Expert Opinion

Cell-penetrating peptides for drug delivery across membrane barriers

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During the last decade, cell-penetrating peptides have been investigated for their ability to overcome the plasma membrane barrier of mammalian cells for the intracellular or transcellular delivery of cargoes as diverse as low molecular weight drugs, imaging agents, oligonucleotides, peptides, proteins and colloidal carriers such as liposomes and polymeric nanoparticles. Their ability to cross biological membranes in a non-disruptive way without apparent toxicity is highly desired for increasing drug bioavailability. This review provides an overview of the application of cell-penetrating peptides as transmembrane drug delivery agents, according to the recent literature, and discusses critical issues and future challenges in relation to fully understanding the fundamental principles of the cell-penetrating peptide-mediated membrane translocation of cargoes and the exploitation of their therapeutic potential.

Keywords: biomacromolecule, cell-penetrating peptides, colloidal carriers, drug delivery, endocytosis, intracellular delivery, nucleic acid, peptide, protein, transduction, translocation

Expert Opin. Drug Deliv. (2008) 5(1):105-117

1. Introduction

One of the major unsolved problems in drug delivery today is how to transport therapeutic biomacromolecules across membrane barriers. The issue will become even more pertinent in the future, as many new drug candidates are biopharmaceuticals that have to pass one or more membrane lipid double layers to reach their pharmacological target site of action. The challenge to overcome is that the size and the hydrophilic nature of biomacromolecules greatly limit their permeation of biological membranes, resulting in low bioavailability.

The identification and exploitation of cell-penetrating peptides (CPPs) during the last two decades of research represents an elegant and promising approach to overcome membrane barriers, inspired by the discovery of naturally occurring CPPs present in shuttling proteins. Reports from 1988 showed that the addition of HIV-1 transactivating (Tat) protein to cell cultures resulted in transactivation of the HIV-1 promoter, succeeding cell membrane permeation [1,2]. The *Drosophila* antennapedia transcription protein was subsequently discovered to possess similar transduction properties [3]. The minimal peptide sequence requirement for the transduction of these transcription factors (called Tat [4] and penetratin [5]) was later deduced and shown to be capable of transporting other macromolecules such as proteins [6] into cells. Since then, many additional CPPs have been identified (for review see [7,8]). These peptides were first called Trojan horses or protein transduction domains, but were also named CPPs after a re-evaluation of the internalisation mechanism, which showed that endocytosis, supplementary to transduction, is a major internalisation pathway [9].

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Multiple studies have consolidated the high efficiency of CPP-mediated cargo delivery *in vitro*, as well as *in vivo* – the first *in vivo* study being the demonstration by Schwarze *et al.* that the fusion of the protein β-galactosidase to the Tat domain lead to ubiquitous delivery of the protein upon intraperitoneal administration in mice [10]. Therefore, CPPs seem to hold great promise as delivery agents for biomacromolecules. However, CPP-mediated delivery is apparently not tissue- or cell-type specific, so for specific targeting purposes additional exipients need to be included in the drug delivery system.

CPPs constitute a very heterogeneous and large group of peptides that is often subdivided into different classes and according to different criteria. Major classes are the polycationic or arginine-rich peptides (e.g., Tat, penetratin, R_8 [octaarginine] [11]) and the amphipatic peptides (e.g., transportan [12]). In addition, CPPs may be classified as naturally occurring fragments of proteins or peptides (Tat, penetratin), as entirely synthetic peptides (e.g., MPG [13], R_8, Pep-1 [14], YTA2 [15]), or as chimaeras such as transportan. In general, all CPPs are short peptide sequences of usually 7 – 16 amino acids. There seems to be little or no homology between primary and secondary structures among CPP classes, but a common feature is that CPPs are often rich in basic residues (Arg, Lys) that can interact with negatively charged cell surface-bound molecules. CPPs are associated with remarkably low toxicity at effective concentrations and have, therefore, been applied for the induction of translocation of a wide range of cargo types into cells. In addition to biomacromolecules (peptides, proteins and nucleic acids) the types of cargo also comprise small molecule drugs, imaging agents and colloidal carriers.

1.1 Mechanisms of internalisation

The puzzling feature of how CPPs – which are often highly cationic charged molecules – can pass non-polar, lipophilic cell membranes has fuelled an intense interest in elucidating their mechanisms of internalisation. The scope of this paper is not a comprehensive review of published mechanistic internalisation data, which has been covered by other recent and excellent reviews [16-18]. The authors' aim here is rather to give an overview of the main findings and key discussion points from both a biological and biophysical perspective.

Despite extensive studies with a number of different sequences, there seems to be no consensus regarding one specific mechanism as a preferred route of CPP uptake [17]. However, it is reasonable to think that the specific route(s) of internalisation depends on a number of factors, with the main being the characteristics of CPPs (e.g., sequence, size, charge, structure, stability), the characteristics of the cargo, and the structure/type of the CPP cargo (e.g., covalent or non-covalent association).

The initial contact between cells and most CPPs involves electrostatic interactions of the positively charged side groups of the basic CPP amino acids with negatively charged, acidic cell-surface proteoglycans, although hydrogen bonding has also been suggested as a likely molecular interaction [19]. Membrane-bound, extracellular matrix proteoglycans are a very heterogeneous group of proteins substituted with long, linear, polysulfated and anionic glycosamninoglycans. Examples of these are chondroitin sulfate, dermatan sulfate and heparan sulfate. The heparan sulfate proteoglycans are ubiquitously expressed and have been shown to be important for the internalisation of arginine-rich peptides such as Tat [19,20]. The fact that CPPs are more than just cationic tags responsible for binding to the negatively charged cell surface, followed by internalisation via constitutive endocytosis, was proven in studies showing that CPPs, in addition to surface binding, stimulate intracellular signalling cascades that induce CPP uptake [20,21]. However, uptake by nonspecific fluid-phase endocytosis requires the CPP to only be in proximity to the cell membrane, and does, thus, apparently not necessarily involve electrostatic interactions.

Internalisation of one of the most studied CPPs, the arginine-rich Tat peptide, was initially believed to be via a non-endocytic pathway, due to the observation of diffuse cytoplasmic localisation upon energy depletion [11]. Subsequent studies concluded that the cytoplasmic localisation observed for Tat *in vitro* was a result of the experimental fixation procedure and that internalisation instead proceeded via endocytic pathways [9,22]. It seems that most fluorescently labelled CPPs and CPPs conjugated to various cargoes are taken up via endocytosis by cells into vesicular structures, even though there appears to be no real consensus in the literature as to how CPPs and CPP-conjugated cargoes are internalised; the internalisation mechanisms remain elusive and highly controversial.

Endocytosis is an energy-dependent process involving vesicle formation that can proceed via phagocytosis (uptake of large particles occurring only in specialised immune cells) or pinocytosis (functional in all cells). Pinocytosis can happen through at least four basic routes of endocytosis that all seem to be involved in the internalisation of CPPs (for review see [16]): clathrin-dependent, caveolae-dependent, clathrin- and caveolae-independent endocytosis and raft-dependent macropinocytosis [23]. The major differences between these endocytosis mechanisms are the size of the vesicles formed upon internalisation, their regulation and the intracellular trafficking route. This implies that the cargo size might be one decisive parameter for the internalisation mechanism and the final intracellular destination.

Many studies (e.g., [24]) show that (fluorescently labelled) arginine-rich peptides may mainly be taken up by macropinocytosis. Macropinocytosis is an actin-dependent endocytic pathway that is active in many cell types (constitutive or inducible) and involves the cellular uptake of large volumes of non-selective bulk fluid in a receptor-independent way (reviewed in [17]). The size of the macropinosomes (1 – 5 μm) suggests that macropinocytosis is the most probable endocytosis pathway for larger CPP conjugates,

as other types of endocytosis in general are characterised by vesicles smaller than 150 nm. Macropinocytosis proceeds via membrane protrusions formed by membrane ruffling that enclose a large volume of extracellular material, followed by pinching off to form intracellular vesicles. The actin polymerisation machinery is involved in the process. Upon internalisation, macropinosomes may undergo various destinies in the cell that are presently poorly understood [17]. Further studies are needed to elucidate whether individual CPPs and CPP–cargo conjugates are taken up by cells into vesicular structures by a specific type of macropinocytosis or by another or additional forms of endocytosis.

Upon internalisation, the cargo has to overcome the bottleneck of escaping the vesicular compartments to avoid degradation in the lysosomes and to enter the cytosol, from where most cargos reach their target site of action [25,26]. How this vesicular escape is achieved is likewise a matter of intense debate, and little experimental data exist that can explain the possible mechanism(s). Two theories for endosomal escape include: i) disruption of vesicular membranes by the CPPs; and ii) hydrophobic counteranion-mediated translocation, which is discussed further below in relation to CPP transduction.

Despite the re-evaluation of the internalisation mechanisms of CPPs as being mainly endocytosis, several studies now suggest that alternative internalisation mechanisms exist by which CPPs can translocate across membranes in an energy-independent and endocytosis-independent way (transduction). Transduction, whereby peptides directly transverse the plasma membrane and enter the cytoplasm, is a physically driven mechanism governed by spontaneous peptide–membrane interactions [27-29]. The transduction mechanism is still elusive, controversial and poorly characterised, but two hypothetical models exist for the transduction mechanism: i) direct membrane penetration [10,30]; and ii) the formation of inverted micelles [31], which both involve membrane interaction, membrane permeation and release of the CPP into the cytosol.

A third hypothesis is related to the translocation of guanidinium-rich, cationic CPPs and is based on counteranion scavenging [18,32,33]. Several studies suggest that the presence of counteranions may lead to charge neutralisation or the inversion of CPPs, which enable CPPs to partition into the nonpolar lipid bilayer of membranes. Transmembrane potentials may then be the driving force for the transport of CPPs with guanidinium groups across the membrane [34,35]. However, there is a need for studies on biological cell plasma membranes to support this hypothesis.

Transduction and endocytosis are not two mutually exclusive pathways for cellular entry, and both processes may occur concurrently. Furthermore, the same CPP may use different endocytosis mechanisms simultaneously, but to different extents depending on the type of CPP and the peptide-to-cell ratio, as shown recently for penetratin, nonaarginine and Tat [36]. Data supports that endocytosis is supplemented at high peptide concentrations with the uptake of CPPs by transduction [36,37]. This suggests that results gained from previous visualisation experiments requiring relatively high concentrations of peptides may be misleading [17]. In this respect it is more relevant to investigate the delivery of the cargo using the pharmacological effect of the drug as a biological read out.

1.2 Peptide stability

The stability of the CPPs is a crucial factor for their application in diagnostics as well as therapeutics. It is essential for the efficiency of the CPPs that they possess the ability to effectively deliver their cargo to the appropriate site in the tissue or inside the cell without being prematurely cleaved by extracellular or intracellular proteases, with subsequent premature release of the cargo. However, whether the stability is the main limiting factor depends on the specific CPP, the cargo and association to the delivery system, the administration site and the specific target site.

The effect of increased stability has been specifically addressed in several studies, which showed that the degradation of fluorophore-labelled MAP [38], penetratin [38,39], Tat and a number of human calcitonin-derived CPPs [39] correlated to a decrease in cellular uptake, indicating that extracellular metabolism affects peptide uptake. The latter study revealed that the degradation pattern was not cell-type dependent, whereas the rate of degradation corresponded to the relative enzyme activity in the three assessed cell culture models [39]. It is generally recognised that increased stability is important for the efficient CPP-mediated delivery of cargo, even though there is not always a correlation between extracellular CPP degradation and translocation efficiency [40].

Intracellular degradation is also a hindrance for the CPP-mediated delivery of cargoes, as sequestration and degradation in endocytic vesicles may, for example, severely limit the cytoplasmic and nuclear delivery of conjugated cargos. As an example, recent results have shown that the intracellular level of free cargo corresponds to the level of intact intracellular CPP [41]. In order to avoid lysosomal degradation of the CPP (and potentially cargo) by the variety of enzymes present in the lysosomes, endosomal escape should be achieved. Recently, Patel *et al.* also addressed the potential importance of a cytosolic proteasome-related degradation pathway in relation to intracellular CPP degradation [16]. In this context, the intracellular processing and degradation kinetics of CPP–cargo conjugates still constitutes a major area of exploitation.

Naturally occurring, as well as synthetic, CPP candidates have been modified in order to increase stability by changing the amino acid configuration (from L to D) [11,42-44], by incorporation of non-α-amino acids [43,45], by altering the peptide sequence [43,44] or by peptide branching [46] and terminal modification [44]. Conjugation to the drug or diagnostic cargo may change the inherent stability of

the CPP and should also be considered. However, the increased stability of D-peptides versus L-peptides might not fully account for an improved cellular uptake of the CPP [44].

The CPP–cargo construct should be appropriately metabolised to release covalently coupled or associated cargo after internalisation in order to obtain the desired effect. Thus, the type of conjugation to the cargo is clearly also of great relevance in order to achieve the most efficient delivery. Some studies have shown that maleimide or amide conjugation is more stable than disulfide binding [43]. In other cases, conditional stability is preferred for optimal cargo release properties. An example is the application of disulfide-linked CPPs and cargoes that are reduced in the cytoplasm, resulting in release of the cargo [47-49]. When optimising the stability and transduction efficacy of CPPs by chemical modification, it is implicit that the issue of safety (i.e., cellular toxicity, physiological clearance and immunogenicity in relation to short and long-term effects) should be specifically addressed.

1.3 Cell-penetrating peptide toxicity

Even though CPPs are associated with little or negligible toxicity at effective concentrations, some tendencies are briefly addressed in the present review. Cellular toxicity is most often evaluated by assays to determine pertubation of the plasma membrane, with consequent leakage from the cell interior [49-51], by assays determining intracellular enzymatic [52,53] or ATP activity [51], or by nucleus staining [54].

In vitro, the cytotoxicity of most CPPs is in or above the micromolar range, with some CPPs even being non-toxic at 100 μM [52]. In some cases, the effect seems to be cell-type dependent [52,55], and also dependent on the type of CPP [52], although some of the differences are minor. In another study, a similar degree of cytotoxicity was observed for Pep-1 in four different cell lines [14], and with Tat and penetratin in two cell lines [53]. When differentiating between the toxicity of different CPPs, a correlation between the degree of amphipathy and membrane leakage has been observed for a range of CPPs [47], and often Tat is shown to be less non-toxic than, for example, transportan, penetratin and polyarginines [50,52].

Recent attention has been paid to the fact that the toxicity of CPP–cargo constructs might be different from the CPP, and the toxicity of Tat has been shown to increase when a peptide cargo (or even rhodamine) is conjugated to CPPs, depending on the specific cargo [52]. Similar findings were recently reported by Cardozo *et al.* [56], who concluded that the degree of cytotoxicity depended on the length of the cargo peptide, as well as the dose applied to the cells. It has also been reported that the increased efficiency of YTA2 complexed with a protein was associated with a higher level of toxicity compared with the corresponding Tat–protein complex [51]. Upon *in vivo* administration, resulting in the successful delivery of insulin, no release of intracellular lactate dehydrogenase was observed from intestinal tissue after the application of Tat [57], which supported the lack of observed toxicity in the initial *in vivo* studies with Tat–cargo constructs.

2. Cell-penetrating peptides as delivery carriers for drug cargoes

In general, two strategies can be used for the association of cargo molecules to CPPs: i) covalent attachment of the carrier and cargo via various types of linker (see [58] for review); and ii) non-covalent self-assembly (ionic interaction), whereby aggregates or nanoparticles are formed (see [18] for review). Most peptides require a crosslinking (chemical coupling) to their cargo for transport. Covalent conjugation involves demanding, time-consuming synthesis and purification steps. In some cases, physical self-assembly is possible, especially with amphipathic peptides [18]. Several studies have shown a membrane-translocating effect of non-covalent complexes of CPP and cargo [14,51,59]. Regardless of the type of conjugation strategy, in general it should be stressed that careful characterisation of the complexes is required in order to fully deduce their biological effect. For covalent conjugates, the purification of conjugates from non-conjugate entities is equally important.

The cargo can affect the cell-penetrating properties of CPPs in various ways, such as alteration of the internalisation mechanism, altered intracellular trafficking and modified membrane destabilising properties, which in the worst case renders the CPP–cargo complexes inactive (reviewed in [7]). An example is a study performed with transportan 10, which suggested that direct membrane effects may cause the membrane translocation of transportan 10 alone and of smaller complexes, but not larger cargoes [60]. The next subsection describes how CPPs have been applied for the transmembrane transport of small molecule drugs, peptides, proteins, nucleic acids and colloidal carrier cargoes, and how the association of cargoes with CPPs affects the cell-penetrating properties of the CPPs.

2.1 Small molecules

CPPs have been conjugated to small molecules (drugs and imaging agents) to enhance their intracellular delivery, although to a minor extent compared with large molecules, probably due to the existence of other and more cost-effective pharmaceutical formulation approaches to modify their absorption properties.

The anticancer drug doxorubicin has been conjugated to penetratin and displayed enhanced uptake across the blood–brain barrier in an *in situ* brain perfusion model [61]. Doxorubicin is a substrate for the efflux pump P-glycoprotein, which is expressed in multi-drug-resistant tumour cells as well as in the blood–brain barrier endothelium. Coupling doxorubicin to CPPs thus allows doxorubicin to bypass P-glycoprotein-dependent drug efflux from the brain, resulting in increased cytotoxicity towards brain tumour cells [62].

Table 1. Examples of delivery of peptides and proteins by cell-penetrating peptides.

CPP	Peptide/protein	Application	Assay	Result evaluation (effect/transduction)	Ref.
Tat	β-galactosidase	Conjugation	*In vivo*, mouse	Uptake and effect	[10]
Tat	GFP	Conjugation	*In vitro*	Uptake	[66]
Tat	Insulin	Conjugation	*In vitro*	Transepithelial transport	[100]
Tat, tenetratin, transportan, MAP	5-amino-acid peptide	Conjugation	*In vitro*	Uptake	[49]
R_7 R_7W	3- or 4-amino-acid peptides	Conjugation	*In vitro*	Uptake	[54]
R_8 R_{16}	Carbonic anhydrase	Conjugation	*In vitro*	Uptake and effect	[11]
R_6 R_8 R_{10}	Insulin	Co-administration	*In situ*, rat	Transepithelial transport and effect	[57]
R_7	Ciclosporin A	Conjugation	*In vivo*, mouse	Effect	[102]
Pep-1	Cell cycle inhibitory protein 27^{kip1}	Complexation	*In vitro*	Effect	[59]
Pep-1	β-galactosidase GFP	Complexation	*In vitro*	Uptake and effect	[14]
Pep-1 YTA2 pVEC	β-galactosidase streptavidin	Co-administration	*In vitro*	Uptake and effect	[51]

CPP: Cell-penetrating peptide; GFP: Green fluorescent protein; R_n: n-Arginine.

Another anticancer agent, methotrexate, which has its therapeutic effect limited by the induction of drug resistance, has demonstrated a fivefold increase in cytotoxicity in breast cancer tumour cells, compared with free drug, upon conjugation to the CPP YTA2 – an approach that seems to overcome the problems related to drug resistance [15]. Imaging agents, such as fluorescein and other fluorophores, can be very hydrophilic and, therefore, impermeable to membrane barriers. There are numerous examples of the intraceullular (cytoplasmic/nuclear) delivery of imaging agents upon conjugation to CPPs that will not be discussed further.

2.2 Peptides and proteins

As previously mentioned, the interest in CPPs as delivery vehicles for peptide and protein delivery was stimulated by the significant distribution of Tat-fused β-galactosidase to most organs – even the brain – in mice upon intraperitonal injection [10]. Following from this, a number of different therapeutic peptides and proteins have been applied together with CPPs, some of which are compiled in **Table 1** (for a more comprehensive overview, see [59,63-65]). Tat is the most widely tested CPP with an active cargo, but also penetratin, the synthetic polyarginines and other CPPs have been used for peptide and protein delivery.

In most cases with a reported successful outcome, the CPP and the cargo are chemically conjugated, but even complexation of the CPP and the cargo or simple co-administration seem to be efficient in some cases. When either administering a conjugated or complexed CPP–cargo construct, these constructs are well defined at the time of dosing; however, after proof-of-concept testing, it will be of utmost value for the future systematic design of optimal constructs to correlate the stability and physical characteristics of the conjugated or complexed structures to their effect. Efficacy assays using end-point determination of cargo activity or biological/pharmacological effect supplementing cellular or tissue localisation are necessary to support the vast number of mechanistic assays carried out with fluorophore-labelled CPPs. There is increasing evidence that not only the type of CPP [49], but also the type of cargo, affects the delivery efficacy of the CPP, which is reflected in the effect on cellular uptake [66] and toxicity [52,56].

2.3 Nucleic acids

CPPs are studied with increasing interest as carriers for nucleic acid-based macromolecules such as oligonucleotides and plasmid DNA, with target sites of action in the cell cytoplasm or nucleus for the modulation of gene expression (reviewed in [67] and [68]). The oligonucleotide cargoes successfully transported by CPPs include peptide nucleic acid (PNA) and short interfering RNA (siRNA). However, transport efficiency appears to be dependent on the charge of

Table 2. Examples of the delivery of nucleic acids by cell-penetrating peptides.

CPP	Nucleic acid	Application	Assay	Result evaluation (effect/transduction)	Ref.
Transportan, penetratin	siRNA	Conjugation	*In vitro*	Uptake and effect	[70]
Penetratin	siRNA	Conjugation	*In vitro*	Uptake and effect	[71]
Tat	siRNA	Conjugation	*In vitro*	Uptake and effect	[72]
Tat, penetratin	siRNA	Conjugation	*In vitro*	Uptake and effect	[47]
Transportan, R_6-penetratin	PNA	Conjugation	*In vitro*	Effect	[48]
MPG	siRNA	Complexation	*In vitro*	Uptake and effect	[73]
MPG	siRNA	Complexation	*In vitro*	Uptake and effect	[74]
EB1	siRNA	Complexation	*In vitro*	Uptake and effect	[75]
Transportan, penetratin	PNA	Conjugation	*In vivo*, rat	Uptake and effect	[76]
Tat, penetratin, R_6-penetratin, R_3-penetratin, R_9-penetratin, K_8, $(R\text{-}Ahx\text{-}R)_4$	PNA	Conjugation	*In vitro*	Uptake and effect	[77]
Tat, R_7, KLA	PNA	Conjugation	*In vitro*	Uptake and effect	[25]
Transportan, $R_{7\text{-}9}$, Tat, penetratin, NLS	PNA	Conjugation	*In vitro*	Uptake and effect	[79]
Transportan 10	Plasmid	Complexation	*In vitro*	Uptake and effect	[80]
Tat	Plasmid	Complexation	*In vitro*	Uptake and effect	[81]
Tat–phage	Plasmid	Conjugation	*In vitro*	Uptake and effect	[82]
Branched Tat	Plasmid	Complexation	*In vitro*	Uptake and effect	[83]

CPP: Cell-penetrating peptide; K_8: Octalysine; NLS: Nuclear localisation signal; PNA: Peptide nucleic acid; R_n: n-Arginine; siRNA: Small interfering RNA.

the nucleic acid cargo and the type of CPP association. **Table 2** lists the examples of CPP-mediated nucleic acid delivery referred to in the sections below.

There are very few published examples of the successful delivery of negatively charged siRNA into cells by covalent attachment of CPPs (reviewed in [69]). Muratovska and Eccles conjugated transportan and penetratin with *N*-terminal cysteins via disulfide bonds to the 5′ end of the sense strand of siRNA, and observed a reduction of luciferase and green fluorescent protein expression in reporter cells by the conjugates [70]. However, a non-specific, 'diamide'-assisted oxidation method of a mixture of free thiol-containing siRNA and peptide was applied, and the complexes were added to cells without further purification and characterisation, leaving an open question of which chemical entity caused the silencing effect. Furthermore, no data on toxicity was reported. Davidson *et al.* linked penetratin to the 5′ end of the sense strand of siRNA, and the construct was delivered efficiently into primary neuronal cells without further purification and caused a downregulation of protein production after 6 h that preceded mRNA degradation [71]. In a third study, Chiu *et al.* conjugated Tat to siRNA through a stable thiomaleimide linkage and purified the conjugate by polyacrylamide gel electrophoresis [72]. The conjugate was able to silence the gene expression of a reporter. However, none of these three reported studies have been reproduced in the literature, which suggests that the careful characterisation of conjugates is important for a reproducible biological effect.

More solid approaches to couple, purify and characterise covalent complexes of CPPs and oligonucleotides/siRNA have recently been reported by Gait and coworkers [47,48].

Disulfide-linked conjugates of CPPs were first synthesised via C-terminal cystein residues to 5′-thiol-containing sense strand oligonucleotides and then annealed to the antisense siRNA strand containing a 3′ fluorescein residue. Formamide was used as a denaturing agent during conjugation and ion exchange HPLC was applied for purification to prevent aggregation and complete separation of excess peptide. This method seems to circumvent the difficulties in coupling a polyanion to a polycation and in annealing sense strand conjugates containing highly cationic peptides to the corresponding negatively charged antisense strand. Some inhibition of P38α MAPK mRNA expression was observed with penetratin/Tat peptide–siRNA conjugates. However, very high levels of conjugates were needed (micromolar concentrations) increasing the risk of nonspecific off-target effects. This shows that the translocation efficiency of the conjugates is inhibited by the multiple anionic charges present on the phosphate backbone, and that a single peptide appears to be insufficiently powerful to trigger enough release from vesicular or endosomal compartments into the cytoplasm.

The non-covalent complexation of siRNA with certain types of CPPs has been shown to be a feasible delivery method at certain molar and charge ratios, where compact, cationic particles are formed. These peptides include MPG [73,74] and the penetratin analog EB1 [75]. Unfortunately, such biological studies are either often performed under serum-free conditions, and therefore do not address the important issue of serum stability of the complexes, and/or use poorly characterised complexes, making interpretation of the results difficult. However, optimising such non-covalent complexes towards serum stability might be a promising approach for siRNA delivery.

Covalent conjugates of CPPs and the electrically neutral nucleic acid analogues, such as PNA, that can hybridise to RNA targets with high specificity [25], appear much more promising than the anionic oligonucleotides. Conjugates of PNA and CPPs such as transportan and R_6-penetratin have been shown to promote PNA translocation and splice correction of target genes [48,76-79]. Some peptide sequences with optimal chemical and structural features might, thus, be able to escape from endosomes in the absence of other endosomolytic agents [26], although additional endosomal membrane-destabilising strategies are required for several CPPs [25].

Finally, few studies have reported the successful delivery of plasmid DNA with CPPs. There seems to be little or no effect with single peptides [80,81]; however, branched CPPs or a combination of CPPs with other delivery agents might be useful for plasmid transfection [80,82,83].

2.4 Cell-penetrating-peptide-mediated delivery of colloidal carriers

CPPs have also been widely tested for the transmembrane delivery of a range of larger-sized/colloidal cargoes, such as liposomes [84,85], polymeric nanoparticles [86,87], polymeric micelles [88], magnetic nanoparticles [89] and quantum dots [90], which have been recently reviewed [64,91] (selected examples are shown in **Table 3**). An inherited problem of applying CPPs for delivery purposes, independently of cargo size, is their lack of cell specificity. Most CPPs will non-specifically associate to membranes of all cell types due to global expression of heparan sulfate proteoglycan [10]. The resulting ubiquitous membrane translocation may be beneficial under some circumstances, but may be undesired in other cases where a targeting of specific cell types or tissues is needed. A way to introduce specificity is to associate CPPs to other exipients of drug delivery systems (e.g., colloidal carriers that by themselves can deliver a drug in a specific way, either by passive targeting via the enhanced permeability and retention [EPR] effect upon systemic administration or by active targeting approaches) [64]. In this section, the authors discuss recently published approaches that combine the membrane-translocating property of CPPs with the specificity of colloidal drug carrier systems in so-called 'smart', stimuli-responsive nanocarriers.

pH-responsive, Tat-modified long-circulating liposomes and micelles have been designed by Torchilin and coworkers [92-94]. These long-circulating poly(ethylene glycol) (PEG)-coated liposomes and micelles are targeted actively to a specific cell or organ via the attachment of an antibody to PEG-phosphatidylethanolamine (PEG-PE) at their surface. PEG-PE is degradable at low pH due to a pH-sensitive bond between PEG and PE. The carriers are furthermore modified with Tat-short PEG-PE derivatives. At normal physiological conditions the longer PEG chains shield or hide Tat on the shorter PEG chains. However, at lower pH, as in tumours, the longer PEG chains are cleaved from the complexes, whereby Tat is exposed and enhances cellular internalisation. In another recent study, polymeric micelles consisting of a hydrophobic core of polylactic acid and a hydrophilic shell of PEG-conjugated Tat were complexed with a pH-sensitive diblock copolymer of poly(methacryloyl sulfadimethoxine) (PSD) and PEG (PSD-*b*-PEG) [88]. The anionic PSD is complexed with the cationic Tat, whereby PSD-*b*-PEG shields the Tat-containing micelles. At low pH the complexes are de-shielded, which exposes Tat, leading to translocation into cells *in vitro*.

Future studies will show if these smart long-circulating drug delivery systems are able to introduce specificity *in vivo* by passive targeting via the EPR effect in tumour tissue (eventually combined with active targeting strategies), where the CPP is exposed and translocates the drug-containing cargo into cells. The above studies investigated highly complex carrier systems that require thorough characterisation to fully understand how individual components of the carrier system contribute to delivery *in vivo*.

Table 3. Examples of the delivery of colloidal carriers by cell-penetrating peptides.

CPP	Colloidal carrier	Application	Assay	Result evaluation (effect/transduction)	Ref.
Transportan 10	PEI	Complexation	*In vitro*	Uptake and effect	[80]
Tat	Phage	Conjugation	*In vitro*	Uptake and effect	[82]
Tat	Liposome	Conjugation	*In vitro*	Uptake and effect	[84]
Tat	Liposome	Conjugation	*In vitro*	Uptake and effect	[85]
Tat	MEND	Conjugation	*In vitro*	Uptake and effect	[86]
Tat	PEG-PEI	Complexation	*In vivo*, mice	Uptake and effect	[87]
Tat	Polymeric micelles	Conjugation	*In vitro*	Uptake	[88]
Tat	Magnetic nanoparticles	Conjugation	*In vivo*, mice	Uptake	[89]
Tat, Pep-1, R_n	Quantum dots	Conjugation	*In vitro*	Uptake	[90]
Tat	Liposomes	Conjugation	*In vivo*	Uptake and effect	[93]
Tat	Liposomes Micelles	Conjugation	*In vitro*	Uptake	[94]

CPP: Cell-penetrating peptide; MEND: Multifunctional envelope-type nano device; PEG: Polyethyleneglycol; PEI: Polyethyleneimine; R_n: n-Arginine.

3. Cell-penetrating peptides for transepithelial drug delivery

Due to the promising, ubiquitous distribution of biomacromolecular cargo when administered together with a CPP *in vivo* [10,61], the delivery of biomacromolecular therapeutics across epithelial or endothelial barriers might also hold great promise. However, most commonly, proliferating cells are used for translocation studies and only a few reports on the use of well-differentiated cell layer(s) exist [21,55,95-98]. Therefore, irrespective of whether the aim is the localised delivery of a cargo into the interior of well-differentiated cells integrated in a certain tissue or to pass a monolayer or stratified epithelial or endothelial barrier, cells in the non-proliferating state should also be considered as test models, as the translocation properties might vary considerably depending on the cell cycle status.

Studies using non-proliferating cells in general show that the uptake of CPP–cargo in differentiated cells is significantly lower or unmeasurable compared with the uptake in the same type of cells in the proliferating state, indicating that transepithelial/transendothelial delivery is less relevant for CPP-assisted cargo delivery. The reasons for this discrepancy in proliferating cells are probably that: i) endocytosis and transcytosis in general are reduced in differentiated and polarised cells; ii) that the presence of microvilli or mucus on the surface limits the seemingly critical contact between the CPP and the plasma membrane; and iii) that the accessible surface area is reduced because, naturally, the epithelium presents a barrier with interconnecting junctions to diffusion. Transportan and transportan 10, but not penetratin, have demonstrated an effect on the tight junctions in Caco-2 monolayers, leading to a slight increase in the transepithelial permeability of the peptides, but not of a co-administered dextran [99].

On the contrary, recent *in situ* studies in rats showed that the co-administration of polyarginines together with insulin significantly enhanced the absorption of insulin from the intestine [57]. This result is supported by the observation that insulin coupled to Tat has shown significantly increased permeability across an intestinal Caco-2 cell model [100]. Interestingly, cutaneous co-administration of a short synthetic peptide has been shown to lead to the increased bioavailability of insulin and growth hormone [101], and the cutaneous delivery of ciclosporin A has been enhanced by conjugation to heptaarginine [102]. So even though the general consensus is that the distribution of therapeutic macromolecules beyond blood vessel endothelia after parenteral administration or uptake after topical administration is sparse, a few promising studies have been reported.

4. Conclusion

As evident from the present review, a significant number of experiments have so far been published on the screening of different types of CPPs with regard to cellular uptake and target validation, with the overall conclusion that CPPs are promising carriers for a vast number of cargoes such as biomacromolecules and nanoparticles. Although some attention has recently been focused on understanding the mechanisms of internalisation, mainly pursued by the use of fluorophore-conjugated CPPs, many issues still have to be resolved. Also, in the rapidly evolving area of nanomedicine,

it is not clear which internalisation mechanisms are responsible for the uptake of the CPPs associated to, for example, a biomacromolecular or colloidal cargo. The present authors anticipate that the present research will lead to delivery systems consisting of a CPP conjugated or associated to small molecule therapeutics, nucleic acids, peptide or protein drugs or formulated in a more complex delivery system. Such CPP-based systems will also constitute valuable tools for research, diagnostics and imaging.

5. Expert opinion

Much evidence shows that the translocation properties of CPPs are mainly through inducing endocytosis or pinocytotosis; this is possibly to some extent in combination with direct penetration/partitioning into the lipid membrane. However, for further elucidating how CPPs, in association with cargoes with different physicochemical properties, can cross plasma and endosomal membranes, biophysical studies on artificial membranes must be supplemented with more mechanistic studies using live cells.

5.1 The structure, uptake and intracellular processing/trafficking of cell-penetrating-peptide-conjugated cargo

What remains to be elucidated is the challenging task of determining the true importance of the molecular structure of CPPs in the vicinity of the membranes of live cells (plasma, endosomal), and to estimate which structural elements are crucial for enhanced uptake when applied together with a therapeutic or diagnostic cargo. Such structure–activity relationships must be deduced in the absence of a fluorophore that might change the behaviour of the CPP, which requires the use of sensitive analytical methods enabling the detection of minute amounts of CPP and cargo upon isolation from cellular organelles.

Such intracellular trafficking studies of CPPs and CPP-associated carriers should be designed and performed to identify and fully understand the rate-limiting steps in CPP-mediated delivery to the specific intracellular target compartment. Endosomal escape is of major importance to this. An increased understanding of the intracellular kinetics of CPP trafficking could reveal ways to target various internalisation pathways.

5.2 Functional studies

In general, there should be increasing focus on not just the level of internalisation, the internalisation mechanisms and intracellular trafficking, but also on the therapeutic effect of applying a drug together with the CPP of interest, which requires the use of sensitive, functional assays with biological read outs depending on the type of cargo (e.g., phenotypic assays such as in [25,103]).

It is still not clear which internalisation mechanisms should be targeted (except in a few studies, e.g., [48]) in order to make use of the biological activity of the cargo. There is a need for systematic studies describing the effect of basic CPP–cargo physicochemical parameters, such as size and charge, the uptake mechanism(s), intracellular trafficking and not least the biological effect. Such clarification on the relationship between uptake and functional studies may reveal the nature of the uptake pathways that lead to biological activity.

5.3 Factors of importance for efficient cell-penetrating-peptide–cargo delivery: stability and concentration

As evident from the present review, the proper purification and/or characterisation of CPP–cargo complexes is a key issue. Addressing and optimising the stability of the CPP and the CPP–cargo complexes under physiological conditions (extracellularly as well as intracellularly in the presence of enzymes and serum) is a prerequisite to increased potency. Such charaterisation and stability studies should be performed prior to *in vitro* and *in vivo* studies to avoid premature and misleading statements, and to ensure reproducibility.

In addition, efforts made to develop CPPs that exert their effect at very low concentrations *in vitro* in order to optimise the chance of efficient *in vivo* delivery. In biological assays, more attention should be given to determining the effective peptide-to-cell ratio and to avoid *in vitro* artifacts caused by the chosen experimental conditions. Many CPP studies are presently performed under serum-free conditions, which might give misleading results due to non-physiological conditions, especially when applying CPP–cargo complexes that are unstable in the presence of serum components. Likewise, the use of proliferating versus non-proliferating cells should be considered.

5.4 Factors of importance for efficient cell-penetrating-peptide–cargo delivery: cell specificity, pharmcokinetics/pharmacodynamics and safety

Efficiency *in vivo* needs to be optimised, for example by formulating the CPPs in a stable drug delivery system that targets tissues and cells with selectivity and controls the release of the CPP/drug molecule in order to obtain an efficient concentration at the pharmacological target site of action. Therefore, the development and characterisation of such multifunctional CPP–drug/carrier conjugates/assemblies, which are often of highly complex composition, is required in order to obtain the desired and reproducible effect *in vitro* as well as *in vivo*.

Some studies – mainly with the Tat peptide associated to nanocarriers designed to accumulate specifically in the required organ or tissue and target specific cells – have reported an increase in the biological effect of a drug molecule *in vitro*, as well as *in vivo* in some case, but generally there is a great need for studies addressing issues such as the biodistribution,

pharmacokinetics and pharmacodynamics of CPP-containing intelligent nanomedicines and biomacromolecular cargoes associated to CPPs to quantify to what extent CPPs enhance cargo delivery *in vivo*. Of major importance is a focus on safety concerns such as the toxicity and immunogenicity of CPPs (or the cargo) when applied together with a biomacromolecular drug or a colloidal carrier.

In vivo trials must be supported by additional studies not just of the CPP–cargo complex, but also of the biological membrane barrier itself in order to resolve the real determinants for CPP-mediated drug delivery across membranes. The potential therapeutic application of CPPs relies on their ability to facilitate the transport of therapeutics across biological membranes, which is still an unexplored area ready for exciting and challenging future research activities.

Acknowledgements

We apologise to our colleagues whose contributions are not cited due to reference limitation.

Declaration of interest

C Foged is supported financially by a grant from the Danish Research Council for Technology and Production Sciences.

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