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Cell-Penetrating Peptides—Uptake, Toxicity, and Applications

Gisela Tünnemann and M. Cristina Cardoso

Abstract

Cell-penetrating peptides (CPPs) mediate the uptake of interconnected cargoes of different types into cells and organisms. Studies in living cells revealed that high-molecular-weight cargoes linked to CPPs are delivered via endocytic routes whereas CPPs by themselves or fused to low-molecular-weight cargoes can in addition enter by a mechanism that does not require vesicle formation. This latter mode of uptake (transduction) allows access to all intracellular compartments. Three models for the mechanism of transduction are proposed.

Most described CPPs experience enhanced uptake due to high-affinity interactions with membrane constituents, but the CPPs can be rather toxic. The subclass of arginine-rich peptides (RRPs), however, mediates high frequencies of transduction with low concomitant toxicity.

Finally, selected examples of applications of CPP-mediated delivery of biological macromolecules in molecular medicine are presented.

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14.1. Introduction—Bits and Pieces of CPP History

In 1988 Frankel and Pabo[1] and Green and Loewenstein[2] reported that the viral protein transactivator of transcription (Tat) from HIV-1 was able to cross biological membranes and to subsequently alter gene transcription.

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This discovery was followed by the mapping of the peptide domain responsible for cellular uptake ability, termed protein transduction domain (PTD), or cell-penetrating peptide (CPP). Subsequently, other PTDs have been identified, for example, the homeobox of the antennapedia protein of *Drosophila melanogaster*, the viral tegument protein VP22 of herpes simplex virus-1,[3–5] human calcitonin-derived peptides[6,7] and the PreS2-domain of hepatitis-B virus surface antigen.[8] The homeobox of antennapedia was the first example of a shuttle that promoted the intracellular delivery of a part of the Rab 3 protein as a cargo into cells in culture.[9] For some of the factors mentioned above the minimal transducing domains were mapped and will hereafter be referred to as CPPs. The Tat CPP was narrowed down to a short peptide motif of nine residues GRKKRRQRRR (amino acids 48–57).[10,11] The well-studied CPP penetratin comprises the third helix of the homeobox of antennapedia RQIKIWFQNRRMKWKK (amino acids 43–58),[12] but a shorter C-terminal segment (52–58) is sufficient to induce translocation.[13] To the panoply of CPPs derived from naturally occurring proteins soon were added artificially designed peptide sequences, which can be categorized as amphipathic or model amphipathic (MAP)[14–16] lysine-, arginine-,[13,17–20] and proline-rich;[21–23] and chimeric[24–26] peptides or peptoids.[20,27] Equipped with this armamentarium, cellular uptake of all sorts of cargoes linked to CPPs, like fluorophores, drugs,[28] peptides,[29] nucleic acids,[30] proteins,[10,31,32] nanoparticles,[33] and liposomes,[34] was achieved. Their internalization seemed to follow an energy-independent, unknown pathway. Importantly, the effects of interconnected cargoes measured in mammalian cells and animals raised hopes that biological membranes could be conquered by any hydrophilic compound, if shuttled by CPPs. Unfortunately, these expectations had to be reconsidered when in 2003 Richard et al. published work showing that the CPPs Tat and penetratin themselves became internalized into mammalian cells by an endocytic mechanism.[35] Even under mild fixation conditions, fluorescently labeled CPPs escaped from the endosomal compartment and were redistributed into the cytoplasm and the nucleus. The cationic nature of CPPs and their consequent strong affinity to negatively charged cell membrane constituents was proposed to lead to their artifactual internalization after treatment of cells with membrane-disruptive methods.[35] Thus, the uptake of CPPs was overestimated in many studies using flow cytometric analysis to quantify the amount of

presumably internalized CPPs. Consequently, published work on CPP-mediated internalization needs to be critically reassessed.

14.2. Influence of Cargo on Mode of Uptake

An analysis of the available data suggests a correlation between size or structure of the cargo and the mechanism of cellular uptake. This is also supported by the observation that peptides and proteins fused to CPPs and simultaneously incubated with mammalian cells end up in different subcellular compartments.[36,37] In addition, some biophysical properties of the cargoes might also interfere with the uptake mechanism.

14.2.1. High-Molecular-Weight Cargoes

Proteins and quantum dots fused to CPPs follow an endocytic route and uptake can be prevented by inhibitors of endocytosis.[36–41] For Tat-fusion proteins caveolin-dependent endocytosis,[38] lipid raft macropinocytosis,[42] or clathrin-dependent endocytosis[43] were suggested as possible mechanisms. Brooks et al. promoted the idea that the CPP Tat, because of its strong adherence to negatively charged membrane constituents, is internalized by any pinocytic process occurring at cell membranes.[44] Recently, arginine-rich peptides (RRPs) were reported to simultaneously use at least three endocytic pathways.[45] Hence, the term adsorptive endocytosis, as already used by the discoverers of Tat-mediated transduction,[46] still applies. The main pathways for pinocytic and endocytic uptake are depicted in **Color Plate 14.1**. For the ionic interaction of the positively charged amino acid residues with cellular membranes, a crucial role of heparin sulfate proteoglycans or phospholipids was established,[36,46–49] but the influence of particular negatively charged membrane constituents on the translocation event may vary for individual CPPs.[40] Endocytic uptake is associated with storage of the internalized CPP species in endosomes or lysosomes for extended periods and thus reduced bioavailability and activity. Nevertheless, a sensitive and non-invasive reporter gene assay based on Cre-mediated recombination after delivery of the protein Tat-Cre

recombinase[50,51] indirectly revealed the presence and activity of the recombinase inside the nucleus.[37,42] In addition, numerous examples of CPP-mediated delivery of high-molecular-weight cargoes like protein and DNA or RNA constructs with functional effects after internalization *in vitro* and *in vivo* have been reported and are reviewed in **ref. 52**.

As the release from cytoplasmic vesicles is the bottleneck for a successful delivery of bioavailable cargoes by CPPs, several studies aimed to increase the efficiency of vesicular release. Using the Tat-Cre recombinase functional assay, lysosomotropic agents like chloroquine or endosome-disruptive agents like PEI (polyethylenimine) or high concentrations of sucrose (1–2 M),[53] as well as the coapplication of the transducible and fusogenic Tat-HA2, peptide together with the Tat-fusion protein markedly enhanced Cre recombinase activity.[42,54] Other methods to destabilize vesicle architecture include Ca^{2+} treatment at millimolar concentrations[55] or photochemical treatment in the presence of photosensitizers like CPPs themselves[53] or membrane soluble chemical compounds,[56] for example, aluminum phthalocyanine enhanced the antisense effect of a peptide nucleic acid conjugate delivered by Tat by two orders of magnitude.[57]

14.2.2. Low-Molecular-Weight Cargoes

Although an endocytic mode of uptake for high-molecular-weight cargoes is generally accepted, the entry route of low-molecular-weight cargoes like peptides smaller than 50 amino acids (aa) attached to CPPs is still a controversial issue. Several groups reported that uptake of CPPs like Tat, oligoarginines, or penetratin did not differ from internalization of high-molecular-weight cargoes fused to CPPs and fell in the category of adsorptive endocytosis (see above).[58–60] From the initial studies defining the minimal transduction domains, a very rapid, energy-independent mechanism of entry was observed concomitantly using non-invasive detection methods, for example, fluorescence microscopy.[11,36,37,59,61,62] This mechanism will hereafter be called transduction. Transduction was not affected by inhibitors of endocytosis and was reported to occur,[11,12,20,61] or even to be enhanced, at lower temperature.[63] In addition, D-amino-acid analogues of CPPs were taken up in the same rapid manner and a receptor-dependent mechanism could therefore be excluded.[20,64–67]

Furthermore, the process of transduction exhibited faster kinetics and led to immediate overall intracellular bioavailability (**Color Plate 14.1**). The uptake kinetics of deca-arginine into HeLa cells was recently shown to start directly after application of fluorescently labeled CPP and reach a plateau after 40 minutes.[67] The membrane potential was proposed as one of the potential driving forces for this mode of uptake of CPPs.[37,68–70] Interestingly, there exists a CPP-specific and cell-type-specific transduction threshold.[37,45] For the CPP Tat coupled to a fluorophore, transduction into mouse myoblasts occurred at or above 1 μM, and when fused to a 20-aa-long peptide, the threshold increased to 7.5 μM. Below these concentration thresholds internalization occurred only via the mechanism of adsorptive endocytosis.[37] Tat, penetratin, and oligoarginine exhibited a critical concentration or transduction threshold in cases where nondegradable D-amino-acid variants were analyzed.[37,45,63] Furthermore, freeze-fracture electron microscopy of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)–liposomes treated with Tat at a ratio of 1:20 (peptide–lipid) showed Tat assembled in small bundles with a spacing of about 5 nm,[71] and in a recent atomic force microscopy analysis of Tat on 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) planar biomembranes, the peptide associated with increasing concentration on the fluid phase.[72] For antimicrobial peptides the realignment and self-assembly of peptides in membranes was reported to be concentration dependent also; for example, a solid-state NMR study with the membrane-disruptive peptide PGLa revealed that with increasing peptide–lipid ratio the peptide changed from a monomeric surface-bound S-state over a dimeric tilted T-state to an oligomeric membrane-inserted I-state.[73] These cooperative effects might explain why for a given CPP both possible uptake modes are controversially reported in the literature.

14.2.3. Special Role of Arginine-Rich Peptides in Cellular Uptake

The unifying feature among most known CPPs is the presence of several lysines or arginines within the individual peptide motifs. The high positive net charge of CPPs leads to an increased local peptide concentration at the cellular membranes driven by electrostatic

interactions.[74,75] CPPs such as transportan, mastoparan, and MAPs form a subgroup of amphipathic CPPs, in which hydrophobic and hydrophilic amino acid side chains are spatially separated in the α-helical peptide structure, a property often found in antimicrobial peptides. Their membrane translocation was reported to occur in an energy-dependent and energy-independent fashion in living cells.[15] A structure-function study astonishingly revealed that membrane association due to positive net charge was not correlated with uptake efficiency and lysines were even dispensable; rather, helical amphipathicity and a length of at least four complete helical turns were essential to allow membrane translocation.[16] Therefore, the mechanism of translocation for this type of CPP might differ from that of Tat and penetratin analogues, where positive charge is absolutely required for the crossing of biomembranes. But charge alone is insufficient to explain the process of transduction. The uptake of oligomers of histidine, ornithine, and lysines, as well as that of branched lysines (loligomers), was demonstrated to be sensitive to temperature, which argues for an endocytic uptake.[20,76,77] On the other hand, a minimum of six consecutive arginines was enough to cause transduction[13,17] and the D form as well as guanidino peptoids worked equivalently.[20] These results, the absence of a common secondary-structure element in known RRPs, and the flexibility in the position of arginines within a given peptide sequence without affecting its transduction efficiency[17] suggest a decisive role for arginine in the mechanism of transduction, which probably resides in its guanidinium function.[20,27]

14.2.4. Relevant Parameters when Measuring CPP Uptake

Several parameters influence the transduction efficiency of CPPs. Among them, the type of CPP, its D- or L–amino-acid composition and its concentration, the application buffer, the cell type, and the administration or application mode can influence the optimal concentrations for transduction. Such methodological details are usually described in a material and methods section. However, only a few publications mention, for example, the final volume of the CPP applied on the cells during experiments, although increasing peptide-to-cell ratio has been shown to influence the mode of uptake. Higher peptide-to-cell ratios

permit, in addition to endocytosis, direct membrane transduction.[78] Therefore, besides the particular CPP concentration, the volume parameter (cubic meter of liquid per square meter of cells) in combination with cell density and cell type should be specified in future work. This is especially important for meaningful comparison of results and drawing conclusions on CPP uptake and its efficiency.

14.3. Models for the Mechanism of Transduction

Although to date the mechanism of transduction (i.e., the non-endocytic mode of CPP uptake with membrane permeation and direct intracellular availability) is still not clear, several models have been developed to explain this intriguing property of CPPs.

14.3.1. Pore Formation

Some CPPs (MAPs[14]) exhibit amphipathicity or adopt α-helical conformation in the presence of artificial micelles (e.g., mastoparan and transportan[79]), which is also a known property of pore-forming antimicrobial or lytic peptides. Such peptides lead to leakage of protons, metal ions, proteins, and so on, finally resulting in cell death due to collapse of the membrane potential.[80] Amphipathic peptides insert into the membrane and multimerize in such a way that hydrophobic residues are exposed and hydrophilic residues form the cavity of a channel (**Color Plate 14.2A**, amphipathic peptides). Thereby short-lived mastoparan pores permit influx of compounds <1000 Da into mammalian cells.[81] Moreover, they are able to traverse the membrane with a flip-flop mechanism.[82] However, this mechanism can apply only for the subgroup of amphipathic CPPs. RRPs do not form pores in both artificial and mammalian cellular membranes,[12,83] and small miscible compounds applied simultaneously do not gain access to the intracellular milieu of the cells upon transduction.[84] Consequently, MAPs exert rather strong toxic effects,[15,16,85] whereas penetratin causes only small membrane perturbations[85] and Tat and oligoarginines are well tolerated by living cells.[11,84,85]

14.3.2. Formation of Inverted Micelles

The interaction of CPPs with artificial membranes has been the subject of several biophysical studies. The observation by ^{31}P-NMR that penetratin induced micelle formation in artificial membranes led to the assumption that CPPs might translocate biomembranes inside the hydrophilic cavity of inverted micelles.[86–88] In **Color Plate 14.2B** the proposed mechanism starts by strong electrostatic interactions of the CPP with the phospholipids or glycolipids, which cause membrane perturbation. The cationic peptide enters the lipid bilayer inside an inverted micelle and is released in the reverse process into the cytoplasm. Further analysis of the interaction of penetratin with artificial zwitterionic and anionic model membranes revealed that penetratin forms a β-sheet hairpin structure and orients parallel to the membrane.[74,89] Penetratin enriches at the lipid–water interface and is anchored by its tryptophan residues inside the lipid bilayer. It causes a membrane curvature and after reaching a certain threshold concentration becomes internalized. The driving force for this event is the electrostatic field created by the differential peptide concentration inside and outside the membrane.[90] However, Tat CPP showed weaker binding to anionic membranes than did penetratin, presumably because no hydrophobic amino acid residue is available for anchoring inside the bilayer.[75] A biophysical study compared the CPPs Tat, octaarginine, octalysine, and other amphipathic peptides and their influence on artificial membranes. Tat induced a pronounced isotropic ^{31}P-NMR signal (indicative of micelles, very small vesicles, or cubic phases) in zwitterionic, but not anionic, membranes. Octa-arginine and to a lesser extent octa-lysine had the same effect, whereas other amphipathic CPPs did not influence the line shape of the ^{31}P-NMR signal. Moreover, freeze-fracture electron microscopy indicated that the changes detected by ^{31}P-NMR were due to the formation of rodlike structures on the membrane surface.[71]

14.3.3. Adaptive Translocation

The superior transduction activity of consecutive arginines over the corresponding lysines, histidines, and ornithines is mainly dependent on the guanidine function of arginine.[20,77,84] The efficiency could be enhanced by using polyarginines prepared from D amino acids or

polyguanidine peptoids with different spacers, comprising at least seven guanidine head groups.[20] Also, highly branched guanidinium-rich oligosaccharides or dendrimers were shown to transduce readily.[66,91,92] In a two-phase partitioning experiment with octanol and water, fluorescent octa-arginine and less-efficient octa-ornithine moved from the aqueous phase into the octanol layer after addition of laurylsulfate. Because the replacement of the amino functions of arginine with one or two methyl groups diminished the partitioning capacity, these results suggest efficient formation of lipophilic ion pairs with abundant negatively charged groups, for example, phospholipids, fatty acid salts, and sulfates via bidentate hydrogen bonds,[69,70] which are able to diffuse into the interior of the hydrophobic lipid bilayer. Authors of a recent molecular dynamics simulation with the RRP Tat stated that the interaction of Tat with the phosphate groups on both sides of an artificial lipid bilayer membrane (DOPC) was crucial for translocation.[93]

The driving force for RRPs to penetrate living cells was found to be membrane-potential dependent. Incubation of living cells with isotonic K^+-enriched buffer abolished uptake of RRPs;[37,68,69] likewise, pretreatment of cells with the membrane-potential-increasing antibiotic valinomycin led to higher intracellular concentration of octa-arginine.[69] Hence, the model depicted in **Color Plate 14.2C** can be described as formation of lipophilic ion pairs and their diffusion along the membrane potential inside the cells. This would also imply that the transport is monodirectional and that internalized cargoes remain inside the cell. In addition, cargo-dependent differences in uptake can be explained because the diffusion rate of higher-molecular-weight cargoes should be limited, if not prevented, by structural constraints.

Likewise, several observations of CPPs themselves and Tat fused to globular proteins suggest that the loss of tertiary structure enhances or permits translocation over the cell membrane. This would be equivalent to the import of cytosolic proteins into cell organelles, which also requires unfolding of the protein to be translocated.[94,95] The protein dihydrofolatereductase (DHFR) fused to the protein Tat was able to translocate into HeLa cells, but the import failed when the tertiary structure of DHFR was stabilized by methotrexate, a folate analogue.[96] In agreement with this observation it was shown that the activity of a variety of proteins genetically fused to the CPP Tat depends on the purification protocol. For Tat-fusion proteins purified under

denaturing conditions, lower concentrations were sufficient to achieve the same effect as their natively extracted counterparts.[31,97] For instance, the enzyme Cu-Zn superoxide dismutase fused to Tat prevented paraquat-induced cell damage in a concentration-dependent manner when the purification scheme included denaturation in urea but had no effect when it was purified natively.[98] The requirement for unfolding for translocation through the membrane is more relevant for proteins fused to CPPs than for CPPs themselves or CPPs fused to short peptides, as the total number of residues in the latter would only permit, if at all, the formation of short secondary structures.

14.4. Toxicity of Cell-Penetrating Peptides

14.4.1. *In Vitro*

A number of studies have assessed the toxicity of CPPs *in vitro*. In general, comparative toxicity studies support the division of CPPs into subgroups, namely, RRPs and amphipathic peptides. Thus, the induction of membrane leakage by amphipathic peptides could be correlated with the hydrophobic moment.[85] The assessment of the toxicity of unmodified CPPs using a lactate dehydrogenase (LDH)-leakage, $DiBAC_4(3)$-(membrane depolarization), and hemolytic assay showed rather severe toxic effects of MAP and transportan 10 as representatives of the amphipathic CPPs, but it showed only mild effects of the RRPs Tat and penetratin.[99] Oligoarginines consisting of a minimum of 5 and a maximum of 12 amino acids at different concentrations were analyzed, using mouse myoblasts, for transduction and concomitant toxicological effects. Nona-arginine was identified as the oligoarginine of choice, combining high transduction frequencies with low short- and long-term toxicological effects.[84] Among the RRPs, toxicity decreases in the series oligoarginine > penetratin > Tat.[100,101] The toxicological properties can be dramatically changed also on attachment of low-molecular-weight cargoes, for example, labels or other peptides.[100,102] The toxicity of Tat fused to the anti-apoptotic nuclear-factor-κB essential modulator (Nemo)-binding domain peptide and to the scrambled variant increased

the detected toxicity in several cell types 100-fold.[102] Because the attachment of high-molecular-weight cargoes shifts the uptake mechanism to an endocytic pathway only, a reduction in toxicity in these cases most likely reflects the lower amount of bioavailable intracellular CPP cargo.[36,37,100]

14.4.2. *In Vivo*

As with *in-vitro* cell-culture systems, *in-vivo* data are difficult to compare because of the variety of CPPs used and the differences between the cargoes attached. In addition, modes of administration of CPP cargoes to animals and the type of animal used further complicates delineation of toxic effects. The RRPs Tat and penetratin alone were shown to reduce p38 mRNA levels in lung tissues after intratracheal administration.[103] The RRP $(RXR)_4$[104,105] (X = 6-aminohexanoic acid) fused to a phosphorodiamidate morpholino oligomer (PMO) cargo was evaluated on the basis of mortality, changes in physical appearance, animal behavior, body weight, and serum biochemistry profile and appeared to be nontoxic below a concentration of 15 mg/kg in Sprague-Dawley rats when administered once by intravenous bolus injection.[106] A Grb7 peptidic inhibitor fused to penetratin was intraperitoneally injected into BALB/c nu/nu mice every 3rd day for 4 weeks at 100 mMol/kg, and no clinically significant adverse effects as assayed by histological changes in major tissues became apparent.[107] Unfortunately, few studies to date add mention of toxicological observations to the biological effect under study.

14.5. CPP-Mediated Intracellular Delivery in Molecular Medicine Applications

The non-invasive CPP-mediated delivery of hydrophilic compounds into living cells has tremendous potential for fundamental research as well as for therapeutics. Furthermore, this delivery method is virtually unlimited with respect to the size of the shuttled cargo, provided that endocytically internalized cargoes can be released from endosomes efficiently. In this

section, we will illustrate the versatile applicability of CPP-mediated delivery approaches on the basis of selected examples.

14.5.1. Labeling and Imaging

CPP-mediated delivery has proved useful in labeling cells or subcellular structures. Tat CPP coupled to magnetic nanoparticles was shown to be taken up by $CD34^+$ cells,[33,108,109] which after intravenous injection into mice, could be tracked *in vivo*. Therefore, endocytic loading of nanoparticles connected to CPPs into particular cells *ex vivo* is a non-invasive cell labeling method allowing subsequent tracking of injected cells *in vivo*.[110] Also, Tat linked to the radiolabel ^{99m}Tc and injected intravenously into mice was detectable in all organs, although at higher levels in the liver and the urinary tract.[111–113] In addition to magnetic and radiolabel Tat species, delivery of fluorescently labeled CPPs has been used for the visualization of tumor xenografts in mice.[114] In the latter study, the specific label of the tumor cells was achieved by release of the positively charged CPP from a negatively charged linked peptide via metalloproteinases presented by the tumor cells. Another interesting application of CPPs in animal imaging is the *in-vivo* uptake via the skin of the low-molecular-weight cargo luciferin coupled to an oligoarginine CPP transporter into living transgenic mice expressing luciferase. Intracellular luciferin is converted by the luciferase to the photo-emitting form oxyluciferin, which can be detected with a cooled charge-coupled-device camera in living animals.[28]

Peptides fused to CPPs have also been used to specifically label subcellular structures in living cells. Decaarginine itself was used as a cell-permeable marker of the nucleolar compartment in a variety of cells,[67] and Tat CPP fused to a peptide derived from human ventricular myosin light chain 1 transduced into primary cardiomyocytes and highlighted sarcomeric structures.[115]

14.5.2. Modulation of Intracellular Function

14.5.2.1. Drug Delivery

Only a few groups have worked on the facilitation of delivery of synthetic macromolecules or therapeutics by CPPs. In a transport feasibility study Tat was shown to be able to shuttle a *N*-(2-hydroxypropyl)methacrylamide

(HPMA) copolymer and the anthracyclin doxorubicin into a human ovarian carcinoma cell line.[116,117] The same drug and benzylpenicillin fused to the CPP SynB1 were able to cross the blood–brain barrier as shown by an *in situ* rat-brain perfusion technique after intravenous administration.[118,119] Cyclosporin A connected to hepta-arginine applied as a lotion entered the epidermis and dermis of murine and human skin. Furthermore, a similar construct that releases cyclosporin A at physiological pH inhibited inflammation in mouse contact dermatitis.[28]

14.5.2.2. Delivery of Peptides and Proteins

To date the vast majority of studies using CPP-mediated intracellular delivery evaluated the uptake of peptides or proteins into living cells or evaluated the corresponding effects on intracellular functions, or both. Several studies have focused on their potential use as antitumor delivery agents, whereas others deal with their advantage to deliver protective agents in model systems of cellular damage (ischemia, neurodegeneration, etc.).

A peptidic inhibitor of growth factor receptor-bound protein 7, a potential tumor therapy agent and a factor highly expressed in metastatic pancreatic cancer, was fused to penetratin. After frequent intraperitoneal injections into mice containing pancreatic cancer cells, it was shown to diminish metastatic nodules to 2% of their original number.[107] Also, the DNA replication licensing protein geminin shuttled by a novel CPP had an antiproliferative effect in cultured cancer cells.[120] Another example of a potential antitumor approach consisted in delivery of the apoptotic $(KLAKLAK)_2$, mitochondrial membrane-disruptive peptide fused to hepta-arginine, which caused rapid cell death when injected subcutaneously into tumor xenografts in mice.[121]

On the other hand, a large number of studies showed cellular protective effects of various CPP fusions. Delivery of Tat-BH4 protein and Tat-Bcl-xL peptides prevented apoptosis in models of sepsis,[122] irradiation,[123] and ischemia or reperfusion.[124] Targeting the JNK pathway with a c-Jun inhibitory peptide fused to Tat minimized the lesion size in a rat model of ischemia. The same peptide was able to cross the blood–brain barrier after intraperitoneal injection.[125] Another neuroprotective Tat fusion to an isozyme specific inhibitor of δ protein kinase C increased the number of microvessels and improved

blood flow in a rat model of ischemia. In addition, prophylactic treatment reduced infarct size in hypertensive Dahl rats following an acute stroke. Another study used delivery of a Tat-Cu-Zn superoxide dismutase to prevent injury caused by reactive oxygen species after transient forebrain ischemia in gerbils.[126] Besides being anti-inflammatory, cavtratin—a chimera from penetratin and a hydrophobic peptide derived from caveolin-1 that negatively regulates the activity of endothelial nitric oxide synthase—reduced microvessel permeability.[127] Intranasal delivery of a STAT6-derived peptide fused to the CPP PTD_4 reduced lung inflammation in murine models of rhinitis and asthma and inhibited many features of allergic airway disease.[128] Strikingly, frequent Tat-mediated intracellular delivery of the protein purine nucleoside phosphorylase rescued the immunodeficiency and neurodegenerative defects of the respective knock-out mice with no apparent toxicity.[129]

Finally, uptake into living adult rat primary cardiomyocytes of a striated muscle-specific human ventricular light chain-1 peptide fused to Tat CPP and its localization to sarcomeric structures was measured. This cell-permeable peptide was able to enhance muscle contractility without affecting the intracellular Ca^{2+}. These properties and the fact that this peptide has targets only in striated muscle make it a novel potential therapeutic tool to improve cardiac function.[115,130]

14.5.2.3. Delivery of Oligonucleotides

Although CPP-mediated pinocytic delivery is independent of cargo size, the introduction of DNA or RNA seems to be technically more demanding. Due to the complexation of the intrinsically basic CPP with the negatively charged phosphate backbone of nucleic acids, the transducing moiety becomes less available for the interaction with negatively charged membrane constituents, which is crucial for the initiation of the internalization event.[114]

Nevertheless, a 16-mer peptide nucleic acid (PNA) stably linked to the CPPs Tat, transportan, and Tp10 inhibited Tat-dependent transactivation,[131] and an 18-mer steric block oligonucleotide (ON) linked to the CPP R_6-penetratin enhanced splice correction activity[132] in a HeLa cell reporter assay.

Use of the RNA analogue PMO replaces the phosphodiester with a neutral phosphorodiamidate linkage and therefore results in a neutral nucleic acid compound that is resistant to nucleases and RNase H. Conjugated via a thioether linkage to several types of RRPs, it redirected the splicing of targeted mRNAs in primary murine leukocytes.[18] With a similar alteration in pre-mRNA splicing, the excision of a nonsense mutation in exon 23 was skipped in a mouse model of muscular dystrophy, and functional dystrophin expression was restored.[133]

Another innovative approach fused Tat to the RNA-binding domain of U1 small nuclear ribonucleoprotein as an adaptor moiety and used this shuttle for delivery of shRNA and siRNA constructs into CHO cells.[134] A further possibility for shuttling ONs is to use complexes of CPPs with ON at optimized ratios, for example, to deliver siRNA[135,136] into cell-culture systems.

14.5.2.4. Gene Therapy

CPPs are also capable of aiding viral mediated gene delivery *in vitro* and *in vivo*. Penetratin mixed with an adenoviral green fluorescent protein (GFP)-expressing construct markedly enhanced infectivity after luminal application into mouse carotid arteries.[137] A recombinant Tat-M_2S (multisubunit DNA binding protein) was mixed with a therapeutic plasmid encoding α-galactosidase A (AGA) and injected into muscles of AGA-knockout mice, suffering from a lysosomal storage disease. This chimera significantly enhanced AGA expression in skeletal muscle in comparison to injection of DNA alone.[138]

14.6. Conclusions and Perspectives

A multitude of studies have demonstrated that delivery of therapeutic molecules by CPPs resulted in functional effects *in vitro* and *in vivo*.

In vitro two different mechanisms of uptake were observed. Proteins, DNA and RNA, peptides, and drugs connected to RRPs were taken up by a CPP-enhanced pinocytic mechanism, but low-molecular-weight

cargoes showed—at higher concentrations—the ability to enter living cells in a nonvesicular mode that guaranteed immediate access to any subcellular intracellular compartment, referred to as transduction. Strikingly, this nonvesicular uptake does not compromise living cells at intermediate concentrations and, at the same time, results in high intracellular bioavailable concentrations.

The use of degradable CPP constructs (composed of L amino acids) has the additional advantage of allowing proteolytic degradation of the non-target-bound excess peptide, whereas nondegradable D–amino-acid-containing CPP constructs forcibly elicits sustained effects. Internalized degradable CPP constructs can be shielded from the intracellular proteolytic machinery when bound to their targets, while the remaining unbound fraction is rapidly degraded, providing a natural control over excess CPP construct. The beauty of this system, of course, does not apply to nondegradable compounds linked to CPPs, for example, drugs, heavy metals, or retro-inverso and other non-L–amino-acid-containing peptides.

Since transduction, in contrast to pinocytosis, is a rapid uptake mode and internalized peptides are immediately bioavailable, CPP-mediated delivery of peptides is well suited for functional studies in primary cells, like human polymorphonuclear neutrophils or cardiomyocytes,[130,139] which are both short lived and resistant to transfection. The latter involves gene expression, and consequently biological effects can be assayed only hours to days after transfection. Illustrating the effectiveness of transduction and the ability to perform immediate functional measurements, a peptide derived from ventricular light chain-1 fused to Tat increased the intrinsic contractility of isolated adult rat cardiomyocytes directly after application of the peptide to the medium.[130]

Although transduction is sometimes described as seemingly energy independent, there has to be a driving force for this kind of uptake. Macromolecular concentration gradients in and out of the cell or the membrane potential are possible parameters, and experimental data supports both.[37,69] This implies that transduction could proceed in only one direction, which has not yet been experimentally demonstrated.

Translation of *in-vitro* CPP transduction onto *in-vivo* applications is feasible, but verification is difficult since the fixation protocols often

used to check for uptake in organs lead to artificial redistribution of the delivered substances. Nevertheless, irrespective of the mode of cellular uptake (transduction or endocytosis) several *in-vivo* studies have demonstrated uptake or biological effects, or both, of CPP-mediated delivery of cargoes (see section above and **Table 14.1**).

A priori, the fast uptake and hydrophilic nature of most CPPs should make their use more suitable for topical delivery to the target tissue, which would also provide specificity, rather than for systemic delivery, which would require deep penetration to reach certain organs and cell types. However, Tat-proteins injected *in vivo* were found in all organs and were even able to cross the blood–brain barrier.[32,111] **Table 14.1** provides a summary of several recent successful CPP-mediated *in-vivo* delivery experiments. How can discrepancies between the marginal penetration of CPP cargoes and the encouraging effects measured in animals in several studies be explained? At least in part, it might be because most studies tackled either inflammatory and apoptotic processes or tumor tissues. The former are associated with enhanced tissue and membrane permeability and the latter with high vascularization and increased interstitial space and the absence of a lymphatic network.[140] Therefore, compromised tissue may be more easily reached by CPP entities circulating in the bloodstream and uptake into harmed cells would be enhanced.

The constraints of the animal experiments do not apply to the exciting possibilities of CPP-mediated cargo delivery in *ex vivo* cell applications, including labeling of cells and subcellular structures, cell-based assays, and modulation of cellular functions. Since all cells so far have proved susceptible to transduction by low-molecular-weight cargoes linked to CPP, this mode of uptake can be used directly. Application of large cargoes (e.g., proteins) that get internalized exclusively by pinocytosis, though, will require optimization of nontoxic strategies to release the CPP cargoes from the vesicles. With recent developments in cell-replacement therapies, this non-DNA-based approach will be extremely useful.

Altogether, CPP-mediated shuttling of hydrophilic compounds over the plasma membranes of living cells provides the fascinating possibility of exploiting the macromolecular repertoire of the cell for molecular medicine.

Table 14.1. Summary of CPP-mediated delivery of peptides and proteins in vivo.

Effect	Specific effect	Cargo	CPP	Animals	Application	Citation
Anti-apoptotic, Anti-inflammatory	Reduced cerebral infarct size	Bcl-x_L	TAT	C57BL/6 mice	i.p.	141
	Resistant to sepsis-induced apoptosis, increased survival	Bcl-x_L, BH4-peptide	TAT	Bcl-x_L overexpressed in T-lymphocytes, transgenic mice	s.c., miniosmotic pumps	122
	Reduced hippocampal damage in excitotoxic seizure model	BH4-domain of Bcl-x_L	TAT	Sprague-Dawley rats	injection into dental gyrus	142
	Blocked inflammation and tumor angiogenesis	Caveolin-1 peptide (amino acids 82-101)	Pen	Swiss mice	pretreated	127
	ROS-reduction	Cu, Zn-superoxide dismutase	PEP-1	gerbil	i.p.	126
	Prevented delayed neuronal cell death after transient global ischemia	FNK (Bcl-x_L)	TAT	gerbil	i.p.	143
	Protection against chemotherapy-induced alopecia	FNK (Bcl-xL)	TAT	Wistar rats	topical	144
	Amelioration of established colitis	NBD-peptide (Nemo-binding domain)	K_8	IL-10 $^{-/-}$ mice	i.p.	145
	Inhibition of caspase-3 activity	D-JNKI1 peptide, c-Jun N-terminal kinase inhibitor	TAT	Wistar rats	i.p.	125
	Decreased infarcted myocardium	p27 (Heat shock protein 27)	TAT	Sprague-Dawley rats	i.p.	146
	Inhibition of OVA-induced lung inflammation	STAT-6 inhibitory peptide	PTD_4	BALB/c mice	i.n.	128
	Reduction of caspase-3 and -9	XIAP (X-linked inhibitor of apoptosis)	Pen	Sprague-Dawley rats	i.p.	147

Anti-diabetic	Elevates insulin levels in diabetic mice	Pdx1 (pancreatic duodenal homeobox-1)	TAT	BALB/c mice	i.p.	148
Anti-neurodegenerative	Suppressed polyglutamine-nducedi neurodegeneration	QBP1 (Aggregate inhibitor peptide poly Q binding peptide 1)	TAT	UAS-MJDtr-Q78 transgenic Drosophila fly line	mixed with food	14
Anti-proliferative	Attenuates cell migration and metastasis	Grb7 (growth factor receptor-bound protein 7) inhibitory peptide	Pen	BALB/c nu/nu mice	i.p.	107
Pro-apoptotic	Apoptotic effects on tumor	Kla-peptide (klaklakklaklak)	R7	athymic nude mice	injected into tumor	121
	Apoptotic effects on tumor		PTD-5	C57BL/6 mice	injected into tumor	150
Protection of microvasculature	Reduced infarct size following an acute stroke	dV1-1 (PKC-derived peptide)	TAT	Sprague-Dawley rats	i.p.	151
Rescue of function	Restores PNP-function in ko-mice	PNP Purine nucleoside phoshorylase	TAT	PNP $^{-/-}$ C57BL/6 mice	i.p.	129
	Restores functional dystrophin	PMO altering pre-mRNA splicing for dystrophin protein	RRP	mdx-mouse model with nonsense mutation in exon 23	i.p.	152
	Delivery over the blood brain barrier restores function	BDNF (brain-derived neurotrophic factor)	TAT	Kung Ming mice	i.v.	153

Abbreviations: i.p. intraperitoneal; i.v. intraveneous; K lysine; s.c. subcutaneous; ROS reactive oxygen species; RRP arginine-rich peptide; Pen penetratin; PTD protein transduction domain, R arginine

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References

1. Frankel AD and Pabo CO. **Cellular uptake of the tat protein from human immunodeficiency virus.** *Cell* 1988, **55**:1189–1193.
2. Green M and Loewenstein PM. **Autonomous functional domains of chemically synthesized human immunodeficiency virus TAT trans-activator protein.** *Cell* 1988, **55**:1179–1188.
3. Elliott G and O'Hare P. **Intercellular trafficking and protein delivery by a herpesvirus structural protein.** *Cell* 1997, **88**:223–233.
4. Murphy AL and Murphy SJ. **Catch VP22: The hitch-hiker's ride to gene therapy?** *Gene Ther* 1999, **6**:4–5.
5. Elliott G and O'Hare P. **Live-cell analysis of a green fluorescent protein-tagged herpes simplex virus infection.** *J Virol* 1999, **73**:4110–4119.
6. Machova Z, Muhle C, Krauss U, Trehin R, Koch A, Merkle HP, and Beck-Sickinger AG. **Cellular internalization of enhanced green fluorescent protein ligated to a human calcitonin-based carrier peptide.** *Chembiochem* 2002, **3**:672–677.
7. Trehin R, Krauss U, Muff R, Meinecke M, Beck-Sickinger AG, and Merkle HP. **Cellular internalization of human calcitonin derived peptides in MDCK monolayers: A comparative study with TAT(47-57) and penetratin(43-58).** *Pharm Res* 2004, **21**:33–42.
8. Oess S and Hildt E. **Novel cell permeable motif derived from the PreS2-domain of hepatitis-B virus surface antigens.** *Gene Ther* 2000, **7**:750–758.
9. Perez F, Joliot A, Bloch-Gallego E, Zahraoui A, Triller A, and Prochiantz A. **Antennapedia homeobox as a signal for the cellular internalization and nuclear addressing of a small exogenous peptide.** *J Cell Sci* 1992, **102**(Pt 4):717–722.
10. Fawell S, Seery J, Daikh Y, Moore C, Chen LL, Pepinsky B, and Barsoum J. **Tat-mediated delivery of heterologous proteins into cells.** *Proc Natl Acad Sci U S A* 1994, **91**:664–668.

11. Vives E, Brodin P, and Lebleu B. **A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus.** *J Biol Chem* 1997, **272**:16010–16017.

12. Derossi D, Joliot AH, Chassaing G, and Prochiantz A. **The third helix of the Antennapedia homeodomain translocates through biological membranes.** *J Biol Chem* 1994, **269**:10444–10450.

13. Fischer PM, Zhelev NZ, Wang S, Melville JE, Fahraeus R, and Lane DP. **Structure-activity relationship of truncated and substituted analogues of the intracellular delivery vector Penetratin.** *J Pept Res* 2000, **55**:163–172.

14. Dathe M, Schumann M, Wieprecht T, Winkler A, Beyermann M, Krause E, Matsuzaki K, Murase O, and Bienert M. **Peptide helicity and membrane surface charge modulate the balance of electrostatic and hydrophobic interactions with lipid bilayers and biological membranes.** *Biochemistry* 1996, **35**:12612–12622.

15. Oehlke J, Scheller A, Wiesner B, Krause E, Beyermann M, Klauschenz E, Melzig M, and Bienert M. **Cellular uptake of an alpha-helical amphipathic model peptide with the potential to deliver polar compounds into the cell interior non-endocytically.** *Biochim Biophys Acta* 1998, **1414**:127–139.

16. Scheller A, Oehlke J, Wiesner B, Dathe M, Krause E, Beyermann M, Melzig M, and Bienert M. **Structural requirements for cellular uptake of alpha-helical amphipathic peptides.** *J Pept Sci* 1999, **5**:185–194.

17. Futaki S, Suzuki T, Ohashi W, Yagami T, Tanaka S, Ueda K, and Sugiura Y. **Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery.** *J Biol Chem* 2001, **276**:5836–5840.

18. Marshall NB, Oda SK, London CA, Moulton HM, Iversen PL, Kerkvliet NI, and Mourich DV. **Arginine-rich cell-penetrating peptides facilitate delivery of antisense oligomers into murine leukocytes and alter pre-mRNA splicing.** *J Immunol Methods* 2007, **325**:114–126.

19. Mi Z, Mai J, Lu X, and Robbins PD. **Characterization of a class of cationic peptides able to facilitate efficient protein transduction in vitro and in vivo.** *Mol Ther* 2000, **2**:339–347.

20. Wender PA, Mitchell DJ, Pattabiraman K, Pelkey ET, Steinman L, and Rothbard JB. **The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: Peptoid molecular transporters.** *Proc Natl Acad Sci U S A* 2000, **97**:13003–13008.

21. Crespo L, Sanclimens G, Montaner B, Perez-Tomas R, Royo M, Pons M, Albericio F, and Giralt E. **Peptide dendrimers based on polyproline helices.** *J Am Chem Soc* 2002, **124**:8876–8883.

22. Fernandez-Carneado J, Kogan MJ, Castel S, and Giralt E. **Potential peptide carriers: amphipathic proline-rich peptides derived from the N-terminal domain of gamma-zein.** *Angew Chem Int Ed Engl* 2004, **43**:1811–1814.

23. Fernandez-Carneado J, Kogan MJ, Pujals S, and Giralt E. **Amphipathic peptides and drug delivery.** *Biopolymers* 2004, **76**:196–203.

24. Lin YZ, Yao SY, Veach RA, Torgerson TR, and Hawiger J. **Inhibition of nuclear translocation of transcription factor NF-kappa B by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence.** *J Biol Chem* 1995, **270**:14255–14258.

25. Langel U, Pooga M, Kairane C, Zilmer M, and Bartfai T. **A galanin-mastoparan chimeric peptide activates the Na+,K(+)-ATPase and reverses its inhibition by ouabain.** *Regul Pept* 1996, **62**:47–52.

26. Chaloin L, Vidal P, Heitz A, Van Mau N, Mery J, Divita G, and Heitz F. **Conformations of primary amphipathic carrier peptides in membrane mimicking environments.** *Biochemistry* 1997, **36**:11179–11187.

27. Rothbard JB, Kreider E, VanDeusen CL, Wright L, Wylie BL, and Wender PA. **Arginine-rich molecular transporters for drug delivery: role of backbone spacing in cellular uptake.** *J Med Chem* 2002, **45**:3612–3618.

28. Rothbard JB, Garlington S, Lin Q, Kirschberg T, Kreider E, McGrane PL, Wender PA, and Khavari PA. **Conjugation of arginine oligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation.** *Nat Med* 2000, **6**:1253–1257.

29. Shibagaki N and Udey MC. **Dendritic cells transduced with protein antigens induce cytotoxic lymphocytes and elicit antitumor immunity.** *J Immunol* 2002, **168**:2393–2401.

30. Astriab-Fisher A, Sergueev D, Fisher M, Shaw BR, and Juliano RL. **Conjugates of antisense oligonucleotides with the Tat and antennapedia cell-penetrating peptides: effects on cellular uptake, binding to target sequences, and biologic actions.** *Pharm Res* 2002, **19**:744–754.

31. Nagahara H, Vocero-Akbani AM, Snyder EL, Ho A, Latham DG, Lissy NA, Becker-Hapak M, Ezhevsky SA, and Dowdy SF. **Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration.** *Nat Med* 1998, **4**:1449–1452.

32. Schwarze SR, Ho A, Vocero-Akbani A, and Dowdy SF. **In vivo protein transduction: Delivery of a biologically active protein into the mouse.** *Science* 1999, **285**:1569–1572.

33. Lewin M, Carlesso N, Tung CH, Tang XW, Cory D, Scadden DT, and Weissleder R. **Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells.** *Nat Biotechnol* 2000, **18**:410–414.

34. Torchilin VP, Rammohan R, Weissig V, and Levchenko TS. **TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors.** *Proc Natl Acad Sci U S A* 2001, **98**:8786–8791.

35. Richard JP, Melikov K, Vives E, Ramos C, Verbeure B, Gait MJ, Chernomordik LV, and Lebleu B. **Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake.** *J Biol Chem* 2003, **278**:585–590.

36. Silhol M, Tyagi M, Giacca M, Lebleu B, and Vives E. **Different mechanisms for cellular internalization of the HIV-1 Tat-derived cell penetrating peptide and recombinant proteins fused to Tat.** *Eur J Biochem* 2002, **269**:494–501.

37. Tunnemann G, Martin RM, Haupt S, Patsch C, Edenhofer F, and Cardoso MC. **Cargo-dependent mode of uptake and bioavailability of TAT-containing proteins and peptides in living cells.** *FASEB J* 2006, **20**:1775–1784.

38. Fittipaldi A, Ferrari A, Zoppe M, Arcangeli C, Pellegrini V, Beltram F, and Giacca M. **Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins.** *J Biol Chem* 2003, **278**:34141–34149.

39. Lundberg M, Wikstrom S, and Johansson M. **Cell surface adherence and endocytosis of protein transduction domains.** *Mol Ther* 2003, **8**:143–150.

40. Nakase I, Tadokoro A, Kawabata N, Takeuchi T, Katoh H, Hiramoto K, Negishi M, Nomizu M, Sugiura Y, and Futaki S. **Interaction of arginine-rich peptides with membrane-associated proteoglycans is crucial for induction of actin organization and macropinocytosis.** *Biochemistry* 2007, **46**:492–501.

41. Ruan G, Agrawal A, Marcus AI, and Nie S. **Imaging and tracking of tat peptide-conjugated quantum dots in living cells: New insights into nanoparticle uptake, intracellular transport, and vesicle shedding.** *J Am Chem Soc* 2007, **129**:14759–14766.

42. Wadia JS, Stan RV, and Dowdy SF. **Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis.** *Nat Med* 2004, **10**:310–315.

43. Richard JP, Melikov K, Brooks H, Prevot P, Lebleu B, and Chernomordik LV. **Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors.** *J Biol Chem* 2005, **280**:15300–15306.

44. Brooks H, Lebleu B, and Vives E. **Tat peptide-mediated cellular delivery: Back to basics.** *Adv Drug Deliv Rev* 2005, **57**:559–577.

45. Duchardt F, Fotin-Mleczek M, Schwarz H, Fischer R, and Brock R. **A comprehensive model for the cellular uptake of cationic cell-penetrating peptides.** *Traffic* 2007, **8**:848–866.

46. Mann DA and Frankel AD. **Endocytosis and targeting of exogenous HIV-1 Tat protein.** *Embo J* 1991, **10**:1733–1739.

47. Tyagi M, Rusnati M, Presta M, and Giacca M. **Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans.** *J Biol Chem* 2001, **276**:3254–3261.

48. Ziegler A and Seelig J. **Interaction of the protein transduction domain of HIV-1 TAT with heparan sulfate: binding mechanism and thermodynamic parameters.** *Biophys J* 2004, **86**:254–263.

49. Goncalves E, Kitas E, and Seelig J. **Binding of oligoarginine to membrane lipids and heparan sulfate: Structural and thermodynamic characterization of a cell-penetrating peptide.** *Biochemistry* 2005, **44**:2692–2702.

50. Peitz M, Pfannkuche K, Rajewsky K, and Edenhofer F. **Ability of the hydrophobic FGF and basic TAT peptides to promote cellular uptake of recombinant Cre recombinase: a tool for efficient genetic engineering of mammalian genomes.** *Proc Natl Acad Sci U S A* 2002, **99**:4489–4494.

51. Nolden L, Edenhofer F, Haupt S, Koch P, Wunderlich FT, Siemen H, and Brustle O. **Site-specific recombination in human embryonic stem cells induced by cell-permeant Cre recombinase.** *Nat Methods* 2006, **3**:461–467.

52. Dietz GP and Bahr M. **Delivery of bioactive molecules into the cell: The Trojan horse approach.** *Mol Cell Neurosci* 2004, **27**:85–131.

53. Maiolo JR III, Ottinger EA, and Ferrer M. **Specific redistribution of cell-penetrating peptides from endosomes to the cytoplasm and nucleus upon laser illumination.** *J Am Chem Soc* 2004, **126**:15376–15377.

54. Caron NJ, Quenneville SP, and Tremblay JP. **Endosome disruption enhances the functional nuclear delivery of Tat-fusion proteins.** *Biochem Biophys Res Commun* 2004, **319**:12–20.

55. Shiraishi T, Pankratova S, and Nielsen PE. **Calcium ions effectively enhance the effect of antisense peptide nucleic acids conjugated to cationic TAT and oligoarginine peptides.** *Chem Biol* 2005, **12**:923–929.

56. Hogset A, Prasmickaite L, Hellum M, Engesaeter BO, Olsen VM, Tjelle TE, Wheeler CJ, and Berg K. **Photochemical transfection: A technology for efficient light-directed gene delivery.** *Somat Cell Mol Genet* 2002, **27**:97–113.

57. Shiraishi T and Nielsen PE. **Photochemically enhanced cellular delivery of cell penetrating peptide-PNA conjugates.** *FEBS Lett* 2006, **580**:1451–1456.

58. Vives E. **Cellular uptake [correction of utake] of the Tat peptide: An endocytosis mechanism following ionic interactions.** *J Mol Recognit* 2003, **16**: 265–271.

59. Kaplan IM, Wadia JS, and Dowdy SF. **Cationic TAT peptide transduction domain enters cells by macropinocytosis.** *J Control Release* 2005, **102**:247–253.

60. Magzoub M, Sandgren S, Lundberg P, Oglecka K, Lilja J, Wittrup A, Goran Eriksson LE, Langel U, Belting M, and Graslund A. **N-terminal peptides from unprocessed prion proteins enter cells by macropinocytosis.** *Biochem Biophys Res Commun* 2006, **348**:379–385.

61. Mai JC, Shen H, Watkins SC, Cheng T, and Robbins PD. **Efficiency of protein transduction is cell type-dependent and is enhanced by dextran sulfate.** *J Biol Chem* 2002, **277**:30208–30218.

62. Ziegler A, Nervi P, Durrenberger M, and Seelig J. **The cationic cell-penetrating peptide CPP(TAT) derived from the HIV-1 protein TAT is rapidly transported into living fibroblasts: optical, biophysical, and metabolic evidence.** *Biochemistry* 2005, **44**:138–148.

63. Fretz MM, Penning NA, Al-Taei S, Futaki S, Takeuchi T, Nakase I, Storm G, and Jones AT. **Temperature-, concentration- and cholesterol-dependent translocation of L- and D-octa-arginine across the plasma and nuclear membrane of CD34+ leukaemia cells.** *Biochem J* 2007, **403**:335–342.

64. Brugidou J, Legrand C, Mery J, and Rabie A. **The retro-inverso form of a homeobox-derived short peptide is rapidly internalised by cultured neurones: A new basis for an efficient intracellular delivery system.** *Biochem Biophys Res Commun* 1995, **214**:685–693.

65. Derossi D, Calvet S, Trembleau A, Brunissen A, Chassaing G, and Prochiantz A. **Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent.** *J Biol Chem* 1996, **271**:18188–18193.

66. Futaki S, Nakase I, Suzuki T, Youjun Z, and Sugiura Y. **Translocation of branched-chain arginine peptides through cell membranes: Flexibility in the spatial disposition of positive charges in membrane-permeable peptides.** *Biochemistry* 2002, **41**:7925–7930.

67. Martin RM, Tunnemann G, Leonhardt H, and Cardoso MC. **Nucleolar marker for living cells.** *Histochem Cell Biol* 2007, **127**:243–251.

68. Bjorklund J, Biverstahl H, Graslund A, Maler L, and Brzezinski P. **Real-time transmembrane translocation of penetratin driven by light-generated proton pumping.** *Biophys J* 2006, **91**:L29–L31.

69. Rothbard JB, Jessop TC, Lewis RS, Murray BA, and Wender PA. **Role of membrane potential and hydrogen bonding in the mechanism of translocation of guanidinium-rich peptides into cells.** *J Am Chem Soc* 2004, **126**:9506–9507.

70. Rothbard JB, Jessop TC, and Wender PA. **Adaptive translocation: the role of hydrogen bonding and membrane potential in the uptake of guanidinium-rich transporters into cells.** *Adv Drug Deliv Rev* 2005, **57**:495–504.

71. Afonin S, Frey A, Bayerl S, Fischer D, Wadhwani P, Weinkauf S, and Ulrich AS. **The cell-penetrating peptide TAT(48-60) induces a non-lamellar phase in DMPC membranes.** *Chemphyschem* 2006, **7**:2134–2142.

72. Shaw JE, Epand RF, Hsu JC, Mo GC, Epand RM, and Yip CM. **Cationic peptide-induced remodelling of model membranes: Direct visualization by in situ atomic force microscopy.** *J Struct Biol* 2007, **162**(1):121–138.

73. Glaser RW, Sachse C, Durr UH, Wadhwani P, Afonin S, Strandberg E, and Ulrich AS. **Concentration-dependent realignment of the antimicrobial peptide PGLa in lipid membranes observed by solid-state 19F-NMR.** *Biophys J* 2005, **88**:3392–3397.

74. Bellet-Amalric E, Blaudez D, Desbat B, Graner F, Gauthier F, and Renault A. **Interaction of the third helix of Antennapedia homeodomain and a phospholipid monolayer, studied by ellipsometry and PM-IRRAS at the air-water interface.** *Biochim Biophys Acta* 2000, **1467**:131–143.

75. Ziegler A, Blatter XL, Seelig A, and Seelig J. **Protein transduction domains of HIV-1 and SIV TAT interact with charged lipid vesicles. Binding mechanism and thermodynamic analysis.** *Biochemistry* 2003, **42**:9185–9194.

76. Sheldon K, Liu D, Ferguson J, and Gariepy J. **Loligomers: Design of de novo peptide-based intracellular vehicles.** *Proc Natl Acad Sci U S A* 1995, **92**:2056–2060.

77. Mitchell DJ, Kim DT, Steinman L, Fathman CG, and Rothbard JB. **Polyarginine enters cells more efficiently than other polycationic homopolymers.** *J Pept Res* 2000, **56**:318–325.

78. Hallbrink M, Oehlke J, Papsdorf G, and Bienert M. **Uptake of cell-penetrating peptides is dependent on peptide-to-cell ratio rather than on peptide concentration.** *Biochim Biophys Acta* 2004, **1667**:222–228.

79. Lindberg M, Jarvet J, Langel U, and Graslund A. **Secondary structure and position of the cell-penetrating peptide transportan in SDS micelles as determined by NMR.** *Biochemistry* 2001, **40**:3141–3149.

80. Dathe M and Wieprecht T. **Structural features of helical antimicrobial peptides: Their potential to modulate activity on model membranes and biological cells.** *Biochim Biophys Acta* 1999, **1462**:71–87.

81. Matsuzaki K, Yoneyama S, Murase O, and Miyajima K. **Transbilayer transport of ions and lipids coupled with mastoparan X translocation.** *Biochemistry* 1996, **35**:8450–8456.

82. Futaki S. **Peptide ion channels: design and creation of function.** *Biopolymers* 1998, **47**:75–81.

83. Thoren PE, Persson D, Karlsson M, and Norden B. **The antennapedia peptide penetratin translocates across lipid bilayers - the first direct observation.** *FEBS Lett* 2000, **482**:265–268.

84. Tunnemann G, Ter-Avetisyan G, Martin RM, Stockl M, Herrmann A, and Cardoso MC. **Live-cell analysis of cell penetration ability and toxicity of oligo-arginines.** *J Pept Sci* 2008, **14**:469–476.

85. Hallbrink M, Floren A, Elmquist A, Pooga M, Bartfai T, and Langel U. **Cargo delivery kinetics of cell-penetrating peptides.** *Biochim Biophys Acta* 2001, **1515**:101–109.

86. Berlose JP, Convert O, Derossi D, Brunissen A, and Chassaing G. **Conformational and associative behaviours of the third helix of antennapedia homeodomain in membrane-mimetic environments.** *Eur J Biochem* 1996, **242**:372–386.

87. Derossi D, Chassaing G, and Prochiantz A. **Trojan peptides: The penetratin system for intracellular delivery.** *Trends Cell Biol* 1998, **8**:84–87.

88. Joliot A and Prochiantz A. **Transduction peptides: From technology to physiology.** *Nat Cell Biol* 2004, **6**:189–196.

89. Magzoub M, Eriksson LE, and Graslund A. **Comparison of the interaction, positioning, structure induction and membrane perturbation of cell-penetrating peptides and non-translocating variants with phospholipid vesicles.** *Biophys Chem* 2003, **103**:271–288.

90. Binder H and Lindblom G. **A molecular view on the interaction of the trojan peptide penetratin with the polar interface of lipid bilayers.** *Biophys J* 2004, **87**:332–343.

91. Luedtke NW, Carmichael P, and Tor Y. **Cellular uptake of aminoglycosides, guanidinoglycosides, and poly-arginine.** *J Am Chem Soc* 2003, **125**: 12374–12375.

92. Chung HH, Harms G, Seong CM, Choi BH, Min C, Taulane JP, and Goodman M. **Dendritic oligoguanidines as intracellular translocators.** *Biopolymers* 2004, **76**:83–96.

93. Herce HD and Garcia AE. **Molecular dynamics simulations suggest a mechanism for translocation of the HIV-1 TAT peptide across lipid membranes.** *Proc Natl Acad Sci U S A* 2007, **104**:20805–20810.

94. Eilers M, Verner K, Hwang S, and Schatz G. **Import of proteins into mitochondria.** *Philos Trans R Soc Lond B Biol Sci* 1988, **319**:121–126.

95. Verner K and Schatz G. **Protein translocation across membranes.** *Science* 1988, **241**:1307–1313.

96. Bonifaci N, Sitia R, and Rubartelli A. **Nuclear translocation of an exogenous fusion protein containing HIV Tat requires unfolding.** *Aids* 1995, **9**:995–1000.

97. Becker-Hapak M, McAllister SS, and Dowdy SF. **TAT-mediated protein transduction into mammalian cells.** *Methods* 2001, **24**:247–256.

98. Kwon HY, Eum WS, Jang HW, Kang JH, Ryu J, Ryong Lee B, Jin LH, Park J, and Choi SY. **Transduction of Cu,Zn-superoxide dismutase mediated by an HIV-1 Tat protein basic domain into mammalian cells.** *FEBS Lett* 2000, **485**:163–167.

99. Saar K, Lindgren M, Hansen M, Eiriksdottir E, Jiang Y, Rosenthal-Aizman K, Sassian M, and Langel U. **Cell-penetrating peptides: A comparative membrane toxicity study.** *Anal Biochem* 2005, **345**:55–65.

100. El-Andaloussi S, Jarver P, Johansson HJ, and Langel U. **Cargo-dependent cytotoxicity and delivery efficacy of cell-penetrating peptides: A comparative study.** *Biochem J* 2007, **407**:285–292.

101. Jones SW, Christison R, Bundell K, Voyce CJ, Brockbank SM, Newham P, and Lindsay MA. **Characterisation of cell-penetrating peptide-mediated peptide delivery.** *Br J Pharmacol* 2005, **145**:1093–1102.

102. Cardozo AK, Buchillier V, Mathieu M, Chen J, Ortis F, Ladriere L, Allaman-Pillet N, Poirot O, Kellenberger S, Beckmann JS, Eizirik DL, Bonny C, and Maurer F. **Cell-permeable peptides induce dose- and length-dependent cytotoxic effects.** *Biochim Biophys Acta* 2007, **1768**:2222–2234.

103. Moschos SA, Jones SW, Perry MM, Williams AE, Erjefalt JS, Turner JJ, Barnes PJ, Sproat BS, Gait MJ, and Lindsay MA. **Lung delivery studies using siRNA conjugated to TAT(48-60) and penetratin reveal peptide induced reduction in gene expression and induction of innate immunity.** *Bioconjug Chem* 2007, **18**:1450–1459.

104. Abes S, Moulton HM, Clair P, Prevot P, Youngblood DS, Wu RP, Iversen PL, and Lebleu B. **Vectorization of morpholino oligomers by the (R-Ahx-R)4 peptide allows efficient splicing correction in the absence of endosomolytic agents.** *J Control Release* 2006, **116**:304–313.

105. Youngblood DS, Hatlevig SA, Hassinger JN, Iversen PL, and Moulton HM. **Stability of cell-penetrating peptide-morpholino oligomer conjugates in human serum and in cells.** *Bioconjug Chem* 2007, **18**:50–60.

106. Amantana A, Moulton HM, Cate ML, Reddy MT, Whitehead T, Hassinger JN, Youngblood DS, and Iversen PL. **Pharmacokinetics, biodistribution, stability and toxicity of a cell-penetrating peptide-morpholino oligomer conjugate.** *Bioconjug Chem* 2007, **18**:1325–1331.

107. Tanaka S, Pero SC, Taguchi K, Shimada M, Mori M, Krag DN, and Arii S. **Specific peptide ligand for Grb7 signal transduction protein and pancreatic cancer metastasis.** *J Natl Cancer Inst* 2006, **98**:491–498.

108. Josephson L, Tung CH, Moore A, and Weissleder R. **High-efficiency intracellular magnetic labeling with novel superparamagnetic-Tat peptide conjugates.** *Bioconjug Chem* 1999, **10**:186–191.

109. Zhao M, Kircher MF, Josephson L, and Weissleder R. **Differential conjugation of tat peptide to superparamagnetic nanoparticles and its effect on cellular uptake.** *Bioconjug Chem* 2002, **13**:840–844.

110. Christian NA, Milone MC, Ranka SS, Li G, Frail PR, Davis KP, Bates FS, Therien MJ, Ghoroghchian PP, June CH, and Hammer DA. **Tat-functionalized near-infrared emissive polymersomes for dendritic cell labeling.** *Bioconjug Chem* 2007, **18**:31–40.

111. Polyakov V, Sharma V, Dahlheimer JL, Pica CM, Luker GD, and Piwnica-Worms D. **Novel Tat-peptide chelates for direct transduction of technetium-99m and rhenium into human cells for imaging and radiotherapy.** *Bioconjug Chem* 2000, **11**:762–771.

112. Bullok KE, Dyszlewski M, Prior JL, Pica CM, Sharma V, and Piwnica-Worms D. **Characterization of novel histidine-tagged Tat-peptide complexes dual-labeled with (99m)Tc-tricarbonyl and fluorescein for scintigraphy and fluorescence microscopy.** *Bioconjug Chem* 2002, **13**:1226–1237.

113. Bullok KE, Gammon ST, Violini S, Prantner AM, Villalobos VM, Sharma V, and Piwnica-Worms D. **Permeation peptide conjugates for in vivo molecular imaging applications.** *Mol Imaging* 2006, **5**:1–15.

114. Jiang T, Olson ES, Nguyen QT, Roy M, Jennings PA, and Tsien RY. **Tumor imaging by means of proteolytic activation of cell-penetrating peptides.** *Proc Natl Acad Sci U S A* 2004, **101**:17867–17872.

115. Haase H, Dobbernack G, Tunnemann G, Karczewski P, Cardoso C, Petzhold D, Schlegel WP, Lutter S, Pierschalek P, Behlke J, and Morano I. **Minigenes encoding N-terminal domains of human cardiac myosin light chain-1 improve heart function of transgenic rats.** *FASEB J* 2006, **20**:865–873.

116. Nori A, Jensen KD, Tijerina M, Kopeckova P, and Kopecek J. **Subcellular trafficking of HPMA copolymer-Tat conjugates in human ovarian carcinoma cells.** *J Control Release* 2003, **91**:53–59.

117. Nori A, Jensen KD, Tijerina M, Kopeckova P, and Kopecek J. **Tat-conjugated synthetic macromolecules facilitate cytoplasmic drug delivery to human ovarian carcinoma cells.** *Bioconjug Chem* 2003, **14**:44–50.

118. Rousselle C, Clair P, Lefauconnier JM, Kaczorek M, Scherrmann JM, and Temsamani J. **New advances in the transport of doxorubicin through the blood-brain barrier by a peptide vector-mediated strategy.** *Mol Pharmacol* 2000, **57**:679–686.

119. Rousselle C, Clair P, Temsamani J, and Scherrmann JM. **Improved brain delivery of benzylpenicillin with a peptide-vector-mediated strategy.** *J Drug Target* 2002, **10**:309–315.

120. Okuyama M, Laman H, Kingsbury SR, Visintin C, Leo E, Eward KL, Stoeber K, Boshoff C, Williams GH, and Selwood DL. **Small-molecule mimics of an alpha-helix for efficient transport of proteins into cells.** *Nat Methods* 2007, **4**:153–159.

121. Law B, Quinti L, Choi Y, Weissleder R, and Tung CH. **A mitochondrial targeted fusion peptide exhibits remarkable cytotoxicity.** *Mol Cancer Ther* 2006, **5**:1944–1949.

122. Hotchkiss RS, McConnell KW, Bullok K, Davis CG, Chang KC, Schwulst SJ, Dunne JC, Dietz GP, Bahr M, McDunn JE, Karl IE, Wagner TH, Cobb JP, Coopersmith CM, and Piwnica-Worms D. **TAT-BH4 and TAT-Bcl-xL peptides protect against sepsis-induced lymphocyte apoptosis in vivo.** *J Immunol* 2006, **176**:5471–5477.

123. Sugioka R, Shimizu S, Funatsu T, Tamagawa H, Sawa Y, Kawakami T, and Tsujimoto Y. **BH4-domain peptide from Bcl-xL exerts anti-apoptotic activity in vivo.** *Oncogene* 2003, **22**:8432–8440.

124. Ono M, Sawa Y, Ryugo M, Alechine AN, Shimizu S, Sugioka R, Tsujimoto Y, and Matsuda H. **BH4 peptide derivative from Bcl-xL attenuates ischemia/reperfusion injury thorough anti-apoptotic mechanism in rat hearts.** *Eur J Cardiothorac Surg* 2005, **27**:117–121.

125. Repici M, Centeno C, Tomasi S, Forloni G, Bonny C, Vercelli A, and Borsello T. **Time-course of c-Jun N-terminal kinase activation after cerebral ischemia and effect of D-JNKI1 on c-Jun and caspase-3 activation.** *Neuroscience* 2007, **150**:40–49.

126. Eum WS, Choung IS, Li MZ, Kang JH, Kim DW, Park J, Kwon HY, and Choi SY. **HIV-1 Tat-mediated protein transduction of Cu,Zn-superoxide dismutase into pancreatic beta cells in vitro and in vivo.** *Free Radic Biol Med* 2004, **37**:339–349.

127. Bernatchez PN, Bauer PM, Yu J, Prendergast JS, He P, and Sessa WC. **Dissecting the molecular control of endothelial NO synthase by caveolin-1 using cell-permeable peptides.** *Proc Natl Acad Sci U S A* 2005, **102**:761–766.

128. McCusker CT, Wang Y, Shan J, Kinyanjui MW, Villeneuve A, Michael H, and Fixman ED. **Inhibition of experimental allergic airways disease by local application of a cell-penetrating dominant-negative STAT-6 peptide.** *J Immunol* 2007, **179**:2556–2564.

129. Toro A and Grunebaum E. **TAT-mediated intracellular delivery of purine nucleoside phosphorylase corrects its deficiency in mice.** *J Clin Invest* 2006, **116**:2717–2726.

130. Tunnemann G, Karczewski P, Haase H, Cardoso MC, and Morano I. **Modulation of muscle contraction by a cell-permeable peptide.** *J Mol Med* 2007, **85**: 1405–1412.

131. Turner JJ, Ivanova GD, Verbeure B, Williams D, Arzumanov AA, Abes S, Lebleu B, and Gait MJ. **Cell-penetrating peptide conjugates of peptide nucleic acids (PNA) as inhibitors of HIV-1 Tat-dependent trans-activation in cells.** *Nucleic Acids Res* 2005, **33**:6837–6849.

132. Abes S, Turner JJ, Ivanova GD, Owen D, Williams D, Arzumanov A, Clair P, Gait MJ, and Lebleu B. **Efficient splicing correction by PNA conjugation to an R6-Penetratin delivery peptide.** *Nucleic Acids Res* 2007, **35**:4495–4502.

133. Moulton HM, Fletcher S, Neuman BW, McClorey G, Stein DA, Abes S, Wilton SD, Buchmeier MJ, Lebleu B, and Iversen PL. **Cell-penetrating peptide-morpholino conjugates alter pre-mRNA splicing of DMD (Duchenne muscular dystrophy) and inhibit murine coronavirus replication in vivo.** *Biochem Soc Trans* 2007, **35**:826–828.

134. Endoh T, Sisido M, and Ohtsuki T. **Photo inducible RNA interference using cell permeable protein carrier.** *Nucleic Acids Symp Ser (Oxf)* 2007:127–128.

135. Simeoni F, Morris MC, Heitz F, and Divita G. **Insight into the mechanism of the peptide-based gene delivery system MPG. implications for delivery of siRNA into mammalian cells.** *Nucleic Acids Res* 2003, **31**:2717–2724.

136. Unnamalai N, Kang BG, and Lee WS. **Cationic oligopeptide-mediated delivery of dsRNA for post-transcriptional gene silencing in plant cells.** *FEBS Lett* 2004, **566**:307–310.

137. Gratton JP, Yu J, Griffith JW, Babbitt RW, Scotland RS, Hickey R, Giordano FJ, and Sessa WC. **Cell-permeable peptides improve cellular uptake and therapeutic gene delivery of replication-deficient viruses in cells and in vivo.** *Nat Med* 2003, **9**:357–362.

138. Lavigne MD, Yates L, Coxhead P, and Gorecki DC. **Nuclear-targeted chimeric vector enhancing nonviral gene transfer into skeletal muscle of Fabry mice in vivo.** *FASEB J* 2008, **22**(6):2097–2107.

139. Chen L, Hahn H, Wu G, Chen CH, Liron T, Schechtman D, Cavallaro G, Banci L, Guo Y, Bolli R, Dorn GW 2nd, and Mochly-Rosen D. **Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC.** *Proc Natl Acad Sci U S A* 2001, **98**:11114–11119.

140. Takakura Y, Mahato RI, and Hashida M. **Extravasation of macromolecules.** *Adv Drug Deliv Rev* 1998, **34**:93–108.

141. Cao G, Pei W, Ge H, Liang Q, Luo Y, Sharp FR, Lu A, Ran R, Graham SH, and Chen J. **In vivo delivery of a bcl-xl fusion protein containing the tat protein transduction domain protects against ischemic brain injury and neuronal apoptosis.** *J Neurosci* 2002, **22**:5423–5431.

142. Ju KL, Manley NC, and Sapolsky RM. **Anti-apoptotic therapy with a Tat fusion protein protects against excitotoxic insults in vitro and in vivo.** *Exp Neurol* 2007, **210**:602–607.

143. Asoh S, Ohsawa I, Mori T, Katsura K, Hiraide T, Katayama Y, Kimura M, Ozaki D, Yamagata K, and Ohta S. **Protection against ischemic brain injury by protein therapeutics.** *Proc Natl Acad Sci U S A* 2002, **99**:17107–17112.

144. Nakashima-Kamimura N, Nishimaki K, Mori T, Asoh S, and Ohta S. **Prevention of chemotherapy-induced alopecia by the anti-death FNK protein.** *Life Sci* 2008, **82**:218–225.

145. Dave SH, Tilstra JS, Matsuoka K, Li F, Karrasch T, Uno JK, Sepulveda AR, Jobin C, Baldwin AS, Robbins PD, and Plevy SE. **Amelioration of chronic murine colitis by peptide-mediated transduction of the IkappaB kinase inhibitor NEMO binding domain peptide.** *J Immunol* 2007, **179**:7852–7859.

146. Kwon JH, Kim JB, Lee KH, Kang SM, Chung N, Jang Y, and Chung JH. **Protective effect of heat shock protein 27 using protein transduction domain-mediated delivery on ischemia/reperfusion heart injury.** *Biochem Biophys Res Commun* 2007, **363**:399–404.

147. Li T, Fan Y, Luo Y, Xiao B, and Lu C. **In vivo delivery of a XIAP (BIR3-RING) fusion protein containing the protein transduction domain protects against neuronal death induced by seizures.** *Exp Neurol* 2006, **197**:301–308.

148. Koya V, Lu S, Sun YP, Purich DL, Atkinson MA, Li SW, and Yang LJ. **Reversal of streptozotocin-induced diabetes in mice by cellular transduction with recombinant pancreatic transcription factor pdx1 - a novel protein transduction domain based therapy.** *Diabetes* 2007, **57(3)**:757–769.

149. Popiel HA, Nagai Y, Fujikake N, and Toda T. **Protein transduction domain-mediated delivery of QBP1 suppresses polyglutamine-induced neurodegeneration in vivo.** *Mol Ther* 2007, **15**:303–309.

150. Mai JC, Mi Z, Kim SH, Ng B, and Robbins PD. **A proapoptotic peptide for the treatment of solid tumors.** *Cancer Res* 2001, **61**:7709–7712.

151. Bright R, Steinberg GK, and Mochly-Rosen D. **DeltaPKC mediates micro-cerebrovascular dysfunction in acute ischemia and in chronic hypertensive stress in vivo.** *Brain Res* 2007, **1144**:146–155.

152. Fletcher S, Honeyman K, Fall AM, Harding PL, Johnsen RD, Steinhaus JP, Moulton HM, Iversen PL, and Wilton SD. **Morpholino oligomer-mediated exon skipping averts the onset of dystrophic pathology in the mdx mouse.** *Mol Ther* 2007, **15**:1587–1592.

153. Zhou JP, Feng ZG, Yuan BL, Yu SZ, Li Q, Qu HY, and Sun MJ. **Transduced PTD-BDNF fusion protein protects against beta amyloid peptide-induced learning and memory deficits in mice.** *Brain Res* 2008, **1191**:12–19.

Fig. 1

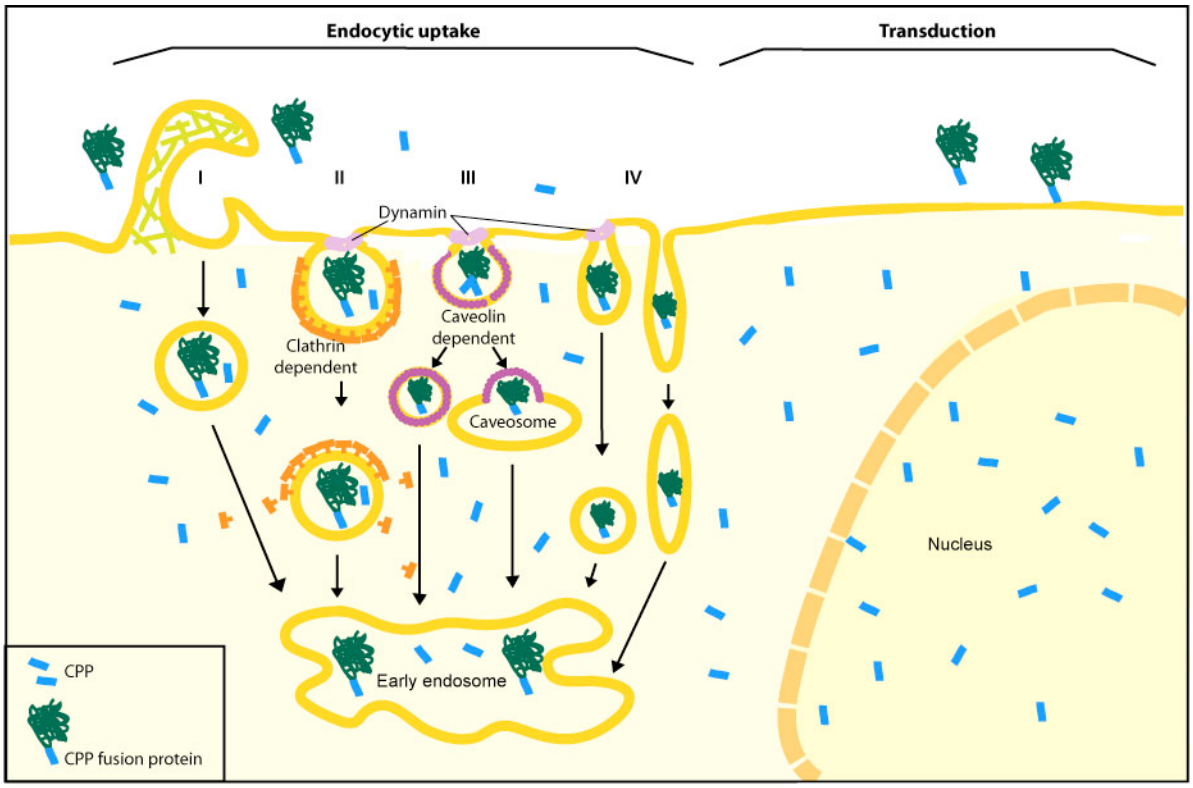
Endocytic uptake
Transduction
I
II
III
IV
Dynamin
Clathrin dependent
Caveolin dependent
Caveosome
Nucleus
Early endosome
CPP
CPP fusion protein

Fig. 2