A conjugate with a poor pharmacokinetic profile and a reduced plasma concentration will result in low delivery, irrespective of the extent to which the cell membrane permeability has been increased because of the peptide vector. Similarly, a peptide vector for which the biodistribution and intracellular routing differs from the intended site of drug delivery will not be efficient in delivering a sufficient amount of drug into the target site. Finally, to develop this strategy at an industrial scale for clinical use in humans, the conjugated drug should be easy to manufacture on a large scale and should be cost-effective.

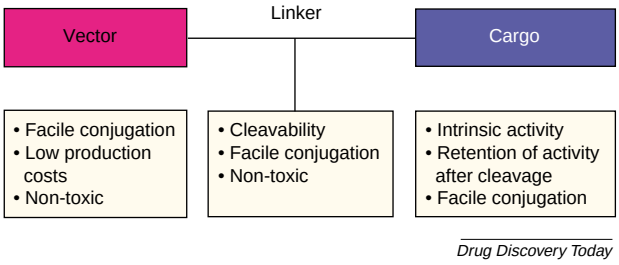

Figure 1. Issues that must be considered in the development of peptide-conjugated cargoes.

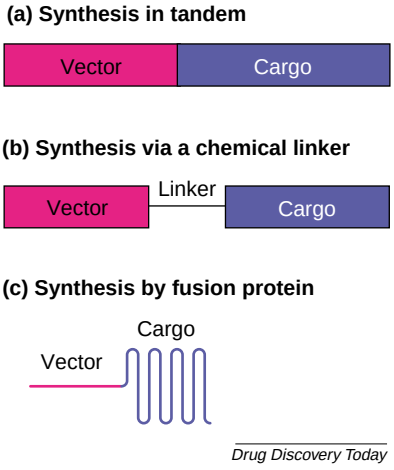

Figure 2. Various modes have been used to couple vectors and cargoes. **(a)** For the majority of peptide cargoes, the cargo was synthesized in tandem with the peptide vector. **(b)** For other molecules, such as small oligonucleotides, a linker was introduced between the peptide vector and the cargo. **(c)** In the case of proteins, the majority of studies have synthesized the complex as a fusion protein in *Escherichia coli*.

Applications of cell-penetrating peptides

Intracellular delivery

The ability to deliver large hydrophilic molecules, such as peptides, proteins, nucleic acids and large particles, into cells is a significant challenge because of the bioavailability restriction imposed by the cell membrane. The plasma membrane of the cell forms an effective barrier that limits the intracellular uptake to those sufficiently non-polar and smaller in size (less than 600 Da). The discovery of CPPs that can translocate efficiently across plasma membrane of cells has, hence, opened up fascinating perspectives for the development of cell delivery [31,32].

Delivery of biologically active peptides and proteins

The discovery of CPPs enables the design of constructs that reach the interior of cells and interact with intracellular proteins. In particular, it was widely established that the direct conjugation of the AntpHD-derived sequence, or its derivatives, with diverse short motifs could provide new peptidic entities that selectively interfere with diverse cellular mechanisms. For example, phosphopeptides linked in tandem with penetratin stimulate a mitogenic response, potentially through the activation of a regulatory protein involved upstream of this process [33]. Similarly, it was demonstrated that such constructs could specifically inhibit ligand-dependent transduction pathways in various cell lines [34–37]. In addition, several studies have focused on the use of penetratin to promote the delivery of fragments of protein-inhibiting cyclin-dependent kinase (Cdk), which is involved in the regulation of the cell cycle [38–40]. Tat peptide was also shown to promote the intracellular delivery of Cdk-inhibiting peptide, enabling arrest of cell proliferation [41].

An interesting application of the CPPs is the design of cytotoxic T lymphocyte (CTL)-inducing vaccines for the treatment or prevention of infectious and malignant

Table 2. Examples of peptide-conjugated cargoes

Cargo	Peptide	Cell type	Refs
Peptide			
PKC inhibitor	Penetratin	Neuron and astrocyte	[79]
	Tat	Isolated rat heart	[80]
PCw3	Penetratin	Macrophage	[81]
Rab 1 and 2	Penetratin	Prolactin cells	[82]
Cdk-4 and Cdk-6 inhibitors	Penetratin	HaCaT cells	[40]
P16 inhibitor	Penetratin	Pancreatic cells	[83]
VHL	Tat	*In vivo*	[84]
Protein			
Grb2SH2	Signal sequence-based peptides	NIH3T3	[85]
RNase A	Tat	*In vivo*	[86]
Bcl-xL	Tat	Neuron and *in vivo*	[75]
Caspases 2 and 8	Tat	Isolated rat heart	[87]
Smac	Tat and penetratin	MCF7 cells	[88,89]
Oligonucleotide			
SOD1	Penetratin	Neuron	[46]
βAPP	Penetratin	Neuron	[90]
MDR-1	Tat	3T3 fibroblasts	[91]
26-mer AsβCom	Signal sequence-based peptides	NIH3T3, HS68 and H9C2	[92]
Large particles			
pCMV-Luc	Tat-branched	Cos-1, 9L,3T3 and PtdCho-3	[53]
Magnetic nanoparticles	Tat	Progenitor cells and *in vivo*	[55]
Tatp-liposome	Tat	NIH3T3 and H9C2	[57]

Abbreviations: βAPP, β amyloid precursor protein; CDK, cyclin-dependent kinase; MDR-1, multi-drug resistance type 1; PKC, protein kinase C; Smac, second mitochondria-derived activator of caspase; SOD1, superoxide dismutase 1; Tat, transactivator of transcription; VHL, Von Hippel–Lindau peptide.

diseases. The CTLs are probably the most effective mechanism that the immune system has to eliminate abnormal cells expressing viral protein of tumour-associated antigen. The use of conventional vaccines, for example, killed pathogens or recombinant proteins, suffers from the inability of antigen-presenting cells (APCs) to trigger a CTL response via the major histocompatibility complex class I pathway. This problem has been addressed by conjugating peptide and protein antigens to CPPs.

Penetratin, SynB and Tat vectors have been shown to facilitate the cytoplasmic uptake of various CTL 8–10-mer epitopes [42–45]. Synthetic peptides were made containing the CPP linked to the CTL peptide: the peptide complexes were shown to internalize into cells rapidly. Furthermore, the use of the conjugates as immunogens resulted in considerably enhanced antigen-specific CTL responses in mice. Other examples of peptide and protein cargoes are cited in Table 2.

Delivery of oligonucleotides

CPPs have been used successfully to deliver oligonucleotides [46–49]. The demonstration that these oligonucleotide–peptide conjugates could specifically block the translation of a gene into a functional protein (antisense strategy) is of particular interest. For example, it has been reported that transportan and penetratin were able to transport a 21-mer peptide nucleic acid (PNA), which was unable to cross the plasma membrane in its original from, into melanoma cells [50]. Once in the cytoplasm, the oligonucleotide blocked the expression of the galanin type I receptor by interacting with the mRNA encoding for this protein. Furthermore, the intrathecal administration of the peptide–oligonucleotide construct in rats resulted in a decrease in galanin binding in the dorsal horn. The rats treated with 100 μM PNA coupled to penetratin had a 40% decrease in ^{125}I-galanin binding in spinal cord sections, as compared with saline or the vectorized scrambled PNA groups. The galanin was then unable to inhibit C-fibre stimulation-induced facilitation of the rat flexor reflex, thus demonstrating that the construct suppresses *in vivo* the expression of functional galanin receptors involved in pain transmission (other examples of oligonucleotide cargoes are cited in Table 2).

Delivery of large particles and DNA

Attempts have been made to use CPPs for the intracellular delivery of DNA [51–54], and even particulates [55–57]. Tung *et al.* [53] have synthesized a series of complexes containing 1–8 Tat moieties and plasmid DNA and evaluated this transport in different cell lines. Only complexes that contain eight molecules of Tat-derived peptide chain per DNA molecule showed transfection capabilities. Similarly,

Ignatovich *et al.* [54] showed that Tat is able to interact with plasmid DNA electrostatically. These interactions result in the formation of polyelectrolytic complexes at various negative:positive charge ratios of plasmid DNA and Tat peptide. The DNA–Tat complexes can be used for delivery of plasmid DNA into mammalian cells [54]. However, a low level of transfection was obtained after intravenous injection into mice: this is probably because of inactivation of DNA–Tat complexes in the bloodstream.

When directly attached to large cargoes, for example, shell-cross-linked (SCL) nanoparticles, or to magnetic nanoparticles, TAT-derived peptide was shown to improve the cell transduction of these molecules [55,56]. The complexes were delivered in a wide variety of cells without modification of viability, differentiation or proliferation. Furthermore, magnetic nanoparticle complexes enabled the tracking of transduced cells within the organism by high-resolution *in vivo* imaging techniques to study cell–cell or cell–tissue interactions [55].

Liposomes have been used to enhance the half-life and solubility of drugs and to decrease their toxicity. However, their slow cell penetration is a major drawback. Tat or penetratin peptides conjugated on liposomes dramatically enhanced their cellular delivery [57–59]. The two different CPPs appear to act by different transfection mechanisms [58]. Indeed, Tat might interact with the cell surface via glycosaminoglycans, whereas penetratin seems to interact directly with the cell surface [58]. Although the cell penetration could be improved by the use of these CPP–liposome complexes, Tseng *et al.* [59] observed that the rapid intracellular release of the encapsulated drugs should be improved to reach the pharmacological efficiency.

Recently, Torchilin *et al.* [60] have used liposomes modified with Tat peptide. This research demonstrated that intratumoural injection of Tat-liposome associated with a DNA coding for green fluorescent protein (GFP) into the Lewis lung carcinoma tumour of mice resulted in the expression of GFP in tumour cells. Gorodetsky *et al.* [61,62] have used 19–21-mer cell-binding peptides, called Haptides, which are equivalent to sequences on the C-termini of fibrinogen β chain (Cβ), γ chain (preCγ) and the extended αE chain of fibrinogen (CαE) [61,62]. They have shown that liposomes complexed with an amphiphilic Haptide are transduced through cell membranes, probably by a non-receptor-mediated process. These results suggest that Cβ or preCγ could be used to augment the cellular uptake of drugs in liposomal formulations.

Brain delivery

The BBB consists of a monolayer of polarized endothelial cells connected by complex tight junctions that separate the blood compartment from the extracellular fluid compartment of the brain parenchyma and poses a formidable obstacle to drug therapy for the central nervous system (CNS). The most important factors that determine the extent to which a molecule will be delivered from blood into the CNS are lipid solubility, molecular mass and charge. Therefore, based simply on lipid solubility and molecular mass, ~95% of the CNS drugs will almost certainly be impeded by the BBB [63].

To overcome the limited access of drugs to the brain, various strategies have been developed that achieve BBB penetration [1,64–66]. These include neurosurgery-based strategies, which bypass the BBB by means of intraventricular drug infusion or disruption of the BBB, pharmacology-based strategies, including lipidation or chemical modification of the drug to mimic an endogenous compound, and physiology-based strategies, which take advantage of specific receptors at the BBB level. However, problems have been encountered with many of these approaches [63–66]. For example, the risk of infection and neurosurgical costs limit the use of neurosurgery-based procedures, whereas increasing the lipophilicity of a drug decreases its solubility in serum and might enhance its accumulation in other non-target sites. Therefore, new and non-invasive methods are urgently needed. An alternative approach, which overcomes many of the drawbacks of the existing methods, is the use of CPPs. Various molecules have been used as a cargo for delivery to brain.

Small molecules

The efficacy of SynB vectors to enhance brain uptake of the anticancer agent doxorubicin was assessed using *in situ* cerebral perfusion in rats and mice [67,68]. This 'vectorization' of doxorubicin to SynB vectors via a succinate linker significantly enhanced its brain uptake without compromising BBB integrity [67]. Interestingly, the SynB-vectorized doxorubicin bypasses P-glycoprotein (P-gp), which has been shown to be present in the luminal membrane of the BBB endothelial cells and restricts the brain entrance of a broad number of therapeutic compounds, including cytotoxic drugs [69]. Similar results were obtained after intravenous administration. The brain concentrations were higher for vectorized doxorubicin compared with that of free doxorubicin [67]. Interestingly, vectorized doxorubicin shows significantly lower levels in the heart, which strongly suggests that cardiotoxicity – the main side effect of doxorubicin – could be reduced using this strategy. The blood clearance of the vectorized doxorubicin was reduced during the first 180 min, enabling the compound to be more exposed to brain and other tissues. The tissue-to-plasma-partition coefficients were calculated and compared with

those of free doxorubicin. The calculated tissue distribution advantage (TDA) was found to be >1 in brain, indicating a more important brain uptake for the vectorized doxorubicin than would have been expected from the observed increase in plasma levels. As a comparison, the brain uptake of doxorubicin conjugated to D-penetratin was also assessed by *in situ* brain perfusion [67]. Although at high concentrations there was an opening of tight junctions, at low concentrations the barrier was intact and a fivefold to sevenfold enhancement in brain uptake was observed compared with free doxorubicin. These data are at odds with the absence of BBB crossing reported by Bolton *et al.* [70]. However, the conditions within the two experiments are different. In particular, in the first study, radioactive doxorubicin was coupled to penetratin composed of D-amino acids and brain accumulation was quantified shortly after administration. By contrast, Bolton *et al.* [70] used a fluorescent compound linked to penetratin and quantified its presence in the brain 24 h after injection. Because D-penetratin coupled to doxorubicin was shown to bypass P-gp [69], this could also explain the enhancement of brain uptake in the first study.

Similar enhancement in brain uptake was obtained with another small molecule – the antibiotic benzyl-penicillin (B-Pc) [71]. β-Lactam antibiotics are often used for treatment of CNS infections, but their poor penetration into the brain does not permit a sufficient efficacy. B-Pc was coupled to SynB1 vector via a glycolamidic ester linker and the brain uptake was measured using *in situ* brain perfusion [71]. The brain uptake of coupled B-Pc showed an average of an eightfold increase in comparison with free B-Pc. This increase was similar for the seven explored grey areas of the rat brain.

Peptides

In a pharmacological application focused on pain management, the brain uptake of the enkephalin analogue dalargin was enhanced significantly after vectorization [72]. Dalargin is a hexapeptide analogue of leu-enkephalin containing D-Ala in the second position and an additional C-terminal Arg. Although the intracerebroventricular injection of this peptide induces analgesic action, its systemic administration shows no activity in central analgesic mechanisms [73]. This is probably because of the poor brain uptake of dalargin. It has been demonstrated by *in situ* brain perfusion that vectorization with SynB vectors markedly enhances the brain uptake of dalargin [72]. Free or conjugated dalargin were also administered intravenously to mice and antinociception was determined using the hot-plate test, an assay known to be mediated by central receptors. The results show that intravenous administration of dalargin to mice exhibited no analgesic activity. By contrast, conjugation of dalargin to SynB vectors led to a considerable enhancement of analgesic activity immediately after the intravenous injection [72].

Proteins

Schwarze *et al.* [74] fused β-galactosidase (β-Gal) to Tat peptide and assessed the tissue distribution after intraperitoneal injection in mice [74]. Administration to mice resulted in readily detectable β-Gal enzymatic activity in the majority of tissues assayed 4 h post-administration. β-Gal activity was strongest in the perfused tissues (e.g. liver, kidney and lung) and, significantly, the fusion peptide crossed the BBB and could be identified in the cell body layers of the brain; the fusion protein did not disrupt the BBB as assayed by co-injection with Evan's blue dye. Subsequently, this strategy has been used to deliver various proteins [75–78]. For example, Cao *et al.* [75] adopted this approach to transport Bcl-xL, which is a well-characterized death-suppressing molecule of the Bcl-2 family. Bcl-xL is expressed in adult neurons of the CNS and might have a crucial role in preventing neuronal apoptosis that occurs during brain development or results from diverse pathologic stimuli, including cerebral ischemia. Bcl-xL was fused to Tat and injected intraperitoneally into murine model of stroke. Administration of the fused protein decreased cerebral infarction in a dose-dependent manner and attenuated ischemia-induced caspase 3 activation in ischemic neurons [75]. These results were corroborated by another study in which intravenous administration (10 min infusion) of Tat–Bcl-xL reduced infarct volume and neurological deficits after long ischemic insults lasting 90 min [76].

Conclusion

The ability to deliver large hydrophilic molecules is a considerable challenge because of the bioavailability restriction imposed by the cell membrane. As rapid advances in cell and molecular biology lead to a proliferation of potent molecules that cannot be effectively delivered to cells and brain by conventional means, a continuing refinement of the new delivery methods will be essential to realize the potential of these molecular drugs. The use of CPPs for drug delivery represents a novel and promising approach. However, several significant points must be considered. First, the cell culture and animal experiments have provided evidence that peptide-conjugated cargoes can successfully transport drugs across the membranes of many different cell types, and furthermore can traverse the more demanding BBB. Second, the approach is broadly applicable to many diseases. Finally, the insights gained during

the past decade have led to the design of more efficient peptide vectors. The optimization of these vectors will also be aided by a greater understanding of the transport mechanisms operating at the cell membranes and the BBB.

The success of the CPP-mediated strategy for clinical use will depend not only on their efficiency and safety but also on the ultimate cost. Large-scale applications and new methodologies are being implemented to increase the yield and reduce the cost. Because of the progress made to date and the tremendous potential of this approach, it is reasonable to state that the beneficial effect of these peptide-conjugated cargoes in humans will come to fruition in the coming years.

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