Chapter

Cell-Penetrating Peptides: A Challenge for Drug Delivery

Sonia Aroui and Abderraouf Kenani

Abstract

Cell-penetrating peptide (CPP) is a term that describes relatively short amphipathic and cationic peptides (7–30 amino acid residues) with rapid translocation across the cell membrane. They can be used to deliver molecular bioactive cargoes due to their efficacy in cellular internalization and also to their low cytotoxicity. In this review we provide an overview of the current approaches and describe the potential of CPP-based drug delivery systems and indicate their powerful promise for clinical efficacy.

Keywords: cell-penetrating peptides, drugs

1. Introduction

A novel approach to overcome cell membrane impermeability and to deliver a large variety of particles and macromolecules into cells has been recently emerged, which is called cell-penetrating peptides (CPPs), also known as protein transduction domains (PTDs) [1, 2]. CPPs are generally short (up to 30 amino acids in length) water-soluble, cationic, and/or amphipathic peptides which make them promising vectors for therapeutic delivery, leading to a considerable amount of research focused on the intracellular delivery of drugs [3–5]. There are two principal types of CPPs that have been utilized for this purpose: (i) cationic CPPs, composed of short sequence of amino acids (arginine, lysine, and histidine). The indicated amino acids give the cationic charge to the peptide and permit its interaction with anionic motifs on the plasma membrane by a receptor-independent mechanism. (ii) amphipathic peptides, which have lipophilic and hydrophilic tails that are responsible for a direct peptide translocation mechanism across the plasma membrane [6].

The most important characteristic of CPPs is that they are able to translocate the plasma membrane at low micromolar concentrations in vivo and in vitro without using any receptors and without causing any significant membrane damage [7, 8]. Other benefits of using CPPs for therapeutic delivery are the absence of toxicity as compared to other cytoplasmic delivery devices, such as liposomes, polymers, etc. [6]. The mechanism for the CPP-facilitated cellular uptake remains not clear and depends on cargo and cellular type [9]. Due to its high density of basic amino acid residues (Arg and Lys), the large charge at physiological pH excludes the passive diffusion of CPPs across the lipid bilayer. Furthermore, it seems that classical uptake mechanisms such as protein-based receptors and transporters are not involved. On the contrary, endocytosis was shown as a common uptake mechanism, but is controversial at the same time. For example, in a number of reports, CPP

uptake was not inhibited at 4°C or in the presence of inhibitors of endocytosis; in contrast, a capture of CPPs in the endocytotic vesicles was observed when soluble heparin sulfate was added [9, 10]. Many other studies indicate that aggregation of the cell surface glycosaminoglycan heparan sulfate (HS) is an important element in the uptake mechanism [2]. The challenge of the strategy using CPPs should take into consideration the size, stability, nonspecific versus specific associations, and potency versus toxicity that all play an important role for the selection of delivery systems [5].

2. History and origin of CPPs

The CPPs are initially discovered in 1965 when it was observed that histones and cationic polyamines such as polylysine stimulate the uptake of albumin by tumor cells in culture. It was shown that the conjugation of polylysine to albumin and other proteins enhances their transport into cells. Moreover, a comparison study of different homopolymers of cationic amino acids demonstrates that medium-length polymers of arginine enter cells more effective than similar-length polymers composed of lysine, ornithine, or histidine [11]. In 1988, it was discovered that the human immunodeficiency virus type 1 (HIV-1) encoded trans-acting activator of transcription (Tat) peptide which also translocates cell membranes and gains intracellular mammalian cells [12, 13]. Covalently the conjugation of Tat peptide to proteins or fluorescent markers allowed these molecules to gain into the cell. A few years later, another discovery was followed when polycationic peptide of natural (VP22 and AntP) and synthetic origin (transportan) was used for the delivery of genes, proteins, small exogenous peptide, or even nanoparticles. Furthermore, it was demonstrated that small domains in these peptides are often responsible for cellular entry [14]. Thus, these translocation sequences could be shortened to a few amino acids in comparison with the first Tat peptide, without affecting cell penetration efficiency [13]. Since that time, the list of synthetic CPPs has increased sharply, and the number continues to rise (**Table 1**). In the last decade, another peptide was described named maurocalcine (MCa), a 33 amino acid residue peptide that has been isolated from the venom of the Tunisian chactid scorpion *Scorpio maurus palmatus.* It folds according to an "inhibitor cystine knot" (ICK) motif and contains three disulfide bridges connected by the following pattern: C1–C4, C2–C5, and C3– C6 [15]. MCa acts on ryanodine receptors resulting in pharmacological activation. These receptors are calcium channels located in the membrane of the endoplasmic reticulum. They control Ca^{2+} release from internal stores and therefore a large number of cell functions [16, 17].

This peptide possesses vector properties when coupled to fluorescent streptavidin. This complex was shown to enter various cell types within minutes and in all cell types tested, a common feature of CPPs. A variety of mutants of MCa were then designed in order to unravel the most active residues for its pharmacological and penetration activities (**Figure 1**) [18, 19].

3. Therapeutic applications of CPPs

3.1 CPP-cargo complex internalization mechanisms

Two distinct advances were shown to be used to bind CPPs to molecular cargoes. One process is non-covalently which connect CPP to its cargoes using electrostatic

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Peptide	Sequence	Origin	Cargoes
Protein transduction domain			
Tat48-60	GRKKRRQRRRPPQ	VIH-1	ADN, peptide, PKC inhibitor
Pénétratin	RQIKIWFQNRRMKWKK	*Drosophila Antennapedia homeodomain*	HSP20 phosphopeptide
Chimeric peptides			
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	Galanin + Mastoparan	Protéine, PNA
Pep-1	KETWWETWWTEWSQPKKKRKV	Rich domain of tryptophan + *spacer* + domain derived from virus SV40-NLS sequence of T antigène	Enzyme
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	Hydrophobic motif derived from HIV-1 gp41 + *linker* + *domain derived* from virus SV40-NLS sequence of T antigène	siARN, oligo-nucléotides
CADY	GLWRALWRLLRSLWRLLWRA	Dérived from PPTG11, variant of JTS1 fusion protéin	siARN
Peptide models			
(Arg)x	(RRRRR)X	Synthetic peptide	siARN, Cyclosporine A
MAP	KLALKLALKALKAALKA	Synthetic peptide	Natural CPPs
Natural CPP			
Maurocalcine	GDCLPHLKLCKENKDCCSKKCKRRGTNIEKRCR	*Scorpio maurus palmatus*	Doxorubicin

Table 1.
Examples of four classes of CPPs and delivered cargoes. The list of cargo is not exhaustive and given for illustration. X = 7, 8, or 9 arginine residues.

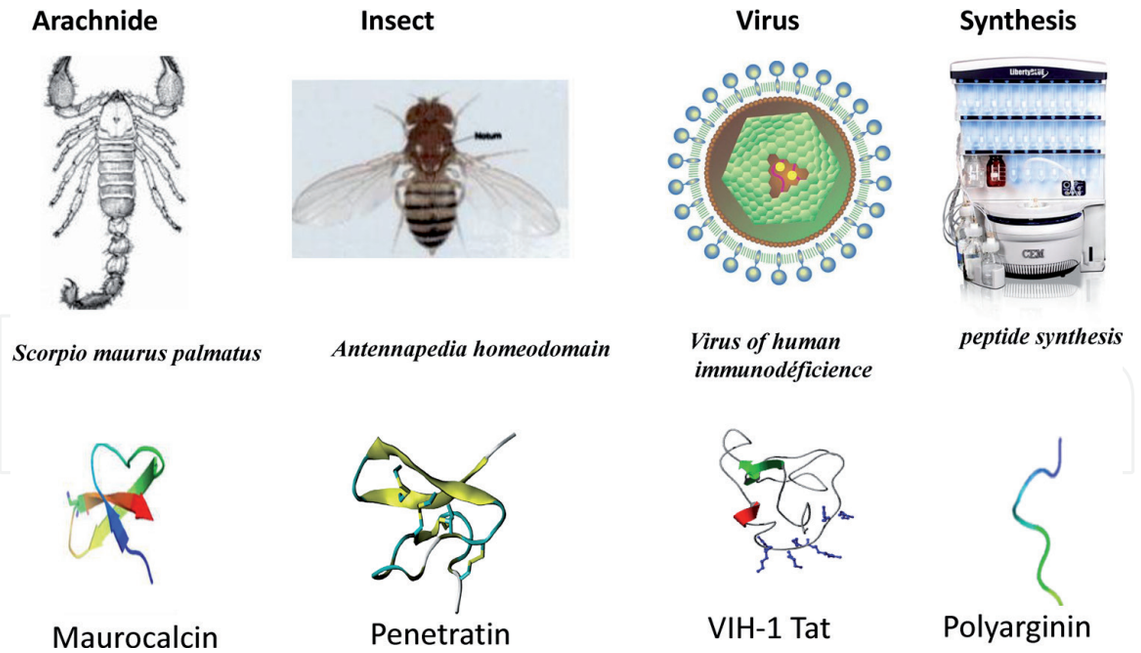

Figure 1.
Example of origin of four CPPs: Maurocalcine, penetratin, tat, and polyarginine. Maurocalcine, penetratin, and tat are derived from natural sequences, but polyarginine was produced by de novo conception in order to obtain a good cellular penetration.

interactions, such as MPG and Pep-1, amphipathic peptides carriers, which link to cargoes beyond any cross linking or chemical changes [20]. The second approach is more frequent and uses a covalent relation between the two compounds. This means has been widely used by different teams and has demonstrated positive advances, especially with TAT, penetratin, or polyarginines [21].

Various mechanisms for CPP internalization have been suggested, but the exact one is still not well known. Yet, many data approve that the energy-dependent tool (endocytosis) and the energy-independent mechanism (direct translocation) or both are involved in the cellular uptake progress [22].

For direct penetration, various mechanisms have been described: the carpet-like model (membrane destabilization) [23] and the pore formation model (barrel-stave) [24]. Positively charged CPPs interact with negatively charged membrane components like phospholipid bilayer or heparan sulfate. Such interaction is dwelling on the first stage of all of these mechanisms, followed by destabilization of the membrane and finished by crossing of the CPP on the lipid membrane.

For endocytosis transduction or cellular digestion, pinocytosis, phagocytosis, and receptor-mediated endocytosis have been reported [25, 26]. A sum-up of CPP transduction systems is shown in **Figure 2**. In pinocytosis, the plasma membrane absorbs solutes, while in phagocytosis it takes great particles. In clathrin-mediated endocytosis, clathrin and also caveolin, which are receptor-mediated endocytosis and cover the intracellular part of the biomembranes, possess a key role in the uptake mechanism. These protein structures are pivotal for the membrane invagination and for the construction of the vesicles after bounding the extracellular molecule to the membrane receptor. Clathrin has a great diameter in comparison with caveolin-coated vesicles and was also considered as a selective route for the translocation of compounds into cells through specific receptors on the surface of the cell [27].

Many determinants influence the internalization process, such as the nature of CPP or the cell type, the cargo, and the experimental conditions (temperature and pH) [22].

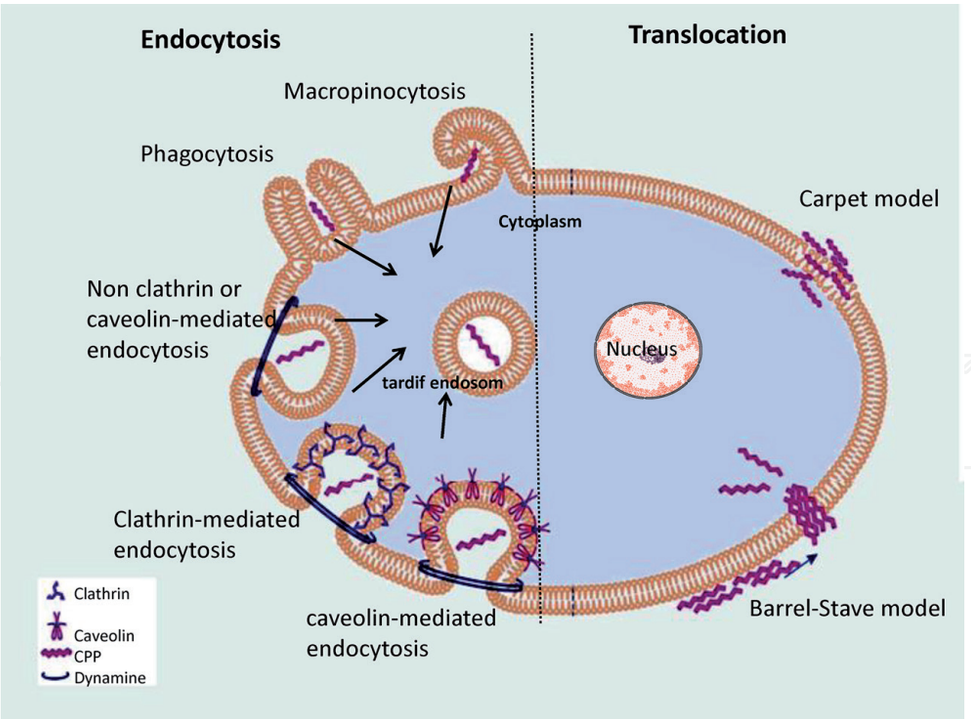

Figure 2.
CPP translocation mechanisms.

3.2 Delivery of chemotherapeutic agents

Chemotherapy used for treatment of cancer has a lot of defects because of the toxicity of the drugs to normal healthy cells and also to resistance developed by tumor cells to the anticancer drug [28]. The major inconveniences with used cancer chemotherapy are the absence of specificity target to tumor cells and thus poor antitumor effect. The challenge in cancer therapy is to know how to deliver a drug intact to the cytosol of every cancer cell, sparing healthy cells.

It was shown that polyarginines carry cargoes that exceed 500 Da by molecular electroporation across the cell membrane which may solve part of the drug delivery problem [29]. However, the use of well-chosen linkers and anions can help target cancer cells and contribute to successful conjugation process. For example, the CXC chemokine receptor 4 (CXCR4) is overexpressed in different types of cancer, including prostate, breast, colon, and small-cell lung cancer. Snyder et al. linked the CXCR4 receptor ligand, DV3, to two transducible anticancer peptides: a p53-activating peptide (DV3-TATp53C′) and a cyclin-dependent kinase 2 antagonist peptide (DV3-TAT-RxL). Treatment of tumor cells expressing the CXCR4 receptor with either the DV3-TATp53C′ or DV3-TAT-RxL targeted peptides resulted in an enhancement of tumor cell killing compared with treatment with nontargeted parental peptides [30]. Furthermore, hypoxia-inducible factor-1 (HIF), the transcription factor central to oxygen homeostasis, is regulated via the oxygen-dependent degradation domains (ODD) of its α isoforms (HIFα). The amino- and carboxyl-terminal sequences of ODD (NODD and CODD) were fused to TAT and injected into sponges implanted subcutaneously (s.c.) in mice by William et al. They demonstrated that this injection causes a markedly accelerated local angiogenic response and induction of glucose transporter-1 gene expression, thus opening additional therapeutic avenues for ischemic diseases [31].

In some cancer cells, such as melanoma (common eye cancers in adults), p53 seems to be inhibited by overexpression of HDM2. A transducible peptide that inhibits HDM2 and Bcl-2 for their ability to induce tumor-specific apoptosis in

these cells was tested [30]. In this study, it was demonstrated that the anti-Bcl-2 peptide induced apoptosis in tumor cells but also caused variable levels of toxicity in normal cells and tissues. On the contrary, the anti-HDM2 peptide induced apoptosis in tumor cells, with little effect on normal cells in a therapeutic dose range. This peptide also caused regression of retinoblastoma in rabbit eyes, with minimal damage to normal ocular tissues. They conclude that the inhibition of HDM2 may be a promising strategy for the treatment of uveal melanoma and retinoblastoma, and that strategy may be an effective technology for local delivery of anticancer therapy to the eye.

Most of the patients with sporadic renal cell carcinomas (RCCs) exhibit mutation of the Hippel-Lindau (VHL) tumor suppressor gene. Conjugation of the protein transduction domain of HIV-TAT protein to the amino acid sequence (104–123) in the beta-domain of the VHL gene product (pVHL) arrested and then reduced proliferation and invasion of 786-O renal cancer cells in vitro. Besides, daily i.p. injections with the conjugate put off and, in some cases, caused partial regression of renal tumors that were implanted in the dorsal flank of nude mice [32].

The tumor suppressor gene *p16INK4A*, an inhibitor of cdk3 4, is often inactivated via intragenic mutation, homozygous deletion, and methylation-associated transcriptional silencing in a large number of human cancers, mainly in pancreatic cancer. Treated animals with the p16-derived synthetic peptide coupled with the Antennapedia carrier sequence, in which we designated as Trojan p16 peptide, showed reduced AsPC-1 and BxPC-3 s.c. tumors, respectively. Thus, we conclude that Trojan p16 peptide system, a gene-oriented peptide coupled with a peptide vector, functions for experimental pancreatic cancer therapy [33].

Recently, it was shown by Sonia et al. that coupling doxorubicin (Dox) to three cell-penetrating peptides Tat, penetratin, and maurocalcine (Dox-CPPs) is a good strategy to overcome Dox resistance in MDA-MB 231 breast cancer cells and CHO cells (**Figure 3**) [3, 34]. We also reported that all conjugates are able to promote cell apoptosis in the breast cancer-resistant cells MDA-MB 231 at lesser concentration needed for Dox alone. Indeed, apoptosis death was shown to be correlated with ladder-internucleosomal degradation, chromatin contraction, caspase activation, Bad and Bax activation by oligomerization on the mitochondrial membrane, and liberation of cytochrome c. Despite the effective Bcl-2 overexpression in apoptosis induced by the Dox alone, such potency was shown to be insufficient in case of Dox-CPP-triggered cell apoptotic death. Otherwise, these results suggest that there are other apoptotic signaling pathways, independent of mitochondrial one, which are implicated in Dox-CPP apoptosis. Moreover, greater effectiveness of Dox when coupled to CPPs is not due only to its higher accumulation on the cells but also to the incitement of other signaling pathways. These pathways include death receptors and activation of the JNK pathway [4, 35].

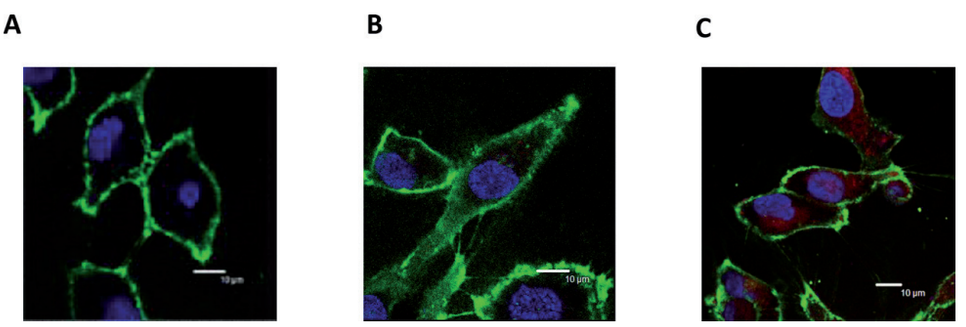

Figure 3.
Cellular internalization of Dox by MCa. MDA-MB231 cells treated with (a) RPMI, (B) Dox alone (red), and (C) Dox coupled to Dox at the same concentration (red).

Another study led by Leslie Walker et al. showed that conjugated Dox to both ELP and SynB1 prevents tumor development in mice. In fact, conjugation of Dox to SynB1-ELP was more efficient in tumor inhibition under hyperthermic condition than Dox alone, which was twofold higher. Such conception was considered hopeful peptide candidates for drug delivery [36]. The anticancer activity of Dox was also enhanced when constructed a drug delivery system by developing 25 nm gold nanospheres (GNSs) conjugated to four α-helical CPPs [37].

A thermally sensitive quantum dot that exhibits an "on-demand" cellular uptake behavior via temperature-induced "shielding/deshielding" of CPP on the surface was synthesized. Poly(N-isopropylacrylamide) (PNIPAAm) and CPP were biotinylated at their terminal ends and co-immobilized onto the surface of streptavidin-coated quantum dots (QDs-Strep) through biotin-streptavidin interaction. Namely, under a lower critical solution temperature (LCST), the hydrated PNIPAAm chains blocked CPP cellular uptake. This effect was broken down when the LCST was raised to allow CPP moieties to be exposed on the cell surface, leading to QD cellular uptake.

Additionally, the "shielding/deshielding" temperature of CPP was also used for siRNA delivery system. Biotinylated siRNA was coupled to the surface of TSQDs. Indeed, the amount of corresponding gene silencing was increased due to the surface exposure of CPP within a rising temperature above the LCST [38].

4. Optimization methods for CPP-mediated cancer therapy and diagnosis

Over the last decade, a great attention has been assigned to the importance of CPP on drug transportation of bioactive molecules in various preclinical studies. In fact, novel computational basics have been made in order to develop knowledge on CPPs [39].

Previously, different researchers have developed a few in silico algorithm approaches for CPP prediction (CPPpred) and screening to facilitate throughput CPP-based research. The in silico screening/prediction methods aimed on the use of scales of chemical characteristic, such as z-descriptors [40, 41]. It is generally followed by experimental validation to make it reliable with less cost and time-consuming approach. Later on, other CPP prediction applied neural network (NN) strategies were developed and consist on introducing an N-to-1 NN. The network proceeds by a sequence of 5 to 30 amino acids in length, as input, and gives a prediction of how probably each peptide is to be cell penetrating [42]. This CPPpred offers an advantage since it was developed with repetition-reduced training and test sets.

Over the years, the commitment therapeutic importance of CPPs motivated other teams to develop the first version of CPP database, i.e., CPPsite which supports broad information on the promising use of CPPs [43]. The CPPsite manually created database of 843 experimentally described CPPs. Each consulting gives us data of the peptide involving peptide sequence, peptide name, nature of peptide, origin, chirality, uptake efficiency, subcellular localization, etc. A deep area of user-friendly tools has been integrated in this database like analyzing and browsing tools. Moreover, they have introduced other informations concerning peptide sequences such as secondary/tertiary structure and physicochemical properties of peptides.

This database version was then developed and updated as a CPPsite 2.0 and holds 1855 entries, including 1012 recent new entries [44]. The renovated version contains further data concerning chemically modified CPPs used on the in vivo model. In addition to other informations on delivered cargoes by CPPs (proteins, molecules, nanoparticles, DNA, RNA, etc.), secondary and tertiary structures of

natural and chemical CPPs (including CPP with D-amino acids) were also predicted in view of their important role in the functionality of CPPs and stored in the database. Numerous tools for information browse and analysis are combined in this database and considered as a useful resource since it is compatible for all users, including smartphone and tablet.

CPP prediction sites are a promising assist to the researchers to design cell penetrating peptide, as well as making different modification and to investigate their effect on cell penetration potency [45].

5. Conclusion

The progressive and continuous application of CPPs shows that they are efficient delivery vectors. Because of the need to ameliorate the drug delivery, a great number of CPP-based applications are still drawing the attention of researchers.

In this review, the current tendency in drug delivery by CPPs is summed up. Conjugation with CPP increases cell-surface affinity and eventual cellular uptake of bioactive molecules.

Author details

Sonia Aroui* and Abderraouf Kenani
Unité de Recherche UR 12ES08 "Signalisation Cellulaire et Pathologies", Faculté de Médecine de Monastir, Monastir, Tunisie

*Address all correspondence to: sonia_aroui2002@yahoo.fr

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