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Cell penetrating peptides: The potent multi-cargo intracellular carriers

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Abstract

Introduction: Cell penetrating peptides (CPPs) known as protein translocation domains (PTD), membrane translocating sequences (MTS) or Trojan peptides (TP) are able to cross biological membranes without clear toxicity using different mechanisms, and facilitate the intracellular delivery of a variety of bioactive cargos. CPPs could overcome

some limitations of drug delivery and combat resistant strains against a broad range of diseases. Despite delivery of different therapeutic molecules by CPPs, they lack cell specificity and have a short duration of action. These limitations led to design of combined cargo delivery systems and subsequently improvement of their clinical applications.

Areas covered: This review covers all our studies and other researchers in different aspects of CPPs such as classification, uptake mechanisms and biomedical applications.

Expert opinion: Due to low cytotoxicity of CPPs as compared to other carriers and final degradation to amino acids, they are suitable for preclinical and clinical studies. Generally, the efficiency of CPPs was suitable to penetrate the cell membrane and deliver different cargos to specific intracellular sites. However, no CPP-based therapeutic approach has approved by FDA, yet; because there are some disadvantages for CPPs including short half-life in blood, and non-specific CPP-mediated delivery to normal tissue. Thus, some methods were used to develop the functions of CPPs *in vitro* and *in vivo* including the augmentation of cell specificity by activatable CPPs, specific transport into cell organelles by insertion of corresponding localization sequences, incorporation of CPPs into multifunctional dendrimeric or liposomal nanocarriers to improve selectivity and efficiency especially in tumor cells.

Keywords: Cell penetrating peptides; bioactive cargos; mechanism of action; biomedical application

Article highlights

- CPPs facilitate the intracellular delivery of a variety of bioactive cargos
- CPPs lack cell specificity and have a short duration of action
- Combination of other delivery systems with CPPs improve their clinical applications
- It is important to predict which CPP is optimal for target of interest

Abbreviations

ACPP: activatable CPP; AHNP: anti-Her-2/neu peptide mimetic; ALL: acute lymphoblastic leukemia; BBB: blood brain barrier; BH4: Bcl-2 homology domain 4; CAT: catalase; CdSe: cadmium selenide; CdTe: cadmium telluride; CPPDs: CPP-drugs; CXCR4: CXC chemokine receptor 4; DCPP: CPP-dendrimer; DDS: drug delivery system; DMD: Duchenne muscular dystrophy; DOX: doxorubicin; ELP: elastin-like polypeptide; GCV: ganciclovir; HDM-2: human double minute-binding protein; K-FGF: Kaposi's sarcoma fibroblast growth factor 1; MDM-2: mouse double minute-binding protein; MMP: matrix metalloproteinase; MSC: mesenchymal stem cells; MT1-MMP: membrane type-1 matrix metalloproteinase; MTSs: membrane-translocating sequences; NBD: NF-κB essential modulator (NEMO)-binding domain; NPCs: nuclear pore complexes; PMO: phosphorodiamidate morpholino oligomers; PNA: peptide nucleic acid; POD: peptide for ocular delivery; PTD: protein transduction domains; siRNA: small interfering RNA; SOD1: superoxide dismutase 1

1. Introduction

Development of novel strategies in the design of bioactive and therapeutic molecules was broadly increased during the recent years. However, the cellular uptake of these therapeutic agents through biological membranes (*e.g.,* nuclear or plasma membranes) was a major barrier for their clinical application. Several delivery systems such as viral and non-viral carriers were developed to overcome low permeability of membranes and to improve delivery of therapeutic molecules. Among these carriers, cell penetrating peptides (CPPs) known as protein transduction domains (PTDs) efficiently penetrate into the cells as well as deliver biologically active cargos [1, 2]. These peptides are small molecules (less than 30 amino acids) [3] which are classified in cationic, amphipathic and hydrophobic groups based on their physicochemical properties [4]. Many CPPs were derived from natural proteins, but other CPPs were either chimeric or completely synthetic. Mechanisms of CPP internalization into cells can occur through an endocytic pathway and/or through direct penetration [2]. Although, CPPs are an

effective approach for delivery of therapeutic peptides and proteins, but however there are some challenges to overcome in clinical trials containing toxicity of CPPs, stability of CPPs (*i.e.,* protection against plasma enzymes), immune responses to CPPs, and tissue-specific targeting by CPPs (*i.e.,* selectivity) **[**5, 6]. Up to now, numerous *in silico* CPP prediction algorithms were established to facilitate screening of peptides. There are 1699 unique CPP sequences that most of them are linear CPPs (94.5%) based on the CPP database site. The major researches on CPPs focus on synthetic peptides (~ 54.8%) [7]. Two methods including CellPPD and CPPpred were applied to predict CPPs (Length: 5-30 amino acids) and design efficient CPPs including quantitative structure-activity relationship models and support vector machines (SVM) [8]. Tang et al. showed that the machine learning model of SVM was suitable for predicting membrane penetrating capability (accuracy: ~95%). In fact, the use of amino acid position as a variable can be considered as a promising method for predicting the ability of CPPs in cell penetration [9]. For example, SVM-based models were developed to predict and design highly effective CPPs. It was possible to recognize CPPs from non-CPPs based on amino acid composition. However, certain amino acids such as Arg, Lys, Pro, Trp, Leu, and Ala were preferred to locate at specific sites [10]. In this review, we attempt to represent an overview of classifications, mechanisms, advantages and limitations, *in vitro/ in vivo* applications, and finally preclinical and clinical uses of CPPs for pharmaceutical development.

2. Discovery of cell penetrating peptides

The first CPPs were identified in 1988 and 1991 which derived from the transactivator protein (Tat) of human immunodeficiency virus type 1 (HIV-1) and the Drosophila antennapedia homeobox protein (pAntp), respectively [2, **Figure 1**]. In 1997, a short peptide carrier (MPG) containing hydrophilic and hydrophobic domains was synthesized to form non-covalent complexes with cargos. In 1998, Langel *et al* designed the first chimeric peptide carrier including the N-terminal fragment of the neuropeptide galanin linked to mastoparan (a wasp venom peptide) entitled as the Transportan peptide [11, 12]. Up to now, more than 100 peptides were identified to deliver a variety of biologically active molecules (*i.e.,* nucleic acids, proteins, peptides, drugs, *etc*.) into eukaryotic and prokaryotic cells [6, **Table 1**]. On the other hand, different preclinical and clinical trials of

CPP-based delivery are recently under investigation. In 2003, the first clinical trial (phase II) was performed by Cell Gate Inc. for topical delivery of cyclosporine linked to polyarginine (**Figure 1**). KAI pharmaceutical Ins. evaluated a fusion of Tat CPP with protein kinase C inhibitor peptide modulator for acute myocardial infarction and cerebral ischemia which entered phase IIb trials. A cell penetrating-based technology (TransMTSTM) was also developed for topical delivery of botulinum toxin and other macromolecules across skin by Revance Therapeutics Inc. which entered phase II trials. Other companies (*e.g.*, Traversa Inc.) evaluated Tat-based non-covalent siRNA delivery at preclinical and clinical trials [12].

3. Classification

CPPs were classified based on a variety of their properties including physicochemical properties (*i.e.,* cationic, amphipathic or hydrophobic), linkage with therapeutic molecules (*i.e.,* covalent or non-covalent binding) and their origin (*i.e.,* natural protein-derived CPP, chimeric or synthetic) [4]. These subclasses were explained as following and summarized in **Tables 1 and 2.**

3.1. Classification based on physicochemical properties

CPPs were divided into three subgroups using their physicochemical properties such as cationic (~ 83%), amphipathic (~ 44%) and hydrophobic (~ 15%) peptides [13].

3.1.1. Cationic CPPs

These peptides are short amino acid sequences containing histidine, arginine and lysine residues, *e.g.,* Tat, poly arginine and poly lysine. The charge of lysine (K) and arginine (R) is positive, but lysine is less effective for cell penetration, alone likely due to the lack of guanidine group. At least eight positive charges are required for effective cellular uptake of cationic CPPs [14]. Although, charged residues are important for cellular penetration, other residues can also be critical. For example, mutation of W_{14} to F in Penetratin (RQIKIWFQNRRMKWKK) reduced its cellular uptake [15]. A special group of cationic CPPs are nuclear localization sequences (NLSs) harboring lysine-, arginine- or proline-rich motifs which enter the nucleus via the nuclear pore complexes (NPCs). NLSs are classified into monopartite (*e.g.,* SV40: PKKKRKV) and bipartite (*e.g.,*

nucleoplasmin: KRPAATKKAGQAKKKL) signals. Other NLSs are TFIIE-β (SKKKKTKV), NF-κB (VQRKRQKLMP), HATF-3 (ERKKRRRE), Oct-6 (GRKRKKRT) and SDC3 (FKKFRKF) [16]. However, NLSs are often covalently linked to a hydrophobic peptide sequence to make an amphipathic CPP with an efficient cell uptake.

3.1.2. Amphipathic CPPs

Amphipathic CPPs have lipophilic and hydrophilic regions for translocation across the cell membrane [14]. They are categorized into primary amphipathic CPPs (*e.g*., Pep-1, pVEC, MPG, penetratin, CADY, ARF or BPrPr), secondary amphipathic α-helical CPPs (*e.g*., hCT18-32), β-sheet amphipathic CPPs (*e.g.,* VT5) and proline-rich amphipathic CPPs (*e.g*., bactenecin-7, SAP) [13, 17].

Some primary amphipathic CPPs including MPG (GLAFLGFLGAAGSTMGAWSQPKKKRKV) and Pep-1 (KETWWETWWTEWSQPKKRKV) are chimeric peptides obtained by covalently linking a hydrophobic domain to the SV40 NLS (PKKRKV) [13]. Membrane translocation of amphipathic CPPs depends on amphiphilicity not on positive charges. For example, replacing lysines with other polar residues in an amphipathic peptide (MAP) (KLALKLALKALKAALKLA) [*i.e.,* the neutral MAP17 peptide: QLALQLALQALQAALQLA and the anionic MAP12 peptide: LKTLTETLKELTKTLTEL] retained its cellular uptake. Moreover, the studies showed that uptake of amphipathic CPPs was severely decreased by single point mutations and deletion (*e.g.,* transportan and MAP mutants) [13].

3.1.3. Hydrophobic CPPs

Hydrophobic peptides (*i.e.,* stapled or prenylated peptides, and pepducins) contain only apolar residues, *e.g.,* the signal sequences from integrin β3 (VTVLALGALAGVGVG), Kaposi fibroblast growth factor (AAVALLPAVLLALLAP) and MAP (KLALKLALKALKAALKLA) [17]. Linear hydrophobic peptides include anionic and cationic pentapeptides such as VPALR, VSALK, PMLKE, IPMLK, VPTLQ, IPALK and VPTLK. The lack of sensitivity to sequence scrambling was determined for hydrophobic

peptides as compared to amphipathic and cationic CPPs [18]. The studies showed that some hydrophobic CPPs can directly translocate via cell membranes and thus eliminate the risk of endosomal entrapment [13].

3.2. Classification based on binding type with cargos

Two main subclasses of CPPs were categorized based on their linkage with therapeutic molecules: **a)** Covalent bonded CPPs: CPPs conjugated to therapeutic molecules through different linkers especially disulfide or thioesters linkages (*e.g.,* Penetratin, R8, Tat, HSV VP22, Transportan, SynB and Buforin I antimicrobial peptides, and polyproline peptides) [19, 20], and **b)** Non-covalent bonded CPPs: CPPs complexed with therapeutic molecules through non-covalent electrostatic and hydrophobic interactions (*e.g.,* mainly primary or secondary amphipathic peptides). For instance, MPG and Pep-1 are primary amphipathic peptides which form stable complexes with oligonucleotides or proteins/peptides, respectively [21, **Figure 2** and **Table 2**].

3.3. Classification based on the source of the peptide

CPPs were classified in three classes based on their origin including: a) natural CPPs derived from DNA-RNA-binding proteins, anti-microbial proteins, viral particle envelope proteins, transactivators of gene transcription, and plant circular skeletal proteins (*e.g.,* Tat, penetratin, VP22); b) Chimeric CPPs generated by combination of natural peptides or of signal peptides with NLS peptides (*e.g.,* transportan, Pep-1, MPG, TP10), and c) Synthetic or artificial CPPs designed based on the naturally-derived CPPs (*e.g.,* polyarginine, MAP) [2]. It is important that the synthetic CPPs should be optimized to enhance their stability in blood circulation, increase cellular internalization, and escape endolysosomal degradation [22]. Indeed, the type of amino acid (mainly arginine and histidine substitution) can affect internalization efficiency of CPPs. The studies showed that the efficiency of cellular uptake was decreased by reducing the number of arginine residues. For example, penetratin-Arg showed higher penetration than that of penetratin-Lys [23, 24]. Moreover, arginine-replacing peptides (*e.g.,* SR9, PR9 and HR9) were able to transport fluorescent proteins into the cells. HR9 peptide is more efficient than SR9 and PR9 peptides, because Histidine (H) motif has buffering ability

under physiological conditions (*i.e.,* pH-responsive), and also endolysosome escape activity (*i.e.,* proton-sponge effect) [22, 25].

4. Antimicrobial peptides as a special class of CPPs

Antimicrobial peptides (AMPs), known as host defense peptides, are short and commonly positive charge peptides which some of them have the ability to kill microbial pathogens directly, whereas others act indirectly by modulation of the host defense systems [26]. Several AMPs are able to translocate into cells without the permanent membrane permeabilization. They were used as effective vectors for intracellular translocation of various active molecules, *e.g.,* Magainin 2 and Buforin 2 [27, 28]. Some AMPs have attracted a special interest because they can enter host cells without damaging their cytoplasmic membrane as well as kill pathogenic agents [28]. Although there are some similar properties between CPPs and AMPs, but their use in treatments especially cancer therapy differ due to their amino acid composition, cell membrane targeting ability, secondary structure manifestation, mode of cell membrane permeabilization, cytoplasmic destination and functional capabilities. While CPPs were involved with cell pore penetration and delivery of different cargos, AMPs were characterized by disruption/destabilization of cell membranes, channel/ pore formation, and enhancement of immune response. As observed, CPPs transports conjugated and/or bound drugs, chemicals, and chemotherapeutic drugs; but AMPs lacks cargo delivery ability, binds metals and dimerizes with peptides and proteins. Moreover, CPPs have no effects on immunity of the host; while AMPs enhance the innate immune response of host and promote chemokine immunomodulation [29]. Thus, it is interesting for combination of CPP and AMP properties, *e.g.,* the design of antimicrobial cell penetrating peptides with bacterial cell specificity. For instance, Iztli peptide 1 (IP-1) was used because of both properties of cell penetrating peptides (CPP) and cationic antibacterial peptides (CAP). IP-1 could make pores in the presence of high electrical potential at the membrane of fungi/human cells as found in bacteria and mitochondria [30].

5. Structure-activity relationship of CPPs

The structure-activity relationship of CPPs interacting with lipid membrane was studied using fluorescence microscopy and spectroscopy techniques. These studies showed the role of secondary structure of peptides, the effect of lipid composition, and the membrane potential in peptide-lipid interactions [31]. A study showed that peptides with α-helical regions can more effectively enter cells. Thus, the efficiency of cargo delivery was improved by modification of the peptide structure to overcome some problems such as poor solubility, aggregation, toxicity and low synthesis amount [19]. CPPs adopt different conformations (*i.e.,* structural polymorphisms) following the interaction with lipids under various experimental conditions including temperature, peptide/lipid ratios and buffer conditions (*e.g.,* ionic strength, pH). For example, penetratin peptide adopted α-helical, β-strand or β-turn structures in model membranes. This peptide mainly showed β-strand and random coil structures in the cytoplasm, and also β-sheet in the nucleus [32, 33]. On the other hand, the role of tryptophan residue is critical for the cellular uptake in arginine-rich CPPs (*e.g.,* RW9: RRWWRRWRR). Indeed, the number of Trp residues, their position in the helix, and the size of the hydrophobic surface in peptides were critical for their cell uptake. The highest internalization occurred for the peptides with three Trp residues that adopted α-helix structure in interaction with lipids [34]. Moreover, incorporation of Trp residues in basic peptide sequences could control the efficiency of CPP internalization through increasing the peptide interaction with negatively charged glycosaminoglycans (GAGs), and subsequently the internalized amounts by endocytosis [35].

Chemical/ structural modifications on CPPs led to the development of their stability, biocompatibility and safety. Generally, structural modifications of proteins and peptides include prodrug, analogous formation, modification of C- or N-terminals (*e.g.,* polymer conjugation, post-terminal modification, site specific modification), conjugation with fatty acids (*e.g.,* irreversible lipidization, reversible lipidization), combination of both site-specific modification and lipidization, hydrophobic ion pairing, complexation with cyclodextrins, and current technologies (*e.g.,* Nobex technology, Emisphere technology) [36]. For feasible delivery of proteins and peptides through oral route, their physicochemical properties (*e.g.,* stability, hydrophobicity and molecular weight) as well as biological barriers (*e.g.,* proteolysis in stomach and poor permeation into cells)

should be considered. These issues could be solved by modification of the physicochemical property or the use of a delivery system. The modification of the primary structure of a peptide through covalent or non-covalent binding led to the improvement of enzymatic stability and mucosal penetration [36]. Chemical modification could provide a more efficient uptake of cargoes across the epithelial barrier of the gastrointestinal tract [37]. Chemical glycosylation was suggested as a method to enhance protein stability and long-term bioavailability. Chemical modification of the peptide structure resulted in an increased skin permeability. Four peptide analogues (Arg0, Arg1, Arg2 and Arg3) dissolved in various propylene glycol and water co-solvents were studied in skin permeation and wrinkle reduction. Two peptides (Arg2 and Arg3) enhanced human skin permeation *in vitro*. On the other hand, the ability of four peptide analogues to reduce wrinkle formation showed that Arg3 was the most effective followed by Arg1, Arg0 and Arg2, respectively [38]. Recently, a novel family of cyclic CPPs containing only a single hydrophobic residue has been generated. The optimal CPP structure included four arginine residues and a hydrophobic residue with a long alkyl chain (*e.g.,* a decyl group) in a cyclohexapeptide ring. The most active member of this family was CPP17 even at high doses of serum protein likely due to the lower protein binding. CPP17 could enter the cell by direct penetration at a relatively low concentration (≥ 5 μm) [39]. The affinity of CPPs for glycosaminoglycans (GAGs) was increased with the number of Trp residues, from 30 nM for a penetratin analog with 1 Trp residue to 1.5 nM for a penetratin analog with 6 Trp residues for heparin (HI). The quantity of peptide internalized into CHO cells enhanced 2 times with 1 Trp residue, 10 times with 2 Trp residues, and 20 times with 3 Trp residues, compared to +6 peptides with no Trp residues. Thus, Trp residues indicated molecular determinants in basic peptide sequences not only for direct membrane translocation but also for efficient endocytosis through GAGs [40]. A nonapeptide series containing only Arg, Trp or D-Trp residues at different positions was designed. The data indicated that to increase the uptake efficiency, Arg could be replaced by Trp in the nonapeptides. The presence of Trp in oligoarginines enhanced the uptake in cells expressing GAGs at their surface. Density functional theory (DFT) analysis showed that salt bridge-π interactions play a main role for the GAG-dependent entry mechanisms [41].

6. Mechanism of cellular uptake

The mechanism of the CPP uptake significantly varies based on cell type, linkage type, incubation time, dose and physiochemical properties (*e.g.,* hydrophobicity and net charge) [42, **Figure 3, Table 2**]. The reports demonstrated that the cellular uptake of CPPs can occur through several approaches such as direct penetration, and clathrin/ caveolae-mediated endocytic uptake depending on the nature of the peptide/ cell membrane interaction [19].

6.1. Direct penetration

Direct penetration as an energy-independent approach includes various mechanisms such as pore formation, inverted micelle formation, the membrane thinning model, and the carpet-like model. In these mechanisms, the first step is the interaction of positively charged CPPs with negatively charged components of membrane (*i.e.,* HS: heparan sulfate) and the phospholipid bilayer. The second step of cellular uptake is dependent on the peptide sequence and dose as well as the lipid structure of the cell membrane. In general, direct penetration further occurs at high doses of CPPs especially primary amphipathic peptides (*e.g.,* MPG or transportan) [43]. Rothbard *et al*. indicated that an increased potential of the cell membrane led to high internalization of CPPs [44]. The "inverted micelle" is another mechanism of the direct penetration as observed for penetratin peptide [45]. Indeed, after primary binding of positively charged residues of the CPP (*i.e.,* lysine and arginine) to the negatively charged phospholipids of the membrane, the CPP traverses the cell membrane toward the cytoplasm forming pocket-like micelles. Then, these micelles go across and invert the cell membrane for the release of the CPP and its cargo into the cells [46]. It seems that the interaction of hydrophobic amino acids (*e.g.,* tryptophan) with the hydrophobic region of the membrane is important in formation of inverted micelles and the efficiency of cell penetration. Thus, the highly cationic CPPs (*e.g.,* Tat peptide) cannot likely use this mechanism [43]. In the membrane thinning model and the carpet-like model, the interaction of cationic CPPs (*e.g.,* Tat peptide at high concentrations) with negatively charged phospholipid led to a thinning and carpeting of the membrane, respectively [2, 43]. Moreover, in pore formation model, the disruption of the lipid membrane occurs

through interaction of the side chains of basic residues in CPP with phospholipid phosphate groups. This model was observed for polyarginine peptides (Arg9) or Mastoparan X (a class of toxic peptides isolated from wasp venom). The peptide-to-lipid ratio can determine the size of pores for cell penetration [47].

6.2. Endocytosis

Endocytosis contains two main mechanisms for the uptake of biomolecules or other cells: phagocytosis in special cells (*e.g.,* macrophages), and pinocytosis in most cells such as macropinocytosis, clathrin-mediated endocytosis or caveolae/lipid raft-mediated endocytosis. The Antp, nona-arginine and Tat peptides simultaneously used three endocytic pinocytosis pathways. However, the endocytic uptake mechanism for CPPs is dependent on cargo type. For instance, Tat peptide conjugated to a protein used lipid raft-mediated endocytosis mechanism while Tat peptide conjugated to a fluorophore utilized clathrin-dependent endocytosis mechanism [6]. A study showed that Tat-mediated delivery of cargos (more than 30 kDa) could proceed through energy-dependent macropinocytosis with an increased endosomal escape into the cytoplasm [48]. Thus, the conjugates of polycationic and amphipathic peptides use a variety of internalization approaches [49]. On the other hand, macropinocytosis was associated with the formation of vesicles called macropinosomes. Dynamin protein was needed for this folding of the membrane [43]. Both Tat-fusion proteins (> 30 kDa) and Tat PTD (1-5 kDa) entered cells by macropinocytosis [17]. In receptor-mediated endocytosis, clathrin-coated vesicles (about a few hundred nanometers in diameter) and caveolin-coated vesicles (about 50-80 nm in diameter) were generated after binding the biomolecules to the membrane receptor leading to cellular uptake [43]. The studies showed that all three mechanisms of the endocytic pathways were independent on the CPP dose and sequence [50].

7. Effective factors in the uptake mechanism

As mentioned above, the mechanisms of the CPP uptake change significantly due to different factors including the dose, hydrophobicity and net charge of the CPPs, cell type, temperature and time of incubation, the size and type of the cargo, and the linkage

method [17, **Table 3**]. For example, lowering temperature as well as depletion of cell energy effectively confirmed the endocytotic mechanism for the internalization of non-covalent protein/ CPP complexes. A study indicated that Tat and transportan CPPs revealed higher efficiency of protein delivery than pVEC or penetratin CPPs [51]. On the other hand, the dose of CPP was important to trigger various cellular uptake pathways. For instance, endocytosis commonly occurred at low CPP concentrations. In contrast, direct penetration occurred at high concentrations especially for primary hydrophobic peptides and many cationic CPPs, but however, the threshold of concentration changes among different types of CPPs, cell lines, and cargos. It was observed that Tat linked to a large cargo was often entrapped within endocytic vesicles; while, Tat fused to a small cargo could escape from endosome and enter the cytosol. Thus, when the cargo is less than 10-20 kDa, the CPP conjugates can directly penetrate the cell membrane under special conditions [52]. The importance of positive charges (arginine residues versus lysine residues) and hydrophobic α-helical structures was proved in the cellular uptake mechanism, as well [53]. The arginine-rich CPPs used different forms of endocytosis as the uptake mechanism at the high peptide concentrations [52]. Administration dose of arginine (R)-rich CPPs has a major role in determining uptake method of these peptides. The researchers showed that there are at least two pathways for CPP internalization including endocytosis and direct translocation, and the latter mode of internalization is highly dependent on administration dose. In this line, other results also confirmed this finding, *e.g.,* the cytosolic translocation (direct penetration) of Tat, Penetratin and R9 was enhanced when HeLa cells were treated at relatively high concentration of these peptides (> 10 *μ*M) [54]. However, low and high concentrations depend on CPP, cargo and cell types. Usually, direct penetration can occur in more than 10-20 μM [55]. Moreover, Meloni *et al*. reported that increasing poly-arginine length improved the cellular uptake, the cytosolic release and subsequently the biological activity [56]. Indeed, dodeca- or hexadeca-arginine peptides indicated higher cellular internalization than octaarginine peptides [57]. The secondary amphipathicity of the peptides could also increase gene delivery [58]. Finally, the length and the conformation of the CPPs affect the uptake mechanism, *e.g.,* efficient translocation of pVEC against scrambled pVEC into various cell lines [43]. Although, some CPP-fusion proteins/

bioactive molecules are entrapped in the endosomal vesicles, several methods were used to facilitate endosomal escape such as chemical agents (*e.g.,* chloroquine, calcium, sucrose, ammonium chloride and sodium azide). Moreover, DMSO was used as an effective penetration enhancer for drugs, anticancer agent and exogenous DNA delivered by Tat peptide [59]. However, it is essential to find chemical agents with lower cytotoxicity and increase the uptake potency of CPPs before clinical use [3]. Ma *et al* showed that the pretreatment with benzisothiazolinone (BIT) augmented the penetrating efficacy of Tat and Tat-protein conjugates [3]. Splicing correction by steric-blocking oligonucleotides (ON) could lead to major clinical applications but needs their effective delivery to cell nuclei. The conjugation of short oligolysine tails was utilized to transfer a correcting peptide nucleic acid (PNA) sequence [$(Lys)_8$-PNA-Lys)] in an endocytic mechanism of internalization. It was observed that a significant and sequence-specific splicing correction was achieved only in the presence of endosome-disrupting agents (*e.g.,* chloroquine or 0.5M sucrose). These agents could overcome the limitations of endosomal trapping for splicing correction by PNA-oligolysine conjugates [60]. The use of CPP-based delivery was limited because of the poor delivery efficiency of CPP conjugated potent cargos. In this line, a study showed that the combination of glucose, sucrose and manntiol (GSM) in the presence of osmoprotectant (glycerol and glycine) could enhance CPP penetration as a novel strategy *in vitro* [61]. On the other hand, chemical linkage of CPPs to other delivery systems is an effective strategy to enhance the uptake of plasmid DNA (pDNA). For example, the conjugation of CPPs (*e.g.,* Tat, penetratin and octaarginine) to thiolated chitosan/pDNA polyplexes improved transfection efficiency of both systems [62]. Other study indicated that the coupling of Tat to chitosan-thioglycolic acid (TGA)/pDNA nanoparticles increased cell penetration and also endosomal escape of nanoparticles [63]. Also, delivery of CPP-peptide nucleic acid (PNA) conjugate into the cells using a variety of treatments (*e.g.,* photodynamic, chloroquine or Ca^{2+} treatment) enhanced the release of CPP conjugate into the cytoplasm leading to the effective antisense effects of CPP-PNA conjugate. The delivery of the CPP-PNA conjugate and its nuclear antisense effects was improved by endosome-disruption treatment [64]. In addition, a fusion protein containing ten arginines fused to residues 253-412 of the translocation domain of *Pseudomonas*

aeruginosa exotoxin A (ETA) facilitated the endosomal escape of enhanced green fluorescent protein (*i.e.,* CPP-ETA-EGFP) as a protein cargo into the cytosol indicating that the linkage of ETA to the CPP-containing protein fusion construct (CPP-EGFP) prevented lysosomal degradation using the delivery of construct from early endosomes to the ER lumen and then into the cytosol [65]. Another study showed that the C-terminus of human papillomavirus (HPV) L2 protein has a conserved cationic CPP leading to the endosomal escape into the cytoplasm, and subsequently virus transport to the trans-Golgi network [66].

8. Cytotoxicity of CPPs

The potential toxicity of CPPs showed a major barrier to their clinical application [67]. The safety was related to CPP and cargo toxicity, clearance and immunogenicity [68]. It was observed that toxicity of CPPs is low at their effective doses [69]. Moreover, their cytotoxicity highly depends on the length and dose of cargo, and the coupling site of cargo within the CPP [67-71]. It seems that the peptide oligonucleotide conjugates showed very little cytotoxicity in therapeutic use [72]. Some studies indicated that the toxicity was dependent on cell type, CPP type and composition, physicochemical properties, and also dose/ frequency/ route of injection [68, 69, 73]. For example, Vives *et al*. reported that the short Tat peptide (aa 37-60) was non-toxic for HeLa cells up to 100 μM concentration and 24 h incubation [74]. Harbour *et al.* showed that the Tat CPP alone was non-toxic in four different tumor cell lines (*i.e.,* WERI retinoblastoma cells, MM-23 uveal melanoma cells, C33A cervical carcinoma cells and U2OS osteosarcoma cells), even at concentrations approximately 300 μM [75]. On the other hand, although penetratin peptide was non-toxic in keratocytes (CC50: 200 μM), but it decreased the survival in HeLa (CC50: 93 μM), Vero (CC50: 70 μM) and TM-1 (CC50: 50 μM) cells [76]. Furthermore, the YTA2 CPP (acetyl-YTAIAWVKAFIRKLRK-amide) was shown to deliver proteins into MDA-MB-231 breast cancer cells with high efficacy and without cytotoxicity even at high doses up to 10 μM concentration [77]. Some studies demonstrated that protein cargos such as GFP and HIV-1 Nef protein were effectively transfected into HEK-293T cells using Pep-1 and Cady-2 amphipathic CPPs at a molar ratio of 20:1 without toxicity through an endosomal pathway-independent mechanism

[5]. Pep-1 also showed no cytotoxicity in L929 cells at high doses [78]. In this line, M918 peptide as a protein carrier was effectively translocated into HeLa and human breast cancer cells without cytotoxicity up to 25 μM concentration [79].

Several amphipathic CPPs with antiviral effects were toxic *in vitro* likely due to pore formation in cell membranes (membrane perturbation triggering the temporal influx of calcium ions and the elevation of intracellular calcium concentration) such as bKLA peptide (b-KLALKLALKALKAALKLA-amide), amphipathic fragment of HIV-1 gp120 and/or the fusogenic domains of viral entry or fusion proteins [67, 73]. The studies showed that there is a correlation between the high numbers of hydrophobic amino acids in the peptide sequence and the induction of toxicity. Holm *et al.* indicated that peptidomimetics containing retro-inversion CPPs composed of D-amino acids induced more cytotoxicity than those composed of L-amino acids due to higher stability against proteolytic enzymes in the cells [80]. However, it was observed that CPP injection systemically did not generate tissue damage at the doses of interest [67]. Generally, cationic CPPs were less toxic than amphipathic CPPs *in vitro* and *in vivo* [81].

9. Delivery of different cargos using CPPs

CPPs were applied for *in vitro* and *in vivo* delivery of various therapeutic molecules, *e.g.,* peptide, protein, DNA, siRNA, drugs, nanoparticles *etc*.

9.1. Peptide and protein delivery

The nature of the cell membrane limits the cellular uptake of drugs to small size (less than 600 Da), and to hydrophobicity. Thus, researchers attempt for effective delivery of proteins and peptides into the cells which have *in vivo* short half-life and poor bioavailability [82]. Recently, the use of CPPs could significantly facilitate the intracellular delivery of a variety of proteins and peptides through their covalent linkage to cargos [83, 84]. For example, delivery of a biologically active protein (β-galactosidase) linked to Tat peptide (Tat-β-galactosidase) across the blood-brain barrier was improved after intraperitoneal administration [85]. On the other hand, delivery of the anti-apoptotic proteins (*e.g.,* Bcl-xL) into cells was increased by their conjugation to CPPs. Cao *et al.* showed that the Bcl-xL protein linked to Tat CPP protected the

neurons in a murine middle cerebral artery occlusion model [86]. The ability to suppress upstream pathway of caspase activation in apoptosis is also crucial. A peptoid inhibitor for the apoptotic protease-activating factor (Apaf-1) was modified by its conjugation to penetratin and Tat. Both CPPs enhanced cellular uptake, but the penetratin conjugate was more effective at inhibiting apoptosis likely due to the toxicity of the Tat conjugate [50]. Mainly, CPP-mediated delivery of peptides and proteins was used to target tumors. A number of tumor suppressor p53-derived peptides were conjugated to CPPs in order to improve cell penetration. For example, injection of the p53-derived peptides conjugated with Tat or polyarginine (R11) to a peritoneal carcinomatosis mouse model increased mice survival. Moreover, the anti-tumor effects of a peptide inhibiting the activity of casein kinase 2 (P15) conjugated with Tat was observed in mice [84]. CPPs were also utilized to induce the generation of pluripotent stem cells as a safer carrier than viral vectors for human use. For instance, the fusion proteins harboring polyarginine CPP (R9) linked to the C-terminal of four proteins involved in cellular reprogramming (*i.e.,* Oct4, c-Myc, Klf4 and Sox2) could be effectively transported into human fibroblasts for their transformation into pluripotent stem cells [87]. On the other hand, CPPs including pAntp, Tat, transportan and polyarginine were broadly used for *in vitro* and *in vivo* delivery of bioactive peptides. A comparison of the delivery efficiency of CPP conjugates revealed that polyarginine = transportan > pAntp > Tat. Also, cellular toxicity showed that pAntp < Tat < transportan < polyarginine [88]. Other studies indicated that hPP10 CPP was able to penetrate into primary cultured cells. Indeed, hPP10 could be considered as a novel vehicle to deliver exogenous proteins or drugs for clinical applications [89]. For example, the hPP10 CPP transported HPV16 E7-GFP fusion protein in HEK-293T cells (~ 63.66%) compared to TurboFect (~ 32.95%). In contrast, the transfection efficiency of hPP10 CPP was low (~ 17.51 and ~ 16.36% in TC-1 and A549 tumor cells, respectively) indicating the importance of cell type *in vitro* [90]. Another study also showed that hPP10 could mediate Cre fusion protein delivery and pDNA transfection simultaneously in the Cre/loxp system *in vitro*. Furthermore, hPP10 fused with an RNA-binding domain could deliver small interfering RNA into cells to silence the reporter gene expression [91]. On the other hand, hPP3 (KPKRKRRKKKGHGWSR) derived from human nuclear body protein could enter cells

in vitro, at a concentration-, incubation time-, serum- and temperature-dependent manner [92]. It was interesting that a CPP (TIP1) derived from toll/interleukin-1 receptor (TIR) domain-containing adapter protein suppressed toll-like receptor-mediated downstream signaling and showed therapeutic potential for TLR-mediated autoimmune and inflammatory diseases [93]. Gros *et al*. improved a novel method for delivery of proteins, peptides and antibodies *in vitro* and *in vivo*, with no chemical conjugation between the cargo and CPP [21]. Indeed, the physical complexation was successfully used to deliver peptides and proteins into cells. A study indicated that the Pep-1 CPP was able to form hydrophobic interactions with the peptide or protein cargos, and transport these cargos into various cell lines [94]. Similarly, Cady-2 peptide showed a high efficacy for delivery of protein cargos (*e.g.,* mRFP and GST-Cdk2), and a group of short peptides (~ 8-24 mer) into the cells [95]. On the other hand, M918 peptide (aa 1-22) derived from the tumor suppressor protein p14ARF could efficiently transport proteins and peptide nucleic acids (PNA) using macropinocytosis mechanism into cells either as a covalent conjugate or a non-covalent complex with the cargo. This peptide was more effective than amphipathic peptides (*e.g.,* TP10) for cargo delivery and also was non-toxic at high concentrations for *in vivo* therapies [79]. Recently, M918 CPP was utilized to enter efficiently HIV-1 Nef and Hsp20-Nef proteins as a candidate antigen into the mammalian cells [96].

An *in vivo* study showed that co-injection of insulin with the penetratin peptide enhanced intestinal and nasal insulin bioavailability to 35 and 50%, respectively [97]. CPPs could also deliver enzymes (*e.g.,* preventing the oxidative damage) into cells as a therapeutic approach for a variety range of diseases (*e.g.,* ischemic injury). For instance, Tat peptide combined with glyoxalase, catalase and superoxide dismutase, and also LMWP CPP conjugated to L-asparaginase could prevent oxidative damage of neuronal cells, and treat oxidative damage of red blood cells in acute lymphoblastic leukemia [2]. Moreover, CyLoP-1 is a cysteine-rich CPP derived from nuclear localization sequence of snake toxin (crotamine) with both cell-penetrating and antimicrobial activities such as killing methicillin-resistant *Staphylococcus aureus*. Its cellular uptake was successfully performed in mammalian cells at very low doses. Indeed, the presence of cysteine residues in the peptide played an important role in biological activity of this peptide [98].

On the other hand, the minimized sequence of Latarcin 1, a spider venom toxin (LDP: Latarcin-derived peptide) conjugated to nuclear localization sequence from Simian Virus T40 antigen (LDP-NLS) could effectively penetrate into HeLa cells without cytotoxicity as compared to LDP with very low uptake and high toxicity. LDP-NLS also successfully transported protein cargos with high molecular weight into the cells [99]. A novel technology described by Salerno *et al* named as CPP-adaptor system increased the intracellular delivery and endosomal escape of protein cargos. This strategy was designed as a CPP-adaptor fusion protein, Tat-calmodulin (Tat-CaM), which non-covalently binds, delivers and releases different protein cargos (*e.g.,* myoglobin, horseradish peroxidase and β-galactosidase) into the cells [100]. The pVEC, an amphipathic CPP (18 aa: LLIILRRRIRKQAHAHSK) derived from murine vascular endothelial-cadherin protein was also able to transport some proteins (*e.g.,* avidin, streptavidin), and oligomers (*e.g.,* hexameric PNA oligomer) in a non-covalent approach into several cell lines as well as bacteria and fungi for killing microbes [101, 102]. Several groups showed CPP-mediated delivery of fusion proteins *in vitro,* but only a few studies successfully used CPPs as protein or peptide vectors *in vivo* [83]. Jo *et al*. used a CPP composed of a hydrophobic signal sequence derived from the fibroblast growth factor 4 to deliver a suppressor of cytokine signaling 3 protein (SOCS3) to immune cells, liver and other organs in mouse model which could effectively suppress the harmful effects of acute inflammation [103]. Moreover, Bleifuss *et al*. applied a CPP derived from the PreS2 domain of the human hepatitis virus B called as translocation motif (~ 12 amino acids) to increase the immunogenicity of antigen cargo in vaccine design [104]. On the other hand, the intraperitoneal injection of 30Kc19 peptide, the first CPP found in the hemolymph of insect (silkworm), into mice could deliver proteins into various tissues of animal model without cytotoxicity [105]. A synthetic guanidine-rich molecular carrier was used for intracellular and transdermal delivery of proteins, as well. For instance, a sorbitol-based carrier with eight guanidine units (Sor-G8) could form the non-covalent complex with GFP, albumin, concanavalin A and immunoglobulin G cargos. These non-covalent cargo-CPP complexes showed an efficient transdermal penetration into the mouse skin. The synthetic Sor-G8 carrier was significantly more effective than Arg8 in the transdermal delivery of proteins [106]. Another study tested

the transdermal delivery ability of IMT-P8, a novel human-derived CPP. IMT-P8 was able to transport effectively GFP and pro-apoptotic peptide (KLA) as IMT-P8-GFP and IMT-P8-KLA fusion constructs into mouse skin following topical application. Moreover, the uptake of IMT-P8-GFP was significantly higher than Tat-GFP in HeLa cell line [107]. Iduna known as RNF146 is a poly (ADP-ribose) (PAR)-dependent E3 ubiquitin ligase that can degrade PARylated proteins via PAR-dependent ubiquitination. The human Iduna-derived peptide was able to deliver macromolecules across the cell membrane. Koo *et al*. showed that the recombinant Iduna-conjugated EGFP (Iduna-EGFP) and its tandem-repeat form (d-Iduna-EGFP) efficiently penetrated Jurkat cells using lipid-raft-mediated endocytosis mechanism. The recombinant d-Iduna-EGFP was more effective than Iduna-EGFP and could be localized in the cytoplasm and nucleus longer than Tat CPP. The effective uptake of the recombinant d-Iduna-EGFP was performed by various tissues such as the spleen, liver and intestine following intravenous injection in C57BL/6 mice [107]. The studies showed that Pep-1 CPP was applied to deliver caspase 3 into the lung of mice, protein kinase A (PKA) into the distal lung epithelial cells of rat in order to repair the defect in a cellular signaling pathway. Moreover, Pep-1 peptide was used to determine the anti-tumor effects of antisense PNAs targeting cyclin B1 as well as to evaluate early embryonic development using the delivery of antibodies and proteins (*e.g.,* p53) into immature bovine and mouse oocytes [108-111].

9.2. Nucleic acid delivery

The first application of CPPs was delivery of nucleic acids into the cells through electrostatic interactions [2]. Generally, CPPs possess several benefits such as: a) to protect nucleic acids from degradation; b) to internalize effectively in specific target cells; c) to improve the release of the cargos in the cytoplasm (*e.g.,* antisense oligonucleotides, siRNA, miRNA) or the nucleus (*e.g.,* plasmid DNA, splice-switching oligonucleotides), d) to show high biological activity at low doses, e) to exhibit no cytotoxicity, and f) to possess a good biosafety for *in vivo* therapeutic studies [112]. The researchers showed the importance of serum proteins or CPP conformation on the delivery of CPP/siRNA complexes into the cells. One of the main restrictions of CPPs is the lack of cell-type specificity. A common approach to overcome this problem was the incorporation of targeting ligands with CPPs to control cell-specific attachment. Fang *et*

al. found that the linkage of a vascular endothelial growth factor receptor-1 (VEGFR-1) targeting peptide to Tat internalized siRNA into tumor cells more effectively than Tat alone [113]. Improved and selective siRNA delivery was reported with other targeting ligands such as mannose, folate and RGD. For example, the nanoparticles coated with folate and penetratin increased siRNA delivery toward folate-expressing tumor cells [2]. CPPs were also combined with viral vectors (*e.g.,* adenovirus gene vector) to infect a large number of cell types [2]. On the other hand, CPPs were easily conjugated covalently or complexed non-covalently with siRNAs. The covalent linkage of siRNAs to Transportan and Penetratin CPPs showed a silencing response in cells [20]. There are some studies about siRNA delivery using CPPs *in vivo*. For instance, the cholesterol-Arg9 complex enhanced siRNA delivery against vascular endothelial growth factors (VEGF) in a mouse tumor model [114]. A small peptide derived from rabies virus glycoprotein (RVG, a ligand for acethylcholine receptor) modified with polyarginine (Arg9) was also demonstrated to transport siRNA into the central nervous system (CNS) for gene silencing *in vitro* and protection against the fatal viral encephalitis in a mouse model [115]. Dowdy *et al*. generated a Tat fusion protein with a double-stranded RNA-binding domain (Tat-DRBD system) to deliver epidermal growth factor receptor (EGFR) and AKT serine/threonine kinase 2 (Akt2) siRNAs efficiently into intracranial glioblastoma tumor mouse model [116, 117].

The first report of non-covalent approach for the delivery of siRNAs was their stable complexes with the MPG peptide (derived from the hydrophobic fusion peptide of HIV-1 gp41 plus the hydrophilic NLS of SV40 large T antigen) [118]. This peptide was used to deliver siRNAs targeting OCT-4 into mouse blastocytes and silencing cyclin B1 (a cell cycle regulator) for reduction of cell differentiation and proliferation, respectively [112]. Also, MPG peptide modified with cholesterol could increase the survival of mice against tumor growth [98]. On the other hand, a variant of MPG (MPG_{α}: Ac-GALFLAFLAAALSLMGLWSQPKKKRKV-Cya) containing five mutations in its hydrophobic domain as α-helical conformation could effectively deliver siRNA cargos [112]. Moreover, an amphipathic CPP named as Cady containing arginine and tryptophan residues could form stable complexes with siRNA, and mediate gene silencing efficiently in different suspension and cell lines such as human osteosarcoma

U2OS, THP1 monocytes, human umbilical vein endothelial and mouse 3T3C cells [119]. Cheng *et al.* developed siRNA delivery with a PEGylated PLGA nanoparticle (NP) using the synergistic activity of two different ligands such as folate (FOL) and penetratin (ANTP) that enhanced knockdown efficacy. ANTP/FOL-NP could enhance cell binding and uptake, protect siRNA, and improve siRNA release [120].

Stearylation of CPPs was useful to improve the potency of siRNA delivery into cells. For instance, stearylated transportan (stearyl-TP10) effectively transported a splice-correcting phosphorothioate 2'-O-methyl RNA (2'-OMe ON) into cells. In addition, a stearyl-TP10 analogue modified with trifluoromethylquinoline was used to increase endosomal escape and effective siRNA delivery in Jurkat cells and human umbilical vein endothelial cells (HUVEC) [121]. Among the stearylation of CPPs, STR-KV peptide (stearyl-HHHKKKVVVVVV) complexed with small interference RNA (siRNA) targeting the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) showed 80-87% gene knockdown efficiency in the cells with low cytotoxicity through a non-endocytic pathway [122]. Moreover, a novel fusion protein containing the tombusviral p19 protein linked to the "Tat" peptide (RKKRRQRRRR) could efficiently deliver siRNAs into the cytoplasm of human hepatoma cells eliciting potent gene knockdown activity without cytotoxicity [123]. Disulfide-constrained cyclic amphipathic peptides increased siRNA penetration into the cells through the formation of non-covalent peptide/siRNA complexes [124]. Recently, amphipathic peptides were developed to self-assemble with siRNAs as peptide-based nanoparticles and to transfect them into cells. A novel CPP named as RICK corresponding to the *Retro-inverso* form of the CADY-K peptide was designed. The data showed that RICK: siRNA self-assembly suppressed siRNA degradation and induced inhibition of gene expression. This novel approach can be considered for targeted anticancer treatment such as knock-down of cell cycle proteins [125]. On the other hand, the influence of the polyethylene glycol (PEG) grafting to RICK NPs was studied on their *in vitro* and *in vivo* siRNA delivery properties. Low PEGylation rates (≤ 20%) of the NPs did not influence their cellular internalization capacity as well as their knock-down specificity *in vitro* as compared to the native RICK: siRNA NPs. After an intra-cardiac injection of the PEGylated NPs in mice, it was shown that 20% PEG-RICK NPs decreased significantly liver and kidney accumulation [126]. Moreover, a novel

family of short (15/16 *mer*) tryptophan (W)- and arginine (R)-rich Amphipathic Peptides (WRAP) could form stable nanoparticles and enroll siRNA molecules into cells. They had several advantages including the rapid encapsulation of the siRNA, the efficient siRNA delivery in several cell types, and the high gene silencing activity even in the presence of serum [127]. Some CPPs such as Tat, transportan and polyarginine were utilized with other non-viral vectors in a single nanocarrier to improve nucleic acid delivery [112]. Poor permeability of the cell membrane to DNA or oligonucleotides led to their low concentrations at their targets. To overcome this problem, CPPs (*e.g.,* polylysine or polyarginine) could bind to DNA via electrostatic interaction and facilitate *in vitro/ in vivo* gene delivery. Moreover, the amphipathic peptides with pH-dependent fusogenic and endosomolytic activities (*e.g.,* GALA, KALA and histidine-rich peptides) could enhance transfection efficiency along with poly-lysine/DNA complexes [20]. Another report indicated that the Tat peptide increased *in vitro* transfection of HIV-1 Nef gene (as Tat-Nef fusion DNA) and subsequently its expression in mammalian cells [128]. Hyndman *et al.* demonstrated that mixing the Tat with liposomes containing DOTAP/ Lipofectin and DNA led to generate the complexes that significantly increased *in vitro* transfection [129]. Morris *et al.* indicated that the non-covalent MPG CPP/DNA complexes could effectively entry cells using an endosomal pathway-independent mechanism. Indeed, the NLS of MPG was involved in both electrostatic interaction with DNA and nuclear targeting [130]. The internalization of MPG-based nanoparticles into COS-7 cells at an N/P ratio of 15:1 (peptide: DNA) was comparable with a commercial transfection reagent (polyethyleneimine) indicating high transfection efficiency of MPG at a certain ratio [131]. In general, the non-covalent linkage of the NLS to DNA improved gene delivery and its expression in cells [42]. Rittner *et al.* showed the efficacy of two novel basic amphiphilic peptides named as ppTG1 and ppTG20 to bind nucleic acids, destabilize cell membranes, and deliver gene *in vitro/ in vivo* [132].

The studies showed that he amphipathic peptides including $(LARL)_6$, GM225.1 (GLFEALLELLESLWELLLEA), KALA (WEAKLAKALAKALAKHLAKALAKALKACEA) and the Hel peptide (KLLKLLLKLWLKLLKLLL) facilitated gene delivery *in vitro*, but their use was limited *in vivo* due to the toxicity and instability in serum [133]. Also, the PepFect14 (PF14) cationic peptide formed stable nanoparticles with DNA (size: 130-

170 nm) which could be internalized through class A scavenger receptors and caveolae-mediated endocytosis [134]. On the other hand, two arginine-rich CPPs such as HR9 and IR9 were able to link non-covalently to plasmid DNA and deliver them into cells and in rotifers without cytotoxicity [135]. The reports represented that peptide modification could increase gene delivery, as well. For instance, stearic acid modification of various arginine-rich peptides including HIV-1 Tat (48-60), HIV-1 Rev (34-50), flock house virus (FHV) coat (35-49), $(RxR)_4$ and oligoarginines (aa 4-16) improved their transfection efficiency through enhancing endosomal escape and nuclear delivery [112]. In this line, stearyl-TP10 increased DNA delivery in different cell lines as similar to lipofection [136]. Effective stearylated Transportan analogues termed as NickFects (NF) were designed to improve DNA transfection efficacy because of long shelf-life, lack of aggregation after reconstitution, high stability against enzymatic degradation, and higher bioactivity *in vivo* [58, 137]. Also, the modified CPPs with cysteine could improve their properties. For example, the branched Tat (BTat), a modified type of Tat (Cys-Tat-Cys-Tat-Cys) with disulfide bonds formed the complexes with plasmid DNA harboring GFP gene and showed higher transfection efficiency (~ 40-fold) than the Tat/ DNA complexes [138]. Saleh *et al*. demonstrated that covalent linkage of membrane active peptide LK15 to Tat peptide improved its gene transfer likely due to the higher uptake of DNA [139]. Moreover, a modified bioreducible branched poly (nona-arginine) CPP (B-mR9) with cysteine residues (Cys-R9-Cys-R9-Cys) could more effectively deliver nucleic acids through endocytosis or direct penetration as compared to its linear type [140]. However, inactivation of some CPP/ DNA complexes (*e.g.,* Tat CPP/DNA complexes) in the bloodstream is important which may be due to their interactions with serum albumin. It was reported that at least eight Tat peptide moieties were necessary to obtain effective gene delivery [112].

On the other hand, CPPs were used to deliver oligonucleotides (ONs) [20]. The use of PNAs was common in CPP-mediated antisense delivery likely due to the peptide backbone of the PNA allowing the formation of CPP-PNA construct as a single polypeptide. PNAs were usually linked to CPPs through a disulfide bridge [53]. Both Transportan and Antp peptides were used to deliver an antisense PNA complementary to the human galanin receptor type 1 mRNA *in vitro* and *in vivo,* leading to modification

of the pain response [53, 141]. Moreover, penetratin modified with arginine residues in its N-terminal region conjugated with PNA ONs (R6Pen-PNA conjugates) was more effective than penetratin alone for promotion of splicing redirection [142]. The non-covalent strategies were also generated by interaction of negatively-charged ONs with positively charged CPPs. For example, a novel generation of PNAs (HypNApPNAs) non-covalently combined with Pep-2 CPP led to the potent delivery of PNAs *in vitro*. Furthermore, the Pep-3 CPP could form stable complexes with both uncharged and charged PNAs promoting their cellular uptake in a variety of cell lines. In this line, PEGylation of Pep-3 significantly improved the delivery efficiency of cyclin B1 anisense ONs to block nervous tumor growth in mouse model [112]. On the other hand, VP22 peptide was also applied to deliver oligonucleotides *in vitro* and *in vivo*. The complexes of VP22 with fluorescein-labeled oligonucleotides (*i.e.,* vectosomes) were effectively internalized by cells and disrupted by light to release the antisense activity. In addition, suppression of the c-Raf1 protein expression was observed by anti-c-raf1 vectosomes potently activated by light leading to reduction of the tumor growth in mice [143].
The recent studies showed new application of CPPs for genome modification. The engineered site-directed nucleases, transcription activator-like effector nucleases, and the clustered regularly interspaced short palindromic repeat-associated nuclease Cas9 (CRISPR/Cas9) were used as cargos for manipulating genes and genomes of organisms. For instance, a recombinant Cas9 protein conjugated through the Cys-terminal residue to poly-arginine (Arg9) resulted in the generation of a cell-permeable Cas9-mR9 chimeric protein. This chimeric protein was combined with a molecular complex formed by single guide-RNA and poly-Arg (sgRNA: 9R) to modify the genome of several human cell types [144].

9.3. Drug delivery

Large molecule drugs (*e.g.,* antibody) have better targeting specificity, prolonged circulation time in the blood and less cytotoxicity effects as compared to small molecule drugs [145]. However, intracellular delivery of drugs is a major problem [146]. Common strategies in drug delivery contain self-assembly, PEGylation, stimulus sensitivity (*e.g.,* pH or temperature), enhanced permeability and retention, and the use of cell-penetrating moieties or of the prodrugs [147]. In recent years, it was shown that CPPs

have ability to deliver drugs into cells [148]. CPPs were conjugated to small molecules (*e.g.,* drugs and imaging agents) to increase their intracellular delivery [69]. However, CPP-mediated delivery was not tissue- or cell-type specific, thus other agents were added in the drug delivery system for specific targeting purposes [69]. The poor membrane permeability of drugs was an important issue in drug design. Intracellular delivery of drugs by CPP was proved to be an important step for overcoming drug resistance [149]. In fact, conjugation of peptide-based drugs (*e.g.,* Shepherdin and p53-derived peptides) with CPPs led to efficiently their internalization into the cells as a promising approach for cancer therapy [150]. It was reported that penetratin crossed the blood brain barrier (BBB) within 10 min and its permeability was 2-3-fold higher than Tat and SynB1 peptides [151]; thus, penetratin can be used as a suitable CPP for facilitating drug delivery in the brain.

9.3.1. CPPs with NLS

Macromolecules are actively translocated across the nuclear membrane via nuclear pore complexes (NPCs) [150]. Herein, nuclear proteins need the short sequences of NLS composed of one (monopartite) or two (bipartite) clusters of basic amino acids for the nuclear import pathway [152]. However, nuclear delivery of anti-cancer agents using systemic injection is a major challenge [153]. Recently, synthetic NLS peptides were utilized to increase DNA delivery into cells. The best NLS sequence is the SV40 large T-antigen NLS (126-PKKKRKV-132) [154]. It was observed that the combination of NLS and cationic metal complexes resulted in the generation of a novel type of ternary delivery systems with high efficiency [17]. On the other hand, signal sequences or membrane-translocating sequences (MTSs) of peptides were recognized by acceptor proteins that transport the pre-protein from the translation machinery into the intracellular organelles. MTSs coupled to NLSs could accumulate in the nuclei [133, 154]. A CPP containing 16 residues from the Kaposi's sarcoma fibroblast growth factor 1 (K-FGF) MTS coupled to the NF-κB NLS (10 residues) or the SV40 T-antigen NLS (12 residues) showed similar efficiency with endogenous NF-κB for nuclear translocation, and consequently inhibited the inflammatory response of NIH-3T3 cells to tumor necrosis factor-alpha (TNF-α) or lipopolysaccharide [154]. Another study demonstrated

that only one NLS linked to the end of a plasmid possesses the most effective nuclear translocation and gene expression as compared to multiple NLSs [152, 155].

9.3.2. Nanocarriers

Different nanocarriers were utilized to enhance the stability of drugs and reduce adverse effects. Among the most common drug carriers were liposomes and micelles for delivery of water-soluble drugs and poorly soluble drugs, respectively [146]. Recently, CPPs including Tat peptide were successfully used to deliver different nanoparticulate pharmaceutical carriers (*e.g.,* nanoparticles, liposomes and micelles) into the cells [156]. The studies showed that the synergistic or combined effects of CPPs with other carriers for delivery of protein/peptide drugs increased their therapeutic effects in various disorders especially cancer [5].

9.3.2.1. Nanoparticles

Using modification of the surface of nanoparticles with CPPs, the cell permeability of nanoparticulate-based therapeutics was increased [146]. The study of CPP-mediated nanoparticulate delivery indicated that the dextran-coated superparamagnetic iron oxide particles (CLIO) coupled to Tat peptide (aa 48-57) generated an effective labeling of the cells (*e.g.,* immune cells) for *in vivo* magnetic resonance imaging (MRI) goals [156]. Tat peptide (aa 48-57) was also conjugated to FITC-doped silica nanoparticles (FSNPs) for bioimaging purposes in human lung adenocarcinoma cell lines (*e.g.,* A-549), and *in vivo* bioimaging in the brain of rats [146].

9.3.2.2. Liposomes

Liposomes are synthetic phospholipid vesicles (size: ~ 50-1000 nm) which can be loaded with some soluble drugs in water [156], and used to increase the half-life of drugs and to reduce their cytotoxicity [157]. A main disadvantage of liposomes is their slow cell penetration [157]. It was reported that long-circulated PEGylated liposomes have the ability to remain in the blood for a long time and to accumulate in different pathological regions (*e.g.,* tumors). In addition, antibodies or cell surface-binding specific molecules were attached to the water-exposed head of PEG chains for specific cell targeting [146]. On the other hand, Tat or penetratin peptides conjugated on

liposomes significantly increased their cellular delivery [157]. Indeed, Tat peptide-modified liposomes (*e.g.,* Tat peptide-lipoplexes) increased the delivery of genes to tumor cells after intratumoral injection without influencing the normal adjacent brain cells [156, 157]. Gorodetsky *et al*. showed that liposomes complexed with an amphiphilic Haptide (*i.e.,* a 19-21-mer cell-binding peptide) could enhance the cellular uptake of drugs in a non-receptor-mediated process [158, 159]. Another study indicated that the translocation of liposomes using Tat, penetratin or Antp peptides was proportional to the number of peptide molecules linked to the liposomal surface. Thus, kinetics of the uptake was dependent on peptide- and cell-type [146, 156]. Moreover, the conjugation of CPPs such as octaarginine to liposomes enhanced the liposome uptake using airway cells upon inhalation [146, 156]. Although, the cell penetration was increased by CPP-liposome complexes with lower toxicity than DOTAP-containing liposomes; but however, the rapid intracellular release of the encapsulated drugs should be improved to obtain the pharmacological efficacy [157].

9.3.2.3. Micelles

Polymeric micelles represented an efficient type of drug carrier. Micelles are colloidal dispersions (~ 5-100 nm) that have ability to enhance the solubility and bioavailability of poorly soluble pharmaceuticals [146]. Their cell penetration could be increased by CPPs. For example, Tat peptide-bearing micellar system was used to target anticancer drugs to solid tumors [156].

10. Specific targeting of CPPs

An ideal drug delivery system (DDS) should specifically penetrate into the target cells, and accumulate in the specific tissue [42]. CPPs are effective tools for drug delivery into cells; but they do not have specificity to cell type [46]. Most CPPs were non-specifically linked to membranes of all cell types due to overall expression of heparin sulfate proteoglycan [69]. Recent efforts were performed using the activatable CPPs (ACPPs), the stimuli-responsive CPPs, and the specific localization sequences to deliver toward the proper cellular organelles [68]. There are different subcellular localization sequences with distinct properties that target a cytosolic protein to a specific organelle such as the endoplasmic reticulum (ER), nucleus, mitochondria, and chloroplast [150].

Recent use of CPPs was focused on development of NLS, pH/ temperature-sensitive targeted delivery, and synergistic effects of targeting ligands and CPPs [17]. In fact, some nanoparticle delivery systems were designed to activate CPPs, and drug release under specific conditions such as hyperthermy (40-42°C), low pH (< 6), light (UV), and interaction with specific enzymes (matrix metalloproteinases, thrombin and legumain) in tumor tissue [5]. Among amino acids used in CPPs, histidine is an essential amino acid with a protonable imidazolyl group which is needed for many enzymatic activities. For example, the replacement of tryptophan (W) by histidine (H) in the antimicrobial peptide sequence R2W2RW2R2 increased the antibacterial activity [160].

10.1. Activatable CPPs

Activatable CPPs (ACPPs) were used to visualize enzymatic reactions as molecular imaging probes [14]. They contain a polycationic CPP (D-Arg9) linked to an inhibitory polyanion (D-Glu9) via a cleavable linker. When this hairpin structure is unbroken, the charge is neutral and mainly covers the attachment of the CPPs [161]. The first ACPP was a protease-activatable CPP, thus a proteolytic cleavage released the activated peptide for cargo delivery to target cells [68]. Activatable CPPs were improved by transiently masking/covering the basic residues or shielding the overall peptide with polyethylene glycol (PEG). Different stimuli such as UV, pH and enzymes were used to remove masking moieties from CPPs leading to the recovery of intact CPP activity, depending on the target site [162]. ACPPs were directed toward extracellular enzymes such as matrix metalloproteinases, elastases and thrombin for *in vivo* detecting their localized enzymatic activity and also for accumulating cargo at the site of target [161]. ACPPs were used to monitor the activity of a family of zinc-dependent endopeptidases (matrix metalloproteinase: MMP) in tumors [17, 163]. These ACPPs with high permeability were sensitive to MMP, and were used to deliver anticancer drugs due to high level of MMP expression in tumor cells. In this line, a conjugate of ACPP with antitumor drug doxorubicin (DOX) sensitive to MMP-2/9 showed high efficiency to deliver antitumor drugs in HT-1080 tumor cells (overexpressing MMPs) as compared to MCF-7 tumor cells (under-expressing MMPs) [14, 164]. Moreover, the peptide sequence DPRSFL from the proteinase activated receptor 1 (PAR-1) was incorporated into an ACPP (DPRSFL-ACPP) for evaluation of thrombin activity in atherosclerosis.

This ACPP as a probe was composed of 9-D-Arg and 9-D-Glu separated by a protease cleavable linker for thrombin cleavage, and accumulation of the DPRSFL-ACPP cleavage product in advanced atherosclerotic lesions in mice [17]. ACPP was utilized to deliver selectively imaging molecules to tumor cells. The fluorescence and gadolinium-labeled ACPPs conjugated to dendrimers (ACPPDs) were developed to detect tumors during surgery. In addition to the improved target specificity of ACPPs, their major advantage against CPPs was the reduction of cytotoxicity likely because of the masked polycationic charge systemically [14].

10.2. Stimuli-responsive peptides

A nanoparticular drug delivery system is able to accumulate by the passive targeting via the enhanced penetration or by the active targeting through antibodies. In this line, the intracellular delivery could be mediated by especial ligands (*e.g.,* folate, transferrin) or by CPPs (*e.g.,* Tat or polyArg) [165]. Indeed, the CPPs were incorporated into ‘‘smart’’ DDS. At the first step of delivery, the non-specific CPP function was sterically protected by an organ/tissue-specific ligand (*i.e.,* a polymer or targeting antibody). Then, for accumulating in the site of target, the protective segment attached to the surface of the DDS through a stimulus-sensitive bond was detached under local environmental/pathological conditions (*i.e.,* abnormal pH or temperature) to reveal the CPP, and subsequently targeted delivery of the carrier and its cargo inside cells [6, 146]. For example, Tat-modified stimuli-sensitive polymeric micelles could significantly interact with cells under acidified conditions to develop gene delivery and tumor specific stimuli-sensitive drug delivery systems [156].

10.2.1. pH-sensitive CPPs

Different systems were designed to develop drug delivery into tumors based on acidic environment. Many chemical linkers (*e.g.,* hydrazine, or amide bonds) could be hydrolyzed under poor acidic conditions but were stable under neutral or poor alkaline conditions [145]. Another strategy was based on the use of materials that undergo conformation change in response to acidic conditions such as the imidazole group of histidine (pKa: 6.5) which is positively charged in the tumor microenvironment [145]. In addition, Kale *et al* designed the smart Tat-modified liposomes with the pH-sensitive

hydrazone bond and Tat modified on the surface of PEGylated long-circulating liposomes. The polyethylene glycol (PEG) chains protected the surface-attached Tat peptide at normal pH. Upon the exposure to the acidic environment of solid tumors, the hydrazone bond could be degraded and subsequently Tat moieties were exposed to deliver drug into tumor cells. Indeed, the liposome-attached Tat peptide residues were exposed and the penetration of the liposomes was enhanced into tumor cells leading to more effective gene delivery [166]. Moreover, Torchilin *et al* designed the pH-responsive, Tat-modified long-circulating liposomes and micelles. These long-circulating PEG-coated liposomes and micelles were targeted actively to a specific organ through the linkage of an antibody to PEG-phosphatidylethanolamine (PEG-PE) at their surface. PEG-PE is degradable at low pH due to a pH-sensitive bond between PEG and PE. The carriers were further modified with Tat-short PEG-PE derivatives. At normal physiological conditions, the longer PEG chains shield Tat on the shorter PEG chains. At lower pH, the longer PEG chains were cleaved from the complexes, thus Tat was exposed to enhance cellular uptake [166-168]. Recently, a multifunctional immunoliposomal nanocarrier was designed including a pH-sensitive PEG-PE component, Tat peptide and tumor cell-specific nucleosome-specific antibody (mAb2C5). This nanocarrier could potentially reduce non-specific interaction with non-target cells, effectively accumulate at tumor cells, and deliver anti-cancer drugs into cells [17]. Furthermore, histidine was broadly used to develop pH-responsive drug delivery systems [169]. Tu *et al*. produced membranolytic peptides with pH-dependent cytotoxicity by changing lysine and arginine residues with histidine in PTP-7 (FLGALFKALSKLL), L5 (PAWRKAFRWAWRMLKKAA) and Citropin (GLFAVIKKVASVIGGL) [170]. Zhang *et al*. also replaced all the lysines in TK peptide (AGYLLGKINLKKLAKL(Aib)LLIL-NH2) with histidines into TH peptide (AGYLLGHINLHHLAHL(Aib)HHIL-NH2), and showed that TH-conjugated camptothecin (TH-CPT) effectively enter cells in acidic environment [171]. Similarly, Jiang *et al*. improved dual-functional liposomes (HA-R6H4-L) with pH-responsive CPPs (R6H4: rich in arginines and histidines) and active targeting by hyaluronic acid (HA) for tumor-targeted drug delivery and decreased drug toxicity. Moreover, paclitaxel (PTX)-loaded

HA-R6H4-L had the strongest antitumor activity against mouse hepatic tumor model [172].

10.2.2. Antibody targeting strategy

Active targeting uses a homing tool including an antibody or a ligand (*e.g.,* oligonucleotides, peptides, vitamins or sugars) leading to attach a drug complex to tumor cells via receptors or antigens on the tumor cell surface. The efficiency of targeting depends on the specificity and affinity of the homing tools as well as the delivery of required dose for inducing considerable effect [46]. In 2013, the potency of efficient drug delivery was proven by fusion to a single-chain variable fragment (scFv) antibody directed towards a mutated K-ras in HCT116 tumor cells with high selectivity. In 2014, an antibody targeting strategy (*i.e.,* a heparin conjugated anti-carcinoembryonic antigen (CEA) monoclonal antibody) and genetically engineered fusion technique (*i.e.,* a CPP-fused chimeric protein: Tat-gelonin toxin inhibiting protein synthesis) were also utilized to treat colorectal cancer with low toxicity to normal tissues. Indeed, for obtaining the selectivity, the monoclonal antibody was linked to fusion construct through reversible electrostatic interaction [81]. It was observed that single-chain FVs (scFVs) antibody fragments accumulate slowly in tumors and eliminate rapidly from circulation leading to the limitation of their efficacy. Thus, CPPs (*e.g.,* penetratin) were used to direct scFV uptake out of circulation, increase rapid internalization into tumors, distribute homogenously the antibody fragment, and improve tumor retention [50].

10.2.3. Temperature-sensitive CPPs

The studies showed that Elastin-like polypeptide (ELP) passively accumulated in solid tumors after hyperthermia (between 39°C and 42°C) [14, 17]. Despite an increased response at high temperature, ELP could not often penetrate in blood vessels and cell membranes due to a relatively large size. The addition of CPPs to these carriers has significantly increased their delivery and antitumor activity [46]. For example, Walker *et al*. conjugated ELP to the anticancer drug doxorubicin (DOX) and used three different CPPs (Bac, Tat and SynB1) for suppressing tumor growth in mice. Tumor eradication under hyperthermic conditions with SynB1-ELP-DOX was two-fold higher than treatment with free doxorubicin at similar dose [173]. Moreover, the ELP-based drug

delivery system conjugated with a lactoferrin-derived L12 peptide was thermally targeted to tumor cells leading to suppression of their proliferation and reduction of systemic toxicity. It was reported that Tat-ELP-L12 along with heat effectively inhibited tumor cell proliferation, and induced cell death by necrosis or apoptosis mechanisms, *in vitro* [17].

10.2.4. Disulfide linkage

Disulfide linkage was broadly used to bind small molecule drugs to CPPs [81]. In one study, the low molecular weight protamine (LMWP) as a CPP was coupled to L-asparaginase through the formation of disulfide linkage. The LMWP-L-asparaginase encapsulated into red blood cells (RBCs) was used to treat acute lymphoblastic leukemia (ALL) [174]. Wender *et al* also showed that R8 CPP conjugated to the drug Taxol using a disulfide linkage was cleaved in the reducing environment of the cytosol, releasing the drug. All Taxol-transporter conjugates increased the sensitivity of human ovarian carcinoma cells resistant to Taxol *in vitro* with low toxicity as compared to Taxol [175].

11. Application of CPPs in preclinical trials

Over 2000 papers were published on the use of CPPs in preclinical trials. However, no CPP or CPP conjugate has achieved to the clinics [6]. Herein, we showed preclinical studies on CPPs in different aspects.

11.1. Antimicrobial activity of mitochondria-penetrating peptides (MPPs)

Mitochondria are a major target for different therapeutic approaches, but mitochondrial matrix- targeting sequences were relatively ineffective for delivering cargos to the mitochondria due to the impermeable structure of the hydrophobic inner membrane [150]. However, lipophilicity and positive charge are two major issues in molecules accumulated in the mitochondria [176]. Peptide-based mitochondrial agents have several advantages such as biocompatibility and easy modification with cargos, *e.g.,* peptide-based antioxidants [176]. To design the effective MPPs, specific thresholds of

charge (cationic residues) and lipophilicity were identified to deliver a variety of bioactive cargos into mitochondria [50]. The potent delivery of a peptide-based antioxidant to mitochondria containing the aromatic and cationic residues indicated that this motif is effective for mitochondrial delivery [176]. For instance, the human antimicrobial peptide Histatin 5 as a CPP found in human saliva could be used for the selective delivery of cargos into fungal and protozoan mitochondria. Its antimicrobial activity is due to the ability to accumulate within the mitochondria and suppress F1F0-ATPase [177, 178] indicating a decrease in mitochondrial membrane potential and in bioenergetic collapse of the parasite. Histatin 5 could be conjugated to leishmanicidal agent and translocated into the parasitic mitochondria as a therapeutic agent with dual antimicrobial activity [50].

11.2. Vaccine

CPPs are important for delivery of antigens into cellular compartments (*i.e.,* antigen presenting cells: APCs) in vaccine development especially DNA- and protein/ peptide-based vaccines [50, 179, 180, **Table 4**]. Different cargos were attached to CPPs for intracellular delivery in vaccine development [180]. Generally, the incorporation of CPPs in vaccine delivery systems may improve antigen uptake by APCs and thus it can be considered as a safe alternative or additive to classical adjuvant formulations. CPPs were suggested as a promising agent for vaccine delivery. CPPs were often fused with antigens to achieve efficient cell membrane translocation, enhancing antigen uptake, processing and presentation by APCs. CPPs were also incorporated into several DNA vaccine candidates to facilitate the transport of genetic material through nuclear and plasma membranes [181]. Indeed, CPPs provided effective means to facilitate intracellular delivery of antigens and induce a cytotoxic T lymphocyte (CTL) immune response [180]. In a study, our group used Pep-1 for *in vitro* and *in vivo* delivery of HPV16 E7 protein as a tumor antigen. Our data indicated that E7/Pep-1 nanoparticles in a certain molar ratio of 1: 20 induced Th1 immune responses and protected mice against TC-1 tumor cells similar to the group immunized with E7 emulsified with Freund adjuvant (~ 80% tumor-free mice) [182]. Moreover, priming with HIV-1 MPER-V3 DNA/ MPG nanoparticles at N/P ratio of 1:10 followed by MPER+V3 peptides as boosting could direct T cell immune responses toward a Th1-type [183]. In this line, HPV16 E7

DNA/ MPG in nanoparticle formulation at an N/P ratio of 10:1 elicited an effective Th1 cellular immune response and completely protected mice against tumors, as well [184]. In another study, the induction of cell-mediated immune response (Th1-biased response) against HCV core and HCV coreE1E2 antigens was stronger in mice immunized with coreE1E2 DNA/MPG and then core DNA/MPG nanoparticles as compared to coreE1E2 and core DNA constructs alone [185]. In addition, the use of Tat CPP fused to Nef antigen (Tat-Nef) in heterologous prime-boost strategy along with Cady-2 CPP significantly induced the Nef-specific T cell responses for development of HIV-1 vaccine [186].

In general, CPPs were used to deliver antigenic peptides or proteins, induce adaptive immune responses and activate both $CD8^+$ and $CD4^+$ T cells [187]. For example, the EBV ZEBRA protein-derived CPPs (Z12, Z13 or Z14) linked to antigenic cargos (*e.g.,* gp100 and TRP2 tumor antigens) were improved as a strong system to break self-tolerance and to elicit therapeutic anti-tumor immune responses *in vivo* [188, 189]. Increased antigen (Ag)-specific immune responses were also reported by linking other tumor antigens (*e.g.,* carcinoembryonic antigen, TRP2, survivin, p53, HPV16 E7, MUC-1 or HER2/*neu*) to a CPP [187]. Wang *et al*. demonstrated that the linkage of TRP2 Ag to CPPs could prolong antigen presentation by dendritic cells (DCs) [190]. On the other hand, the herpes simplex virus (HSV-1) VP22 CPP could facilitate intercellular spreading of the attached cargo. For example, DNA vaccination with VP22 linked to HPV16 E7 or E6 (VP22-E7 or VP22-E6) significantly induced $CD8^+$ T cell responses and anti-tumor immunity against the E7-expressing tumors in mice [180]. These findings were confirmed in DNA vaccines expressing VP22 fused to antigens from other diseases including bovine herpesvirus 1, influenza virus and porcine reproductive and respiratory syndrome virus [180]. A finding showed that DNA vaccines encoding E7 conjugated to Tat or Antp (Tat-E7 or Antp-E7) could not elicit potent $CD8^+$ T cell responses as observed by HSV-1 VP22-E7 DNA vaccine [191].

The efficacy of several CPPs (*e.g.,* MPG, Cady-2, Pep-1, P28 and hPP10) was studied to improve DNA- or protein-based therapeutic vaccines against HPV infection. The data showed that E7 DNA + MPG prime/E7 protein + P28 boost-based nanovaccines significantly induced Th1 immune responses, and completely protected mice against

TC-1 tumor cells. Indeed, P28 and MPG peptides were effective protein and gene delivery systems, respectively [192]. Moreover, the NT-gp96 fused to E7 (NT-E7 fusion DNA as an antigen) in combination with IP-10 chemokine and PEI600-Tat delivery system significantly increased the efficiency of HPV DNA vaccines against HPV-related cancers [193]. On the other hand, CPP-antigen-based DC vaccination could enhance CTL responses against cancer and infectious diseases without receptor targeting. For example, penetratin or poly-arginine (R9) CPPs linked to the CD4 or CD8 specific OVA epitope-pulsed DCs successfully induced T cell proliferation and immune responses against OVA expressing tumor cells and reduced tumor size in mice. Moreover, mature Tat-Her2/*neu*-pulsed DCs elicited Her2/*neu*-specific $CD8^+$and $CD4^+$ T cell responses in a breast tumor model [194]. As known, the p53 protein is a potent tumor antigen in both mouse and human cancer vaccines. A study showed that immunization with Tat-p53-pulsed DCs in HLA-A-0201/Kb transgenic mice induced antigen-specific $CD4^+$ T cell responses [180]. In addition, a major use of CPPs is the ability to deliver synthetic multiple epitopes in peptide vaccines [180]. For instance, Dakappagari *et al*. showed that immunization with a multi-epitope peptide harboring Pep-1 linked to three HLA-A2 restricted epitopes of Her-2/*neu* peptides induced higher CTL responses than the multiepitope peptide alone in HHD HLA-A2 transgenic mice [195]. The immunostimulatory properties of HIV-1 Nef DNA and protein constructs were evaluated using small heat shock protein 20 (sHsp20) and Freund's emulsion as an adjuvant, and four CPPs (HR9, MPG, M918, and penetratin) as a gene or protein carrier in BALB/c mice. The data indicated that a heterologous Hsp20-Nef DNA + MPG prime/ rHsp20-Nef protein+M918 boost regimen significantly elicited higher levels of IgG2a, IgG2b, IFN-gamma, and Granzyme B directed toward Th1 responses in a long period (3 months) after the last immunization compared to other groups. These findings demonstrated that the simultaneous use of M918 and MPG CPPs as protein and gene carriers improves HIV-1 Nef-specific B- and T-cell immune responses as a promising approach for development of HIV-1 monovalent vaccine [196]. Similarly, other data showed that HR9, and Cady-2 could form stable nanoparticles with HCV NS3 and heat shock protein 27 (Hsp27)-NS3 genes, and proteins, respectively and enhance their delivery into HEK-293T cells in a non-covalent approach. Furthermore, the heterologous

Hsp27-NS3 DNA+HR9 prime/rHsp27-NS3+Cady-2 protein boost elicited a higher Th1 cellular immune response with a predominant IgG2a, IgG2b, IFN-γ profile and strong Granzyme B secretion than those induced by other groups. Briefly, the combination of a natural adjuvant (Hsp27) and CPPs (HR9 and Cady-2) could significantly stimulate effective immune responses as a promising approach for development of HCV therapeutic vaccines [197].

11.3. Imaging agents

Quantum dots (QDs, size: 1-6 nm) as fluorescence probes with high intensity were used not only for *in vitro*/ *in vivo* imaging studies, but also for disease diagnosis (*e.g.,* cancer) [198]. The most important QDs are Cadmium Selenide (CdSe) and Cadmium telluride (CdTe) [14]. However, delivery of QDs into cells is difficult due to the cell membrane barrier. The mixture of CPPs with QDs could solve this problem [198]. Indeed, the CPP-mediated delivery was used to label cells with QDs such as CPP-modified QD-loaded polymeric micelles [156]. The uptake mechanism of the non-covalent CPP-QD complexes into cells was through endocytosis [198]. Tat-QD conjugates could label mouse endothelial cells *in vitro* and reveal tumor neovascularization *in vivo* [156]. Tat peptide was used to deliver QDs into rat brain tissue, as well [14]. An arginine-rich CPP (SR9) facilitated the delivery of QDs into A549 cells in a non-toxic approach [14]. In addition, the non-covalent mixture of CPPs (*e.g.,* $G(SG)_4TP10$ or $F(SG)_4TP10$) with CdSe/ZnS QD increased the membrane permeability more effectively than QD alone [198]. On the other hand, labeling of QDs using octaarginine (R8) could be used for *in vivo* imaging [14]. Generally, the cellular uptake of the QD-CPP complexes depends on the dose of QD and CPP as well as the cell type [14]. Except to QDs, a H_2O_2 targeting mechanism was developed based on activatable CPPs (H_2O_2-ACPP) to monitor the oxidative burst of promyelocytes *in vitro,* and lung inflammation *in vivo* [161]. Moreover, ACPPs were used to detect the pathological process of stroke *in vivo* as gelatinase-specific non-invasive probes [163].

11.4. Treatment

Several studies have focused on the use of CPPs for delivering bioactive agents into tissues as well as on the study of their therapeutic applications in a variety of disorders [46].

11.4.1. Neurodegenerative diseases

A variety of strategies have been developed to increase BBB penetration and access drugs to the brain including neurosurgery-based strategies, pharmacology-based strategies, and physiology-based strategies. However, there are some problems for these approaches such as the risk of infection and neurosurgical costs as well as drug accumulation in non-target sites due to its high lipophilicity [157]. Therefore, novel and non-invasive approaches are required to overcome these problems such as the use of CPPs. For example, the brain uptake of doxorubicin conjugated to D-penetratin or SynB CPP was increased by *in situ* brain perfusion in rats and mice [199]. As known, prion diseases are fatal neurodegenerative disorders in humans which are caused by a misfolded prion protein (PrPsc) instead of its normal isoform (PrPc). Recently, a CPP composed of 28 amino acids was determined in PrPc which possess a region for specific interaction with the PrPsc to prevent further conversion of PrPc to PrPsc [50]. Moreover, it was observed that an increase in the anti-apoptotic Bcl-XL protein in rodent brain led to increase the resistance against ischemic injury. The studies indicated that a Tat-HA-Bcl-XL or Tat-Bcl-XL fusion protein reduced the infarct volume and increased neuroprotective effects in mice [86, 200, 201]. On the other hand, Yang *et al*. showed that the intranasal delivery of a NF-κB peptide inhibitor fused to Tat CPP (Tat-NBD) effectively diminished NF-κB signaling, microglial activation and Hypoxic-ischemic (HI) brain injury in animal models [202]. Moreover, the Bcl-2 homology domain 4 (BH4) of Bcl-XL fused to Tat CPP (Tat $_{48-57}$-BH4) was able to reduce neural cell death *in vitro* by regulating the efflux of intracellular calcium, and also improve the survival of ALS transgenic mice after chronic treatment [203].

11.4.2. Cardiovascular Diseases

The intracellular delivery of GATA4 transcription factor using VP22 CPP showed a positive effect after myocardial infarction in Lewis rats by improved myocardial function

[50]. In another study, treatment with the Antp-NBD peptide was able to improve cardiac function in mice lacking dystrophin and its homolog utrophin. Previous studies indicated that two fusion proteins containing Pep-1 CPP linked to zinc superoxide dismutase (Pep-1-SOD1) or catalase (Pep-1-CAT) could transduce the myocardium and protect it against ischemia-reperfusion (IR)-induced damage. Furthermore, the combination of Pep-1-SOD1 and Pep-1-CAT was more effective than each peptide by increasing expression of the anti-apoptotic Bcl-2 protein, removing Reactive oxygen species (ROS) and subsequently protecting the heart against IR injury [203].

11.4.3. Bacterial sepsis

CPP-mediated modulation of the immune response was observed to overcome bacterial sepsis. Herein, CPPs were used to deliver anti-apoptotic proteins as a treatment for sepsis. It was reported that *in vivo* injection of the Bcl-xL or its BH4 domain conjugated to Tat reduced sepsis-induced lymphocyte apoptosis and immune system depletion as well as improved survival following sepsis [50].

11.4.4. Duchenne muscular dystrophy

CPPs have been recently used to deliver phosphorodiamidate morpholino oligomer (PMO) into mouse models of Duchenne muscular dystrophy (DMD) which possess a nonsense mutation in the dystrophin gene [50]. Activation of NF-κB signaling was reported in DMD patients, thus NF-κB was proposed as a possible molecular target for treatment of this disorder [203]. Peterson *et al*. showed that mice treated with a peptide inhibitor of NF-κB containing NBD fused to the Antennapedia PTD (Antp-NBD) improved motor performance and progressive weakness of skeletal muscles [204].

11.4.5. Cancer therapy

Tumor-targeted drug delivery systems are an important approach for cancer treatment [205]. Recently, CPPs were used to deliver chemotherapeutic drugs (*e.g.,* Taxol, cyclosporine A and methotrexate) and pro-apoptotic proteins into resistant cells and improve their activity [50]. For example, the VP22-p53 chimeric protein induced apoptosis in p53-negative human osteosarcoma cells [206]. Moreover, targeting the

tumor tissue was performed through specific tumor-related biomarkers which showed some problems such as the heterogeneity of the tumor tissues and the development of resistance [160]. To overcome these problems, multifunctional CPPs were developed with their physicochemical properties [160]. For enhanced cytosolic delivery of the anticancer drug Bleomycin (BLM), R8-modified fusogenic DOPE liposomes (R8-DOPE-BLM) were developed to induce cell death and DNA damage *in vitro* [14]. Another strategy to target tumor is the use of tumor homing domains linked to CPPs (*e.g.,* PEGA homing domain conjugated to the pVEC CPP). Indeed, the homing domain was naturally cell impermeable but its linkage to the CPP led to the effective and selective uptake into tumor in mice [50]. Different types of cancer were treated with irinotecan, a prodrug that is converted into its active metabolite SN38 (7-ethyl-10-hydroxycamptothecin) by the action of liver carboxylesterases. However, SN38 cannot be administered directly due to its high insolubility [203]. Meyer-Losic *et al* generated DTS-108, a novel water-soluble compound including SN38 linked to a highly charged oligopeptide of human origin named as DPV1047 which significantly released high levels of SN38 after intravenous injection in dogs [207]. On the other hand, peptides and protein domains derived from cyclin-dependent kinases inhibitors (*i.e.,* p21, p16Ink, p27kip or p15) conjugated to CPPs (*e.g.,* p27kip-Tat or p16Ink-penetratin) suppressed tumor growth *in vivo* [208].

11.4.6. Transplant rejection

A key factor in transplant rejection is the proto-oncogene c-Myc expressed in different tissues including liver, lung and cornea. Recently, CPPs have been used for treatment of transplant rejection. Hosseini *et al.* showed the ability of a novel compound named as AVI-5126 peptide (formulation: c-Myc antisense PMO linked to an arginine-rich CPP) to prevent corneal rejection in rat [209].

11.4.7. Ocular medication

Recently, a cell penetrating peptide for ocular delivery (POD CPP acting as a NLS) was designed to deliver small molecules such as fluorescent dyes (*e.g.,* GFP) into retinal cells *in vitro* and *in vivo*. The studies showed that the HIV Tat and HSV VP22 CPPs

were able to deliver recombinant proteins to human embryonic retinoblasts *in vitro* but not *in vivo*. Thus, POD-fusion proteins were more effective for penetration of macromolecules in the retina as compared to Tat- fusion proteins or VP22- fusion proteins for therapeutic applications [5].

12. Application of CPPs in clinical trials

In spite of the large number of preclinical and clinical trials that are currently underway, no CPP has approved by US Food and Drug Administration (FDA) [46]. A reason is the slow release of the free drug to compete with its clearance. Up to now, few CPP-linked drugs have entered the clinic for both topical and systemic administration [5, 210]. The first compound that entered phase II clinical trial was a cyclosporine A (CsA)-polyarginine conjugate (PsorBan1; CellGate, Inc.) for the topical treatment of psoriasis [203]. AZX-100 is a CPP that mimics heat shock protein 20 function (HSP20) entered phase II clinical trial leading to relaxation of smooth muscle and prevention of dermal scarring after topical application [6]. Most clinical trials have involved HIV Tat CPP, but none of them were approved by the FDA [5, 51, 183, **Table 5**]. For example, a peptide inhibitor of c-Jun N-terminal kinase conjugated to Tat (termed as XG-102 in clinical trial) was used to protect against apoptotic cell death in cerebral ischemia [5]. Moreover, Phase I clinical trial studies of azurin-derived p28 CPP in treating patients with progressive central nervous system (CNS) tumors and refractory disease were reported [NCT00914914, 46, 203]. Phase II clinical studies of DTS-108 were also performed in patients with metastatic colon or rectal cancer for intravenous application [46]. On the other hand, a Phase I clinical trial for an HIV vaccine based on HIV-1 Tat and V2-deleted Env proteins was conducted by Istituto Superiore di Sanita and Novartis (ISS P-002) [6]. In 2016, a Phase I/II clinical trial of TransMTS1-botulinum toxin A (RT002) for the treatment of glabellar lines confirmed its safety and efficacy (NCT02303002) as well as a currently ongoing Phase II study of the treatment of cervical dystonia (NCT02706795) [203]. An initial Phase Ib/II clinical trial to evaluate the safety and efficacy of a CPP-PMO conjugate (AVI-5126: (R-Ahx-R) 4AhxB-PMO targeted to human c-myc) for the *ex-vivo* treatment of vein tissue in coronary artery bypass grafts has started in Poland and Ukraine and designed to prevent inappropriate cell proliferation

that can occur after the grafting procedure [211]. Kinali *et al* also studied the safety and efficacy of intramuscular administration of AVI-4658, a CPP-PMO designed to induce the expression of dystrophin locally in treated muscles in Phase I/II clinical study of DMD patients (NCT00159250). A Phase I/II clinical trial in 2015 assessed the safety and efficacy of repeated doses of systemic intravenous AVI-4658 in DMD patients (NCT00844597) and the compound was well tolerated. A Phase III clinical trial was started, as well (NCT02255552) [203, 212].

13. Conclusion

The efficiency of CPPs to penetrate the cell membrane and deliver different cargos to specific intracellular sites is a suitable approach for delivery of chemotherapeutics. Although many preclinical studies showed the promising results through the CPP-mediated delivery of therapeutic molecules in treating cancer and other diseases, no CPP-based therapeutic approach has approved by FDA, yet. Some disadvantages for CPPs are their short half-life in blood, and non-specific CPP-mediated delivery to normal tissue. These problems could be improved by coupling CPPs to other carriers (*e.g.,* liposomes), and the use of endogenous (*e.g.,* specific enzymes or pH value) and exogenous stimuli (*e.g.,* mild heat), respectively for increasing CPP-mediated therapeutic efficacy. Indeed, the stimuli could enhance the release of cargos and/or the specific accumulation of CPP-delivered drugs to minimize toxic effects in normal tissues and improve treatment efficiency.

14. Expert opinion

Due to low cytotoxicity of CPPs as compared to other carriers and final degradation to amino acids, they are suitable for preclinical and clinical studies. It was observed that cationic CPPs were less toxic than amphipathic CPPs *in vitro* and *in vivo*. Up to now, a large number of these peptides have been identified with different sequences. CPPs were divided into three subgroups using their physicochemical properties such as cationic (~ 83%), amphipathic (~ 44%) and hydrophobic (~ 15%) peptides. They have various uptake mechanisms to transport different cargos at a low micromole range. However, it is important to predict which CPP is optimal for target of interest. Up to now,

numerous *in silico* CPP prediction algorithms were established to facilitate screening of peptides. There are 1699 unique CPP sequences that most of them are linear CPPs (94.5%) based on the CPP database site. The major researches on CPPs focus on synthetic peptides (~ 54.8%). The mechanism of the CPP uptake significantly varies based on cell type, linkage type, incubation time, dose and physiochemical properties (*e.g.,* hydrophobicity and net charge). The cellular uptake of CPPs can occur through several approaches such as direct penetration, and clathrin/ caveolae-mediated endocytic uptake depending on the nature of the peptide/ cell membrane interaction. Direct penetration as an energy-independent approach includes various mechanisms such as pore formation, inverted micelle formation, the membrane thinning model, and the carpet-like model. Direct penetration further occurs at high doses of CPPs especially primary amphipathic peptides (*e.g.,* MPG or transportan). An increased potential of the cell membrane led to high internalization of CPPs. Endocytosis contains two main mechanisms for the uptake of biomolecules or other cells: phagocytosis in special cells (*e.g.,* macrophages), and pinocytosis in most cells such as macropinocytosis, clathrin-mediated endocytosis or caveolae/lipid raft-mediated endocytosis. Lowering temperature as well as depletion of cell energy effectively confirmed the endocytotic mechanism for the internalization of non-covalent protein/ CPP complexes. The nature of the cell membrane limits the cellular uptake of drugs to small size (less than 600 Da), and to hydrophobicity. Thus, researchers attempt for effective delivery of proteins and peptides into the cells which have *in vivo* short half-life and poor bioavailability. The studies showed that the synergistic or combined effects of CPPs with other carriers for delivery of protein/peptide drugs increased their therapeutic effects in various disorders especially cancer. Some methods were used to develop the functions of CPPs *in vitro* and *in vivo* including the augmentation of cell specificity by activatable CPPs, specific transport into cell organelles by insertion of corresponding localization sequences, incorporation of CPPs into multifunctional dendrimeric or liposomal nanocarriers to improve selectivity and efficiency especially into tumor cells. In general, further studies are required to overcome some problems related to CPPs for using in clinical trials. Recently, CPPs were proposed for delivery of antigens into APCs in vaccine development. These CPPs were used to deliver DNA, peptide and protein into the cells

such as Pep-1, MPG, VP22, Tat, Cady-2, P28, hPP10, penetratin, and poly-arginine. In this line, it seems that CPPs complexed with antigen are more effective than CPPs linked to antigen due to direct penetration and the lack of endosomal escape. On the other hand, recently, a combination of small molecules was discovered to push the highly efficient intracellular delivery of native proteins, independent of any transduction peptide. This process was termed as ‘‘iTOP’’ for induced transduction by NaCl-mediated hyperosmolality in combination with a transduction compound (*i.e.,* propanebetaine). It was observed that iTOP allows the highly efficient delivery of recombinant cytoplasmic and nuclear proteins into a broad variety of primary cell types [354]. Thus, it will be interesting to compare the efficiency of iTOP method with cell penetrating peptides for protein delivery.

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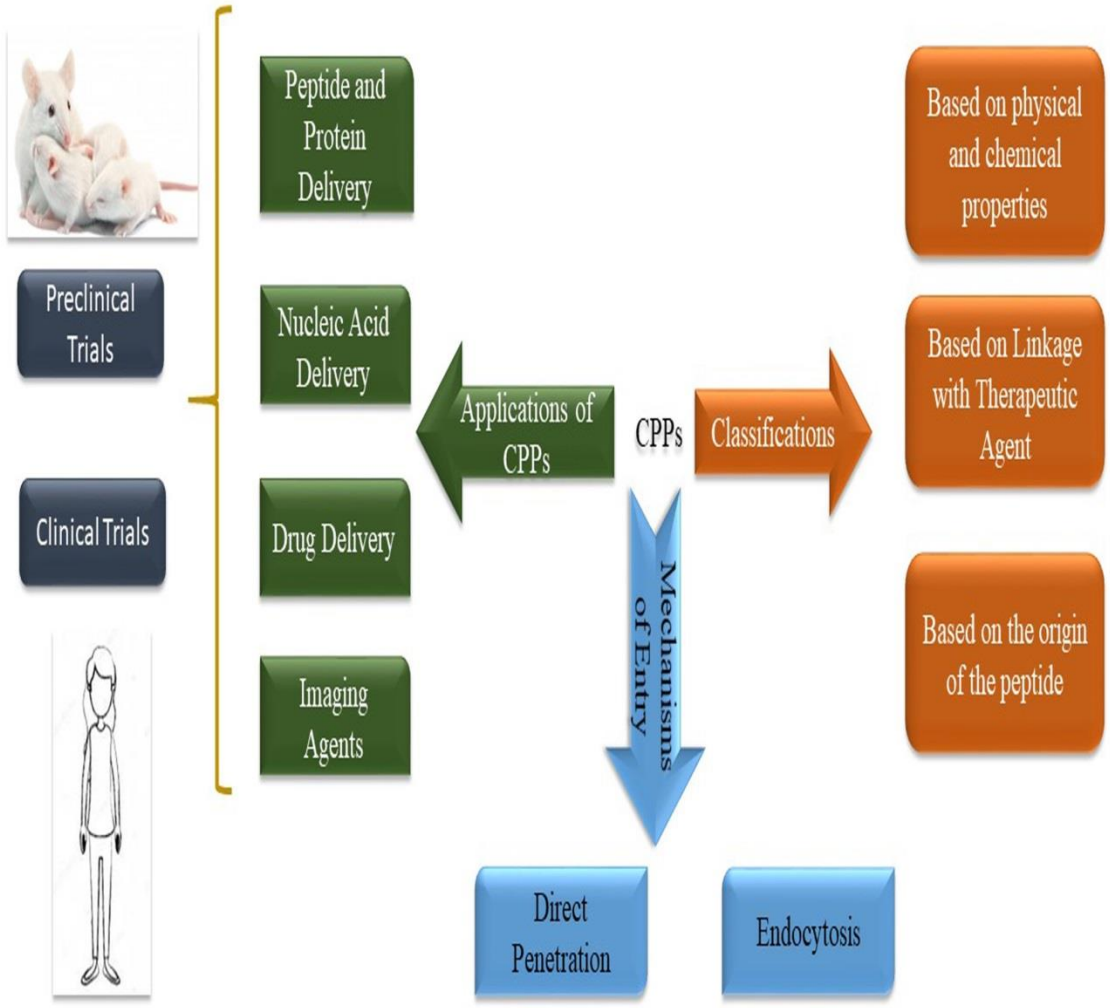
Figure Legends

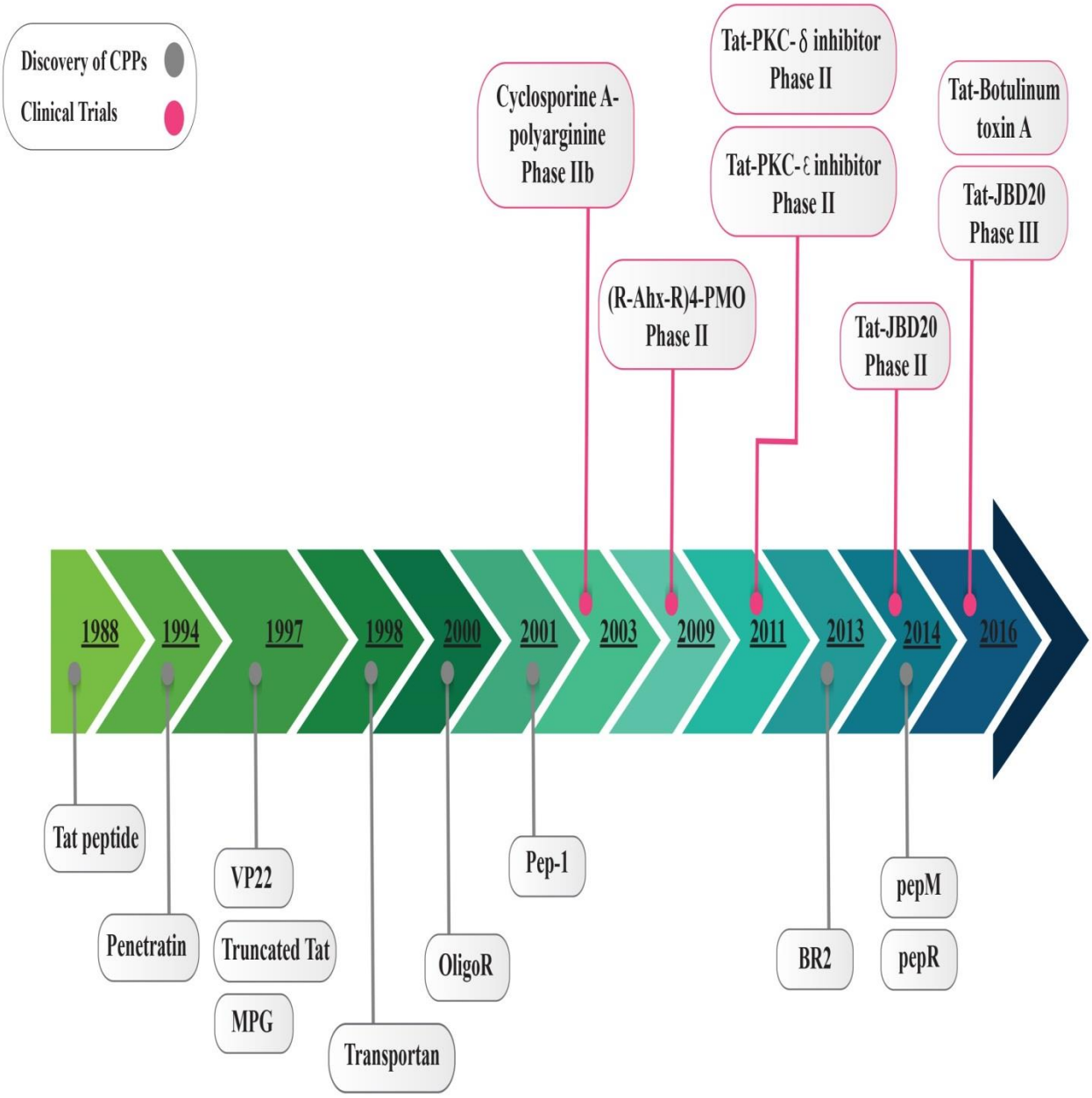
Figure 1: Schematic model of discovery and clinical trials of several main CPPs; PKC, Protein kinase C; PepM and PepR: CPPs derived from two domains of the dengue virus for delivery of nucleic acids into cells.

Figure 2: Classifications of CPPs based on Applications and Linkage

Figure 3: Mechanisms of CPP uptake across the cellular membrane: Different uptake mechanisms were proposed to explain the internalization of free or cargo-conjugated CPPs. CPPs along with small cargoes may enter cells quickly via direct translocation in addition to the endocytic pathway. Uptake of large molecules attached to these peptides tended to be mediated by macropinocytosis in an energy-dependent manner with slower rates for larger compounds.



Graphical abstract


Discovery of CPPs
Clinical Trials
Cyclosporine A-polyarginine Phase IIb
Tat-PKC-δ inhibitor Phase II
Tat-Botulinum toxin A
Tat-PKC-ε inhibitor Phase II
Tat-JBD20 Phase III
(R-Ahx-R)4-PMO Phase II
Tat-JBD20 Phase II
1988
1994
1997
1998
2000
2001
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2013
2014
2016
Tat peptide
Penetratin
VP22
Truncated Tat
MPG
Transportan
OligoR
Pep-1
BR2
pepM
pepR

Figure 1

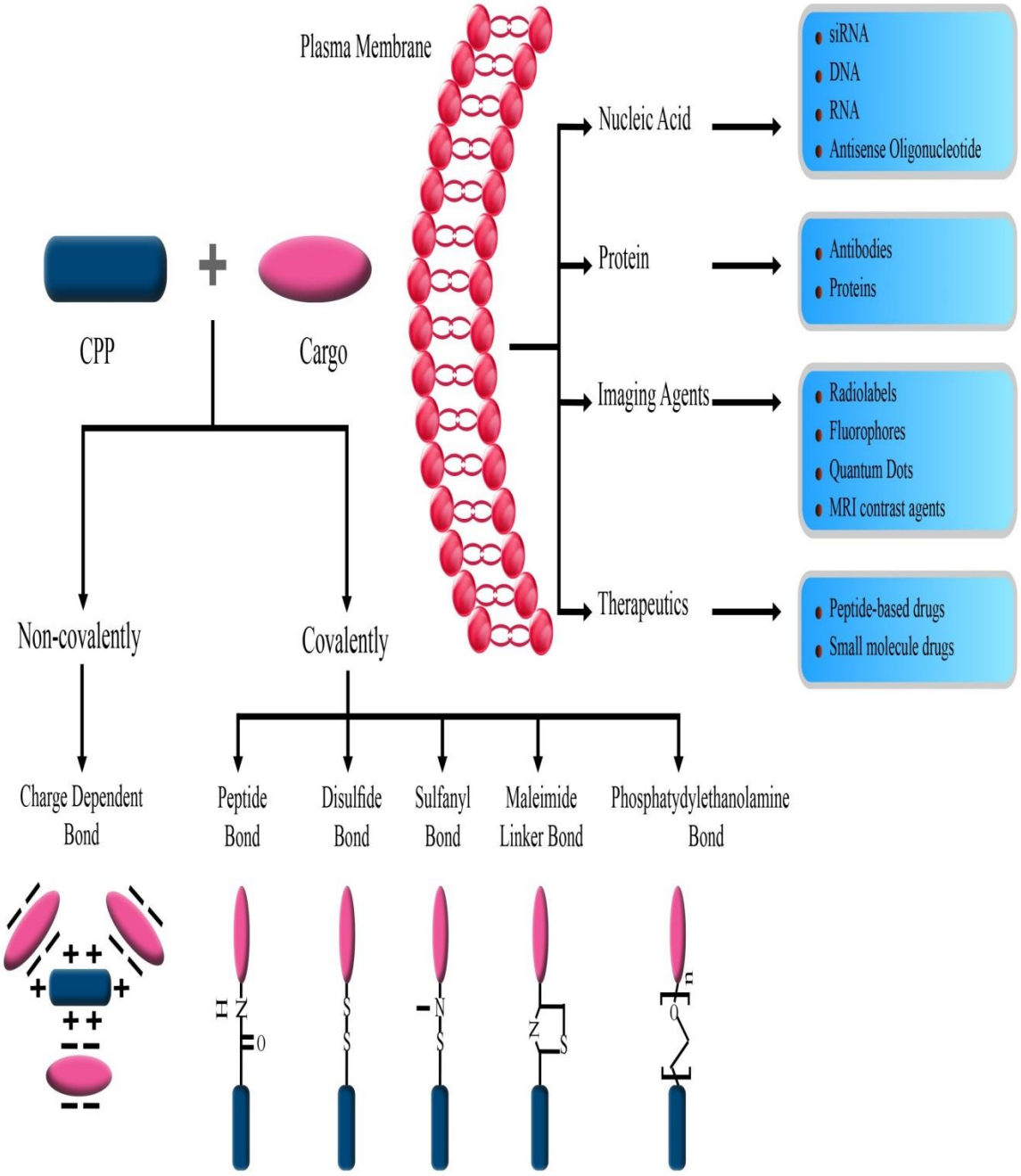
Plasma Membrane
CPP
Cargo
Non-covalently
Covalently
Charge Dependent Bond
Peptide Bond
Disulfide Bond
Sulfanyl Bond
Maleimide Linker Bond
Phosphatydylethanolamine Bond
Nucleic Acid
Protein
Imaging Agents
Therapeutics
siRNA
DNA
RNA
Antisense Oligonucleotide
Antibodies
Proteins
Radiolabels
Fluorophores
Quantum Dots
MRI contrast agents
Peptide-based drugs
Small molecule drugs

Figure 2

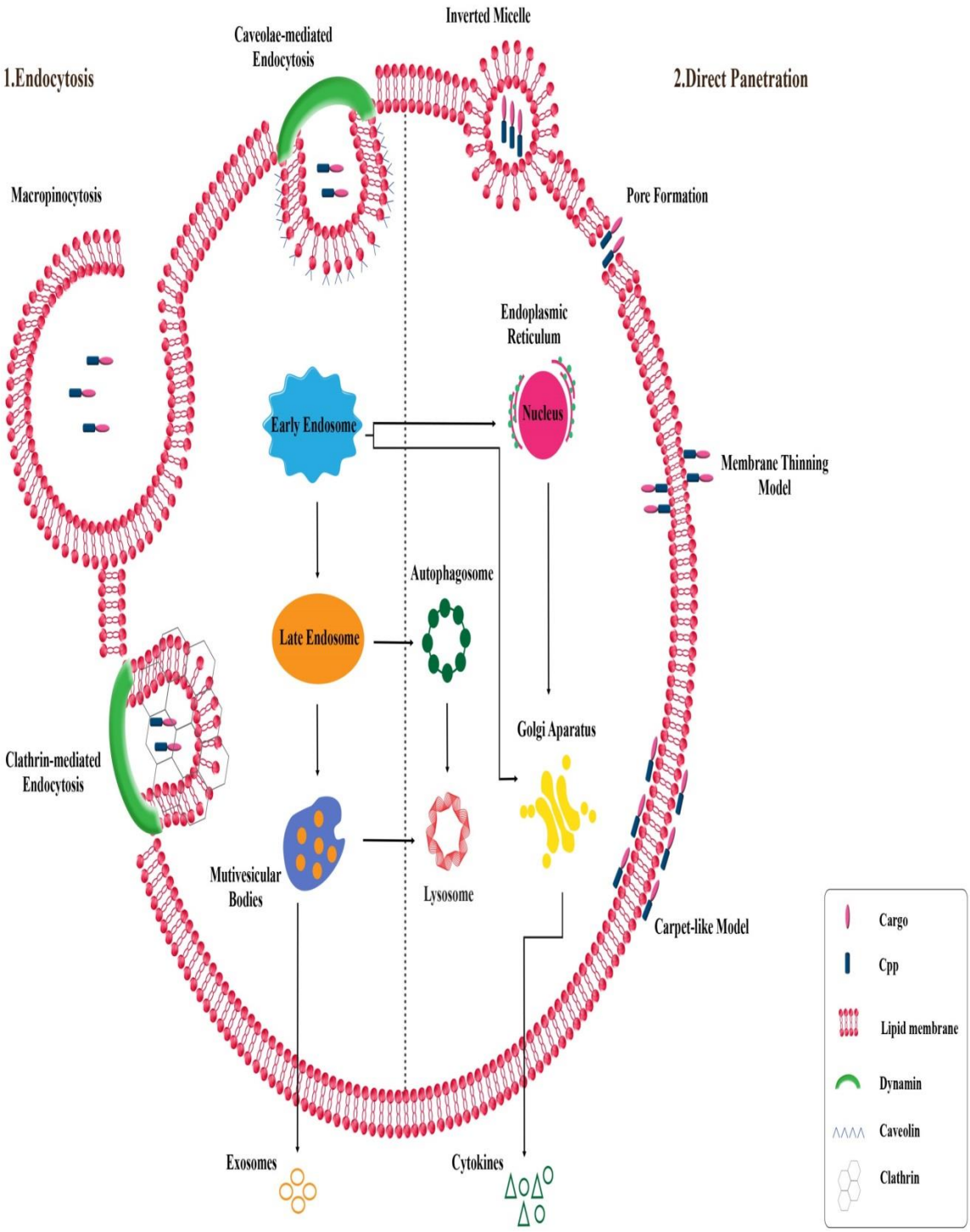
1.Endocytosis
Caveolae-mediated Endocytosis
Inverted Micelle
2.Direct Panetration
Macropinocytosis
Pore Formation
Endoplasmic Reticulum
Early Endosome
Nucleus
Membrane Thinning Model
Autophagosome
Late Endosome
Golgi Aparatus
Clathrin-mediated Endocytosis
Mutivesicular Bodies
Lysosome
Carpet-like Model
Exosomes
Cytokines
Cargo
Cpp
Lipid membrane
Dynamin
Caveolin
Clathrin

Figure 3

Table 1: Physicochemical properties of CPPs

CPP	Sequence*	Length	No. of Arg	No. of Lys	Net Charge**	Water Solubility**	Iso-electric point**	MW (g/mol)**	Origin	Ref.
Tat (48–60)	GRKKRRQRRRPPQ	13	6	2	+8	Good	12.81	1719.02	HIV-Tat domain	[43]
Tat (47-57)	YGRKKRRQRRR	11	6	2	+8	Good	12.41	1559.83	HIV-Tat domain	[213]
Tat (46-57)	CYGRKKRRQRRR	12	6	2	+7.9	Good	12.13	1662.98	HIV Tat Protein	[214]
Tat (49–57)	RKKRRQRRR	9	6	2	+8	Good	12.81	1339.61	HIV-1 Tat protein transduction domain	[215]
HIV-1 Rev (34–50)	TRQARRNRRRRWRERQR	17	10	---	+9	Good	12.7	2437.74	HIV-1 protein	[23, 216]
Penetratin (Antp)	RQIKIWFQNRRMKWKK	16	3	4	+7	Good	12.45	2246.73	Drosophila antennapedia gene homeobox	[217]
pVEC	LLIILRRRIRKQAHAHSK	18	4	2	+6.2	Good	12.59	2209.69	Murine vascular endothelial cadherin protein	[218]
M918	MVTVLFRRLRIRRACGP-PRVRV	22	7	--	+6.9	Good	12.5	2652.3	Peptide derived from the tumor suppressor protein p14ARF	[79]
ARF(1–22)	MVRRFLVTLRIRRACG-PPRVRV	22	7	---	+6.9	Good	12.5	2652.3	p14ARF protein	[219]
Mastoparan	INLKALAALAKKIL-amide	14	---	3	+4	Poor	14	1478.91	Isolated from the hornet venom of Vespa affinis	[220, 221]
TP10	AGYLLGKINLKALAAL-AKKIL	21	---	4	+4	Poor	10.78	2182.73	Derived from mastopara	[222]

									n	
NLS	CGYGPKKKRKVGG	13	1	4	+4.9	Good	10.82	1377.66	SV40 NLS peptide	[16]
NLS	PKKKRKV	7	1	4	+5	Good	11.79	883.14	SV40 NLS peptide	[223]
LDP-NLS	KWRRKLKKLRPKKKR-KV	17	4	8	+12	Good	12.63	2276.91	Latarcin1	[99]
LDP	KWRRKLKKLR	10	3	4	+7	Good	12.45	1411.79	Latarcin1	[99]
hCT(9–32)	LGTYTQDFNKFHTFPQT-AIGVGAP	24	---	1	+0.1	Poor	7.74	2610.87	A hormone secreted by the C cells of the thyroid	[224]
DPV3	RKKRRRESRKKRRRES	16	8	4	+10	Good	12.34	2212.58	Human heparin binding proteins and/or anti-DNA antibodies	[225]
Secretin	HSDGTFTSELSRLRDSA-RLQRLLQGLV	27	4	---	+1.1	Good	10.28	3056.39	Human	[226]
LL-37	LLGDFFRKSKEKIGKEFK-RIVQRIKDFLRNLVPRTES	37	5	6	+6	Good	11.15	4493.26	Human Cathelicidin Family	[227]
Lactoferrin sequences	SQPEATKCFQWQRNMRK-VRGPPVSCIKRDSPIQI	34	4	3	+4.9	Good	10.85	3985.63	derived from human lactoferrin	[228]
RGD	GRGDSY	6	1	---	0	Good	6.59	653.64	Various circulating proteins	[229]
Sweet arrow peptide (SAP)	(VRLPPP)3	18	3	---	+3	Good	12.4	1997.48	N-terminal domain of γ-zein	[17]
hLF	KCFQWQRNMRKVRGP-PVSCIKR	22	4	3	+6.9	Good	11.75	2718.29	Antimicrobial peptides	[17]
Bac7 (1-24)	RRIRPRPPRLPRPRPRPL-PFPRPG	24	9	---	+9	Good	13	2938.54	Bacteneicin family of antimicrobial peptides	[230]

Buforin IIb	RAGLQFPVG[RLLR]3	21	7	---	+7	Good	12.88	2560.15	histone H2A-derived antimicrobial peptide	[231]
sC18	GLRKRLRKFRNKIKEK	16	4	5	+8	Good	12.17	2070.54	derived from cationic antimicrobial protein, found in rabbit leukocytes	[232]
Protegrin-1	RGGRLCYCRRRFCVCVG-R	18	6	---	+5.7	Good	10.7	2160.63	Isolated from porcine leukocytes	[199, 233]
BPrPp (1–28)	MVKSKIGSWILVLFVA-MWSDVGLCKKRP	28	1	4	+3.9	Poor	10.67	3192.95	The N-terminus of the unprocessed bovine prion protein	[234]
DPRSFL	DPRSFL	6	1	---	0	Good	6.68	733.81	Proteinase activated receptor 1 (PAR-1)	[235]
VP22	NAATATRGRSAASRPTQ-RPRAPARSASRPRRPVQ	34	9	---	+9	Good	13	3656.05	Herpes simplex virus (HSV)	[236]
transcription factor (267–300) VP22	DAATATRGRSAASRPTE-RPRAPARSASRPRRPVE	34	9	---	+6	Good	12.2	3659.01	Herpes simplex virus (HSV)	[236]
vT5	DPKGDPKGVTVTVTVT-VTGKGDPKPD	26	---	4	0	Good	6.91	2608.89	Viral proteins	[237]
FGF	PIEVCMYREP	10	1	---	-1.1	Good	4.15	1236.46	Cellular and viral proteins	[238]
C105Y	CSIPPEVKFNKPFVYLI	17	---	2	+0.9	Poor	8.93	1994.4	The residues 359–374 of 1-antitrypsin	[239]

p28	LSTAADMQGVVT DGM-ASGLDKDYLKPDD	28	---	2	-4	Good	3.41	2914.18	Azurin	[17]
PFV	PFVYLI	6	---	---	0	Poor	3.84	750.92	C105Y	[240]
SG3	RLSGMNEVLSFRW L	14	2	---	+1	Poor	10.39	1708	A randomized peptide library	[241]
Pep-7	SDLWEMMMVSLA CQY	15	---	---	-2.1	Poor	0.67	1807.15	CHL8 peptide phage clone	[242]
CyLoP-1	CRWRWKCCKK	10	2	3	+4.8	Good	10.56	1396.76	Natural cationic polypeptide crotamine	[243]
MK2i	WLRRIKAWLRRIK ALN-RQLGVAA	23	5	2	+7	Good	12.71	2789.38	MAPKAP Kinase 2 (MK2)	[244]
Influenza HA-2	GLFGAIAGFIENG WEGM-IDGWYG	23	---	---	-3	Poor	0.57	2460.72	Chimeric from Influenza HA	[245]
Influenza HA-2 (1–20) KALA sequence	WEAKLAKALAKA LAHL-AKALAKALKACE A	29	---	6	+4	Good	10.4	3003.65	A segment of influenza virus hemagglutinin	[246-249]
p28	LSTAADMQGVVT DGM-ASGLDKDYLKPDD	28	---	2	-4	Good	3.41	2914.18	Protein derived from cupredoxin family	[250]
CPP-C	PLEVCMYREP	10	1	---	-1.1	Good	4.15	1236.46	Specific Domain of FGF12	[238]
Bax-inhibiting peptides (BIP)	VPTLK	5	---	1	+1	Good	10.1	556.7	Natural	[13]
PTD-5	RRQRRTSKLMKR	12	5	2	+7	Good	12.71	1615.96	Protein Transduction Domain	[251]
q-NTD	KGRKKRRQRRRPP Q	14	6	3	+9	Good	12.81	1847.19	Protein derived	[252]
FHV coat (35–49)	RRRRNRTRRNRRR VR-amide	15	11	---	+12	Good	14	2163.52	Flock house virus coat	[23, 216]

									proteins	
KLA sequence	Acetyl-KLALKLALKALKAALKL-A-amide	18	---	5	+5	Good	14	1918.5	KLA peptide[1]	[247, 253-255]
Translocation motif (TLM)	PLSSIFSRIGDP	12	1	---	0	Good	7.08	1288.45	Derived from the hepatitis B virus	[104]
Substance P and analogs	RPKPQQFGLM-amide	10	1	1	+3	Good	14	1200.46	Neuropeptide	[247, 256, 257]
Crotamine	YKQSHKKGGKKGSG	14	---	5	+5.1	Good	10.9	1489.68	Rattle snake toxin derived from NrTP6	[258-260]
R9	RRRRRRRRR	9	9	---	+9	Good	13	1423.69	Designed	[261]
ppTG1	GLFKALLKLLKSLWKL-LLKA	20	---	5	+5	Poor	11.28	2296.96	Designed	[132]
KALA	WEAKLAKALAKALA-KHLAKALAKALKACEA	30	---	7	+5	Good	10.56	3131.83	Designed	[132]
Pen-Arg	RQIRIWFQNRRMRWRR	16	7	---	+7	Good	12.88	2358.79	Designed	[262]
R6H4	RRRRRHHHHH	10	6	---	+6.4	Good	12.8	1503.60	Designed	[172]
CADY	GLWRALWRLLRSLWR-LLWRA	20	5	---	+5	Poor	12.7	2622.17	Designed	[263]
KAFAK	KAFAKLAARLYRKALA-RQLGVAA	23	3	3	+6	Good	11.91	2487	Designed	[264]
Pep-1	KETWWETWWTEWSQP-KKKRKV-Cya***	24	1	5	+2.9	Good	9.92	3185.61	Designed	[265]
ppTG20	GLFRALLRLLRSLWRLL-LRA	20	5	---	+5	Poor	12.7	2437.03	Designed	[132]
BR2	RAGLQFPVGRLLRRLLR	17	5	---	+5	Good	12.7	2021.46	Designed	[266]
R4	RRRR	4	4	---	+4	Good	12.58	642.76	Designed	[267]

R6	RRRRRR	6	6	---	+6	Good	12.8	955.13	Designed	[267]
R10	RRRRRRRRRR	10	10	---	+10	Good	13.05	1579.88	Designed	[267]
R12	RRRRRRRRRRRR	12	12	---	+12	Good	13.14	1892.25	Designed	[267]
MPG	GALFLGWLGAAGSTM-GAPKKKRKV	24	1	4	+5	Good	11.76	2444.94	Designed	[268]
HR9	CHHHHHRRRRRRRRR-RHHHHHC	21	9	---	+9.9	Good	12.4	3001.38	Designed	[269]
Pep-3	ac-KWFETWFTEWPKKR-K-Cya	15	1	4	+3	Good	10.67	2097.42	Designed	[270]
4K	KKKK	4	---	4	+4	Good	11.15	530.7	Designed	[271]
MPG β	ALFLGFLGAAGSTMGA-WSQPKKKRKV	26	1	4	+5	Poor	11.76	2750.27	Designed	[183]
R8 (8-Arginine)	RRRRRRRR	8	8	---	+8	Good	12.94	1267.5	Designed	[215]
8-Lysine	KKKKKKKK	8	---	8	+8	Good	11.52	1043.39	Designed	[271]
6K	KKKKKK	6	---	6	+6	Good	11.37	787.05	Designed	[271]
10K	KKKKKKKKKK	10	---	10	+10	Good	11.63	1299.74	Designed	[50]
12K	KKKKKKKKKKKK	12	---	12	+12	Good	11.71	1556.08	Designed	[271]
5RQ	RRQRR	5	4	---	+4	Good	12.58	770.89	Designed	[271]
8RQ	RRQRRQRR	8	6	---	+6	Good	12.8	1211.39	Designed	[271]
11RQ	RRQRRQRRQRR	11	8	---	+8	Good	12.94	1651.89	Designed	[271]
MPGΔNLS	GALFLGFLGAAGSTM-GAWSQPKSKRKV	27	1	3	+4	Poor	11.68	2766.23	Designed	[263]
R15	RRRRRRRRRRRRRRR	15	15	---	+15	Good	13.25	2360.81	Designed	[272]
H8R15	HHHHHHHHRRRRRR-RRRRRRRRR	23	15	---	+15.8	Good	13.25	3475.92	Designed	[272]
H16R8	HHHHHHHHHHHHHHHH-HRRRRRRRRRRRRRRR	31	15	---	+16.6	Good	13.25	4555.04	Designed	[272]
NYAD-41	ISFDELLDYYGESGS	15	---	---	-4	Good	0.57	1694.74	Designed	[273]

AcD4	GYGYGYGYGYGY GYG-YKKRKKRKKRKK RKQ-QKQQKRRK	38	6	12	+18	Good	11.16	4767.51	Designed	[274]
RICK	KWLLRWLSRLLR WLAR WLG	19	4	1	+5	Poor	12.58	2523.08	Designed	[125]
WRAP	LLWRLWRLLWRL WRLL	16	4	-	+4	Poor	12.58	2292.86	Designed	[127]
MAP	KLALKLALKALKA ALK-LA	18	---	5	+5	Good	11.28	1877.45	Chimeric	[275]
Chimeric dermaseptin S4 and SV40 'S413-PV'	ALWKTLLKKVLK APKK-KRKVC	21	1	8	+8.9	Good	11.44	2480.2	Chimeric	[276]
Transportan	GWTLNSAGYLLG KINL-KALAALAKKIL	27	---	4	+4	Poor	10.77	2841.44	Chimeric	[277]

*Green: hydrophobic uncharged residues, like F, I, L, M, V, W, A and P; Red: acidic residues, like D, E; Blue: basic residues, like R, K, H; Black: other residues, like G, S, T, C, N, Q and P

** Calculated by Pepcalc.com [76]

*** Cya: Cysteamine

1: The term KLA peptide, also denoted MAP (model amphipathic peptides) designates a group of peptides derived from the α-helical amphipathic model peptide [87].

Table 2: Mechanisms of action for a variety of CPPs

Cargo	CPP	Sequence	Formulation Approach	Classification	Internalization mechanism	Origin	Ref.
Gene	ppTG1	GLFKALLKLLKSLWKLLLKA	Noncovalent	Amphipathic	Endocytosis	Synthetic	[132]
	KALA	WEAKLAKALAKALAKHLAKALAKALKACEA	Noncovalent	Amphipathic	Endocytosis	Synthetic	[132]
	ppTG20	GLFRALLRLLRSLWRLLLRA	Noncovalent	Amphipathic	Endocytosis	Synthetic	[132]
	Influenza HA-2	GLFGAIAGFIENGWEGMIDGWYG	Noncovalent	Amphipathic	Endocytosis	Chimeric from Influenza	[278]
	Secretin	HSDGTFTSELSRLRDSARLQRLLQGLV	Noncovalent	Cationic	Based on receptor	Human	[226]
	TAT (47-57)	YGRKKRRQRRR	Noncovalent	Cationic	Direct penetration	HIV TAT Protein	[138]
	MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	Noncovalent	Amphipathic	Clathrin-dependent endocytosis	Chimeric (HIV Gp41-SV40 NLS)	[12]
	Loligomer	$(TPPKKKRKVEDPKKKKK)^{-}_{8}$	Noncovalent	Cationic	Endocytosis	Synthetic	[279]
	Hel	KLLKLLLKLWLKLLKLLL	Noncovalent	Cationic Amphipathic	Endocytosis	Synthetic	[280]
	VP22	DAATATRGRSAASRPTERPRAPARSASRPRRPVE	Covalent	Amphipathic	Endocytosis	Herpes simplex V	[281]
	TP10	AGYLLGKINLKALAALAKKIL	Noncovalent	Amphipathic	Endocytosis	Chimeric	[19]
	C105Y	CSIPPEVKFNKPFVYLI	Covalent	Hydrophobic	Clathrin- and caveolin-independent	Synthetic	[239]
	R8	RRRRRRRR	Noncovalent	Cationic	Direct penetration	Synthetic	[121]
	LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	Noncovalent	Amphipathic	Pore formation	Human antimicrobial protein	[227]
	Scp01-b	VSRRRRRRGGRRRRGGGSYARVRRRGPRRGYARVRRRGPRR	Noncovalent	Amphipathic	Endocytosis	Synthetic	[282]
	B-mR9	CRRRRRRRRRCRRRRRRRRRC	Noncovalent	Cationic	Endocytosis, Direct penetration	Synthetic	[140]
siR	MPG	GALFLGFLGAAGSTMGAWSQPKKKR	Noncov	Amphip	Clathrin-	Chimeri	[20

NA		KV	alent	athic	dependent endocytosis	c (HIV Gp41-SV40 NLS)	, 112]
	Penetratin (pAntp)	RQIKIWFQNRRMKWKK	Covalent	Amphipathic	Endocytosis	Antennapedia	[283]
	Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	Covalent	Amphipathic	Endocytosis, Direct penetration	Chimeric (Galanin and mastoparan)	[20]
	PepFect 14	AGYLLGKLLOOLAAAALOOLL-NH_2	Noncovalent	Amphipathic	Endocytosis	Synthetic	[121]
	PepFect 6	AGYLLGK(εNH^{QN})INLKALAALAKKIL-NH_2	Noncovalent	Amphipathic	Endocytosis	Synthetic	[121, 284]
	R9	RRRRRRRRR	Noncovalent	Cationic	Direct penetration	Synthetic	[46]
	Tat-DRBD	TAT-TAT-HA-TAT-DRBD	Noncovalent	Cationic	Macropinocytosis	Chimeric (dsRNA binding domain (DRBD) fused to a Tat-based PTD)	[117]
	STR-KV	stearylation- HHHKKKVVVVVV	Noncovalent	Amphipathic	Direct penetration	Synthetic	[122]
	CADY	GLWRALWRLLRSLWRLLWRA	Noncovalent	Amphipathic	Direct penetration	Synthetic	[285]
	TP10	AGYLLGKINLKALAALAKKIL	Noncovalent	Amphipathic	Endocytosis	Chimeric	[286]
	TAT_{48-60}	CYGRKKRRQRRR	Covalent	Cationic	Direct penetration	HIV TAT Protein	[287]
	EB1	LIRLWSHLIHIWFQNRRLKWKKK	Noncovalent	Amphipathic	Endocytosis	Synthetic (analog of penetratin)	[288]
ON-PNA	MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	Noncovalent	Amphipathic	Clathrin-dependent endocytosis	Chimeric (HIV Gp41-SV40 NLS)	[289]

	LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	Noncovalent	Amphipathic	Pore formation	Human cathelicidin family	[227]
	Pep-3	ac-KWFETWFTEWPKKRK-Cya	Covalent	Amphipathic	Direct penetration	Chimeric	[270]
	TP10	AGYLLGKINLKALAALAKKIL	Covalent	Amphipathic	Endocytosis	Chimeric	[290]
	M918	MVTVLFRRLRIRRACGPPRVRV	Noncovalent	Amphipathic	Endocytosis	Synthetic	[79]
	Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	Covalent	Amphipathic	Endocytosis, Direct penetration	Chimeric (Galanin and mastoparan)	[141]
Protein and peptide	Bac7 $_{(1-24)}$	RRIRPRPPRLPRPRPRPLPFPRPG	Noncovalent	Amphipathic	Receptor-mediated/ pore formation	Bactenecin family	[230]
	TAT_{48-60}	GRKKRRQRRRPPQ	Covalent	Cationic	Direct penetration	HIV TAT Protein	[84]
	R9	RRRRRRRRR	Covalent	Cationic	Direct penetration	Synthetic	[84]
	CPP-C	PIEVCMYREP	Covalent	Hydrophobic	Endocytosis	Derived Form FGF12	[238]
	BIP	VPTLK	Covalent	Hydrophobic	Endocytosis	Natural	[291]
	Penetratin-pAntp (43-58)	RQIKIWFQNRRMKWKK	Covalent	Cationic	Endocytosis	Antennapedia	[292]
	HR9	CHHHHHRRRRRRRRRHHHHHC	Noncovalent	Cationic	Direct penetration	Synthetic	[135]
	VP22	DAATATRGRSAASRPTERPRAPARSASRPRRPVD	Covalent	Amphipathic	Using actin cytoskeleton	Herpes simplex V	[236]
	M918	MVTVLFRRLRIRRACGPPRVRV	Covalent or non-covalent complex	Amphipathic	Macropinocytosis	Protein-derived peptides	[79]

	Pep-1	KETWWETWWTEWSQPKKRKV	Noncovalent	Amphipathic	Direct penetration	Chimeric	[182]
	CADY-2	Ac-GLWWRLWWRLRSWFRLWFRA-Cya	Noncovalent	Amphipathic	Direct penetration	Chimeric	[95]
	pVEC	LLIILRRRIRKQAHAHSK	Covalent	Amphipathic	Direct penetration	Murine vascular endothelial-cadherin protein	[101]
	CyLoP-1	CRWRWKCCKK	Noncovalent	Cationic	Endocytosis, Direct penetration	NLS of snake toxin	[98]
	LDP-NLS	KWRRKLKKLRPKKKRKV	Noncovalent	Cationic	Endocytosis	Chimeric (Latarcin-derived peptide conjugated with NLS)	[99]
	Sor-G8	sorbitol-based molecule with 8 guanidine units	Noncovalent	Electrostatic and hydrogen bonding	Macropinocytosis	Synthetic	[106]
	AA3H	MASIWVGHRG	Covalent	Hydrophobic	Endocytosis	Derived from N-terminal sequences of annexin	[293]
	Iduna (RNF146)	RRRKIKR	Covalent	Cationic	Endocytosis	Derived from PolyADP-ribosylation	[108]

Table 3: Physicochemical properties that influence the CPP uptake pathway

Entrance Pathway	Temperatures	Concentrations	Internalization	Energy	Cargo size
Direct	low temperatures (approximately 4^{o}C)	high concentrations*	electrostatic attraction	Energy-independent	Small cargo
Endocytosis	high temperatures (approximately 37^{o}C)	low concentrations*	electrostatic attraction	Energy-dependent	Large cargo

* Low and high concentrations depend on CPP, cargo and cell types. Usually, direct penetration can occur in more than 10-20 μM

Table 4: Preclinical vaccination studies using CPPs

CPP	Cargo	Therapeutic Use	Model	Ref
MPG	HCV core	Hepatitis C virus	BALB/c Mice	[185]
MPG	HCV coreE1E2	Hepatitis C virus	BALB/c Mice	[185]
CL22	TAAs (tumor-associated antigens)	Cancer	C57BL/6 Mice	[294]
TAT	Chicken ovalbumin (OVA)	Cancer	C57BL/6 Mice	[295]
TAT	Murine tyrosinase-related protein 2 (Trp2)	Cancer	C57BL/6 Mice	[296]
VP22	Amastin-enhanced green fluorescent protein (EGFP)	Leishmaniasis	BALB/c Mice	[297]
TAT	Leishmania homolog of receptors for activated C kinase (LACK)	Leishmaniasis	C57BL/6 Mice	[298]
TAT	p53	Cancer	C57BL/6 Mice	[299]
TAT	Her2/neu	Cancer	FVB/N Mice	[300]
TAT	Carcinoembryonic antigen	Cancer	C57BL/6 Mice	[301]
Penetratin	CD4 or CD8 specific OVA epitope	Cancer	C57BL/6 Mice	[302]
Penetratin	SIINFEKL (AntpSIIN)	Cancer	C57BL/6 Mice	[303, 304]
VP22	Human papillomavirus type 16 E7	Cancer	C57BL/6 Mice	[305]
Pep-1	HPV16 E7	Cancer	C57BL/6 Mice	[182]
$VP22_{1–267}$	HPV-16 E7	Cancer	C57BL/6 Mice	[191, 306]
MPG	HPV16 E7	Cancer	C57BL/6 Mice	[184]
PEI600-Tat	HPV16 E7	Cancer	C57BL/6 Mice	[193]
MDV-1 UL49	HPV-16 E7	Cancer	C57BL/6 Mice	[191]
VP22	C-terminal OprF	Pseudomonas aeruginosa	BALB/c Mice	[307]
TAT	Nef	HIV-1	BALB/c Mice	[186]
MPG	HIV-1 MPER/V3	HIV-1	BALB/c Mice	[183, 308]
pVP22	NP (nucleoprotein) gene	Influenza	BALB/c Mice	[309]

Table 5: Preclinical and clinical trials of CPPs in treatment

CPP	Cargo	Therapeutic Use	Preclinical	Clinical	Ref.
R7	Cyclosporine A	Psoriasis	-	Phase IIb discontinued 2003	[310, 311]
(R-Ahx-R)4	PMO	Cardiovascular disease Coronary artery bypass	-	Phase II discontinued 2009	[312]
TAT	PKC-δ inhibitor	Myocardial infarction	-	Phase II completed 2011	[313]
TAT	PKC-ε inhibitor	Pain: postherpetic neuralgia, spinal cord injury, postoperative	-	Phase II completed 2011	[314]
p28	p28	Recurrent or progressive central nervous system (CNS) tumors	-	Phase I completed 2014	[315]
TAT	JBD20	Hearing loss	-	Phase II completed 2014	[316]
TAT	JBD20	Hearing loss	-	Phase III compeleted in 2016	ClinicalTrials.gov ID: NCT02561091 NCT02809118
TAT	botulinum toxin A	Glabellar lines	-	Phase I/II completed 2016	[317]
TAT	JBD20	Inflammation	-	Phase I completed 2012	[318]
TAT	JBD20	Intraocular inflammation and pain	-	Phase III completed 2016	[319, 320]
TAT	PKC-ε inhibitor	Ischemia	-	Phase I	[84, 321]
TAT	Botulinum toxin A	Cervical dystonia	-	Phase II	[322]
TAT	Botulinum toxin A	Excessive underarm sweating	-	Phase II	[84, 321]
PTD	HA–Bcl-XL	Cerebral ischemia	Mice	-	[86]
PTD	FNK	Cerebral ischemia	Gerbils	-	[200]
TAT	JBD20 (D-JNKI-1)	Cerebral ischemia	Mice	-	[323]
TAT	δ PKC inhibitor	Cerebral IR injury	Rats	-	[324]
TAT	NBD	Perinatal infection in HI brain injury	Neonatal Rats	-	[202]
TAT $_{48–57}$	BH4	ALS	hSOD1^{G93A} Mice	-	[325]

TAT	JBD20 (D-JNKI-1)	Alzheimer Disease (AD)	TgCNRD8 Mice	-	[326]
Antp	NBD	Duchenne muscular dystrophy (DMD)	Mice	-	[204, 327]
PEP-1	SOD1	Myocardial IR injury	Rats	-	[328, 329]
PEP-1	CAT	Myocardial IR injury	Rats	-	[328, 329]
TAT $_{48–60}$	BH4	Myocardial IR injury	Mice	-	[330]
RI-TAT	p53C′	Cancer	Mice	-	[331]
DPV1047 (Vectocell)	SN38	Cancer	Beagle dog, Mice and Rats	-	[207]
MPG-8	siRNA	Cancer	Mice	-	[332]
TAT-DRBD	siRNA	Cancer	Mice	-	[333]
(R-Ahx-R)4	PMO	Corneal transplant rejection	Rats	-	[209]
TAT	Antibody (Tumoricidal immunoglobulins as Fab fragment)	Tumor therapy	3T3-L1 cells	-	[68, 84, 334]
TAT	B-gal, RNase A, Horseradish peroxidase, Pseudomonas exotoxin A domain III	Heterologous protein delivery	Hela cells/ BALB/c mice	-	[68, 84, 334]
SynB	Doxorubicin	Cancer	Rats and Mice	-	[199, 335]
D-penetratin	Doxorubicin	Cancer	*In situ* brain perfusion	-	[199]
SynB1	Antibiotic benzyl-penicillin (B-Pc)	CNS infections	*In situ* brain perfusion	-	[336]
SynB	Dalargin	Brain uptake	Mice	-	[337]
TAT	BH4	Bacterial sepsis	Mice	-	[338]
TAT	Bcl-x_L	Bacterial sepsis	Mice	-	[338]
R8-modified fusogenic DOPE liposomes	Bleomycin (BLM)	Cancer	BALB/c mice bearing 4T1 tumors	-	[339]
TAT	anti- Her-2/neu peptide mimetic, AHNP	Breast Cancer	Breast cancer cells	-	[340]
TAT	SpA (the B domain of staphylococcal protein A)	Cancer	murine F9 teratocarcinoma-bearing mice	-	[341]
pVEC-PEGA homing domain	Chlorambucil	Cancer	Breast cancer cell lines	-	[342]
VP22	p53	Cancer	p53-negative	-	[206]

			human osteosarcoma cells		
Penetratin	PNC-28	Cancer	Nu/Nu mice	-	[343, 344]
R9	LDP	Glioma therapy	Carcinoma cell lines	-	[345]
Penetratin	p16Ink	Cancer	Tumor-bearing mice	-	[208]
TAT	P15	Cancer	C57BL6 mice bearing day 7-established solid tumors		[346]
TAT	Chitosan/Dox	Cancer	Tumor bearing mouse	-	[347]
TAT–DRBD	epidermal growth factor receptor (EGFR) siRNA	Cancer	Intracranial glioblastoma cancer mouse models	-	[333]
TAT–DRBD	AKT serine/threonine kinase 2 (Akt2) siRNA	Cancer	Intracranial glioblastoma cancer mouse models	-	[333]
iRGD	PEG-PLA nanoparticles loaded with paclitaxel	Cancer	Nude mice bearing intracranial C6 glioma	-	[348]
tLyp-1	PEG-PLA nanoparticles loaded with paclitaxel	Glioma therapy	Mice bearing intracranial C6 glioma	-	[349]
Penetratin	single-chain FVs (scFVs) antibody fragments	Cancer	mice bearing human colon cancer xenografts	-	[350]
POD (peptide for ocular delivery)	siRNA	Ocular Medication	Human embryonic retinal cells	-	[351]
VP22	Recombinant proteins	Ocular Medication	Human embryonic retinoblasts *in vitro* and retinal tissues *in vivo*	-	[352]
TAT	Recombinant proteins	Ocular Medication	Human embryonic retinoblasts *in vitro* and retinal tissues *in vivo*	-	[353]

JDB20: The 20- amino-acid JNK-binding motif (JBD20) of JNK-interacting protein-1/islet-brain 1; PMO: phosphorodiamidate morpholino oligomers