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REVIEW PAPER

Application of Cell Penetrating Peptides as a Promising Drug Carrier to Combat Viral Infections

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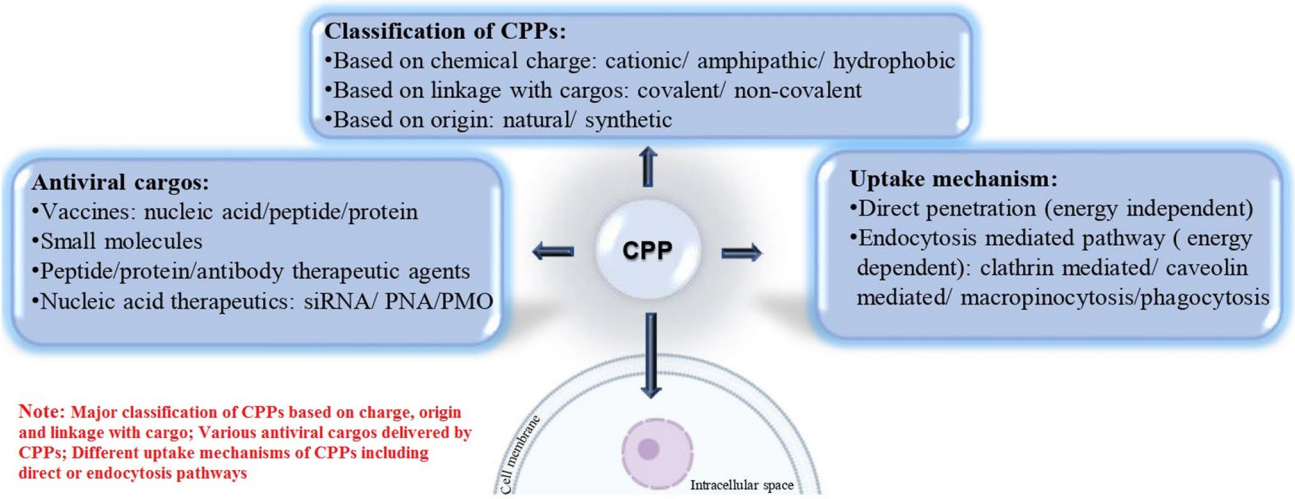
Abstract

Novel effective drugs or therapeutic vaccines have been already developed to eradicate viral infections. Some non-viral carriers have been used for effective drug delivery to a target cell or tissue. Among them, cell penetrating peptides (CPPs) attracted a special interest to enhance drug delivery into the cells with low toxicity. They were also applied to transfer peptide/protein-based and nucleic acids-based therapeutic vaccines against viral infections. CPPs-conjugated drugs or vaccines were investigated in several viral infections including poliovirus, Ebola, coronavirus, herpes simplex virus, human immunodeficiency virus, hepatitis B virus, hepatitis C virus, Japanese encephalitis virus, and influenza A virus. Some studies showed that the uptake of CPPs or CPPs-conjugated drugs can be performed through both non-endocytic and endocytic pathways. Despite high potential of CPPs for cargo delivery, there are some serious drawbacks such as non-tissue-specificity, instability, and suboptimal pharmacokinetics features that limit their clinical applications. At present, some solutions are utilized to improve the CPPs properties such as conjugation of CPPs with targeting moieties, the use of fusogenic lipids, generation of the proton sponge effect, etc. Up to now, no CPP or composition containing CPPs has been approved by the Food and Drug Administration (FDA) due to the lack of sufficient *in vivo* studies on stability, immunological assays, toxicity, and endosomal escape of CPPs. In this review, we briefly describe the properties, uptake mechanisms, advantages and disadvantages, and improvement of intracellular delivery, and bioavailability of cell penetrating peptides. Moreover, we focus on their application as an effective drug carrier to combat viral infections.

Graphical Abstract



Keywords Antiviral therapy · Cell penetrating peptide · Drug · Intracellular delivery · Physicochemical properties · Uptake mechanism

Extended author information available on the last page of the article

Introduction

Cell penetrating peptides (CPPs) with 5–30 amino acids can deliver a variety of biomolecules into the cells [1] such as small molecules, small interfering RNA, proteins, peptide-nucleic acid (PNA), imaging agents, and vaccines [2–4]. Human immunodeficiency virus transactivating regulatory protein (HIV TAT) was reported as the first CPP with the membrane translocating property [5–7]. After that, other CPPs with different properties in charge, polarity, and structure were reported to deliver bioactive molecules as classified in the CPPsite 2.0 database (https://webs.iiitd.edu.in/raghava/cppsite/index.html). CPPs are capable of delivering therapeutic substances (drugs or vaccines) into cellular compartments using the covalent or non-covalent linkage [8–11].

In recent years, different viral infections with high variability have been represented as major threats to the global health. Some of them led to a significant public health burden and huge economic loss [12–14]. Viruses can latch onto host cells as seen in several outbreaks including current coronavirus (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [15]. Antiviral drugs including small molecules that may interfere with viral replication, mRNA-, DNA-, or RNA interference (RNAi)-based vaccines that is said to be the potential vaccines to fight against viruses, and using antibodies that could act as both therapeutic and prophylactic have the capacity to prevent or treat viral infections [15, 16]. The diagnosis of viral infections can be performed by viral culture, antigen detection, serological tests, and viral nucleic acid detection. The molecular technology performed by polymerase chain reaction (PCR) is widely used in the detection and identification of pathogenic viruses [17, 18]. Recently, innovative methods such as biosensing technology (*e.g.*, graphene/zinc oxide nanocomposite-based electrochemical genosensors) enable the researchers to detect nucleic acids in a faster and inexpensive way [19]. In spite of advances in the development of therapeutic strategies, it is recommended to optimize the delivery system rather than investing time and resources into developing new antiviral therapeutic agents. As known, most of the therapeutic targets of antiviral agents (drug or vaccine) are located inside the cells, thus development of a potent and safe intracellular delivery vehicle (such as CPPs) is necessary for increasing their effects *in vivo* [9]. For example, the incorporation of CPPs in subunit or nucleic acid-based vaccines could improve antigen uptake by antigen-presenting cells (APCs). Furthermore, CPPs could be harnessed to develop new therapeutics and considered as a safe alternative or additive to classical adjuvant formulations. Indeed, they can improve the properties of current antiviral drugs and vaccines [20, 21]. In this review, we briefly describe the properties of CPPs, their mechanisms, and their variety of applications in drug delivery with a focus on the management of viral infections.

Classification of CPPs

Cell penetrating peptides were classified based on their type of origin, chemical charge (cationic, amphipathic, and hydrophobic groups related to their uptake mechanism), physicochemical properties (*e.g.*, charge, hydrophobicity, and distribution of the residues in the peptide sequence), and the extent of modifications and design efforts (Fig. 1). Cationic CPPs are the largest group of cell penetrating peptides containing basic amino acids that are responsible for their cellular uptake and suppression of helical folding formation [*e.g.*, polyarginine, TAT, and DNA- and RNA- and heparan-binding proteins of viruses, protamines, histones, and nuclear localization signal (NLS)] [22–25]. Some studies showed that oligoarginine peptides (the optimal length: 8–10 residues) penetrate into cells remarkably better than oligolysine peptides with the same length and charge [26, 27]. Increasing the length of oligoarginine peptides decreased their potency of delivery due to the cell toxicity and irreversible interaction with plasma membrane [28]. In addition, more than 40% of CPPs are amphipathic peptides with negative overall charge [29]. These CPPs were divided into primary (mainly chimeric or synthetic peptides derived from natural proteins such as tumor-suppressor p14ARF protein or prion proteins) [30–32], and secondary (*e.g.*, MAP or M918) [33] groups based on the peptide sequence, length, and association with lipids. In general, transition to a β-sheet structure led to forming the amphipathic structure and cell penetration [34]. The lowest number of CPPs contains hydrophobic CPPs with only apolar residues derived from signal peptide sequences (*e.g.*, transportan, stapled peptides, prenylated peptides, pepducins, SG3, Pep-7, and fibroblast-growth factor) [35–41]. On the other hand, CPPs can be classified based on the linkage with therapeutic agent into covalently (as fused to a recombinant protein cargo or conjugated by a linker to the cargo including TAT, penetratin, polyarginine, VP22, Buforin I, transportan, and SynB peptides) [42, 43] or non-covalently (as stable complexes with peptide/protein and oligonucleotide cargos through non-covalent electrostatic and hydrophobic interactions including Pep-1 and MPG peptides)-bonded forms [44, 45]. Table 1 provides a summary of main CPPs and their characteristics. Additionally, the classification of CPPs as protein-derived CPPs (*e.g.*, viral proteins, mammalian DNA/RNA-binding proteins, and transcription factors), chimeric CPPs, and synthetic CPPs is very useful to design more efficient

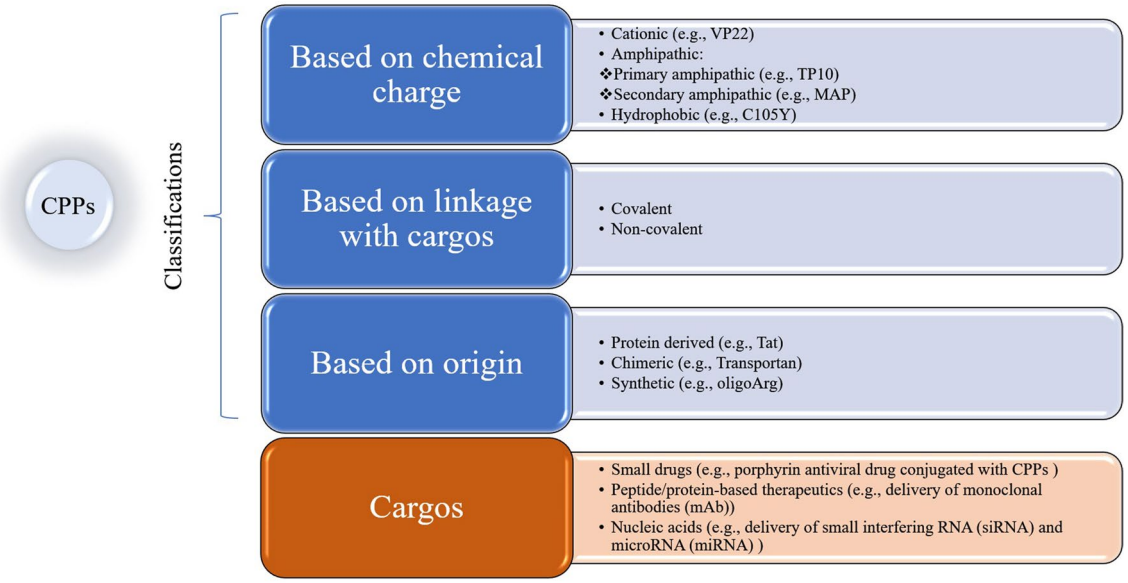


Fig. 1 A schematic diagram illustrating the types of CPPs and their cargos: CPPs can be classified based on chemical charge, linkage with cargo, and also their origin. Different kinds of antiviral cargos including vaccines, small molecules, and peptide/ protein and nucleic acid therapeutics can conjugate with CPPs via covalent or non-covalent binding

CPPs being able to increase the cellular uptake, and delivery of the related cargos. In recent years, chemical modification of CPPs was used to enhance cargo delivery [46–56].

Applications of CPPs

The intracellular delivery of therapeutic agents (*e.g.*, nucleic acids or proteins) is difficult due to their large size and hydrophilic nature. Several physical (*e.g.*, electroporation or microinjection), viral (*e.g.*, lentiviral vectors), and non-viral/ chemical (*e.g.*, polymers and liposomes) approaches were applied to enhance their cellular uptake [77]. However, some drawbacks such as low efficiency and immunogenicity, high toxicity, and poor specificity limited their use for delivery of different cargos [78]. In recent years, CPPs have been used to promote the delivery of drugs into cells due to their high efficiency in crossing cell membranes without the aid of any specific receptor, and low toxicity [79]. Moreover, CPPs have been applied in many fields of medicinal applications including imaging and biosensing applications, enzyme replacement therapy, anti-inflammation therapy using antisense peptide nucleic acids (PNAs), cancer therapy, and vaccine development [80]. Among these applications, antiviral drug delivery using CPPs attracted a special interest as follows. In general, current antiviral therapy mainly relies on small molecules to inhibit multiple targets involved in the viruses' life cycle. The main limitation of currently used antiviral drugs is inefficient delivery into the infected cells *in vivo* due to their pharmacokinetics and pharmacodynamics properties which limit their applications [81]. Therefore, CPPs were proposed as an effective delivery system for different types of biotherapeutics such as peptides/proteins and nucleic acids in antiviral therapy [82].

Delivery of Therapeutic Small Molecule Drugs

Small molecule drugs inhibit the activity of cellular or viral proteins involved in different stages of the virus life cycles [83]. Although most of small molecule drugs can pass the cell membrane, in some cases, bioavailability of these molecules is limited by their high degree of hydrophilicity which diminishes the ability of crossing the cell membrane [84]. Thus, conjugation of CPPs with these small molecule drugs could enhance their cellular uptake [*e.g.*, delivery of small molecules through the blood–brain barrier (BBB)], and improve their pharmacokinetics and pharmacodynamics profile (*e.g.*, the increased solubility and bioavailability of small molecule drugs in body fluids) [85]. For instance, porphyrin antiviral drug conjugated with CPPs can cross the BBB, and inhibit brain-resident HIV virus causing HIV-associated neurocognitive disorders (HAND) *in vitro* [86].

Delivery of Peptide/Protein-Based Therapeutics

A large number of peptide/protein-based therapeutics (*e.g.*, enzymes and antibodies) were clinically used to treat various viral diseases. However, some physiochemical properties of peptides/proteins such as size and hydrophilicity could limit their intracellular accumulation. Thus, CPPs

Table 1 Main CPPs and their characteristics

Type	Name	Amino acid sequence	Origin	Structure	Reference
Cationic peptides	HIV-TAT (aa: 47–57)	YGRKKRRQRRR	HIV-1 transcriptional activator protein	Random	[6, 7]
	Penetratin	RQIKIWFQNRRMKWKK	DNA binding protein (Homeoprotein) of *Drosophila antennapedia*	Random/ β-sheet	[34]
	Oligoarginines (R_9-R_{12})	RRRRRRRRR/RRRRRRRRRRR	Synthetic	Random	[27]
	$S4_{13}$-PV	ALWKTLLKKVLKAPKKKRKVC	Chimeric peptide with AMP dermaseptin S4 and NLS	α-helix	[24]
	VP22	DAATATRGRSAASRPTERPRAPARSASRPRRPVE	HSV-1 protein	α-helix/ β-sheet/Random	[49]
Amphipathic peptides	Transportan (Primary)	GWTLNSAGYLLGKINLKALAALAKKIL-amide	Galanin-Lys-mastoparan protein	α-helix	[35]
	TP10 (Primary)	AGYLLGKINLKALAALAKKIL-amide	Synthetic	α-helix	[57]
	Pep-1 (Primary)	Ac-KETWWETWWTEWSQPKKKRKV-NH-CH_2-CH_2-SH	Chimeric of SV40 NLS and HIV-1 reverse transcriptase	α-helix	[51]
	MAP (Secondary)	KLALKLALKALKAALKLA	Synthetic	Random/ α-helix	[58]
	MPGα (Primary)	Ac-GALFLAFLAAALSLMGLWSQPKKKRKV-NH-CH_2-CH_2-SH	Chimeric of HIV gp 41 and NLS from SV40	Random/ β-sheet	[59]
	MPGβ (Primary)	Ac-GALFLGFLGAAGSTMGAWSQPKKKRKV-NH-CH_2-CH_2-SH	Chimeric of HIV gp 41 and NLS from SV40	Random/ β-sheet	[59]
	MPG8 (Primary)	βAFLGWLGAWGTMGWSPKKRK-NH-CH_2-CH_2-SH	Chimeric of HIV gp 41 and NLS from SV40	Random/β-sheet	[59]
	Buforin II (secondary)	TRSSRAGLQWPVGRVHRLLRK	Histon H2A-derived AMP from the stomach of the Korean common toad *Bufo bufo gargarizans*	Random/ α-helix	[60]
	CADY (Secondary)	Ac-GLWRALWRLLRSLWRLLWKA-NH-CH_2-CH_2-SH	PPTG1 peptide	α-helix	[52]
	SynB1 (Secondary)	RGGRLSYSRRRFSTSTGR	Protegrin	–	[61]
	ARF (1–22) (Primary)	MVRRFLVTLRIRRACGPPRVRV	N-terminal part of p14ARF	–	[30]
	PepFect6 (Secondary)	Stearyl-AGYLLGK(ε-TMQ)INLKALAALAKKIL	Synthetic	α-helix	[62]
	PepFect14 (Secondary)	Stearyl-AGYLLGKLLOOLAAAALOOLL-amide	Synthetic	α-helix	[62]
	PepFect15 (Secondary)	Stearyl-AGYLLGK(K_3QN_4)LLOOLAAAALOOLL-amide	Synthetic	α-helix	[62]
	NickFect NF61 (Secondary)	Stearyl-AGYLLGOINLKALAALAKKIL-amide	Synthetic	α-helix	[62]

Table 1 (continued)

Type	Name	Amino acid sequence	Origin	Structure	Reference
	VT5 (Secondary)	DPKGDPKGVTVTVTVTVTGKG-DPKPD	Synthetic	β-sheet	[63]
	KALA (Secondary)	WEAKLAKALAKALAHL-AKALAKALKACEA	Synthetic	α-helix	[64]
	KLA (Secondary)	Acetyl-KLALKLALKALKAAL-KLA-amide	Synthetic	α-helix	[64]
	pVEC (Primary)	LLIILRRRIRKQAHAHSK-amide	Murine vascular endothelial cadherin protein	β-sheet	[47]
Hydrophobic peptides	*Kaposi* FGF	AAVALLPAVLLALLAP	Signal peptide of Kaposi's fibroblast growth factor	β-sheet	[65]
	C105Y	CSIPPEVKFNKPFVYLI	C terminal tail of α-anti-trypsin	α-helix	[66]
	SG3	RLSGMNEVLSFRWL	Synthetic	–	[39]
	Grb2 (SH2 domain)	AAVLLPVLLAAP	Signal sequence of SH2 domain	β-sheet /α-helix	[67]
	BIP	VPMLKE	Synthetic	–	[68]
	Pep-7	SDLWEMMMVSLACQY	Synthetic	α-helix	[40]
Antimicrobial peptides	Protegrin-1 (Cationic arginine-rich)	RGGRLCYCRRRFCVCVGR	AMP from cathelicidin family	β-sheet /α-helix	[69]
	Lactoferrin (Cationic)	VSQPEATKCFQWQRN-MRKVRGPPVSCIKRDSPIQI	AMP from human milk protein	α-helix	[70]
	LL-37 (Amphipathic)	LLGDFFRKSKEKIG-KEFKRIVQRIKDFLRN-LVPRTESC	AMP from human cathelicidin	α-helix	[71]
	Bac 7 (Amphipathic proline-rich)	RRIRPRPPRLPRPRPRPLPFPRPG	Bactenecin family of antimicrobial peptides	–	[72]
	PR39 (Proline/arginine-rich)	RRRPRPPYLPRPRPPPFFPPRLP-PRIPPGFPPRFPPRFP	AMP from cathelin family	–	[73]
N-terminal prion peptides	Bovine Prpr (1–30) (Primary amphipathic)	MVKSKIGSWILVLFVAMWSD-VGLCKKRPKP	N-terminal of unprocessed bovine prion protein	–	[31]
	Bovine Prpr (1–24) (Primary amphipathic)	MVKSKIGSWILVLFVAMWSD-VGLC	N-terminal of unprocessed bovine prion protein	–	[31]
Poly-α-amino acids	SAP (Proline-rich)	$(VRLPPP)_3$	N-terminal of γ-zein	Polyproline II helix	[74]
pH-sensitive CPPs	pHLIP (Secondary amphipathic)	AEQNPIYWARYADWLFTTPLLL-LELALLVDADEGT	Synthetic	Unstructured at pH = 7.4 but folds as a transmembrane helix at pH = 6	[75]
	GALA (Secondary amphipathic)	WEAALAEALAEALAEHLAEAL-AEALEALAA	Synthetic	α-helix	[76]

AMP an antimicrobial peptide; *ARF* ADP-ribosylation factor; *Bovine Prpr* bovine prion protein; *gp41* glycoprotein; *HIV* human immunodeficiency virus; *HSV* herpes simplex virus; *MAP* model amphipathic peptide; *NLS nuclear localization* signals; *PPTG1* lysine-rich cell membrane destabilizing peptide bound to plasmid DNA; *SAP* sweet arrow peptide; *SV40* simian virus 40; *TAT* Trans-activator of transcription; *TP10* Transportan 10

were extensively utilized as shuttles for intracellular delivery of a variety of peptides/proteins Incorporation of CPPs (*e.g.*, penetratin, SynB, and TAT) in cytotoxic T lymphocytes (CTL)-inducing therapeutic vaccines is one of their interesting applications for delivery of peptide/protein-based therapeutics [87–89]. Moreover, several antiviral peptides (*i.e.*, natural proteins-derived peptides or synthetic peptides) have emerged as ideal therapeutic agents against viral infections in clinical trials. Many of these peptides could exert their antiviral activity by interfering with various steps of virus life cycles. Enfuvirtide is the only approved peptide drug for HIV-1 treatment. Conjugation of antiviral peptide drugs with CPPs was highly suggested to overcome their poor cell permeability [90]. Table 2 shows successful delivery of CPP-conjugated peptides/proteins against related viral infections.

On the other hand, remarkable target specificity and low immunogenicity of antibodies make them as a promising therapeutic agent. More than 80 therapeutic antibodies were approved by the Food and Drug Administration (FDA) and hundreds more are in various phases of clinical trials. Thus, therapeutic antibodies have become one of the predominant and fastest-growing classes of new drugs developed in recent years [91]. However, large molecules including antibodies are notoriously hard to be delivered. TAT cell penetrating peptide showed the significant translocation potency for delivery of antibodies into the cells (*e.g.*, delivery of monoclonal antibodies (mAb) for radioimmunotherapy and radioimmunodetection). Moreover, CPPs were used as shuttles for delivery of single-chain variable fragments (ScFv) which are the engineered proteins generated by fusion of the variable heavy (VH) and light (VL) domains of an antibody. The delivered antibody could maintain its functional conformation to interact with the target in the cell [92]. Some studies have demonstrated effective internalization, and significant antiviral activity of antibody fragments fused to CPPs. For instance, one study showed that mAb targeting HIV capsid protein p24, fused to κFGF-MTS CPP (KAAVALLPAVLLALLP) efficiently internalized into the cells, and inhibited the HIV-1 replication in cell culture [93]. Another study indicated that the TAT-fused antibody targeting intracellular HBV X protein (HBx) effectively internalized into the cells, and reduced intracellular HBx *in vitro* and *in vivo* [94]. Table 2 represents successful delivery of antibodies by CPPs against related viral infections.

Delivery of Nucleic Acids and Oligonucleotides in Gene Therapy

Gene therapy is efficient delivery of genetic material into the cell, tissue or whole organ without causing pathogenic effects [95]. However, poor permeability of the plasma membrane of eukaryotic cells to DNA led to low concentration of DNA and other oligonucleotides at their targets. To overcome this issue, polylysine and polyarginine peptide carriers with the membrane-destabilizing properties could bind to DNA through electrostatic interaction (*i.e.*, non-covalently bonded form), and facilitate gene transfer into cells [96]. For example, cationic polymers such as polyethylenimine (25 kDa PEI) were utilized for gene delivery due to the formation of nanometer-sized particles with the negatively charged plasmid DNA. The linkage of PEI to TAT CPP through a hetero-bifunctional polyethylenglycol (PEG) spacer (*i.e.*, the TAT-PEG-PEI conjugate) could significantly increase the efficiency of gene delivery in lung, and reduce *in vivo* toxicity [97].

On the other hand, the therapeutic potential of small interfering RNA (siRNA) and microRNA (miRNA) against various types of viruses (*e.g.*, HIV, HCV, HBV, influenza, Ebola, HSV, and poliovirus) was reported through sequence-specific suppression of gene expression (*e.g.*, transcription or translation) [98]. Cell penetrating peptides could easily be conjugated covalently and non-covalently with siRNAs for their effective delivery into the cells. However, non-covalent strategies were more effective than covalent strategies for siRNA delivery. For example, MPG peptide could significantly improve the efficiency of siRNA delivery and its safety in the target cells [99, 100]. Additionally, MPG-based particles enter the cell independently of the endosomal pathway, and can efficiently deliver siRNAs in a fully biological active form into a variety of cell lines and *in vivo* [101].

Due to overcoming some problems of siRNAs and miRNAs (*e.g.*, poor pharmacokinetics and sensitivity to enzymatic degradation), a series of antisense oligonucleotides (AOs) with improved properties (*i.e.*, high stability, high affinity, and low toxicity) has been developed for antisense therapy. Peptide nucleic acids (PNAs) and phosphorodiamidate morpholino oligomers (PMO) are such AOs widely used for therapeutic applications. For instance, the CPP-PMO conjugates (PPMOs) were shown to reduce viral replication, and increase the survival rate of infected mice. These *in vitro* and *in vivo* therapeutic experiments were performed against various types of viruses (*e.g.*, poliovirus, Ebola, SARS, HSV, HIV, HBV, HCV, measles, Japanese encephalitis virus, and influenza A virus) [102–110]. Some studies showed that the efficacy of PNA and PMO conjugated to CPPs was only significant in the presence of endosomolytic agents (*e.g.*, chloroquine and calcium ions) [111, 112]. However, most of the endosomolytic agents are too toxic for *in vivo* applications limiting the use of CPP-PNA or CPP-PMO [113, 114]. Table 2 includes successful delivery of oligonucleotide-based antiviral therapeutics.

Table 2 Potential of CPPs for delivery of antiviral therapeutic agents

CPP	Cargo	Virus	Activity	Reference
TAT	I24	HSV, SV40, CMV, Ad5, Vaccinia	Suppressed viral infection *in vitro* via inhibition of viral entry and gene expression	[118]
R_7	NBS	HBV	Suppressed viral infection *in vitro* via blocking of nucleocapsid assembly	[120]
HPV-16 L2	RBS	HPV	Suppressed viral infection *in vitro* and *in vivo* via inhibiting virus trafficking during virus entry	[121]
TAT	Casp3	HIV-1	Induced apoptosis in HIV-1 infected cells	[122]
TAT	P53	HBV	Suppressed HBV transcription and expression *in vitro* and *in vivo*	[123]
TAT	P27SJ	HIV-1	Suppressed transcription and replication of HIV-1 *in vitro*	[124]
R_9, TAT	Mx2	VSV,Mucosal influenza	Inhibited viral replication	[125]
R_9, TAT	AZP	HPV-18	Suppressed HPV18 replication	[126]
κfgf-mts	IgG antibody targeting capsid protein p24 antigen	HIV	Inhibited virus replication *in vitro*	[93]
TAT	ScFv antibody targeting HIV-1 Tat protein	HIV	Inhibited virus transcription *in vitro*	[127]
TAT	mAb targeting HBcAg	HBV	Inhibited virus replication in vitro	[128]
TAT	mAb targeting HBx protein	HBV	Suppressed viral transcription, replication, and protein production both *in vitro* and *in vivo*	[94]
R_9	ScFv antibody targeting NS5A	HCV	Inhibited virus replication *in vitro*	[129]
Penetratin	NS4B	HCV	Inhibited virus replication *in vitro*	[130]
Penetratin	ScFv antibody targeting M1 matrix protein	influenza	Interfered with the replication of the virus *in vitro* and *in vivo*	[131]
R_9	ScFv antibody targeting VP40	Ebola	Inhibited virus assembly	[132]
TAT	siRNA targeting *tat* and *rev* genes	HIV-1	Inhibited virus replication *in vitro*	[133]
T_9	siRNA targeting NP gene	Influenza	Inhibited replication of the virus *in vitro* and *in vivo*	[134]
TAT	siRNA targeting 5'-UTR	HCV	Suppressed virus infection *in vitro*	[135]
TAT	PNA targeting direct repeat sequence of HBV RNA	HBV	Inhibited virus replication *in vitro* and *in vivo*	[136]
TAT	PNA targeting X-RNA sequence	HCV	Inhibited virus replication *in vitro*	[107]
R_9	PNA targeting HIV-1 TAR sequence	HIV-1	Inhibited HIV-1 TAT-dependent transactivation	[137]
TAT	PNA targeting-1PRF	SARS-CoV	Suppressed SARS-CoV replication	[138]
$(RXR)_4XB$	PNA targeting MeV mRNA of virus nucleocapsid	Measles	Suppressed virus infection *in vitro*	[108]
TAT	PMO targeting 3'- and 5'- UTR of mRNA and genome cyclization motifs	JEV	Suppressed virus proliferation	[109]
$(RXR)_4XB$	PMO targeting ICP27 or ICP0 mRNA	HSV-1	Inhibited HSV-1 ocular infection *in vivo*	[105]
R9F2	PMO targeting translation start site of VP35 gene	Ebola	Suppressed virus infection *in vitro* and *in vivo*	[139]
P_7	PMO targeting critical regions in viral genome RNA	Influenza A	Suppressed various subtypes of influenza A *in vitro*	[140]

Ad5 adenovirus serotype 5; *Casp3* caspase 3 (a crucial mediator of apoptosis); *CMV cytomegalovirus; HbcAg* Hepatitis B core antigen; *ICP27 and ICP0*, two nonstructural immediate-early genes essential for HSV-1 replication; *JEV* Japanese encephalitis virus; *NBS* nucleocapsid binding subunits; *NS4B* non-structural protein 4B (an essential protein for the HCV infectious cycle); *NS5A* non-structural protein 5A (an essential protein for the HCV RNA replication); *PNA* peptide nucleic acids; *PMO* phosphorodiamidate morpholino oligomers; *PRF* programmed ribosomal frameshifting; *RBS* retromer-binding site; *$(RXR)_4XB$* R = Arginine: X = 6-aminohexanoic acid: B = β-alanine; *UTR* untranslated region; VSV vesicular stomatitis viruses*; X-RNA* a highly conserved 98 nucleotide sequence in RNA-dependent RNA polymerase (RdRp) of HCV

CPPs with Antiviral and Antimicrobial Properties

Antimicrobial peptides (AMPs) are promising antimicrobial agents that influence cellular membrane of microorganisms. Some of CPPs have antibacterial properties. Antibacterial CPPs can penetrate to cytoplasm, bind to nucleic acids, inhibit protein synthesis, and finally cause bacterial cell death [115]. For example, PEP27-2 (MWKWFHNVLSWGWLLADKRPARDYNRK-NH2) is a potent antimicrobial CPP which reduced skin abscess formation during *Staphylococcus aureus* infection in mouse when used as combined with antibiotics. Indeed, PEP27-2 inhibited cellular processes by interrupting DNA metabolism in bacterial cells [116]. Moreover, some of CPPs possess antiviral properties. For instance, interaction of TAT peptide with CXCR4 co-receptor inhibited the replication efficiency of the virus strains such as HIV-1 and HSV, and showed the antiviral activity [117]. The advantage of these CPPs with antiviral activity is their synergistic antiviral effects, and their role as a delivery vehicle for other antiviral agents. Indeed, the net positive charge of these peptides could interact with the negatively charged components of viruses, and prevent viral infection at the attachment or entry step [106]. For example, TAT-I24 (GRKKRRQRRRPPQCLAFYACFC) demonstrated inhibitory activity against DNA viruses. This peptide could suppress the early step of viral replication cycle at the level of viral entry, and gene expression. The mechanism of action is the direct binding of the peptide to the viral envelope and/or affecting the membrane structures [118]. Another study showed that the anti-HIV-1 virucidal activity of Transportan-PNA conjugate was efficient to block HIV-1 infection or inactivate virus in the plasma before attachment and entry the cells [119].

Mechanism of CPP Internalization

Although the exact mechanism of CPP internalization with or without cargo has not been completely revealed, their uptake pathways have been classified as non-endocytic (*i.e.*, the energy-independent direct delivery of cargo to cytoplasm) and endocytic (*i.e.*, the energy-dependent delivery of cargo to lysosomes) pathways (Fig. 2). Most CPPs utilize two or more uptake mechanisms depending on the type of CPP, cell line, cargo, concentration, temperature and time of incubation. For example, TAT peptide was internalized through three endocytic pathways such as macropinocytosis, clathrin- and caveolae/lipid raft-mediated endocytosis based on cargo type. TAT peptide conjugated with protein was internalized through lipid raft-medicated endocytosis, while TAT peptide embedded with fluorophore used clathrin-dependent endocytosis mechanism [141].

The non-endocytic pathway or direct translocation includes the interaction of positively charged CPPs with negatively charged components of phospholipid bilayer [142]. Direct translocation was divided into inverted micelle formation (*e.g.*, penetratin) [48, 143], pore formation mechanisms through barrel-stave or toroidal models (*e.g.*, Pep-1) [144, 145], and the carpet model (*e.g.*, TAT, and dermaseptin as an antimicrobial and amphipathic peptide) [146]. In contrast, endocytic pathway was classified into four dominant pathways including macropinocytosis (a lipid raft-dependent and receptor-independent endocytic pathway; *e.g.*, CPPs-attached growth factors) [147], clathrin-mediated (a receptor-dependent and dynamin-required process; *e.g.*, arginine-rich CPPs) [148] or caveolae-mediated endocytosis (a lipid raft endocytosis associated with caveolin and cavin-1 interaction; *e.g.*, proline-rich CPPs, transportan and transportan-10) [149, 150], and phagocytosis (*e.g.*, the CPP cargo tagged by opsonins such as IgG and complement components enabling CPPs to be recognized by immune cells such as macrophages and dendritic cells) [151].

CPPs with endocytic uptake pathways should escape from endosomes. For example, positively charged CPPs (*e.g.*, TAT) could interact with negatively charged phospholipids in the endosomal membrane leading to a pore/leakage, and finally the release of CPPs [152]. Moreover, the formation of ionic pairs between CPPs and negatively charged membrane lipids occurred in oligo-arginine peptides. Three strategies are available for improving the endosomal release of CPPs including: a) the use of fusogenic lipids (*e.g.*, dioleoylphosphatidyl-ethanolamine (DOPE)) [153], conjugation of the carrier with viral fusion sequence (*e.g.*, HA2 peptide as a pH-sensitive fusogenic peptide derived from influenza virus) [154], and increasing the osmotic pressure within endosomes through the proton sponge effect (*e.g.*, histidine residues) [153].

Advances in Enhancing the CPP Potency

CPPs showed high potential for delivery of a wide range of molecules, but they have some drawbacks including non-specific internalization, fast elimination from the body, and intracellular/vesicular entrapment. However, our knowledge regarding the mechanism and structure–activity relationship of internalization is growing. The most common feature among CPPs is the presence of positive charges such as arginine and lysine. In addition, hydrophobicity plays a major role in the translocation process [56]. The studies showed that the replacement of arginine residues of polyarginine peptides (*e.g.*, TAT, penetratin, transportan, MPG, Pep-1,

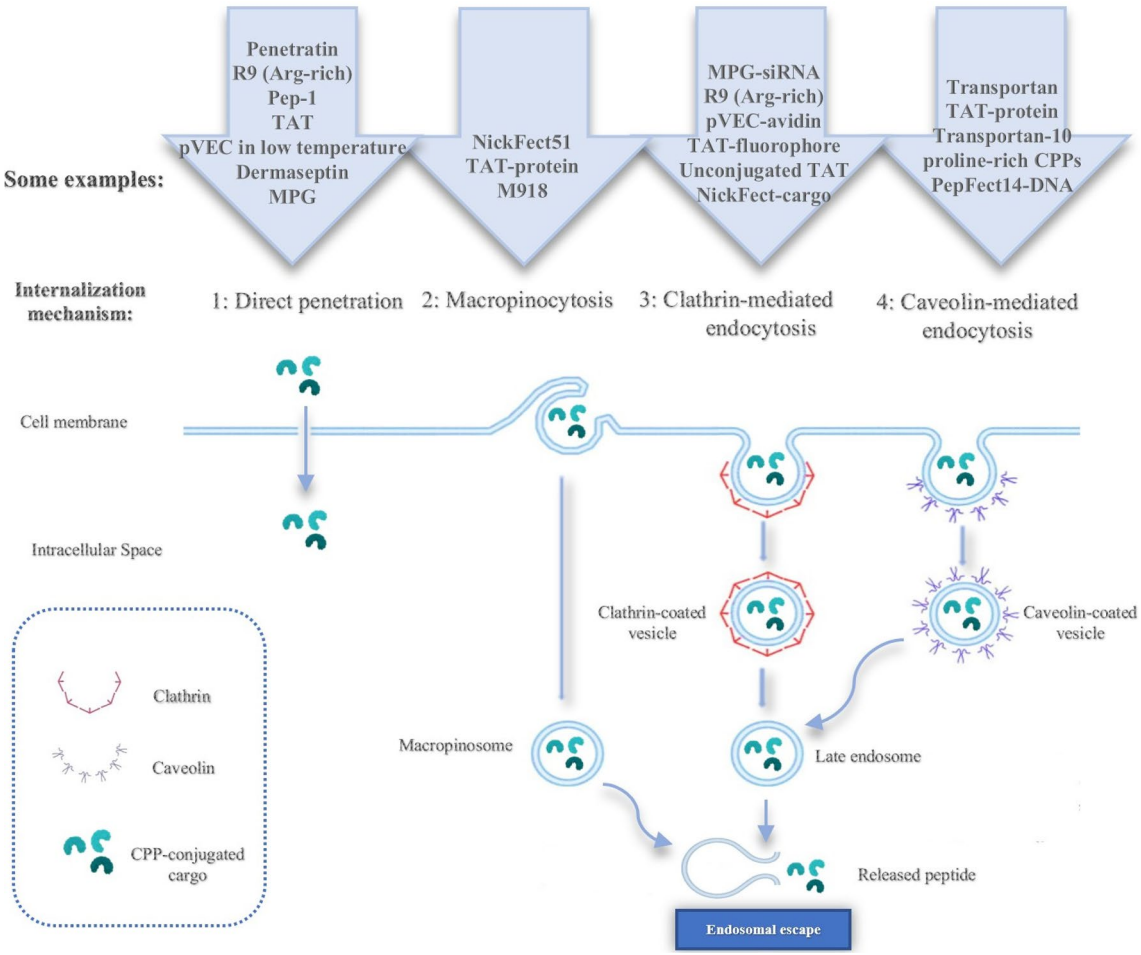


Fig. 2 Schematic illustration of uptake mechanisms of CPPs, and some major examples: The CPP uptake mechanism can be directed by non-endocytic (direct penetration) and endocytic pathways. As the cell membrane is impermeable to hydrophilic substances, the delivery into cells can be facilitated by linking the cargos to CPPs. Most CPPs utilize two or more uptake mechanisms depending on the type of CPP, cell line, cargo, concentration, temperature, and time of incubation

and pVEC) with lysine residues showed a weaker affinity to the cell surface, and remarkably reduced intracellular translocation. Thus, the presence of arginine in the peptide chain is a desirable modification [25, 155–157].

On the other hand, a specific class of CPPs was based on polyproline secondary structure (*e.g.*, SAP and PR39 as an AMP). Indeed, proline residue has a secondary amine chain that does not participate in intra- or inter-molecular hydrogen bonding. Thus, the internalization of polyproline peptides is due to the amphipathicity of the secondary structure rather than of the primary sequence [73, 149]. Additionally, Tryptophan is a very important amino acid in CPP structure being able to transport cargo across cell membranes (*e.g.*, CADY and Pep-1). In fact, the number of tryptophan residues, their position in the helix, and the size of the hydrophobic face formed by them are important for the cell internalization [158, 159]. For example, increasing the number of tryptophan up to three amino acids could enhance cellular uptake. Above this number, the intracellular translocation activity was decreased due to low solubility of the peptide [160]. Tryptophan substitution can also enhance the antimicrobial activity of CPPs. For example, PEP27-2, an analog of PEP27 with Trp substitution, showed stronger antimicrobial activity against a variety of bacteria [116]. Furthermore, polyhistidine in CPP structure (*e.g.*, LAH4) facilitated direct membrane translocation of peptides into cells. Indeed, substitution of amino acids with histidine residues could provide endosomal disruption by the proton sponge effect in the acidic endosomal compartments provoking the endosomal escape [161]. For example, protonated histidines enhanced the delivery of nucleic acids to their targets through osmotic swelling with lysis of endosomes, unpacking of the carrier complex, and the release of nucleic acids [162, 163]. Other properties of histidine include its ability to stabilize nanoparticles through hydrogen bonding and aromatic interactions [162].

Generally, different modifications on CPPs could increase their internalization and/or change the mechanism of penetration such as amino acid substitute [157, 160, 161], and functional group modification [2, 164]. Amino acid substitution of CPPs is one of the ways of achieving variability of the physicochemical parameters (*e.g.*, hydrophobicity or cationic nature) especially changes in dissolution properties [157]. Modification of functional groups (*e.g.*, the alteration of CPPs, α-helicity through hydrocarbon stapling) is another approach for increasing the CPP efficiency [2]. For example, modification of MAP CPP with citraconic anhydride blocked forming acid-labile amide linkage to mask the cationic charge, decreased the non-specific binding and uptake, and thus significantly improved the targeted drug delivery [164].

Also, there is a need for improvement in rational design of CPPs to tackle the possibility of *in vivo* toxicity. Synthetic tools have paved the way to explore new approaches to improve the cell penetration of CPPs and CPP-therapeutic conjugates. For example, cyclization and stapling increases the metabolic stability and internalization efficiency due to increased structural and conformational rigidity. Thus, multivalency of covalent dimers (primary structure), stabilized helices (secondary and tertiary structure) and even quaternary structure can help to improve the internalization [165]. However, the lack of methodologies for systemic rational design and optimization of new CPPs is one of the key impediments. Fortunately, molecular dynamics (MD) simulation (a simulation-guided rational design approach) is now able to predict structure-based rational fine-tuning of functional properties. Although the next challenge of designing suitable CPPs is to find proper engineering techniques to control the morphology to improve selectivity and specificity [165]. Thus, chemistry and rational design could contribute to the CPP field.

Current Status and Challenges of CPPs in Viral Infections

CPPs have been used to increase drug delivery efficiency [166]. However, it is required to determine the pharmacokinetic properties of CPPs through assessing toxicity, tissue distribution, cell selectivity, solubility and stability, immunogenicity and endosomal degradation [167]. On the other hand, the mechanism of CPP internalization is non-specific binding to bilayer phospholipids on the cell membrane, which severely limits the clinical application of CPPs. There are several solutions for enhancing the CPP's specificity, and decreasing possible adverse effects of therapeutics such as: a) the design of cell and tissue-specific CPPs. For example, two tumor targeting peptides including RGD (Arg-Gly-Asp) and NGR (Asn-Gly-Arg) can be used for improving specificity in virus-related tumors [168], b) conjugating/coupling of the CPP with various cell-specific targeting ligands (*e.g.*, folic acid, specific antibodies, and transferrin) through covalent and non-covalent bonds [169–172], and c) masking the cell-penetrating effect with a stimulus-sensitive cleavable linker (*e.g.*, pH-sensitive, enzyme-sensitive, temperature-sensitive, and magnetism-sensitive or light sensitive cleavable linkers). These linkers can be cleaved, and the CPP restores its normal activity. However, the activation process is generally irreversible and often occurs at off-target sites instead of on-target sites [172].

To date, no CPP-conjugated drugs have been approved by FDA; although several clinical trials on cancer have been evaluating them, one of the issues is the lack of sufficient *in vivo* studies on stability [149]. The rapid blood clearance of therapeutic agents may be a drawback as the enzymic degradation is happening before reaching the therapeutic site. Moreover, the assessment of immunological and pharmacokinetic studies of CPPs need validation in animal models [21]. The polypeptide CPPs may increase the risk of undesired immune response [173]. Also, the off-target absorption of the therapeutics by any normal tissues and cells can cause cellular toxicity. Thus, the exact dosing of each CPP should be measured in animal models before officially getting applied to the patients [174]. On the other hand, the endosomal degradation is another drawback and CPPs should be designed to have effective endosomal escape to speed up the release of the carriers [175]. Molecular imaging of intracellular and intranuclear targets can be helpful for understanding the CPP internalization mechanism and intracellular trafficking [176]. To our best of knowledge, there is no ongoing clinical trial to investigate the delivery system of CPPs in viral infections.

Conclusion and Future Prospects

CPPs have the potential to transport numerous types of therapeutic agents into a variety of cells. Several biophysical factors including charge, amphipathicity, shape, complexity, and compactness of the structures play an important role in entry of CPP/cargo into the cells. Extensive *in vitro* and *in vivo* studies have shown the successful delivery of nucleic acid- and peptide/protein-based drugs and vaccines. To date, no CPP or composition containing CPPs has been approved by the FDA due to the lack of sufficient *in vivo* studies on stability, immunological assays, toxicity, and endosomal escape of CPPs; but several ongoing clinical trials in different phases are evaluating them (mainly for drug delivery in tumor cells). Despite the many advantages, some serious drawbacks such as non-target tissue-specificity, instability and suboptimal pharmacokinetics

features limit their clinical applications. However, three distinct delivery strategies are proposed to enhance the CPP's specificity including designing cell/ tissue-specific CPPs, conjugation of CPPs with targeting moieties, and modulation of CPP uptake by a stimulus-sensitive signal. Also, the endosomal entrapment issue of CPPs can be solved by the use of fusogenic lipids via destabilizing the endosome membrane, and the use of histidine by creating the proton sponge effect and generating lysosome osmotic swelling. In general, the success of the CPP-based strategy for clinical use depends on their efficiency, safety, and also ultimate cost. Large-scale applications and new methodologies are being implemented to increase the yield and reduce cost. With increasing our knowledge of various aspects of CPPs, along with the development of new efficient CPPs overcoming some limitations, CPPs are expected to become an important part of pharmaceutical agents, especially in antiviral therapies.

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Declarations

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