Therapeutic applications of the cell-penetrating HIV-1 Tat peptide

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Over the past decades, many new therapeutic approaches have been developed for several conditions, including neurodegenerative diseases. However, efficient biodistribution and delivery at biological target sites are hampered by the presence of cell and tissue barriers, and a clinical therapy is prevented by the requirement of invasive administration routes. Candidate drug conjugation to cell-penetrating peptides, which are able to cross cellular membranes and reach biological targets even when administered systemically, represents a promising tool to overcome this issue. Here, we review the biology, classification and mechanisms of internalization of cell-penetrating peptides. We focus our attention on the cell-penetrating peptide: HIV-derived Tat peptide, and discuss its efficient but controversial use in basic, preclinical and clinical research from its discovery to the present day.

Introduction

Many human diseases, including neurodegenerative disorders, are currently incurable. New therapeutic approaches able to correct identified causative genetic defects or early pathogenic mechanisms are strongly needed. The presence of cell and tissue barriers, such as the blood–brain barrier (BBB) in the specific case of neurodegenerative diseases, represents a real drawback for systemic drug delivery, precluding the ability of therapeutic molecules to reach their own targets. A promising strategy to increase tissue biodistribution of therapeutics is represented by their conjugation with cell-penetrating peptides (CPPs) derived from proteins that are able to cross biological membranes. CPPs would be able to bear different therapeutic molecules, conveying them to their specific target and increasing their concentration in difficult-to-access tissues. Consequently, their therapeutic efficiency might also be augmented. This approach has the potential to revolutionize the treatment of a wide spectrum of human disorders.

One of the most promising and most studied CPPs is the HIV-1 transactivator of transcription peptide (pTat). pTat can be efficiently linked to different potential therapeutic molecules, including small molecules and antibodies, peptides, liposomes, nanoparticles and nucleic acids; it represents an extremely powerful tool to increase tissue biodistribution and the efficiency with which targets are reached. pTat is a promising strategy for the treatment of various human diseases, and particularly for neurodegenerative diseases. In this review, we will first provide an analysis of CPP biology and we will discuss CPP chemical structure and classification as well as their mechanisms of internalization. Then, we will focus our attention on pTat, which represents one of the first peptides identified that is currently widely studied and used.

The biology of CPPs

The integrity of biological membranes is crucial for tissue homeostasis. Some membranes, such as the BBB, are physically selective barriers to pathogenic bacteria, viruses and large hydrophilic molecules, whereas they allow the penetration of small or hydrophobic molecules [1]. By contrast, tissue barriers represent an obstacle to the use of a systemic administration protocol; they impair the ability of therapeutics to reach their targets when administered in the bloodstream. Therefore, the potential for CPP-conjugated molecules to deliver drugs to target tissues would usher in a new era in the treatment of neurological and non-neurological disorders, and would increase the possibility of rescuing the pathological phenotype of many diseases.

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CPPs as biological delivery agents

During the past 20 years, studies about internalization mechanisms have identified more than 100 peptidic sequences in the range 5–40 amino acids in length that are able to conduct active molecules, cargo and drug delivery vectors [2]. In addition, high-throughput screening of DNA-encoded peptide libraries has resulted in the discovery of many CPPs, for a detailed review see [3]. pTat was the first CPP identified; pTat peptide is the basic domain of the Tat protein, the shortest amino acid sequence that can efficiently enter cells and promote HIV viral gene expression [4]. Another peptide called Penetratin has also been identified; it naturally enters nerve cells and regulates neural morphogenesis through a short sequence with cell-penetrating properties derived from the third helix of the Antennapedia homeodomain, a transcription factor of *Drosophilia melanogaster* [5]. Many other peptides have been identified and studied because they represent a promising tool for drug delivery, particularly in neurological disorders, owing to their potential to overcome biological membranes and release therapeutic biomacromolecules at pharmacological target sites. These peptides were initially termed Trojan horses or protein transduction domains, and were named CPPs after a review of their internalization pathways, which mainly involve endocytosis and transduction [6].

As with peptides in general, the main concern with CPPs can be connected to their short duration of action and lack of oral biodisponibility. Medicinal chemistry can overcome these troubles through the use of artificial amino acids, conformational stabilization of the 3D structure and the use of alternative routes of administration to bypass poor oral bioavailability [7].

The physicochemical classification of CPPs

CPPs constitute a broad heterogeneous group of peptides derived from proteins, chimeric sequences resulting from the merger of two natural sequences or the synthetic result of structure–activity software prediction studies. Classification criteria are based on the physicochemical features of the sequences [8]; the main three classes are cationic (83%), amphipathic (44%) and hydrophobic (15%) sequences [3].

The cationic class is the largest, and the best-known member of this class derives from the HIV-1 protein Tat. pTat, Penetratin and nuclear localization sequences (NLSs) also belong to this class. The arginine-rich sequence requires at least eight arginine residues (named octaarginine, R8) for cellular uptake [9]. The typesetting of the sequence, particularly the relative abundance of arginines, is crucial to peptide transduction properties. Owing to the specific guanidine group, arginine residues can easily penetrate cells at physiological pH. Although lysines present the same positive net charge as arginine, they are less competent at internalization because they lack the guanidine head group [2]. Because these short cationic sequences develop electrostatic interactions with negatively charged glycoproteins on the cellular surface, they demonstrate great potential as transmembrane carriers for therapeutic compounds [10].

In the second CPP class, primary chimeric amphipathic peptides such as MPG and Pep-1 are included as well as peptides derived from natural proteins such as vascular endothelial cadherin peptide (pVEC). Secondary amphipathic α-helical CPPs in which hydrophobic and hydrophilic amino acids occupy different faces of the helix, such as model amphipathic peptides (MAP) peptide and transportan, are also enclosed. Finally, β-sheet amphipathic CPPs, such as VT5, and proline-rich amphipathic peptides, such as Bac7 and sweet arrow peptide (SAP), are part of this group, for a detailed review see [3].

The third class is the hydrophobic class, which includes peptides based on natural amino acids or chemically modified peptides. The latter are further divided into rigid blocking peptides, structure peptides (stapled peptides), prenylated sequences and pepducins. Hydrophobic CPPs appear to cross the cell membrane directly, avoiding endosomal degradation. Moreover, CPPs can be characterized as derived from natural proteins or peptides, such as heparin-binding proteins, DNA-binding and RNA-binding proteins (e.g. Tat peptide), homeoproteins (e.g. Penetratin), signal peptides, antimicrobial peptides and viral proteins.

Mechanisms of internalization

CPPs are translocated into cells by several mechanisms that are independent but can occur simultaneously (Fig. 1). The protein or peptide from which CPPs derive can often provide information about the mechanism of internalization. Short, positively charged, arginine-rich CPPs such as pTat increase cellular drug uptake by interacting with the negatively charged plasma membrane and activating permeabilization of the cell membrane through a receptor-independent pathway, which results in endocytosis of the cargo [11]. At the beginning, the interface between CPPs and the cell membrane involves an electrostatic interaction between basic amino acids and negatively charged proteoglycans, mainly substituted with anionic heparan sulfate associated with arginine-rich peptide. Nevertheless, nonspecific fluid-phase endocytosis appears not to involve electrostatic interactions but to require only CPP contiguity with the cell membrane for uptake [10]. Moreover, CPPs stimulate intracellular signaling cascades that enrich the biological pathway of the uptake process [12]. Endocytosis is a natural, energy-dependent, cellular process that can begin with electrostatic interactions with proteoglycans at the cellular surface or by direct destabilization interactions across the lipid bilayer [8]. CPPs and CPP–drug conjugates can penetrate cells using different endocytotic pathways (and in particular pinocytosis), including macropinocytosis, clathrin-mediated endocytosis, caveolae or lipid-raft-mediated endocytosis, and clathrin- or caveolae-independent endocytosis [13]. The choice between single and multiple endocytic uptake mechanisms depends on chemical and physical peptide sequence properties, on its cargo-molecule-conjugated features and on cell-specific target characteristics [14]. Peptide stability is essential to deliver the drug efficiently to the cellular target site without being prematurely cleaved by proteases; delivery of conjugates is limited by the extracellular metabolism, which involves peptide uptake, and intracellular degradation in endocytic vesicles. Endosomal escape is essential to avoid degradation in lysosomes and allow the cargo to reach its biological target [8].

Many studies display alternative internalization mechanisms to endocytosis through which CPPs can cross the membranes using an energy-independent pathway known as the direct translocation pathway, which is based on spontaneous peptide–membrane interactions. Several hypotheses have been developed suggesting that the translocation mechanism involves direct membrane penetration, such as the pore formation model, electroporation-like permeabilization, entry at microdomain boundaries or shaping of inverted micelles [8]. An additional hypothesis about the internalization of

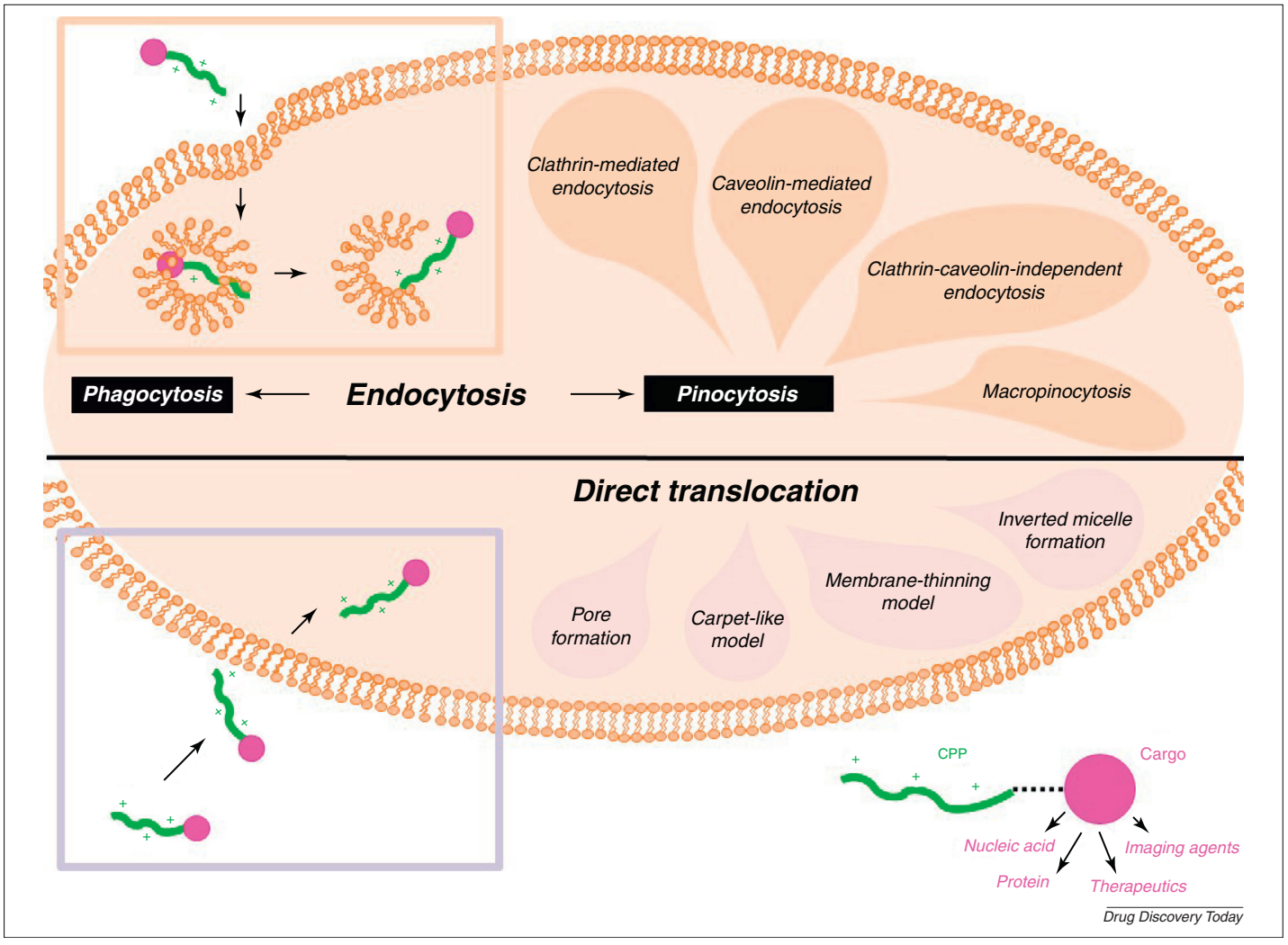

FIGURE 1

Two different mechanisms for cell-penetrating peptide (CPP) internalization: direct translocation and/or endocytosis. CPPs and CPP–drug conjugates can penetrate cells using different endocytotic pathways, in particular pinocytosis which includes macropinocytosis and clathrin-mediated, caveolae or lipid-raft-mediated and clathrin- or caveolae-independent endocytosis or phagocytosis. Alternatively, CPPs can cross the membranes using an energy-independent pathway known as the direct translocation pathway, which is based on spontaneous peptide–membrane interactions.

guanidinium-rich CPPs (adaptive translocation) foresees counter anion scavenging with charge neutralization or CPP inversion using transmembrane potential as the driving force across the membrane lipid bilayer [10]. However, all of the hypothesized direct translocation mechanisms can be clustered into four pathways: inverted micelle formation, pore formation, the carpet-like model and the membrane-thinning model [15].

Although direct translocation and endocytic processes can co-exist at the same time, a specific CPP can use a different endocytosis pathway, as has been reported for Penetratin, nonarginine and Tat peptides [16]. Recent research reveals that, in addition to the two main mechanisms of internalization outlined above, there are other specific pathways of entry mediated by receptors such as scavenger receptors [17].

HIV-1 Tat peptide

Since the discovery of CPPs, many studies have been conducted to transport a wide variety of therapeutic molecules within the target cells. After the characterization of cellular and molecular mechanisms needed to support HIV infection, Tat protein was identified for its great ability to move across cells. Tat protein is a 14 kDa, RNA-binding protein that recognizes the transactivator response element (TAR), a specific sequence from the viral genome (Fig. 2) [18]. Tat stimulates HIV-1 gene expression during transcription initiation and elongation, enhancing the processivity of RNA polymerase II complexes and stimulating the efficient elongation of viral transcripts [19]. It contains a very strong transcriptional activation domain composed of a cysteine-rich region and a hydrophobic core motif, along with an arginine-rich RNA-binding motif (ARM) that specifies the binding of Tat. Tat is able to increase membrane permeability through different mechanisms, including severe vascular modifications in the expression patterns of claudins, occludins and junction adhesion molecules (JAMs) of the endothelial tight junctions [20]. Moreover, Tat protein is able to influence tight junction morphology by regulating matrix metalloproteinase (MMP)-9 [21] and exploiting the Rho signaling pathway associated with the c-AMP response-element-binding protein (CREB)-dependent response [22]. Tat can

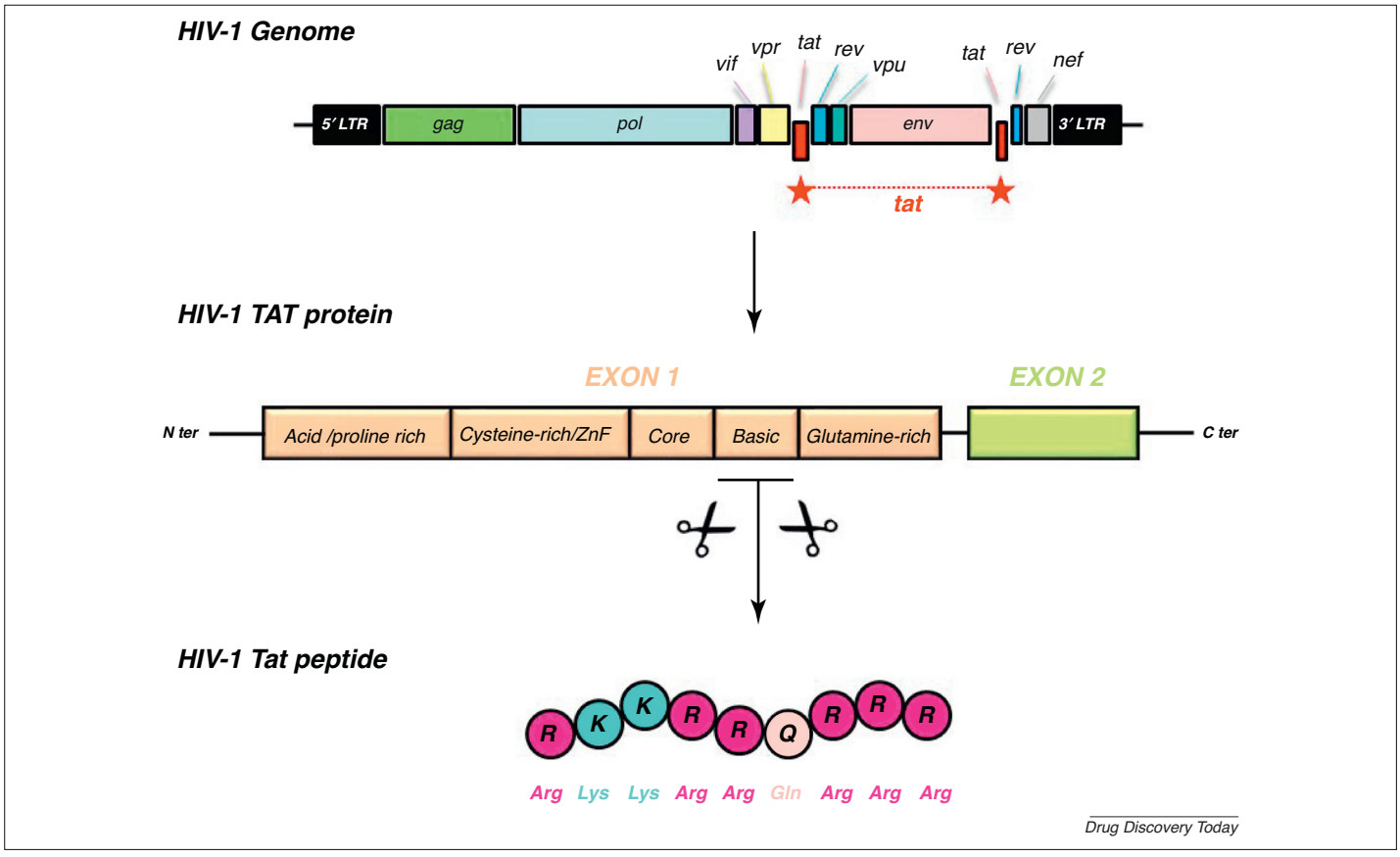

FIGURE 2

Tat peptide derivation from HIV-1 Tat protein. Tat peptide is derived from the basic domain of the Tat protein encoded by the HIV-1 genome and it is the shortest amino acid sequence that can efficiently enter cells and promote HIV viral gene expression.

also develop a non-receptor transport-mediated mechanism to enter the bilayer, owing to its highly cationic transduction domain, which is responsible for the endocytosis of high molecular weight molecules [23].

pTat has been derived from HIV-1 Tat protein. Exploiting its ability to overcome cellular and tissue barriers, pTat has been conjugated with molecules of a variety of sizes to facilitate their delivery to target cells, thereby enhancing the likelihood of an efficient pharmacological action. In particular, pTat has been used for the transmembrane transport of small molecules, antibodies, therapeutic peptides and proteins, and has also been conjugated to liposomes, nanoparticles, small interfering (si)RNAs and antisense oligonucleotides. In the next part of this review, we provide a systematic analysis of the various compounds that could be conjugated with pTat (Table 1).

Small molecules, antibodies and miscellaneous delivery agents

Small molecules (e.g. drugs and imaging agents) have been linked to pTat in an attempt to increase their bioavailability. The bioavailability of these molecules has been limited by their high degree of hydrophilicity, which impairs their ability to cross the lipid bilayer. In particular, the imaging agents oxotechnetium V and oxorhenium V have been conjugated with pTat with good results, as shown by high intracellular concentrations [24]. Paramagnetic labels complexed with pTat can be detected in mammalian cells through magnetic resonance imaging (MRI) [25]. *In vitro* assays and *in vivo* biodistribution studies display a direct correlation between fluorescence intensity and scintigraphic and radiometric data, demonstrating that pTat is capable of efficient cargo internalization for molecular imaging applications [26].

The use of CPPs was also considered to transport antibodies that are unable to cross the phospholipid bilayer. For instance, pTat has been used to convey tumoricidal immunoglobulins inside cells, resulting in the increased uptake of antitumor antibody, such as the Fab fragment, by tumor cells [27]. In the antitumor antibody field, *in vivo* biodistribution studies of pTat–antibody conjugates demonstrated good cellular uptake of CPP-conjugated cargo; however, at the same time, the CPP-conjugated antibodies displayed a significant reduction in the ability to recognize targets compared with unconjugated antibodies [28]. pTat has also been used to deliver specific anti-tetanus toxoid (TET) antibodies required for tetanus toxin neutralization in the nervous system, providing a new therapeutic strategy for neuroprotection against a neurotoxin [29]. The protein transduction domain of HIV-1 pTat has also been used to deliver antibodies into cells through a genetically engineered fusion protein with a specific staphylococcal protein domain named SpA [30]. Despite its success in that context, when Tat transduction domain was fused to a specific diphtheria toxin fragment (dtA) it was unable to deliver the enzymatically active bound fragment to the cytosol efficiently [31].

TABLE 1

The different compounds conjugated with pTat and their applications.

	Categories	Cargo	Application	Refs
pTat conjugates	Small molecules	Imaging agents as oxotechnetium V and oxorhenium V, paramagnetic labels	Molecular imaging	[24–26,58]
	Antibodies	Tumoricidal immunoglobulins as Fab fragment	Tumor therapy	[27,28]
		Specific antibodies as anti-TET antibody, toxin fragment as SpA or dtA	Neuroprotection against neurotoxins	[29–31]
	Peptides and proteins	Tat-βgalactosidase, horseradish peroxidase, RNase A, *Pseudomonas* exotoxin A domain	Heterologous protein delivery	[33]
		Exogenous proteins	Protein-based vaccine administration	[34]
		Recombinant antigen	Dendritic-cell-based immunotherapy	[35]
		Apoptin	Selective cancer cell apoptosis	[36]
		Antiapoptotic proteins as Bcl-X(L) and PEA-15, insulin	Diabetes	[37,38]
		Bcl-xL molecule, FNK cytoprotective protein	Neuroprotection against cerebral ischemic injury	[39,40]
		Cu,Zn-superoxide dismutase (Cu,Zn-SOD), liver catalase (CAT), HSP70, other heterologous genes	Disorders related to oxidative stress	[41]
		Super-repressor mutant IkBα (srIkBα)	Chronic inflammatory conditions	[41]
	Liposomes	Plain and PEGylated liposomes as carriers for drugs and DNA	Tumor studies	[46,47]
		Targeted long-circulating PEGylated liposomes and PEG-PE micelles	Stimuli-sensitive nanocarrier development	[48]
		Covalent link with cholesterol for doxorubicin-loaded liposome formulation	Brain tumor therapy	[49,50]
	Nanoparticles	Dextran-coated superparamagnetic iron oxide particle, superparamagnetic-derivatized nanoparticles	Cell magnetic labeling through MR imaging	[56–58]
		Fluorescein-doped silica nanoparticles (FSNPs)	Brain bioimaging and therapeutics	[59]
		Tat-BMPs-PAMAM complexed with small interfering RNA expression plasmid (psiRNA)	Brain tumor gene therapy	[60]
		Modified nano micelles (MPEG-PCL)	Nose to brain siRNA and drug delivery	[61,63]
	Polymer-based approach	Polymer-bound anticancer drug (doxorubicin)	Human ovarian carcinoma cell drug delivery	[32]
	Gene therapy	Thymidine kinase (TK)	Cancer and other hyperproliferative disorders	[64]
		Recombinant λ phage particles carrying mammalian marker genes	Therapeutic gene transfer	[65]
		Plasmid DNA	Gene transfer	[66]
	Antisense oligonucleotides	Peptide nucleic acid (PNA)	Antisense therapy	[68]
		Short interfering RNA (siRNA)	Gene expression	[69]
		2′-*O*-Methyl phosphorothioate oligonucleotides	Targeting splicing machinery	[82]
		Phosphorodiamidate morpholino oligomers (PMO)	Gene expression	[79]

Furthermore, pTat has been employed in the field of oncology to convey chemotherapeutic agents such as anthracyclines to their targets. *In vitro* studies of human ovarian carcinoma cells treated with copolymer-pTat conjugated with doxorubicin showed that the anticancer drug was delivered into the cell through a pathway different from endocytosis, supplying new tools for the development of a synthetic-polymer-based system approach [32].

Peptides and proteins

The conveyance of proteins and peptides *in vivo* is a difficult goal to reach, and many studies have been designed to investigate the possibility of using CPPs as a delivery system for these molecules. CPPs can be covalently linked or complexed with the cargo, although properties of the peptide and the cargo influence the efficiency with which the complex is delivered, and therefore the related toxicity. Many heterologous proteins, such as horseradish peroxidase, RNase A and a specific domain of *Pseudomonas* exotoxin A, have been conjugated to pTat through chemical cross-linking and successfully tested for *in vitro* delivery, whereas *in vivo* assays of pTat–β-galactosidase chimeras revealed that they have little or no activity in the kidney and brain [33]. pTat can be used to administer protein-based vaccines; in this way, exogenous proteins that are usually unable to reach the cell can be processed by the class I major histocompatibility complex (MHC), stimulating specific cytotoxic T cell responses [34].

pTat is also suitable for the design of selective cancer therapies, such as dendritic-cell-based immunotherapy [35], or a protein-based approach in which the peptide is fused to apoptin. This latter strategy is very interesting from a therapeutic point of view, because it targets only cancer cells; therefore pTat–apoptin conjugates induce apoptosis in cancer cells only, defending normal cells from the apoptotic pathway [36].

pTat has also been applied to enhance the survival of transplantable Langherans islets in diabetic patients. The efficiency of the system relies on the *ex vivo* delivery of antiapoptotic proteins such as Bcl-x(L) and PEA-15, leading to the escape of islet cells from the apoptosis pathway and the preservation of insulin cell secretion [37]. Moreover, pTat displayed the potential to improve oral insulin absorption through the gastrointestinal mucosa epithelial layer; researchers observed an effective gain in hormone uptake compared with unbound insulin [38].

Taking advantage of the ability of pTat to cross the BBB, research studies have also been directed to deliver *in vivo* Bcl-xL in an animal model of focal ischemia/reperfusion, confirming the neuroprotective effect of this cargo in cerebral ischemic injury [39]. For the same application, pTat was fused to an engineered cytoprotective protein called FNK, derived from the antiapoptotic Bcl-x gene, to reduce *in vitro* ischemic damage of hippocampal neurons [40].

The fusion of pTat with various proteins has been applied in many other contexts, ranging from disorders associated with oxidative stress to inflammatory conditions, in attempts to overcome the historical difficulty (presently mainly *in vivo*) of delivering proteins into cells, for a detailed review see [41].

Liposomes

The conjugation of HIV-1 pTat with liposomes represents a very promising drug delivery system. The potential of this liposome-based approach lies in its ability to combine the membrane-crossing properties of CPPs with the specificity of these carrier systems loaded with drugs [10]. Liposomes are vesicles comprising phospholipids that can be filled with different cargos, reducing the intrinsic drug toxicity and improving molecule biodistribution. Because conventional liposomes are quickly degraded by the reticuloendothelial pathway, second-generation liposomes have been designed with a more stable lipid composition and a polyethylene glycol (PEG)-coated surface that stabilizes these formulations. The new generation of liposomes displays a good pharmacokinetic profile and reduced systemic toxicity but displays a delay in liposome uptake; therefore, CPPs have been used to enhance their poor intracellular delivery. The uptake efficiency is related to the number of peptide molecules linked to the liposome surface and to the specific type of target cells [42]. In fact, the presence of a large number of peptide molecules attached to the liposome surface allows cellular drug delivery through direct contact between pTat and the cell surface [43] in an energy-independent internalization process [44].

pTat conjugates at the vesicular surface can also be used as a safe gene delivery system with an high transfection rate. pTat-modified liposomes have been used for gene therapy in tumor cell cultures and *in vivo* [45,46] for human brain tumor studies in nude mice [47]. pTat-modified liposomes coated with PEG have also been linked with an antibody specifically to target a cell type or an organ affected by tumors, increasing the specific cellular uptake of the antibody [48]. In cancer therapy, cholesterol (an electrically neutral element of the liposome) has been used as a linker sequence to bind pTat through PEG; moreover, liposomes coated with PEG are an efficient and sturdy delivery system. The cationic charge of pTat–liposome conjugates increases brain release *in vitro* and *in vivo*; pTat covalently conjugated with cholesterol has been subsequently used to prepare liposomes filled with doxorubicin for the treatment of brain glioma. Biodistribution findings revealed a higher efficiency in brain and heart delivery with low cardiotoxic risk [49,50].

Nanoparticles

To overcome the BBB in a noninvasive way, different biodegradable supramolecular nanodevices have been developed. Liposomes, niosomes, nanogels and cyclodextrins are some colloidal drug delivery systems that can target and release bioactive specific molecules in the central nervous system (CNS) [51]. In the field of brain disorders, lipid nanoparticles have always been studied because they ensure improved drug loading, storage stability, ease of production and safety of formulations for pharmaceutical drug delivery [52].

Superparamagnetic nanoparticles have interesting potential in the biomedical field. They work as specific imaging contrast agents, useful labeling or tracking tools for cell detection and specific purification instruments based on magnetic separation, for reviews see [2,53]. pTat-conjugated nanoparticles revealed their ability in cell transduction [54] and showed improvement in vascular clearance [55]. The first biocompatible nanosystem was linked to multiple pTat sequences to enhance cell magnetic labeling for *in vivo* cell target detection through MRI [56]. Linking a greater number of peptide molecules to the magnetic nanoparticles increased the degree of uptake and improved *in vivo* signaling [57,58]. pTat-modified nanoparticles can also be used to deliver therapeutic agents across the BBB, such as fluorescein-doped silica nanoparticles (FSNPs) derivatized with pTat, which displayed an ability to label cerebral blood vessels and could be used as potential tools for bioimaging and therapeutic applications in the brain [59]. In the context of brain tumors, polyamidoamine dendrimer (PAMAM) and pTat were conjugated to bacterial magnetic nanoparticles (BMPs) called magnetosomes to build a nanoscale magnetic gene delivery system for siRNA expression plasmid (psiRNA) [60]. pTat has also been shown to enhance the delivery rate of block copolymers to the brain in mice, when the Tat-polymer conjugated with nano micelles was administered intranasally through a noninvasive and effective nose-to-brain delivery system, overcoming the BBB [61]. This approach has also been studied to improve the delivery of siRNA to the brain as a therapy for several neurological disorders [62].

Nucleic acids

CPP-based gene therapy has been used to overcome difficulties with introducing genetic material into target cells, which arise because of the low safety profile of viral vectors and the inefficiency associated with nonviral methods, for a complete review see [63]. CPPs such as pTat have been exploited for cancer treatment with suicide gene therapy approaches, delivering an enzyme encoding a gene that is able to produce a cytotoxic effect [64]. CPPs have also been used for plasmid DNA transfection [65,66] and oligonucleotide delivery, including peptide nucleic acid (PNA) [67,68] and siRNA [62,69]. Other synthetic CPPs with the ability to bind DNA and transport it into cells have also been developed [70].

Antisense oligonucleotides

Antisense oligonucleotides (ASOs) are modified nucleotides that bind specific complementary mRNA sequences. This binding can

mark a specific mRNA for degradation or linkage to specific *cis*-acting splicing regulatory motifs. Different ASOs utilizing various chemistries [71–73] have been studied in cell and animal models. A variety of sequences have been targeted by three different ASO chemistries: 2?-*O*-methyl phosphorothioate (2OMePS) oligonucleotides; the more stable variant, 2?-*O*-methoxyethyl (MOE) phosphorothioate oligonucleotides; and phosphorodiamidate morpholino oligomers (MOs). Oligonucleotide studies revealed that the sugar–phosphate structure is important for membrane crossing. Changes to the backbone, such as the introduction of a morpholine ring instead of deoxyribose and phosphorodiamidate linking groups, render these modified oligos resistant to nuclease attack [74]. Chemically modified ASOs resistant to cellular endonuclease activity and insusceptible to RNase-H degradation have already been used to develop therapeutic strategies in animal models of neuromuscular disorders, including Duchenne muscular dystrophy and spinal muscular atrophy; they have also recently been translated in clinical trials [72,75,76]. Although extremely promising, ASOs (particularly MOs) do not readily cross cell membranes. Although they can work very well in neonatal mice, they can be less effective in adult animals, which limits their use during the symptomatic phase of diseases. Nevertheless, the delivery efficiency of ASOs and MOs can be strongly increased by their conjugation with cationic CPPs, producing a high transfection efficiency rate that could be further increased owing to the use of peptide NLSs [66,77]. In fact, the inadequate cellular delivery rate of MOs has been increased using cationic CPP conjugates, which increase the volume of distribution of MOs to muscles throughout the entire body and promote their internalization through an active process that improves the bioavailability of ASOs and MOs [78–81]. Although cationic peptides can interact electrostatically with the anionic backbones of many antisense structural types that form hairpin structures or intermolecular aggregates, uncharged antisense oligomers such as MOs would not have dangerous electrostatic interactions. pTat is a cationic CPP and its ability to enhance biodistribution of ASOs, for example phosphorothioate, 29-*O*-methylphosphorothiate oligonucleotide (ODN) [82] and PNA [68], has been studied *in vitro*. pTat has great potential owing to its ability to target different cell types successfully. Covalent conjugation of pTat to MOs significantly enhances MO uptake, although the resulting pTat–MO conjugate is a bit more toxic than the unconjugated peptide [79].

pTat and clinical trials

In the past decade, pTat has proceeded to various phases of human clinical trials, although no therapy employing pTat has been approved by the FDA. Revance Therapeutics has completed a Phase II clinical trial using pTat to deliver botulinum toxin type A in a topical ointment for the removal of wrinkles (RT-001). Subcutaneous infusion of protein kinase Cδ inhibitor conjugates with pTat has been studied in Phase I/II clinical trials to evaluate the efficacy of the drug in: pain caused by post-herpetic neuralgia, spinal cord injury or post-operative pain (KAI-1678); blood flow restoration after a heart attack (KAI-9803); and prevention of ischemic injury (KAI-1455). A Phase I clinical trial evaluating the safety profile and immunogenicity of a vaccination with recombinant HIV-1 Tat and V2-deleted Env after intramuscular and intradermal injection was recently terminated (ISS P-002). For all of these trials, no study results are posted on ClinicalTrials.gov. The first and the unique successful clinical trial for the use of pTat closed in 2012 with good results (NCT00728182). pTat was conjugated with NA-1 (Tat-NR2B9c), a compound that disrupts pro-death signaling pathways that involve postsynaptic density-95 protein and can prevent damage in the brain caused by reduced blood flow. The Canadian biotechnology company NoNO conducted a Phase II clinical trial to assess the safety and efficacy of NA-1 in reducing small embolic strokes in patients that underwent neurosurgery to repair aneurysms. The intravenous infusion of NA-1 resulted in a neuroprotective effect of the drug with a reduction in the amount of brain damage [83]. Based on these results, pTat conjugated with NA-1 can be tested in later-stage clinical trials for stroke and subarachnoid hemorrhage.

Tat pitfalls

Although different strategies of pTat conjugation with several compounds have resulted in very interesting, selective delivery to target cells, many pitfalls and critical aspects have emerged from basic, preclinical and clinical studies. Generally speaking, the stability of the peptide is fundamental to ensure the delivery of the cargo molecule to the target site. In fact, CPP cleavage by extracellular proteases influences the peptide uptake the most, whereas intracellular metabolism in lysosomal vesicles should be avoided by endosomal escape. Hence, even if stability depends on the specific peptide and the associated cargo molecule, the entire delivery system conducted by CPP and administered *in vivo* has been modified over time. For instance, peptide moiety has been modified to use a D-amino acid configuration, which is less susceptible to protease activity than the natural L-form. Peptide stability can also be increased by using peptide mimics to increase the ability to reach the biological target. The challenge is to achieve a good compromise between avoiding degradation to the biological target and obtaining a sufficient drug release rate from the CPP complex, for a detailed review see [10]. To release the associated drug after cellular internalization, the linker strategy (e.g. maleimide, amide, thiolmaleimide, thioether, thiazolidine, oximine, hydrazine and disulfide bonds) should be carefully considered, especially if it depends on a chemical approach. The coupling is usually a covalent connection based in most cases on disulfide binding, which is suitable for cargo release because of the reduction of intracellular bonds [84]. Additional covalent strategies include the employment of bifunctional crosslinker molecules, peptide bonds or producing chimeric fusion protein in bacteria. Noncovalent linkage takes advantage of electrostatic interactions between the cationic peptide and the negatively charged nucleic acid backbone or exploits streptavidin–biotin attachment [63].

Toxicity is another issue to be considered. The nature of toxicity is not yet well understood. The safety landscape is directly connected to CPP and cargo toxicity, clearance and immunogenicity. The potential immune response is mainly related to the genesis of peptides, because these molecules often derive from non-human proteins. CPP toxicity could be cell-type-related, dose-dependent and influenced by chemical–physical features; overall, the final toxic effect results from the perturbation of plasma membrane dynamics that occurs at high peptide dosages [85]. Moreover, toxicity is associated with the amino acid CPP composition and

the dose or frequency of administration [77]. Intratracheal or intraperitoneal CPP administration produces pulmonary toxicity *in vivo* [86] and epithelial cell damage *in vitro* [87]. CPP length influences the magnitude of the neurotoxic effect, as demonstrated by pTat, the shorter isoform of which produces greater neurotoxic damage than the full-length protein [88]. The cysteine-rich domain and the basic region of pTat are required for neurotoxicity. Peptide toxicity increases if CPP is conjugated to a peptide cargo, depending on the length of the peptide and the dose used [89]. As described by Moulton *et al.* [79–81] for MOs, Tat peptide toxicity is dose-dependent and the conjugated construct was more toxic than either CPP or MO administered alone. Moulton's group found that unconjugated free pTat is toxic by itself, and they speculated that a dose threshold might exist that should not be exceeded to ensure safety. Overall, the observed pTat toxicity poses a challenge for the determination of an effective and safe dose regimen in humans.

The route of administration represents another concern. As already mentioned above, some CPPs are associated with a specific organ toxicity [86,87]. Moreover, oral administration can lead to immediate degradation in the gastrointestinal system, resulting in a shorter active plasma half-life, which can interfere with the achievement of the final target at a meaningful pharmacological dosage. For neurotherapies, all brain delivery should be addressed without invasive methods (e.g. intracerebroventricular injection or special intracranial implants). An alternate route of delivery can be parenteral administration; however, this strategy can be a problem for translating peptides into the clinic because of low rates of patient compliance and adherence to therapy. Another alternative to intravenous drug delivery into the brain is represented by intranasal administration, which exploits the wide surface area and rich vascularity of the olfactory region [90].

The low cell, tissue and organ selectivity of CPPs is another huge drawback for the therapeutic applications of these carriers. However, recent promising developments have been made using activatable CCPs and stimuli-responsive peptides, or by the insertion of specific localization sequences to address the delivery toward the proper cellular organelles. Internalization mechanisms take advantage of tissue- and organ-specific ligands for different receptors. In addition, because each organ expresses a specific set of molecules (also called 'zip code' system) on their vasculature, the insertion of one of these homing sequences into the CPP–cargo could represent a strategy for the efficient and cell-specific CPP delivery allowing the translocation across the cellular membrane, increasing the potential of the first-generation CPPs [91]. Another strategy to overcome the low CPP specificity is the recent development of activatable CPPs (ACPPs) that become active depending on the biochemical properties of the target sites. The first ACPP was developed in 2009 and was a protease-activatable CPP: a proteolytic cleavage releases the activated peptide that can lead the cargo to target cells [92]. Moreover, pH, transmembrane potential, hypoxia and the uptake pattern can represent other specific microenvironment features, detectable for example in tumoral affected tissues, that should be investigated for exploiting different stimuli-responsive carriers for cargos delivery [93]. The expertise in selective intracellular transport through specific localization sequences represents a great potential for the intracellular direct transport to the nucleus by NLS or to different cell organelles such as mitochondria, lysosomes or Golgi apparatus by specific peptides [93].

Concluding remarks

As summarized in this review, many studies have been conducted with pTat. Despite several promising lines of preclinical evidence that have demonstrated its ability to overcome biological barriers to deliver several types of drugs to target tissues, many clinical trials involving pTat have failed and some critical issues have emerged. However, as a CPP, pTat represents an interesting and promising tool to improve the biodistribution of drugs and to allow their systemic administration owing to its good cell-penetrating capacity and the fact that it is nonimmunogenic and barely toxic. Moreover, the development of CPPs from the second generation aiming to overcome the low cell and tissue specificity of the first CPP generation lays the groundwork for a feasible cell-type-selective therapy or even organelle-specific approach suitable for Tat, from a diagnostic point of view and for therapeutic purposes.

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