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Review

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Therapeutic delivery using cell-penetrating peptides

Abstract: Intracellular delivery of promising therapeutic agents as well as nanocarriers presents a unique challenge. However, with the discovery of the cell-penetrating peptides (CPPs), overcoming this obstacle seems more plausible. In many cases, CPPs conjugated with therapeutic agent or therapeutic agent loaded-nanoparticles have shown promising results via increased cellular uptake. In this review, the current status of CPPs for the intracellular delivery of not just potential therapeutic small molecules but also large molecules like peptides, nucleic acids and nanocarriers is discussed. In addition, the design of 'smart stimuli-sensitive nanocarrier' to overcome the non-target-specificity of CPPs is also described.

Keywords: cell penetrating peptides; nanocarrier; nucleic acids; stimuli-sensitive liposomes.

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CPPs: Tools for crossing the cell membrane and molecular mechanism

The cell membrane, a fundamental necessity for a cell, presents a challenge for intracellular delivery for many therapeutic molecules. Most therapeutic molecules, whether small or large, more often, have their molecular target located intracellularly. Although such molecules, like proteins, peptides, nucleic acids and various kinds of nanocarriers hold greater promise for improved therapeutic effects, their intracellular delivery remains challenging.

During the past 20 years, a new family of short peptides that readily transport across the biological membrane, known as cell-penetrating peptides (CPPs) has emerged. This discovery has been regarded as a potentially important step in the development of novel strategies to increase the intracellular availability of molecules of high therapeutic interest but low membrane permeability, including peptides, proteins and nucleic acids.

Classification of CPPs

Based on their origin, CPPs can be divided into three classes: protein-derived peptides, model peptides and designed peptides (1, 2). Protein-derived peptides such as transactivator of transcription (TAT) (3) and penetratin (4), also referred to as protein transduction domains (PTDs), are the short stretches of the protein domain that are primarily responsible for their translocation ability. Model peptides mimic the translocation properties of known CPPs such as model amphipathic peptide (MAP) (5). Designed CPPs are produced by the fusion of hydrophilic and hydrophobic domains from different sources. These include transportan (a fusion of galanin and mastoparan) (6), MPG (the chimeric peptide from the fusion sequence of HIV-1 glycoprotein (gp)41 protein and peptide from the nuclear localization sequence (NLS) of simian vacuolating virus (SV)40 T-antigen) (7). Synthetic peptides such as polyarginines also show potential for translocation (8).

Based on the peptide sequences and binding properties to the lipids, CPPs can also be divided into three classes: primary amphiphatic, secondary amphiphatic and non-amphiphatic CPPs (9). The primary amphiphatic CPPs include transportan (6) or TP10 (10) and contain more than 20 amino acids. They have both hydrophobic and hydrophilic residues along their primary structure (9). Secondary amphiphatic CPPs such as penetratin (4), vascular endothelial-cadherin (pVEC) (11) and M918 (12) contain smaller number of amino acids compared to primary amphiphatic CPPs and their amphiphatic property is revealed when they form an alpha-helix or beta sheet structure upon interaction with a phospholipid membrane. The non-amphiphatic CPPs are short and contain high amount of cationic amino acids such as arginine, for example

arginine-9 (R_9) (8) and TAT (3, 13). All the CPPs are highly positively charged due to the contribution of basic residues such as lysine or arginine. MAP has the fastest cellular uptake and cargo delivery efficiency, followed by transportan, TAT, and penetratin (14).

The successful use of these peptides for intracellular delivery of electrostatically or covalently linked cargos, including proteins, nucleic acids (15–18), liposomes (19–21), micelles (22, 23) and other nanoparticles (24–26), has led efforts to identify CPPs with greater cell penetrating activity. Such efforts have resulted in the identification of CPPs with diverse amino acid sequences (Table 1).

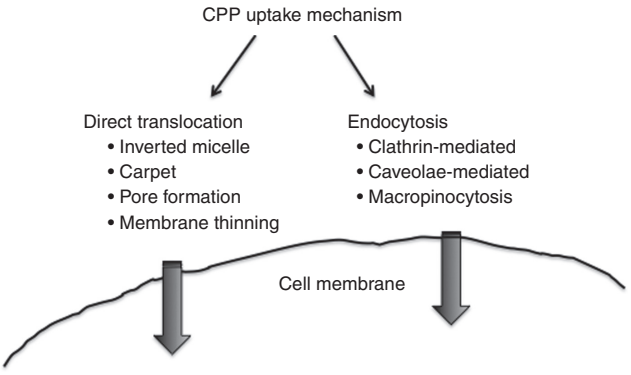

Figure 1 Scheme of different suggested pathways of CPP uptake mechanisms.

Mechanism of uptake of CPPs

Despite of multiple studies to elucidate the mechanism by which CPPs enter the cells (27–31), the mechanism of uptake of CPPs is still not completely clear. Different uptake mechanisms are involved in different systems, and in some cases, the mechanism is cell type- or cargo type-specific. This difficulty in elucidating these mechanisms may be due to the fact that in many cases CPPs can interact with multiple cell surface molecules. Therefore, CPPs can be taken up by cells via multiple pathways (28, 32, 33). There is evidence of both energy-dependent vesicular mechanisms, referred to as endocytosis, or direct processes involving translocation through the lipid bilayer by CPPs (34, 35). Also, all three major types of endocytosis (i.e., clathrin-mediated, caveolin-mediated and macropinocytosis) (36–40) appear to be involved for different CPPs or CPP-attached cargo (Figure 1).

Direct translocation via energy independent pathways may involve different mechanisms such as inverted micelle formation (41), the carpet-like model (42), pore formation (42) and membrane thinning model (43). The interaction of the positively charged CPP with negatively charged cellular membrane components such as heparan sulfate and the phospholipid bilayer constitutes the first step in all these mechanisms. These internalization mechanisms are highly dependent on the peptide concentration, peptide sequence and lipid composition in each model membrane study. It has been reported that the direct translocation mechanism is most likely to occur at high CPP concentrations and for primary amphiphatic CPPs such as transportan analogues and MPG (28, 44, 45). The inverted micelle model was suggested for direct translocation of penetratin (46). Since this mechanism was shown to involve interaction between hydrophobic residues such as tryptophan and the hydrophobic part of the membrane, this mechanism is not probable for the highly cationic CPPs such as TAT. For pore formation mechanism certain threshold concentration of peptide is required, which is different for different peptides. The carpet-like model and membrane thinning model also involves interactions between negatively charged phospholipid and cationic CPPs that leads to a carpeting and thinning of the membrane, respectively. This is followed by translocation of the CPP, only when CPP concentration is above a threshold concentration.

From the recent studies, it can be concluded that three types of endocytic uptake mechanisms are involved: clathrin-mediated (30), lipid raft-mediated through the formation of caveolae (39) and macropinocytosis (36, 47, 48). The peptides reported in Table 1 can be also divided based on hydrophobicity such as hydrophilic

Table 1 Examples of CPPs.

CPP	Amino acid sequence	Total charges	References
Polyarginines (R_9)	RRRRRRRRR	+9	(8)
TAT_{47-60}	GRKKRRQRRRPPQ	+8	(3)
M918	MVTVLFRRLRIRRACGPPRVRV	+7	(12)
Penetratin (Antennapedia, pAntp)	RQIKIWFQNRRMKWKK	+7	(4)
TP10	AGYLLGKINLKALAALAKKIL	+4	(10)

or arginine-rich CPPs (TAT and R_9), intermediate hydrophobic CPPs (Penetratin, Pen-Arg, M918, pVEC) and hydrophobic (TP10). Since these peptides have different number of arginine residues and positive charges, they could use different cellular uptake mechanisms. Guterstam et al. suggested direct translocation mechanism for arginine-rich CPPs such as R_9 and TAT in presence of high pyrenebutyrate concentration (50 μM). At lower pyrenebutyrate concentration, translocation of R_9-attached oligonucleotide occurred by macropinocytosis (49). Macropinocytosis was demonstrated to be implicated in the internalization of polyarginines (50), and to a much less extent of penetratin. Inhibition of macropinocytosis also led to a decrease in the uptake for TAT, suggesting macropinocytosis as a route of entry (29, 47). Dowdy et al. also confirmed macropinocytosis as the route for TAT uptake (51). In contrast some other study has shown involvement of an energy- and temperature-independent pathways for arginine-rich CPPs (52). Different types of endocytic uptake mechanisms are also reported for arginine-rich CPPs alone or conjugated to cargo (37, 39, 47, 53, 54). Thus, type of cell lines and nature of cargo may affect their uptake mechanism.

For intermediately hydrophobic peptides such as pVEC, M918 and penetratin different cellular entry pathways are reported. It has been shown that pVEC is able to translocate into different cell lines (11). The involvement of clathrin dependent endocytotic pathway is also suggested (55). M918 is able to deliver various cargo molecules into different cell lines. The presence of endocytotic pathways (especially macropinocytosis) was confirmed as the uptake mechanism. However, glycoaminoglycans on the cell membrane are not involved in cellular uptake mechanism (12). For penetratin, endocytosis was suggested as the uptake mechanism both in the absence or presence of the cargo molecules. However, different types of endocytotic pathways for penetratin and its cargo conjugates have been reported (28).

For hydrophobic CPP like TP10 alone and with cargo attached, different cellular uptake and translocation mechanisms have been demonstrated. However, the cellular uptake is suggested to occur mainly via the endocytotic pathway (56).

CPPs and endosomal escape

After endocytosis, the entrapment in the endosomal compartment is the major challenge for CPPs and their cargoes. They could be digested by lysosomal hydrolysis before reaching a target. Increasing hydrophobicity of the complex is one way used to overcome the endosomal membrane barrier (57). A variety of methods such as those using fusogenic lipids (58–61), membrane disruptive peptides (47, 54, 62, 63) and polymers (64, 65), and lysomotropic agents (47, 66, 67) have been reported to enhance the endosomal escape of CPP-attached cargoes. Recently, a new CPP called PepFect6 (PF6), which has four chloroquine-analogs (trifluoromethylquinoline, QN) covalently, attached to the peptide via a succinylated lysine-tree is reported (68). This modification dramatically increased the efficiency compared to its parent peptide stearyl-TP10 in the delivery of siRNA, due to the CPP's increased ability to escape the endosomal compartment. Later, PF14 was designed with stearyl-TP10 as a backbone but with the lysine residues replaced with the non-coded amino-acid ornithine to improve complex formation with oligonucleotides (69). This peptide was more resistant to protease degradation. This was further modified by designing a peptide combining the transfection efficiency of PF14 with an moieties to facilitate endosomal escape (70).

After the endosomal escape, intracellular trafficking of the cargo may be influenced by the presence or absence of the carrier. If both the carrier and the cargo remain attached then the high positive charge on carrier such as TAT and R_8, may direct the CPP-conjugate to the nucleus where the positively charged carrier is likely to interact with the negatively charged DNA. To avoid this possibility, some CPP conjugates have been designed so that the cargo is released from the CPP after reaching the cytosol by using a reducible disulfide bond between the CPP and cargo (71).

Since the discovery over 20 years ago of CPPs, CPP-conjugated cargoes have found applications in a variety of disease areas such as cancer, cardiology, stroke and pain. The relative lack of both toxicity and cell specificity has enabled the use of CPP technology in various preclinical models (72).

Delivery of therapeutics using CPPs

CPPs have shown great potential and can be used for range of therapeutic applications (as can be seen in Figure 2), both in vitro and in vivo. CPPs have delivered various cargoes to challenging target areas such as brain, eye, heart, immune system, intestinal wall and skin. More detailed information on current status of delivery of various therapeutics and nanocarrier-based systems using CPPs is given below.

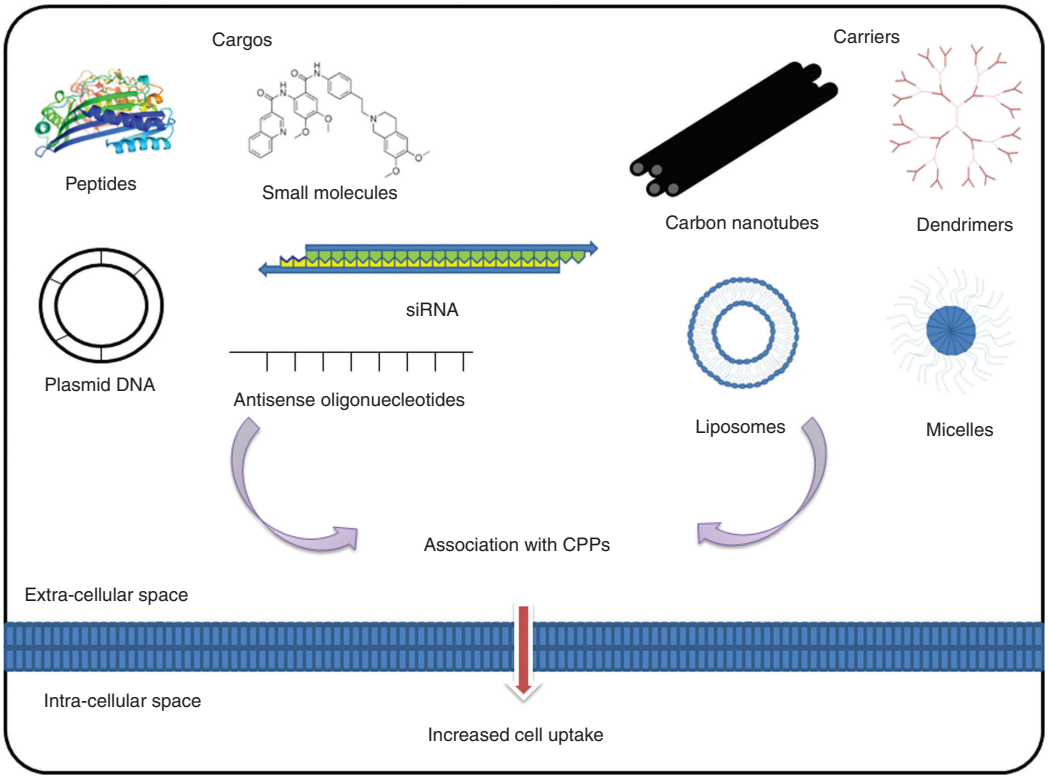

Figure 2 Potential targets for intracellular delivery using CPPs.

Delivery of small molecules

Conjugation with CPPs can enhance the intracellular delivery of small molecules. Doxorubicin (DOX) is one of the most commonly used drugs to treat various types of cancers. Dox-CPPs were found to induce apoptosis in MDA-MB-231 breast cancer cells at much lower doses than free drug (73). The cells treated with free DOX usually over express the anti-apoptotic protein Bcl-2. However, this protein was inefficient in preventing DOX-CPP-induced apoptosis. In another study, coupling of DOX to penetratin helped DOX to bypass the P-glycoprotein (Pgp)-dependent drug efflux from the brain and thus enhanced the uptake across the blood-brain barrier in an in situ brain perfusion model (74). Methotrexate, an anticancer agent, demonstrated a five-fold increase in cytotoxicity with a breast cancer cell line after conjugation with the CPP, YTA2 (75). Recently, Zhang et al. reported a new analogue of transportan 10 (TP10) known as TP10–5 (TK), a CPP with a remarkable capacity for membrane translocation (76). However, low levels of specificity and high toxicity limited its successful use for drug delivery applications. Further modification of TK by replacement of all lysines with histidines resulted in development of a new type of acid-activated CPP (TH) with more cellular uptake at acidic pH 6.0 compared to a typical physiological pH. After attachment of camptothecin (CPT) to TH, this conjugate exhibited increased cytotoxicity to cancer cells in a pH-dependent manner compared with free CPT and TK-CPT.

The conjugation of CPPs to small molecule cytotoxic drugs has also been used to alter the in vivo distribution and to improve the efficacy profile of the parent molecule. When DOX was attached via a non-cleavable linker to several CPPs derived from heparin-binding proteins or anti-DNA antibodies (named as ‘Vetocell’ peptides) (77), one of such conjugate (DTS-101, see Table 2) exhibited increased antitumor efficacy and reduced systemic cytotoxicity compared to the parent drug. In another study, a conjugation of Vetocell with an active metabolite of irinotecan showed greater efficacy and higher plasma levels than the parent molecule (78).

Delivery of proteins and peptides

The nature of the cell membrane restricts cellular drug uptake to small (<600 Da) and to hydrophobic molecules. This offers several challenges for intracellular delivery of proteins and peptides which have short in vivo half-lives and poor bioavailability. Thus, methods that enable

Table 2 Clinical status of CPP-conjugated compounds.

Company (Compound)	CPP	Target/Indication	Status
Capstone Therapeutics (AZX100)	PTD4	HSP20/Keloid scarring	Phase 2
KAI Pharmaceuticals			
(KAI-9803)	TAT PTD	Protein kinase Cδ inhibitor/Myocardial infarction	Phase 2b
(KAI-1678)		Protein kinase Cε inhibitor/Pain	Phase 2a
(KAI-1455)		Protein kinase Cε inhibitor/Ischemia	Phase 1
Xigen	TAT PTD	c-Jun-N-terminal kinases/Hearing loss,	Phase 2
(XG-102)		Stroke	Phase 1
Revance Therapeutics	TAT PTD	Transdemal delivery of Botulinum toxin type A/	
(RT001)		Wrinkles	Phase 2b
		Excessive sweating	Phase 1
Diatos SA (DTS-108)	Anti-DNA antibody	Nuclear delivery of cytotoxin/Cancer	Preclinical

their efficient intracellular delivery and successful in vivo application are needed.

CPPs and protein delivery

The use of CPPs greatly facilitates the intracellular delivery of a wide range of proteins (79–82). The best example of a biologically active protein delivered by a CPP is the TAT- β-galactosidase (gal) fusion protein. Delivery across the blood-brain barrier is usually restricted to very small (<6 amino acids) highly lipophilic peptides, but after fusion with TAT, this CPP carried 480 kDa β-gal across the blood-brain barrier after intraperitoneal injection (83). The peptide-mediated cytoplasmic delivery of macromolecules had not been achieved until these studies.

CPPs also have potential in the treatment of stroke. Cerebral ischemia is usually the result of a blood flow blockage in the brain. Death of the neuronal cells after cerebral ischemia is associated with apoptosis. The apoptosis is mediated by the release of pro-apoptotic proteins and anti-apoptotic proteins such as Bcl-xL that counteract the pro-apoptotic process. However, failure to deliver these anti-apoptotic proteins to cells has been a challenge. Cao et al. used the anti-apoptotic protein Bcl-xL conjugated to TATp for neuroprotection in a murine middle cerebral artery occlusion (MCAO) study (84). The hemagglutinin (HA)-tagged compound known as PTD-HA-Bcl-xL was administered intraperitoneally up to 45 min after the start of reperfusion. The infarct size was significantly reduced in a dose-dependent manner measured 3 days after the start of reperfusion. PTD-HA-Bcl-xL also decreased ischemia-induced caspase-3 activation in ischemic neurons. This indicated that the effect was mediated partly by a reduction in the cellular apoptosis response following ischemia.

Cyclosporine A (CsA) is an immune suppressant and anti-inflammatory agent used for a number of indications. However, the topical application of this drug is limited since it cannot readily penetrate the epidermis. When conjugated to a polyarginine CPP by a pH-sensitive linker (7-mer, called R_7-CsA), it resulted in enhanced penetration into the dermis of mouse and human skin and human skin grafted onto mice (85). The conjugate was taken up by dermal T-lymphocytes and reduced the secretion of the pro-inflammatory cytokine, interleukin (IL)-2 and significantly reduced cutaneous inflammation in this mouse model of contact dermatitis.

Use of TAT has also enabled the delivery of antibodies for radiotherapeutic application (86). Morris et al. developed a new technique for protein delivery based on the peptide, Pep-1 (87). Pep-1 aided delivery of different peptides, proteins, and antibodies inside different cells in vitro and in vivo, without any chemical coupling between the cargo and Pep-1 (79). CPPs also have great potential to facilitate insulin permeation from the intestinal lumen or nasal cavity into systemic circulation via efficient uptake by epithelial cells. In fact, the co-administration of insulin with the peptide penetratin increased intestinal and nasal insulin bioavailability to 35 and 50%, respectively (88).

CPPs and peptide delivery

Recently, Boisguerin et al. studied four CPPs (TAT, $(RXR)_4$ (an oligoarginine analog, which has an α-aminohexanoic acid linker (x) between arginine residues), Bpep and Pip2b) conjugated to the BH_4-peptide, derived from the

BH_4 domain of the Bcl-xL anti-apoptotic protein (89). TAT-BH_4 and Pip2b-BH_4 conjugates decreased apoptosis in vitro in primary cardiomyocytes. When injected intravenously at a low dose at the onset of reperfusion in a surgical model of myocardial ischemia and reperfusion injury, they induced a large decrease in infarct size when measured after 24 h reperfusion. These effects were not observed following the injection of the CPP alone or a scrambled version of BH_4. A peptide inhibitor of c-Jun-N-terminal kinase conjugated to TAT (now in clinical development by Xigen as XG-102), has also been shown to protect against apoptotic cell death in both in vitro and in in vivo models of cerebral ischemia (90).

Protein kinase (PK) enzymes play a major role in cardiac cellular function by transducing signals from the cell membrane to intracellular locations (91). δ-PKC inhibition during the reperfusion period led to restoration of cellular energy stores, enhanced recovery from intracellular acidosis, preservation of mitochondrial function and reduced damage to myocytes and endothelial cells after an ischemic insult (92). KAI-9803 is a novel peptide derived from the δV1-1 portion of δPKC, conjugated reversibly to the cell penetrating peptide TAT via a disulfide bond that inhibits δ-PKC activity by disrupting binding of δ-PKC to its receptor for activated C kinases, thereby preventing localization of δ-PKC to the mitochondria during periods of myocardial ischemia and reperfusion (93, 94). In ex vivo studies, KAI-9803 inhibited global cardiac injury following ischemia and reperfusion in rat hearts (95) as well as in excised human cardiac tissue (96). Administration of KAI-9803 at the beginning of the reperfusion period led to much greater recovery of contractile function following ischemia than with a TAT CPP control group.

The clinical development status of several CPP-conjugated compounds is presented in Table 2.

Delivery of nucleic acids

Current advances in genetics have led to the recognition of nucleic acid-based therapies as a promising tool for the treatment of various pathological conditions resulting from unregulated expression of genes. To mediate their efficient cellular uptake, however, there is an urgent need for the delivery systems that prevent nucleic acid degradation and promote intracellular delivery of loaded cargo. Their unique properties that enhance cellular uptake shared by most of CPPs make them particularly suited to act as gene delivery vectors. The following section describes the recent advances in the use of CPPs for the delivery of different types of nucleic acids such as DNA, siRNA and oligonucleosides.

DNA delivery

DNA delivery into cells has been identified as one of the most promising approaches to treat diseases related to unregulated gene expression. Overcoming physiological barriers that affect stability of DNA and uptake of DNA by targeted cells, is still the prime requirement for the success of DNA delivery. An approach utilizing CPPs for DNA delivery has been widely studied because of the very low toxicity profile of CPPs, low immunogenicity of CPPs, their ability to protect and transport loaded cargo in the intracellular space and a unique property shared by most CPPs to accumulate inside nucleus. Numerous researches have been carried out on CPPs for DNA delivery as outlined in Table 3.

The potential of peptides for DNA delivery was demonstrated as early as 1998 by Pooga et al. (109). In their study, DNA condensed with Transportan peptide successfully suppressed expression of functional galanin receptors. Later, simple complexes formed by luciferase encoded plasmids and oligoarginines showed a slight improvement in transfection efficiency in comparison to plasmid-only controls (110). This was improved by N-terminal modification of octa-arginine (R_8) by hydrophobic moiety such as stearic acid, referred to as stearyl-R_8 (110). This strategy was later extended to another arginine-rich peptide, $(RxR)_4$ for delivery of pDNA and showed significant increase in gene expression in variety of cell lines than regular stearyl-oligoarginine (57). Recently, stearyl-TP10 was reported to mediate efficient gene delivery in vitro and in vivo without any toxicity or immunogenicity (111). Lo et al. reported a TAT peptide analog modified with histidines and cysteins (C-5H-TAT-5H-C), which showed enhanced luciferase expression in a variety of cell lines. It also showed increased luciferase gene expression after direct injections to the brain and spinal cord (112). An interesting study was carried out by Liu et al., where the macro-branched cell penetrating peptides POLYTAT1, POLYTAT 2 and POLYTAT 3 were designed for gene delivery. All three compounds demonstrated higher transfection efficiency in mammalian cell lines when condensed with DNA compared to TAT alone (113). Rajpal et al., showed synthesis of various novel CPP candidates from already known pVEC (101). It was found that the presence of histidines along with 9 arginines and secondary amphipathicity showed efficient DNA delivery with low toxicity even in absence of chloroquine in multiple cell lines.

Table 3 CPPs used for nucleic acid delivery.

Peptide	Sequence	Interaction	References
DNA delivery			
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	Non-covalent	(97)
KALA	WEAKLAKALAKALAKHLAKALAKALKACEA	Non-covalent	(98)
ppTG1	Gly-Leu-Phe-Lys-Ala-Leu-Leu-Lys-Leu-Leu-Lys-Ser-Leu-Trp-Lys-Leu-Leu-Lys-Ala	Non-covalent	(99)
TAT-λ phage	TAT peptide linked to phage protein along with RGD peptide, NLS domain of SV40 T antigen	Non-covalent	(100)
S_9R	Modified pVEC	Non-covalent	(101)
siRNA delivery			
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	Non-covalent	(102)
$MPG\Delta^{NLS}$	GALFLGFLGAAGSTMGAWSQPKSKRKV	Non-covalent	(102)
MPGα	GALFLAFLAAALSLMGLWSQPKKKRKV	Non-covalent	(7, 103)
Activated Penetratin 1	RQIKIWFQNRRMKWKK	Thiol linkage	(104)
R_9	RRRRRRRRR	Non-covalent	(105)
Chol-R_9	Cholesteryl-RRRRRRRRR	Non-covalent	(106)
CADY	Ac-GLWRALWRLLRSLWRLLWRA-cysteamide	Non-covalent	(107)
LMWP	VSRRRRRRGGRRRR	Non-covalent	(108)

MPG, MPG is a bipartite amphipathic peptide derived from both the fusion peptide domain of HIV-1 gp41 protein and the NLS (nuclear localization sequence) of SV40 large T antigen. $MPG\Delta^{NLS}$, NLS mutant of MPG.

siRNA delivery

RNA interference (RNAi) has been recognized as a vital tool for manipulation of gene expression in cells. Numerous studies have exploited the usefulness of CPPs for intracellular delivery of siRNAs. Initial attempts were made to link siRNAs with CPPs either by covalent or non-covalent approaches. Numerous studies have since been carried out to evaluate both approaches for the silencing of the target gene (Table 3).

The first successful delivery of siRNA using cell penetrating peptide was achieved with the MPG peptide (102). Non-covalently linked siRNA/MPG complexes resulted in significant downregulation of the target protein, GAPDH. This RNAi effect was further increased when siRNA was complexed with $MPG\Delta^{NLS}$, a NLS mutant of MPG. Application of the MPG peptide was also evaluated for the delivery of siRNA in vivo. This attempt resulted in successful downregulation of targeted genes and ultimately a reduction in tumor growth (114). In 2006, Sandra and coworkers tested the ability of MPGα, a variant of MPG, for the delivery of siRNA (103). The interesting part in their study was the discovery of significant gene silencing with the MPGα/siRNA complex even in presence of several endocytosis inhibitors. Lundberg et al. reported a histidine-modified penetratin analog, an EB1 peptide, with endosomolytic properties, which vectorized siRNA in non-covalent manner and showed significant gene silencing in cell cultures (115). Modified polyarginine peptides, cholesteryl oligoarginine, have also been exploited for the delivery of siRNA (106). Complexation of Chol-R_9 with siRNA as well as DNA resulted in a significant increase in cell transfection compared to plain R_9 in vitro. They observed that the in vivo delivery of siRNA/Chol-R_9 complex resulted in decreased expression of intratumoral vascular endothelial growth factor (VEGF, the target protein), which resulted in a decreased tumor volume. Stearylation was also reported to improve siRNA delivery efficiency into cells using CPPs. Langel et al. reported that stearylated transportan TP-10 efficiently delivered a splice-correcting phosphorothioate 20-O-methyl RNA (20-OMe ON) into cells (10, 116). Later the novel PF6 carrier peptide (a stearyl-TP10 analogue modified with the chloroquine derivative trifluoromethylquinoline) was developed to improve endosomal escape (68). The PF6 peptide showed efficient transfection in human umbilical vein endothelial cells (HUVEC) and Jurkat cells with siRNA. Moreover, the high RNAi response produced by PF6/siRNA was retained in the presence of serum. The same group also developed PepFect 14 (PF14), by substitution of stearly-TP10 lysines with ornithines (69). PF14/siRNA nanocomplexes prepared by a solid dispersion

technique displayed comparable RNAi activity with that of the freshly prepared nanocomplexes in solution (117).

Dowdy et al. reported an interesting approach for the delivery of siRNA using a fusion protein with three repeats of a TAT peptide containing a double-stranded RNA binding domain (PTD-DRBD) (118). PTD-DRBD showed efficient gene knockdown in various hard-to-transfect primary and transformed cells, including T cells, HUVEC and human embryonic stem cells without any cytotoxicity or immunogenicity. TAT-DRBD also showed tumor reduction in a tumor xenograft model after intratumoral delivery in mice (119).

Crombez et al., also demonstrated the successful intracellular delivery of siRNA when complexed with a synthetic peptide, CADY (107). This peptide had very high affinity for siRNA (K_d=15.2 nM) which resulted in the formation of stable complexes. The cellular uptake mechanism of CADY/siRNA complex was partially dependent on the endosomal pathway. However, it was apparently associated with interaction between the cell membrane and trypsin residues of the peptide. Low molecular weight protamine (LMWP), derived from natural protamine, evaluated for the delivery of siRNA, showed formation of stable complexes of LMWP with siRNA which resulted in gene silencing, both in vitro and in vivo (108). Interestingly, the treatment with/siRNA complex did not result in an increase in the serum level of inflammatory cytokines including interferon IFN-α and interleukin IL-12 indicating a minimum immunostimulatory effect.

Delivery of pharmaceutical nanocarriers

Various types of pharmaceutical nanocarriers have been used successfully to increase the stability of drugs, alter their pharmacokinetics and ultimately reduce the undesired side-effects with improvement in the efficacy of the loaded drugs (120, 121). The most commonly studied nanocarriers are liposomes and micelles. These nanocarriers can be loaded with a wide variety of drugs and can be functionalized with various ligands (120, 122). CPPs including TAT have been used to functionalize various nanocarriers (liposomes, micelles, nanoparticles) to increase the intracellular delivery of the cargo.

The chemistry utilized to join the peptide and nanocarrier together has a direct effect on subsequent function. Ideally, whatever chemistry is used to attach the peptide to a nanocarrier should result in a uniform display of the peptides on the nanocarrier surface with their active regions clearly extended and available for interaction. For example, it is very important that the positive charge on the oligoarginine motifs are clearly available for direct interaction with the extracellular membranes (123). Sterically blocking of the oligoarginine or other nanocarrier surface-attached ligands can result in mixed functional avidity with some nanocarriers undergoing high efficiency uptake while others do not. Further, for efficient uptake of CPP-modified nanocarrier, it is also important to have a high density, or ratio, of CPP on nanocarrier surfaces. However, low modification levels may not be sufficient for uptake, especially when applying small concentrations of nanocarrier materials to cells (123). While developing smart stimuli-sensitive nanocarrier, it is important to control the peptides affinity to the nanocarrier surface. It has become increasingly evident that the CPP peptides should be attached to the cargos in such a way that allows them to be released intracellularly at a specific organelle where their activity is highest. This can be achieved with a linkage that is responsive to external stimuli (e.g., light or heat) or other environmental cues (e.g., changes in pH or presence of a reducing agent/protease) (124). Also, particular attention should be paid to the characteristics of the nanocarrier material itself, other ligands and functional groups on their surface, the peptide sequence itself and the final required utility. There are currently four general strategies commonly applied for attachment of peptides to nanoparticle materials (Table 4).

Various in vitro and in vivo examples of TAT-modified nanocarriers that can serve as the basis for cellular fluorescence and magnetic labeling reagents have been reported. The first example of use of TAT peptide as a vector for nanoparticle delivery was described in 1999 (132). TAT functionalized iron oxide nanoparticles efficiently labeled cells, to serve as a tool for magnetic resonance imaging (MRI) or magnetic separation of cells in vivo.

Modification of liposomes with TAT enhanced the delivery of liposome into cells, such as murine Lewis lung carcinoma (LLC) cells, human breast tumor BT20 cells, and rat cardiac myocytes H9C2 (19). The TAT was attached to liposomes via the spacer, p-nitrophenylcarbonyl-PEG-PE, at a density of a few hundreds of TAT per single 200 nm liposome vesicle. Only those TAT-liposomes, which provided direct contact of TAT residues with cells, displayed an enhanced uptake by the cells (133). TAT-modified liposomes also enhanced binding and endocytosis in ovarian carcinoma cells (20). Ant $_{(43–58)}$ and TAT coupled to small unilamellar liposomes accumulated in higher proportions within tumor cells and dendritic cells than unmodified control liposomes (134). Application of CPP-modified liposomes for aerosol lung delivery was investigated with three CPPs: TAT, Ant, and R_8 conjugated to neutral liposomes. Efficient internalization of

Table 4 Strategies applied for attachment of peptides to nanoparticle materials.

Conjugation strategy	Requirements	Examples	References
Electrostatic interaction	– Simple mixing of oppositely charged peptide and nanocarrier.	– Positively charged lysine-rich 21-residue Pep-1 peptidyl sequence noncovalently associated with commercial streptavidin-conjugated CdSe/ZnS core/shell quantum dots, which facilitated their delivery to HeLa cells.	(125)
		– Negatively charged citrate-stabilized Au-nanoparticles associated with a positively charged, coiled peptide with a pH change used as a stimulus to alter the electrostatics and control assembly kinetic.	(126)
Direct interaction	– Direct binding of certain peptide motifs to nanocarrier via high-affinity interactions, such as binding of thiols to a Au-nanoparticle surface, or to quantum dots with a His_n-appended peptide (commonly a His6).	– Peptides assembled with quantum dots to create sensors for monitoring proteolytic activity for CPP-facilitated cellular delivery.	(127) (128)
Secondary interaction	– Mixing of peptide sequences with synthetic insertion of a biotin groups and nanocarrier functionalized with avidin/streptavidin. – Mixing of nitriloacetic acid functionalized nanocarriers with a His_n –appended peptide.	– A variety of functionalized nanocarriers such as Au-nanoparticles, FePt magnetic nanoparticles or semiconductor quantum dots.	(129) (130) (131)
Covalent linkage	– The most commonly available groups on both nanocarrier and peptides such as amines, thiols and carboxyl groups are used for conjugation. – Use of chemistry including 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/ N-hydroxysuccinimide (EDC/NHS) to join amines to carboxyls, maleimides to target reduced thiols, and thiol exchange, which also target thiols.	– Modification of liposomes and micelles with TATp.	(19, 23)

the modified liposomes was observed by airway epithelial cells in culture and delivered cargo (e.g. dextran) to the cytoplasm of the cells (135).

The requirement for direct unhindered contact of CPP with cells was also shown for TAT-modified micelles (23). These micelles, when loaded with the anticancer drug paclitaxel, demonstrated increased cytotoxicity in vitro with various cancer cell lines. This was considered to be a result of an increased cell interaction of TAT-modified micelles compared to non-modified-micelles.

The treatment of central nervous system diseases such as brain glioma is a major challenge due to the presence of the blood–brain barrier (BBB). The cationic charge of TAT can facilitate interaction with the normally negatively charged BBB, triggering permeabilization of the cell membrane via a receptor/transporter-independent pathway which results in endocytosis of the sequence (41). TAT crossed the BBB and accumulated in the CNS (136). Qin et al. covalently conjugated TAT with cholesterol to prepare DOX-loaded liposomes for brain glioma therapy (137). Results of the biodistribution study showed higher efficiency of brain delivery and lower cardiotoxic risk. The survival time of the glioma-bearing rats treated with TAT-modified liposome was much longer than controls.

TAT was also used to modify thiocholesterol-based cationic lipids (TCL)-based nanolipoparticles (NLPs). The TAT-modified NLPs had a zwitterionic surface and a higher transfection efficiency than the non-modified cationic NLPs (138). The TAT-modified lipoplexes were internalized by cells mainly *via* a cholesterol-dependent clathrin-mediated pathway (139).

The potential of TAT-modified liposomes to enhance the delivery of a model gene, a plasmid encoding for the GFP (pEGFP-N1), to human brain tumor U-87 MG cells was investigated both in vitro and in vivo in an intracranial model in nude mice (140). An enhanced selective delivery of pEGFP-N1 to tumor cells and effective transfection was observed after intratumoral injection compared to plain plasmid-loaded lipoplexes. No transfection (green fluorescence of GFP) was noted in the normal brain adjacent to tumors. Thus, TAT–lipoplexes may be used to augment the delivery of genes to tumor cells when injected

intratumorally, without affecting the normal adjacent brain.

We have reported a double–targeted delivery system using low cationic DNA lipoplexes modified with TAT and/or with monoclonal anti-myosin monoclonal antibody 2G4 (mAb 2G4) specific for cardiac myosin for targeted gene delivery to ischemic myocardium (141). Increased transfection was observed in vitro of both normoxic and hypoxia damaged cardiomyocytes in the presence of TAT. Modification of these lipoplexes with mAb 2G4 antibody increased the transfection further in the case of hypoxic, but not normoxic cardiomyocytes. After in vivo administration, an increased accumulation of mAb 2G4-modified TAT lipoplexes in the ischemic rat myocardium led to significantly enhanced transfection of cardiomyocytes in the ischemic zone. Thus, the genetic transformation of hypoxic cardiomyocytes can be enhanced with lipoplexes modified with TAT and/or mAb 2G4.

We recently reported R_8-modified pegylated liposomal DOX (R_8-PLD) for the treatment of non-small cell lung cancer, for which the primary treatment modality currently consists of surgery and radiotherapy. The treatment of non-small cell lung cancer cell line, A549 monolayers with R_8-PLD increased the level of cell death marker lactate dehydrogenase (LDH) secretion confirming higher cytotoxicity of R_8-PLD than PLD. R_8-PLD induced greater level of apoptosis to A549 tumor xenograft and dramatic inhibition of tumor volume and tumor weight reduction (21).

The arginine octamer (R_8) modified liposomes were studied for cellular delivery of siRNA. The R_8-liposomal siRNA had a very high stability in serum and produced a highly elevated transfection efficiency of SK-MES-1 lung tumor cells otherwise resistant to Lipofectamine 2000-mediated transfection. The siRNA in R_8-liposomes effectively inhibited the targeted gene and significantly reduced the proliferation of cancer cells (142). Arginine-9 (R_9) was also investigated as a potential carrier for siRNAs (143). Harashima et al. have developed R_8-modified liposomes with condensed DNA cores coated with lipid membranes. These liposomes are prepared by programmed packaging known as multifunctional envelope-type nanodevices (MEND) and showed high transfection efficiency and little cytotoxicity (59, 144). This system was also developed for delivery of siRNA (145). The MEND system developed with stearyl-R8 showed highest RNAi response. Later, improved endosomal escape of R_8-MEND was reported by surface decoration with a pH-dependent fusogenic peptide GALA and a optimized lipid mixture for endosomal fusion (146) or with a new pH-sensitive cationic lipid, YSK05 (147).

A traceable micellar system was constructed from degradable poly(ethylene oxide)-block-poly(ε-caprolactone) (PEO-b-PCL) block copolymers for simultaneous intracellular delivery of DOX and siRNA against P-gp expression in multidrug-resistant MDA-MB-435 human tumor models that overexpress P-gp (148). The functional group on the PCL block incorporated short polyamines for complexation with siRNA or chemically conjugated DOX via a pH-sensitive hydrazone linkage. These micelles were surface-modified with integrin Rvβ3-specific ligand (RGD4C) for active cancer targeting and TAT peptide for intracellular delivery. The micelles were further tagged with near-infrared fluorescent imaging probes. Compared to plain micelles, TAT- and RGD-micelles significantly increased cellular uptake of FAM-siRNA and DOX. Also the level of reversal of resistance was higher for RGD4C and TAT-modified micelles.

Kanazawa et al. prepared methoxy poly(ethylene glycol) (MPEG)/polycaprolactone (PCL) diblock copolymers conjugated with a TAT peptide via a disulfide linkage and evaluated their ability as an siRNA carrier. The MPEG-PCL-SS-TAT/anti-VEGF siRNA complexes achieved a high anti-tumor effect in tumor-bearing mice after i.v. administration (149).

After endocytosis, the sequestration and entrapment of the majority of internalized material within endocytic vesicles represents the major limitation of CPP-mediated delivery of biologically active molecules. When the endosomolytic peptide HA2 was covalently conjugated with TAT to induce the release of a TAT-coupled cargo from endocytic bodies, it exerted a more significant biological effect at lower concentrations (47). Lundberg et al. attempted a similar strategy by noncovalent packaging of siRNA duplexes with an endosomolytic peptide that is potentially capable of enhancing the effectiveness of CPP/siRNA complex delivery by inducing its release from endosomes (115).

Recently, a tumor-penetrating peptide, iRGD (CRGDK-GPDC), was reported to increase vascular and tissue penetration in a tumor-specific and neuropilin-1-dependent manner, as compared to conventional RGD peptides (150–152). The iRGD homes to tumor sites by binding to integrins, which are highly expressed in tumor endothelium. After binding, the iRGD peptide is proteolytically cleaved to produce CRGDK fragment, which favors binding to neuropilin-1 receptor, facilitating the penetration of drugs into the tumor. This lead to enhanced therapeutic effect of antitumor drugs. The iRGD when conjugated to liposomes enhanced the antitumor efficacy in breast tumor cells, including multidrug-resistant cells (153). Gu et al. co-administered iRGD with MT1-AF7p peptide (which

presents high binding affinity to membrane type-1 matrix metalloproteinase) functionalized paclitaxel-loaded PEG-poly(lactic acid) (PLA) nanoparticles. This resulted in significantly improved nanoparticles extravasation across the blood-brain tumor barrier and accumulation in glioma parenchyma and longer median survival time of the nude mice bearing intracranial C6 glioma (154).

Smart stimuli-sensitive multifunctional nanocarriers

Although, CPPs have shown immense potential for intracellular delivery of variety of cargoes, their in vivo application is usually limited because of nonspecificity leading to risk of unwanted tissue distribution and drug-induced toxic effects on normal tissues. Thus, the nonselective in vivo penetration is of major pharmacological concern. To solve this problem we introduced the idea of 'smart stimuli-sensitive multifunctional nanocarrier'. An ideal multifunctional nanocarrier should circulate in blood for a prolonged time, bear specific cell-surface targeting moieties (monoclonal antibodies, Fab fragments, phage display peptides) and respond to certain stimulus characteristics of the pathological site (to either release an entrapped drug or expose "hidden" active moieties (such as non-specific CPPs) by surface-attached pH- or temperature-sensitive coatings) and finally, deliver the cargo intracellularly.

We have prepared targeted long-circulating PEGylated liposomes and PEG–phosphatidylethanolamine (PEG–PE)-based micelles possessing several functionalities as can be seen in Figure 3 (155). These nanocarriers were surface modified with a monoclonal antibody (infarct-specific antimyosin antibody 2G4 or cancer-specific antinucleosome antibody 2C5) and cell penetrating TAT moieties with TAT-(short PEG)–PE derivatives. In addition, the low pH-sensitive PEG_{2000} or $_{5000}$-hydrazone (Hz)-PE was used for liposome surface modification or for micelle preparation to shield the TAT moieties at normal pH. At pH 7.5–8.0, both liposomes and micelles had high specific binding with antibody substrates, but showed very limited internalization of NIH/3T3 or U-87 cells. However, after brief incubation (15-to-30 min) at low pH (pH 5.0–6.0) these nanocarriers lost their protective PEG shell by acidic hydrolysis of PEG–Hz–PE and acquired the ability to get internalized effectively by cells *via* exposed TAT moieties.

Recently, we reported multifunctional liposomes prepared by modification of Doxil with TAT moieties and cancer-specific mAb 2C5. TAT was conjugated with a short PEG_{1000}-PE spacer, and mAb 2C5 was attached to a long PEG chain (2C5-PEG_{3400}-PE) (156). The TAT moieties were shielded with pH-sensitive PEG_{2000}-Hz-PE. This multifunctional immuno-Doxil® preparation increased cytotoxicity of B16-F10, HeLa and MCF-7 cells when pre-incubated at lower pH, indicating TAT exposure and cell penetration activity. Based on the above idea, a liposomal delivery system modified with TAT-PEG_{2000}-PE and a protective longer cysteine (Cys)-cleavable $PEG_{(5000)}$ to modulate the function of TAT was studied recently (157). TAT-modified stimulus-sensitive polymeric micelles prepared to enhance interaction with cells under acidified conditions have been described (22, 158).

We also expanded the applicability of this concept by using other stimulus sensitive bonds such as enzyme sensitivity. In particular, the levels of matrix metalloprotease 2 (MMP 2) are upregulated in the extracellular matrix of the tumor. We reported a novel matrix metalloprotease-2 **(**MMP 2)-sensitive multifunctional immunoliposome comprised of a TAT peptide function shielded sterically by long-chain PEG, mAb 2C5 for active tumor targeting attached to the liposomal surface and a MMP 2-cleavable octapeptide (Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln) as a labile bond between long chain PEG and lipid. The

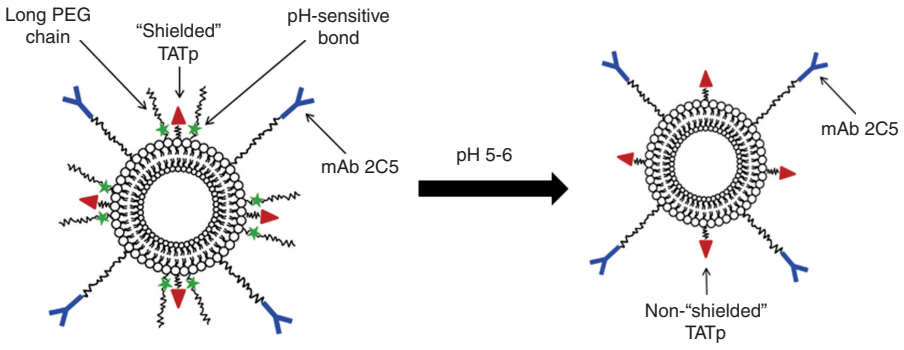

Figure 3 'Smart' nanocarrier system exposing targeting antibody while protecting CPPs.

octapeptide linker was degraded by the extracellular MMP2 in the tumor cells, exposing the TAT moiety resulting in increased cellular uptake (159).

Harris et al. developed the magnetofluorescent dextran-coated iron oxide nanoparticles modified with long-chain PEG via the MMP 2 cleavable spacer to mask CPP (160). This system was able to selectively accumulate at the xenograft tumor via the enhanced permeability and retention (EPR) effect, followed by detachment of PEG due to cleavage of the linker by endogenous MMP 2. As a consequence, CPP function was exposed and cell penetration of the iron oxide nanoparticles was activated. Similarly, Mok et al. developed MMP2-senstive, PEG- and CPP-modified quantum dots (QD) for cancer diagnosis (161).

Jiang et al. group developed a "activatable cell penetrating peptides" (ACPPs) system for tumor imaging (162). In this system, a MMP2/9 substrate peptide, XPLGLAG, was selected as the cleavable linker, and poly Glu (E9) was chosen as the inhibitory polyanionic moiety to interact with CPP. The CPP-bearing payload was delivered into tumor cells after the linker was cleaved by MMP2/9. It provided sharp-contrast images that distinguished the high-uptake regions from those with low uptake. This corresponded well to the MMP activity distribution (163). This system was further modified by adding a large- molecular-weight carrier (dendrimer, PAMAM) to the polyarginine CPP of the ACPP. This system provided amplified signals because of the presence of more than one contrast agent per peptide (164). The same group recently reported activatable cell-penetrating peptide (dtACPP) system that is dual-triggered by the lowered pH and MMP2. The dtACPP-modified nanoparticles used to co-deliver plasmid expressing interfering RNA targeting VEGF (shVEGF) and DOX resulted in effective shutdown of blood vessels and cell apoptosis within the tumor (165). Based on this strategy, an activatable low molecular weight protamine (ALMWP, E10-PLGLAG-VSRRRRRRGGRRRR) in which the positive charges on the LMWP necessary for transduction were initially masked by a polyanionic peptide (E10) sequence, a MMP-2/9 cleavable peptide linker sequence PLGLAG16 used as a linker. This ALMWP when used as tumor-targeting ligand to modify PEG-PLA nanoparticles exhibited an enhanced MMP-dependent accumulation in HT-1080 (human fibrosarcoma) cells via both energy-independent direct translocation and clathrin-mediated, cytoskeleton-dependent endocytosis (166). Pharmacokinetic and biodistribution study in HT-1080 tumor-bearing mice showed that ALMWP-modified paclitaxel-loaded nanoparticles significantly increased the accumulation of paclitaxel in the tumor site but not the nontarget tissues and also exhibited improved antitumor efficacy over that by unmodified nanoparticles and LMWP-functionalized nanoparticles.

A prodrug strategy based on electrostatic interaction, ATTEMPTS (antibody targeted, [protamine] triggered, electrically modified prodrug-type strategy) was reported. This system is based on the charge neutralization of CPP by heparin and the competitive binding between CPP and protamine with heparin. The system comprised of conjugate of the polycationic CPP and the protein drug as 'drug compartment' and 'targeting compartment made of a heparin-modified antibody. There is strong electrostatic binding between polyanionic heparin motif on the antibody and the polycationic CPP, resulting in formation of polyelectrolyte Drug-CPP Hep-Ab prodrug style complex. The CPP function is inhibited due to binding with heparin and is expected to remain stable during targeting with no detachment of the two compartments. The release of the active CPP-drug conjugates is triggered by systemic injection of protamine (167).

Shamay et al. reported a system in which, CPP was modified with photolabile caged mol- ecules, thereby providing light-dependent cell internalization (168). In this system, the positive charges of lysine residues on the CPP (52-RRMKWKK-58) were temporarily masked by photocleavable groups, Nvoc(6-nitroveratrylcarbonyl), thus forming the caged CPP (cCPP, Ac-KRRMKNvocWKNvocK-Nvoc). After illuminated with the UV light, the protecting groups (Nvoc) were cleaved and CPP function is exposed.

Concluding remarks and future perspectives

Discovery of various CPPs and their ability to deliver attached cargo intracellularly could benefit hundreds of potential therapeutic molecules including large molecules like peptides and nucleic acids. The progress in the use of CPPs would be further benefited by more detailed understanding of their mechanism of entry into cells which might lead to development of cell or tissue specific CPPs. Currently, the idea of 'smart stimuli-sensitive multifunctional nanocarrier' serves the purpose of protecting these non-specific CPPs till reaching the target. More detailed studies on bioavailability of CPP-modified nanocarrier, possible toxicity mechanisms are also important considerations, which might depend on the nature of the CPP, the linker design, and the specific cargo. Thus, thoughtful CPP-conjugate design will be high priority to the development of safe and effective therapeutics. The production of multifunctional nanocarriers economically

and on large scale is also challenging. The important criteria that should be kept in mind while designing multifunctional nanocarriers include: (1) the use of biocompatible, biodegradable materials, (2) the use of simple and reproducible bioconjugation techniques for the surface modification of nanocarriers, (3) a simple multifunctional nanocarrier assembly process, (4) the optimization of biophysicochemical properties of the nanocarrier to achieve optimal drug loading/release, a long circulation half-life, enhanced target tissue accumulation and (5) optimal nanocarrier stability.

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