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Review

Cell-Penetrating Peptides: From Basic Research to Clinics

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The presence of cell and tissue barriers together with the low biomembrane permeability of various therapeutics often hampers systemic drug distribution; thus, most of the available molecules are of limited therapeutic value. Opportunities to increase medicament concentrations in areas that are difficult to access now exist with the advent of cell-penetrating peptides (CPPs), which can transport into the cell a wide variety of biologically active conjugates (cargoes). Numerous preclinical evaluations with CPP-derived therapeutics have provided promising results in various disease models that, in some cases, prompted clinical trials. The outcome of these investigations has thus opened new perspectives for CPP application in the development of unprecedented human therapies that are well tolerated and directed to intracellular targets.

Trends

Many human pathologies present unmet medical needs because of the presence of cell and tissue barriers that prevent therapeutics from reaching their specific intracellular targets.

Conjugating therapeutics with peptidic delivery factors, particularly cell-penetrating peptides (CPPs), could enhance their cellular internalization and therapeutic efficacy.

Medical application of CPPs has been considered for the delivery of various types of therapeutic molecules, such as antimicrobial, anti-inflammatory, antineoplastic, and neuroprotective agents.

Since their discovery, numerous preclinical evaluations have been performed in animal models to investigate applications of CPP-derived therapeutics. Some of these peptides have entered into Phase I, Phase II, and, in some cases, Phase III clinical trials.

CPPs: A Historical Overview

The introduction of recombinant proteins into medical practice dates back to the early 1980s, when recombinant human insulin was first approved by the US FDA for the treatment of diabetes mellitus. Since then, the use of biologically active molecules – such as proteins, but also peptides and oligonucleotides – has tremendously expanded in a wide range of therapeutic areas. The advantages of these agents are several-fold as they are generally highly specific, well tolerated, and more easily transferrable to clinical development than chemical drugs. However, the hydrophilic nature of these bioactive molecules often limits their therapeutic value because of their low membrane permeability, which prevents their access to intracellular targets. An even more difficult task, for example in the specific case of neurodegenerative disorders, is to deliver such hydrophilic molecules across the **blood–brain barrier (BBB)** (see Glossary), a complex biological interface that prevents transport of most drugs from the vasculature into the parenchyma of the nervous system.

Opportunities to overcome these constraints have arisen with the discovery of the shuttling properties of specific proteins [1]. In 1988 two independent groups reported that the *trans*-activator of transcription (TAT) protein of HIV-1 could be efficiently internalized by cells *in vitro* [2,3]. A few years later, in 1991, the homeodomain of Antennapedia, a homeoprotein of *Drosophila melanogaster*, was also shown to enter the cells [4]. The acknowledgement of the entry capacity of these proteins prompted extensive structure–function investigations to identify the minimal amino acid sequence able to enter into the cell. Derossi and colleagues described the translocation properties of a short, 16-amino-acid peptide named pAntp(43–58), or penetratin, corresponding to the third helix of the Antennapedia homeodomain [5].

Truncated versions of TAT were later investigated by Vivès and colleagues, who discovered that the domain responsible for **cellular uptake** is a basic region of 13 amino acids extending

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from residue 48 to 60 ($TAT_{48–60}$) [6]. Park and collaborators further showed that the key motif for transduction could be reduced even more, to residues 49–57 ($TAT_{49–57}$) [7]. A few years after the discovery of the TAT and Antennapedia homeodomain internalization features, a plethora of peptides with translocation capacities were identified that are commonly defined as **CPPs** [8–12]. CPPs are a family of various peptides, typically comprising 5–30 amino acids, that can pass through tissue and cell membranes via energy-dependent or -independent mechanisms with no interactions with specific receptors [13].

Table 1 provides a list of the most structurally and functionally characterized CPPs, the majority of which are currently in preclinical or clinical development.

These peptides have been extensively shown to be capable of transporting into cells a wide variety of biologically active conjugates (cargoes) including proteins, peptides, DNAs, **siRNAs**, and small drugs, and thus are considered peptidic delivery factors [14]. Cargoes can be conjugated to CPPs either by covalent bonds or by non-covalent complex formation. The first

Table 1. Examples of CPPs and Their Sequences, Origins, and Physical–Chemical Properties

CPP name	Sequence	Origin	Class	Refs
HIV-1 TAT protein, $TAT_{48–60}$	GRKKRRQRRRPPQ	HIV-1 TAT protein	Cationic	[6]
HIV-1 TAT protein, $TAT_{49–57}$	RKKRRQRRR	HIV-1 TAT protein	Cationic	[7]
Penetratin, pAntp(43–58)	RQIKIWFQNRRMKWKK	Antennapedia *Drosophila melanogaster*	Cationic	[4,5]
Polyarginines	Rn	Chemically synthesized	Cationic	[10,36]
DPV1047	VKRGLKLRHVRPRVTRMDV	Chemically synthesized	Cationic	[37]
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	HIV glycoprotein 41/ SV40 T antigen NLS	Amphipathic	[43]
Pep-1	KETWWETWWTEWSQPKKKRKV	Tryptophan-rich cluster/SV40 T antigen NLS	Amphipathic	[43]
pVEC	LLIILRRRIRKQAHAHSK	Vascular endothelial cadherin	Amphipathic	[44]
ARF(1–22)	MVRRFLVTLRIRRACGPPRVRV	p14ARF protein	Amphipathic	[45]
BPrPr(1–28)	MVKSKIGSWILVLFVAMWSDVGLCKKRP	N terminus of unprocessed bovine prion protein	Amphipathic	[46]
MAP	KLALKLALKALKAALKLA	Chemically synthesized	Amphipathic	[11]
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	Chimeric galanin–mastoparan	Amphipathic	[9]
p28	LSTAADMQGVVTDGMASGLDKDYLKPDD	Azurin	Amphipathic	[47,48]
VT5	DPKGDPKGVTVTVTVTVTGKGDPKPD	Chemically synthesized	Amphipathic	[49]
Bac 7 ($Bac_{1–24}$)	RRIRPRPPRLPRPRPRPLPFPRPG	Bactenecin family of antimicrobial peptides	Amphipathic	[50,51]
C105Y	CSIPPEVKFNKPFVYLI	α1-Antitrypsin	Hydrophobic	[53]
PFVYLI	PFVYLI	Derived from synthetic C105Y	Hydrophobic	[53]
Pep-7	SDLWEMMMVSLACQY	CHL8 peptide phage clone	Hydrophobic	[54]

Glossary

Activatable cell-penetrating peptides (ACPPs): peptides comprising a polycationic CPP and a neutralizing polyanion separated by a protease cleavable linker.
Blood–brain barrier (BBB): dynamic interface that separates the brain parenchyma from the vasculature protecting the CNS from potential external damage and regulating the transport of essential molecules to maintain a stable environment.
Cell-penetrating peptides (CPPs): short peptides able to pass through tissue and cell membranes via energy-dependent or -independent mechanisms; used to transport into cells a wide variety of biologically active conjugates (cargoes).
Cellular uptake: various mechanisms for the transport of molecules into cells.
Ischemia–reperfusion (IR) injury: deleterious effects – such as formation of oxygen free radicals, calcium overload, and neutrophil-mediated myocardial and endothelial injury – induced by reperfusion therapy following ischemia.
Middle cerebral artery occlusion (MCAO): blockage of middle cerebral artery that causes oxygen deprivation in brain tissue; commonly used to create an animal model of ischemia or hypoxia.
Nuclear factor kappa B (NF-κB): multifunctional transcription factor that controls the expression of several proinflammatory and stress response mediators.
Nuclear factor kappa B (NF-κB) essential modulator (NEMO)-binding domain (NBD): selective inhibitor peptide of NF-κB; blocks the interaction of NEMO with the IκB kinase complex, preventing NF-κB activation.
Nuclear localization signal (NLS): short sequence of positively charged amino acids that mediates the nuclear import of proteins by binding to its receptors, known as importins.
Peptide nucleic acids (PNAs): synthetic homologs of nucleic acids in which the phosphate–sugar polynucleotide backbone is replaced by a flexible pseudopeptide polymer to which the nucleobases are linked.
Percutaneous coronary intervention (PCI): non-surgical procedure, commonly known as coronary angioplasty, used to treat

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strategy is used for delivering cargoes like **phosphorodiamidate morpholino oligomers (PMOs)**, **peptide nucleic acids (PNAs)**, peptides, proteins, and small drug molecules. These agents can be covalently coupled to CPPs by chemical linkage (mainly disulfide or thioester bonds) or by cloning and subsequent expression of CPP fusion proteins. However, a major risk of the covalent CPP technology is that, in some cases, it may alter the biological activity of conjugates. In these instances non-covalent strategies appear more appropriate. Non-covalent complex formation relies on electrostatic and/or hydrophobic interactions between large, negatively charged cargoes, such as oligonucleotides, and positively charged CPPs (Figure 1). The advantages of this method mainly relate to the capacity of peptides to protect the bioactive conjugates from protease or nuclease degradation, thereby increasing the serum half-life of cargoes [15–17].

Since their discovery, CPPs have been increasingly used in basic research – for example, as one of the most effective tools for transfection in various cell types – and in translational research. In the latter case, CPPs have emerged as key elements in increasing medicament concentrations in tissue and cell compartments that are difficult to access, thereby enhancing their therapeutic effectiveness. They have been exploited to deliver, in a variety of disease contexts, various types of therapeutics. These include antimicrobials, anti-inflammatory drugs, and antineoplastic and neuroprotective agents [18]. Of note, preclinical evaluations with CPP-derived therapeutics provided promising results in various disease models that, in some cases, prompted clinical trials [19–35]. The outcome of these investigations has thus opened new perspectives for CPP application in the development of unprecedented human therapies.

In this review we provide an overview of the physical–chemical properties of the various peptidic agents and describe the mechanisms that CPPs exploit to enter cells. Furthermore, we explore the potential therapeutic applications of CPPs, as highlighted by preclinical studies performed in animal models of various diseases, and discuss the ongoing CPP-based clinical trials.

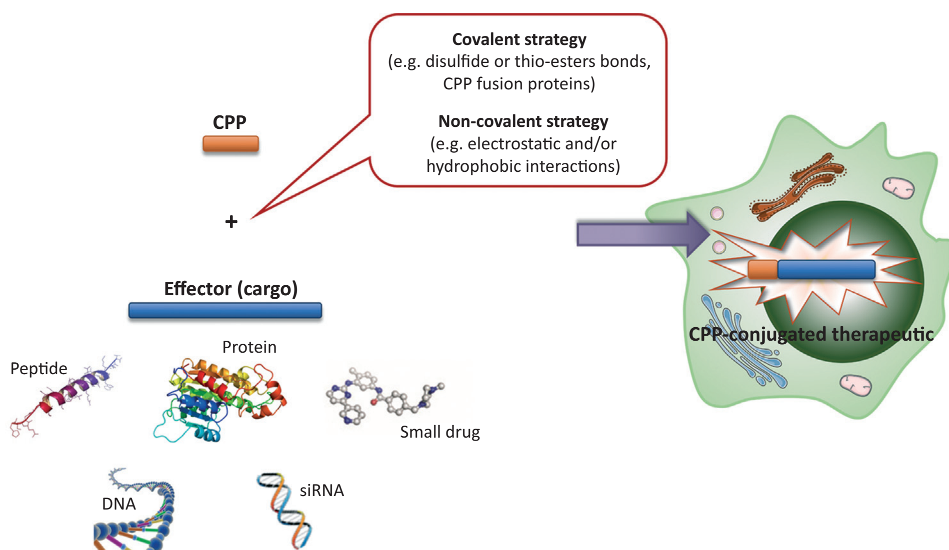

Figure 1. Schematic Drawings Representing Cell-Penetrating Peptide (CPP)-Based Technologies. The hydrophilic nature of effectors/cargoes (blue), such as peptides, proteins, nucleic acids, or small drugs, can prevent their cellular uptake and hamper their access to intracellular targets. Conjugating the effector (cargo) to a CPP (orange) by covalent bonds or noncovalent complex formation enables the CPP–effector conjugate (CPP–conjugated therapeutic) to cross the cell membrane and reach intracellular areas that are difficult to access, thereby enhancing the therapeutic effectiveness.

stenotic coronary arteries of the heart.
Phosphorodiamidate morpholino oligomers (PMOs): synthetic DNA analogs that inhibit gene expression in a sequence-dependent manner.
Small interfering RNA (siRNA): class of double-stranded RNA molecules that mediate gene silencing by degradation/blockage of translation of the target mRNA.

Classification of CPPs

Although several different criteria have been proposed for the classification of CPPs based on their origin, sequence, function, or mechanism of uptake, no unified taxonomy of these peptides presently exists. According to their physical–chemical properties, they can be simply categorized into three main classes; that is, cationic, amphipathic, and hydrophobic peptides.

Cationic Class

The cationic class comprises peptides with highly positive net charges at physiological pH that primarily originate from the basic short strands of arginines and lysines. Peptides belonging to this class include TAT-derived peptides, penetratin, polyarginines, and Diatos peptide vector 1047 (DPV1047) (Vectocell®) [5–7,36,37] (Table 1). The structural requirements for the cellular uptake of cationic CPPs have been extensively explored in multiple studies by quantitatively evaluating the ability to enter cells of a series of fluorescently labeled and truncated analogs of TAT_{49-57} as well as polyarginines (R5–R12) [36,38]. These reports showed that truncated analogs are much less able to enter cells than nonamers of arginine (R9). It was therefore proposed that the number and order of the amino acids within the peptide sequence, mostly arginines, is critical in determining the transduction properties of CPPs. These analyses further revealed that arginine residues contribute more to cellular uptake than lysines [36,38]. This was ascribed to the capacity of the guanidine head group of arginine to form bidentate hydrogen bonds with the negatively charged carboxylic, sulfate, and phosphate groups of cell membrane constituents, leading to cellular internalization of CPPs under conditions of physiological pH [39]. At variance, the amino acid lysine does not contain the guanidine head group and thereby displays a lower capacity to penetrate the plasma membrane [40].

Amphipathic Class

Amphipathic CPPs contain both polar (hydrophilic) and nonpolar (hydrophobic) regions of amino acids. Besides lysine and arginine, which are distributed throughout the sequence, they are also rich in hydrophobic residues, such as valine, leucine, isoleucine, and alanine [41]. Amphipathic peptides are generally classified into primary, secondary, and proline-rich CPPs [42]. Several primary amphipathic CPPs are chimeric peptides obtained by covalently binding a hydrophobic domain, which is essential for efficient targeting to cell membranes, to a **nuclear localization signal (NLS)**. The latter is a short cationic peptide based on lysine-, arginine-, or proline-rich motifs that target peptide cargoes into the cell nucleus through the nuclear pore complex. This CPP family includes MPG and Pep-1, two peptides that are generated by fusing HIV glycoprotein 41 or a tryptophan-rich cluster, respectively, to the NLS of the simian virus 40 (SV40) large T antigen (KKKRKV) [43] (Table 1). Other primary amphipathic CPPs originate from natural proteins. These include a peptide derived from vascular endothelial cadherin (pVEC) [44], the p14 alternative reading frame (ARF) protein-based ARF(1–22) [45], and the N-terminus of the unprocessed bovine prion protein, BPrPr(1–28) [46]. Secondary amphipathic CPPs generally exhibit a peculiar structure, assuming an α-helical conformation with hydrophilic and hydrophobic residues grouped on different sides of the helix. Examples include model amphipathic peptide (MAP) [11], Transportan [9], and the azurin-derived p28 peptide [42,47,48]. Alternatively, they can present their amino acid sequence in a β-sheet structure on interaction with a phospholipid membrane. Similarly to α-helical CPPs, amphipathic β-sheet peptides, such as VT5, comprise one hydrophobic and one hydrophilic expansion of amino acids. The ability to form β-sheets is essential for their cellular internalization, as single amino acid mutations that reduce the tendency to adopt this conformation are sufficient to decrease their cellular uptake [49]. Another interesting class of amphipathic peptides comprises proline-rich CPPs, which have been reported in various families that differ in sequence and structure but all of which contain a proline pyrrolidine template. Proline displays unusual features among the 20 genetically encoded amino acids due to the rigidity conferred by its pyrrolidine ring. Furthermore, it cannot donate a hydrogen bond (in the peptide structure) to stabilize an α-helix

or a β-sheet because of the lack of a hydrogen on the α amino group. Unlike other amino acids, which exist almost exclusively in the *trans* form in polypeptides, proline can exist in the *cis* configuration in peptides.

Examples of proline-rich peptides are synthetic fragments of Bac 7, a 59-residue antimicrobial protein with four 14-residue repeats from the bactenecin family. The dual functions of cell permeability and antimicrobial activity of Bac 7 colocalize at the N-terminal 24 residues (Bac $_{1–24}$) [50,51] (Table 1).

Hydrophobic Class

Hydrophobic CPPs mainly contain nonpolar residues, resulting in a low net charge. These hydrophobic motifs, and their high affinity for the hydrophobic domains of cellular membranes, are crucial for the process of cellular internalization. As yet only a limited number of hydrophobic peptides has been discovered and their internalization mechanisms have been poorly studied compared with the cationic and amphipathic classes. However, it has been proposed that this family of peptides could spontaneously translocate across membranes in an energy-independent manner, thereby exhibiting a behavior that differs from the other classes of CPPs [52].

Examples of hydrophobic CPPs are the C105Y peptide and its PFVYLI C-terminal portion [53] as well as the Pep-7 peptide [54] (Table 1). Besides these CPPs, which are based on natural amino acids, the hydrophobic class also includes chemically modified peptides [42]. Peptide modifications can be achieved by various strategies, such as peptide stapling to stabilize α-helices [55], prenylation to promote membrane interactions [56], and pepducin technology to modulate G protein-coupled receptor signal transduction [57,58].

Cellular Uptake Mechanisms of CPPs

Although the mechanisms for cellular internalization of CPPs have been the subject of intense investigation, the pathways involved in this process have not been fully clarified. The difficulties encountered in the comprehension of the cellular uptake of these peptides are mostly ascribed to the differing physicochemical properties, size, and concentration of the diverse CPPs and/or CPP–cargo conjugates. These features can, indeed, have significant impact on the efficiency of cellular entry [59]. Nonetheless, it has become clear that a single CPP can exploit different routes to enter the cell and that these routes may occasionally operate concomitantly, depending on the context of the experimental conditions. These entry routes are broadly divided into two groups: energy-independent direct penetration of the plasma membrane and energy-dependent endocytosis. While direct translocation across the cell membrane occurs in some cases, mainly at high concentrations of the peptide [60,61], it is generally accepted that most CPPs and CPP–cargo conjugates enter cells by endocytosis [62–64].

Direct Penetration

The process of direct penetration (or ‘membrane transduction’) has peculiar features as not only is it energy independent, but it can occur even at low temperatures and in the presence of endocytotic inhibitors. In addition, it involves multiple entry routes that are initially based on the interaction of positively charged CPPs with the negatively charged membrane components and phospholipid bilayer. This interaction is followed by peptide entrance via various mechanisms that have been described as causing transient pore formation or membrane destabilization. In the proposed transient pore formation models, CPPs were reported to shape either ‘toroidal pores’ or ‘barrel-stave pores’ within the plasma membrane. In the former case, CPPs accumulate on the outer leaflet of the phospholipid bilayer causing distortion of the membrane and leading to the formation of a transient pore [65,66]. Recent studies addressing the cellular uptake of cationic CPPs suggested a novel mechanism of internalization based on the ubiquitous interplay among guanidinium groups, fatty acids, and the plasma membrane pH

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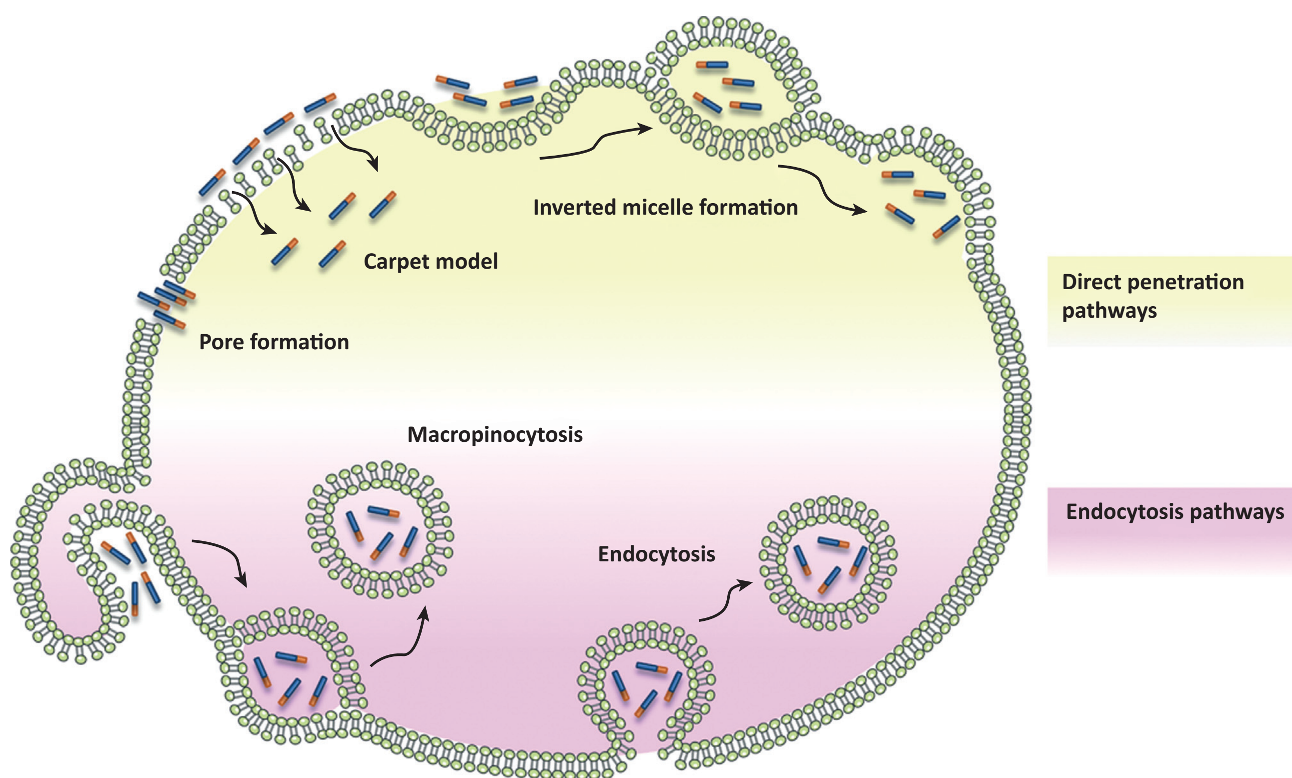
gradient. More specifically, it was proposed that, at high pH, fatty acids bind guanidinium groups of extracellular CPPs and mediate their transport across the plasma membrane by nucleating a transient toroidal pore. In contact with the lower cytosolic pH, cell membrane fatty acids release CPPs into the cells and the transient pore closes [67]. According to the barrel-stave pore model, peptides assume an α-helical structure in the membrane with their hydrophilic side chains forming the internal face of the pore. This enables the entrance of the CPPs' hydrophilic elements (Figure 2) [68]. In addition, several direct translocation mechanisms have been proposed to occur by membrane destabilization through: (i) the 'carpet-like' model [69,70], which is characterized by a transient increment in membrane fluidity following the interaction of the positively charged amino acids of CPPs with the negative charges on the membrane surface; or (ii) the 'inverted-micelle' mechanism, which is based on invagination of the phospholipid bilayer with the formation of inverted micelles encapsulating the peptide [71]. CPP translocation across the cell membrane occurs within the micelles, which release the peptide into the cytosol by inversion once they have entered the cell (Figure 2). Recent investigations have added further insights into these mechanisms by suggesting that the electrostatic interactions between CPPs and the lipid bilayer can trigger conformational alterations in the peptide followed by its incorporation into the lipid bilayer and, finally, changes of membrane physical properties [72].



Trends in Pharmacological Sciences

Figure 2. Schematic Representation of Proposed Mechanisms for Cell-Penetrating Peptide (CPP) Internalization. The diagram illustrates that the involved pathways can be divided into two groups: direct penetration of plasma membrane (yellow) and endocytic pathways (purple). The first type of process involves several energy-independent models including membrane insertion of CPPs through pore formation and membrane destabilization through the carpet-like model or inverted micelle formation. Endocytic internalization of CPPs is an energy-dependent process that comprises macropinocytosis and endocytosis.

Endocytosis

Although direct penetration was first proposed as the main mechanism of CPP internalization [5,6], it later became evident that some experimental approaches, such as cell fixation, could lead to artifactual redistribution of CPPs into the cell. This prompted reevaluation of the mechanism of CPP uptake, and data obtained from the ensuing investigations suggested that endocytosis is the principal internalization route for many CPPs and CPP–cargo complexes [73]. Endocytosis is a natural and energy-dependent process occurring in all cells. It may involve several different pathways that can be classified as macropinocytosis, clathrin- or caveolin-mediated endocytosis, and clathrin/caveolin- independent endocytosis (Figure 2) [40]. Whether one or another pathway is predominant mostly depends on the size and physicochemical nature of the cargo molecule [74]. While CPPs can be directly localized into the cytosol after translocation by non-endocytic pathways, there is evidence suggesting that peptides remain trapped in endosomes during endocytosis [18]. Therefore, after endocytic internalization CPPs and CPP–cargo complexes must escape from endosomes to the cytosol to avoid degradation from lysosomes, to reach their target sites and to exert their biological activity. Escape from endocytic vesicles appears to be the principal limiting factor in the efficient intracellular delivery of functional macromolecules [75]. The precise mechanism of endosomal escape remains elusive, although some hypotheses to explain this process have been put forward. For example, one model suggests that the CPP's positive charges can interact with the negatively charge components of the endosomal membrane. This binding causes stiffening of the membrane, determining its rupture and the release of the vesicle's contents [76]. Another paradigm evokes the importance of the pH gradient across the endosomal membrane, as acidification enhances the interaction of CPPs with the membrane and, consequently, their transduction. A third model suggests that increases in the concentration of vesicle contents might enhance endosomal escape. While several data indicate that these models may be applicable to macromolecular cargoes, such as nucleic acids transduced to cells as non-covalent complexes with amphipathic CPPs [77–79], there is evidence suggesting that they may not apply to cationic CPPs covalently bound to large cargoes, which are more likely to remain entrapped in endocytic vesicles [75,80]. Thus, various agents have been developed to improve the efficiency of endosomal escape of various CPP–cargo complexes, including endosomolytic peptides and polymers [81–83]. One approach that has shown promising results is based on the introduction into the peptide sequence of pH-sensitive domains that destabilize lipid membranes at the acidic pH of endosomes, thus facilitating the escape of CPPs from endocytic vesicles [84]. Another, similar method is based on the insertion into CPPs of histidine moieties, which adsorb protons at endosomal pH leading to an increase of osmotic pressure in the endosomal vesicle. This causes rupture of the membrane and the release of contents ('proton-sponge effect') [85]. Recently, Ülo Langel and collaborators reported other strategies to overcome the entrapment of CPP–cargo in endosomal compartments. These are based on the use of PepFects (PFs), a series of peptides originally derived from the amphipathic CPP Transportan 10 (TP10). The latter was originally altered by N-terminal stearylation, a modification that significantly promoted endosomal escape and improved delivery efficiency compared with the unmodified peptide [86]. This novel peptide, named stearyl-TP10, was used as a backbone to create other similar peptides, among which are PF6 and PF15. Since the lysosomotropic agent chloroquine (CQ) is well known to enhance endosomal escape of CPPs, PF6 was generated by covalently incorporating four CQ analogs (i.e., trifluoromethylquinoline) into stearyl-TP10 by a succinylated lysine-tree structure. This modification further increased the endosomolytic properties and the efficiency of cargo delivery compared with stearyl-TP10 [79]. PF15 was later designed using PF6 as a backbone and replacing lysine residues with ornithines to enhance the transfection efficiency [12]. The presence of CQ analogs was then critical to increase the capacity for endosomal escape [87].

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Preclinical Studies

To identify potential applications for CPP-derived therapeutics, several preclinical studies have been performed in areas of major unmet medical needs (Figure 3 and Table 2). Many of these investigations provided promising results that, in some cases, were translated to human disorders. In this review we provide some examples of prospective uses for CPP-derived peptide therapeutics in various areas of clinical interest.

One of the most common neurological disorders in humans is cerebral ischemia, in which the cerebral blood flow is hindered by the obstruction of an artery causing oxygen deprivation in brain tissue and, consequently, neuronal cell death. Early investigations revealed that an increase in the antiapoptotic proteins Bcl-2/Bcl-X_L in rodent brains was associated with enhanced resistance to ischemic injury [88–90]. This observation inspired Cao and colleagues to evaluate the therapeutic potential of Bcl-X_L in a mouse model of transient focal cerebral ischemia produced by **occlusion of the middle cerebral artery (MCAO)**. To this end they generated a PTD-hemagglutinin (HA)–Bcl-X_L fusion protein in which Bcl-X_L was conjugated to the protein transduction domain (PTD) $TAT_{47–57}$ (YGRKKRRQRRR). Intraperitoneal administration of PTD-HA–Bcl-X_L before the onset of ischemia reduced the infarct volume in a dose-dependent manner and improved neurological scores in mice. Furthermore, delayed treatment with PTD-HA–Bcl-X_L continued to reduce the infarct volume when administered shortly after the completion of the ischemic event [21]. Consistent with these data, another group of investigators confirmed a neuroprotective effect *in vivo* for a mutant form of the Bcl-X_L protein conjugated with $TAT_{47–57}$ (referred to as PTD–FNK) after intraperitoneal injection before ischemia [91].

Since c-Jun N-terminal kinase (JNK) was reported to be involved in mediating neuronal cell death during ischemia, another group of investigators addressed the impact of a peptide inhibitor of JNK in two animal models of MCAO. This peptidic molecule, comprising the 20-amino-acid JNK-binding motif (JBD_{20}) of JNK-interacting protein-1/islet-brain 1 fused to

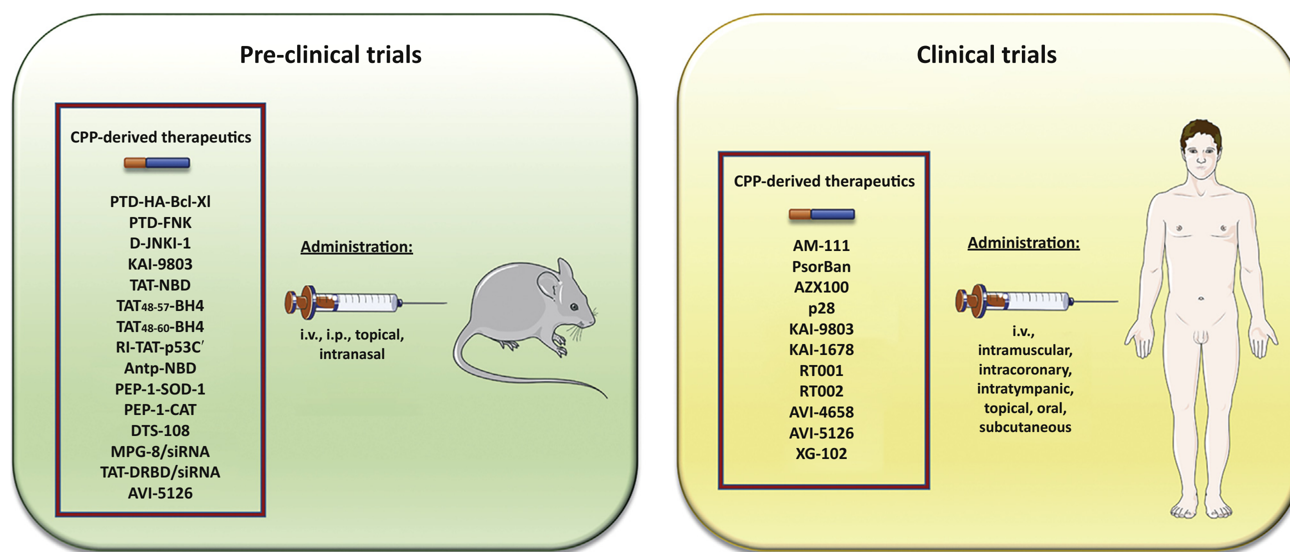

Figure 3. Schematic Representation of Preclinical and Clinical Evaluations of Some Cell-Penetrating Peptide (CPP)-Derived Therapeutics. Numerous studies have been performed to investigate the therapeutic applications of various CPPs both in animal models of various diseases and in humans. Administration of CPP-derived therapeutics can be undertaken through relatively noninvasive administration routes, such as intravenous (i.v.), intraperitoneal (i.p.), intranasal, topical, intramuscular, *per os*, intracoronary, intratympanic, and subcutaneous.

Table 2. Recently Proposed Therapeutic Applications for CPP–Cargo in Animal Models of Various Diseases

CPP–*cargo*	Animal model	Therapeutic use	Refs
PTD-HA–*Bcl-X*$_L$	MCAO in mice	Cerebral ischemia	[21]
PTD–*FNK*	Cerebral ischemia in gerbils	Cerebral ischemia	[91]
TAT–*JBD*$_{20}$ (D-JNKI-1)	MCAO in mice	Cerebral ischemia	[92]
TAT–*δPKC inhibitor* (KAI-9803)	MCAO in rats	Cerebral IR injury	[94]
TAT–*NBD*	Infection-sensitized HI brain injury in neonatal rats	Perinatal infection in HI brain injury	[32]
TAT$_{48–57}$–*BH4*	hSOD1^{G93A} mice	ALS	[28]
TAT–*JBD*$_{20}$ (D-JNKI-1)	TgCNRD8 mice	AD	[30]
Antp–*NBD*	*mdx* mice *utrn*$^{-/-}$;*mdx* mice	DMD	[29,24]
PEP-1–*SOD1* PEP-1–*CAT*	Myocardial IR injury in rats	Myocardial IR injury	[101,102]
TAT$_{48–60}$–*BH4*	Myocardial IR injury in mice	Myocardial IR injury	[103]
RI-TAT–*p53C′*	Terminal peritoneal carcinomatosis/lymphoma in mice	Cancer	[105]
DPV1047–*SN38* (DTS-108)	Healthy beagle dog Xenografted tumor in mice and rats	Cancer	[35]
MPG-8/*siRNA*	Xenografted tumor in mice	Cancer	[107]
TAT-DRBD/*siRNA*	Intracranial glioblastoma cancer in mice	Cancer	[110]
(R-Ahx-R)$_4$–*PMO* (AVI-5126)	Corneal transplantation in rats	Corneal transplant rejection	[34]

hSOD1^{G93A}, human copper, zinc superoxide dismutase G93A; p53C′, peptide derived from C terminus of p53; SN38, active metabolite of chemotherapeutic agent irinotecan; (R-Ahx-R)$_4$, arginine-rich peptide.

TAT$_{48–57}$ (GRKKRRQRRR) (D-JNKI-1), emerged as a potent neuroprotectant *in vivo* with a remarkably long therapeutic window and a strong effect on both functional outcome and lesion size [92].

There is evidence suggesting that, in some cases, the injury initially caused by ischemia may be exacerbated by post-ischemic reperfusion. An event that has been implicated in ischemia–reperfusion (IR) damage in multiple organs is the activation of delta protein kinase C (δ-PKC) [19,93]. This evidence prompted the investigation of the role of the δ-PKC isozyme in brain **IR injury** using, in rats with MCAO, the δ-PKC-selective inhibitor peptide δV1-1 conjugated by a disulfide bond to TAT$_{47–57}$ (KAI-9803). After intra-arterial or intraperitoneal administration, the peptide significantly reduced the brain infarct area in animals treated at the onset or after the onset of reperfusion, although it did not confer protection when it was administered before the ischemic period. Furthermore, delivery of the peptide reduced the neurological deficit in animals after MCAO injury [94].

Hypoxic–ischemic (HI) brain injury is an important cause of neonatal mortality and neurological disabilities. The occurrence of this event seems to be conditioned by the exposure of the immature brain to inflammatory stimuli during fetal and early postnatal life. In addition, inflammation of the central nervous system (CNS) can by triggered by systemic infections arising at any time during pregnancy or neonatal life [95,96]. Since the transcription factor **nuclear factor kappa B (NF-κB)** was implicated in microglial activation in this pathological condition, Yang and colleagues investigated the therapeutic potential of interfering with NF-κB activity. They examined the effect of intranasal delivery of a NF-κB peptide inhibitor comprising the

NF-κB essential modulator (NEMO)-binding domain (NBD) fused to $TAT_{47–56}$ (YGRKKRRQRR) (TAT–NBD) in two animal models of neonatal infection-sensitized HI. Administration of the peptide markedly attenuated NF-κB signaling, microglial activation, and brain damage triggered by HI in both models [32].

Besides its implication in ischemia, the antiapoptotic $Bcl\text{-}X_L$ protein is also involved in amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder affecting the motor system. In this pathological context, our laboratory investigated the effect of the Bcl-2 homology domain 4 (BH4) of $Bcl\text{-}X_L$ in counteracting the process of neural cell death. We found that BH4 fused to $TAT_{48–57}$ ($TAT_{48–57}$–BH4) was able to reduce cell death *in vitro* by regulating the efflux of intracellular calcium. Furthermore, chronic treatment with $TAT_{48–57}$–BH4 prevented neural cell death and improved both motor performance and survival of ALS transgenic mice [28].

Another neurodegenerative disorder that has been considered for treatment with CPP-derived peptide therapeutics is Alzheimer's disease (AD). AD involves, as a first feature, synaptic dysfunction, which causes synaptopathy and neuronal cell death ultimately leading to cognitive impairment. In the TgCNRD8 mouse model of AD, Sclip and colleagues investigated the pathway leading to synaptopathy, and showed that JNK signaling was activated in the postsynaptic compartment before the onset of the disease. Thus, they chronically treated TgCNRD8 transgenic mice with the cell-penetrating JNK inhibitor D-JNKI-1. This treatment completely reverted spine alterations, preventing biochemical alterations of postsynaptic density and abolishing the postsynaptic activation of the apoptosis effector caspase-3. Furthermore, D-JNKI-1 reverted the morphological alterations of the spines, promoting structural rescue of excitatory synapses [30].

Together these studies suggest that the abovementioned CPP-conjugated peptide therapeutics can cross the BBB and penetrate the brain parenchyma, thereby reaching their therapeutic targets.

Some preclinical trials have been conducted on another neuromuscular pathology, Duchenne muscular dystrophy (DMD). This is a deadly genetic disease caused by the absence of the dystrophin protein and characterized by progressive weakness of skeletal muscles. Because activation of NF-κB signaling was reported in DMD patients and was shown to promote disease progression in the dystrophin-deficient *mdx* genotypic mouse model of DMD [97,98], NF-κB was proposed as a possible molecular target for therapeutic intervention in this disorder. Peterson and colleagues thus assessed the efficacy *in vivo* of a peptide inhibitor of NF-κB comprising NBD fused to the Antennapedia PTD (Antp–NBD). *mdx* mice treated intraperitoneally with this fusion peptide showed improvement in motor performance and partial rescue of diaphragm contractile capacity [29]. The same group of researchers then investigated whether treatment with the Antp–NBD peptide was able to improve cardiac function in addition to diaphragm function in mice lacking dystrophin and its homolog utrophin ($utrn^{-/-}$;*mdx*). The outcome of this trial revealed an improvement in both the active force development of cardiac muscles and the frequency-dependent behavior of muscles. However, no variations were observed in the histological features, such as inflammation markers or collagen content [24].

Other CPP-derived peptide therapeutics were conceived and developed for the treatment of cardiac diseases, including acute myocardial infarction (AMI). AMI is one of the primary causes of mortality and is triggered by an interruption in the bloodstream to the myocardium that leads to the loss of cardiomyocytes, causing heart failure. The tissue injury initiated during the ischemic episode can be continued by reperfusion. The latter is necessary to limit the infarct size, improving functional myocardial recovery and increasing survival. However, similar to cerebral ischemia, reperfusion can also induce multiple deleterious effects, globally defined as

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IR injury. For example, re-entry of oxygenated blood into ischemic tissue can result in the formation of reactive oxygen species (ROS) that lead to further cardiomyocyte death [99]. Under physiological conditions, the antioxidant enzymes copper, zinc superoxide dismutase (SOD1) and catalase (CAT) protect cells from oxidative damage, removing the ROS. However, the activity of these enzymes is severely reduced after IR [100]. Previous studies reported that two fusion proteins comprising the cell-penetrating peptide Pep-1 linked to SOD1 (Pep-1–SOD1) or CAT (Pep-1–CAT) were individually able to transduce the myocardium after intraperitoneal administration and to protect it against IR-induced damage [101,102]. Furthermore, combined use of Pep-1–SOD1 and Pep-1–CAT had a greater effect than the individual peptides, reducing myocardial markers of injury and increasing expression of the antiapoptotic protein Bcl-2. Pep-1–SOD1 and Pep-1–CAT cooperatively protected the heart against IR injury by removing ROS and reducing myocardial apoptosis, thus limiting the infarct size and improving functionality [102]. Another group of researchers evaluated the capacity to inhibit apoptosis of a fusion peptide comprising the BH4 of Bcl-X_L coupled to various CPPs, including TAT_{48-60} (TAT_{48-60}–BH4), in a murine model of IR. They found that a single intravenous injection of a low dose of TAT_{48-60}–BH4, at the onset of reperfusion, drastically reduces infarct size and prevents apoptosis in the left ventricle. These cardioprotective effects were lost when the peptide was administered after the onset of reperfusion, making critical the time window for drug application [103].

Since traditional chemotherapy usually lacks specificity and may result in toxicity, CPPs have also been used to develop novel approaches for the treatment of oncological pathologies. Major attention was focused on the protein p53, which is responsible for cell cycle arrest and apoptosis following oncogenic stress. Malignant advancement depends on the loss of p53 function due to mutations of the gene encoding the protein itself, which are present in various human cancers, or through impairments in the signaling pathways interacting with p53 [104]. Thus, to restore the endogenous proapoptotic activity of p53 in tumor cells, Snyder and collaborators developed a transducible and proteolytically stable peptide, named RI-TAT–p53C′. This compound comprises a retroinverso D-isomer peptide derived from the C-terminal regulatory domain of p53 linked to TAT_{47-57}.The C-terminus of p53 is able to induce apoptosis by activating wild-type p53 and by restoring the transcriptional *trans*-activating function of at least some mutant p53 proteins. Systemic delivery of the RI-TAT–p53C′ peptide in preclinical terminal peritoneal carcinomatosis and peritoneal lymphoma models resulted in significant increases in lifespan and the generation of disease-free animals [105].

Various types of cancer are treated with the chemotherapeutic agent irinotecan, a prodrug that following administration is converted into its active metabolite SN38 by the action of liver carboxylesterases. Although SN38 has a greater efficacy than irinotecan, it cannot be administered directly due to its high insolubility [106]. Meyer-Losic and colleagues thus generated DTS-108, a new water-soluble compound comprising SN38 linked to a highly charged oligopeptide of human origin, named DPV1047 (Vectocell®) [37]. Studies in dogs showed that DTS-108 liberates significantly higher levels of free SN38 than irinotecan after intravenous infusion without causing gastrointestinal toxicity. Furthermore, a dose-dependent improvement in the antitumoral efficacy of DTS-108, as evaluated in mouse and rat models of colon, mammary, and lung human tumors, was also observed compared with irinotecan [35].

An optimized and shorter version of the amphipathic peptide carrier MPG, called MPG-8 (AFLGWLGAWGTMGWSPKKKRK), was developed by Crombez and coworkers. This novel CPP forms stable nanoparticles with siRNA thereby improving its delivery *in vivo*. Such an approach was exploited to target cyclin B1, a regulatory protein involved in mitosis and exhibiting altered expression in various forms of cancer. The efficacy of local MPG-8-mediated administration of cyclin B1 siRNA in preventing tumor growth was then investigated in

xenografted tumor mouse models. To improve the bioavailability and stability of the MPG-8/siRNA particles and to render them more suitable for systemic administration, the surface of these particles was functionalized with a cholesterol moiety. The functionalized compound was injected intravenously in mice bearing xenografted tumors and a significant reduction in tumor size was observed [107]. An alternative approach to efficiently deliver siRNA for therapeutic purposes in cancer models was developed by the group of Steven F. Dowdy. They generated a TAT fusion protein with a double-stranded RNA-binding domain (TAT–DRBD) that binds siRNAs with high avidity and serves as an excellent vehicle for siRNA delivery [108,109]. The TAT–DRBD system was used to deliver epidermal growth factor receptor (EGFR) and AKT serine/threonine kinase 2 (Akt2) siRNAs into intracranial glioblastoma cancer mouse models to induce synthetic lethal RNAi responses that significantly enhance longevity [110].

Another field in which CPPs can be used is the treatment of transplant rejection; for example, in corneal transplantation. A key factor in graft rejection and immunomediated damage after transplantation is the proto-oncogene c-Myc, which is expressed in various tissues such as lung, liver, and cornea. In a rat corneal transplantation model, Hosseini and collaborators investigated the ability of a novel compound, named AVI-5126, to prevent corneal rejection when administered before or after transplantation. AVI-5126 is a peptide comprising a c-Myc antisense PMO linked to an arginine-rich CPP $(R\text{-}Ahx\text{-}R)_4$. They observed that graft rejection was significantly delayed when graft corneas were maintained before transplantation in a solution containing AVI-5126 followed by topical application of the compound post-transplantation [34] (Table 2).

Clinical Applications of CPPs

The significant achievements in the preclinical evaluation of various CPP-derived peptide therapeutics, during the past decades, have revealed a remarkable potential for clinical application. Thus, several pharmaceutical companies have undertaken the clinical development of CPPs for local and systemic administration of various therapeutic molecules (Figure 3 and Table 3).

Among CPP-derived therapeutics successfully tested in animal models, KAI-9803 is one that has moved to the clinical stage. Since this selective δ-PKC inhibitor exhibited antiapoptotic properties in animal models of MCAO [94] and AMI [93], KAI Pharmaceuticals (incorporated into Amgen, Inc.) decided to evaluate this compound in the Direct Inhibition of δ-Protein Kinase C Enzyme to Limit Total Infarct Size in Acute Myocardial Infarction (DELTA-MI) Phase II clinical study. This clinical trial, completed in 2011, was intended to investigate the safety, tolerability, and activity of different KAI-9803 doses administered by intracoronary injection during primary **percutaneous coronary intervention (PCI)** in patients with acute ST-segment elevation myocardial infarction. This peptide exhibited an acceptable safety and tolerability profile. Furthermore, signs of drug activity with KAI-9803 were suggested by trends for consistent nonsignificant reductions in creatine kinase MB, a biomarker for myocardial cell death. Patients receiving the peptide displayed faster recovery of ST-segment elevation near baseline and smaller infarct size. KAI-9803 may therefore represent a novel myocardial protection approach to optimize the results of reperfusion therapy by primary PCI (NCT00785954) [19]. KAI Pharmaceuticals initiated three Phase II clinical trials with KAI-1678, an inhibitor of ε-PKC conjugated to $TAT_{47–57}$. Among the various isoforms of PKC, ε-PKC was identified in the neuronal system involved in pain processing. The objective of these clinical studies was therefore to evaluate the safety and the analgesic properties of subcutaneous administration of KAI-1678 in the treatment of neuropathic pain associated with post-herpetic neuralgia (NCT01106716), spinal cord injury (NCT01135108), or postoperative pain (NCT01015235). All of these trials were completed in 2011 but only results regarding the first clinical trial mentioned above are currently available. The peptide was safe and well tolerated by patients, although it

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Table 3. Examples of CPP-Conjugated Therapeutics Under Clinical Development

Pharmaceutical organization	Compound	CPP-*cargo*	Therapeutic use	Status	ClinicalTrials.gov ID	Refs
Auris Medical	AM-111	TAT–*JBD_{20}* (D-JNKI-1)	Hearing loss	Phase II completed 2014	NCT00802425	[113]
				Phase III recruiting 2015	NCT02561091 NCT02809118	N/A
CellGate, Inc.	PsorBan®	R7 *-cyclosporin A*	Psoriasis	Phase IIb discontinued 2003	N/A	[114,115]
Capstone Therapeutics	AZX100	PTD_4–*HSP20 phosphopeptide*	Scar prevention/ reduction	Phase IIa completed 2012	NCT00451256 NCT00892723 NCT00811577	[27]
CDG Therapeutics, Inc.	p28	*p28*	Cancer	Phase I completed 2014	NCT00914914	[31]
KAI Pharmaceuticals	KAI-9803	TAT–*δPKC inhibitor*	Myocardial infarction	Phase II completed 2011	NCT00785954	[19]
	KAI-1678	TAT-*εPKC inhibitor*	Pain: postherpetic neuralgia, spinal cord injury, postoperative	Phase II completed 2011	NCT01106716 NCT01135108 NCT01015235	[23]
Revance Therapeutics, Inc.	RT001	MTS–*botulinum toxin A*	Lateral canthal lines Crow's feet Facial wrinkles	Phase II completed 2013	NCT01064518 NCT00968942 NCT00968825 NCT00884234 NCT01124552 NCT01124565 NCT00907387 NCT00888914 NCT01940991	[20,116]
	RT002	TransMTS® *-botulinum toxin A*	Glabellar lines	Phase I/II completed 2016	NCT02303002	[117]
			Cervical dystonia	Phase II recruiting	NCT02706795	N/A
Sarepta Therapeutics	AVI-4658	N/A	Duchenne muscular dystrophy	Phase I/II completed 2010 and 2015	NCT00159250 NCT00844597	[22,26]
				Phase III recruiting	NCT02255552	N/A
	AVI-5126	$(R\text{-}Ahx\text{-}R)_4$–*PMO*	Cardiovascular disease Coronary artery bypass	Phase II discontinued 2009	NCT00451256	[120]
Xigen SA	XG-102	TAT–*JBD_{20}* (D-JNKI-1)	Inflammation	Phase I completed 2012	NCT01570205	[25]
			Intraocular inflammation and pain	Phase III completed 2016	NCT02235272	[111,33]

R7, polyarginine, *n* = 7.

did not improve clinical pain scores compared with placebo. However, according to the authors the results should be interpreted with caution because the trial had several limitations, such as a small number of participants, possible subtherapeutic doses, and inability to demonstrate that the peptide selectively inhibits ε-PKC because of the lack of pharmacodynamic markers. Finally, it was suggested that chronic treatment with KAI-1678 may be necessary for manifestation of therapeutic effects [23].

In the context of pain and inflammation, Xigen SA moved into clinical development XG-102 (previously known as D-JNKI-1), a CPP inhibitor of JNK that was already shown to have potential for the treatment of intraocular inflammation in rat models of uveitis [33,111]. In 2012 a Phase I clinical trial with XG-102 was completed. The primary objectives of this study were to evaluate the safety, tolerability, and pharmacokinetics following single intravenous administration of different doses to healthy male volunteers. The incidences of adverse effects in subjects who received XG-102 or placebo were similar. No trends suggesting a relationship with XG-102

dose were apparent for any adverse effect (NCT01570205) [25]. Afterward, the compound was evaluated in a Phase III clinical study to investigate its ability to reduce intraocular inflammation and pain in patients undergoing cataract surgery. The results of this study, completed in 2016, are expected to be published soon (NCT02235272).

Auris Medical employed XG-102, formulated in a biocompatible and fully biodegradable gel (AM-111), for the treatment of acute sensorineural hearing loss caused by cochlear injury, based on findings in animal models [112]. In a Phase II clinical trial concluded in 2014, AM-111 was administered as a single-dose intratympanic injection into the middle ear. From there, the drug can diffuse through the round window membrane into the cochlea. AM-111 and the intratympanic injections were well tolerated. Furthermore, the drug showed statistically significant, clinically relevant, and persistent improvements in hearing and speech discrimination and greater tinnitus remission compared with placebo in patients with severe-to-profound hearing loss (NCT00802425) [113]. The Phase III clinical development program for AM-111 includes two trials that are currently ongoing (NCT02561091, NCT02809118).

Other clinical evaluations with CPP-derived peptide therapeutics were developed for the treatment of dermatological diseases. The first compound that entered a clinical trial was a cyclosporine–polyarginine conjugate (PsorBan®; CellGate, Inc.) for the topical treatment of psoriasis by transdermal delivery of cyclosporine A (CsA). Attempts at topical application of CsA in a range of inflammatory skin disorders had previously proven ineffective because of the poor absorption. However, this problem was overcome by conjugating CsA to a polyarginine [114]. In Phase I clinical studies of healthy volunteers, PsorBan® penetrated the skin and retained biological activity. The compound was thus entered into Phase IIa, showing potential benefit in patients with mild-to-moderate psoriasis without the adverse effects associated with systemic administration of cyclosporine. However, despite the efficient uptake, the release of the free drug was not rapid enough to compete with clearance, so the subsequent clinical studies (Phase IIb) were interrupted in 2003 [115].

Capstone Therapeutics developed AZX100, a compound comprising a phosphorylated peptide analog of heat shock protein 20 (HSP20) linked to PTD_4 (YARAAARQARA), an optimized version of $TAT_{47–57}$. Because preclinical testing of AZX100 revealed its potential to reduce excessive scarring and fibrotic disorders [27], the company started Phase IIa clinical trials to evaluate the safety and efficacy of the compound in reducing post-excision keloid scars and trocar site scars in patients undergoing arthroscopic shoulder surgery (NCT00451256, NCT00892723, NCT00811577). Study results have not yet been published.

Revance Therapeutics, Inc. developed a compound, named RT001, for the topical treatment of lateral canthal lines, crow's feet, and facial wrinkles. This molecule comprises two parts, a 150 kDa-botulinum toxin type A (BoNTA) molecule and a macromolecule transport system (MTS) comprising a positively charged lysine-rich central peptidic domain situated between two $TAT_{49–57}$ domains to enable large molecules to cross the skin [18]. Phase II clinical trials confirmed the safety and efficacy of RT001 in penetrating the skin and delivering BoNTA with temporary blockage of neuromuscular synaptic transmission and paralysis of facial muscles lateral to the eye (NCT01064518, NCT00968942, NCT00968825, NCT00884234, NCT01124552, NCT01124565, NCT00907387, NCT00888914, NCT01940991) [20,116]. However, in Phase III clinical trials the topical gel preparation of RT001 did not achieve its co-primary and other endpoints. Therefore, Revance Therapeutics, Inc. decided to develop a new, injectable formulation of BoNTA generated using the proprietary TransMTS® carrier-peptide delivery system (RT002). This compound was meant to be a long-acting neurotoxin. In 2016 a Phase I/II clinical trial of RT002 for the treatment of glabellar lines was concluded and confirmed its safety and efficacy with an extended duration of action (NCT02303002) [117]. The

clinical development program for RT002 also includes a currently ongoing Phase II study of the treatment of cervical dystonia (NCT02706795).

Clinical trials with CPP-derived therapeutics have also been conducted on neuromuscular degenerative diseases. Sarepta Therapeutics, for example, has developed a set of arginine-rich CPPs conjugated with PMOs, some of which were tested in DMD. Progressive muscle degeneration in DMD is due to frame-shift mutations disrupting the open reading frame of the gene encoding dystrophin, which compromises its expression. Chemically modified oligonucleotides such as PMOs have been used to modify splicing and induce exon skipping [118,119], thus restoring the open reading frame and, consequently, the production of the functional protein. Kinali and coworkers studied the safety and efficacy of escalating doses of intramuscular administration of AVI-4658, a CPP–PMO designed to skip exon 51 in the dystrophin mRNA in DMD patients [26]. This Phase I/II clinical study revealed the safety of the compound and its ability to induce the expression of dystrophin locally in treated muscles (NCT00159250). This proof-of-concept study led to a Phase I/II clinical trial, concluded in 2015, in ambulant DMD patients, assessing the safety and efficacy of repeated doses of systemic intravenous AVI-4658 (NCT00844597). Even in this case, the compound was well tolerated. Furthermore, AVI-4658 resulted in significant restoration of dystrophin expression and in a dose-dependent reduction of inflammatory infiltrates in muscles, where the protein was restored [22]. A Phase III clinical trial was consequently started and patients are currently being recruited (NCT02255552).

Another arginine-rich CPP conjugated with PMO, developed by Sarepta Therapeutics, is AVI-5126, directed against c-Myc and previously tested in animal models to prevent graft rejection following corneal transplantation [34]. In a Phase II clinical trial, researchers evaluated whether immersion of the saphenous vein in AVI-5126 solution, before transplantation in heart bypass graft surgery, could prevent graft failure after 1 year [120]. This study was discontinued in 2009 because efficacy would not have been achieved (NCT00451256).

Clinical evaluations of CPP-derived peptide therapeutics were also developed for the treatment of cancer. CDG Therapeutics isolated p28, a 28-amino-acid peptide derived from the bacterial protein azurin. After penetration of cancer cells, this peptide enters the nucleus where it binds a region within the DNA-binding domain of the tumor suppressor protein p53, inhibiting its degradation. This causes an intracellular increase of p53 that leads to inhibition of the cell cycle, preventing cancer cell proliferation. Following promising preclinical results [121], p28 entered a Phase I clinical trial to test for possible adverse effects and the maximum tolerated dose for intravenous administration in patients with p53-positive solid tumors resistant to standard methods of treatment. This compound was well tolerated with no immunogenicity or severe adverse effects. Furthermore, it appeared to be effective in patients with refractory disease who had refused prior treatments (NCT00914914) [31].

Concluding Remarks

Data obtained from multiple preclinical and clinical trials have clearly demonstrated the ability of CPPs to transport therapeutic molecules of various types across cell and tissue barriers, thereby allowing them to reach their targets. However, it seems that further experimental studies are needed to elucidate some critical aspects of CPP-mediated delivery (see Outstanding Questions).

A drawback of using CPPs in clinical settings is their general lack of cell and tissue specificity. The cellular uptake mechanism of CPPs has not been fully elucidated. There is a consensus that the first contact between CPPs and the cell surface occurs through electrostatic interactions between the peptide and membrane components. Nonetheless, it has been postulated that

Outstanding Questions

The approaches used to study CPP uptake are often based on evaluation of the total peptide internalized, using fluorescent or radioactive labels. How can cytosolic, vesicular, and nuclear localization be distinguished to discern the different pathways involved in the process of internalization?

What methodological advances will increase selective cell and tissue drug delivery by CPPs?

Which approaches can be used to select the drug delivery system to target not only specific tissues or the cell cytosol but also subcellular compartments?

What are the advantages of small CPPs in delivering peptide therapeutics? Is there a direct proportionality between the length of each CPP and its toxicity?

What are feasible methods to increase CPP stability in the extracellular environment?

What are the best linker strategies to permit the release of the cargo from the CPP after cellular internalization?

What are the available effective and nontoxic agents able to facilitate the endosomal escape of CPP–cargo complexes?

CPP internalization could be limited to certain cell types and may depend on cell-specific membrane constituents or lipid composition [122]. In addition, it has been reported that only moderate CPP translocation can occur through tight junction-forming epithelial cell layers under physiological conditions [123–125], whereas CPP uptake could be enhanced in inflammatory circumstances, when tight junctions are compromised [126]. Thus, it is reasonable to postulate that greater understanding of the internalization mechanisms exploited by these peptides may facilitate the design of CPPs capable of targeting specific cell types or tissues. The direct consequence of this achievement would be the optimization of their therapeutic efficacy and, possibly, the reduction of the toxic side effects caused by nonspecific actions after systemic administration. Because each tissue expresses specific markers, such as proteins or receptors, on its vasculature (called 'vascular bed-specific zip codes'), one possibility to improve the specificity of CPP-derived therapeutics was proposed to be the insertion into CPP–cargo complexes of homing peptide ligands, which are meant to target tissue- or cell-specific receptors, thereby enabling selective drug delivery. A similar approach can also be exploited to address CPP-derived therapeutics toward intracellular organelles, such as mitochondria, lysosomes, the Golgi apparatus, or the nucleus [127].

A groundbreaking advance to improve CPP specificity was achieved by the recent development of **activatable CPPs (ACPPs)**, which comprise peptides whose adsorption and cellular uptake is minimized by a covalently attached inhibitory domain. Cleavage of the linker connecting the inhibitory and CPP moieties by tissue-specific proteases dissociates the inhibitory domain, thereby enabling the cleaved ACPP to enter cells. This strategy has been mainly used in tumor-affected tissues, which are characterized by a specific microenvironment with upregulated proteases, acidic pH, lower transmembrane potential, and hypoxia. These elements can be exploited to selectively activate ACPPs [128]. In 2009 the group of the Nobel Prize winner Roger Y. Tsien developed the first protease ACPP able to target many xenograft tumor models from various cancer sites as well as a transgenic model of spontaneous breast cancer. Membrane-bound and secreted proteases of cancer cells – mostly matrix metalloproteases – can cleave the linker between the polycationic CPP and the polyanionic neutralizer thereby activating the CPP. After activation the released peptide can transport cargoes into tumor and metastatic cells [129,130].

Beside specificity, another important element that should be carefully considered is the short blood plasma half-life of CPPs administered *in vivo*. Indeed, extracellular proteases may degrade the delivery carriers before they reach the target site, thus reducing the efficacy of the conjugated drugs. Greater stability can be achieved by using non-natural amino acids or D enantiomers that are less sensitive to enzyme degradation than the L form [68]. CPP–cargo complexes that are internalized by endocytosis can also be subject to degradation by acidic pH as they traffic into late endosomes or lysosomes. Thus, it should be considered that endosomal escape must be highly efficient to facilitate the early release of therapeutic cargoes from endosomes into the cytosol [131]. Although some obstacles remain to be overcome before CPPs can be generally used in the generation of novel therapeutics, it is clear that CPP techniques have greatly improved and the development of CPP-conjugated peptide therapeutics in human therapies appears increasingly feasible.

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