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Cell-penetrating peptides: achievements and challenges in application for cancer treatment

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Abstract

One of the major hurdles to cure cancer lies in the low potency of currently available drugs, which could eventually be solved by using more potent therapeutic macromolecules, such as proteins or genes. However, although these macromolecules possess greater potency inside the cancer cells, the barely permeable cell membrane remains a formidable barrier to exert their efficacy. A widely used strategy is to use cell penetrating peptides (CPPs) to improve their intracellular uptake. Since the discovery of the first CPP, numerous CPPs have been derived from natural or synthesized products. Both *in vitro* and *in vivo* studies have demonstrated that those CPPs are highly efficient in transducing cargoes into almost all cell types. Therefore, to date, CPPs have been widely used for intracellular delivery of various cargoes, including peptides, proteins, genes, and even nanoparticles. In addition, recently, based on the successes of CPPs in cellular studies, their applications *in vivo* have been actively pursued. This review will focus on the advanced applications of CPP-based *in vivo* delivery of therapeutics (e.g., small molecule drugs, proteins, and genes). In addition, we will highlight certain updated applications of CPPs for intracellular delivery of nanoparticulate drug carriers, as well as several ‘smart’ strategies for tumor targeted delivery of CPP-cargoes.

Keywords

Cell penetrating peptides; Cancer; Macromolecule; Nanoparticle; *In vivo*

INTRODUCTION

In the past decades, numerous biomaterials have been newly developed and drawn enormous interest for their potential use in a wide variety of fields, encompassing medicine, biology, chemistry and tissue engineering.[1, 2] To date, one of the specific fields that those biomaterials have made a significant impact is the cancer therapy. The intervention of biocompatible peptides (e.g., cell penetrating peptides (CPPs)), proteins, biopolymers and

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various nanocomposites in delivery of anti-cancer drugs has revolutionized the paradigm of drug delivery. [3–6]

The first record of cancer can be traced to an Egyptian papyrus dating back to about 3000 B.C.[7] Although cancer has been known for much of our recorded history, an effective cure for this life-threatening disease is yet to be found. Efforts during the past century have led to enormous advances in our understanding of cancer pathogenesis, detection techniques, surgical methods and also the development of anticancer drugs. Despite these advances, the primary treatment of cancer has yet been limited to chemotherapy intended for relief of pain or extension of life span, especially for metastatic cases. A major hurdle for treatment of cancer by current anticancer drugs lies in the poor efficacy and inadvertent systemic toxicity caused by the narrow therapeutic window of these drugs. To this regard, drug delivery systems (DDS) have been pursued to address the toxicity issue, and significant progress has been made in this area, resulting in the approval of several DDS formulated drugs for clinical applications. The second thrust of effort, meanwhile, has been concentrated on the search for new drug entities with greater efficacy. To this issue, macromolecules (e.g., protein, peptides or genes) have gained enormous interest due to their unmatched potency and specificity compared to current anticancer drugs. The delivery of these macromolecular drugs, however, remains a formidable challenge. Many of these macromolecular drugs act upon specific processes found within the cell which require internalization of the molecules and their delivery to appropriate cellular compartments. Yet, the greater size and lower lipophilicity of macromolecular drugs, compared to small molecule counterparts, hinders their efficient cellular internalization.

An interesting finding made by Frankel and Pabo in 1988 was that transcription transactivating protein (TAT) derived from HIV virus has the ability to penetrate cellular membranes.[8] This discovery was a prelude to the identification and characterization of various cell penetrating peptides (CPPs) that possess the ability to enter cells. More interestingly, it was later found that the coupling of CPPs to different cargoes (e.g., small molecules, peptides or proteins, genes or nanoparticles) enabled efficient internalization of these cargoes into cells.[9–13] The discovery of CPPs and their ability to ferry cargoes into cells has produced a revolutionary impact on the development of macromolecule-and nanoparticle-based treatments.

In this review, we will briefly discuss the nature of various CPP-mediated drug delivery, as well as the suggested mechanisms for their cellular uptake. We will then look at the application of the CPPs for cancer treatment, focusing specifically on advanced *in vivo* studies, followed by discussing a few 'smart' strategies for targeted delivery of CPP-cargoes.

CELL PENETRATING PEPTIDES

CPPs, also known as protein transduction domains (PTDs), are a group of peptides sharing several common characteristics as follows.[14–16] First, they are peptides consisting of less than 35 amino acid residues. Second, they can efficiently internalize into cells and also carry coupled cargoes into cells with them. Furthermore, they do not pose any reported cytotoxicity. The peptides which fall in this category originate from a wide variety of sources (e.g., humans, mice, viruses or synthesis). Based on their structural characteristics, CPPs can be simply divided into 2 classes: arginine-rich CPPs and amphipathic CPPs.[17]

Arginine rich CPPs

Commonly explored arginine-rich CPPs include **TAT** peptide (TATp), oligoarginine, penetratin, low molecular weight protamine (LMWP) and pVEC.[10, 18–21] It is widely

believed that the basic arginine and lysine residues of these peptides play a crucial role in the cell internalization process.

TATp is an 11-mer peptide (YGRKKRRQRRR) derived from the transcription transactivating protein (TAT) encoded by human immunodeficiency virus type 1 (HIV-1). A study of TATp (47–57) in 1998 revealed this to be the minimum TAT protein length that retained the cell entry capability.[18] Since then, the TATp has become one of the most extensively studied CPPs.[13, 15, 22, 23]

Since the initial discovery of TATp, various researches have elucidated the critical role of cationic amino acids (i.e., arginine, lysine, and histidine) in the cell entry of TATp. Further studies have shown that oligoarginines composed of 8–10 arginine residues (Arg) can also efficiently internalize into cells.[19] It was found that among the cationic amino acids, arginine possessed the greatest potential for cell penetration, possibly attributed to its more basic nature (pKa 12.5) and the formation of strong hydrogen bonds between the guanidine groups and anionic glycosaminoglycans on the cell membrane.[24] Interestingly, it has also been demonstrated that nona-arginine (RRRRRRRRR) is the most efficient oligoarginine for the cell entry.[19, 25] Further mechanistic studies showed that D-isoform and even the branched forms of oligoarginine retained the cell penetrating ability, which suggested the possible involvement of receptor-independent cell entry pathways.[19]

LMWP is a 14-mer peptide (VSRRRRRRGGRRRR) derived by thermolysin-mediated digestion of protamine.[20] Compared with TATp, LMWP retains similar CPP motif in structure and has shown equivalent cell penetrating activity. LMWP, however, possesses several important and attractive characteristics compared to other CPPs, such as: (1) efficient mass production by simple enzymatic digestion of natural protamine; (2) proven low toxicity and immunogenicity in animal model studies; and (3) ensured safety as it originates from protamine, a widely used clinical heparin antidote.[20] LMWP has been successfully utilized to translocate various cargos into different types of cells.[20, 26–29]

While TAT protein was the first identified cell permeable protein, the first CPP discovered was penetratin. Penetratin is a 16-mer peptide (RQIKIWFQNRRMKWKK) present in the third helix of *Antennapedia homeodomain*.[4] Homeo-proteins are a class of transcription factors that bind DNA through a 60 amino acid length homeo-domain composed of three α-helices and a β-turn. Studies have shown that arginine and lysine residues are important for the cellular translocalization of penetratin.[10] Also, tryptophan residues (Trp48 and 56) are reported to play an important role in the initiation of the translocation.[10, 30, 31] Since the discovery of penetratin, a number of studies have been designed to use this CPP for delivery of various cargoes, including small molecules, proteins, oligonucleotides, peptide nucleic acid (PNA), and nanoparticles.[32–34]

pVEC, an 18-mer peptide (LLIILRRRIRKQAHAHSK) corresponding to residues (615-632) of the murine vascular endothelial cadherin (VE-cadherin), has been shown to translocate into various cell lines, even including the plant cells.[21] A non-endocytic pathway for the cell entry has been proposed, as the rate of translocation is not affected by temperature or endocytosis inhibitors.[21] Interestingly, pVEC was found to also possess antimicrobial activity at micro-molar concentrations.[35]

Amphipathic CPPs

Amphipathic CPPs are structurally distinguished from arginine-rich CPPs in that they have fewer, or completely lack of arginine residues and in that they are amphipathic in nature. Amphipathicity and lysine residues are considered to play a crucial role in the cellular translocation of this class of CPPs. They can be further divided into 2 groups: 1) peptides

with primary amphipathicity (e.g., transportan, MPG, and pep-1) – having distinct hydrophobic and hydrophilic domains separately located in the peptide sequence;[36–38] and 2) peptides with secondary amphipathicity (e.g. MAP) – having an α-helix structure with polar and non-polar residues located on the opposite sides.[39]

Transportan, a 27-mer chimeric peptide (GWTLNSAGYLLGKINLKALAALAKKIL) composed of 13 N-terminal residues of galanin neuropeptide with substitution of proline to lysine at 13th amino acid and 14 residues of mastoparan peptide from wasp venom, is able to translocate green fluorescence protein, antibody, or gold particles into cells.[36] Interestingly, a truncated form of transportan named TP10 (AGYLLGKINLKALAALAKKIL) was found to also possess equivalent cell penetrating ability to the parent transportan.[40] EL-Andaloussi *et al.* showed that a hybrid of TP10 and peptide nucleic acid (PNA), conjugated with a double stranded oligodeoxynucleotide against a transcription factor, Myc, was efficiently taken up by mouse neuroblastoma cells (N2a) and human breast cancer cells (MCF-7).[41]

Both the 27-mer MPG peptide (GALFLGFLGAAGSTMGAWSQPKKKRKV) and the 21-mer pep-1 peptide (KETWWETWWTEWSQPKKKRKV) are chimeric peptides with primary amphipathicity constructed of an N-terminal hydrophobic region and a C-terminal hydrophilic domain containing the nuclear localization signal (PKKKRKV) of the SV40 large T antigen. The hydrophobic region of MPG originates from HIV-gp41 coat protein and that of pep-1 consists of a tryptophan rich domain.[37] It was suggested that the hydrophobic residues may contribute to the peptide's interaction with, and destabilization of the cellular membrane. A truncated MPG (MPG-8: β-AFLGWLGAWGTMGWSPKKKRK-Cysteamide) was also reported to have the CPP activity.[37, 38]

Model amphipathic peptide (MAP) is an 18-mer peptide (KLALKLALKALKAALKLA) with secondary amphipathicity. Through mechanistic *in vitro* studies, MAP has been shown to internalize into cells by a non-endocytic pathway,[42] and has successfully been used to deliver PNA into cells.[43] Interestingly, it was found that MAP's cell-internalization ability was retained even after depriving of its amphipathicity.[42]

CELL ENTRY MECHANISM OF CPPs

While the mechanism of CPP-mediated cell entry has been the subject of a large number of studies, there is still no consensus on the mechanism of this translocation process. A pioneering mechanism appeared to be a passive, energy-independent process that was not sensitive to endocytosis inhibitors.[19, 21, 44] Recent studies re-evaluating the cellular entry mechanisms of CPPs or CPP-cargoes, however, have indicated the involvement of endocytosis.[45] It has been suggested that properties of CPPs (e.g., amphipathicity or charge delocoalization) and the attached cargoes can affect the cell entry pathway.[46, 47] Cell lines and incubation conditions have also been shown to affect the cell entry of CPPs.[48] This review will briefly discuss two major intracellular uptake mechanisms of CPPs: the energy-independent (non-endocytosis) pathways and the endocytic pathways. These CPP internalization pathways are illustrated in Fig. 1.

Energy independent pathways

Different energy independent cell entry pathways for CPPs/CPP-cargoes have been suggested, such as the (1) inverted micelle model,[49–51] (2) pore formation model,[52] (3) carpet model,[53, 54] and (4) membrane thinning model.[55] The first step in all of these models requires the positively charged CPPs to interact with the negatively charged components of the cell membrane, such as heparan sulfate (HS) and the phospholipid bilayer. Binding of the CPPs on the lipid bilayer then leads to temporary destabilization of the plasma

membrane. Subsequent steps in the internalization process vary depending upon the type and concentration of CPPs, cargoes, cell lines and incubation conditions.

The inverted micelle model was proposed based on NMR studies of the interaction of penetratin with phospholipid membranes. According to the hypothesized mechanism, the positively charged CPP interacts with the negative heads of the membrane phospholipids, followed by a reorganization of the lipid bilayer and membrane shuttling aided by interactions of the hydrophobic residues of the CPP with the membrane. Subsequently, the CPP moves into the bilayer while being entrapped in an inverted micelle, resulting in the move of this micelle to the opposite side of the bilayer and release of its contents directly into the cytosol. Because this model assumes the involvement of the hydrophobic residues of CPPs in the formation of the inverted micelle, it cannot be used to describe the translocation of CPPs that have no hydrophobic segments (e.g., TATp and oligoarginines). Moreover, Lewin *et al.*,[56] based on their studies with CPP-conjugated iron oxide nanoparticles (~45 nm), raised serious doubts on the formation of such “inverted micelles”. Additionally, the inverted micelle structures have not been visualized microscopically.

The pore formation model, also called the barrel-stave model, describes one way in which antibacterial peptides weaken the bacterial membrane, which is followed by depolarization of the membrane. Pore formation is the result of bundles formed by the amphipathic α-helical structure of CPPs. In this arrangement, outwardly facing hydrophobic residues interact with the lipid membrane, while hydrophilic groups form a central lumen to create the pore.

The carpet model was described as a model for translocation of some antimicrobial peptides, but was also applied to describe CPP toxicity at higher concentrations.[53, 54] In the carpet model, positively charged CPPs, monomeric or oligomeric, cover the negatively charged membrane in a carpet-like manner, which results in a change in the CPP secondary structure as the basic residues turn towards the membrane surface and the hydrophobic residues interact with the hydrophobic membrane core. When a concentration threshold is reached, the CPP permeates the membrane by locally disrupting the bilayer structure. Although this model has been suggested for CPP translocation, its dependence on hydrophobic residues seems to generate some controversy since most CPPs are primarily basic and hydrophilic.

An alternative to the carpet model is the so-called “membrane-thinning” effect, which was first suggested for a peptide toxin, magainin.[55] In this model, following carpet formation by CPPs, perturbation caused by charge interaction in the outer leaflet results in a lateral rearrangement of negatively charged lipids, followed by a thinning of the membrane. Interaction, or aggregation, of CPPs on the membrane surface produce a reduction of the local surface tension and allows CPPs to intercalate within the membrane. The membrane then reseals after internalization of CPPs onto intracellular targets.

Although all these mechanisms explain certain aspects of membrane translocation by CPPs, none of them has provided a satisfactory or complete description of the internalization pathway applicable to all types of CPPs. Moreover, none of these models adequately predicts the cellular uptake of large cargoes (up to 100-fold their own size) mediated by these CPPs.

Endocytosis

Endocytosis, including phagocytosis and pinocytosis,[57, 58] is a highly regulated process of internalization of solutes and fluids in the extracellular matrix. Phagocytosis is a complex process used to uptake large particles and pinocytosis is mainly used to bring in smaller particles. This pinocytosis can be further classified into four different pathways: 1) clathrin-

mediated, 2) caveolae/lipid raft-mediated, 3) clathrin and caveolae-independent endocytosis and 4) macropinocytosis.[59–61]

According to most of the initial mechanistic studies, the cellular uptake of CPPs was considered to follow a non-endocytic pathway. Later studies, however, showed that experimental artifacts could affect the study results.[59] For instance, the fixation of cells with methanol/formaldehyde could allow the redistribution of the CPPs bound on the cell surface, but not internalized. With further studies, there are now some agreements on the involvement of various types of endocytosis during the cell entry of CPPs or CPP-cargoes.[40, 62]

The endocytosis pathway for a particular CPP seems to strongly depend on its properties as well as that of the conjugated cargoes (type, size, charge or/and hydrophobicity). For example, TATp has been shown to enter cells *via* lipid raft-mediated endocytosis when conjugated to a protein, but through a clathrin-dependent endocytosis when the cargo was a fluorophore. When conjugated to large cargoes (MW >30 kDa), the CPP-cargoes entered cells *via* macropinocytosis. Macropinocytosis is an actin-dependent form of endocytosis that can occur in all cell lines at different rates.[60, 61, 63, 64] It is distinguished from other types of endocytic pathways by the membrane ruffling event and the size of the vesicles formed.

APPLICATION OF CPPS AS DELIVERY VEHICLES FOR CANCER THERAPY

CPP-based drug delivery has been explored to treat various diseases, including neuronal disease, asthma, ischemia, diabetes, and cancer.[22, 65–67] There are currently more than 300 published studies related to CPP-based drug delivery.[15] The most principal application among all studies has been the cancer. As an efficient carrier, the CPP has been shown to successfully deliver cytotoxic drugs (e.g., small anticancer drugs, toxins, tumor suppressor proteins, siRNA, *etc*) into tumor cells to induce apoptosis.[11, 20, 23, 68] Despite the success reported through *in vitro* studies, only a few studies have shown treatment efficacy in animal models. Here, we cover a few of the *in vivo* works conducted using CPPs to treat cancers. Additionally, recent progress in CPP-coupled intracellular delivery of nanoparticles is briefly mentioned.

CPP-coupled delivery of small molecule drugs

While small molecule anticancer drugs generally diffuse into tumor cells with great efficiency due to their small size and lipophilicity, there is a frequent occurrence of multidrug resistance (MDR) after repeated exposure of tumor cells to the same drugs.[69] To overcome this problem, studies have explored the coupling of these drugs with CPPs. For an example, Dubikovskaya *et al*. conjugated octaarginine (R8) to taxol, an anticancer drug, *via* a disulfide bond, which displayed the effect of preferentially releasing taxol inside the cells.[70] Both R8-taxol and taxol alone were tested against ovarian cancer models with OVCA-429 (taxol-sensitive) or OVCA-429T (taxol-resistant) cells implanted in the peritoneal cavity of mice. According to their results, R8-taxol and taxol had similar effects against taxol-sensitive tumors. However, when tested against a taxol-resistant tumor model, R8-taxol treated mice had significantly higher survival rates compared with mice treated with taxol alone. Interestingly, this study showed several more merits of coupling small molecule drugs with CPP, aside from the potential to overcome the MDR. One was the increased aqueous solubility of the drug, enabling use of the drug without adding surfactants (e.g., cremophore EL). The investigators also observed a high drug concentration near the injection site due to the increased adherence of CPP-drug to the surrounding tissues. Although this property might not be favorable for systemic administration of the CPP-drug, it can improve therapeutic efficacy of the drug in case of direct intra-tumor injection.

For enhanced cytotoxicity and targeted delivery of the anticancer drug, Lee *et al*. synthesized a chitosan/doxorubicin/TAT hybrid by chemically conjugating doxorubicin and TAT to the polymeric chitosan backbone. Compared with free doxorubicin or chitosan/doxorubicin without TAT, the chitosan/doxorubicin/TAT hybrid displayed more efficient cell internalization and, thereby, yielded significantly higher anti-tumor effects. This enhanced cell internalization of the chitosan/doxorubicin/TAT hybrid could further alter the biodistribution profiles of the doxorubicin, resulting in augmented tumor localization and eventually leading to significant inhibition of tumor growth in CT26 xenograft bearing mice.[71] In addition, Myrberg *et al.* designed a chimeric peptide by coupling a breast tumor homing peptide (so-called 'PEGA peptide'; 9-mer cyclic peptide: cCPGPEGAGC) with a CPP (pVEC) and then chemically conjugated this peptide to an anticancer drug, chlorambucil.[72] *In vitro* studies showed higher cellular uptake and cytotoxicity level of PEGA-pVEC-chlorambucil conjugate against breast cancer cells, than chlorambucil alone. Further *in vivo* studies proved that fluorescence dye-labeled PEGA-pVEC could target the vessels of breast tumors with lower accumulation in non-tumor tissues, compared with pVEC alone. This study exemplifies a rational design for targeted delivery of CPP-coupled drugs. However, caution must be taken when applying targeting ligands with a CPP, as previous studies showed that CPP-mediated uptake was so efficient that it could abolish targeting specificity of the conjugated antibodies.[73] In addition, Shokolenko *et al.* reported that the order of the targeting ligands and CPPs could affect the targeting efficiency of chimeric peptides.[74]

Not only CPP modification could enhance cellular internalization, but remarkably it could also augment the delivery of cargoes across the blood brain barrier (BBB) without compromising its integrity. Rousselle *et al.* showed that CPP (SynB1 or D-penetratin) modification enabled doxorubicin to bypass the P-glycoprotein-mediated efflux from the BBB, thereby increasing the transport of doxorubicin into brain parenchyma by approximately 20-fold. Interestingly, CPP modification also altered the tissue distribution profile of doxorubicin. Notably, compared with free doxorubicin, significantly lower (10-fold lower) concentration of CPP-modified doxorubicin was observed in the heart, which could potentially reduce the cardiotoxicity concerns.[75]

CPP-coupled delivery of therapeutic peptides or proteins

Apoptosis is induced by various cellular stresses and this process is regulated by certain tumor suppressor proteins, such as p53 or p16. In many human cancers, the tumor suppressor proteins are altered or dysfunctional due to mutations that occur during tumorigenesis.[76, 77] Therefore, attempts have been made to treat cancer by restoring functions of these proteins by means of delivering the full length proteins or peptides that correspond to the crucial residues of these proteins.

Snyder *et al.* constructed a retro-inverso analogue of TATp-p53 C-terminus chimeric peptide.[78] Tested against a terminal peritoneal carcinomatosis mouse model by intraperitoneal (i.p.) injection, the treatment group (mean survival time: >70 days) showed 6-fold increase of lifespan over mutant or vehicle treated mice (mean survival time: 10 days). Also, in a terminal peritoneal lymphoma model, treatment group showed 50% longer in survival time than those of the control groups (mean survival time for vehicle and mutant peptide treated groups: 35 and 33 days, respectively); with half of the mice surviving for more than 200 days. Another successful anti-tumor application was realized by using a chimeric peptide consisting of p53 peptide from its mdm-2-binding domain (residues 17–26; named PNC-28) and penetratin.[79] Programmed release of PNC-28 from an osmotic pump implanted under the skin or in the peritoneal cavity effectively blocked the subcutaneously (s.c.) or i.p. implanted pancreatic tumor growth *in vivo*.

In an attempt to restore p16 activity, Hosotani *et al.* prepared 'Trojan p16' peptide which was a disulfide bridged conjugate consisting of 20 amino acid residues of the p16 protein and penetratin, and then tested the efficacy against a pancreatic tumor animal model.[80] In both s.c. or i.p. implanted tumors, the Trojan p16-treated groups exhibited a significant inhibition of tumor growth (treatment group: 79 ± 17 mg *vs*. control vehicle only: 139 ± 15 mg *vs*. p16 only: 151 ± 15 mg *vs*. Trojan peptide only: 149 ± 12 mg) and an improved survival rate (mean survival time benefit of 6 days for treatment group) over the controls.

Fulda *et al.* attempted a different approach to induce apoptosis of tumor cells.[81] These investigators prepared a chimeric peptide consisting of N-terminal 7 amino acid residues of second mitochondria-derived activator of caspase (SMAC) and TATp. SMACs are mitochondrial proteins which play important roles in mitochondrial regulation of apoptosis.[82] Induced by apoptotic signals, SMACs are released from the mitochondria and deactivate the inhibitor of apoptosis proteins, allowing the apoptosis to proceed. *In vitro* studies showed that SMAC-TATp by itself was not effective enough to kill tumor cells, but could sensitize these cells to apoptotic stimuli, such as TNF-related apoptosis inducing ligand (TRAIL), CD95 or doxorubicin. In an intracranial U87 human glioma xenograft mouse model, delivery of a combination of SMAC-TATp and TRAIL significantly inhibited tumor growth, and complete eradication of the tumor was observed when 0.6 or 2 μg of TRAIL was administered with the SMAC-TATp.

A rather direct approach being explored to treat cancer is to deliver highly toxic enzyme drugs into tumor cells. Due to their repetitive mode of reaction, enzyme drugs offered an unmatched therapeutic potency over conventional, small cytotoxic drugs, rendering them an attractive candidate for cancer drug therapy. Gelonin, a plant origin toxin which belongs to ribosome inactivating proteins (RIPs), is highly potent in inhibiting protein translation with IC_{50} concentrations being at pico-molar levels.[83] In spite of this exceptional potency, however, the anti-cancer activity of gelonin is rather negligible because it cannot efficiently internalize tumor cells. To overcome this membrane barrier, Park *et al.* prepared a chemical conjugate of gelonin with either TATp or LMWP.[20] In cell culture studies, both the TATp- and LMWP-gelonin conjugates displayed strong anti-tumor activity when being tested against a mouse model harboring s.c. murine CT26 colorectal cancer cells. A complete inhibition of tumor growth was observed when the animals were injected with a total dose of 100 μg of either CPP-gelonin conjugates.

Apart from gelonin, caspase-3, asparaginase and a proapoptotic peptide (KLA; peptide sequence: KLAKLAKKLAKLAK) have also been conjugated with CPPs and studied *in vivo*. In the respective rat ascite model, acute lymphoblastic leukemia mouse model and lung carcinoma mouse s.c. model, CPP-caspase-3, CPP-asparaginase and CPP-KLA conjugates all yielded significantly improved survival rates over those of the control counterparts.[29, 84, 85]

CPP-coupled delivery of genes

Delivery of genes, antisense oligodeoxynucleotides (ODNs), or small interference RNA (siRNAs) could be a powerful method for cancer therapy.[86–88] Indeed, RNA interference (RNAi) has emerged as a particularly attractive therapeutic strategy, due to its great specificity against the target gene and unmatched tumor-inhibiting efficacy. However, there remain many obstacles for the delivery of naked siRNA *in vivo*. A major hurdle of RNAi treatment lies in the poor cellular permeability of siRNA drugs, due to their strong charges and relatively large sizes. Various approaches have been attempted to overcome this obstacle, such as utilizing cationic liposomes, cationic polymers, or CPPs. Among these carriers, CPPs have drawn great interest primarily because of their demonstrated low toxicity under both *in vitro* and *in vivo* conditions.

The two major methods used to attach siRNA to CPP are *via* covalent conjugation or non-covalent complex formation.[15] Although covalent conjugation may be the most ideal approach, it is technically difficult to conjugate and purify siRNA-CPP because of their strong charge-charge interactions – often leading to the formation of tightly bound complexes and aggregates. Cellular uptake of these ionic siRNA-CPP aggregates is much poorer comparing to that of the 1:1 siRNA-CPP covalent conjugates, because the exceeding negative charges of siRNA could easily neutralize the cationic amino acids of the CPP, rendering both siRNA and CPP to partially lose their biological functions. Despite such disadvantages, non-covalent, ionic complexation of siRNA with CPP has still been preferably employed in most of the published studies, primarily due to its simplicity. While most of the studies regarding siRNA-CPP have focused on proving the feasibility under *in vitro* conditions, the number of reports demonstrating success in animal models is continuously growing.

Vascular endothelial growth factor (VEGF) plays a major role in angiogenesis which is essential for the growth of tumors. Blocking the VEGF receptor (e.g., with an antibody targeted against the VEGF receptor – Bevacizumab)[89] or silencing the VEGF gene are effective ways to treat cancers.[90] Kim *et al.* used cholesterol-R9 conjugate as a carrier and prepared a complex with siRNA against VEGF (siVEGF).[91] The complex was able to silence the VEGF in cellular studies and when tested in an s.c. CT26 murine colon cancer xenograft model, the cholesterol-R9/siVEGF complex yielded significant inhibition in the growth and vascularization of the tumor compared with the complex formulated with scrambled siRNA. Another successful application of siVEGF/CPP complex was performed by Choi *et al*., who prepared the complex and tested the anti-cancer activity in an s.c. SK-HEP1-LUC xenograft mice model.[27] The siVEGF/LMWP complex significantly reduced the tumor size, which was already relatively large (700–750 mm^3) when the treatment was started.

Cyclin B, a mitotic cyclin involved in mitosis by forming a complex with cyclin-dependent kinase, has also been of interest for RNAi treatment.[92] Essential for cell division, cyclin B is often deregulated in tumors. Crombez *et al.* have prepared a siRNA against cyclin B1 in complex with a truncated version of MPG (named ‘MPG-8).[93] The siRNA/MPG-8 complex was able to down-regulate cyclin B1 levels and induce arrest of the cell cycle, leading to inhibition of the proliferation of various human cancer cell lines. Furthermore, in an s.c. xenograft tumor model, significant inhibition of tumor growth was observed after intra-tumor or intravenous (i.v.) injection of the siRNA/MPG-8 complex.

Another attractive target for RNAi-based treatment is the epidermal growth factor receptor (EGFR) which is frequently altered in various tumors, including high-grade gliomas.[94] Han *et al.* tested a complex of plasmid based EGFR siRNA (psiEGFR) and TATp-polyamidoamine dendrimer conjugated bacterial magnetic carrier against U251 glioma xenograft model *via* intra-tumor injection.[95] The treatment group showed significant inhibition of tumor growth, which was equivalent to positive control group (lipofectamine 2000/psiEGFR). Michiue *et al.* devised a fusion peptide composing of a TATp and a double stranded RNA-binding domain (referred to as PTD-DRBD) that strongly binds to any double stranded siRNA. This versatile peptide was utilized as a carrier for siRNAs of EGFR and Akt2. In an intracerebral glioblastoma mouse model, these PTD-DRBD/siRNA complexes induced significant tumoricidal activity and led to increase in longevity.[96] Kim *et al.* prepared a complex of siRNA against Human Epidermal Growth Factor Receptor 2 (HER2) and oligoarginine (R15) and tested the effect in a SK-COV3 ovarian xenograft tumor model *via* intra-tumor injection.[95] Compared with the scrambled siRNA/R15 complex or naked siRNA, the siHER2/R15 complex displayed significant reduction of tumor growth.

CPP-coupled delivery of nanoparticles

Although the translocation mechanisms of CPPs/CPP-cargoes remain highly elusive, these peptides have been widely and successfully exploited for enhanced intracellular delivery of a variety of nanoparticles, such as magnetic nanoparticles,[56, 97–101] lipid-based formulations,[33, 102–107] micelles,[108] gold nanoparticles,[109, 110] quantum dots,[111] and others.[112–116] A number of such applications of CPP-mediated intracellular delivery of nanoparticles have been summarized in Table 1.

In 1999, Josephson *et al.* first reported the conjugation of CPPs to nanoparticles.[97] In this study, TATp was conjugated to CLIOs (cross-linked iron oxide particles). The TATp-CLIO conjugate yielded efficient labeling of cells, offering the potential to function as a tool for MRI or in magnetic separation of homed cells *in vivo*. In all cell lines tested, the intracellular delivery of TATp-CLIO, with average size of 41 nm and TATp moieties of 6.7/particle, was about 100-fold higher than that of the control group (non-TATp conjugated CLIOs). Later on, the effect of TATp density on the particle surface and the sensitivity of MRI detection were investigated in mouse lymphocytes using different TATp/CLIO ratios.[100] A non-linear increase profile in cellular uptake of TATp–CLIO was observed with increasing TATp/CLIO ratio. Compared with the control, a maximum of about 100-fold increase in cell uptake was achieved using 15 TATps/CLIO particles. Because of the advancement in intracellular uptake by the TATp moieties, cells could be monitored at 100-fold lower concentrations using a higher ratio of the TATp/CLIO conjugates.

Another successful study regarding to cell labeling by CPP modified nanoparticles was reported by Stroh *et al*. These investigators prepared TATp-modified micelles constructed with polyethylene glycol-phosphatidyl ethanolamine (PEG-PE) containing entrapped quantum dots.[111] By incubation of these TATp-modified micelles with primary bone marrow lineage-negative cells, the cells could be successfully labeled *ex vivo*. When those quantum dot-labeled cells were injected through the carotid artery of MCaIV tumor bearing mice, their recruitment by the tumor vasculature could be clearly tracked *via* imaging of the quantum dots.

Modification of liposomes with CPPs such as TATp or penetratin was also proven successful in enhancing intracellular delivery over a variety of cell lines (rat cardiac myocyte H9C2 cells, murine Lewis lung carcinoma cells and human breast tumour BT20 cells).[117] Direct interaction between CPPs and cell surface appeared to be essential for the cell entry, as shielding of CPPs severely inhibited the uptake. Results showed that approximately five CPPs per particle were sufficient to enhance the intracellular delivery of liposomes. Nevertheless, the efficiency in cell uptake of liposomes was proportional to the density of the conjugated CPPs. Interestingly, the kinetics of the uptake was dependent upon the type of the CPP and cell lines. Compared with the TATp–liposomes which exhibited relatively slow cell internalization, the penetratin-liposomes internalized into cells very rapidly and reached the maximum uptake within 1 hr.

An attractive application of CPP-modified nanoparticles lies in their use as a transfection agent. Combining the high binding capacity of the nanoparticles and great translocation ability of CPPs, highly efficient gene transfection is often observed. Torchilin *et al.* prepared a non-covalent complex between TATp-liposomes and DNA.[105] Compared with the commonly used Lipotectin®, the TATp-liposome/DNA complex showed a significantly higher transfection and lower cytotoxicity *in vitro*, when incubated with mouse fibroblast NIH 3T3 cells and cardiac myocytes H9C2 cells. These complexes also displayed an efficient transfection by means of intra-tumor injection. A multifunctional envelope-type nanodevice (MEND), consisting of a condensed DNA core and a surrounding lipid envelope, was also synthesized for tumor gene delivery.[118] The MEND containing octa-

arginine on the envelope exhibited a 1000-fold higher transfection activity than the control DNA/(poly-L-lysine/lipid) complex.

Encouraged by the successes in CPPs for intracellular delivery of various nanoparticles in vitro, there has been rising interest in the use of CPP-conjugated nanoparticles for improved delivery of anti-cancer drugs *in vivo*. By combined effects of tumor targeting *via* enhanced permeability and retention (EPR) effect and CPP-mediated cell penetration, these nanoparticles can provide effective means for safer delivery of potentially toxic anti-cancer drugs with greater therapeutic efficacy. Balzeau *et al*. synthesized a lipid nanocapsule functionalized with a glioblastoma-specific CPP (NFL-TBS.40-63). When paclitaxel was loaded to this CPP-modified lipid nanocapsule and administered to GL261 glioma brain tumor bearing mice,[119] it showed selective and efficient penetration into glioblastoma cells and yielded the best therapeutic effect among all the tested groups in inhibiting tumor growth (75%). Jiang *et al*. devised a dual-functional liposome consisting of pH-responsive CPP (R6H4) and exterior hyaluronic acid coating (readily degradable by hyaluronidase rich in certain tumor milieu).[120] The negatively charged hyaluronic acid coating of the liposome was intended to mask the positive charge of CPPs during targeting and selectively expose the CPPs to tumor cells. Paclitaxel-loaded dual-functional liposome yielded significant tumor size reduction in Heps xenograft tumor mouse model, which excelled the therapeutic effect by clinically used Taxol®. In addition, a CPP (F3 peptide)-functionalized cisplatin-hydrogel nanoparticle (named “F3-Cis-Np”) was developed and evaluated for ovarian cancer treatment in both solid and i.p. tumor models.[121] The results showed that F3-Cis-Np sufficiently and selectively bound to the tumor vessels and induced significant tumor regression *via* anti-angiogenesis.

SMART STRATEGIES FOR TUMOR-LOCALIZED DELIVERY OF CPP-COUPLED CARGOES

Even though CPPs themselves were not reported to produce toxicity below the level of hundreds of micro-molar concentration, nonspecific cellular internalization of CPP-coupled cargoes might result in unexpected toxicity caused by the cargoes. In an *in vivo* study, Schwarze *et al.* reported that TATp-β-galactosidase fusion protein could be found in various organs all over the body, including the brain.[122] The general lack of the target specificity of CPPs demands that special considerations must be taken when delivering drugs with low tissue selectivity. Although the mechanism for CPP mediated cellular internalization is yet unclear, previous studies have shown that the initiation of the cell entry requires the interaction of CPPs with the cell surface glycosaminoglycans that are negatively charged. Based on this property, a key motif for achieving selective delivery of the CPP-drugs would be to utilize a “smart” strategy that could block the CPP activities during circulation until it reaches the target site, and then reverse the block at the target to expose the CPPs so that it could exert the cell penetrating activity locally.

To date, only a few DDS using the above strategy for targeted delivery of CPP-coupled cargoes have been established and tested in animal models. The blocking of CPPs was usually achieved by either directly forming complexes between cationic CPPs and anionic counterparts or by physically shielding CPPs with PEG chains. Different methods for reversing the block have been designed, including utilizing the tumor microenvironment or applying an external triggering agent.

ACPP (Activatable CPP) prodrug strategy

ACPP is a construct developed by Dr. Tsien and coworkers for selective delivery of CPPs.[123–125] The scheme of the construct is depicted in Fig. 2. The construct is composed of

a polycationic CPP (r9; nine D-form arginine residues) fused with an polyanionic counterpart (e8; eight D-form glutamate residues) *via* a peptide linker cleavable only by certain specific proteases (MMP-2 or 9) present in the tumors. While the linker is intact, r9 and e8 form an intra-molecular hairpin structure that neutralizes the CPP activity of r9. However, once the linker is cleaved in the presence of MMP-2 or 9, the r9 is dissociated from e8 due to a weakened interaction. Enhanced cellular uptake of ACPP was obvious in 2D and 3D cell culture models and even in human specimens, after cleavage of the linker by MMP-2 which was added externally or secreted from HT1080. In an *in vivo* study, investigators found that r9 could induce severe systemic toxicity with doses above 5 μmol/kg following i.v. injection, which eventually led to death of mice due to respiratory failure. However, injection of ACPP produced a much milder toxicity even with 4-fold of the tolerable dose.

CPP-modified ATTEMPTS (Antibody targeted triggered electrically modified prodrug type strategy)

The ATTEMPTS is a drug delivery strategy utilizing active targeting and prodrug feature for selective delivery of macromolecules to the disease site.[126] The scheme of the DDS is illustrated in Fig. 3. The underlying principle of this DDS is by forming a plasma-stable tight complex between the targeting component, the antibody-heparin conjugate, and the drug component, a CPP-coupled drug. The complex is formed by electrostatic interaction between the anionic heparin and the cationic CPP. With the complex formation, the charge of the CPP is reversibly masked by heparin, leading to an improved antibody targeting as well as plasma stability of CPP against endogenous proteases. The drug delivery strategy works in two steps. In the first step, the DDS is administered and allowed to accumulate in the tumor site *via* antibody-mediated targeting. In the second step, protamine is systemically injected as a triggering agent to reverse heparin inhibition on the CPP, when the tumor to non-tumor concentration ratio of the DDS is optimal. Protamine is a small cationic protein that has been used clinically as the antidote for heparin. Due to the stronger binding between heparin and protamine compared to that between heparin and the CPP, protamine triggers the release of the CPP-drug from the DDS, and the CPP-drug is now free to penetrate through the tumor cell membrane. The feasibility of the DDS was demonstrated in an *in vitro* study by Kwon *et al*.[126] Based on the confocal microscopy and FACS results, cell uptake of LMWP-asparaginase conjugate was shown to be effectively blocked by heparin, and this blockage was successfully reversed by the addition of protamine. Further studies are currently ongoing in Yang's lab by utilizing an anti-CEA antibody for targeting colorectal cancer and TATp modified-gelonin.

Reversal shielding by pH-sensitive PEG

A modified Doxil®-based nanocarrier (doxorubicin-loaded PEGylated liposome) was reported recently.[127] This strategy was by incorporating a 2C5 monoclonal antibody with long-protective PEG chains as well as short PEG chains linked with TATp; both were linked *via* the pH-responsive hydrazone bond. Compared with the unmodified, commercial Doxil®, this multifunctional liposomal formulation significantly augmented the cytotoxicity and intracellular uptake of the drug, when being examined against four different cancer cell lines under the pre-exposure to low pH conditions. Other studies of multifunctional TATp-coupled liposomes and micelles by utilizing similar strategies were also reported elsewhere.[128] At pH 7.5–8.0, these liposomes or micelles displayed very limited internalization by either NIH-3T3 or U-87 cells. However, in contrast, effective internalization into both NIH-3T3 and U-87 cells was achieved after brief incubation (15–30 min) at a low pH (pH 5.0–6.0) under which the nanocarriers shed their PEG shielding by hydrolysis of the hydrazone bonds.

TATp-modified and GFP-loaded liposomes were also prepared and studied *in vivo* by means of intra-tumor injection.[129] The administration of liposomes without pH-degradable PEG coating resulted in very limited transfection of tumor cells, simply because of the TATp moieties were being shielded by the PEG chains. As a comparison, the administration of liposomes with pH-sensitive PEG showed a much higher transfection of tumor cells. Removal of the PEG shield by the acidic tumor environment led to the exposure of TATp on the liposome surface and, as a consequence, yielded much enhanced cellular uptake of the model gene, pEGFP-N1. The scheme of this approach is illustrated in Fig. 4.

Tumor specific protease cleavable PEG shielding

In the tumor microenvironment, the levels of certain enzymes (so-called tumor specific proteases) such as MMP-2 or 9 are often elevated in the extracellular space of the tumors. This up-regulated enzyme activity provides a unique strategy to expose CPP molecules conjugated on the surface of nanoparticles selectively to tumor cells, by masking the nanoparticles with PEG chains that are cleavable by these tumor specific proteases. Harris *et al.* prepared a magnetic nanoparticle conjugated with PEG chains *via* MMP-2 cleavable peptide linkers and polyarginine peptides (NH2-RRRRGRRRRK(FITC)GC).[101] When the veiled polyarginines under the PEG chains were exposed by cleavage of the PEG chains *via* MMP-2, 40-fold higher cell accumulation of nanoparticles were observed *in vitro*. The *in vivo* feasibility of this system was further confirmed by MRI and fluorescence imaging. The scheme of this approach is also depicted in Fig. 4.

CONCLUSION

CPPs have been exploited in a wide variety of theranostic applications, including the intracellular delivery of small anticancer drugs, macromolecules, and nanoparticle systems. Although the mechanism of CPP translocation remains unclear, new CPPs including modification of existing CPPs are being designed and tested, which will no doubt extend the therapeutic applications of CPPs. The potential of CPPs to deliver highly potent and highly specific macromolecular drugs is of particular interest. The ability of CPPs to enter cells of almost any type, however, still possesses significant toxicity concerns that must be appropriately addressed. To overcome such toxicity issues, different types of “smart” strategies have been designed to activate the CPPs only within diseased tissues. Although it may be too early to predict clinical applications of CPP-cargoes, the current achievements in the CPP-mediated delivery of drugs *in vivo* are inspiring, and the future of CPP-based effective and safe chemotherapy indeed looks very promising.

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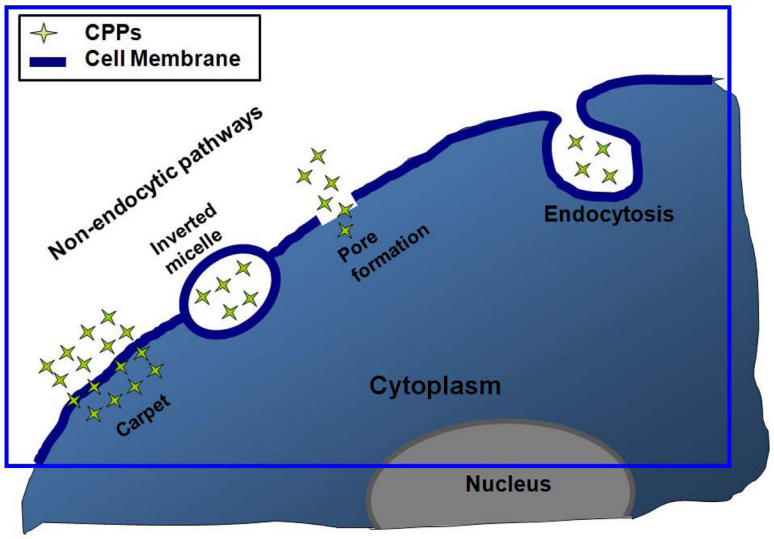

FIGURE 1.
Schematic illustration of cell entry mechanism of CPPs. Reproduced with permission from Ref. [23].

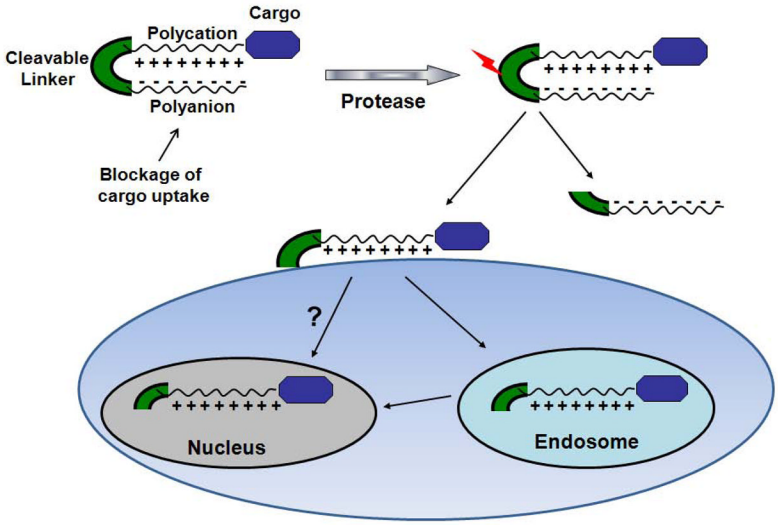

FIGURE 2.

Schematic illustration of ACPPs. CPP is blocked by a polyanionic peptide covalently attached *via* a MMP2/9-cleavable linker. Cleavage of the linker induces exposure of CPP and enables cell internalization. Reproduced with permission from Ref. [123].

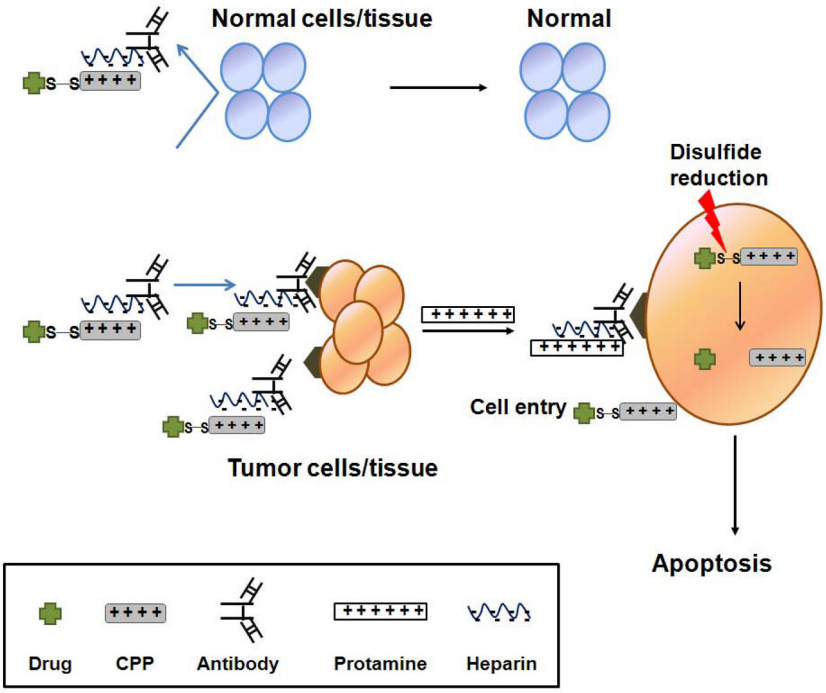

FIGURE 3.
Schematic diagram of the CPP-modified ATTEMPTS delivery system. Reproduced with permission from Ref. [126].

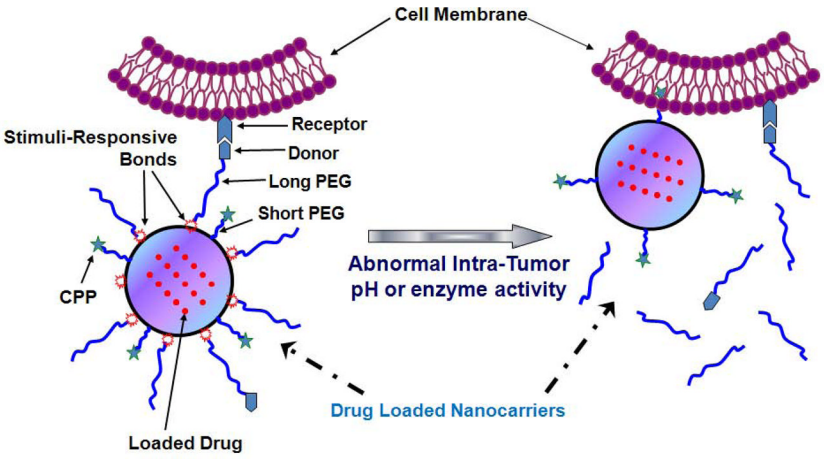

FIGURE 4.
Schematic illustration of tumor-environment-responsive exposure of CPPs on ‘smart’ nanocarriers. Reproduced with permission from Ref. [13].

Table 1

Summary of CPP modified nanoparticles and their cellular applications.

Nanoparticles	CPPs	Cell lines and/or Effects	Refs
Liposomes	TATp, Penetratin, Octa-arginine	Calu-3 12- to 17-fold enhanced cell uptake of liposomes	102
	TATp	COS-7	103
	Poly-arginine	NCI-H446, A549, SK-MES-1 Enhanced siRNA intracellular delivery and tumor cell growth inhibition	104
	TAT, Penetratin	SK-BR-3, MCF-7, HTB 9, ADR, A431,C26 12-fold increase of Dox intracellular uptake	33
	TATp	LLC, BT20, H9C2 Enhanced cell uptake of liposomes	107
CLIO (MION)	TATp	Mouse lymphocytes, human natural killer, HeLa, human hematopoietic CD34+, mouse neural progenitor C17.2, human lymphocytes CD4+, T-cells, B-cells, macrophages, stem cells MRI imaging and magnetic separation of homed cells	56, 97–99
pH sensitive PEG-polylactic acid micelles	TATp	MCF-7 Enhanced cytotoxicity at acidic environment (pH < 7)	108
Gold nanoparticles	TATp Other NLS peptides	NIH3T3, HepG2, HeLa, hTERT-BJ1 Enhancedcytosolic and nuclear uptake of particles	109,110
Quantum dot-loaded micelles	TATp	MS1, lineage-negative bone marrow cells Dynamic imaging and tracking of labeled cells	111
PEI-PEG-TAT/DNA nanoplexes	TATp	SH-SY5Y Enhanced gene expression	112
	TATp	A549 Enhanced gene transfection of lung cells	113
Boron carbide nanoparticles	TATp	EL4, B16F10 Boron neutron capture therapy	114
Dendrimers	TATp, Penetratin	MDR3T3, MES-SA/Dx5 Enhanced ODN intracellular delivery	115
Solid lipid nanoparticles	DimericTATp	16HBE14o- Enhanced gene transfection	116