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Cell-penetrating peptides and their analogues as novel nanocarriers for drug delivery

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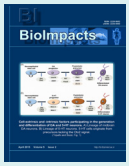

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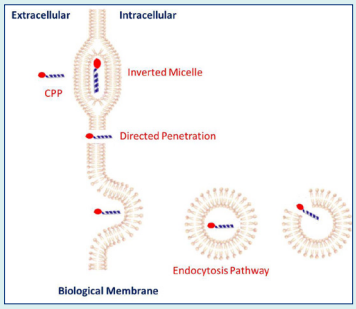
Abstract



Introduction: The impermeability of biological membranes is a major obstacle in drug delivery; however, some peptides have transition capabilities of biomembranes. In recent decades, cell-penetrating peptides (CPPs) have been introduced as novel biocarriers that are able to translocate into the cells. CPPs are biologically potent tools for non-invasive cellular internalization of cargo molecules. Nevertheless, the non-specificity of these peptides presents a restriction for targeting drug delivery; therefore, a peptidic nanocarrier sensitive to matrix metalloproteinase (MMP) has been prepared, called activatable cell-penetrating peptide (ACPP). In addition to the cell-penetrating peptide dendrimer (DCPP), other analogues of CPPs have been synthesized.

Methods: In this study, the most recent literature in the field of biomedical application of CPPs and their analogues, ACPP and DCCP, were reviewed.

Results: This review focuses on CPP and its analogues, ACPP and DCPP, as novel nanocarriers for drug delivery. In addition, nanoconjugates and bioconjugates of these peptide sequences are discussed.

Conclusion: DCCP, branched CPPs, compared to linear peptides have advantages such as resistance to rapid biodegradation, high loading capacities and large-scale production capability.

Introduction

Although the existence of phospholipid membrane is necessary for cells survival and their function, it is a major obstacle for intracellular cargo delivery. Until recently, transport of hydrophilic macromolecules into cells was not possible without interruption of the plasma membrane. This problem was resolved with the discovery of peptides. Cell-penetrating peptides (CPPs) are one promising class of peptide carriers that indicate transition capability through biomembranes.[1,2]

CPPs are positively-charged short peptide sequences, rich in lysine or arginine. These peptide sequences are also known as protein transduction domains (PTDs), protein translocation domains, membrane translocating sequences and Trojan peptides.[3] These cationic peptides can facilitate cellular internalization of therapeutic agents; this is attributed to the interaction between the negatively-charged plasma membrane and the positively-charged carrier.[4,5] A CPP/cargo combination can block the endocytosis pathway and translocate directly into cells without consuming energy.

These peptide carriers have advantages such as high internalization, ease of synthesis, potential for sequences modification and low cytotoxicity. HIV TAT (HIV-1transcriptional activator protein) peptide and penetratin are the primary peptides that can cross the biomembranes.[6-8]

Today, with the arrival of nanotechnology in the medical field, the limitations of traditional drug delivery are being overcome. Nanomedicine has been focused on bioimaging, drug delivery systems and new drug therapies using nanoparcticles (NP), which are ultrafine particles in

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the range of 1-100 nanometers in size.[9-14]

These particles have dominant physicochemical properties including high surface-to-volume ratio and small size. The former results in a high therapeutic molecule load on the surface of NPs and the latter lead to passage of material from barriers such as the blood–brain barrier [BBB], the central nervous system (CNS), the gastrointestinal (GI) tract, the capillaries and the lymphatic system.

The conjugation of NPs, including gold nanoparticles,[15,16] quantum dots (QDs),[17,18] magnetic nanoparticles,[19-22] polymeric nanoparticles,[23-25] polymeric micelles,[26,27] lipid nanoparticles[28] and solid lipid nanoparticles (SLNs),[29] to CPPs has been a matter of interest in many recent studies. In this review, we briefly describe CPPs, their uptake mechanism and conjugation of them to biomolecules and nanomaterials, as well as introducing two new promising CPPs called the activatable cell-penetrating peptide (ACPP) and the cell-penetrating peptide dendrimer (DCPP).

Classification of cell-penetrating peptides

In the literature, there are various classifications for CPPs. Due to their physicochemical properties, CPPs are divided into three classes: cationic, amphipathic and hydrophobic. Most CPPs are cationic because of their positive charge. Amphipathic CPPs are sequences that contain non-polar and hydrophobic amino acids. Hydrophobic CPPs include only non-polar sequences, low net charge and/or a hydrophobic motif. Hydrophobic CPPs are fewer than cationic and amphipathic CPPs.[30] Some examples of these categories accompanied by their sequences are presented in Table 1.

In another classification, CPPs are categorized based on the origin of the peptide:

- Derived CPP
- Chimeric CPP
- Synthetic CPP

Examples of derived peptides are TAT and penetratin. Chimeric peptides include two or more motifs from different peptides, such as transportan, derived from mastoparan and galanin, and its shorter analogue TP10. Synthetic peptides for example the polyarginine family are another type in this category.[35,36]

Uptake mechanism of cell-penetrating peptides

Although the mechanism of internalization of CPPs into the cells is obscure, researchers have proposed three main possibilities for CPPs translocation across the biological membranes:[37-39]

1) Direct penetration: This pathway involves interactions between positively charged CPPs, the phosphate groups on both sides of the lipid bilayer of cellular membrane, the formation of cavities in order to facilitate transition and the direct penetration of CPPs into the cytoplasm.
2) Endocytosis mediated translocation (energy-dependent pathway): During the process of endocytosis, cells capture materials from the outside of the membrane and absorb them.
3) Translocation through the formation of a transitory structure: This mechanism is based on the formation of the inverted micelles that are also known as aggregates of colloidal surfactants. The structure of the inverted micelles allows the peptide to be stable in a hydrophilic environment. In this model, a penetratin dimer combines with the negatively-charged phospholipids that lead to the formation of an inverted micelle inside of the lipid bilayer.

In most of the literature, there are indications that endocytosis pathway is the dominant mechanism of CPPs uptake. Fig. 1 schematically demonstrates the proposed models of the internalization of CPPs with cargo across the cell membrane.

Table 1. Examples of cationic and amphipathic CPPs with their sequences

Type of CPP	Example	Ref.
Cationic	Tat: GRKKRRQRRRPPQ Penetratin: RQIKIWFQNRRMKWKK	31,32
Amphipathic	Transporant: GWTLNSAGYLLGKINLKALAALAKKIL Pep-1: KETWWETWWTEWSQPKKKRKV MAP: KLALKLALKALKAALKLA	33,34

CCP, cell-penetrating peptide

Conjugation of nanoparticles with the cell-penetrating peptides

With the advent of nanotechnology and its widespread applications in the medical field, enormous advances have been made in the treatment of various diseases including different types of cancers, AIDS and hepatitis.

Nanoparticles refer to materials with a size in the range of 1-1000 nanometers and include a group of compounds such as metals, semiconductor quantum dots (QDs), oxides, polymers, vesicles (e.g., micelles/liposomes), carbon-based materials (e.g., nanotubes, fullerenes and nanodiamonds) and protein- and nucleic acid-based particles. In recent decades, nano-sized materials, because

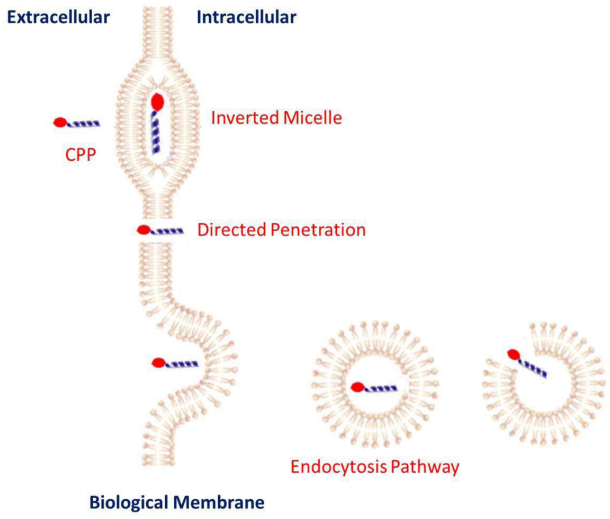

Fig. 1. Translocation mechanisms of CPPs into the cells.

of their dominant properties such as large surface areas, their binding to a large number of surface functional groups, appropriate distribution, controllable absorption and their release properties, have attracted increasing attention in medical science.[40,41]

The following briefly introduces conjugated nanoparticles with CPPs and their analogues such as activatable cell-penetrating peptide (ACPP) and cell-penetrating peptide dendrimer (DCPP).

Conjugation of quantum dots with cell-penetrating peptides

QDs are fluorescent colloidal semiconductor nanocrystals. These inorganic nanoparticles, because of their remarkable properties including broad excitation, dependence of fluorescent emission on the QD composition and core size and narrow size distribution, are used widely in drug delivery, labelling and imaging fields.[42,43]

CCP functionalized QDs have been used for effective intracellular delivery of fluorescent proteins, including yellow fluorescent protein (YFP) and the multichromophore b-phyco-erythrin complex (b-PE). Delivery of these proteins with direct microinjection of QD-protein cargos into live cells bypassed the endolysosomal system and resulted in a more homogeneous distribution of conjugates throughout the cytosol, while conjugates of QD-peptide-protein were distributed within the endosomal compartments.

Recently, Liu et al demonstrated that interaction of QDs with chimeric IR9 CPP (IR9: combination of INF7 fusion peptide and nona-arginine, R9) and formation of stable IR9/QD complexes led to the efficient localization of these complexes inside cells. Electrostatic interactions of IR9/cargo complexes, due to the cationic nature of IR9, with the plasma membrane play an important role in cellular internalization. IR9 and IR9/cargo complexes are not cytotoxic at their low concentrations for cells; therefore, these new chimeric CPPs could be a powerful tool in the study of biological processes, such as gene expression.[44]

Conjugation of superparamagnetic iron oxide nanoparticles with cell-penetrating peptides

Superparamagnetic iron oxide nanoparticles (SPIONs), due to their valuable magnetic properties and low toxicity, have been extensively studied in drug delivery, gene delivery and contrast-enhancing agents in MRI.[45-47] Most available SPIONs cannot penetrate into cells; therefore, in order to develop uptake of these nanoparticles by cells, their surfaces have to be modified. Wang et al suggested that conjugation of TAT peptide to SPIONs could increase the translocation of these nanoparticles.[48] Flow cytometry assays revealed that TAT-decorated SPIONs have higher cellular uptake, and their greater accumulation over the unmodified SPIONs is due to the positive charge on the TAT surface and its positive zeta potential.

In another study, conjugation of ɣ-amino-proline-derived cell-penetrating peptide, a novel synthetic CPP, with SPIONs demonstrated higher translocation of these nanoparticles into the HeLa and COS-1 cells compared to the analogue TAT–SPION. Therefore, this new CPP was used to design efficient bimodal imaging nanoagents.[49] The stability of these types of peptides towards protease degradation, imparted by the ɣ-peptide skeleton and also their low toxicity are important advantages of this new nanocarrier.

Conjugation of gold nanoparticles with cell-penetrating peptides

Among metal nanoparticles, gold nanoparticles (GNPs) command great interest in drug delivery systems. Due to their special properties, such as induced minimum toxicity, high solubility, easy synthesis, bioconjugation, strong absorption, efficacious clearance from the body and scattering, they have encouraged many medical researchers.[50-52] Conversely, GNPs have a positive charge; therefore, they can accompany cationic CPPs in translocating into the cells by an energy-independent method. The α-helix peptides 17-amino acids conjugated to gold nanoparticles are used as carriers for delivery of the anti-cancer drug doxorubicin (DOX); it has been shown that this system has higher efficiency in cell-selective drug delivery than free DOX, which is attributed to the cell-selective internalization activity of the chosen peptides.[16]

Conjugation of polymeric nanoparticles with cell-penetrating peptides

Proper cellular uptake and high transfection efficiency have important roles in safe and effective gene delivery. Chitosan (CS) is a natural cationic copolymer that due to good biocompatibility, biodegradability and low cytotoxicity, has been extensively studied as a suitable carrier for drug delivery.[53-56] However, application of this polymer in gene delivery has been limited by its low gene transfection efficiency.

Penetratin, pAntp peptide, is a peptide sequence with 43.75% basic amino acids derived from drosophila antennapaedia homeodomain. Layek and Singh employed CPP and penetratin conjugated CS as a promising non-viral vector for gene delivery. Results demonstrated that linoleic acid and penetratin dual functionalized chitosan (CS-Lin-Pen), a modified CS, has been applied successfully for transfection of plasmid DNA (pDNA). The modified CS exhibited the impressive protection of pDNA from DNase I attack and ~34–40-fold higher transfection compared to unmodified CS.[57]

The blood–brain barrier (BBB) is a major obstacle in brain drug delivery. The BBB consists of endothelial tight junctions; crucially, it restricts the diffusion of therapeutic molecules into the central nervous system (CNS).[58,59] CPPs linked to nanoparticles have been introduced as an appealing carrier for improving brain-targeted delivery, though because of their positive charge the brain delivery efficiency of these carriers could be cancelled out by their rapid systemic clearance.[60,61]

It has been reported that penetratin functionalized poly (ethylene glycol) – poly (lactic acid) (PEG–PLA)

nanoparticles successfully was used for brain drug delivery. Results revealed that PEG–PLA coupled to the CPP reduces systemic clearance of nanoparticles. Moreover, penetratin conjugation on the surface of nanoparticles could enhance their cellular uptake.[62]

Conjugation of lipid nanoparticles with cell-penetrating peptides

Efficacious and lucrative internalization of small interfering RNA (siRNA) in the gene therapy depends on plasma half-life and biodistribution of siRNA, because naked RNA is easily degraded by RNase in the body. One non-invasive technique in the transition of siRNA is the entrapment of siRNA within nanoparticles for protection from enzymatic degradation.[63,64]

Recently, protamine peptide coupled cholesterol nanoparticles have been designed as an effective vector for improvement of delivery half-life and efficiency of siRNA delivery. Interaction of CPP with the endosomal membrane as well as the positive zeta potential of CPP-lipid nanoparticles could facilitate the transfer of siRNA into the cytoplasm.[65]

Bioconjugates of cell-penetrating peptides

In recent years, diagnostics and treatment of diseases using oligonucleotides has attracted great attention. Due to the negative charge of plasma membrane and oligonucleotides, a positively-charged vector is necessary for efficient delivery of these biomaterials. The cationic nature of CPPs facilitates transduction and promotes stability of nucleic sequences.[66-68]

High molecular weights, hydrophilicity and enzymatic degradation of peptides and proteins decrease the parameters of intestinal absorption in oral delivery of peptide and protein drugs.[69-71] Morishita et al demonstrated that the co-administration of insulin as a peptide drug and D-R8 (D-form arginine octamer, a typical CPP) enhances intestinal absorption of the peptide drugs because of intermolecular binding between the D-R8 and the insulin.[72]

Efficient intracellular delivery of antibodies is confined because of the hydrophobic nature and large size of these biomolecules. On the other hand, the antibody molecule is degraded within the lysosome; therefore, to prevent the lysosomal degradation, it needs to be released into the cytoplasm by fracturing the endosome.[73]

There are two dominant approaches for effective entrance of antibody fragments to targeted compartments. The first is delivery of DNA encoding for an antibody fragment within the cell. The second is the delivery of the antibody molecule into the cytoplasm by suitable vectors. Diverse vectors are used for the enhancement of transition efficacy of antibodies into cells such as quantum dots,[74,75] carbon nanotubes,[76,77] gold nanoparticles[78] and polymeric nanoparticles.[79,80]

Recently, CPPs have been employed as a promising vector for introducing the above mentioned biomolecules into cells.[81,82] Montrose et al constructed the Xentry complex (a CPP derived from an N-terminal region of the X-protein of the hepatitis B virus), using an antibody and siRNA, in order to enhance the uptake of antibodies and increase the capacity for killing B-raf-dependent melanoma cells.[83]

RNA interference technology has been demonstrated as an effective therapeutic modality in vivo for the reduction of pathological molecules in neurons, for the treatment of neurodegenerative diseases. Neurodegeneration is the general term for the progressive loss of structure and function of neurons. Malhotra et al utilized TAT oligopeptide, as a model CPP, covalently conjugated to the chitosan (CS)-PEG copolymer for siRNA delivery targeting neurodegenerative diseases.[84]

Particle size and surface charge are two key factors in the intracellular delivery of siRNA. Due to the positive charge of the nanoparticles and the negative charge of cell membranes, the cells take up these biomolecules through an adsorptive mechanism. The results showed that the unmodified CS nanoparticles were greatly toxic, though the conjugation of PEG and TAT on CS nanoparticles significantly reduced the toxicity and improved the intracellular delivery of siRNA.

Activatable cell-penetrating peptides

CPPs, despite their many advantages, have limited in vivo application due to their non-specificity. Lately, a novel strategy of CPPs, called activatable cell- penetrating peptides (ACCPs), is used in targeted cargo delivery. ACPPs, new CPPs with high permeability, are composed of a polycationic cell-penetrating peptide attached to a polyanionic peptide through a cleavable linker and sensitive to metalloproteinase (MMP).[6,85] As a result of the high level of expression of MMP in tumour cells, ACPPs can be used for site-specific targeting and delivery of anticancer drugs. Indeed, MMPs are disease biomarkers which can be used for improvement of diagnosis or application in image-guided surgery with radiolabeled MMP binding ligands, such as antibodies or small molecules.[87,88] Detection of MMP activity in tumours by radiolabeled ACPPs has been identified as a strong enhancement of tumour retention in vivo. Fig. 2 schematically demonstrates ACCP and its internalization into the cell.

Paclitaxel (PTX) is used in cancer chemotherapy as one of the most appropriate antiproliferative agent which prevents cell proliferation by stabilization of microtubules and tubulin polymerization, leading to cell apoptosis.[89] Xia et al investigated ACCPs functionalized nanoparticles (NPs) with enhanced permeability for site-specific targeting of PTX in the tumour tissue.[90] The findings demonstrated that PTX loaded by ACCPs-NP had a higher accumulation in the tumour site than unmodified nanoparticles, and therefore, these systems could enhance antitumor efficacy over the CPP-PTX-NP, PTX-NP and PTX.[90]

Cell-penetrating peptide dendrimer (DCPP)

Therapeutic peptides have a typically linear structure, but branched peptides are also found in nature. Highly

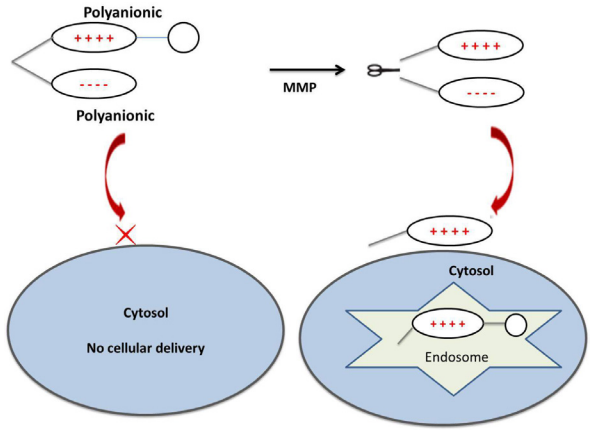

Fig. 2. ACPP and its internalization into the cells.

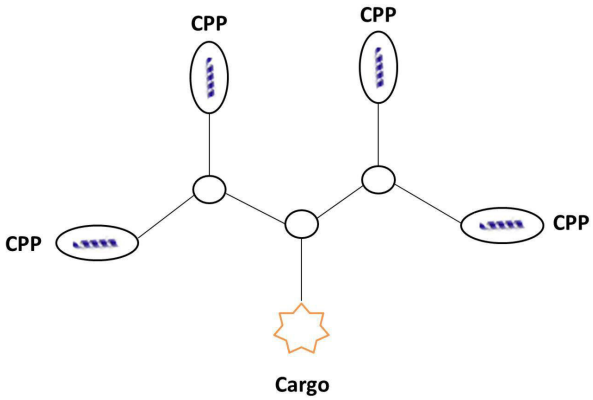

Fig. 3. Structure of cell penetrating peptide dendrimer.

branched structure, the so-called dendrimer, is derived from the Greek word, *dendron*, meaning tree. In the last few years, CCP dendrimers (DCCPs) as novel peptide carriers have attracted a great deal of attention. Dendrimers, in comparison to many linear peptides, have advantages such as resistance to rapid biodegradation, high loading capacities and large-scale production capability.[91,92] Fig. 3 demonstrates the dendritic structure of DCCP. In a study, polyester-based dendritic guanidine with a focal point alkyne conjugated to Fe_3O_4 nanoparticles has been used for enhancement of the cellular uptake of these particles. Investigations demonstrated that the functionalization of Fe_3O_4 nanoparticles with branched guanidine led to the increase of cell uptake and the improvement of the ability to detect the cells by MRI.[93]

Zhao et al employed different generations (G) of lysine dendrimers (G1–G3) as the carriers for delivery of anticancer drug 5-fluorouracil.[94] Flow cytometric analysis of these new peptide sequences showed that they have the capacity for effective translocation into cells. In addition, investigations demonstrated that these new CPPs have advantages such as stable drug release, low toxicity to normal cells, and moderate inhibition of tumour cells.

Final remarks and expert opinions

Transition of hydrophilic macromolecules into the cellular compartments without interruption of biological membrane is the main goal of the novel drug delivery systems. The effective passage of therapeutic agent across the biological membrane could decrease the quantity of administrated drug as well as its side effect on the normal tissues. Incapability of the traditional drug delivery techniques in the transport of drug molecules into the target cells together with a need to the design of novel carriers for drug targeting have led to widespread researches in this field. Various methods have been investigated to overcome biological barriers, among which peptidic carriers have attracted great attention. Short peptide sequences with positive charge, CPPs, are one of these promising and attractive biocarriers.

CPPs have potential for non-invasive cellular internalization of the drugs, imaging probes, oligonucleotides, peptides, proteins and antibodies by forming nanoparticulate carriers. Furthermore, CPPs have considerable ability in the transduction and transfection in gene therapy. A wide range of studies has been successfully carried out using CPPs for intracellular cargo delivery in vitro (Table 2). The transcellular delivery ability of these peptide sequences could increase drugs bioavailability and subsequently improve therapeutic efficiency of these molecules. Recently, CPPs conjugated nanostructures have been frequently investigated for cargo delivery into the cells because of suitable physicochemical properties such as large surface areas, their binding to a large number of surface functional groups, appropriate distribution, controllable absorption and appropriate release properties.

Despite the extensive use of CPPs for delivery purposes, the exact mechanism of these peptides is still obscure. However, most of researchers have proposed endocytosis pathway and energy-dependent pathway for these peptide sequences. Indeed, interaction between the negatively-charged plasma membrane and these cationic carriers (i.e., CPPs) facilitates their cellular internalization.

Regardless of several in vitro researches on CPPs, their in vivo applications are still restrained due to the non-specificity of these peptides and their accumulation both in targeted and non-targeted cells. Thus, extensive efforts were performed for design of site-specific peptides.

ACPPs, activatable CPPs, are new CPPs with high permeability. ACPPs are composed of a polycationic cell-

Table 2. Some applications of amphipathic and cationic CPPs in biomedical field

Type of CPP	Application	Ref.
Amphipathic	Gene delivery (siRNA delivery, plasmid DNA delivery)	95-97
Cationic	Drug delivery (anticancer drugs)	51,98,99
	Protein delivery (neuropilin-1 delivery)	100
	Imaging probes (fluorescently labeled bovine serum albumin, protease-activated peptides)	101,102

CCP, cell-penetrating peptide

penetrating peptide attached to a polyanionic peptide through a cleavable linker, which is sensitive to matrix metalloproteinase (MMP). High level of expression of MMP in cancer, atherosclerosis, and heart failure together with the undemanding recognition of these proteases by radiolabeled ACPPs make them a suitable biomarker in diagnostic of these diseases. Therefore, ACPP strategy has attracted noteworthy attention for site-specific targeted delivery of anticancer drugs. Conjugation of ACPP-nanoparticles (NP) with anticancer drugs reveals higher permeability of these biocarriers and enhances antitumor efficacy of the drugs compared to CPP-NP- drugs and NP-drugs.

Although, therapeutic peptides commonly have linear structure, DCPPs could also be considered as novel peptidic carriers due to their resistance to rapid biodegradation, high loading capacities, scaled-up capability and monodispersity. The dendritic structure of DCPPs leads to a stronger interaction with the cellular components and consequently effective translocation of these peptidic compounds; nevertheless, the larger sizes of these molecules initiate their increased cytotoxicity. Accordingly, utilization of DCPPs as a carrier requires more attention and consideration.

Investigation of literature has exhibited that ACPPs are safer, non-toxic and more effective carriers in targeted cargo delivery in comparison with CPPs and DCPPs.

Ethical issues

The authors declare no ethical issues.

Competing interests

The authors declare no conflict of interests.

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Review Highlights

What is current knowledge?

√ Description of CPPs and their classification.
√ The uptake mechanism of CPPs.
√ Conjugation of CPPs to biomolecules and nanomaterials.

What is new here?

√ An introduction to two attractive analogues of CPPs; Activatable CPPs (ACPPs) and CCP dendrimers (DCPPs).
√ A review on the advantages of ACPPs and DCPPs.

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