**NOVEL APPROACHES IN DEVELOPMENT OF CELL PENETRATING PEPTIDES**

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**ABSTRACT**
Therapeutic cargos which are impermeable to the cell can be delivered by cell penetrating peptides (CPPs). CPP-cargo complexes accumulate by endocytosis inside the cells but they fail to reach the cytosolic space properly as they are often trapped in the endocytic organelles. Here the CPP mediated endosomal escape and some strategies used to increase endosomal escape of CPP-cargo conjugates are discussed with evidence. Potential benefits can be obtained by peptides such as reduction in side effects, biocompatibility, easier synthesis and can be obtained at lower administered doses. The particular peptide known as cell penetrating peptides are able to translocate themselves across membrane with the carrier drugs with different mechanisms. This is of prime importance in drug delivery systems as they have capability to cross physiological membranes. This review describes various mechanisms for effective drug delivery and associated challenges.

**INTRODUCTION**
The lipid bilayer is hydrophobic in nature so it is impermeable for most hydrophilic molecules and renders protection from extracellular matrix. Internalization of large molecules such as protein occurs via endocytosis [1]. Some viral and non-viral delivery vectors have been developed to increase transport of active agent into the cells. Limitations of viral vectors are problem in production and immunogenicity [2]. These can be overcome by non-viral vectors but they also have some limitations such as cellular toxicity and cargo-vector complex instability [3]. In recent years some short, amphipathic or cationic peptides called cell penetrating peptides (CPPs) and protein transduction domains (PTDs) being able to translocate into mammalian cell by energy and receptor independent mechanism gained more attention as they have been used successfully to transport peptides, protein, siRNAs, antisense oligonucleotides, plasmids and large particles like liposomes into the cell both in-vivo and in-vitro [4,5]. CPPs were discovered in 1988 from Human immunodeficiency virus type

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1(HIV-1) encoded on trans-activator of transcription (Tat) peptide [6,7] which can cross cell membranes. Few years later, small exogenous peptide was delivered using penetratin [8–11].

One Study suggest that the cellular uptake is mainly due to the small domains in these peptides so shortening of these translocation sequences without losing cellular uptake efficiency could be possible [12]. As the selection of CPP mainly depend on the application type, the most commonly used peptides are Tat, Pep-1, penetratin, polyarginine, transportan which can deliver small bio- molecules [13], nucleic acids [14], proteins [15–17].

**Figure 1** Types of endocytosis mechanisms

**CLASSIFICATION**

**Based on linkage with cargo:** [18][19]

Covalent bonding between CPP and cargo:
The covalent linkage present between CPPs and cargo made by chemical cross linking or cloning with expression of fusion protein of CPP [20] such as polyarginine Arg8 sequence, Tat peptide, transportan, antimicrobial peptides SynB and buforin I, VP22 protein from Herpes Simplex Virus(HSV) [17,21,22].

Non-covalent bonding between CPP and cargo:
To improve intracellular delivery, CPPs are non-covalently bonded with biomolecules having polar and nonpolar domains.

**Based on physicochemical property of CPP** [18]

**Cationic**
This class has positive charge and polyarginine groups in primary sequence. E.g. Tat in HIV-1 contains lysine and arginine residues [23].

**Amphipathic**
This class has amphipathicity because of lysine residues. E.g. Transportan.

**Hydrophobic**
This class has very less importance as carriers and has only non-polar/hydrophobic sequences E.g. SG3, Pep-7

**Based on origin of CPPs**

**Chimeric CPPs**
They are made up of two or more motifs from different peptides. Example, Transportan derived from mastoparan and galanin.

**Protein derived CPPs**
Transactivators of gene transcription [24], viral envelope protein [25,26], antimicrobial peptides [27,28], DNA /RNA binding proteins [29], plant skeletal proteins [30] which can cross plasma membranes are natural proteins. E.g. Penetratin and Tat are derived from natural proteins.

**Artificial CPPs**
They are synthesized and designed on the basis of structure of naturally derived CPPs [31,32]. Example, polyarginine which mimics with arginine and is made artificially by many arginine residues as it helps in transduction mechanism [33].

**STRUCTURAL REQUIREMENTS FOR CPPS**

For the cellular entry, the first step is electrostatic interaction between proteoglycans charged negatively and phospholipids present on the surface of cell with basic CPPs [34][35–39]. Membrane binding and insertion can lead to direct translocation or endocytic pathways especially for amphipathic CPPs [40–42]. Hydrophobic or electrostatic interaction is affected by positive charge, hydrogen bonds and density [18,43,44].

**Magic arginine and positive charge**
Studies suggest that the residues of arginine were more effective for internalization than lysine residues, so by replacing the residues of lysine with arginine enhanced the uptake rates [45,46]. But the uptake efficiency is not only because of positive charge but also because of guanidinium head group of side chain of arginine and also number of residues of arginine. This is important as with 7-15 residues, it gives optimal uptake [7,45,47,48].

**Hydrophobicity and “tryptophan power”**
Translocation across the plasma membrane bilayer can be increased by presence of hydrophobic residues in CPPs [49–51]. Studies suggest that by adding fluorescein isothiocynate (FITC) or tryptophan residue to the Tat peptide will increase translocation but caution must be observed because the deep
insertion in lipid bilayer membrane may decrease internalization as peptide will be stuck in the plasma membrane [52–54].

**CELLULAR UPTAKE MECHANISM OF CPPS**

For cellular uptake, it has been reported that positively charged amino acids such as arginine and lysine interact with acidic motifs containing proteoglycans of plasma membrane in receptor-independent manner [19,55–57]. After this interaction, peptides undergo internalization which will be dependent on type of CPPs, size and charge of cargo [58,59]. One study has indicated that Tat and Antp CPP internalization can be mediated by caveolin-dependent endocytosis, clathrin-dependent endocytosis, macro pinocytosis and direct intracellular translocation [60].

**Endocytosis**

*Receptor mediated endocytosis*

In this type of mechanism, the cell surface receptors first recognize the ligand and uptake mechanism is mediated by invagination of plasma membrane to from vacuole which is the energy dependent process and depends on clathrin for mediation of invagination process and also involves actin and microtubule filaments.

*Pinocytosis*

In this mechanism ions or molecules gain entry in the cytoplasm but only small molecules can be engulfed by this mechanism which occurs continuously.

*Macropinocytosis*

Larger molecules can be engulfed into the cell without formation of endocytic vesicle using RhoGTPases, form the irregular, large vesicles at the site of membrane ruffling because of the closure of lamellipodia. The ruffling of membrane in this process is predominantly actin-driven and so there is no need of clathrin.

*Potocytosis / caveolae mediated uptake*

Flask shaped regions of the plasma membrane characterized by the filamentous caveolins coat lining the inner surface known as caveolae used to transport both large and small molecules. It is not associated with the clathrin and can transport the molecule into the cell bypassing the lysosome in that way it differ from endosomes [61].

*Receptor independent uptake*

In this mechanism, specific cell surface receptors are not required but the overall charge of peptide is important [62]. Arg residues are important in mediating translocation which was demonstrated by the structural studies of Tat PTD [34,45].

**Direct penetration**

Direct penetration can be possible only when the concentration of CPPs is high, while endocytosis is possible in almost all the cases [31,63–65]. It is energy independent process so occurs only when there is high concentration of CPPs and even in the presence of endocytosis inhibitors and at low temperature (4°C). It involves interaction of negatively charged cell membrane components and positively charged CPPs.

*Barrel stave model*

It requires CPP with helix conformation where hydrophilic residue forms the internal environment of pore and hydrophobic residue bind with hydrophobic tail of lipid bilayer. Such as alamethicin, which forms the trans-membrane pores having 3-11 parallel helical structure [66].

*Toroidal pore model*

It also dependent on the helix conformation of CPP but differs in the mechanism of pore formation. Pore formation occurs because of peptides associated with the polar head groups of the lipid inside the cell membrane. The hydrophilic core of the toroidal pore is lined with the inserted peptides and hydrophilic head group of the phospholipid cell membrane [67]. Magainins, protegrins and melittin induce the formation of toroidal pores [66].

*Carpet like model*

It is based on the cell membrane destabilization and reorganization because of electrostatic interactions between cationic charged particles and anionic head groups of phospholipids [68]. The concentration of CPPs must be high to form the carpet like membrane coating then only permeation across the membrane occurs. Main difference is absence of peptide internalization into the hydrophobic core which was shown in barrel stave model [69].

*Inverted micelle formation*

When the lipid bilayer is converted into a micelle, it will lead to formation of transient hole. The interaction between hydrophobic part of cell membrane and hydrophilic residue of CPP and also interaction between cationic charged CPPs and anionic charged membrane is responsible [70]. Octa-arginine is internalized effectively with inverted micelle formation.

**Endosomal escape of CPPs**

The drawbacks of endocytosis include endosomal accumulation and degradation in the endosome. The CPP-cargo complex can interact with the phospholipid known as Bis (Monoacylglycerol) Phosphate (BMP), which is a part of endosomal membrane.
Strategy to improve endosomal escape

Multivalent CPPs enhance the endosomolytic activity by strong interaction with the BMPs in membranes, and they can escape efficiently from the endosomes than monomeric CPPs. This approach is based on increasing the local concentration of the CPP by presenting large number of copies of CPPs on a delivery vector where the peptide interacts with cellular components [71,72,81–84,73–80]. Multivalency can be achieved by chemical conjugation of CPPs to dendrimers, by conjugating a protein oligomerization domain to the CPP, or by attaching CPPs to the branched oligopeptides, such as the fork-like structure of glutamic acid or lysine. Limitations of this approach are higher risk of immunogenic properties and difficulty in chemical synthesis of multivalent CPPs. It is important to balance the number of branches of CPP, to obtain a strong but not too extensive reaction. Cyclization of CPPs rich in arginine led to efficient cellular uptake process and delivery to the nucleus and cytoplasm [65,85,86].

![Mechanisms of CPP’s entrance through cellular membrane](https://example.com/image1)

**Figure 2** Mechanisms of CPP’s entrance through cellular membrane

[Source of figure: Derakhshankhah H, Jafari S. Cell penetrating peptides: A concise review with emphasis on biomedical applications. Biomed Pharmacother 2018; 108:1090–6. https://doi.org/10.1016/j.biopha.2018.09.097.]

![Mechanisms of direct penetration](https://example.com/image2)

**Figure 3** Mechanisms of direct penetration
Figure 4 Schematic models of direct penetration of CPPs through cell membrane. The hydrophilic parts of the peptides are red colored and the hydrophobic parts of the peptides are blue colored

[Source of figure: Böhmová E, Machová D, Pechar M, Pola R, Venclicková K, Janoušková O, et al. Cell-penetrating peptides: A useful tool for the delivery of various cargoes into cells. Physiol Res 2018;67:s267–79.]

Figure 5 Schematic Representation of Proposed Mechanisms for Cell-Penetrating Peptide (CPP) Internalization

[Source of figure: Guidotti G, Brambilla L, Rossi D. Cell-Penetrating Peptides: From Basic Research to Clinics. Trends Pharmacol Sci 2017;38:406–24. https://doi.org/10.1016/j.tips.2017.01.003.]
METHODS TO STUDY MECHANISM OF CPPS
Various biophysical and biological methods are used to quantify CPPs and cargos inside the cell and to study the internalization mechanism.

In cellulo approaches
These methods are indirect and used to detect the biological activity of the cargos by fluorescence [87]. Direct method had been developed for quantification of intact CPPs bound to the cellular membranes or inside the cells, based on matrix-assisted laser desorption-time of flight mass spectrometry (MALDI-TOF MS) [88,89]. Other biophysical methods are also used in the living cells, like electron microscopy to study the distribution of peptides and membrane structures induced by CPPs [90,91] and in cell Raman spectroscopy to know the secondary structure of peptide in the cellular compartments [92].

Fluorescence-based protocols
These are the most commonly used methods. In fluorimetry the fluorophore is covalently attached with the peptides and the measurement of fluorescence will directly quantify the peptides. In the confocal microscopy there is localization of the probes inside the living cells. It is convenient but has some limitations such as quenching of the fluorescence because of the accumulation in the subcellular compartments therefore may give inaccurate results [93].

Functional assays in cells
These methods are used to detect the biological activity of cargoes or conjugated molecules [94,95]. These approaches are very useful for the biotechnological and therapeutic applications [96,97].

APPLICATIONS
Cell-penetrating peptides as delivery vectors
Major challenge in the drug delivery is often the inability of drug to cross the lipid membrane of the cell but CPP can transport different cargos across the lipid membrane.

Peptides as cargo
Use of small peptide is better than the full length protein in several ways, such as purification of peptide is easy as can be synthesized daily while purification of protein is money and time consuming process. Peptides have great therapeutic potential in the treatment of several diseases such as diabetes, cancer, influenza, neurodegenerative disorders with less side effects [16,98]. Therefore, in future impermeable bioactive peptides can be used for therapy both in vivo and vitro using CPPs for the delivery [17,99].

Delivery of other cargo by CPP
Liposomes have been used to enhance the solubility and half-life and reduce the toxicity but the cell penetration is very slow which limits their use. Conjugation of Antp or Tatp on the surface of the liposomes improves the cellular delivery and show efficient and fast translocation into cytoplasm [100–102]. Fluorescent microscopic observation of the markers trapped inside the liposomes showed the liposomes remain intact for few hours in the cytoplasm and then migrate towards the nucleus slowly and release the contents into cytoplasm. Tatp-liposomes used as vectors for gene delivery, result as with high in-vitro transfection efficiency and are less cytotoxic [102]. Peptide-based imaging agents OxorheniumV and Oxotechnetium V can be delivered by CPPs into the cellular compartments to achieve high intracellular concentrations to carry out radio therapy and imaging [103]. The intracellular uptake of the paramagnetic nanoparticles can be significantly improved by Tatp which can be detected easily through magnetic resonance imaging (MRI) [34,104,105].

Cell penetrating peptides in biopharmaceuticals
The membrane of the cell prevents the entry of peptides, proteins and drug carriers into the cell unless transported by an active transport [106]. So CPPs are used to promote the delivery of biopharmaceutical agents into the Cell which includes SiRNA delivery, Antisense oligonucleotide delivery and delivery of drug carriers.

SiRNA delivery
CPPs and siRNA can be conjugated non-covalently or covalently easily. Covalently linked siRNAs to Penetratin or Transportan associated with the silencing response have high reproducibility [107]. Non-covalent complexes with siRNA have net positive charge [108]. However the non-covalent strategy is more efficient for delivery of siRNA [109,110].

Antisense oligonucleotide delivery
Antisense technology is based on the use of oligonucleotides (ONs) specific to sequence that can hybridize with the complementary mRNA strands lead to mRNA degradation by activation of the cellular enzymes belongs to the RNaseH family or translational arrest and prevent the gene expression [111]. The therapeutically potential ONs include aptamers, ribozymes, antisense ONs, triplex-forming ONs, immunostimulatory CpG motifs. CPPs can be used for the delivery of ONs with the
therapeutic agent by either a non-covalent or covalent linkage [111,112].

**Delivery of drug carriers**

Nanoparticles, liposomes, and other different types of nanocarriers have been used to modulate their biodistribution and pharmacokinetics, improve the drugs stability, decrease side-effects and increase efficacy [106]. However, the main challenge is intracellular delivery of these large molecules because of their hydrophobic/hydrophilic nature and three-dimensional structure [113]. CPPs have been used to deliver the therapeutic molecules which are 200 times larger than the current bioavailability size restriction [114].

**Imaging applications**

The technology is similar to that used for cancer therapeutics. CPPs having peptide with the transduction ability labeled fluorescently and attached with the cleavable linker for example, proteases expressed by tumor tissue recognize the cleavable site present in the linker. The neutralizing peptide is cleaved off when exposed to tumor tissue and its associated proteases, giving high concentration of CPP locally which leads to increased uptake by the tumor tissue [115,116]. Quantum dots (QD) are photostable, semiconductor nanocrystals, having diameter of 1–6 nm, brightly fluorescent, used mainly for biological imaging. Benefits of QDs over the traditional dyes are narrow emission peak, high quantum yield, resistance to the photo-bleaching and dependent on the size, broad photoluminescence. But major limitation is their inability to cross the plasma membrane. To overcome this limitation, CPPs, most commonly Tat, has been used. Dynamic confocal imaging studies suggested that Tat-QD conjugates were internalized by macropinocytosis which was triggered by the binding of the conjugate to the negatively charged cell membranes [117–119].

**Application of CPPs in gene therapy**

CPPs offer many advantages for cellular delivery, for example *in-vivo* efficacy, applicability in various types of cell, favorable nuclear targeting, no restriction for cargo size, non-immunogenic [62]. CPPs can also able to deliver nucleic acids, peptides into the bacterial cells. For treatment of genetic disorders therapeutically active genes are incorporated to cure the mutation. The major challenge is DNA delivery across the biological membranes- plasma membrane and nuclear membrane with minimum cell toxicity. Viral gene delivery vectors have efficient capability of gene transfer but some drawbacks which limit their use are oncogenicity, pathogenicity and stimulate ions of immunological responses in the host [111]. Non-viral gene delivery vectors are safe but the limitation is their inefficiency. With the use of CPP based delivery systems problems with non-viral gene therapy can be solved and also there is improvement in the viral gene therapy to some extent [120–123].

**Delivery of DNA to the intracellular environment through synthetic CPPs**

Oligonucleotides have been modified in many ways, such as with modification of chemical group, changes in the sequence, and the use of analogues of nucleotide show varying antisense activity. Modification in the sugar-phosphate backbone of the oligonucleotides is very important as it plays a role in gene silencing and membrane translocation [120]. Improvements to ON modifications are constantly being developed [124]. The degradation issues of naturally occurring oligonucleotides can be overcome by using ONs containing nucleotide analogues. For example, morpholinos, which are modified ONs, having standard nucleic acid bases, but are bound to the morpholine rings instead of deoxyribose rings and attached through the phosphorodiimidate groups instead of phosphates [125]. These changes make them resistant to the nuclease degradation and prevent immune responses. Synthetic CPPs have been developed to overcome the problem of inefficient gene transfer by non-viral vectors. Synthetic CPPs have been designed in the way that they can condense the DNA and transport it into the cell through the bilayer of lipid, either via endosome where CPP destabilize the endosomal lipid bilayer at low pH and mediate the plasmid release or directly in the case of amphipathic CPPs [126].

**Suicidal gene therapy approaches**

It is widely used for treating hyperproliferative disorders and cancer. It is based on introduction of gene into the target cells which encode the enzyme that converts inactive prodrug to the potent cytotoxic agent. Various prodrug /enzyme combinations have been developed, but the most commonly used is HSV-1 thymidine kinase (TK)/ganciclovir (GCV) combination [34,127].

**Transdermal delivery with CPPs**

Skin act as barrier for the macromolecules to deliver across the skin [128]. The barrier function of skin is because the stratum corneum has highly organized structure [129]. It protects the body from the outside environment, but also acts as epidermal
permeability barrier for the delivery of therapeutic agents for treatment of skin diseases. The drugs for the treatment of primary cutaneous disease are administered systemically because of poor absorption of drug through skin and very low topical bioavailability [130]. Topical delivery of peptides has been studied because these compounds are important in the treatment of skin diseases and improvement in the skin properties in case of cosmeceuticals. Administration of several peptides by topical route would be better, such as TGF-b, IGF-1, leptin for wound healing, interferon as antiviral, bacitracin for the skin infection, cyclosporine for the treatment of autoimmune diseases [130,131]. Several peptides have been studied as antigens by applying to the skin for the development of topical vaccines [132].

Anti-inflammation therapy
Antisense peptide nucleic acids (PNAs) have been demonstrated to prevent the growth of E. coli and gene expression and are good anti-inflammatory agent. For the efficient delivery of PNAs, PNAs are conjugated with the CPPs (CPP-PNA complex) [133]. For example, administration of acpP-targeting PNA conjugated with the CPP into E. coli K-12-infected BALB/c mice enhanced survival of the infected mice, prevented the fatal infection and reduced bacterial blood counts [134].

Tumor therapy
Conventional chemotherapy cause severe side effects because of lack of specificity to the tumor cell and has low concentration of drug at the local tumor areas. Efficient strategies for targeting tumor have been developed to overcome these limitations. Conjugation of antitumor agents with the CPPs has increased the efficiency of tumor therapy. CPPs can be used in tumor therapy as the conjugated antitumor therapeutics have increased permeability through the cellular membrane so targeting of tumor cells can be possible [135]. Bleomycin (BLM) is extensively used as an anticancer agent, but its effect is dependent on the cytosolic accumulation. The artificial R8-DOPE-BLM conjugate can enter into cytosol resulting in strong induction of tumor cell death and in vitro DNA damage compared to BLM. Similar results have been obtained by combination of CPP with Taxol, doxorubicin, methotrexate [136–138]. These data indicate that CPP conjugated antitumor agents can improve the treatment by increase in the concentration of drug at the tumor tissue.

Figure 6 Schematic diagram of routes for topical delivery of cell-penetrating peptides (CPP)/Cargo complexes via human skin [Source of figure: Nasrollahi SA, Taghibiglou C, Azizi E, Farboud ES. Cell-penetrating Peptides as a Novel Transdermal Drug Delivery System. Chem Biol Drug Des 2012;80:639–46. https://doi.org/10.1111/cbdd.12008.]

Protein and nucleic acid delivery
Large macromolecules, such as proteins and nucleic acids are not able to penetrate the plasma membrane so are unable to enter into the cells. CPPs can be used as a delivery tool for proteins and nucleic acids as they enhance the cellular uptake of the large molecules. For the treatment of infectious diseases, cancer and genetic disorders, siRNA can be used for gene silencing [139]. CPPs can overcome the problem of low permeability and may
lead to the internalization of siRNA [140]. A CPP-siRNA complex was synthesized by disulfide shown to efficiently decrease the expression of reporter transgenes in several mammalian cells [141].

**Biomedical applications of CPPs**

The cell membrane act as barrier for peptides, proteins and drug carriers and prevent them from entering into the cells except an active transport mechanism is involved. CPPs can easily deliver the drugs intracellularly as they can carry cargos without injury to the cell. CPPs can also be used in biomedical applications such as direct action as antifungal, antimicrobials, imaging, and anti-parasitic and as a carrier to deliver the drugs, nucleotides, small interfering RNA (siRNA), peptides and proteins [114,142].

**Antifungal and antimicrobial action of CPPs**

From studies it is found that the CPPs have capability to disrupt the cell membranes of fungi and bacteria. Antimicrobial peptides and CPPs have similar structural features such as positive charge and size which increases their interactions with anionic bio-membranes. Antimicrobial activity of CPPs and their derivatives is because of arginine residue in the peptide sequence. The antimicrobial activity and uptake of CPPs can be enhanced in the presence of multiple guanidinum groups. Number of studies has been investigated for the antibiotic activity of penetratin and Tat against Gram-negative and Gram-positive bacteria [143].

**CPPs improving intracellular delivery of anti-parasitic drugs**

Protozoan parasites cause serious human infections such as leishmaniasis, malaria. The therapeutic application of anti-parasitic drugs is limited by increasing levels of resistance and poor intracellular access. Here, CPPs have been used to carry the active compounds across the parasite membranes, for improving the efficacy of anti-parasitic drugs. Miltefosine was the first orally active leishmanicidal drug but its clinical application is limited because of resistance mechanisms. But leishmaniasis resistance to miltefosine can be treated by conjugation to Tat in which the conjugate was internalized into the R40 Leishmania strain efficiently where Miltefosine alone was not permeable, resulting in fast killing of parasites [144,145].

**CPPs-modified pH-sensitive delivery**

The exploitation of the acidic pH can improve the cytoplasmic delivery of cargo molecules performed with CPPs. CPPs with nanocarriers have triggered exposure mechanisms, such as degradation of enzyme of the protective coat, acid degradable cross-links, allows their controlled effect at the site of tumor microenvironment, because of the presence of pH gradient between physiological environment and the tumor milieu. So the therapeutic efficiency of nanocarriers enhanced and reduced toxicity [146].

**ADVANCES IN CELL PENETRATING PEPTIDE DEVELOPMENT**

**Chemical modification of CPP for enhanced delivery**

**Amino acid substitution**

By the amino acid substitution in the cell penetrating peptides, desired properties of peptide like cationic nature or hydrophobicity can be achieved. This strategy has resulted in increased intracellular internalization by certain CPPs. Kaeko et al. conducted a comprehensive search for novel CPPs using an in vitro virus library of peptides consisting of 15 amino acids and reported improved intracellular translocation efficiency at low concentrations due to the effect of cationic amino acids. As the amino acid Arginine has strong affinity to the surface of the cell, substituting it with another amino acid in the peptide chain such as Lysine improved the intracellular translocation significantly even at low concentration[147,148].

**Modification in functional group**

It involves the formation of linkage or masking groups to the highly reactive sites in the peptide chain. The peptide bonds formed should be weak and so that can be easily broken by simple variation of physiological conditions for regeneration of the cell penetrating peptide [114,149].

**CHALLENGES OF CPPS**

Biosafety and cytotoxicity are the main challenges for CPPs. The studies have been found that CPPs are less cytotoxic; but it should be considered that everything can become cytotoxic in a certain dose threshold. CPPs generally show two types of cytotoxic effects: 1) cytotoxic effects arising from the specific interaction of CPPs with cellular components 2) cytotoxic effect on the cell and also organelle membranes [150,151].

**LIMITATIONS AND FUTURE DIRECTION OF CELL-PENETRATING PEPTIDE-BASED STRATEGIES**

CPPs based therapies pose three main limitations. The first limitation is the absence of specificity to the tumor cells over normal cells in case of anticancer therapy. Most anticancer drugs
interfere with the replication of cell therefore show similar effects on the proliferating tissues. The interaction with targeted tumor cells would result in therapeutic functions of the drug, but interaction with normal cells can cause toxic side effects. The second limitation is the rapid clearance of water soluble drugs having low molecular weight from the bloodstream, and immunogenicity and/or proteolytic degradation of large protein or nucleic acid-type drugs. The third limitation is the difficulty in penetrating through cell membrane. The first limitation of insufficient tumor selectivity can be overcome by attaching the drug to the targeting component such as a peptide ligand or an antibody. A combination of prodrug and targeted delivery system is a solution to this limitation, as drug remains in inactive form during the targeting and delivery process and then converted to the active form at the targeted site [112]. The second limitation, which is related to the pharmacokinetic properties of the drugs, can be managed. In the natural systems, the pharmacokinetic behavior of many small drugs is regulated by series of transport proteins [152]. So, binding of such drugs to the macromolecule or a carrier would prolong their circulation time [99].

CONCLUSION

CPPs can transport the wide range of therapeutic agents and macromolecules across the biomembranes, enabling their localization to the cell nucleus, cytoplasm and various tissues for execution of their different functions: permeation through the skin mucosa to develop percutaneous delivery of nucleic-acid and protein drugs for clinical use, penetration through intestinal mucosa to increase oral bioavailability and the rate of drug absorption. The penetration capacity of the CPPs can be used for the study of the functional effects and intracellular mechanisms of biomolecules.

The main limitations of CPPs are easy degradation by plasma proteases, and lack of specificity, which may lead to loss of membrane permeation ability of CPPs. Modified CPPs can facilitate the endosomal escape, increase drug permeating efficiency, improve the tumor targeting and stimulus responsive controlled release of drug specific for the tumor microenvironment. Use of CPPs will lead to convenient and effective multifunctional drug delivery system which is important in the clinical applications and promote the research of new drugs.

ABBREVIATIONS

as = Antisense
CPPs = Cell-penetrating peptides
HA = Hemagglutinin
HSV-1 = HERPES Simplex virus type 1
ON = Oligonucleotide
Pen = Penetratin
PG = Proteoglycan
PNA = Peptide nucleic acid
PTD = Protein transduction domain
siRNA = Small interfering RNA
TP = Transportan
ACPPs = Activatable CPPs
Antp = Antennapedia homeodomain
DNA = Deoxyribonucleic Acid
FITC = Fluorescein Isothiocyanate
MRI = Magnetic Resonance Imaging
mRNA = Messenger Ribonucleic Acid
RNA = Ribonucleic Acid
Tatp = Trans-activator of transcription (Tat) peptide
US = Ultrasound

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Nil

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Vatsal Shah compiled and organized the data of the work and contributed to the drafting of the manuscript. Yamini Shah conceptualized the work and interpreted the data. Mansi Athalye contributed to the writing of the final manuscript. All authors read and approved the final manuscript.

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REFERENCES

[1] Conner SD, Schmid SL. Regulated portals of entry into the cell. Nature, 422, 37–44 (2003).
[2] Miller DG, Adam MA, Miller AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell. Biol.*, **10**, 4239–42 (1990).

[3] Luo D, Mark, W S. Synthetic DNA delivery systems. *Nat. Biotechnol.*, **18**, 33–7 (2000).

[4] Yacoub MD. The κ-μ distribution and the η-μ distribution. *IEEE Antennas Propag. Mag.*, **49**, 68–81 (2007).

[5] Järver P, Langel Ü. The use of cell-penetrating peptides as a tool for gene regulation. *Drug Discov. Today*, **9**, 395–402 (2004).

[6] Derossi D, Calvet S, Trembleau A, Brunissen A, Chassaing G, Prochiantz A. Cell internalization of the third helix of the antennapedia homeodomain is receptor-independent. *J. Biol. Chem.*, **271**, 18188–93 (1996).

[7] Futaki S, Suzuki T, Ohashi W, Yagami T, Tanaka S, Ueda K, Sugiura Y. Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J. Biol. Chem.*, **276**, 5836–40 (2001).

[8] Oren Z, Shai Y. Mode of action of linear amphipathic α-helical antimicrobial peptides. *Biopolymers*, **47**, 451–63 (1998).

[9] Matsuzaki K, Yoneyama S, Miyajima K. Pore formation and translocation of melittin. *Biophys. J.*, **73**, 831–8 (1997).

[10] Thorén PEG, Persson D, Isakson P, Goksör M, Önfelt A, Nordén B. Uptake of analogs of penetratin, Tat(48-60) and oligoarginine in live cells. *Biochem. Biophys. Res. Commun.*, **307**, 100–7 (2003).

[11] Lundberg M, Wikström S, Johansson M. Cell surface adherence and endocytosis of protein transduction domains. *Mol. Ther.*, **8**, 143–50 (2003).

[12] Ferrari A, Pellegrini V, Arcangeli C, Fittipaldi A, Giacca M, Beltram F. Caveolae-mediated internalization of extracellular HIV-1 Tat fusion proteins visualized in real time. *Mol. Ther.*, **8**, 284–94 (2003).

[13] Console S, Marty C, García-Echeverría C, Schwendener R, Ballmer-Hofer K. Antennapedia and HIV transactivator of transcription (TAT) “protein transduction domains” promote endocytosis of high molecular weight cargo upon binding to cell surface glycosaminoglycans. *J. Biol. Chem.*, **278**, 35109–14 (2003).

[14] Sandgren S, Wittrup A, Cheng F, Jönsson M, Eklund E, Busch S, Belting M. The Human Antimicrobial Peptide LL-37 Transfers Extracellular DNA Plasmid to the Nuclear Compartment of Mammalian Cells via Lipid Rafts and Proteoglycan-dependent Endocytosis. *J. Biol. Chem.*, **279**, 17951–6 (2004).

[15] Drin G, Cottin S, Blanc E, Rees AR, Temsamani J. Studies on the internalization mechanism of cationic cell-penetrating peptides. *J. Biol. Chem.*, **278**, 31192–201 (2003).

[16] Wadia JS, Stan R V., Dowdy SF. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat. Med.*, **10**, 310–5 (2004).

[17] Holm T, Langel Ü. Cell-Penetrating Peptides: Mechanisms and Applications. *Curr. Pharm. Des.*, **11**, 3597–611 (2005).

[18] Milletti F. Cell-penetrating peptides: Classes, origin, and current landscape. *Drug Discov. Today*, **17**, 850–60 (2012).

[19] Heitz F, Morris MC, Divita G. THEMED SECTION: VECTOR DESIGN AND DRUG DELIVERY REVIEW Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. *Br. J. Pharmacol.*, **157**, 195–206 (2009).

[20] Zatsenin TS, Turner JJ, Oretskaya TS, Gait MJ. Conjugates of Oligonucleotides and Analogues with Cell Penetrating Peptides as Gene Silencing Agents. *Curr. Pharm. Des.*, **11**, 3639–54 (2005).

[21] Pujals S, Fernández-carneado J, López-iglesias C, Kogan MJ, Giralt E. Mechanistic aspects of CPP-mediated intracellular drug delivery: Relevance of CPP self-assembly. *Biochim. Biophys. Acta*, **1758**, 264–79 (2006).

[22] Murriel CL, Dowdy SF. Influence of protein transduction domains on intracellular delivery of macromolecules. *Expert Opin. Drug Deliv.*, **3**, 739–46 (2006).

[23] Kim H, Kitamatsu M, Ohtsuki T. Bioorganic & Medicinal Chemistry Letters Enhanced intracellular peptide delivery by multivalent cell-penetrating peptide with bioreducible linkage. *Bioorg. Med. Chem. Lett.*, **28**, 378–81 (2018).

[24] Vives E. Cellular uptake of the Tat peptide: an endocytosis mechanism following ionic interactions AN IMPROVED DELIVERY UPON. *J. Mol. Recognit.*, **16**, 265–71 (2003).
[25] Hew K, Dahlroth S, Pan LX, Cornvik T. VP22 core domain from Herpes simplex virus 1 reveals a surprising structural conservation in both the Alpha - and Gammaherpesvirinae subfamilies. J. Gen. Virol., 96, 1436–45 (2015).

[26] Yu X, Li T, Xia Y, Lei JUN, Wang YAN, Zhang L. Herpes simplex virus type 1 VP22-mediated intercellular delivery of PTEN increases the antitumor activity of PTEN in esophageal squamous cell carcinoma cells in vitro and in vivo. Oncol. Rep., 35, 3034–40 (2016).

[27] Splith K, Neundorf I. Antimicrobial peptides with cell-penetrating peptide properties and vice versa. Eur. Biophys. J., 40, 387–97 (2011).

[28] Plaza JGR, Diener C, Gonzalez ZD, Teresa M, Ortiz L, Blake IO, Pantoja O, Chem JB. Microbiology: Cell Penetrating Peptides and Cationic Antibacterial Peptides : two sides of the same coin Rudolf Volkmer, Edda Klipp, Andreas Supplemental material: Poon GMK, Gariépy J. Cell-surface proteoglycans as molecular portals for cationic peptide and polymer entry and aggregation and subsequent dissociation of negatively charged phospholipid vesicles. FEBS Lett., 505, 307–12 (2001).

[29] Nakase I, Hirose H, Tanaka G, Tadokoro A, Kobayashi S, Takeuchi T, Futaki S. Cell-surface Accumulation of Flock House Virus-derived Peptide Leads to Efficient Internalization via Macropinocytosis. Mol. Ther., 17, 1868–76 (2009).

[30] Peptides CC, Cascales L, Henriques T, Kerr MC, Huang Y, Sweet MJ, Daly NL, Craik DJ. Identification and Characterization of a New Family of. J. Biol. Chem., 286, 36932–43 (2011).

[31] Copolovici DM, Langel K, Eriste E, Langel Ü. Cell-penetrating peptides: Design, synthesis, and applications. ACS Nano, 8, 1972–94 (2014).

[32] Macewan SR, Chilkoti A. Harnessing the power of cell-penetrating peptides: activatable carriers for targeting systemic delivery of cancer therapeutics and imaging agents. Wiley Interdiscip. Rev. Nanomedicine Nanobiotechnology, (2012).

[33] Bechara C, Sagan S. Cell-penetrating peptides: 20 years later, where do we stand? FEBS Lett., (2013).

[34] Wagstaff KM, Jans DA. Protein Transduction: Cell Penetrating Peptides and Their Therapeutic Applications. Front. Med. Chem. (Volume 5), 98–126 (2012).

[35] Yang H, Liu S, Cai H, Wan L, Li S, Li Y, Cheng J, Lu X. Chondroitin sulfate as a molecular portal that preferentially mediates the apoptotic killing of tumor cells by penetratin-directed mitochondria-disrupting peptides. J. Biol. Chem., 285, 25666–76 (2010).

[36] Letoha T, Keller-Pintér A, Kusz E, Kolozsi C, Bozsó Z, Tóth G, Vizler C, Oláh Z, Szilák L. Cell-penetrating peptide exploited syndecans. Biochim. Biophys. Acta - Biomembr., 1798, 2528–65 (2010).

[37] Poon GMK, Gariépy J. Cell-surface proteoglycans as molecular portals for cationic peptide and polymer entry into cells. Biochem. Soc. Trans., 35, 788–93 (2007).

[38] Persson D, Thorén PEG, Nordén B. Penetrin-induced aggregation and subsequent dissociation of negatively charged phospholipid vesicles. FEBS Lett., 505, 307–12 (2001).

[39] Tiriveedhi V, Butko P. A fluorescence spectroscopy study on the interactions of the TAT-PTD peptide with model lipid membranes. Biochemistry, 46, 3888–95 (2007).

[40] Walrant A, Correia I, Jiao C, Lequin O, Bent EH, Goadsou N, Lacombe C, Chassaing G, Sagan S, Alves ID. Biochimica et Biophysica Acta Different membrane behaviour and cellular uptake of three basic arginine-rich peptides. BBA - Biomembr., 1808, 382–93 (2011).

[41] Alves ID, Goadsou N, Correia I, Aubry S, Galanth C, Sagan S, Livielle S, Chassaing G. Membrane interaction and perturbation mechanisms induced by two cationic cell penetrating peptides with distinct charge distribution. Biochim. Biophys. Acta, 1780, 948–59 (2008).

[42] Eiríksdóttir E, Konate K, Langel Ü, Divita G, Deshayes S. Biochimica et Biophysica Acta Secondary structure of cell-penetrating peptides controls membrane interaction and insertion. BBA - Biomembr., 1798, 1119–28 (2010).

[43] Lundin P, Johansson H, Gutermast P, Holm T, Hansen M, Langel Ü, Andaloussi SEL. Distinct Uptake Routes of Cell-Penetrating Peptide Conjugates. Bioconjug. Chem., 19, 2535–42 (2008).

[44] Deshayes S, Decaffmeyer M, Brasseur R, Thomas A. Structural polymorphism of two CPP : An important parameter of activity. Biochim. Biophys. Acta, 1778, 1197–205 (2008).

[45] Mitchell DJ, Kim DT, Steinman L, Fathman CG, Rothbard JB. Polyarginine enters cells more efficiently than other polycationic homopolymers. J. Pept. Res., 56, 318–25 (2000).

[46] Wender PA, Mitchell DJ, Pattabiraman K, Pelkey ET, Steinman L, Rothbard JB. The design, synthesis, and
evaluation of molecules that enable or enhance cellular uptake: Peptoid molecular transporters. *Proc. Natl. Acad. Sci. U. S. A.*, **97**, 13003–8 (2000).

[47] Nakase I, Takeuchi T, Tanaka G, Futaki S. Methodological and cellular aspects that govern the internalization mechanisms of arginine-rich cell-penetrating peptides. *Adv. Drug Deliv. Rev.*, **60**, 598–607 (2008).

[48] Rothbard JB, Jessop TC, Lewis RS, Murray BA, Wender PA. Role of Membrane Potential and Hydrogen Bonding in the Mechanism of Translocation of Guanidinium-Rich Peptides into Cells. *J. Am. Chem. Soc.*, **126**, 9506–7 (2004).

[49] Takayama K, Nakase I, Michiue H, Takeuchi T, Tomizawa K. Enhanced intracellular delivery using arginine-rich peptides by the addition of penetration accelerating sequences (Pas). *J. Control. Release*, **138**, 128–33 (2009).

[50] Elmquist A, Hansen M, Langel Ü. Structure – activity relationship study of the cell-penetrating peptide p VEC. *Biochim. Biophys. Acta*, **1758**, 721–9 (2006).

[51] Carrigan CN, Imperiali B. The engineering of membrane-permeable peptides. *Anal. Biochem.*, **341**, 290–8 (2005).

[52] Mishra A, Hwee G, Schmidt NW, Sun VZ, Rodriguez AR, Tong R, Tang L. Translocation of HIV TAT peptide and analogues induced by multiplexed membrane and cytoskeletal interactions. *Proc. Natl. Acad. Sci. U. S. A.*, **108**, 16883–16888 (2011).

[53] Magzoub M, Goran LE, Graslund A. Comparison of the interaction, positioning, structure induction and membrane perturbation of cell-penetrating peptides and non-translocating variants with phospholipid vesicles. *Biophys. Chem.*, **103**, 271–88 (2003).

[54] Walrant A, Vogel A, Correia I, Lequin O, Olausson BES, Desbat B, Sagan S, Alves ID. Biochimica et Biophysica Acta Membrane interactions of two arginine-rich peptides with different cell internalization capacities. *BBA - Biomembr.*, **1818**, 1755–63 (2012).

[55] Edenhofer F. Protein Transduction Revisited: Novel Insights Into the Mechanism Underlying Intracellular Delivery of Proteins. *Curr. Pharm. Des.*, **14**, 3628–36 (2008).

[56] Duchardt F, Fotin-Mleczek M, Schwarz H, Fischer R, Brock R. A comprehensive model for the cellular uptake of cationic cell-penetrating peptides. *Traffic*, **8**, 848–66 (2007).

[57] Gump JM, Dowdy SF. TAT transduction: the molecular mechanism and therapeutic prospects. *Trends Mol. Med.*, **13**, 443–8 (2007).

[58] Li H, Tsui TY, Ma W. Intracellular delivery of molecular cargo using cell-penetrating peptides and the combination strategies. *Int. J. Mol. Sci.*, **16**, 19518–36 (2015).

[59] Fawell S, Seery J, Daikh Y, Moore C, Chen LL, Pepinsky B, Barsoum J. Tat-mediated delivery of heterologous proteins into cells. *Proc. Natl. Acad. Sci. U. S. A.*, **91**, 664–8 (1994).

[60] Ditler B, Schroeder J. Anti-Cancer Therapies that Utilize Cell Penetrating Peptides. *Recent Pat. Anticancer. Drug Discov.*, **5**, 99–108 (2010).

[61] Anderson RGW, Kamen BA, Rothberg KG, Lacey SW. Potocytosis: Sequestration and transport of small molecules by caveolae. *Science (80-. ).*, **255**, 410–1 (1992).

[62] Lundberg P, Langel Ü. A brief introduction to cell-penetrating peptides. *J. Mol. Recognit.*, **16**, 227–33 (2003).

[63] Guidotti G, Brambilla L, Rossi D. Cell-Penetrating Peptides: From Basic Research to Clinics. *Trends Pharmacol. Sci.*, **38**, 406–24 (2017).

[64] Reissmann S. Cell penetration: Scope and limitations by the application of cell-penetrating peptides. *J. Pept. Sci.*, **20**, 760–84 (2014).

[65] Böhmová E, Machová D, Pechar M, Pola R, Vencliková K, Janoušková O, Etrych T. Cell-penetrating peptides: A useful tool for the delivery of various cargoes into cells. *Physiol. Res.*, **67**, s267–79 (2018).

[66] Brogden KA. Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.*, **3**, 238–50 (2005).

[67] Matsuzaki K, Murase O, Fujii N, Miyajima K. An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation. *Biochemistry*, **35**, 11361–8 (1996).

[68] Graslund A, Madani F, Lindberg S, Langel Ü, Futaki S. Mechanisms of cellular uptake of cell-penetrating peptides. *J. Biophys.*, **2011**, (2011).

[69] Shai Y. Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-
lytic peptides. *Biochim. Biophys. Acta - Biomembr.*, **1462**, 55–70 (1999).

[70] Islam MZ, Sharmin S, Moniruzzaman M, Yamazaki M. Elementary processes for the entry of cell-penetrating peptides into lipid bilayer vesicles and bacterial cells. *Appl. Microbiol. Biotechnol.*, **102**, 3879–92 (2018).

[71] Angeles-Boza AM, Erazo-Oliveras A, Lee YJ, Pellois JP. Generation of endosomolytic reagents by branching of cell-penetrating peptides into lipid bilayer vesicles and bacterial cells. *Bioconjug. Chem.*, **21**, 2164–7 (2010).

[72] Kawamura KS, Su R-C, Nguyen LT, Elford AR, Ohashi PS, Gariépy J. In Vivo Generation of Cytotoxic T Cells from Epitopes Displayed on Peptide-Based Delivery Vehicles. *J. Immunol.*, **168**, 5709–15 (2002).

[73] Medina SH, El-Sayed MEH. Dendrimers as carriers for delivery of chemotherapeutic agents. *Chem. Rev.*, **109**, 3141–57 (2009).

[74] Pantos A, Tsiourvas D, Nounesis G, Paleos CM. Interaction of functional dendrimers with multilamellar liposomes: Design of a model system for studying drug delivery. *Langmuir*, **21**, 7483–90 (2005).

[75] Kang H, DeLong R, Fisher MH, Juliano RL. Tat-conjugated PAMAM dendrimers as delivery agents for antisense and siRNA oligonucleotides. *Pharm. Res.*, **22**, 2099–106 (2005).

[76] Kawamura KS, Sung M, Bolewska-Pedyczak E, Gariépy J. Probing the impact of valency on the routing of arginine-rich peptides into eukaryotic cells. *Biochemistry*, **45**, 1116–27 (2006).

[77] Soo-Jin L, Yoon SH, Doh KO. Enhancement of gene delivery using novel homodimeric tat peptide formed by disulfide bond. *J. Microbiol. Biotechnol.*, **21**, 802–7 (2011).

[78] Rudolph C, Schillinger U, Ortiz A, Tabatt K, Plank C, Müller RH, Rosenecker J. Application of novel solid lipid nanoparticle (SLN)-gene vector formulations based on a dimeric HIV-1 TAT-peptide in vitro and in vivo. *Pharm. Res.*, **21**, 1662–9 (2004).

[79] Ainoto S. Synthesis of proteins by native chemical ligation. *Tanpakushitsu Kakusan Koso.*, **52**, 1804–5 (2007).

[80] Singh D, Bisland SK, Kawamura K, Gariépy J. Peptide-based intracellular shuttle able to facilitate gene transfer in mammalian cells. *Bioconjug. Chem.*, **10**, 745–54 (1999).

[81] Singh D, Kiarash R, Kawamura K, LaCasse EC, Gariépy J. Penetration and intracellular routing of nucleus-directed peptide-based shuttles (loligomers) in eukaryotic cells. *Biochemistry*, **37**, 5798–809 (1998).

[82] Sheldon K, Liu D, Ferguson J, Gariépy J. Loligomers: Design of de novo peptide-based intracellular vehicles. *Proc. Natl. Acad. Sci. U. S. A.*, **92**, 2056–60 (1995).

[83] Kim J Bin, Choi JS, Nam K, Lee M, Park JS, Lee JK. Enhanced transfection of primary cortical cultures using arginine-grafted PAMAM dendrimer, PAMAM-Arg. *J. Control. Release*, **114**, 110–7 (2006).

[84] Hassane FS, Ivanova GD, Bolewska-Pedyczak E, Abes R, Arzumanov AA, Gait MJ, Lebleu B, Gariépy J. A peptide-based dendrimer that enhances the splice-redirecting activity of PNA conjugates in cells. *Bioconjug. Chem.*, **20**, 1523–30 (2009).

[85] Appelbaum JS, Larochelle JR, Smith BA, Balkin DM, Holub JM, Schepartz A. Arginine topology controls escape of minimally cationic proteins from early endosomes to the cytoplasm. *Chem. Biol.*, **19**, 819–30 (2012).

[86] Ma Y, Gong C, Ma Y, Fan F, Luo M, Yang F, Zhang YH. Direct cytosolic delivery of cargoes in vivo by a chimera consisting of D- and L-arginine residues. *J. Control. Release*, **162**, 286–94 (2012).

[87] Holm, T., Andaloussi, EL., Langel Ü. Comparison of CPP Uptake Methods. Ülo Langel (ed.), *Cell-Penetrating Pept. Methods Protoc. Methods Mol. Biol.*, **683**, 21–9 (2011).

[88] Burlina F, Sagan S, Bolbach G, Chassaing G. Quantification of the cellular uptake of cell-penetrating peptides by MALDI-TOF mass spectrometry. *Angew. Chemie - Int. Ed.*, **44**, 4244–7 (2005).

[89] Burlina F, Sagan S, Bolbach G, Chassaing G. A direct approach to quantification of the cellular uptake of cell-penetrating peptides using MALDI-TOF mass spectrometry. *Nat. Protoc.*, **1**, 200–5 (2006).

[90] Hirose H, Takeuchi T, Osakada H, Pujals S, Katayama S, Nakase I, Kobayashi S, Haraguchi T, Futaki S. Transient focal membrane deformation induced by arginine-rich peptides leads to their direct penetration into cells. *Mol. Ther.*, **20**, 984–93 (2012).
[91] Palm-Apergi C, Lorents A, Padari K, Pooga M, Hällbrink M. The membrane repair response masks membrane disturbances caused by cell-penetrating peptide uptake. *FASEB J.*, **23**, 214–23 (2009).

[92] Ye J, Fox SA, Cudic M, Rezler EM, Lauer JL, Fields GB, Terentis AC. Determination of penetratin secondary structure in live cells with Raman microscopy. *J. Am. Chem. Soc.*, **132**, 980–8 (2010).

[93] Ziegler A, Seelig J. High affinity of the cell-penetrating peptide HIV-1 Tat-PTD for DNA. *Biochemistry*, **46**, 8138–45 (2007).

[94] Dietz G. Cell-Penetrating Peptide Technology to Deliver Chaperones and Associated Factors in Diseases and Basic Research. *Curr. Pharm. Biotechnol.*, **11**, 167–74 (2010).

[95] Gaynor JW, Cosstick R. Therapeutic Oligonucleotides, ch 2: Diverse Dinucleotides Containing 3′-S-Phosphorothiolate Linkages. *Methods Mol. Biol.*, **764**, 17–30 (2011).

[96] Bechara C, Sagan S. Cell-penetrating peptides: 20 years later, where do we stand? *FEBS Lett.*, **587**, 1693–702 (2013).

[97] Ponnappan N, Budagavi DP, Chugh A. CyLoP-1: Membrane-active peptide with cell-penetrating and antimicrobial properties. *Biochim. Biophys. Acta - Biomembr.*, **1859**, 167–76 (2017).

[98] Lindsay MA. Peptide-mediated cell delivery: Application in protein target validation. *Curr. Opin. Pharmacol.*, **2**, 587–94 (2002).

[99] Suk Choi Y, Yeon Lee J, Sook Suh J, Jin Lee S, C. Yang V, Pyoung Chung C, Jeong Park Y. Cell Penetrating Peptides for Tumor Targeting. *Curr. Pharm. Biotechnol.*, **12**, 1166–82 (2011).

[100] Tseng YL, Liu JJ, Hong RL. Translocation of liposomes into cancer cells by cell-penetrating peptides penetratin and Tat: A kinetic and efficacy study. *Mol. Pharmacol.*, **62**, 864–72 (2002).

[101] Torchilin VP, Rammohan R, Weissig V, Levchenko TS. Tat peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. *Proc. Natl. Acad. Sci. U. S. A.*, **100**, 1972–7 (2003).

[102] Polyakov V, Sharma V, Dahlheimer JL, Pica CM, Luker GD, Piwnica-Worms D. Novel Tat-peptide chelates for direct transduction of technetium-99m and rhenium into human cells for imaging and radiotherapy. *Bioconj. Chem.*, **11**, 762–71 (2000).

[103] Lewin M, Carlesso N, Tung CH, Tang XW, Cory D, Scadden DT, Weissleder R. Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. *Nat. Biotechnol.*, **18**, 410–4 (2000).

[104] Bhorade R, Weissleder R, Nakakoshi T, Moore A, Tung CH. Macrocyclic chelators with paramagnetic cations are internalized into mammalian cells via a HIV-tat derived membrane translocation peptide. *Bioconj. Chem.*, **11**, 301–5 (2000).

[105] Trabulo S, Cardoso AL, Mano M, de Lima MCP. Cell-penetrating peptides-mechanisms of cellular uptake and generation of delivery systems. *Pharmaceuticals*, **3**, 961–93 (2010).

[106] Kim WJ, Christensen L V., Jo S, Yockman JW, Jeong JH, Kim YH, Kim SW. Cholesteryl Oligoarginine Delivering Vascular Endothelial Growth Factor siRNA Effectively Inhibits Tumor Growth in Colon Adenocarcinoma. *Mol. Ther.*, **14**, 343–50 (2006).

[107] Zeineddine D, Papadimou E, Chebli K, Gineste M, Liu J, Grey C, Thurig S, Behfar A, Wallace VAA, Skerjane IS, Pucéat M. Oct-3/4 Dose Dependently Regulates Specification of Embryonic Stem Cells toward a Cardiac Lineage and Early Heart Development. *Dev. Cell*, **11**, 535–46 (2006).

[108] Crombez L, Divita G. A non-covalent peptide-based strategy for siRNA delivery. *Methods Mol. Biol.*, **683**, 349–60 (2011).

[109] Nguyen QN, Chavli R V., Marques JT, Conrad PG, Wang D, He W, Belisle BE, Zhang A, Pastor LM, Witney FR, Morris M, Heitz F, Divita G, Williams BRG, McMaster GK. Light controllable siRNAs regulate gene suppression and phenotypes in cells. *Biochim. Biophys. Acta - Biomembr.*, **1758**, 394–403 (2006).

[110] Glover DJ, Lipps HJ, Jans DA. Towards safe, non-viral therapeutic gene expression in humans. *Nat. Rev. Genet.*, **6**, 299–310 (2005).
[112] Kwon YM, Li Y, Naik S, Liang JF, Huang Y, Park YJ, Yang VC. The ATTEMPTS delivery systems for macromolecular drugs. Expert Opin. Drug Deliv., 5, 1255–66 (2008).

[113] Koch AM, Reynolds F, Merkle HP, Weissleder R, Josephson L. Transport of surface-modified nanoparticles through cell monolayers. ChemBioChem, 6, 337–45 (2005).

[114] Munyendo WLL, Lv H, Benza-Ingoula H, Baraza LD, Zhou J. Cell penetrating peptides in the delivery of biopharmaceuticals. Biomolecules, 2, 187–202 (2012).

[115] Olson ES, Jiang T, Aguilera TA, Nguyen QT, Ellies LG, Scadeng M, Tsien RY. Activatable cell penetrating peptides linked to nanoparticles as dual probes for in vivo fluorescence and MR imaging of proteases. Proc. Natl. Acad. Sci. U. S. A., 107, 4311–6 (2010).

[116] Nguyen QT, Olson ES, Aguilera TA, Jiang T, Scadeng M, Ellies LG, Tsien RY. Surgery with molecular fluorescence imaging using activatable cell-penetrating peptides decreases residual cancer and improves survival. Proc. Natl. Acad. Sci. U. S. A., 107, 4317–22 (2010).

[117] Lei Y, Tang H, Yao L, Yu R, Feng M, Zou B. Applications of mesenchymal stem cells labeled with tat peptide conjugated quantum dots to cell tracking in mouse body. Bioconjug. Chem., 19, 421–7 (2008).

[118] Ruan G, Agrawal A, Marcus AI, Nie S. Imaging and tracking of Tat peptide-conjugated quantum dots in living cells: New insights into nanoparticle uptake, intracellular transport, and vesicle shedding. J. Am. Chem. Soc., 129, 14759–66 (2007).

[119] Zahid M, Robbins PD. Cell-type specific penetrating peptides: Therapeutic promises and challenges. Molecules, 20, 13055–70 (2015).

[120] Pooga, M., Somets, U., Rezaei, K., Kahl, U. et al. Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo. Nat. Biotechnol., 16, 857–61 (1998).

[121] Morris MC, Vidal P, Chaloin L, Heitz F, Divita G. A new peptide vector for efficient delivery of oligonucleotides into mammalian cells. Nucleic Acids Res., 25, 2730–6 (1997).

[122] Wyman TB, Nicol F, Zelphati O, Scaria P V., Plank C, Szoka FC. Design, synthesis, and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers. Biochemistry, 36, 3008–17 (1997).

[123] Gratton JP, Yu J, Griffith JW, Babbitt RW, Scotland RS, Hickey R, Giordano FJ, Sessa WC. Cell-permeable peptides improve cellular uptake and therapeutic gene delivery of replication-deficient viruses in cells and in vivo. Nat. Med., 9, 357–62 (2003).

[124] Kurreck J. Antisense technologies: Improvement through novel chemical modifications. Eur. J. Biochem., 270, 1628–44 (2003).

[125] Ito E, Sweterlitsch LA, Bui-Vinh Tran P, Rauscher FJ, Narayanan R. Inhibition of PC-12 cell differentiation by the immediate early gene fra-1. Oncogene, 5, 1755–60 (1990).

[126] Morris MC, Chaloin L, Heitz F, Divita G. Translocating peptides and proteins and their use for gene delivery. Curr. Opin. Biotechnol., 11, 461–6 (2000).

[127] Tasciotti E, Zoppè M, Giacca M. Transcellular transfer of active HSV-1 thymidine kinase mediated by an 11-amino-acid peptide from HIV-1 Tat. Cancer Gene Ther., 10, 64–74 (2003).

[128] Simpson CL, Patel DM, Green KJ. Deconstructing the skin: Cytarchitectural determinants of epidermal morphogenesis. Nat. Rev. Mol. Cell Biol., 12, 565–80 (2011).

[129] Madison KC. Barrier Function of the Skin. J. Invest. Dermatol., 121, 231–41 (2003).

[130] Rothbard JB, Garlington S, Lin Q, Kirschberg T, Kreider E, McGrane PL, Wender PA, Khavari PA. Conjugation of arginine oligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation. Nat. Med., 6, 1253–7 (2000).

[131] HouYW, Chan MH, Hsu HR, Liu BR, Chen CP, Chen HH, Lee HJ. Transdermal delivery of proteins mediated by non-covalently associated arginine-rich intracellular delivery peptides. Exp. Dermatol., 16, 999–1006 (2007).

[132] Partidos CD, Beignon AS, Mawas F, Belliard G, Briand JP, Muller S. Immunity under the skin: Potential application for topical delivery of vaccines. Vaccine, 21, 776–80 (2003).

[133] Deshayes S, Konate K, Aldrian G, Crombez L, Heitz F, Divita G. Structural polymorphism of non-covalent peptide-based delivery systems: Highway to cellular uptake. Biochim. Biophys. Acta - Biomembr., 1798, 2304–14 (2010).
[134] Tan XX, Actor JK, Chen Y. Peptide nucleic acid antisense oligomer as a therapeutic strategy against bacterial infection: Proof of principle using mouse intraperitoneal infection. Antimicrob. Agents Chemother., 49, 3203–7 (2005).

[135] Hegedüs R, Manea M, Orbán E, Szabó I, Kiss É, Sipos É, Halmos G, Mez G. Enhanced cellular uptake and in vitro antitumor activity of short-chain fatty acid acylated daunorubicin-GnRH-III bioconjugates. Eur. J. Med. Chem., 56, 155–65 (2012).

[136] Dubikovskaya EA, Thorne SH, Pillow TH, Contag CH, Wender PA. Overcoming multidrug resistance of small-molecule therapeutics through conjugation with releasable octaarginine transporters. Proc. Natl. Acad. Sci. U. S. A., 105, 12128–33 (2008).

[137] Aroui S, Mili D, Brahim S, Waard M De, Kenani A. Doxorubicin coupled to penetratin promotes apoptosis in CHO cells by a mechanism involving c-Jun NH2-terminal kinase. Biochem. Biophys. Res. Commun., 396, 908–14 (2010).

[138] Walker L, Perkins E, Kratz F, Raucher D. Cell penetrating peptides fused to a thermally targeted biopolymer drug carrier improve the delivery and antitumor efficacy of an acid-sensitive doxorubicin derivative. Int. J. Pharm., 436, 825–32 (2012).

[139] Kanasty R, Dorkin JR, Vegas A, Anderson D. Delivery materials for siRNA therapeutics. Nat. Mater., 12, 967–77 (2013).

[140] Eguchi A, Dowdy SF. siRNA delivery using peptide transduction domains. Trends Pharmacol. Sci., 30, 341–5 (2009).

[141] Guo Z, Peng H, Kang J, Sun D. Cell-penetrating peptides: Possible transduction mechanisms and therapeutic applications (review). Biomed. Reports, 4, 528–34 (2016).

[142] Gao H, Zhang Q, Yang Y, Jiang X, He Q. Tumor homing cell penetrating peptide decorated nanoparticles used for enhancing tumor targeting delivery and therapy. Int. J. Pharm., 478, 240–50 (2015).

[143] Derakhshankhah H, Jafari S. Cell penetrating peptides: A concise review with emphasis on biomedical applications. Biomed. Pharmacother., 108, 1090–6 (2018).

[144] De La Torre BG, Hornillos V, Luque-Ortega JR, Abengózar MA, Amat-Guerri F, Ulises Acuña A, Rivas L, Andreu D. A BODIPY-embedding miltefosine analog linked to cell-penetrating Tat(48-60) peptide favors intracellular delivery and visualization of the antiparasitic drug. Amino Acids, 46, 1047–58 (2014).

[145] Gallo M, Defaus S, Andreu D. 1988–2018: Thirty years of drug smuggling at the nano scale. Challenges and opportunities of cell-penetrating peptides in biomedical research. Arch. Biochem. Biophys., 661, 74–86 (2019).

[146] Wang F, Wang Y, Zhang X, Zhang W, Guo S, Jin F. Recent progress of cell-penetrating peptides as new carriers for intracellular cargo delivery. J. Control. Release, 174, 126–36 (2014).

[147] Kucerz M, Konopińska D, Rosiński G. Insect gonadotropic peptide hormones: some recent. J. Pept. Sci., 1, 16–26 (2007).

[148] Mason AJ, Leborgne C, Moulay G, Martinez A, Danos O, Bechinger B, Kichler A. Optimising histidine rich peptides for efficient DNA delivery in the presence of serum. J. Control. Release, 118, 95–104 (2007).

[149] Kersemans V, Cornelissen B. Targeting the tumour: Cell penetrating peptides for molecular imaging and radiotherapy. Pharmaceuticals, 3, 600–20 (2010).

[150] Simmaco M, Mignogna G, Barra D. Antimicrobial peptides from amphibian skin: What do they tell us? Biopolymers, 47, 435–50 (1998).

[151] Ganz T, Lehrer RI. Antimicrobial peptides of vertebrates. Curr. Opin. Immunol., 10, 41–4 (1998).

[152] Russell-Jones GJ, Alpers DH. Vitamin B12 transporters. Pharm. Biotechnol., 12, 493–520 (1999).