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REVIEW ARTICLE

Cationic and amphipathic cell-penetrating peptides (CPPs): Their structures and *in vivo* studies in drug delivery

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Abstract Over the past few decades, cell penetrating peptides (CPPs) have become an important class of drug carriers for small molecules, proteins, genes and nanoparticle systems. CPPs represent a very diverse set of short peptide sequences (10−30 amino acids), generally classified as cationic or amphipathic, with various mechanisms in cellular internalization. In this review, a more comprehensive assessment of the chemical structural characteristics, including net cationic charge, hydrophobicity and helicity was assembled for a large set of commonly used CPPs, and compared to results from numerous *in vivo* drug delivery studies. This detailed information can aid in the design and selection of effective CPPs for use as transport carriers in the delivery of different types of drug for therapeutic applications.

Keywords cell penetrating peptides, amphipathic peptides, drug delivery

1 Introduction

The term "cell-penetrating peptide" (CPP) was first used about 30 years ago, and originated from findings that a short cationic sequence in the HIV-1 Tat protein, termed Tat peptide, could carry the protein into the cytosol and nucleus of cells to transactivate reporter genes [1,2]. Since then, the list of CPPs has been expanded to over 200 sequences, including protein derived, synthetic sequences, or chimeric sequences containing a protein derived sequence fused to a nuclear localization sequence (NLS) (Table 1). The most attractive aspect of these small peptides is their capability to carry other molecules, regardless their molecular size, into intracellular compartments including the nucleus and cytoplasm [3,4]. Obviously, CPPs can be developed into carrier-mediated delivery systems for various types of drug from small molecules to proteins and genes [5–7]. CPP is one of many terms that have been used to describe peptides with transmembrane transport properties. It appears to be a more generally acceptable term than others, such as membrane transduction peptide (MTP) and protein transduction domain (PTD), which imply a specific type of transport process at the cell membrane. However, in a broad view, CPP represents a large diversity of peptides that possess different mechanisms, efficiency and intracellular targets in transmembrane transport [3,8–10]. The diversity of CPPs makes the comparison of transport mechanisms and application in drug delivery difficult. Therefore, a systematic classification of CPPs based on their molecular and transport characteristics is a useful way to enable selection of a certain CPP as a transport carrier to deliver a specific drug with well-defined molecular properties and intracellular target of action. In general, CPPs have been divided into 2 major types, i.e., cationic and amphipathic peptides. However, within each type, there are other subtypes which add further diversity of CPPs in their transport characteristics. In this review, we will separate CPPs into different classes based on more detailed chemical structures. We believe that such a classification will correlate more accurately with the mechanism and effectiveness of a CPP as a transport carrier in drug delivery. We will focus on the results recently obtained from *in vivo* studies to justify our classification of CPPs.

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2 Sequence, structure and membrane-binding of cationic and amphipathic CPPs

2.1 Cationic CPPs

Cationic peptides, including synthetic polylysine and natural protamines, have a long history as carriers for

Table 1 Examples of CPPs

Sequence	Peptide name	Origin	%[a)]	NC[b)]	Helical[c)]	Ref.
Protein derived CPPs						
GRKRKKRT	6-Oct	Transcription factor Oct-6	0%	+ 6	No	[11,12]
RRIRPRPPRLPRPRP	Bac-1-15	Bactenecin 7	13%	+ 7	No	[13]
KMDCRWRWKCCKK	Crot (27–39)	Rattlesnake venom (Crotamine)	46%	+ 5	No	[14]
RKKRRRESRKKRRRES	DPV3	Human superoxide dismutase	0%	+ 12	No	[15]
RRRRNRTRRNRRRVRGC	FHV coat (35–49)	RNA binding peptides	11%	+ 11	No	[16–18]
ASMWERVKSIIKSSLAAASNI	FHV γ peptide	Flock house virus	52%	+ 2	Yes (8 THR)	[19,20]
LGTYTQDFNKFHTFPQTAIGVGAP	hCT (9–32)	Human calcitonin (hCT)	33%	0	No	[21]
GGVCPKILKKCRRDSDCPGACICRGNGYCGSGSD	MCoTI-II(MCo)	*Momordica cochinchinensis* trypsin inhibitor II	32%	+ 3	No	[22]
VQRKRQKLMP	NF-kB	Transcription factor NF-kB	30%	+ 4	No	[12]
NAKTRRHERRRKLAIERGC	P22 N	RNA binding peptides	26%	+ 6	No	[16]
RQIKIWFQNRRMKWKK	Penetratin (Antp)	Antennapedia homeodomain of drosophila	37%	+ 7	Yes (3 THR)	[14,20,21, 23–33]
LLIILRRRIRKQAHAHSK	pVEC	Murine vascular endothelial-cadherin protein	44%	+ 6	Yes (4 THR)	[34]
PKKKRKV	SV40	Transcription factor SV40	14%	+ 5	No	[12]
RRLSYSRRRF	SynB3	Protegrin	30%	+ 5	No	[31]
GRKKRRQRRRPPQC	HIV-1 Tat (47–57)	HIV-1	7%	+ 8	No	[13–15,17, 21,29,33, 35–43]
VSRRRRRRGGRRRR	LMWP	Protamine	7%	+ 10	No	[44]
KAAPAKKAAAKKAPAKKAAAKK	HBHAc	*Mycobacterium tuberculosis*	44%	+ 10	No	[45]
Chimeric CPPs						
CHHHHHRKKRRQRRRHHHHHC	mTat	Tat + histidine	5%	+ 6	No	[46]
KETWWETWWTEWSQPKKKRKV	Pep-1	Trp rich cluster + SV40 NLS	28%	+ 3	Yes (5 THR)	[47]
KETWFETWFTEWSQPKKKRKV	Pep-2	Trp rich cluster + SV40 NLS	28%	+ 3	Yes (5 THR)	[48]
KWFETWFTEWPKKRK	Pep-3	Trp rich cluster + SV40 NLS	33%	+ 3	Yes (3 THR)	[48]
PKKKRKVALWKTLLKKVLKA	PV-S4(13)	SV40 NLS + dermaseptin S4 peptide	45%	+ 9	Yes (7 THR)	[40]
AAVALLPAVLLALLAPVQRKRQKLMP	SN50	Signal sequence of K-FGF + NLS of NF-kB p50 subunit	65%	+ 4	Yes (6 THR)	[49]
AGYLLGKINLKALAALAKKIL	Transportan; TP10	Fusion of neuropeptide galanin and wasp venom peptide	61%	+ 4	Yes (9 THR)	[26,29,50]
Synthetic CPPs						
WEAALAEALAEALAEHLAEALAEALEALAA	GALA	n/a	73%	−7	Yes (17 THR)	[51]
IRQRRRR	IRQ	n/a	29%	+ 5	No	[52]
WEAKLAKALAKALAKHLAKALAKALKACEA	KALA	n/a	66%	+ 5	Yes (16 THR)	[53]
KLALKLALKALKAALKLA	MAP	n/a	72%	+ 5	Yes (12 THR)	[28,54,55]
l-R_n, d-R_n (n = 5–16)	Oligoarginine	n/a	0%	+ n	No	[14,16,23, 29,30,35,41, 42,55–58]
RALARALARALRALAR	RALA	n/a	69%	+ 5	No	[59]

(*Continued*)

Sequence	Peptide name	Origin	%[a)]	NC[b)]	Helical[c)]	Ref.
Synthetic CPPs						
WRWRWRWRWRWRWR	RW	n/a	50%	+7	No	[18]
CVQWSLLRGYQPC	S41	n/a	46%	+1	No	[60]

a) %: %Hydrophobicity, determined as the percentage of hydrophobic amino acids/total amino acids
b) NC: Net charge
c) Helicity determined by the number of total hydrophobic residues (THR) on the same surface; Peptides with 3 or more THR may form α-helices

intracellular delivery of proteins [61–63], DNA [64,65] and small molecule drugs [66–68]. In the late 1980s, however, it was found that arginine and lysine-rich sequences in natural proteins were capable of carrying the protein into the cytosol or nucleus of cells, initiating the CPP field [1,2]. One of the first, and most well studied, CPPs is the Tat peptide [47–57] (YGRKKRRQRRR), which is the 11-amino acid sequence derived from the protein transduction domain in HIV-1 virus transcriptional factor [1,2]. The abundant arginine residues in Tat peptide subsequently prompted the investigation into various lengths of synthetic oligoarginine [23,42,43,58,69] (Table 1). It was suggested that, due to the bidentate hydrogen-bonding capacity, the guanidinium moieties in oligoarginines may provide a stronger binding to carboxylic, sulfate and phosphate groups on the surface of cell membranes (Fig. 1) [70]. The difference between oligoarginine and oligolysine on membrane translocation was observed in the early works on CPPs, where studies of the comparison of oligoarginine and oligolysine demonstrated the unique properties of the guanidinium group in arginine in proteoglycan binding, including both the affinity and the clustering of binding [71,72]. Many studies have suggested that the binding of CPPs to glucosaminoglycan moieties in proteoglycans is an important step for the delivery of the peptide into the cytoplasmic compartment in the cells [71,73]. It was further substantiated in later studies that both the cellular uptake and the intracellular localization of these 2 cationic oligopeptides were very different [42,74]. Using a subcellular fractionation method or by comparison of the internalization of 4 °C *versus* 37 °C, it was found that oligoarginine was localized in the cytoplasmic fraction while oligolysine was mainly vesicular (Table 2). Other arginine-rich CPPs, such as Tat peptide, behave similar to oligoarginine, rather than oligolysine (Table 2). Interestingly, when oligolysine was guanidinylated to convert the ε-amino group to guanidine (Fig. 1), the intracellular localization shifted from vesicular to cytosolic fraction (Table 2). Further, methylation of oligoarginine (Fig. 1), which maintains the positive charge but disrupts the bidentate binding ability, also results in reduction of the cytosolic localization (Table 2). Taken together, the inclusion of arginine, rather than simply cationic charge from a primary amino acid-containing side group such as lysine, in the CPP sequence has been shown to be important for access to the cytosolic compartment. This difference in intracellular localization may explain the different effectiveness when a biological active cargo was carried by oligolysine or oligoarginine [75], even though both oligopeptides are equally positively charged. Thus, arginine-rich, rather than lysine-rich, cationic CPPs are preferred as carriers for the delivery a biologically active molecule into the cytoplasmic compartment.

The sequence of cationic charges in a CPP sequence is mainly oriented in clusters of 3 or more arginine or lysine residues adjacent to each other, as in Tat peptide, 6-Oct, DVP peptides, SV40 protein derived CPPs and synthetic oligoarginine CPPs (Table 1). There are only a few examples where the cationic CPPs are distributed throughout the CPP sequence, such as in crotamine derived CPP

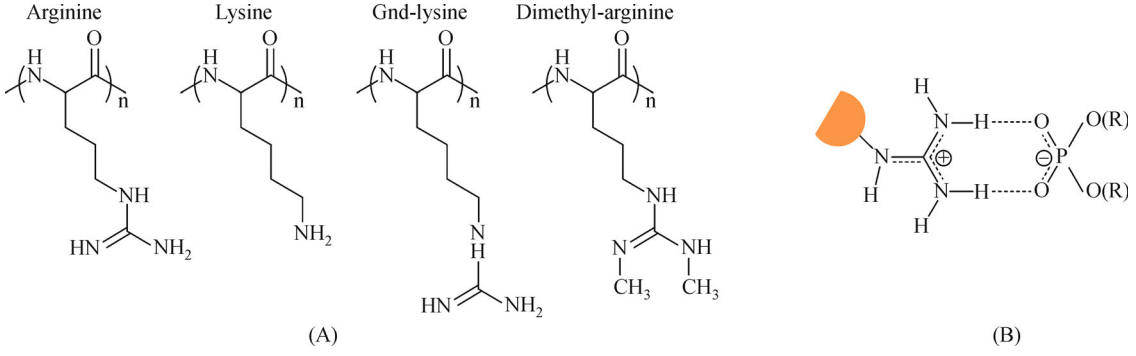

Fig. 1 Guanidine structure in CPPs. (A) Structural comparison of arginine and lysine side chains, along with guanidinated (Gnd)-lysine and methylated arginine; (B) Proposed hydrogen bonding of the guanidine group of arginine with phosphate groups present at the cell surface

Table 2 Comparison of CPPs for cytosolic *versus* vesicular accumulation

CPP	Direct translocation	Vesicular	Ref.
Antp	15%	85%	[74][b)]
$(RW)_9$	30%	70%	[74][b)]
Tat	75%	25%	[74][b)]
Tat	92%	8%	[42][c)]
$(R)_9$	50%	50%	[42][c)]
$(R)_9$	82%	18%	[42][c)]
$(K)_9$	21%	79%	[42][c)]
Gnd-$(K)_9$[a)]	53%	48%	[42][c)]
(Methylated-$R)_9$	n.d.[d)]	> 100%	(unpublished findings)
MAP	n.d.[d)]	> 100%	[55][c)]

a) 50% guanidine : amine modification
b) Calculated by comparing amount internalized at 4 °C *versus* 37 °C
c) Calculated by subcellular fractionation to separate the vesicular from cytosolic localization of internalized CPP
d) Not detectable

and MCoTI-II (Table 1). Previously, it has been shown that the cationic CPPs with a clustered orientation had higher internalization than those with a mixed orientation [76], which may be related to the internalization mechanism and membrane binding properties.

Membrane-binding is an important factor in determining the effectiveness of using a CPP as a drug carrier because it will determine the efficiency in transmembrane transport and the cytotoxicity of the delivery system. Membrane-binding can be considered as the first step in the transport of CPPs into the cells. The positive charge, which is the fundamental characteristic of most CPPs, is thought to provide an anchor force for CPPs to adhere to the negatively charged cell surface. Generally, the structure of oligoarginine and oligolysine in aqueous solution is in a random conformation [77–79]. As such, ionic interactions have been the primary focus for their binding to cell membranes. However, after careful analysis of the energy requirement for membrane binding, it was found that the force for the membrane-binding of a positively charged polypeptide was mostly hydrogen-bonding rather than ionic interactions [80]. The contribution from hydrogen-bonding was especially predominant in the case of oligoarginine compared to oligolysine, suggesting the important role of hydrogen-bonding in the superiority of oligoarginine in membrane transport [80].

It has long been reported that both polyarginine and polylysine can acquire an alpha-helical conformation upon complexation with heparin [71,81]. It has also been reported that polylysine-heparin complexes exhibited an unique membrane binding and transport mechanism which was different from polylysine in cell cultures [82]. In fact, it has been demonstrated that oligoarginine binds tightly with the heparan sulfate moiety in proteoglycans, with a cationic/anionic charge ratio close to 1 [81]. Therefore, it is likely that the binding of cationic CPPs to the sulfate groups in proteoglycan may maintain the peptide structure in an α-helical conformation. However, the effect of such conformation on the membrane binding and transmembrane transport of cationic CPPs is not known at the present time.

As will be further discussed in the next section, due to the negative charges in phospholipids, cationic oligoarginine and oligolysine can also interact directly with lipid bilayers [80], even though the binding to phospholipid is much lower than that to heparan sulfate proteoglycan [71]. It was demonstrated by using solid-state NMR method that, at physiological temperature, the binding of phosphoryl group in phospholipid to guanidinyl group in arginine is much stronger than to the ε-amino group in lysine [83]. This finding is consistent with the general observation that arginine, rather than lysine, plays an important role in the cross-membrane transport of cationic CPPs.

2.2 Amphipathic CPPs

While the arginine and lysine residues in most cationic CPPs are clustered together, they are evenly distributed throughout the sequence in a majority of amphipathic CPPs (Table 1). In addition to lysine and/or arginine residues, amphipathic sequences are also rich in hydrophobic residues such as valine, leucine, isoleucine, alanine, etc. Of all of the protein derived peptides, about half of them are amphipathic, where the other half is cationic. Interestingly however, almost all of the synthetic CPPs, as well as chimeric sequences which contain a protein derived sequence fused to a NLS, are amphipathic (Table 1). The binding of amphipathic CPPs to the lipid bilayers in cell membrane is very different from that of cationic CPPs. The strong hydrophobic interaction and the insertion into the lipid bilayer are observed in amphipathic, but not in

cationic, CPPs [80]. In fact, for primary amphipathic CPPs, CPPs with an α-helical conformation in aqueous solution, the binding of the hydrophobic region on a CPP molecule to the lipid surface of the cell membrane is considered as the major force of interaction [84]. In this case, the CPP molecule will insert into the lipid bilayer regardless the strength of the ionic interaction. In contrast to cationic CPPs which can be found in the cytosolic fractions, amphipathic CPPs tend to be associated with vesicles (Table 2) [55,74]. These findings can be related to the internalization mechanism, or due to their high affinity of the amphipathic CPPs for the lipid membranes. On the other hand, charge interaction is important for the membrane binding of secondary amphipathic CPPs, which are CPPs with a random conformation in aqueous solution but exhibit an α-helical conformation after binding to polyanionic molecules. These molecules contain several hydrophobic residues that are displayed on the same surface in a helical wheel, indicating their propensity to form helical structures. In most cases for secondary amphipathic CPPs, the hydrophobic cluster will only be formed when a CPP molecule complexes with anionic components on the cell surface, such as proteoglycans and phospholipids. Therefore, the insertion of a secondary amphipathic CPP into the lipid bilayer on the cell surface is dependent on the charge interaction, which makes this type of CPPs a hybrid between primary amphipathic and cationic CPPs. One of the most well studied and effective secondary amphipathic CPPs is penetratin (Table 1). Many of the unique properties of penetratin as an effective CPP are due to both the charge and amphipathicity of this peptide [85].

3 Cellular uptake pathways of CPPs in drug delivery

The lack of a clearly elucidated mechanism for the transport of CPPs across cell membrane is an indication that cellular uptake of CPPs involves more than one pathway. In general, there are two major pathways that are used to describe how a CPP can be transported into the cells, i.e., endocytosis and direct penetration through the plasma membrane (termed "membrane transduction") (Fig. 2) [39,86]. For example, macropinocytosis [87,88], micropinocytosis [89], and clathrin-mediated [38,73] and caveolae/lipid raft-mediated [90,91] endocytosis, have been proposed as the mechanism of CPP internalization. Although the formation and the intracellular processing of various endocytotic pathways are very different from each other, the main localization of the CPP following internalization is vesicular. The mechanism that can induce transmembrane transport, presumably resulting in the direct access of the CPP and its cargo into the cytosolic compartment, is even more diverse. Due to the differences in intracellular localization, the classification of CPPs into endocytosed or membrane transductive polypeptides is useful for the design of drug delivery systems to reach the desired intracellular compartment to achieve biological activity.

For CPP-conjugates that are internalized by endocytosis, one of the most critical factors in determining the effectiveness in drug delivery is the escape of either the intact CPP-conjugates or the drug cargo from the endosome to the cytosol [92]. The escape from endosomes has 2 implications, i.e., to avoid lysosomal degradation and

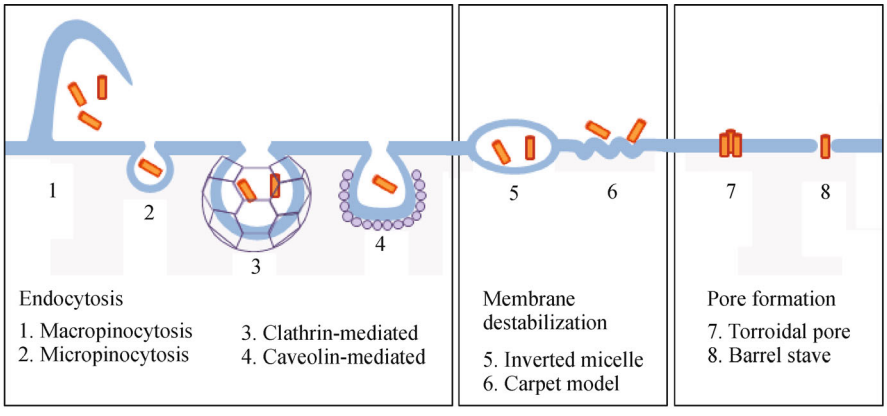

Fig. 2 Proposed mechanisms of internalization. Endocytic internalization mechanisms including (1) macropinocytosis, (2) micropinocytosis, (3) clathrin-mediated endocytosis, and (4) caveolar-mediated endocytosis have been shown to be involved in CPP uptake into cells. Several endocytosis-independent mechanisms have also been proposed, including membrane destabilization through (5) inverted micelle formation, or the (6) "carpet" model describing perturbation of the phospholipids to increase membrane fluidity; or pore formation through (7) membrane insertion following interaction with the anionic groups of the phospholipid membrane to form toroidal pores, or (8) formation of an α-helical structure within the membrane where hydrophilic side chains form the inner face of the port to form a barrel stave pore

to reach cytosolic target. Many CPPs can facilitate the transport of cargo across endosomal membrane by either endosomolysis [93], membrane fusion [94], or channel formation [95]. The linker between the cargo and CPP can also play an important role in the trans-endosomal membrane transport. For example, in cytochrome c-MAP conjugates, free cytochrome c was detected in cytosolic fraction only when the cargo is conjugated to MAP via a disulfide, but not thioether, linker [96]. In this case, MAP as a CPP is internalized by the cell via endocytotic pathway and is localized intracellularly in vesicular and nuclear, but not cytosolic, compartments [97]. The disulfide linkage can be reduced on the endosomal membrane to release the intact cargo, cytochrome c, into the cytosol [96].

The direct penetration, or "membrane transduction" models for CPP internalization involve strong binding of the CPP on the outer leaflet of the lipid bilayer, followed by entry through several different proposed mechanisms including membrane lysis through transient formation of pores or membrane destabilization (Fig. 2). In the proposed transient pore formation models, the CPPs insert into the membrane through interaction with the anionic groups of the phospholipid membrane ("toroidal pore"), or assume an α-helical structure within the membrane where their hydrophilic side chains form the inner face of the port ("barrel stave pore") [98–100]. The destabilization methods include the "inverted micelle" model [25,101], which postulates that the CPP remains in contact with the membrane surface during the entire translocation, and the "carpet" model where membrane fluidity is transiently increased by the association of the cationic peptide with the anionic cell surface [102]. The pore formation models, although proposed for both cationic and amphipathic CPPs, is generally more acceptable for the amphipathic CPPs, since it is difficult to explain how sequences lacking hydrophobic amino acids can interact with the lipid bilayer. The pore formation and the cytolytic activity of antimicrobial and cytolytic peptides, such as magainins [103–105] and melittin [106,107], has been studied extensively both experimentally and by molecular modulations. Although this type of internalization may be more efficient, as studies have shown that the internalization of amphipathic CPPs is generally much higher than cationic CPPs [55,108], it is also much more toxic [109,110]. Membrane destabilization models, on the other hand, have gained more interest for cationic CPPs lacking hydrophobic residues, since the CPPs remain associated with the polar groups of the lipid bilayer and do not contact its hydrophobic interior. Since these mechanisms do not lyse the membrane, they are considered to be less toxic, but also a less efficient pathway for cell entry. Taken together, support for all of the direct penetration models is mainly via *in vitro* studies showing that CPP internalization is energy independent, occurs at low temperatures that inhibit endocytosis, and/or occurs in the presence of various different types of agents that inhibit endocytosis. However, a majority of the mechanistic studies are done using artificial membranes, so it is not clear if the processes occur in live cells. Further, the membrane destabilization and formation of pores is generally thought to be transient, and therefore difficult to capture and measure.

Further complicating the understanding of the internalization mechanisms of CPPs is that most studies show that CPPs are internalized via multiple pathways (different types of endocytosis and/or membrane transduction) concurrently and may depend on several factors including the size and type of CPP, concentration used, and cell line [111–119]. A major component in the debate over the transport mechanism is the difficulty in quantitatively evaluating CPP internalization. The methodology used to study CPP transport relies on the measurement of the total intracellular internalization using a radioactive or fluorescent label, the use of confocal microscopy of live cells, or the downstream activity of a cargo molecule delivered to the cytosolic or nuclear site [117,120]. The major pitfall in the measurement of total internalization is the lack of separation of the cytosolic, vesicular, or nuclear localization. Since the CPPs are distributed to different extents in each of the compartments, measuring the total intracellular CPP does not distinguish each pathway independently. The intracellular localization of cell-internalized CPPs can be determined by the separation of the homogenized cells into organelles and cytosol fractions, with the calibration of the degree of organelle rupture during the homogenization [42,43,55]. As described in Section I, cationic CPPs with high content of arginine residues, such as oligoarginine and Tat, are mostly found in cytosolic fraction, while CPPs with amphipathic properties, such as MAP, are localized exclusively in vesicular compartments (Table 2). Similar results were obtained when the internalization of CPPs is compared at 4 °C, where endocytosis is inhibited, *versus* 37 °C. For example, it was found that the internalization the amphipathic CPP penetratin was mainly through the vesicular route (70% vesicular), while cationic CPPs Tat and oligoarginine were not (25% and 50% vesicular, respectively) (Table 2) [74]. However, when applied in drug delivery, the intracellular localization of the carrier CPP may not reflect the location of the drug molecules.

There are several reasons that a drug-CPP conjugate may differ from CPP in their transport pathway. First, the charge and size of the drug will affect the transport of the CPP in both membrane transduction and endocytosis. If the drug molecule is negatively charged under the physiological conditions, the positive charge of CPP will be neutralized. This charge neutralization effect is most obvious when a cargo of a macromolecule, such as oligoglutamic acid [121], is conjugated to a CPP. In fact, anionic oligopeptides have been successfully applied to prevent the non-specific uptake of CPPs when used as a drug carrier [76,122–126]. The charge neutralization is a concern when CPPs are used for highly negative charged

oligonucleotide delivery such as in gene and siRNA therapies, and generally the CPP in the conjugate or complex should be in excess [93,127,128]. When proteins are the macromolecular cargoes in CPP conjugates, the charge neutralization is usually not an issue. On the other hand, the size of a macromolecular cargo may not affect the internalization of CPP by endocytosis due to the size of early endosomes. However, macromolecular drugs can change the membrane transduction of the CPP significantly, not only by the size but also by the hydrophilicity of the cargo, which can interfere the insertion of peptide into lipid bilayers [4]. Small molecular drugs, including small peptides [75], can be transported predominately via membrane transduction when conjugated to an arginine-rich cationic CPP.

4 *In vivo* studies of CPP-mediated drug delivery

4.1 Small molecular drug conjugates

As shown in Table 3, most of the small molecules that have been conjugated to CPPs for *in vivo* studies were imaging markers such as fluorophores or PET tracers. The imaging studies provided not only a measurement of the biodistribution of the CPPs, but also the potential as diagnostic agents. There were very few successful studies on simple drug-CPP conjugates in therapeutic investigation because conventional small molecular drugs generally do not have problem in membrane transport. However, CPPs have been considered as carriers for drugs that are impermeable to cell membrane or desirable for targeted delivery in cancer treatment. Cationic oligopeptides, such as oligolysines and oligoarginines, have long been used to increase the cellular uptake of small impermeable molecules or to overcome the multiple drug resistance [68,129]. For small molecular drugs, the mechanism of the cellular uptake of a drug-peptide conjugate is not very critical if the linkage between the drug and CPP can be cleaved either in endosomal-lysosomal pathway or in cytosolic compartment. Therefore, oligoarginines or Tat-peptides are most commonly selected CPPs for small molecular drugs due to their easy accessibility and chemical conjugation. For the transport across blood-brain barrier, the hydrophobicity of a CPP, as shown in SynB3 for endomorphin-1 delivery [130], could be an important factor.

Conjugation of CPPs to small molecule drugs is generally made via the thiol of an incorporated cysteine residue, or to an epsilon- or α-amine in the sequence. Stable linkages such as maleimide [125,126,130,131,137] or amide [125,126,130–134] are commonly utilized (Fig. 3). More recently, click chemistry reactions, such as copper-catalyzed azide-alkyne 1,3-dipolar cycloadditions (CuAAC) are also being applied (Fig. 3) [139]. Alternatively, in order to ensure an intracellular release of the active drug, small molecular drugs are also conjugated to CPPs by either disulfide linker [138] or acid-sensitive

Table 3 CPP-small molecule conjugates for *in vivo* delivery[a)]

CPP	% Φ	NC	Helical[b)]	Drug /cargo	Studies	Animal model	Ref.
SynB3	30%	+ 5	No	Endomorphin-1	Brain delivery; analgesic effect	Mouse	[130]
R_9-linker-E_9 (linker: cleavable by MMP2/9)	0%	+ 9	No	Cy5/Cy7	Tumor targeting	Mouse (HT-1080 xenograft)	[125]
R_9-linker-E_9 (linker: cleavable by MMP2/9)	0%	+ 9	No	Cy5/Cy7	Tumor targeting	Mouse (orthotopic HNSCC xenograft, tongue)	[131]
R_{11}	0%	+ 11	No	FITC	Brain delivery	Mouse	[132]
R_8-linker-E_8 (linker: cleavable by MMP2)	0%	+ 9	No	^{125}I/^{177}Lu-DOTA	Infarcted myocardium uptake	Mouse model of myocardial infarction	[133]
hCT(9-32)				^{68}Ga-DOTA	Biodistribution; metabolism	Mouse	[134]
^{14}C-R8	0%	+ 8	No	n/a	Biodistribution; metabolism	Rat	[135]
Tat	7%	+ 8	No	Porphyrin	Tumor accumulation	Mouse (PC-3M xenograft)	[136]
R_8-linker-E_8 (linker: cleavable by MMP2)	0%	+ 8	No	Cy5	Detection protease activity	Mouse (asthma model)	[137]
R_9-linker-E_9 (linker: H_2O_2-cleavable small molecule)	0%	+ 9	No	Fluorescein/Cy5	Imagining of inflammation	LPS mouse model of lung inflammation	[126]
TAT, R_{11}	7%, 0%	+ 8, + 11	No	Sodium undecahydro-mercaptocloso-dodecaborate	Tumor reduction via BNCT	Glioma-bearing mouse	[138]

a) BNCT: boron neutron capture therapy; Cy5: cyanine fluorescent dye no. 5; Cy7: cyanine fluorescent dye no. 7; DOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; Ga: gallium; H_2O_2: hydrogen peroxide; Lu: lutetium; MMP2/9: matrix metalloprotease 2/9; NC: Net charge; Φ: %Hydrophobicity, determined as the percentage of hydrophobic amino acids/total amino acids

b) Helicity determined by the number of total hydrophobic residues (THR) on the same surface; Peptides with 3 or more THR may form α-helices

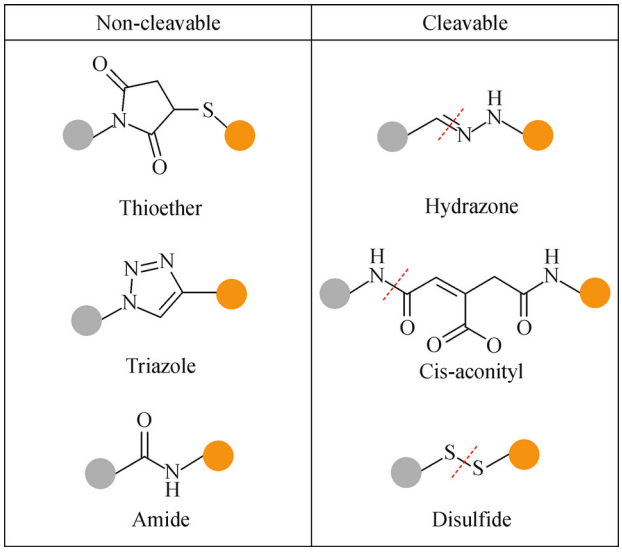

Fig. 3 Examples of linkers in small molecule-CPP conjugates. Stable linkers for small-molecule CPPs include the thioether linkage, which is achieved utilizing a maleimido-reactive group, the triazole linker by use of copper-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) click chemistry, and the amide linkage using carbodiimides and/or *N*-hydroxysuccinimide esters. Cleavable linkers include those cleaved in acidic pH such as the hydrazone linker, obtained by reaction of an aldehyde with a hydrazide, and the cis-aconityl linker, obtained by reaction of citraconic anhydride with an amine. Disulfide linkers formed between two thiols, which are cleaved under reducing conditions are also used

linker [140,141] (Fig. 3). Since the release of free drug from the conjugate is independent of the degradation of CPPs, there may be advantages of using D-form of CPP that is resistant to proteolytic degradation and can increase the stability and prolong the accumulation inside the target cells [41,142].

The lack of specific uptake by tumor cells is the major concern in the application of CPP as a simple drug carrier in cancer treatment [143–145]. Most CPPs show a non-specific biodistribution in highly fenestrated organs such as liver and spleen, possibly due to the charge and size of the oligopeptides [146]. In many cases, this non-specific distribution can over-power the active targeting recognition. For example, when conjugated with a tumor specific antibody [147], or antibody fragment [148], CPP can reduce the selective binding to the tumor antigen and decrease the tumor localization. Therefore, a drug-CPP conjugate may not achieve any therapeutic advantages, unless the non-specific biodistribution due to the cationic charge can be blocked by different masking mechanisms, such as charge neutralization by an anionic oligopeptide [76,122–126]. The targeted delivery effect of the masked CPP will rely on the selective reactivation of the CPP at the target site. For example, targeted delivery of CPPs has been demonstrated if the removal of the masking anionic oligopeptide can be achieved by either the presence of specific proteases [149,150] or the mild-acidic microenvironment [151–153] within the microenvironment inside a solid tumor. For example, an activatable CPP, R_n-linker-E_n (n = 8−9), originally developed by Tsien et al. [124,125,131,137,154,155] showed the cell binding and internalization of oligoarginine were masked by the oligoglutamic acid while the linker was intact. Following selective cleavage of the linker, the cell penetrating properties oligoarginine were revealed at the target site (Fig. 4(A)). Based on these studies and others, the use of enzyme specific activation of the R_9/E_9 complexes has been demonstrated as a promising approach to enhance the *in vivo* application of CPPs. There have been several studies, however, that have shown that the enzymatic activation *in vivo* is not tumor selective and may also occur in the vascular compartment [156]. An alternate approach utilizes a pH-sensitive masking peptide containing histidine-glutamic acid repeats ("$(HE)_n$") fused to a cationic or amphipathic CPP (oligoarginine or model amphipathic peptide, respectively) [122,123,157]. In this design, the $(HE)_n$ sequence is net-negative at physiological pH 7.4, thus masking the cationic charge of the CPP sequence. However, at mildly acidic pH < 7.0, the protonation of the histidine residues neutralizes the masking sequence to reveal the cationic charge of the CPP (Fig. 4(B)). The CPP along with its fused cargo (i.e., small molecule drug or diagnostic imaging agent, peptide or protein drug) can then exert its high binding and internalization directly at the mildly acidic site.

4.2 Proteins and peptides

One of the major hurdles in the development of protein and peptide drugs is the poor absorption across biological barriers due to the size and hydrophilicity [160]. Conceivably, CPP technology is an ideal approach to

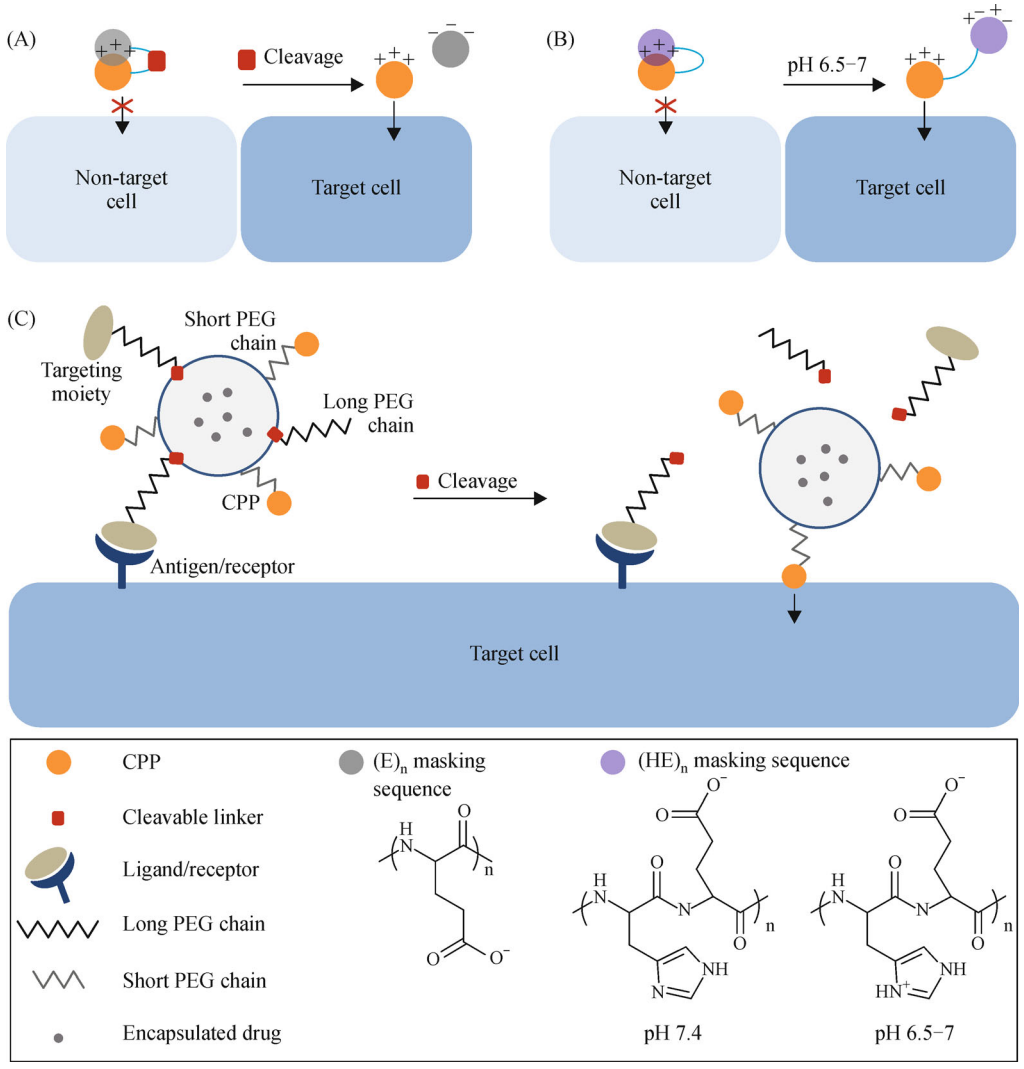

Fig. 4 Examples of masking strategies to target CPP-drug delivery systems. Due to their high cationic charge, CPPs are non-specifically internalized in many types of cells and tissues. This non-specific distribution can overpower the targeting recognition when combined with other active targeting approaches. One approach in overcoming this issue is by shielding the charge of the CPP until the complex reaches the target site. (A) In this example, the cationic charge of oligoarginine was masked by linkage to oligoglutamic acid through a protease-sensitive peptide sequence [124,125,131,137,154,155], or a hydrogen peroxide sensitive group [126]. Upon cleavage, the oligoarginine and oligoglutamic acid dissociate, revealing the cationic charge of oligoarginine. (B) Alternatively, the cationic charge of the CPP was masked by linkage to a histidine-glutamic acid copolymer sequence [122,123,157]. At physiological pH 7.4, the histidine is neutral while the glutamic acid is negative; therefore the net anionic copolymer masks the cationic charge of the CPP. With cationic histidine and anionic glutamic acid at mildly acidic pH 6.5–7, the copolymer net charge is neutral, unmasking cationic charge of the CPP. (C) In this example, the small chain PEG-CPPs are linked to lipids in a liposome via a stable linkage, and also targeted by conjugation with a long chain PEG-antibody via a cleavable linker (e.g., protease sensitive [158] or acidic pH-labile [159]). In this design, the CPP conjugated to a small PEG chain is masked by the longer PEG chain linked to a targeting agent. Once the liposomes accumulate near the surface of the target site, the targeting agent-long PEG chain conjugate is cleaved, revealing the CPP to drive the internalization

overcome the absorption limit. Proteins or peptides can be linked to CPP by chemical conjugation methods. In fact, chemical conjugation with a CPP carrier has been demonstrated to increase the pulmonary absorption of insulin *in vivo* [41]. There are also promising *in vivo* and *in situ* studies on the use of Tat peptide and low molecular weight protamine to deliver insulin across intestinal epithelia [161–163].

However, chemical conjugation of a protein with a CPP most likely will produce a heterogeneous product. Therefore, recombinant technology to fuse a protein drug with a CPP domain is a common technology to prepare a fusion product with one protein drug domain and one CPP domain. When constructing a CPP-fused recombinant protein, several factors have to be considered. First, the order of CPP and protein in the fusion protein may be important for the expression efficiency. Generally, the protein domain can be located in either end of the CPP

sequence. However, it has been observed in *E. coli* expression system that, in the preparation of fusion proteins with MAP, an amphipathic CPP, the MAP domain must be placed after the protein. Fusion proteins with MAP at the *N*-termini could not be expressed, possibly due to the cytotoxicity of MAP [164]. Another factor to be considered for the construction of CPP-fusion proteins is the interference of charges and hydrophobicity of CPP on the conformation or bioactivity of the protein drug. Therefore, a proper linker between the CPP and the protein drug may be required in order to achieve a highly active fusion protein for *in vivo* studies [165].

Due to the large molecular size, proteins either conjugated or fused with CPP are internalized mostly by endocytotic pathway regardless the CPP properties. Therefore, a mechanism allowing protein and peptide drugs to escape the endosomes and reach intracellular target sites is an important factor for the biological activity of the conjugate or fusion proteins. This factor has already been discussed in Section III. Therefore, it is not surprising that more than 30% of all CPPs in fusion proteins with the successful *in vivo* delivery, as shown in Table 4, are hydrophobic or amphipathic in nature. If Tat peptide, a prototype of CPP, will be removed from Table 4, the majority of CPP in the fusion protein production are amphipathic, i.e., penetratin and Pep-1, which possess the endosomolytic activity [8,166].

4.3 Non-covalent complex formation

Due to the high positive charge and, in some cases, hydrophobicity, CPPs can form stable complex with other macromolecules, including polypeptides and polynucleotides. A majority of the non-covalently linked complexes for CPP-mediated drug delivery *in vivo* use negatively charged DNA or siRNA along with mainly cationic CPPs, however there are a few examples of CPP-protein complexes as well (Table 5).

CPPs are capable of condensing DNA or RNA to form polyplexes [93,128]. Despite their extensive evaluation *in vitro*, there are few examples of their application for *in vivo* delivery. The main hindrances in the successful application of CPP-DNA/RNA complexes are (i) low stability of the polyplexes *in vivo* [188] (ii) the low efficiency of transport to the nucleus (DNA polyplexes) or cytosol (siRNA polyplexes) [188], (iii) the charge neutralization of the CPP which reduces its cell internalization properties [76]. Therefore, simply mixing CPPs and DNA/RNA is not sufficient for successful drug delivery, and many of the CPP-polyplexes consist of multiple components with multifunctional properties. In early works, a large excess of CPP was used to maintain a ratio of multiple CPP to each polynucleotide in the complex formation [93,127,128]. However, this type of complex may still exhibit a poor stability in the plasma. In fact, many early studies of siRNA-CPP complex were carried out in cell cultures with serum-free medium [189], which would be difficult to translate into *in vivo* application. In order to avoid the requirement of a large excess of CPP in the complex, one approach was to form a polyplex of siRNA from a non-CPP polycations, e.g., oligolysines, with a defined number of grafted CPP (Fig. 5) [190]. The advantages of this type of complex are (i) a molar charge ratio of 1 : 1 between the phosphate group in siRNA and amino-group in oligolysine can be achieved so that the charge on CPP will not be neutralized, (ii) the number of grafted CPP can be determined to provide efficient cellular uptake with less toxicity, and (iii) oligolysine is readily digested by cellular proteases and thus a naked siRNA can be released inside the cell to deplete specific mRNA. Like protein-CPP fusion protein, siRNA and CPP complex is internalized by mammalian cells mostly through the process of endocytosis. The complex must be able to escape from endosome in order to reach its target, i.e., cytosolic compartment. Therefore, CPPs with amphipathic properties, such as penetratin or MAP, showed a much better functional uptake by the cell, presumably due to the endosomolytic activity that allowed the siRNA escaped from endosomes. CPP-mediated cellular uptake of oligonucleotide is currently one of the most promising approaches in siRNA delivery [191]. In another approach by Yang et al., multifunctional polyplexes included a cationic polymer to aid in DNA condensation, polyethylene glycol (PEG) and poly(γ-glutamic acid) for stabilization, a histidine-modified Tat CPP to increase uptake, and a nuclear localization sequence to improve nuclear transport [46]. This delivery platform was able to achieve *in vivo* Luciferase gene transfection efficiency in Balb/c mice.

The examples of non-covalent CPP complexes with peptide drugs tested *in vivo* are aimed at improving their oral delivery. Oral delivery of protein or peptide drugs remains an unanswered challenge, mainly due to the poor absorption across the intestinal epithelium and the rapid proteolytic degradation of proteins in the gastrointestinal track [192]. The ability of CPPs to increase the oral bioavailability of peptide drugs has been tested *in vivo* following co-mixing of the CPP and peptide cargos. In a study by Nielsen et al., an orally dosed mixture of insulin and the CPP penetratin in mice exhibited a blood glucose lowering effect [186]. The study showed that, following non-covalent mixing of the protein and CPP, hypocalcemic and blood glucose lowering effects could be achieved. The increased bioavailability was attributed to both the increased stability in the GI track, as evidenced by the increased half-life of the CPP/INS mixture compared to INS alone, and the enhanced absorption across the intestinal epithelium via the CPP.

4.4 Nano particles

For the application of CPP to nanoparticle delivery, the major goal is to increase the uptake by target cells.

Table 4 CPP-fusion proteins for *in vivo* delivery[a)]

CPP	% Φ	NC	Helical[b)]	Drug /cargo	Delivery system	Studies	Animal model	Ref.
Tat	7%	+ 8	No	Gelonin	Tat-gelonin + CEA-heparin complex	Tumor targeting & reduction	Mouse (LS174T xenograft)	[167]
Tat	7%	+ 8	No	HaFGF	Fusion protein	Intranasal delivery	Rat	[168]
R_8-linker-E_8 (linker: cleavable by MMP2)	0%	+ 8	No	MMAE	Fusion protein with integrin targeting cyclic peptide	Tumor regression	Mouse (mDA-MB-231 xenograft)	[154]
Tat	7%	+ 8	No	β-galactosidase	Fusion protein	PK; tissue distribution	Mouse	[169]
Tat	7%	+ 8	No	BCL6 peptide inhibitor	Fusion protein	Tissue penetration; tumor regression	Mouse (DLBCL xenograft)	[170]
Penetratin	37%	+ 7	Yes	p53(17−26)	Fusion protein	Tumor regression	Mouse (TUC-3 xenograft)	[171]
Tat	7%	+ 8	No	$[(KLAKLAK)_2$-DEVD$]_3$	Fusion protein	Apoptosis at tumor site	Mouse (B16-F10 xenograft)	[172]
Tat	7%	+ 8	No	AHPN	Fusion protein	Tumor growth inhibition	Mouse (435.eB xenografts)	[173]
Penetratin	37%	+ 7	Yes	NBD peptide	Fusion protein	Anti-inflammatory effect	Colitis-induced mouse model	[174]
Penetratin	37%	+ 7	Yes	NBD peptide	Fusion protein	Prevention of nigrostriatal degeneration	Parkinson's disease mouse model	[175]
Tat	7%	+ 8	No	BH4 peptide	Fusion protein	Immunosupression	Mouse sepsis model	[176]
Tat	7%	+ 8	No	STAT-6-IP	Fusion protein	Inhibition of lung inflammation	Allergic rhinitis and asthma mouse model	[177]
d-R9	0%	+ 9	No	Insulin	Fusion protein	Decreased blood glucose levels	Diabetic rat model	[41]
$(HE)_{10}$-MAP-GST	72%	+ 5	Yes	GST	Fusion protein	Tumor targeting	Mouse (MDA-MB-231 xenograft)	[122]
Bac	13%	+ 7	No	p21 peptide	ELP-fusion protein	Tumor targeting; survival time	Mouse (S2013 xenograft)	[178]
d-Tat	7%	+ 8	No	AlexaFluor-488 (fluorophore), QSY7 (quencher)	Fusion peptide with activatable probe containing DEVD sequence	Imaging of caspase activity	Rat	[179]
Pep-1	28%	+ 3	Yes	HO-1	Fusion protein	Prevention of intestinal ischemia	Rat	[180]
Pep-1	28%	+ 3	Yes	HO-1	Fusion protein	Reduced myocardial infarct size	Ischemic rats	[181]

a) AHNP: anti-HER-2/*neu* peptide mimetic; BCL6: B-cell lymphoma 6 protein; BH4: Bcl-2 homology domain 4; CEA: anti-carcinoembryonic antigen monoclonal antibody; DEVD: caspase recognition sequence; ELP: elastin-like polypeptide; GST: glutathione S-transferase; HaFGF: human acidic fibroblast growth factor; HO-1: Heme oxygenase-1; MMAE: monomethyl auristatin E; MMP2: matrix metalloprotease 2; NBD: NC: Net charge; NF-kB essential modulator (NEMO)-binding domain; PK: pharmacokinetics; STAT-6-IP: STAT-6 inhibitory peptide; Φ: %Hydrophobicity, determined as the percentage of hydrophobic amino acids/total amino acids
b) Helicity determined by the number of total hydrophobic residues (THR) on the same surface; Peptides with 3 or more THR may form α-helices

Therefore, it is not surprising that all of the *in vivo* studies as listed in Table 6 used highly cationic CPP as a transport enhancer. Another issue in conjugation of CPP to the surface of nanoparticle is that it should not interfere with the stability of nanoparticle, especially self-assembled nanoparticles. This could be a concern when hydrophobic and amphipathic CPPs will be used for the surface modification of nanoparticles because the self-assembly of many nanoparticles is mediated by hydrophobic interaction.

CPP-based nanoparticles mainly involve the delivery of liposomes, but also include quantum dots (QDs), polymeric nanoparticles composed of poly(lactic-co-glycolic acid) (PLGA), polycaprolactic acid (PLA), or polyethylene glycol (PEG), and gold or silver nanoparticles. For liposomal delivery, the majority of the applications combine CPPs along with other stabilizers (e.g., PEG, chitosan, PLGA), or targeting agents (e.g., integrin, transferrin, angiopep-2) into the delivery system. There are several examples of utilizing these multifunctional liposomes as targeted carriers, where the lipids of the liposomes are fused to a CPP-small PEG chain conjugate

Table 5 Non-covalent CPP-complexes for *in vivo* delivery[a)]

CPP	% Φ	NC	Helical[b)]	Drug /cargo	Other components	Studies	Animal model	Ref.
RALA	66%	+ 5	Yes	pDNA	n/a	Gene expression	Mouse	[182]
R_8	0%	+ 8	No	P53 pDNA, AVPI, and Dox	n/a	Gene expression; tumor regression	Mouse (MCF7 xenograft)	[183]
Mannose-Tat-PEI1800 + DNA	7%	+ 8	No	DNA	Mannose-CPP-PEI conjugate complexed with DNA	Dendritic cell targeting following transdermal microneedle delivery	Mice	[184]
mTat, Importin NLS				DNA	DNA complex with PPMS, PEG, PGA	Gene transfection efficiency	Mouse	[46]
R9-linker-PEG (linker: cleavable by MMP2)	0%	+ 9	No	siRNA	n/a	Tumor targeting & regression	Mouse (orthotopic MDA-MB-231 xenograft)	[185]
Penetratin	37%	+ 7	Yes	Insulin	n/a	Oral delivery	Mouse	[186]
Tat	7%	+ 8	No	sCT	Mixture with poliovirus coat protein peptide	Oral delivery	Rat	[187]

a) AVPI: apoptotic peptide; DNA: deoxyribonucleic acid; Dox: doxorubicin; FITC: fluorescein isothiocyanate; MMP2/9: matrix metalloprotease 2/9; NC: Net charge; PEG: polyethylene glycol; PGA: polyglutamic acid; PEI: poly(ethyleneimine); PPMS: polyamine-coester; sCT: salmon calcitonin. Φ: %Hydrophobicity, determined as the percentage of hydrophobic amino acids/total amino acids

b) Helicity determined by the number of total hydrophobic residues (THR) on the same surface; Peptides with 3 or more THR may form α-helices

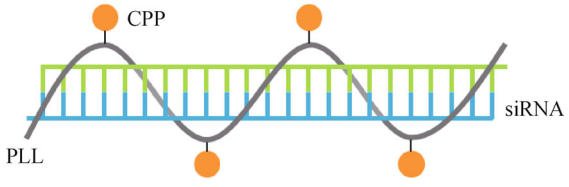

Fig. 5 Poly-L-lysine (PLL)-CPP siRNA Polyplexes. In order to overcome the issue of charge neutralization of the CPP upon complexation with siRNA, a multi-component polyplex consisting of siRNA, 21mer PLL modified to incorporate CPP conjugation sites, and a CPP was designed. PLL was first reacted with the amine-to-thiol crosslinker, *N*-succinimidyl 3-(2-pyridyldithio) propionate to form pyridyldithiol (PDP)-activated PLL, and then complexed with siRNA to form a neutral polyplex. Cysteine-terminal CPPs were then conjugated to the siRNA-polyplex via a reducible disulfide bond in order to allow for separation of the carrier CPPs from the PLL-siRNA polyplex following access to the reductive cytosolic compartment [190]

via a stable linkage, and also to a targeting agent-long PEG chain conjugate via a linkage that is cleavable (e.g., protease sensitive [158], acidic pH-labile [159]) (Fig. 4 (C)). In this design, the CPP conjugated to a small PEG chain is masked by the longer PEG chain linked to a targeting agent. Once the liposomes accumulate near the surface of the target site, the targeting agent-long PEG chain conjugate is cleaved, revealing the CPP to drive the internalization (Fig. 3).

Although there are not yet many examples of their *in vivo* use, QD have been extensively studied *in vivo* due to their value in serving as an alternative to traditional fluorescent dyes for animal imaging assays. QDs are 1–6 nm sized nanoparticles exhibiting broad absorption spectra with high quantum yields, narrow emission peaks and resistance to photobleaching. QDs can also be surface functionalized by a variety of reactive moieties, enabling simple conjugation with CPPs [193].

Similar to CPP-RNA/DNA complexes, CPP-nanoparticles are mainly internalized via endocytosis due to their large size. Therefore, one of the main concerns with CPP-mediated nanoparticle delivery is escape from the endosome. Several groups are working on strategies to incorporate an endosome-disrupting component into the nanoparticle delivery system. In these examples, endosomolytic agents such as sucrose and chloroquine, or fusogenic peptides such as melittin and GALA which form pores in the mildly acidic endosomal environment, are incorporated into the design of the nanocarrier systems in order to improve endosomal escape [194].

5 Summary and future perspective

Over the past few decades, CPPs have grown into an important field for drug delivery applications to carry molecules into the cytosol or nucleus of cells. Due to the large number of CPPs, diversity of their sequences and differences in internalization mechanisms, there is no systematic approach in selecting a specific CPP for a specific drug. Depending on the intracellular site of action for a bioactive drug, different CPPs may be more optimal carriers to achieve biological activity. Cationic CPPs such as oligoarginine and Tat generally have lower internalization efficiency than amphipathic CPPs such as penetratin or MAP. However, cationic CPPs are generally favored over amphipathic CPPs due to their lower toxicity. For most *in vivo* applications including small molecular drugs, non-covalent complexes, and nanoparticles, the CPPs

Table 6 CPP-modified Nanoparticles for *in vivo* delivery[a)]

CPP	% Φ	NC[b)]	Helical	Drug/cargo	Nanoparticle	Studies	Animal model	Ref.
R_9-linker-E_8 (linker: cleavable by uPA)	0%	+9	No	PTX	PLA-PEG	Tumor targeting & reduction	Glioma-bearing mouse	[195]
R_8-linker-E_8 (linker: cleavable by MMP2)	0%	+8	No	Coumarin-6	LRP1 coated PEG-PCL	Tumor targeting	Mouse (U87 xenograft)	[196]
IRQ				sCT	SLN, CSK-targeted	Oral delivery	Rat	[197]
R_8	0%	+8	No	sCT	Chitosan-modified liposome	Oral delivery	Rat	[198]
R_8-linker-E_8 (linker: cleavable by MMP2)	0%	+8	No	Dox	Angiopep-2 targeted PEG-PCL	Tumor targeting & regression	Glioma-bearing mouse	[199]
Tat	7%	+8	No	Dox	Disulfide-linked PEG-liposome	Tumor targeting & regression	Mouse xenograft	[200]
Tat	7%	+8	No	Dox	Transferrin-liposome	Tumor targeting & regression	Glioma-bearing mice	[201]
R_8	0%	+8	No	PTX	Integrin-targeted liposome	Tumor targeting; survival time	Glioma-bearing mice	[202]
Tat	7%	+8	No	DiD dye	Transferrin and cholesterol-PEG modified liposome	Tumor targeting	Mouse (HepG2 xenograft)	[203]
R_{11}	0%	+11	No	p53 gene	Ad5	Tumor regression	Mouse (EH-GB2 xenograft)	[204]
R_8	0%	+8	No	Dox	PEG-modified liposome	Tumor regression	Mouse (C26 xenograft)	[205]
Penetratin	37%	+7	Yes	Dox	NGR-targeted thermosensitive Liposome	*In vivo* tumor regression	Mouse (HT-1080 xenograft)	[200]
R_8	0%	+8	No	Dox	HPMA copolymer with CPP-polyanion (heparin sulfate, hyaluronic acid, fucoidan, polyglutamic acid)	Survival time	Mouse (B16-F10 xenograft)	[206]
R_7	0%	+7	No	Vincristine sulfate	PLGA-PEG folate conjugate	Tumor regression	Mouse (MCF7 xenograft)	[207]
Tat	7%	+8	No	Dox	PEG-PE-linked to liposome via Hz-linker	Tumor regression	Mouse (SKOV-3 xenograft)	[159]
R_8	0%	+8	No	α-galactosyl-ceramide	Liposomes	Improved immune response	Mouse	[208]

a) Ad5: adenovirus type 5; CSK: C-Src kinase; DiD: dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine-4-chlorobenzenesulfonate; Dox: doxorubicin; HPMA: *N*-(2-Hysroxypropyl) methacrylamide; Hz: hydrazone pH sensitive linker; LRP1: low density lipoprotein receptor-related protein; MMP2: matrix metalloprotease 2; NC: Net charge; NGR peptide: asparagine-glycine-arginine; targets vascular antigen aminopeptidase N; PCL: polycaprolactic acid; PE: phosphatidylethanolamine; PEG: polyethylene glycol; PLA: polylactic acid; PLGA: poly(lactic-co-glycolic acid); PTX: paclitaxel; sCT: salmon calcitonin; SLN: solid lipid nanoparticle; uPA: urokinase-type plasminogen activator; % Φ: %Hydrophobicity, determined as the percentage of hydrophobic amino acids/total amino acids
b) Helicity determined by the number of total hydrophobic residues (THR) on the same surface; Peptides with 3 or more THR may form α-helices

utilized are cationic CPPs. In the case of small molecules, the size of the cargo is not as likely to reduce the internalization efficiency of the CPP, and therefore the less efficient cationic CPPs are sufficient to obtain bioactivity. Non-covalent complexes with anionic DNA or RNA rely heavily on charge-charge interactions, and therefore the cationic CPPs are also predominantly utilized as carriers for these types of macromolecules as well. However, care must be taken in these applications to ensure the net cationic charge of the CPP is not completely neutralized. For nanoparticle applications, the design allow for attachment of many CPPs to the surface. Therefore, the lower efficiency can be overcome by increasing the number of CPPs per nanoparticle. For proteins and peptide drugs, however, the majority of CPPs utilized have some hydrophobic components incorporated. In order to retain

biological activity, there is usually a limited number of modification sites allowed on a protein or peptide. Therefore, recombinant fusion protein technique, rather than the chemical linkage, is generally applied for the preparation of protein-CPP conjugates. CPP fusion proteins are mostly internalized by endocytosis process and their escape from endosomes is critical for reaching the cytoplasmic target. Therefore, due to the higher internalization efficiency and endosomolytic activity, the amphipathic CPPs are generally necessary to attain sufficient bioactivity of protein-CPP fusion proteins.

In this review, we focus on the selection of different classes of CPP as effective carriers for *in vivo* delivery of various types of drug cargoes. The selection of appropriate CPPs is important for the increase of cellular uptake and intracellular delivery of specific types of drug molecules, and is the first step in the design of an effective CPP-mediated delivery system. However, there are other factors that can also influence the success of using CPPs as *in vivo* drug carriers. One of the challenges is to increase the localization of CPP-targeted drug at the disease tissues, for example at tumor sites for anti-cancer therapeutics. Most, if not all, CPPs have non-selective biodistribution and are preferentially taken up by tissues with high permeability, such as liver and spleen. The non-selective tissue distribution can override other active targeting action, such as antigen- or receptor-mediated drug delivery. Several approaches have been proposed for the design of tumor-selective CPPs, as we have discussed in Section 4.1 of this review. Even though the effectiveness of those targeting strategies in cancer therapy is still yet to be demonstrated, these recent studies have indicated the feasibility of tumor-targeted localization of CPP in animal models (see Section 4.1). Therefore, with the combination of the selection of an appropriate CPP for the drug and a design of an effective mechanism for tumor targeting, it can be anticipated that a CPP-mediated drug delivery in cancer treatment with clinical applications will be achieved in the near future.

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