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Review article

Chances and pitfalls of cell penetrating peptides for cellular drug delivery

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Abstract

Over the past decade, several classes and/or prototypes of cell penetrating peptides (CPP) have been identified and investigated in multiple aspects. CPP represent peptides, which show the ability to cross the plasma membrane of mammalian cells, and may thus give rise to the intracellular delivery of problematic therapeutic cargos, such as peptides, proteins, oligonucleotides, plasmids and even nanometer-sized particles, which otherwise cannot cross the plasma membrane. Most of the currently recognized CPP are of cationic nature and derived from viral, insect or mammalian proteins endowed with membrane translocation properties. The exact mechanisms underlying the translocation of CPP across the cellular membrane are still poorly understood. However, several similarities in translocation can be found. Early studies on CPP translocation mechanisms tended to suggest that the internalization of these peptides was neither significantly inhibited by low temperature, depletion of the cellular adenosine triphosphate (ATP) pool, nor by inhibitors of endocytosis. Moreover, chemical modification of the peptide sequence, such as the synthesis of retro-, enantio- or retroenantio-analogs, appeared not to affect the internalization properties. Therefore, translocation was concluded to result from direct, physical transfer through the lipid bilayer of the cell membrane. Later studies, however, showed convincing evidence for the involvement of endocytosis as the dominating mechanism for cellular internalization. In addition to describing the general properties of the commonly recognized classes of CPP, in this review we will also point out some limitations and typical pitfalls of CPP as carriers for therapeutics. In particular we will comment on emerging discrepancies with the current dogma, on cell-to-cell variability, biological barrier permeability, metabolic fate, toxicity and immunogenicity of CPP.

Keywords: Cell penetrating peptides; Cellular drug delivery; Endocytosis; Permeation; Biological barriers; Toxicity

1. Introduction

Over the past decade, an increasing number of potential protein- or DNA-based drugs have been suggested for therapeutic application. Often these biomacromolecules show a limited ability to cross the plasma membrane resulting in poor cellular access, which largely prohibits them from reaching intracellular targets and from crossing epithelial or endothelial barriers. Several approaches have been proposed to overcome such limitations, including micro-injection [1,2], electroporation [3], viral delivery systems [4], liposomes [5,6], encapsulation in polymers, and receptor mediated endocytosis [7]. Unfortunately, these approaches are often plagued with limited efficiency, or cause appreciable cellular toxicity. The discovery of several peptides with the ability to translocate the plasma membrane of eukaryotic cells by a possibly receptor- and endocytosis-independent mechanism, has opened a new avenue in biomedical research [8–10]. Among the so-called cell penetrating peptides (CPP), Tat, penetratin and VP22 are the most widely known ones and have been intensely studied since their discovery. In addition to these, further peptide sequences featuring cell penetrating properties have been described in the literature and are summarized in Table 1. Several naturally occurring CPP are constituents of hydrophobic signaling sequences and tend to accumulate in the cell nucleus when coupled to a nuclear localization sequence (NLS). Examples are sequences derived from Kaposi's sarcoma fibroblast growth factor 1 (K-FGF) [11], human β_3 integrin [12], HIV-1 gp41 [13], or the signal sequence of the variable immunoglobulin light chain Ig(v) from *Caïman crocodylus* [14] conjugated to NLS peptides originating from the nuclear transcription factor κB (NF-κB) [15], Simian virus 40 (SV40) T-antigen [16] or K-FGF [17]. On the other hand, engineered CPP are often chimeric

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Table 1
Sequences of selected cell penetrating peptides

Names	Sequences	Ref.
Protein-derived peptides		
Tat(49–57)	RKKRRQRRR	[35]
Penetratin(43–58)	RQIKIWFQNRRMKWKK	[26,44]
VP22	DAATATRGRSAASRPTERPRAPARSASRPRRPVD	[27]
Kaposi FGF signal sequences	AAVALLPAVLLALLAP, AAVLLPVLLAAP	[11,17,130]
Human β3 integrin signal sequence	VTVLALGALAGVGVG	[15,20]
gp41 fusion sequence	GALFLGWLGAAGSTMGA	[13,16]
Caiman crocodylus Ig(v) light chain	MGLGLHLLVLAAALQGA	[14]
hCT derived peptide	LGTYTQDFNKFHTFPQTAIGVGAP	[22,71]
Synthetic/chimeric peptides		
Transportan	GWTLNSAGYLLKINLKALAALAKKIL	[18]
Loligomer	$(TPPKKKRKVEDPKKKKK)_8^-$	[19]
Arginine peptide	RRRRRRR	[20,131]
Amphiphilic model peptide	KLALKLALKALKAALKLA	[21]

and contain sequences of various functions for reaching synergistic translocation efficiency. For example, the chimeric 27 amino acid peptide transportan is a combination of the N-terminal fragment of the neuropeptide galanin and the membrane interacting wasp venom peptide, mastoparan [18]. Another example is the branched peptide loligomer, which is a hybrid of the SV40 NLS and a lysine pentapeptide acting as cytoplasmic translocation signal [19]. Homooligopeptides containing more than seven arginines, but less than 15 [20], as well as an 18-mer amphiphilic model peptide [21] also represent efficient CPP. Finally, another family of CPP under investigation derives from the C-terminal fragment of human calcitonin (hCT) from residues 9 to 32, i.e. hCT(9–32), down to residues 18–32, i.e. hCT(18–32) [22–24]. These peptides are modestly cationic and feature a hydrophobic C-terminal domain.

In the present work, we will mainly focus on a discussion of Tat derived peptides, penetratin, VP22 and address their use as carrier peptides. In addition, we will discuss some limitations of their application as cellular shuttles that have recently come under scrutiny. In this context we will also review selected aspects of hCT derived peptides.

2. Mechanisms of internalization

Because of its hydrophobic interior, the lipid bilayer of the plasma membrane represents a highly impermeable barrier to most polar molecules. In order to translocate small molecules and ions into or out of cells, the plasma membrane contains gated ion channels and pumps. For certain molecules, cellular influx and efflux are regulated by highly selective transmembrane transporter proteins. For large molecules and particles, either receptor-dependent or -independent endocytosis is involved in cellular internalization. In contrast to these mechanisms, CPP were initially suggested to directly penetrate cell membranes by an energy-independent route. According to early studies on the cellular translocation of Tat derived peptides, penetratin and VP22, none of the classical receptor-, transporter-, or endocytosis-mediated processes seemed to be involved [25–28]. Instead, passive transfer (Model 2, Fig. 1) through the lipid bilayer was postulated as the potential translocation mechanism. Nevertheless, according to recent re-evaluations of the cellular entry of certain CPP and cargo-conjugated CPP, more and more evidence is brought up suggesting the involvement of endocytic processes (Model 3, Fig. 1). Overall, however, evidence about the exact mechanisms of entry is scarce, and often controversial or elusive.

2.1. Passive transfer through the lipid bilayer

2.1.1. Tat derived peptides

The 86 amino acid Tat protein is a nuclear transcription-activating protein from the HIV-1 virus [29] and organized in three different functional domains [30]: (i) an acidic N-terminal region important for trans-activation; (ii) a cysteine-rich DNA-binding region (amino acids 22–37) featuring a zinc-finger motif; and (iii) a basic region (amino acids 49–58) responsible for nuclear import featuring an NLS. Tat derived peptides, largely studied in the literature, are truncated versions of the Tat protein that were reported to be rapidly taken up by cells in culture and subsequently concentrate in the nucleus due to their NLS sequence [28,31,32]. A Tat sequence containing residues 38–60 adopts, under certain conditions, an α-helical amphipathic conformation in the range of amino acid 38–45, whereas the basic region 49–57 remains in a random coil conformation [33]. Another study [28] showed that the amphipathic helix was in fact not required for cellular peptide uptake, whereas the whole basic region was a prerequisite [34]. A detailed study on the structure–activity relationship of Tat(49–57) revealed that further truncations of the 9mer sequence led to a reduction in the propensity to penetrate the cell plasma membrane. In conclusion, the minimal sequence necessary

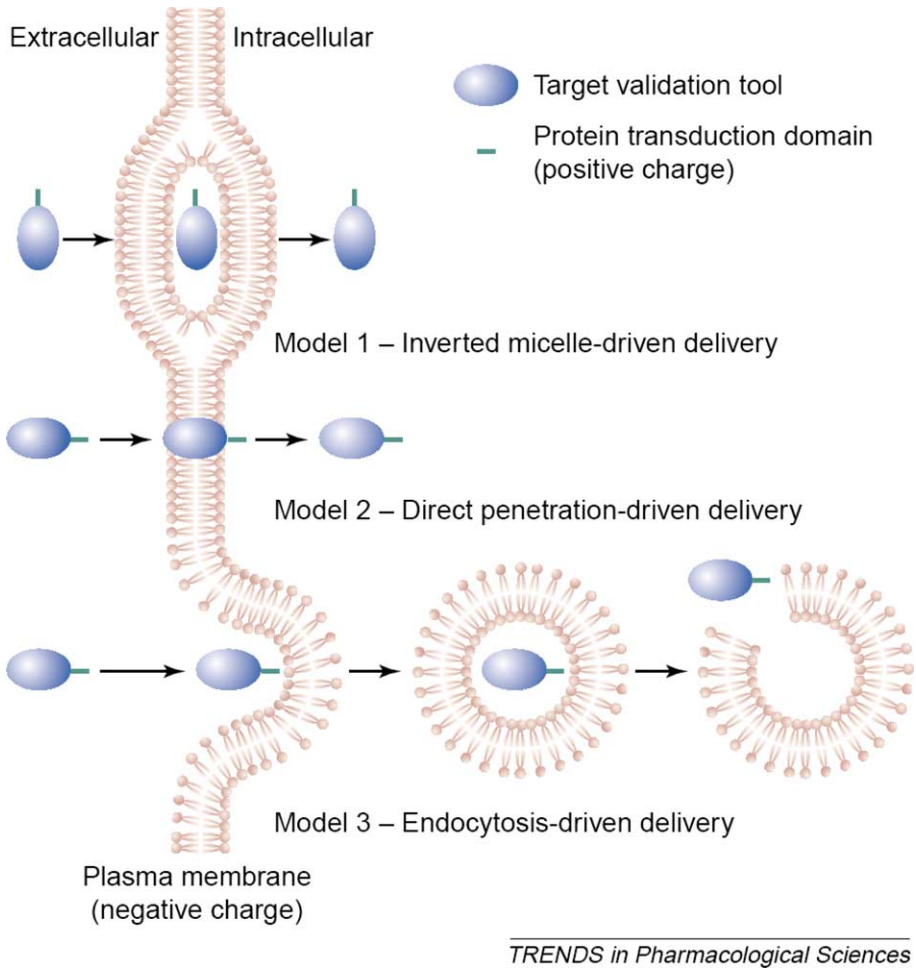

Fig. 1. Proposed mechanisms of cellular delivery of cargos mediated by cell penetrating peptides (CPP). Cationic CPP conjugated to macromolecules such as peptides, proteins or oligonucleotides seem to firstly bind to the plasma membrane via an initial rapid electrostatic interaction. Subsequently the complexes are internalized by cells through various hypothetical mechanisms. Model 1 represents a mechanism that is assumed to occur through the formation of an inverted micelle. Model 2 suggests direct penetration of the plasma membrane. Both mechanisms were considered to occur via rapid, energy- and receptor-independent pathways. However, more recent observations imply that the uptake of a majority of CPP and cargo-conjugated CPP results from endocytosis by which small quantities of the conjugates are released via an unknown mechanism (Model 3). Figure reprinted from Ref. [132], with permission from Elsevier.

and sufficient for membrane translocation is the basic region, i.e. amino acids 49–57 [35,36].

In order to investigate the mechanism by which Tat derived peptides are taken up, retro-, enantio-, and retroenantio-modified sequences were synthesized and their internalization followed in cell lines [35,36]. In fact, these modifications maintained the membrane translocation efficiency of the peptides and even had a beneficial effect. However, the presence of arginine was demonstrated to be crucial since by deleting one arginine residue in the sequence Tat(49–57), translocation ability decreased by 50%, and the deletion of two arginine residues resulted in a 75% loss of activity [35,36]. The use of endocytosis inhibitors did not block cellular uptake. Moreover, it was demonstrated that uptake even occurred at low temperatures [28]. Therefore, Tat derived peptides seemed to follow a receptor-, energy- and endocytosis-independent mechanism of internalization. Alternatively, a mechanism involving direct penetration of the lipid bilayer was proposed (Model 2, Fig. 1), caused by the localized positive charge of the vector [37]. The involvement of heparan sulfate (HS) or chondroitin sulfate (CS) proteoglycans, constituents of the extracellular matrix (ECM) of most eukaryotic cells, was discussed. Internalization of Tat(48–60) was suppressed by heparan sulfate (HS) lyase or treatment with anti-HS antibody, or by addition of soluble sulfated polysaccharides, as well as by addition of chondroitin sulfates A, B and C [32]. It was thus suggested that the negatively charged proteoglycans would take part in the concentration of the basic Tat(48–60) peptide on the plasma membrane followed by an internalization of the peptide [32,38]. Contrastingly, Silhol et al. [39] observed internalization of Tat(48–60) in Chinese hamster ovary (CHO) mutant cell lines deficient of the expression of HS proteoglycans. In addition, the uptake of Tat(48–60) was not altered in cells treated with heparinase III, an enzyme which degrades HS receptors. The authors concluded from these two studies that more than one

mechanism might be involved in the translocation of Tat derived peptides.

2.1.2. Antennapedia derived peptides, penetratin

The homeodomain of the Antennapedia (Antp) transcription factor of *Drosophila* is composed of three α-helices, with a β-turn between helices 2 and 3 [40]. The third α-helix ranging from amino acid 43 to 58 exhibits the capacity to translocate across the plasma membrane of cells and is further internalized into the nucleus [41,42]. Therefore, the minimal sequence that was necessary and sufficient for the translocation of biological membranes was named *penetratin* [25,43]. Subsequently, Fisher et al. [44] described the internalization of a C-terminal fragment (52–58) of penetratin, raising the possibility that even shorter Antp-based vectors may be developed. Penetratin that is poorly structured in water, adopted an α-helical structure in a hydrophobic environment [25]. However, helicity did not appear to be a prerequisite for successful internalization, because the introduction of three helix-breaking prolines into the sequence did not prevent internalization [26]. On the contrary, the two tryptophan residues in positions 48 and 56 played a crucial role in the translocation process, as their substitution by phenylalanines aborted the internalization of the peptide [25], raising the possibility that the internalization of penetratin may not be solely based on its general hydrophobicity. This result was corroborated by the Ala-scan mapping study of Drin et al. [45], successively substituting each amino acid by an alanine. In the same study, the mutation of charged residues (R, K) within the sequence resulted in a significant decrease in cellular uptake demonstrating that the basic amino acids seemed to be a prerequisite for efficient peptide translocation. To investigate the presence of a chiral receptor, two peptides were synthesized, a retro- and an enantio-modified sequence. These two peptides were successfully internalized by cells precluding the presence of a specific chiral receptor [26]. Interestingly, homeodomain internalization appeared to be favored in cells expressing α-2,8-polysialic acid (PSA) at their surfaces [46]. It is thus possible that α-2,8-PSA enhanced the internalization of Antp homeodomain peptides by increasing the local concentration of the homeodomain, or by stabilizing the interaction of the homeodomain with plasma membranes [46]. However, Antp homeodomain peptides are also internalized by cells that do not express α-2,8-PSA. Therefore, other complex sugars or negative charges that are exposed at the cell surface could be proposed to explain this function [47]. The cellular internalization of penetratin in cortical-striatal cells was also shown to be effective at both 37 and 4 °C, indicating an energy-independent character of the uptake process [25,26].

Prochiantz and colleagues [41,42,48] proposed a model for the internalization of penetratin. The authors argued that internalization of penetratin may depend on the formation of a transient, inverted micellar phase consisting of bilayer lipids and penetratin (Model 1, Fig. 1). An initial charge–charge interaction between the peptide and the bilayer would cause bilayer disruption, resulting in the move of the peptide into the bilayer while being entrapped in an inverted micelle [42,49]. This micelle was then suggested to pass to the opposite side of the bilayer and release its contents directly into the cytosol. To challenge this hypothesis, Thoren et al. [50] investigated the uptake of a fluorescent-labeled penetratin across pure phospholipid multilamellar and unilamellar vesicles in vitro. By this approach, the authors managed to avoid any additional complexity of membrane proteins or sub-structures such as caveolae or cholesterol rafts. As a result, they observed an affinity of penetratin for the negatively charged lipid bilayer, and its uptake into the vesicles. More importantly, leakage of a fluorescent dye out of the vesicles was negligible, thus demonstrating that bilayer integrity was not compromised. In contrast, the bee venom peptide melittin, having no CPP properties (but with membrane-disrupting properties through pore formation) enhanced the leakage of entrapped dye. While these experiments cannot rule out the possibility that penetratin can interact with other components of the cell membrane as well, the evidence suggested that it could cross pure phospholipid bilayers. In contrast with the results of Thoren et al. [50], Drin et al. [45,51] indicated that penetratin (residues 43–58) was unable to pass across a non-cellular phospholipid bilayer neither by a translocation-pore formation process [45], nor by diffusion [51]. The authors showed that even after 10 min of incubation, penetratin remained bound to the outer leaflet of the lipid vesicles [51]. Therefore, penetratin probably interacts with other cell surface components to cross cell membranes. Nevertheless, it is likely that its lipid-binding ability (related to its hydrophobicity and amphipathicity) also contributes to its cell membrane interaction prior to internalization.

2.1.3. VP22 vector peptide

VP22 is a transcription factor of Herpes simplex virus type 1 (HSV-1) that interacts with chromatin and elements of the cytoskeletal matrix [52]. The mechanism of internalization of VP22 has not been thoroughly investigated. The 38 kDa structural protein has been demonstrated to provide the remarkable property of intercellular transport [27]. Transfer was found to be so efficient that, following expression in a subpopulation, the protein spread to every cell of the monolayer, where it concentrated in the nucleus and bound chromatin despite lacking a NLS [27]. The uptake of VP22 did not involve the standard route of endocytosis, but appeared to follow a novel, cytochalasin D sensitive pathway, seemingly by interaction with the actin cytoskeleton. Moreover, its intercellular trafficking apparently occurred, in spite of lacking a signal sequence, by a Golgi-independent mechanism termed non-classical secretion [53]. It was also noticed that the C-terminal 34 residues of VP22 are crucial for cellular binding and entry, indicating that this region is involved in the interaction between VP22 and its cellular receptor. Elliot et al. [27]

postulated that the VP22 receptor may be one of the cell-surface proteins that make contact either directly or indirectly with actin microfilaments since actin is required in VP22 uptake.

2.2. Endocytosis

In contrast to the hypotheses involving passive transfer through the lipid bilayer as outlined in the previous paragraph, CPP internalization has also been alternatively associated with endocytosis (Model 3, Fig. 1), an energy-dependent mechanism.

2.2.1. Full-length Tat protein

The full-length Tat protein was suggested to follow an absorptive endocytic mechanism when internalized [54]. Cellular uptake was prevented by low temperature and lysomotropic agents, such as chloroquine [54,55]. Moreover, live cells treated with rhodamine-labeled Tat protein showed punctuated cell surface staining similar to that seen in receptor-mediated endocytosis. After several hours, significant nuclear staining and punctuated cytoplasmic staining was observed. The punctuated cytoplasmic staining suggested that Tat protein localized within either endosomes or lysosomes [54]. It is interesting to note that these early observations contrast sharply with the later hypotheses on the mechanism involved in Tat derived peptide uptake [28,56]. When Fawell et al. [57] conjugated Tat protein to four different cargo proteins, they demonstrated that most of the conjugates were confined inside cytoplasmic vesicles with some diffuse fluorescence in the cytoplasm and the nucleus. More recently, Silhol et al. [39] confirmed that the conjugate green fluorescent protein (GFP)–Tat protein may enter cells by an adsorptive-mediated endocytic mechanism. The involvement of HS proteoglycans was also postulated to affect the mechanism of internalization of Tat protein since Tyagi et al. [58] observed inhibition of Tat protein uptake in the presence of soluble heparin. Contrasting with a Tat derived peptide (residues 48–60), the internalization of Tat protein was not inhibited by chondroitin sulfates (CS), another type of sulfated polysaccharides [58]. Suzuki et al. [32] suggested that a distinct secondary or tertiary structure is required in the case of Tat protein, which distinguishes HS from the CS. In addition, Rusnati et al. [59,60] have taken interest in the interaction between Tat protein and other polyanions, e.g. suramin and pentosan sulfate, and found an inhibitory role for these compounds in Tat protein internalization. Other authors suggested that Tat protein, which contains a highly conserved Arg-Gly-Asp (RGD) sequence in its C-terminal domain, may interact with cellular integrins. In fact, RGD sequences were identified by integrin-mediated cell adhesion processes, and could, thus, possibly be involved in the cellular uptake of the full-length Tat protein [61]. More recently, the cellular uptake of GFP fused to Tat protein was demonstrated to occur through a temperature-dependent endocytic pathway that originates from cell membrane lipid rafts and follows caveolar endocytosis [62,63]. The same authors suggested that the internalization of Tat protein might occur following its interaction with the sugar moiety of the glypicans, one of the two families of cell surface HS proteoglycans [62,63].

2.2.2. Tat derived peptides, penetratin, VP22 and others

In addition to the full-length Tat protein, additional CPP and their cargo conjugates were recently claimed to follow an endocytic mechanism for their cellular internalization. Clearly, these results are in contradiction with the dogma that describes a rapid, receptor- and endocytosis-independent pathway as a mechanism governing CPP internalization. For example, Richard et al. [64] demonstrated that the internalization of Tat(48–60) and poly-arginine $(R)_9$ peptides was strongly inhibited by low temperature or by depletion of the cellular pool of ATP. Moreover, the kinetics of peptide uptake was as slow as that typical for endocytosis. After incubation of live cells with these peptides or their peptide nucleic acid (PNA) conjugates, using fluorescence microscopy, the authors observed a punctuated distribution pattern that is characteristic of endocytosis, as well as the colocalization with common endocytic markers. These results strongly support the involvement of endocytosis as significant route for the internalization of Tat(48–60) and $(R)_9$. Moreover, Koppelhus et al. [65] described a vesicular uptake of C-Tat(48–60) and modified penetratin(42–58) conjugated to PNA that was time-, temperature- and concentration-dependent in a variety of human cell lines, thus reinforcing the hypothesis of a (receptor-mediated) endocytic process. More recently, Lundberg et al. showed that MG-Tat(47–57)-G, VP22, polyarginine $(R)_8$ and polylysine $(K)_8$ fused to the GFP efficiently and strongly adhered to the cell surface and were internalized by endocytosis [66]. Moreover, biotinylated SG-Tat(47–57)-C and SG-penetratin(43–58)-C fused to avidin or streptavidin promoted cellular uptake of cargos through endocytosis [67]. Console et al. [67] also showed that the uptake of Tat fusion protein was blocked by heparan and dextran sulfate while the uptake of penetratin fusion protein was only blocked by heparan sulfate. This illustrates the interaction between the cationic CPP and the negatively charged complex of sulfated glycosaminoglycans exposed on the surface of the cells. Moreover, Sandgren and co-workers [68] investigated the role of heparan sulfate proteoglycans in Tat-mediated cellular uptake in detail. They showed that cell surface proteoglycans play a pivotal role in the uptake of Tat(48–60) polyanion complexes exhibiting a relatively high peptide to polyanion ratio, corresponding to a net positive charge of the complexes. These conclusions are consistent with those of Fittipaldi et al. [63] who recently demonstrated that Tat(48–58) fused to GFP is internalized by the cells through a temperature-dependent endocytic pathway that originates from cell membrane lipid rafts and follows caveolar endocytosis. Similar to Tat protein, Tat(48–58) seemed to interact with

glypicans prior to its internalization [63]. Another study suggested that penetratin(43–58) enters cells using a common adsorptive-mediated endocytosis pathway rather than by a passive translocation mechanism [45,51]. Overall, the volume of adverse hypotheses is intriguing.

Endocytosis was also claimed for transportan conjugated to gold particles. When this complex was incubated with cells, both vesicular and diffuse cytoplasmic distribution was observed [18]. This suggests that endocytosis occurred side-by-side with passive direct transfer of large transportan-cargo complexes across the plasma membrane. Again relative to transportan, Hällbrink et al. [69] observed that the rate of cell penetration decreased when the size of the cargo increased, while that of endocytosis was more or less constant. The authors concluded that the delivery process consisted of two mechanisms: fast cellular penetration and the 'normal' slower endocytosis.

Endocytosis was also suggested for the delivery of doxorubicin into the brain via a SynB domain. SynB is a peptide sequence which was demonstrated to cross efficiently cell membranes without any cytolytic effect [70]. The authors demonstrated a saturable transport mechanism as well as an inhibition of uptake by inhibitors of endocytosis. They concluded adsorptive-mediated endocytosis to explain the uptake of doxorobucin-SynB complex. The same conclusion was given for SynB labeled with a fluorophore [51].

2.2.3. hCT derived peptides

In our group, we have suggested hCT derived peptides as potential CPP for cellular drug delivery. The aspects of delivery into or across epithelial barriers were particularly investigated. The C-terminal fragment of hCT featuring residues 9–32, i.e. hCT(9–32), as well as human calcitonin itself were previously demonstrated by our group to be internalized into bovine nasal epithelium in vitro. The mechanism involved in this process seemed to be driven by endocytosis [22,71]. More recently, we found that truncated sequences of hCT, from hCT(9–32) to hCT(18–32), penetrated the plasma membrane and demonstrated a sectoral, punctuated cytoplasmic distribution in Madin–Darby canine kidney (MDCK) monolayers, a renal columnar-type epithelium. The uptake process appeared to be temperature-, time- and concentration-dependent, suggesting an endocytic mechanism [24]. Moreover, hCT derived peptides were co-localized with FITC-dextran, a marker for fluid phase endocytosis, supporting once again the endocytic nature of the cellular uptake of these peptides [24]. The peptide hCT(9–32) also depicted a weak punctuated cytoplasmic distribution in TR146, a human buccal, squamous-type epithelial model [72].

Overall, many recent observations imply that CPP uptake could be explained by initial rapid electrostatic interaction with the plasma membrane followed by endocytic uptake and partial intracellular release (Model 3, Fig. 1). However, without further experimental evidence, it is yet elusive to entirely eliminate the possibility of uptake via a passive transfer. If the more recent mechanistic studies are correct, strategies promoting endosomal escape will have to be developed to prevent cleavage of the cargo in the endosomal/lysosomal compartments. Recent studies demonstrated both increased in vitro and in vivo delivery of Tat-plasmid complexes from endosomes when used in combination with the cationic polymer polyethylenimine [73] or cationic liposomes [74], which are commonly employed for plasmid DNA delivery. Therefore, a better understanding of the cellular pathways of CPP to cross the cell plasma membrane would be beneficial to develop more efficient delivery systems.

3. Cargo delivery using cell penetrating peptides

Whereas purely mechanistic studies are often restricted to CPP which are labeled with fluorescent tags rather than carrying a therapeutic cargo, the proof-of-concept requires more realistic cargo models. A related selection of references is given in Table 2. The strategies for covalent coupling of CPP to their cargos generally fall into two broad categories: the production of CPP–cargo chimeras, or the separate development of CPP and cargo followed by subsequent linkage. Alternative to covalent linkage is the non-covalent, physical interaction between CPP and cargo. This third category has previously been introduced for the cellular delivery of nucleotides [13]. Specifically, the authors used a 27 amino acid CPP, named MPG, which was derived from the sequences of a hydrophobic domain of HIV gp41 in combination with a cationic nuclear localization signal (NLS) of the SV40 large T-antigen, separated by a tripeptide spacer. To produce the CPP–cargo construct, the MPG can simply be mixed with the oligonucleotide cargo to form a complex through electrostatic interactions

Table 2
Examples of cargos delivered by CPP

CPP	Cargos	Ref.
Tat-derived peptides	Fluorescence tag	[28,35]
	Antibody	[75,76]
	Peptide ($p16^{INK}$)	[78]
	Protein (caspase 3, GFP, β-galactosidase)	[31,79,82,83]
	Nucleic acid (oligonucleotides, plasmid DNA)	[84,85]
	Particles	[86]
	Liposomes	[87]
Penetratin	Peptide (LFG, $p21^{WAF1}$)	[88,89]
	Protein (GFP)	[90]
	Nucleic acid (oligonucleotides)	[84,95,96]
	PNA	[92]
	Drug	[93,94]
VP22	Protein (GFP)	[97,98]
hCT(9–32)	Protein (GFP)	[23]

between the positively charged domain of the peptide's NLS and the negatively charged regions of the nucleotide phosphate backbone.

3.1. Cargo delivery using Tat derived peptides

In numerous studies, Tat derived peptides have been utilized to carry peptides and larger proteins into cells and tissues. Their capacity was first assessed by delivering a Fab antibody fragment into tumor cells, using Tat(37–62) [75,76]. Tat derived peptides (residues 1–72 or 37–72) were also used in vitro to deliver protein cargos such as β-galactosidase, horseradish peroxidase, RNAse A or a *Pseudomonas* exotoxin A fragment [57,77]. To examine its biodistribution, β-galactosidase conjugated to Tat derived peptides (residues 1–72 or 37–72) was injected intravenously into mice [57]. Histological staining showed high tissue-associated activity in the liver, spleen and heart, but no staining in the brain. Contrastingly, intraperitoneal injection of the 120 kDa Tat(47–57)/β-galactosidase fusion protein in mice resulted in the cellular internalization of the biologically active fusion protein in multiple tissues including the brain [31]. This demonstration stands as a benchmark for the delivery of polypeptide cargos in terms of size and tissue permeability. Its results were considered to open a new avenue for the direct delivery of proteins into patients in the context of protein therapy, which will be discussed in more detail below.

Additional bioactive peptide and protein cargos that were rendered cell permeable by association with Tat derived peptides, include a 20-residue peptide derived from the tumor suppressor $p16^{INK}$ [78] and caspase-3, a key regulatory protease in cell death [79]. Vocero-Akbani et al. [79] utilized caspase-3 in designing an anti-HIV therapeutic approach. Tat derived peptide and caspase-3 were connected by an HIV protease substrate sequence. The Tat derived peptide was able to non-selectively deliver the fusion protein into a variety of cultured cells. If the cells had previously been infected by HIV, HIV protease cleaved the recognized substrate sequence, thus releasing the caspase-3 which induced further cell death [79]. On the other hand, non-infected cells that lack the HIV protease could not cleave the fusion protein and were thus not affected. Other examples for protein delivery using Tat derived peptides include RhoA [80], $p27^{kip1}$ [81], or green fluorescence protein (GFP) [82,83]. As a widely used biomarker in various biological studies, GFP has been anchored to Tat derived peptides [82], or full-length Tat protein [83] in order to study trafficking, intracellular localization and protein interaction.

Short Tat derived peptides were also used in order to introduce nucleic acids into cells [84]. Tat derived peptides covalently attached to an oligonucleotide against cell surface P-glycoprotein showed significant inhibition of the protein's expression [84]. Tung et al. [85] have synthesized a series of complexes containing 1–8 Tat moieties and plasmid DNA, and evaluated them as transfection enhancers in a variety of cell lines. They found that only molecules containing eight Tat derived peptide chains showed significant transfection capabilities.

Larger cargos such as magnetic nanoparticles have also been effectively transduced by Tat derived peptides. The complex was taken up by human hematopoietic CD34 + cells, mouse neural progenitors, human CD34 + lymphocytes and mouse splenocytes, suggesting that even such extraordinarily large nanoparticulate cargos in the order of ~45 nm could be delivered into a wide variety of cells [86]. When quantified, the level of iron uptake was as high as 30 pg of superparamagnetic iron per cell. Importantly, such uptake levels did not alter viability, differentiation or proliferation of the labeled cells. Therefore, it was possible to track CD34 + cells in vivo using conventional magnetic purification protocols to re-isolate the cells from their hosts [86].

To extend the size of translocated cargos even further, Torchilin et al. [87] recently demonstrated that the attachment of multiple (i.e. ~500) Tat derived peptides to 200 nm liposomes enhanced their delivery into a variety of cell lines involving an energy-independent process. More recently, Console et al. [67] showed that the uptake of SG-Tat(47–57)-C-tagged liposomes in CHO cells was dramatically enhanced when compared with control liposomes. Tat-derivatized liposome uptake was indiscriminately blocked by inhibitory polysaccharides such as dextran sulfate and heparin, demonstrating the likely involvement of an interaction with the ECM in the process of uptake.

3.2. Cargo delivery using Antp homeodomain derived peptides or penetratin

Similar to Tat derived peptides, many proteins, peptides and oligonucleotides have been internalized into various cells using Antp homeodomain derived peptides or penetratin [48]. For example, Chen et al. [88] fused penetratin to a peptide domain, LFG, that serves as a docking site of cyclin–cyclin-dependent-kinase (cyclin–Cdk) complexes. Owing to the necessity of cyclin–Cdk activity for cell-cycle progression, the complexes—when applied to tumorigenic cell lines—resulted in specific apoptosis of cells. In another example, two functional peptide fragments derived from the cyclin inhibitor $p21^{WAF1}$ were conjugated to penetratin and used to inhibit cancer cell growth through cell cycle inhibition [89]. Similar to Tat derived peptides, GFP was anchored to penetratin [90] to study trafficking, intracellular localization and protein interaction. Moreover, penetratin was ligated to liposomes, and their uptake observed in CHO cells. As with Tat derived peptide, penetratin-derivatized liposome uptake was indiscriminately blocked by inhibitory polysaccharides such as dextran sulfate and heparin [67].

It is worth mentioning that the penetratin approach has also been tested in vivo. The first in vivo application was

the induction of a T-cell response by specific antigenic peptides linked to penetratin and internalized by antigen-presenting cells [91]. The rationale was to introduce the epitope into the cell cytoplasm and to allow its presentation by the MHC-I complex. Another in vivo application was with PNAs. PNAs represent oligonucleotides in which the sugar–phosphate backbone is replaced with a neutral peptide backbone. This modification maintains the specificity of antisense oligonucleotides for hybridization in combination with an increase in resistance towards chemical inactivation. Pooga et al. [92] showed that PNAs directed against type-1 galanin receptor (Gal-R1) and linked to penetratin were successfully internalized in vivo. Following internalization, they specifically downregulated the synthesis of Gal-R1, reducing the physiologic activity of galanin. Galanin is a polypeptide with a wide range of physiologic functions, from regulating food intake to pain perception. Recently penetratin has been shown to act as a delivery vehicle for the transfer of doxorubicin, an antineoplastic agent, across the blood–brain barrier (BBB) [93,94]. Doxorubicin attached to a D-amino acid modified penetratin was shown to allow significant improvements in delivery in various BBB models. When the doxorubicin–peptide conjugates were injected into mice, their uptake was significantly increased within 30 min.

Combining Antp homeodomain derived peptide vector conjugates and antisense oligonucleotides showed not only successful cellular delivery, but also a clear antisense effect, e.g. inhibition of neurite outgrowth due to suppression of amyloid precursor protein neosynthesis [95] and cell death due to down-regulation of Cu/Zn superoxide dismutase (SOD1) [96]. In the latter example, apoptotic cell death was conferred with a 100-fold increase in efficacy versus penetratin free delivery. Another example showed that when Antp homeodomain derived peptide was covalently ligated to an oligonucleotide against cell surface P-glycoprotein, the oligonucleotide–peptide construct exhibited significant inhibition of P-glycoprotein expression [84].

3.3. Cargo delivery using VP22

The utility of VP22 as a transport vector was demonstrated using fusion proteins with the 27 kDa GFP. The GFP fusion protein was expressed in COS-1 cells and was observed to spread into surrounding untransfected cells in the same way as wild-type VP22. Although the intercellular transport of GFP–VP22 constructs was discussed controversially [97], recent reports have confirmed intercellular transport, not only to proliferating cells, but also to terminally differentiated cells [98–101]. Furthermore, transport of GFP–VP22 was quantified [102] and in vivo delivery of the complex demonstrated [103]. VP22-mediated protein transport has also been suggested to deliver thymidine kinase (TK) to neuroblastomas in mice, rendering the tumors sensitive to the TK-activated prodrug gangciclovir and causing tumor regression [104]. Additionally, VP22 was linked to the entire p53 protein and the complex was shown to spread to surrounding cells and retained its proapoptotic activity, as well as a widespread cytotoxic effect in p53 negative human osteosarcoma cells [105]. Normal and supernormal levels of p53 are usually not harmful to normal cells. Unwanted side effects were expected to be minimal with this type of cancer treatment.

3.4. Cargo delivery using hCT derived CPP

To assess the potential of hCT derived peptides for cellular internalization of a model protein, enhanced green fluorescence protein (EGFP) was fused to the C-hCT(9–32) fragment [23]. The introduction of the cysteine residue at the N-terminus of hCT(9–32) was in order to perform the ligation with EGFP thioester. Internalization of EGFP-C-hCT(9–32) by excised bovine nasal mucosa was monitored by confocal laser scanning microscopy. The EGFP conjugate revealed specific mucosal internalization, whereas EGFP alone was not capable of any uptake. The internalized construct demonstrated a sectoral distribution in about 5–10% of the living cells of the tissue. The same range of stained cells was observed with the non-ligated fragment C-hCT(9–32) alone labeled with carboxyfluorescein and used as control. At this point the mechanism of translocation of this construct remains unknown.

4. Limitations and pitfalls in the investigation of CPP

The cellular transfer of organic or inorganic cargos by means of CPP represents an important milestone in the field of cellular drug delivery. However, substantial limitations need to be pointed out as well. As recently observed and discussed in more detail below, related data, even when published in good impact life sciences journals turned out to be plagued by artifacts. Moreover, examples demonstrating the therapeutic value of the CPP approach are still rare, and toxicity concerns of this approach have not yet been given sufficient attention. Finally, permeation of cellular barriers and the cellular metabolism of CPP when in contact with those barriers have so far only found minor consideration. Here we will discuss a representative selection of problematic issues.

4.1. Potential artifacts associated with experimental techniques

4.1.1. Fixation of cells

So far, the majority of fluorescence imaging studies dealing with intracellular delivery of CPP have been performed with cell cultures that were chemically fixed to allow histological staining or preserve the samples. Recently, authors of a number of studies became increasingly suspicious about the ability of cell-fixation procedures to induce artifactual intracellular redistribution of CPP.

As early as 1992, Melan et al. [106] observed the cellular redistribution of various proteins into inappropriate locations upon fixation and permeabilization. In 1999, the intracellular localization of fluorescein-labeled oligonucleotides was found to be influenced by the protocols for fixation [107]. Oligonucleotides were detected as cytoplasmic punctuated, bright objects when cells were observed with or without prior paraformaldehyde (PFA) fixation. Further, no fluorescence was detected in the nuclei. In contrast, fixation with an ethanol/acetic acid mixture, methanol or acetone showed cells which were almost devoid of any fluorescent spots in the cytoplasm, whereas the nuclei showed extremely bright fluorescence. The conclusion drawn by the authors was an artifactual permeabilization activity of ethanol, methanol and acetone by dissolving membrane lipids, whereas PFA appeared to preserve the internal structure of the cells. More recently, VP22 has been a subject of controversy. Lundberg et al. [108] showed that VP22 adheres to the cell surface of living cells and relocates to the cell nucleus during fixation due to its affinity for DNA. The same authors were not able to detect nuclear GFP fluorescence in cells incubated for up to 24 h with VP22–GFP in the absence of fixation. However, spontaneously after methanol fixation, the cells exhibited nuclear GFP fluorescence [109]. Apparently, methanol caused disruption of the cell membrane, explaining the contrasting results. In a comparative study, the intercellular transport of GFP–VP22 was evaluated after methanol or PFA fixation. The spread of GFP–VP22 into surrounding cells was observed after methanol fixation, whereas only little spread was detected after PFA fixation. In addition, the uptake mechanism of Tat(48–60) and poly-arginine $(R)_9$ has recently been re-evaluated [64]. Even mild cell-fixation protocols commonly used to evaluate uptake by fluorescence microscopy caused an artifactual redistribution of these peptides into the nucleus. In living cells both CPP depicted a punctuated cytoplasmic distribution predominantly co-localized with some markers of endocytosis. However, after mild fixation with 3.7% of formaldehyde the distribution of Tat(48–60) and $(R)_9$ changed, giving rise to the characteristic nuclear localization reported in most published data. As an explanation, the authors suggest that cationic Tat(48–60) and $(R)_9$ first bind to negatively charged proteoglycans on the plasma membrane by electrostatic interaction and subsequently localize into both the cytoplasm and the nucleus following plasma membrane disruption due to fixation. The authors conclude that endocytosis is the major route for the internalization of Tat(48–60) and $(R)_9$, and propose their nuclear location to represent a fixation artifact. In a recent study, Lundberg and co-workers claimed that MG-Tat(47–57)-G, VP22, polyarginine $(R)_8$ and polylysine $(K)_8$ fused to GFP strongly adhered to the cell surface, were not removed by washing, but were internalized and bound nuclear DNA upon membrane disruption by methanol fixation [66]. The same conclusion was given by Drin et al. [51] for penetratin(43–58) and SynB peptides.

4.1.2. Fluorescence detection—bound versus internalized

Several investigators have used flow cytometry analysis (fluorescence-activated cell sorting; FACS) of living cells to determine the uptake of CPP [31,79,86,110]. Although this technique does not involve fixation, it may be unsuitable to discriminate between fluorescence bound to the cell surface versus internalized fluorescence. CPP often tend to adhere to the cell surface and may thus lead to an overestimation of uptake when analyzed by flow cytometry. Under this aspect, a number of previously published studies may deserve re-evaluation. To prevent such artifacts, cell membrane-adsorbed CPP could be removed with a mild protease digestion step that needs to be included in the protocol before evaluating cellular uptake by FACS. Richard et al. [64] showed that a trypsin treatment prior to FACS analysis removed surface-bound Tat(48–60) and $(R)_9$ by digestion of peptide and/or membrane proteins and led to a more accurate estimation of peptide uptake, whereas the absence of trypsin treatment gave rise to a significant overestimation of cellular uptake. Treatment with other proteases may also be used to decrease binding of peptides to the cell surface. Alternative approaches were recently proposed by Drin et al. [45]. They conjugated penetratin to 7-nitrobenz-2-oxo-1, 3-diazol-4-yl (NBD), whose fluorescence could be quenched by dithionite, a membrane impermeable compound. Cell association could therefore be discriminated from cell uptake. In addition to the study of Richard et al. [64], Lundberg et al. [108] demonstrated that VP22 remained attached to the cell membrane even after extensive washing and concluded that FACS analysis was likely to reflect cell surface adherence rather than intracellular accumulation of the investigated CPP. The same group showed in a recent study that CHO cells incubated for 5 min with Tat–GFP, VP22–GFP, $(K)_8$–GFP or $(R)_8$–GFP and washed with heparin prior to flow cytometry analysis resulted in complete loss of cellular fluorescence, indicating that CPP–GFP fusion proteins were located on the cell surface and had not traversed the cell membrane [66]. Heparin in solution was understood to have competitively removed the CPP–GFP fusion proteins from the cell surface.

4.2. Discrepancies with the dogma

4.2.1. Poor cellular uptake

So far, the discussion of CPP in the literature has been focused on their capability to cross the plasma membrane by rapid, receptor- and endosome-independent uptake. However, some recent studies suggested that under certain experimental conditions, even well-studied CPP such as Tat derived peptides, penetratin and VP22 and/or their cargo conjugates were weakly or even not at all internalized by cells. For example, Koppelhus et al. [65] evaluated the uptake of C-Tat(48–60) and modified penetratin(42–58) in five cell lines, HeLa (cervical carcinoma), SK-BR-3 (breast carcinoma), IMR-90 (fetal lung fibroblast), H9 (lymphoid) and U937 (monocytic). The two CPP demonstrated either

poor or no uptake in the investigated five cell lines. The authors concluded that the results could be either explained by a very low rate of CPP uptake, or by an unusually quick degradation of the fluorescent-labeled CPP in the cells. In addition Violini et al. [111] and Krämer et al. [112] demonstrated that Tat(48–57) and Tat(44–57), respectively, were not internalized in MDCK cell monolayers. In another study, Tat(47–57) and VP22 were fused to the diphtheria toxin A-fragment (dtA), an extremely potent inhibitor of protein synthesis, and the translocation of the complexes into cell cytosol was evaluated [113]. The two complexes bind heparin on the surface of the cells, however no cytotoxicity due to dtA was detected, indicating that Tat(47–57) and VP22 were unable to efficiently deliver enzymatically active dtA into the cytosol. When CPP were used to deliver biologically active molecules into cells, high concentrations (typically 100 nM and higher) were commonly applied. In contrast, Falnes et al. [113] used concentrations as low as 1 nM and lower. The authors go as far as to argue, that since relatively high concentrations were required before biological effects could be observed, CPP would not yet represent sufficiently established and efficient vehicles for the intracellular delivery of macromolecules; also they question whether there are relevant intrinsic membrane penetrating activities. As an explanation, Falnes et al. [113] suggested that cationic CPP elicit massive association with the cell surface, possibly through interaction with cell-surface heparans, and that only a very small fraction of the cell-associated molecules are able to reach the desired destination where a biological effect is exerted. This would explain, as the authors point out, that when only a small amount of CPP was used, no efficient internalization occurred. Similarly, Leifert et al. [114] concluded from a study on Tat(47–57) and VP22 that these CPP would neither enhance release from nor entry into cells. Instead, CPP would enhance binding of free protein to the cell membrane. In addition to the results of Leifert et al. [114], Fang et al. [97] suggested that the use of VP22 to mediate intercellular trafficking of transgene products is limited. Limitations for the intracellular delivery of cargo in vivo have also been described. In mice, muscles injected with G-Tat(47–57)-G conjugated to GFP showed intense labeling of the ECM, suggesting that, although these Tat fusion proteins can transduce muscle fibers, their binding by components of the ECM surrounding myofibers could interfere with the intracellular transduction process [82].

Despite various examples reporting on poor or absent uptake of CPP and cargo-conjugated CPP, many studies demonstrated successful in vitro or in vivo uptake and cargo delivery into cells (see paragraph 3). As mentioned above, the mechanisms of internalization of most CPP seem to occur via endocytosis. The inconsistencies in the capabilities of the CPP to deliver their cargo into cells would therefore depend on the capability of the cargo to be released from the endosomes. Not yet fully discussed is the issue that when CPP are linked to a fluorophore such as carboxyfluorescein or FITC, fluorescence detection might be compromised by fluorescence quenching due to the low pH in endosomes in combination with increased fluorophore concentration.

4.2.2. Cellular uptake is cell line dependent

There is substantial evidence in literature supporting the hypothesis that Tat derived peptides are capable of passing cell membranes irrespective of cell type [37,115]. Koppelhus et al. [65] argued that often the intracellular presence of Tat derived peptides could have been demonstrated by activation of genes controlled by the HIV-1 LTR promoter. Activity of the HIV-1 LTR promoter is a very sensitive reporter of the presence of Tat derived peptides and could thus compromise precise quantitative assessments of the amount of internalized Tat. Also the authors demonstrated that C-Tat(48–60) and modified penetratin(42–58) showed distinct differences in the uptake pattern relative to the tested cell line. Therefore, they concluded that the internalization of the two CPP would be limited to certain cell types and depend on cell-specific membrane components or lipid compositions. An additional study showed that the kinetics of cellular uptake and cargo delivery by CPP, are probably dependent not only on the peptides themselves, but also on the lipid composition of the cell membrane which varies from cell line to cell line [69]. Moreover, Mai et al. [116] claimed that CPP-mediated transduction is influenced by the cell type tested.

Equally, we found by comparison of three different epithelial cell culture models, namely confluent MDCK, Calu-3 and TR146 cell layers, that the nature of the biomembranes had a distinct effect on the type and extent of CPP uptake [72]. CPP derived from hCT showed a punctuated cytoplasmic pattern in MDCK, but not in Calu-3 and only weakly in TR146. The uptake of Tat(47–57) and penetratin(43–58) was clearly punctuated and cytoplasmic in MDCK, whereas in Calu-3 cells the very same peptides depicted both a punctuated cytoplasmic and extracellular staining of the paracellular space. In TR146 cells, however, Tat(47–57) showed only extracellular staining and penetratin(43–58) a punctuated cytoplasmic pattern.

In conclusion, what appeared to be an event of broad applicability for many types of cells and biological barriers, now appears to be an individual cellular feature that needs to be investigated case-by-case rather than under a general concept.

4.2.3. No permeation through cellular barriers

In vivo studies in mice showed that intraperitoneal injection of a fusion protein consisting of Tat(47–57) fused to β-galactosidase resulted in the distribution of biologically active fusion protein to practically all tissues in mice, even across the BBB [31]. Therefore, practically unlimited systemic access of CPP could have been envisaged from this study. However, more recently and in sharp contrast, Violini et al. [111] described that well-differentiated

epithelial cells, MDCK and Caco-2, were essentially non-permeable to Tat(48–57) under physiologic conditions, and restricted to a level similar to a paracellular macromolecular marker, [^{14}C]inulin. Moreover, in our group we recently showed poor permeation of carboxyfluorescein-labeled hCT derived peptides and Tat(47–57) through three well-differentiated epithelial cell lines, MDCK, Calu-3 and TR146 [72]. The observed permeability coefficients of the tested peptides were in the range or even lower than those of markers of comparable molecular weights, which are commonly used to assess passive transport, such as [^{3}H]-mannitol, [^{3}H]-polyethyleneglycol 900 (PEG900) and [^{3}H]-PEG4000. The general expectations raised after the study of Schwarze et al. [31] in mice, must, therefore, be reconsidered. The investigated CPP sequences seem to have no relevant potential for systemic drug delivery across epithelia. Nevertheless, their distinct patterns of cellular localization in either MDCK, Calu-3 or TR146 [72] may offer potential for localized epithelial delivery.

4.3. Metabolic degradation of CPP

The metabolic stability of CPP is an important biopharmaceutical factor since the peptides should carry their cargo to its target before they are metabolically cleaved. To our knowledge, only three studies have so far investigated the cellular metabolism of CPP. The first study was performed by Elmquist et al. [117] on *p*VEC, a CPP derived from murine vascular endothelial cadherin. The stability of *p*VEC inside and outside human aorta endothelial cells and murine A9 fibroblasts was assessed in order to evaluate its potential usability as a drug delivery vector. As a result, *p*VEC was found to be rapidly degraded when incubated with both cellular models. By contrast, when replacing all L-amino-acid residues of its sequence by their non-natural D-counterparts, *p*VEC was no longer subject to any degradation regardless of media composition and incubation time [118]. In the second study, the authors focused on the stability of transportan; a transportan analogue [119], transportan 10 (TP10) and penetratin (43–58). The stability of the peptides was shown to be in the order of transportan > TP10 > penetratin [120]. The third study was carried out in our laboratory with hCT derived peptides, Tat(47–57) and penetratin(43–58) [72]. The tested CPP were incubated with the three epithelial models, MDCK, Calu-3 and TR146. We could demonstrate that in all three epithelial models, the metabolic stability of Tat(47–57) was superior to that of hCT derived peptides and penetratin(43–58). Among the three cell lines, Calu-3 revealed the highest level of peptidase activity leading to rapid cleavage of most of the peptides. The peptide hCT (9–32) was initially degraded within its N-terminal domain by endopeptidases. The resulting metabolites were subsequently further degraded by aminopeptidases, endopeptidases and/or carboxypeptidases. Identical or similar patterns of metabolization for this peptide were found in all three epithelial cell lines, suggesting a general pathway. Stabilization may be achieved by the replacement of the naturally occurring L-amino-acids of hCT(9–32) sequence by their non-physiologic D-counterparts. Nevertheless, in addition to a negative connotation, metabolization of CPP is also a prerequisite for the release of chemically ligated cargos after internalization, and has important consequences for the physiologic clearance of CPP and their acute and chronic toxicity when used for therapeutic purposes. We expect the balance between both aspects to require the development of carefully engineered CPP, in order to avoid premature cleavage and to free the cargo once internalized.

4.4. Biological effects: toxicity and immunogenicity

Full-length Tat protein exerts a toxic action on primary rat neuronal cultures inducing neuronal cell death that is correlated with the time of exposure [121]. The neurotoxic effects of Tat protein were confirmed by Nath et al. [122]. Interestingly, the authors demonstrated that the length of Tat derived peptides appeared to be important in mediating neurotoxicity since Tat(31–61) produced significantly higher levels of neurotoxicity than the full-length Tat protein whereas increasing the length of the 31-mer Tat(31–61) to the 41-mer Tat(31–71) decreased the degree of neurotoxicity [122]. Tat(31–61) includes the core region (positions 32–47) and the basic region (positions 48–57); both regions seem to be essential for neurotoxicity [122]. Benjouad et al. [123] reported the neurotoxic effect of the basic region Tat(49–57) alone. Moreover, Jia et al. [124] demonstrated that the cysteine-rich and basic domain of Tat protein induces endothelial cell apoptosis. Vives et al. [28] showed that 100 μM of Tat(37–60) and Tat(37–53) decreased cell viability of HeLa cells, whereas Tat(43–60) and Tat(48–60) did not induce any significant toxicity during a period of 24 h. The results of Vives et al. [28] are consistent with those of Nath et al. [122], who were also unable to demonstrate any toxicity with the peptides Tat(43–57), Tat(48–62) and Tat(48–85) in spite of the presence of the basic region of Tat protein. For penetratin(43–58), intrastriatal injection of 10 μg caused neurotoxic cell death [125], and a 50 μM solution induced a cytotoxic effect in U2OS osteosarcoma cells [126]. Drin et al. [51] mentioned in a recent publication that penetratin(43–58) starts to induce cell-lysis at a concentration of 40 μM. We investigated the toxicity of selected hCT derived peptides, Tat(47–57) and penetratin(43–58) in contact with MDCK, Calu-3 and TR146 using the MTS/PMS and LDH release assays [24,72]. With the MTS/PMS test, for concentrations of up to 100 μM, hCT derived peptides and Tat(47–57) turned out to be non-toxic, only penetratin(43–58) revealed a slightly toxic effect, but exclusively in TR146 cells. However, at concentrations as high as 1 mM Tat(47–57) and penetratin(43–58) demonstrated a toxic effect in all cell lines [72]. The non-toxic effect of hCT derived peptides on MDCK cell monolayers

was also confirmed by the LDH release test [24]. Another CPP, poly-L-arginine, induced cell membrane damage, resulting in increased permeability and loss of cell–cell contacts in epithelial cells in vitro [127]. Moreover, injection of poly-L-arginine into the pleural cavity or into the trachea induced inflammatory responses in rat lungs in vivo. The authors concluded that the lethality after intratracheal administration is due to electrostatic interaction of the polycation with anionic surfaces present in the pulmonary epithelium [128].

Immunogenicity may be another problem with CPP. So far, in particular, it is only documented for full-length VP22 chimeras [9]. Viral VP22 is know to be a T-cell antigen [129], and VP22-reactive T-cells may play a role in the control of recurrent herpes simplex virus (HSV) infection. To our knowledge, except for VP22, the literature lacks further data on CPP immunogenicity. However, it needs to be mentioned that most of the well-described CPP are derived from non-human proteins and could potentially induce an immune response when injected in the human body, at least when bearing a large size cargo.

5. Conclusions

Over the past decade, a variety of CPP have been developed and evaluated for their ability to deliver therapeutic agents that do not normally cross the plasma membrane, including inherently selective macromolecules such as proteins and oligonucleotides. Although prominent studies claimed practically unrestricted cellular access of these CPP and their conjugated cargos into cells via a rapid, receptor- and endocytosis-independent mechanism, some restrictions in the understanding of these shuttles have been pointed out more recently. The concept of an endocytic mechanism has been given additional support by the latest reports, sharply contrasting with earlier studies that suggested a passive, direct transfer through the plasma membrane. In some cases misleading artifacts but no uptake of CPP could be detected, and several studies demonstrated neurotoxicity with selected CPP. On the basis of such recent studies, the results of some of the previous studies need to be revisited. Therefore, a more complete understanding of the mechanisms involved in the translocation of CPP through cell membranes is still needed, as well as further information about the biological limits of their usage. These are prerequisites to improve the development of optimized CPP suitable for future therapeutic applications. Typically, today's CPP research lacks significant numbers of in vivo studies that could demonstrate viable therapeutic potential. So far, CPP research is largely dominated by basic science aiming at the biochemistry, biophysics and cell biology of CPP translocation. More data are definitely needed to approach a meaningful preclinical stage that could be further developed towards the clinic. Shifting gears to therapeutically more relevant cell culture and animal models, and related metabolism, immunogenicity and toxicity studies could be a first step from concept to prototype.

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