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Expert Review

Cell Penetrating Peptides: Intracellular Pathways and Pharmaceutical Perspectives

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Abstract. Cell penetrating peptides, generally categorized as amphipathic or cationic depending on their sequence, are increasingly drawing attention as a non-invasive delivery technology for macromolecules. Delivery of a diverse set of cargo in terms of size and nature ranging from small molecules to particulate cargo has been attempted using different types of cell penetrating peptides (CPPs) *in vitro* and *in vivo*. However, the internalization mechanism of CPPs is an unresolved issue to date, with dramatic changes in view regarding the involvement of endocytosis as a pathway of internalization. A key reason for the lack of consensus on the mechanism can be attributed to the methodology in deciphering the internalization mechanism. In this review, we highlight some of the methodology concerns, focus more on the internalization pathway and also provide a novel perspective about the intracellular processing of CPPs, which is a crucial aspect to consider when selecting a cell penetrating peptide as a drug delivery system. In addition, recent applications of cell penetrating peptides for the delivery of small molecules, peptides, proteins, oligonucleotides, nanoparticles and liposomes have been reviewed.

KEY WORDS: cell penetrating peptides; endocytosis; intracellular pathways; membrane transduction peptides; protein transduction domains; transduction.

INTRODUCTION

Cell-penetrating peptides (CPPs), also known as protein transduction domains (PTDs) or membrane transduction peptides (MTPs), are of interest due to their ability to translocate across cellular membranes. CPPs consist of 30 or less amino acids and are classified as either cationic or amphipathic in nature. The cationic CPPs contain clusters of primarily arginine and also lysine residues, including the nuclear transcription activator Tat protein, Tat-(47–57) (YGRKKRRQRRR) the regulator of expression of virion Rev protein, HIV-1 Rev-(34–50) (TRQARRNRRRR WRERQR) both encoded by HIV-1, the Drosophila Antennapedia protein, Antp(43–58) (RQIKIYFQNRRMKWKK), flock house virus (FHV) coat (35–49) (RRRRNRTRRNRR RVR), small oligoarginine, $(R)_n$, and small oligolysine, $(K)_n$, (reviewed in (1)). Amphipathic CPPs contain mostly lysine cationic residues, have an even distribution of hydrophobic and hydrophilic amino acids and exhibit an α-helical structure including the model amphipathic peptide MAP, (KLAL KLALKALKAALKLA) and its analogues, and transportan (reviewed in (2)).

In general, studies in the evolving field of CPPs have focused on one of the following three inter-related research areas: 1. Defining the structural properties of CPPs that afford the capability to translocate the membrane barrier, which has led to the emergence of several new classes of CPPs and also helped engineer CPPs with optimum activity. 2. Elucidating the mechanism of entry using various approaches since several controversies regarding the mechanism exist in the literature. 3. Exploiting the cell penetrating property to deliver a wide range of impermeable macromolecules that modify cellular functions in the cytoplasm, as well as the nucleus, that can consequently be therapeutic targets. While a great potential for CPPs as a practical delivery system is projected and currently being tested, there are critical concerns at the basic level that need to be addressed. Several pitfalls exist in the fundamental methodology as well as interpretation of results obtained from studies in this field (3). Some of the important limitations of the methodology are highlighted in this review and illustrated in Fig. 1.

Cationic CPPs except oligolysine, contain at least one residue of arginine in their primary sequence. Thus the structural aspects of arginine in polymeric chain form have been studied in detail to determine the defining properties of

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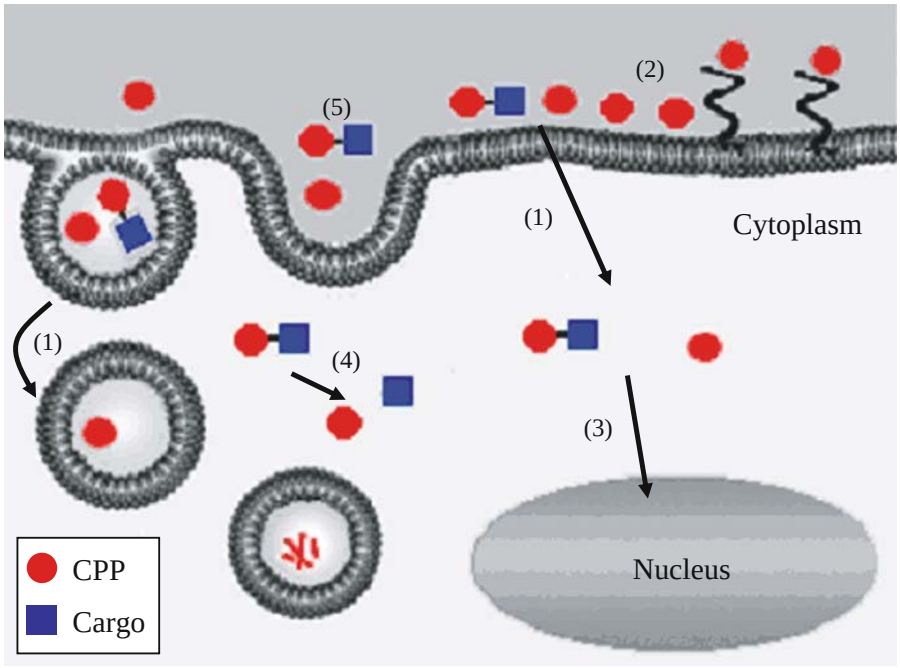
ABBREVIATIONS: CHO cells, Chinese hamster ovary cells; CLIO, cross-linked iron oxide nanoparticles; CPPs, cell penetrating peptides; EGFP, enhanced green fluorescent protein; GAGs, glycosaminoglycans; GFP, green fluorescent protein; HIV-1, human immunodeficiency virus–1; MAP, model amphipathic peptides; MTPs, membrane transduction peptides; MTS, mitochondrial targeting signal; NPC, nuclear pore complex; PCI, photochemical internalization; PNA, peptide nucleic acid; PTDs, protein transduction domains; USPIO, ultra small paramagnetic iron oxide nanoparticles.



	Methodological Challenges	Solutions and Suggestions
1	**Transduction and concurrent endocytosis** Both processes can occur concurrently depending on the CPP and cell line, causing an overestimation of transduction efficiency	▪ Differentiate transduction from endocytosis by methods such as subcellular fractionation (4) or image analysis algorithms (145)
2	**High surface binding** A simple saline wash does not remove all cell surface-bound CPP, leading to overestimation in internalization	▪ Protease digestion step (trypsin wash) (16) ▪ Competitive binding to negative charge on cell surface (heparin wash) (27) ▪ Both protease digestion and competitive binding (18) ▪ Fluorescence quenching of external, cell-associated CPP-fluorophore (32, 82)
3	**Cell fixation** Methanol or formaldehyde destabilize cell membrane and lead to artifactual redistribution of CPP into nucleus where they are retained by the negatively charged DNA	▪ Live cell imaging (16, 44, 82)
4	**Stability of CPP and CPP-cargo linkage** Digestion of the CPP by proteases or an unstable fluorescence label on the CPP may lead to false interpretation of the kinetics and localization of the intact CPP	▪ Use of protease inhibitors and shorter incubation times ▪ Substitute L-isoform of peptide with protease resistant D-isoform
5	**Cargo attachment** Attaching different sizes of cargo may change the internalization pathway to different degree	▪ Establish size limit for cargo delivery by transduction vs endocytosis for different types of CPP

Fig. 1. Difficulties in measurement of internalization of cationic oligopeptides and problems with current methodology.

CPPs. The effects of charge, primary sequence, length, linearity, chirality and peptide backbone have been investigated (4–9). The key structural feature of arginine implicated in the internalization is the guanidine moiety. Homopolymers of lysine that have the same net charge as homopolymers of arginine but do not have the guanidine head group were not efficiently internalized (8). Moreover, homopolymers of citrulline, which is an analogue of arginine with a urea head group rather than guanidine, have no cell penetrating properties (8). The guanidine group is thought to form bidentate hydrogen bonds with the anionic groups on the surface of the cell, similar to the hydrogen bonds formed between arginine and the phosphate backbones of RNA (10). This membrane interaction facilitates their subsequent internalization into the cell. In contrast, amphipathic peptides like MAP and transportan are devoid of arginine, and as a result, their internalization is not dependent on the guanidine moiety. Instead, the most decisive structural requirement has been attributed to the secondary amphipathicity (11). The presence of separate hydrophilic and hydrophobic regions in an α-helical conformation of the peptide at neutral pH is considered to be necessary for membrane destabilization and subsequent internalization (12). These structural differences in the various classes of CPP suggest that different mechanisms of internalization may be involved when they are used as delivery vectors for macromolecular drugs.

The exact mechanism of cellular entry of CPPs is still elusive and remains a major controversy. Until recently, it was believed that endocytosis was not involved in the internalization of CPPs as indicated by receptor-, energy- and temperature-independent uptake (7,13–15). In 2003, the report by Richard *et al.*, suggested that the methods used to

study translocation of peptides, namely, FACS analysis and flow cytometry, were inherently flawed (16). Even mild fixation of cells with formaldehyde or methanol leads to artifactual redistribution of the membrane bound fluorescently labeled peptides into the cytoplasm and nucleus. Since then, the view has shifted from a complete non-endocytosis pathway (13–15) to more involvement of endocytosis as the main pathway of internalization in live cells, for fluorescently labeled CPPs (16–21) and CPPs conjugated to various cargo molecules (22–24). However, few have considered the contribution of more than one pathway of internalization. The cationic oligopeptides are able to strongly adsorb to the cell surface through non-specific electrostatic interactions with the negative charges present. Therefore, it is likely that CPPs are entering the cell through the unique transduction mechanism and concurrently through non-specific endocytosis (Fig. 2). We have previously reported a novel method based on cell fractionation that can quantitatively separate the amount of cationic oligopeptides internalized by endocytosis versus that internalized directly by transduction (4). When comparing oligopeptides with different cationic amino acids, or with altered distributions of charges, the ratio of internalization via endocytosis and transduction will be changed. This ratio may also be dependent upon the distribution of amino acids, the size of peptide, and the cell-type under investigation (4).

Despite the presence of multiple internalization pathways, the majority of the *in vitro* assays used, including fluorescence and confocal microscopy (7), flow cytometry (25), and measurement of biological activity (reviewed in (26)), measure the total cellular uptake of the oligopeptides, and therefore do not effectively measure only the membrane transduction efficiency. For example, when comparing CPPs with varying lengths, while the total internalization may be

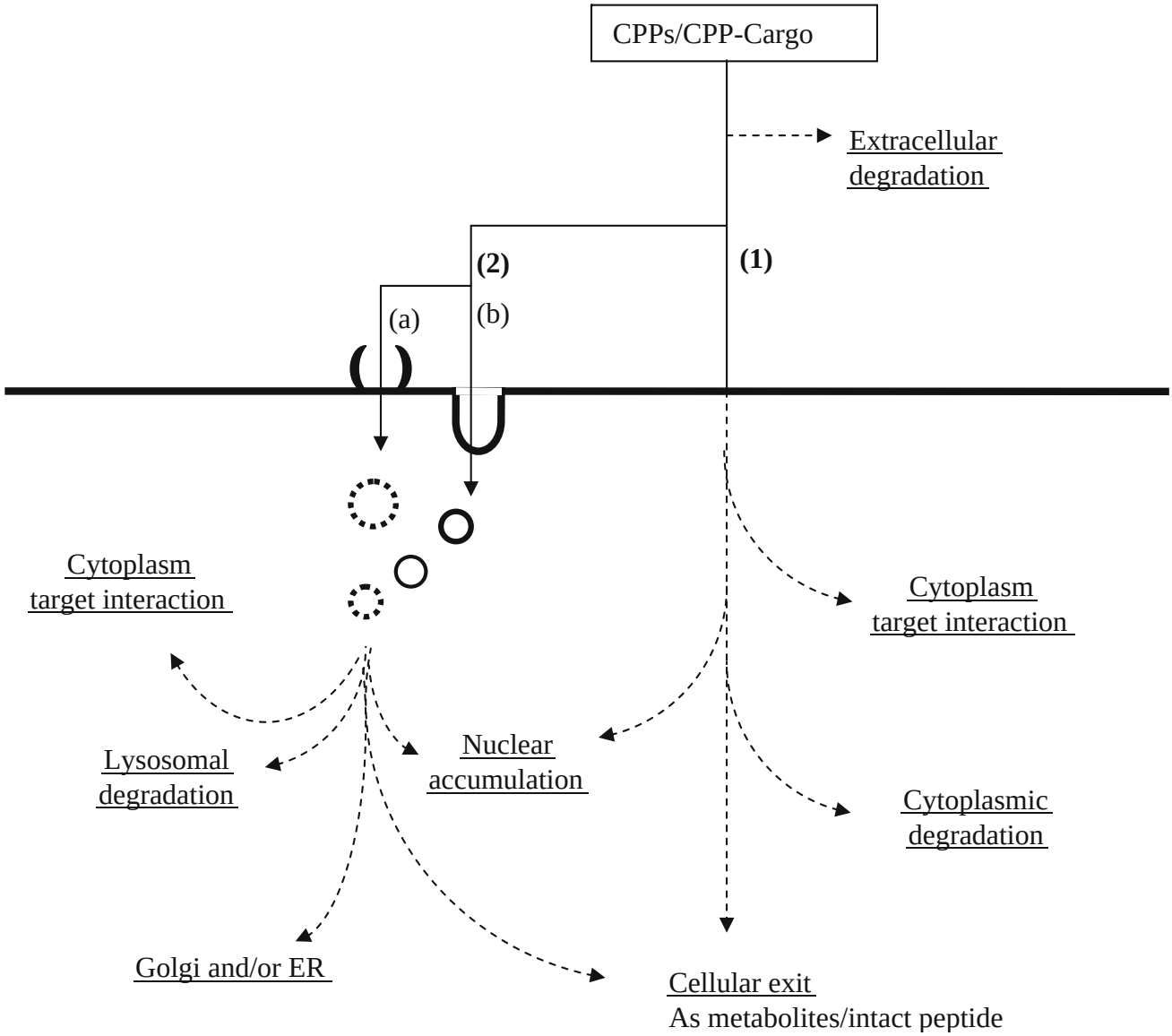


Fig. 2. Schematic representation of the internalization pathway and intracellular processing that CPP or CPP-cargo can undergo in the cell. (*1*) Represents transduction with direct cytosol access and (*2*) collectively represents endocytosis, with (*a*) depicting macropinocytosis characterized by outward protrusions for the formation of macropinosomes whereas (*b*) represents all the other types of endocytosis characterized by invagination of plasma membrane to form vesicles.

similar to each other, they may be preferentially endocytosed or transduced (4). Additionally, the use of various known inhibitors of cellular processes may only affect endocytosis, while the transduction remains unchanged (27). If the CPP under investigation is primarily endocytosed, then the total internalization may be significantly inhibited, while transduction is not altered. Furthermore, the biological activity of cargo macromolecules may not accurately measure the membrane transduction efficiency because, as mentioned, the attachment of large macromolecules with varying chemical properties alters the internalization properties (28–31), possibly changing transduction and endocytosis to different extents. Also, as mentioned earlier, the protocols involving cell fixation for *in vitro* confocal microscopy assays cause artifactual redistribution of the oligopeptides leading to a false cytosolic and nuclear localization (16,32). This problem is addressed in current studies by using live cells. The high membrane surface binding further complicates the quantitative transduction measurement, resulting in an overestimation of internalization measurement. The numerous problems with the current methodology, summarized in Fig. 1, are a highly probable reason why controversies still exist with respect to the structure and size requirements for CPPs and the cargos, as well as the transduction mechanism across cell membranes.

The cationic CPPs have been shown to deliver a wide range of cargo from small molecular weight molecules, proteins, antisense oligonucleotides to liposomes both *in vitro* (recently reviewed in (33)) and *in vivo* (34–39). However, the fact that the attachment of large macromolecules changes the internalization properties of the conjugates has not been fully examined, and often overlooked. After conjugation of Tat to antisense oligodeoxynucleotides or DNA, the internalization has been shown to be clearly affected by decreased temperature and inhibitors of caveolae formation (13,25,40). Furthermore, attempts have been made to deliver large proteins such as those containing CD8+ T-cell epitopes, lymphocytic choreomeningitis virus nucleoprotein, and 58 kDa holotoxins into cytoplasm through conjugation with the Tat peptide with little success (18,28). On the other hand, the conjugation of large monoclonal antibodies, anti-fullerene and anti-HIV-1 Gag, to large molecular weight poly-L-arginine (10,750 MW) resulted in cytoplasmic and nuclear delivery, while the use of smaller octaarginine and Tat had no success (41). CPP delivery systems for a large range of molecular weight cargos have been researched without the establishment of the limitations of the transport process. Therefore, it is important to understand the internalization and intracellular processing of various CPPs, as well as their conjugates, in order to develop an effective drug carrier system. This review will focus on the intracellular pathways of CPPs and their potential implications in drug delivery.

INTERNALIZATION PATHWAYS AND INTRACELLULAR PROCESSING

The presence of different internalization pathways is of paramount importance when considering CPPs for use as a drug carrier. Simply altering the oligopeptide by the attachment of molecules may change the internalization pathway depending on the size and hydrophobicity, and affect the efficiency of the delivery system for cytosolic or nuclear targeting (42,43). Furthermore, the pathway followed governs the intracellular processing, kinetics and final fate of the CPP and its cargo. There are several scenarios that can exist as depicted in Fig. 2. If the CPP is transduced directly into the cytoplasm, it could interact with its cytoplasmic target, be imported to the nucleus, be degraded by cytoplasmic proteases, or it may be redirected out of the cell either intact or after degradation. On the other hand, if it is internalized by endocytosis, depending on the type of endocytosis, it could be targeted for lysosomal degradation, may be able to escape lysosomal degradation and enter the cytoplasm and possibly the nucleus, or be taken to the Golgi apparatus or the endoplasmic reticulum, or transcytosed out of the cell. The pathway followed will be highly governed by several factors including; the type of CPP, type of cargo attached, the nature of linkage between the cargo and CPP and the cell system under investigation. For example, peptides with a nuclear localization signal would be preferentially directed to the nucleus, whereas peptides rich in lysine may be ubiquitinated for proteasome degradation.

Non-endocytotic Pathway/Transduction

Several lines of evidence suggest that transduction pathway for CPP uptake exists and is different from endocytosis. The energy- and receptor-independent uptake of CPPs that was refuted as a mere artifact of the methodology has been reevaluated in living cells and confirmed to be energy independent (44,45). Biophysical studies in cell independent systems such as unilamellar vesicles that lack cell membrane proteins further supports the role of the non-endocytic pathway (46,47). We have shown that transduction and endocytosis are two different pathways with the Tat peptide and oligoarginine being preferentially transduced, whereas oligolysine is endocytosed in Chinese hamster ovary (CHO) and HeLa cells (4,27,42). Moreover the physiological relevance of such a pathway is exemplified by entry of functionally intact transcription factors from which the cell penetrating peptides, namely Tat and Antp, were originally derived. With most attention now directed towards endocytosis, few efforts are aimed towards dissecting the transduction mechanism, limiting it to two hypothetical models with relatively less experimental evidence. These models include direct membrane penetration and inverted micelle (15,39,47,48). Both models follow a 3-step process of internalization: (a) membrane interaction, (b) membrane permeation and (c) release of CPP into the cytosol. The main difference between transduction and endocytosis lies in step 2 and 3. Whereas transduced CPPs directly localize in cytoplasm after traversing the plasma membrane, endocytosed CPPs are confined in vesicles and may or may not be released into the cytosol.

The direct penetration model was based on the finding that a Tat fusion protein can interact electrostatically with the cell surface in the denatured, high-energy form and penetrate directly into the cytosol with subsequent protein refolding with the help of chaperones (39,48). The inverted micelle model is more prevalent with Antp peptides in which

the peptides bind to the membrane by electrostatic attraction causing a transient formation of inverted micelles that carry the peptides and release them into the cell (49,50). Biophysical studies using isothermal titration calorimetry support this model but are not supported by others (47,51,52). Furthermore, the hydrophobic residue tryptophan is considered to play an important role in internalization. However, cationic CPPs such as oligoarginine and Tat peptide do not contain any hydrophobic residues, so tryptophan importance may only be applicable to Antp peptides and cannot be generalized for all CPPs.

A more recently introduced hypothesis concerning translocation of guanidinium-rich cationic CPPs is based on counterion scavenging (53–55). It is hypothesized that the presence of different counter anions (amphiphilic or hydrophilic anions) will lead to charge neutralization or charge inversion of the CPP, consequently altering the lipophilicity and solubility of the CPP to different degree. This dynamic change in the physical properties of the CPP will enable it to partition into the lipid bilayer (membrane penetration) or out of the bilayer (cytosol release) depending on the counter ions present (54,55). Phase transfer experiments using liquid and artificial bilayer membranes and several different counter ions including phosphate, chloride, HEPES (hydrophilic anions) and SDS, cholesterol sulfate, pyrenebutyrate (amphiphilic anions), have been performed to support this hypothesis (54,55), however, validating such a phenomenon in a biological system or even extrapolating it to cell plasma membranes may be a challenge.

Endocytotic Pathways

Endocytosis is a collective term for a regulated cellular process of macromolecule internalization that is energy dependent and is characterized by vesicle formation. It is broadly categorized as phagocytosis, for uptake of large particles and pinocytosis, for uptake of fluids and solutes. Phagocytosis is restricted to specialized cells like macrophages and leucocytes, but pinocytosis occurs in all cells. Pinocytosis can occur through at least four different endocytosis pathways; clathrin mediated, caveolin mediated, clathrin and caveolin independent endocytosis and macropinocytosis. All four processes have been implicated as pathways for internalization of different CPPs with different types of cargo (17,18,21,22,32,56). This heterogeneity can be attributed to the various CPP-cargo used for one, but also, these pathways differ with regards to the size of endocytic vesicles formed, the intracellular trafficking of the vesicles and the factors regulating vesicle formation, thus influencing their degree of involvement in different systems. For instance, the different pinocytosis pathways result in different sizes of vesicles. Clathrin coated vesicles are roughly 120 nm in diameter, caveolae pits are ~50–80 nm in diameter, vesicles formed via clathrin and caveolin independent endocytosis are ~90 nm and macropinosomes resulting from macropinocytosis range from 1 to 5 μm (57). Although the size of vesicles is not rigid, one may speculate that the variation in size of vesicles resulting from different types of endocytosis, is likely to play a role in restricting entry of cargo that exceeds the size limit of the vesicle. It would be hard to imagine 200 nm-sized liposomes tagged with CPPs, internalized by classical endocytosis.

Macropinocytosis

Recent studies on internalization pathway of cationic CPPs have focused on macropinocytosis as the major route of cell internalization (18,19,22,58,59). Macropinocytosis is an actin-dependent form of endocytosis that can occur in all cells at different rates, either constitutively or it can be induced by stimulation with growth factors or phorbol esters. Two aspects that distinguish macropinocytosis from other forms of endocytosis is the membrane ruffling event and the size of vesicles formed. Membrane ruffles and filopodia are dynamic, actin-rich protrusions or outgrowths of the cell that collapse and fuse with the plasma membrane to form large vesicles referred to as macropinosomes, as opposed to invagination as a process of vesicle formation in endocytosis. The physiological role of macropinocytosis is suggested to be important for directed cell migration and immune surveillance by sampling large volumes of the extracellular fluid but has also been exploited by pathogens as a mechanism of cell invasion.

Macropinocytosis seems like an attractive pathway to explain the uptake because it involves internalization of significant volumes of extracellular fluids and since CPPs are concentrated on the surface of the cell, they will indiscriminately be internalized with the extracellular fluid. It is possible that macropinocytosis is involved to a certain degree in the uptake of some CPP-cargo, especially large macromolecules, but whether it is the only pathway involved is debatable. For example, Tat coated liposomes were shown to penetrate into cells intact and without any signs of membrane fusion. The size of the liposomes (200 nm) precludes entry by other forms of endocytosis other than macropinocytosis since the vesicles of other forms of endocytosis are much smaller than the size of the liposomes (60). Khalil *et al* showed that liposomes modified with a high density of octaarginine were preferably internalized by macropinocytosis, but liposomes modified with a low density of octaarginine resulted in uptake by clathrin-mediated endocytosis (58). The authors suggest that a higher density of oligoarginine is actually required for stimulating macropinocytosis through enhanced interaction of the CPP with the cell membrane while a similar concentration of free peptide did not stimulate macropinocytosis. Dowdy *et al* have implicated macropinocytosis as a pathway of internalization for the Tat peptide (18) and Tat-Cre fusion protein (22). They show that in Namalwa cells, Tat peptide was not only internalized by macropinocytosis but also was important in inducing macropinocytosis (18). Additionally, the uptake of Tat-cre fusion protein was inhibited by amiloride a macropinocytosis inhibitor (22). Interestingly, although macropinosomes are known to be inherently leaky, the Tat-Cre fusion protein was not released from the macropinosomes for up to 24 h, which suggests an inefficient process of macropinosome escape for the fusion protein. In HeLa cells, fluorescently labeled octaarginine was internalized by macropinocytosis as determined by inhibition studies (19). Recently, Zaro *et al*., have reported that there was no increase in cytosolic localization of ^{125}I-oligoarginine in HeLa cells after the co-incubation with epidermal growth factor, a known stimulator of macropinocytosis

(61). They found that neither macropinosome nor filopodia formation correlates with the membrane transduction of ^{125}I-oligoarginine (61). This discrepancy can be attributed to several factors such as the effect of the markers, i.e., the small tracers like ^{125}I versus larger cargo such as Cre-protein, attached to CPPs and different experimental designs, i.e., to detect the amount of the oligopeptide in cytosolic compartment or the biological activity of the cargo molecules in the cell.

Inhibitors of Endocytosis

The involvement of different types of endocytosis in CPP internalization is specified by either looking for protein markers that colocalize with the CPP or by using chemical compounds known to inhibit a specific type of endocytic process. The common feature for all types of endocytosis is that it is an energy driven process, and therefore temperature dependence assays are carried out to implicate endocytosis. At 4°C cellular processes including ATP hydrolysis are arrested, so internalization by endocytosis can be blocked. Moreover at this temperature the membrane fluidity is also affected. Since transduction mechanism is thought to occur through the direct interaction with the cell membrane, it is conceivable that inhibition of the membrane fluidity also inhibits the non-endocytic pathways of internalization. This hypothesis has been tested by determining the uptake of oligoarginine at 4, 16 and 37°C in CHO cells (4). While the amount of peptide internalized by endocytosis is reduced significantly at 16°C and 4°C compared to 37°C control, the amount transduced at 16°C is not statistically different from that at 37°C but at 4°C, transduction is inhibited by approximately 40% of control. This indicates clearly that while membrane fusion events, a prerequisite for vesicle formation that is inhibited at 16°C, are necessary for endocytosis, they have no effect on membrane transduction. However, inhibition of membrane fluidity at 4°C can affect both endocytosis and membrane transduction (4,27). Similarly, when examining the effect of various endocytosis inhibitors on cellular uptake, it is important to distinguish between the uptake by endocytosis versus the uptake by transduction. Moreover, the various endocytosis pathways are not mutually exclusive; they may contribute unequally to the overall internalization with one pathway more dominant than others.

In addition, the fact that certain inhibitors may affect more than one endocytosis pathway can further complicate interpretation of the results. For example, Dowdy *et al*. used methyl-β-cyclodextrin as an inhibitor of lipid raft formation to conclude that internalization of fluorescently labeled Tat peptide is mediated by lipid raft dependent macropinocytosis in Namalwa cells (18). However, this inhibitor is also known to affect the formation of caveolin-coated vesicles and would therefore limit any potential internalization by caveolin-dependent endocytosis (62). In HeLa, HepG2 and CHO cells, the internalization of fluorescently labeled Tat peptide was suggested to result from clathrin dependent endocytosis (17,21). Using potassium depletion and chlorpromazine as specific inhibitors of clathrin-dependent endocytosis, ~50% inhibition of uptake for Tat peptide was shown, whereas transferrin, a classical marker of clathrin dependent endocytosis was inhibited by 85% (17). Such a large variation in inhibition between the endocytic marker and the Tat peptide uptake suggests that Tat peptide is only partially internalized by clathrin-dependent endocytosis and other pathways could also be involved. Caveolin-dependent endocytosis in HeLa and HL3T1 cells was shown for Tat-avidin, Penetratin-avidin and fusion protein of Tat and enhanced green fluorescent protein (EGFP) by colocalization with cholera toxin B subunit and caveolin-1 protein markers, and also by inhibition of uptake in the presence of methyl-β-cyclodextrin (20,23,62).

Membrane Interaction

The first step in cellular internalization whether it is through endocytosis or transduction, involves some sort of interaction between the macromolecule and the surface of the cell. If a specific cognate receptor for the ligand exists, the ligand will bind to its receptor to form a ligand-receptor complex and be internalized by receptor-mediated endocytosis, as is the case with physiological ligands, transferrin and LDL. On the other hand, the macromolecule can interact with cell surface non-specifically through electrostatic attraction and be internalized by adsorptive endocytosis (e.g. polylysine). Alternatively, macromolecules can be engulfed due to their proximity to the cell membrane, along with the extracellular fluid lining the cell surface through non-specific fluid-phase endocytosis (e.g. horse radish peroxidase (HRP) or dextran).

It is widely accepted that a cognate receptor is not involved in the internalization of cationic CPPs. This is evidenced by equal or higher efficiency of internalization of the D-isoform of both Tat and Antp peptides and their derivatives compared to the L-isoform (8,15). Futhermore, transduction has been shown to occur in most cells examined and even in artificial unilamellar vesicles (46,47). On the other hand, the amount of ^{125}I-oligoarginine transported by membrane transduction was inhibited in the presence of unlabeled oligoarginine (4), suggesting that there are limited binding sites on the cell surface for CPPs. The binding was specific for guanidine groups on oligoarginine, but without stereospecificity for D- and L-isoforms (9). Several studies have focused on the role of the negatively charged cell surface proteoglycans in the electrostatic membrane interaction of CPPs due to evidence obtained from experiments with the full-length Tat protein (63–66).

Proteoglycans are glycoproteins with one or more glycosaminoglycan chains (GAGs) covalently attached to a core protein. The GAGs are long linear polysaccharides that bear a negative charge at physiological pH due to the presence of polysulfates on their chains and include the chondroitin sulfate, dermatan sulfate and heparan sulfate. The ubiquitously expressed heparan sulfate proteoglycans (HS) have been implicated in translocation of full length Tat protein based on competition studies with exogenous proteoglycans, enzymatic degradation of extracellular HS and use of mutant cells, deficient in all proteoglycans (CHO A-745) or HS (CHO-D677 cell line) (17,25,63,67,68). Furthermore, the binding affinity of the full length Tat protein to heparin, a soluble analogue of HS, has been shown to be very high with a dissociation constant in the nanomolar range (68). The truncated Tat peptide and oligoarginine either alone or

as conjugates also interact with HS but with relatively lower affinity than the full length Tat protein (67,69–71).

It is not surprising that CPPs can be endocytosed as a result of their electrostatic interaction with the negatively charged cell surface. Long before the discovery of cell penetration of Tat protein (72,73), it was shown that cationic macromolecules such as polylysine could be used as intracellular transport carriers by virtue of their non-specific adsorptive endocytosis facilitated by the electrostatic attraction between the cationic polypeptides and the anionic surface of the cells (74,75). Moreover, proteoglycans the postulated binding sites for CPPs, are shown to be internalized in the cells by endocytosis with subsequent lysosomal degradation as a means of their turnover (76). Therefore, cationic peptides electrostatically bound to proteoglycans that do not dissociate before vesicle fusion, could likely be endocytosed with the proteoglycans and meet the same intracellular fate as the proteoglycans. The key questions then are: What ratio of total CPP accessible to the cells is internalized by endocytosis? Are different CPPs with the same net positive charge endocytosed to the same extent? And, how can the CPP be used effectively to deliver its cargo to the desired target instead of accumulating in the endocytic compartments?

Endosomal Escape for CPPs Internalized by Endocytosis

Following membrane interaction, vesicle fusion and budding events occur in endocytosis, capturing the macromolecules into early endosomal compartment. Different endocytic pathways will then target vesicular contents to different cellular destinations. In clathrin-mediated endocytosis, some of the endosomes are recycled back to the plasma membrane, while others are routed from the early endosomes to the late endosomes and ultimately to lysosomes (17,57). In caveolin-mediated endocytosis, the endosomes are targeted to the golgi apparatus or to the endoplasmic reticulum by retrograde transport (20,57). The intracellular fate of macropinosomes resulting from macropinocytosis is different in different cells. In macrophages, the macropinosomes are shown to shrink and merge with lysosome, whereas in cells like human A431 and HeLa cells, the macropinosomes show little interaction with endosomal compartments and mainly recycle their contents back to the extracellular space (57,77). Overall, CPPs internalized by endocytosis are enclosed in vesicles from which they must escape to reach the target sites of their cargo before they are sorted to the cellular destination specified by the type of endocytosis.

For fluorescently labeled CPP, some reports suggest that endosomal escape is inefficient and the CPP is confined in vesicles as evidenced by punctate staining patterns with confocal laser scanning microscopy (20,67). Others reported punctate as well as diffuse staining of the CPPs in the cytoplasm and to some extent the nucleus (21). The diffuse staining was attributed to endosomal escape because co-incubation with ammonium chloride, bafilomycin A or chloroquine, all inhibitors of endosome acidification, resulted in only punctate staining in HeLa or MC57 cell lines (21). The conclusion drawn was that the pH change in the endosome alters the conformation of the peptide and thus facilitates its escape from endosomes. One cannot rule out the possibility that the peptides are degraded within the endosomes and the smaller metabolites with the fluorescent tag escape the endosomes resulting in a diffuse staining pattern.

For biologically active cargo, the need for endosomal escape, as well as retention of functional activity is paramount. Since endocytosis is the major pathway when higher molecular weight bioactive cargo is attached to CPPs, an efficient and feasible method to disrupt endosomes and liberate the cargo has to be employed. The N-terminal domain of influenza virus hemagglutinin-2 (HA2), a pH-dependent fusogenic peptide that induces lysis of membranes at a low pH, within endosomes, has been utilized to promote the escape of Tat-Cre fusion protein and also p53 conjugated to oligoarginine (22,78). Photochemical internalization (PCI), a strategy that depends on endosome disruption by reactive oxygen species (ROS) generated by photosensitizers upon irradiation, has been shown to increase the intracellular release of endocytosed toxins, plasmids and adenovirus vectors. Similarly, fluorescently labeled CPP (79) and a peptide nucleic acid (PNA)-CPP conjugate entrapped in endosomes were treated with PCI to enhance their release (80). However, it is not clear whether endosomal rupture is required for the cytosolic delivery of CPPs under normal physiological conditions.

Nuclear Localization

Nucleocytoplasmic trafficking of macromolecules across the nuclear envelope is considered to be mediated by the nuclear pore complex (NPC) that allows free diffusion of cargo up to 9 nm in diameter (~50 kDa) (81). In principle, the size of most CPPs (<30 kDa) would allow their free diffusion from the cytoplasm to nucleus provided that the size of the cargo attached to the CPP does not exceed the size limit, but it would also depend on other factors such as the proximity to the nucleus, metabolic stability, cytoplasmic concentration and other cellular events that may block nuclear entry. Since nuclear entry is secondary to cellular internalization, whether it occurs after endocytosis or transduction will determine whether the rate-limiting step is escape from endocytic vesicles or diffusion across the cytoplasm to the nucleus.

Nuclear accumulation of fluorescently labeled CPPs was shown in fixed cells but was later considered an artifact of cell fixation (16). Studies in living cells did however confirm nuclear localization but with different kinetics. For example, FITC labeled Tat peptide (47–57), was reported to accumulate within seconds in the nucleus of living fibroblasts as seen by time-lapse confocal microscopy by Ziegler *et al.*, which is consistent with what was reported by Potocky *et al.* in HeLa cells (21,51,82). However, while the study by Ziegler indicated rapid nuclear localization, the latter concluded that nuclear localization was a result of release from endocytic vesicles. This suggests that nuclear transport can occur following different pathways in different cell models. Also, it is important to note that the relatively small size of the fluorescent molecule does not hinder kinetics of tat peptide and can therefore be detected in the cell rapidly. A comparison of half-times ($t_{0.5}$) of internalization of various CPPs with fluorophore cargoes shows that cell entry generally occurs in less than 20 min with first order kinetics (83). On the other hand, attaching larger cargoes such as plasmid DNA or protein

will substantially decrease CPP internalization rate possibly due to a predominantly endocytic pathway. The problem with fluorophores as cargo for nuclear localization though, is that they are qualitative rather than quantitative. In other words, they might be helpful in cellular trafficking visualization, but to determine the efficiency of the CPP for nuclear delivery and establish a proof-of-concept, functional-activity based cargos are more relevant, except for fluorescence derived from a reporter gene like the gene encoding EGFP.

Cargos with biological activity targeted to the nucleus include plasmid DNA or gene regulating -proteins, -oligonucleotides and -peptides. Most of these cargos exceed the size limit for free diffusion when coupled to CPPs and therefore have to depend on active transport mediated by the NPC of cargo larger than 60 kDa (9–40 nm). Active transport requires the presence of a nuclear localization signal (NLS) in the cargo that binds to nuclear import machinery, importin-α/β (a.k.a. karyopherins) and subsequently translocates across the NPC (81). CPPs such as the Tat peptide and Antp are derivatives of transcription factors and are known to have NLS embedded in their sequence (84,85). Several studies show that plasmid DNA or oligonucleotides complexed or conjugated to CPPs are able to efficiently target the nucleus both in *in vitro* and *in vivo*. However, as is the case with other gene delivery carriers like the non-viral vectors-lipoplexes and polyplexes, several factors have to be considered and optimized when using CPPs to target the bioactive molecule to the nucleus.

It must be noted that during mitosis the nuclear envelop breaks down, and thus rapidly dividing cells have an extra entry point to the nucleus as compared to non-dividing cells. Whether the biological activity of cargo is indeed due to the ability of CPP to penetrate intact nuclear envelope rather than opportunistic entry during mitosis would have to be proven through other methods.

Cytoplasmic or Endosomal Degradation

Premature degradation of the CPP before it reaches its target, whether cytoplasm, nucleus or extracellular, has to be avoided for it to serve as a good delivery carrier, however it should also be able to metabolize and release chemically ligated cargo after internalization. The major degradation pathway for proteins in the cytosol is the ubiquitin-proteasome pathway (86). Two successive steps are involved in this pathway; covalent attachment of ubiquitin to the target protein followed by the 26S proteasome-mediated degradation of the ubiquitinated substrate and the subsequent release of ubiquitin that is recycled (86). The proteasomes are located abundantly throughout the cytoplasm and hence are likely to encounter CPPs that localize in the cytoplasm. Whether CPPs are a substrate for ubiquitination is not known. Ubiquitination occurs on the lysine residue of proteins (87), and therefore CPPs rich in lysine can be speculated to be targets. Moreover, if the cargo carried by the CPP is a full-length protein that is a substrate of the proteasome like cyclins or cyclin dependent kinases, there is likely to be competition between degradation of the cargo and assertion of its bioactivity. In addition to the ubiquitin pathway, the 26S proteasome mediates degradation through ubiquitin independent pathways with chymotrypsin-like (Tyr or Phe), trypsin-like (Arg or Lys), and caspase like specificity. To our knowledge, no study has focused on the proteasomal degradation of CPPs. It would be interesting to determine what role the proteasome plays in degradation of various CPPs, if any, and also how modulation of the proteasome activity would affect the cytosolic accumulation of CPP and its cargo, since it is an irreversible pathway. Other than the proteasome, there are several other cytosolic peptidases such as tripeptidylpeptidase II that could digest CPPs (88). Trehin *et al.* studied the metabolic stability of three different CPPs (Tat, penetratin and hCT) in three different epithelial cell lines. Rapid cleavage of the peptides was noticed, with endopeptidases digesting the N-terminal domain followed by further degradation of the metabolites by aminopeptidases and carboxy peptidases in all the three cell models (89). Another study focused on the degradation of pVEC, a CPP derived from a vascular endothelial cadherin, and found that it was rapidly degraded when incubated inside and outside of human aorta endothelial cells as well as murine A9 fibroblasts (45). As mentioned earlier, rapid degradation of the CPP is undesired and use of the D-isoform of the peptides or addition of proteases inhibitors in *in vitro* experiments as strategies to prevent it have been applied, On the other hand, for *in vivo* applications of the D-isoforms, it will be necessary to account for physiological clearance of the peptides as well as their acute and chronic effects.

The fraction of CPPs localized in endosomes as a result of endocytosis will also encounter the degradation pathway of the lysosome if they are unable to escape from the endosomes. Lysosomal proteases are well characterized. With more than 40 hydrolytic enzymes including proteases, nucleases glycosidases, lipases, phospholipases, phosphatases and sulphatases associated with the lysosomes, CPPs or the cargo attached to CPP are bound to be substrates for some of these enzymes.

Transcellular Transport

CPPs have been widely used to translocate across the cell plasma membrane barrier to reach the cytoplasm or nucleus of the cell, but surprisingly fewer studies have focused on traversing intact cellular barriers like the endothelial barrier (BBB) or epithelial barriers (intestinal or pulmonary epithelium). These cellular barriers restrict the entry of most compounds into the brain or blood circulation. Paracellular transport is restricted by expression of tight junctional proteins that afford high resistance to the cell, whereas transcellular transport is partly prevented by the activity of efflux pumps like P-glycoprotein. Considering that during HIV infection, the Tat protein is shown to be released from infected cells and driven into neighbouring cells (90), it is conceivable that CPP added on one side of the cell monolayer can be internalized by the cells and retrieved on the opposite side of the monolayer. Langel et al. showed that transportan (a chimeric peptide from the neuropeptide galanin and the wasp venom peptide, mastoparan) and its analogue transportan10 could permeate through Caco-2 epithelial cells transcellularly with minimum effect on the transepithelial resistance (91). On the other hand, both L and D isoforms of the Tat peptide labeled with Technitium showed low permeability in MDCK and Caco-2 epithelial cells (92), while fluorescently labeled human calcitonin-derived CPPs showed intracellular accumulation but insignif-

icant permeability across MDCK, Calu-3 and Tr146 cells (93). Interestingly, various reports show that upon conjugation of CPP to cargo, the permeability of the conjugate is much higher across the cellular barriers than cargo alone. Cyclosporine A conjugated to heptaarginine was able to permeate both the epidermal and dermal layer of the skin (37). Insulin covalently linked to the Tat peptide showed a 6-fold increase in transport across Caco-2 cells compared to native insulin (94). Cross-linked iron oxide (CLIO) nanoparticles functionalized with either the Tat peptide or octaarginine were shown to cross Caco-2 cells with a 2–3 h lag phase followed by a faster steady state transport of up to 8 h (95). Similarly doxorubicin conjugated to D-penetratin or SynB vector (derived from protegrin-1) led to 6-fold increase in uptake and a 20-fold increase in the amount of doxorubicin transported across the BBB in an in situ rat brain perfusion model (96). A fusion protein of Tat and neutrophin GDNF (97), Tat and β-galactosidase (39), and conjugates of Tat and quantum dots (98) are among the macromolecules shown to reach the brain parenchymal tissue by overcoming the blood brain barrier. Our own studies show that insulin conjugated to the D-isoform of nonaarginine led to a 22-fold increase in transport across primary cultured rat alveolar epithelial cells (unpublished results). Collectively, these studies indicate that the quantitative transport of CPP attached to different cargo can differ from free CPP. Whether this is a result of difference in translocation efficiency, difference in sensitivity of the assay, or involvement of different pathways that compete to transport the CPP and cargo is not yet clear.

PHARMACEUTICAL PERSPECTIVES

The utilization of CPPs as carriers in the intracellular delivery of cargo molecules both *in vivo* and *in vitro*, have been recently reviewed (1,99). A comprehensive list of more than 300 applications of different CPPs with a diverse set of cargo was published in 2004 (100). But since this is a rapidly growing field, several studies applying CPPs as carriers for various macromolecules are continuously being added to the existing literature, which makes it rather difficult to keep track of all the applications. One property of CPPs that is clearly being exploited is its ability to deliver cargo that varies greatly in size and nature irrespective of the pathway of internalization. Additionally, the relative lack of toxicity and cell specificity has enabled its widespread use in preclinical models, while only a few have made it to the clinical trial stages. In this review, we highlight some of the very recent advances in CPP mediated delivery. Table I shows some of the applications of CPP based on compartment and type of macromolecules delivered.

Small Molecules

The plasma membrane poses as a serious barrier not only to hydrophilic substances but also to small hydrophobic molecules. For example, fluorescein, a hydrophilic dye widely used to label CPPs is highly impermeable. Through conjugation with various CPPs it has been shown to reach the cytoplasm as well as the nucleus (20,21). The effectiveness of the anticancer agent, methotrexate is limited because tumor cells develop drug resistance. Methotrexate prevents tumor proliferation by impairing the synthesis of purine nucleotides, the building blocks of DNA through inhibition of the enzyme dihydrofolate reductase in the cytoplasm. By conjugating methotrexate to a CPP YTA2, a five-fold increase in cytotoxicity was seen compared to methotrexate alone, with EC50 values of 3.8 and 18.5 μM respectively in MDA-MB-231 cells (101). Doxorubi-

Table I. Examples of Different Macromolecules Delivered *In Vitro* and *In Vivo* Using CPPs

	Cargo Type	Example	CPPs	Reference
Cytoplasm	Small molecules	Methotrexate	YTA2, YTA4	(101)
	Peptides	p16	R9, K9, Tat, Antp	(42,106,107)
	Proteins	Survivin(Thr34), Bcl-X_L, exonuclease III	Tat	(110,111,114)
	Antisense	PNA against ank RNA	TP10	(124)
	SiRNA	Against VEGF, luciferase, GFP	R9, penetratin, transportan,	(125,126)
	Liposomes	DOTAP	Tat, R8, Antp	(58)
	Nanoparticles	USPIO	Tat	(131)
Nucleus	Small molecules	Metalloporphyrin, rhodium DNA intercalator	MAP, R8	(102,103)
	Proteins	HSV thymidilate kinase 1	Tat	(115)
	Plasmid DNA	Luciferase gene	Tat, R8	(116,117)
	Liposomes (perinuclear localization)	PEGylated liposomes with GFP plasmid	Tat	(143)
	Nanoparticles	Gold	Tat	(130)
Transcellular	Small molecules	Doxorubicin	Penetratin, synB1	(96,144)
	Peptides	Cyclosporin A	oligoarginine	(37)
	Proteins	Insulin	Tat	(94)
	Nanoparticles	CLIO nanoparticles	Tat, R8	(95)
	Quantum dots	CdS:Mn/ZnS	Tat	(98)
In Vivo	Peptides	cJNK, NMR2, p53, SmacN7	Tat, R8	(132,133,138,139)
	Proteins	β-gal	Tat	(39)
	Plasmid DNA	Luciferase gene	Tat	(116)
	Liposomes	VEGF, GFP	R9, Tat	(125,143)

cin another anticancer agent was shown to be transported transcellularly in BBB models when conjugated to the CPP, synB vector. The pathway of transcellular transport however, was suggested to be by adsorptive endocytosis as evidenced by competitive inhibition in transport with protamine and polylysine (96). Metalloporphyrin a chemical nuclease, linked to MAP was shown to follow the nuclear pathway and efficiently kill tumor cells compared to free metalloporphyrin (102). To boost the nuclear delivery of a DNA intercalator based on transition metal complex rhodium, Brunner and Barton conjugated the organometallic compound to D-octaarginine and demonstrated efficient nuclear localization of the conjugate in HeLa cells, while retaining the site-specific DNA binding activity of the intercalator (103).

Although a proof-of-concept is established for delivery of small molecules using CPPs, practically and for commercial purposes, attaching CPPs to small molecules may not be the favored method of choice to improve delivery of lead molecules, because of their vulnerability to steric hindrances and orientation effects. Furthermore other tactics of delivery improvement such as formulation manipulation can easily be employed.

Peptides

Several cytoplasmic proteins involved in cell cycle progression and regulations have been identified as targets for multiple types of cancer. Peptide sequences that correspond to the crucial region of the protein are sometimes used for therapeutic intervention instead of the full-length protein. For example, the p16 protein, a cyclin dependent kinase inhibitor, controls cell cycle progression by competitively binding to CDK4 thus preventing the CDK4-cyclinD complex formation in the late G1 phase of the cell cycle (104). However in tumors, the growth inhibitory effect of p16 protein is abolished. A synthetic peptide corresponding to residues 84–103 of the full-length protein has been shown to be effective in restoring the function of p16 protein (105). This peptide was conjugated to Tat peptide as well as Antp and delivered effectively to the cytosol (106,107). The importance of understanding the pathways of CPP internalization to select the right CPP for the appropriate target was shown in our study using p16 peptide. While both oligolysine and oligoarginine p16 conjugates showed similar internalization, oligoarginine conjugate was more effective in anti-proliferative activity owing to its direct transduction into the cytosol compared to endosomal trapping of oligolysine conjugate (42).

Another cyclin inhibitor peptide derived from p21 ^{WAFI} was conjugated to penetratin and shown to prevent cancer cell growth by inhibiting cell cycle progression. Calpastatin peptide derived from the endogenous peptide calpastatin was linked to 11 meroligoarginine and shown to significantly inhibit calpain activity in living neuronal cells. Calpain is a calcium-dependent cysteine protease that regulates the function of several intracellular proteins that contribute to the pathogenesis of neuronal diseases such as Alzheimer's and Parkinson's disease (108). An amphipathic antimicrobial peptide KLA, that induces apoptosis by disrupting the mitochondrial membrane was attached to heptarginine and shown to exert its activity in several tumor cell lines as well as *in vivo* (109). To prevent rapid intracellular degradation, the D-isoform of KLA and heptaarginine was used in this study.

Proteins

Delivery of full-length proteins intracellularly is hampered by their size and the use of plasmid-based genetic recombinant techniques to express proteins in the cell, has its own set of limitations such as variable transfection efficiency and cytotoxicity. Successful CPP-mediated delivery has been shown for several full-length proteins that retain their biological activity. With the exception of Pep-1, a CPP that is physically mixed with protein cargo, CPPs are coupled to proteins through covalent chemical ligation or more commonly through fusion constructs produced by transfected bacteria.

Survivin, a cytoplasmic protein involved in cell cycle regulation, is a novel member of the inhibitor of apoptosis family and a crucial target for anti cancer therapy. A phosphorylation defective mutant of survivin (Thr34) acts as an antagonist by dissociating the caspase-9 survivin complex resulting in apoptosis. A fusion protein of Tat and mutant survivin was generated and shown to facilitate apoptosis in cancer cell lines but not normal cells (110). Similarly, when fused to Tat peptide the anti-apoptotic protein Bcl-X_L of the BCL2 family was able to prevent apoptosis in retinal ganglion cells (111).

Cell penetrating peptides have been shown to resist penetration through the mitochondrial phospholipid bilayers (112,113). Therefore, to deliver exonuclease III, a mtDNA repair enzyme to its target, the mitochondrial matrix of breast cancer cells, Shokolenko *et al.* constructed a fusion protein that has a mitochondrial targeting signal (MTS) on the N-terminal and a Tat peptide (CPP) on the C-terminal end (114). This study exemplifies a rational design that applies a combination of transduction properties of CPP and targeting signals, not only to deliver the protein intracellularly but also to the intended target. They also note that the order of the peptides in the fusion construct is crucial. Tat peptide on the N terminus followed by the MTS and then the functional protein did not show any mitochondrial delivery (114).

A functionally active and stable fusion protein of Tat and HSV thymidylate kinase 1 was shown to localize in the nucleus in human hepatoma cells and sustain the activity of the prodrug gancyclovir due to its bystander effect (115). According to the authors, endocytosis was not involved in internalization and the stability of the fusion protein was attributed to the lack of lysosomal involvement.

Plasmid DNA and Oligonucleotides

Current methods of gene delivery including viral and non-viral methods (lipoplexes, polyplexes) and physical methods like electroporation or microinjection have certain limitations that prevent their effective use. Since CPPs share some properties similar to polyplexes and lipoplexes, like their cationic nature and high cellular association, it has been postulated that they may serve as good DNA or oligonucleotide (ON) delivery agents. Also, because nuclear delivery is necessary for biological activity, the nuclear localization property of some of the CPPs can be exploited for this purpose.

One study used the monomeric form of Tat peptide to complex with DNA, and showed similar efficiency in transfec-

tion as compared to commonly used polylysine while demonstrating that the plasmid DNA-Tat complex was internalized by endocytosis (116). Octaarginine however, did not show good transfection efficiency until it was modified with hydrophobic lipid molecules (117). The current trend is the use of branched CPP to complex with DNA (118–121) or alternatively, functionalizing particles that carry ON or DNA with the CPP. Some studies use CPP in addition to the conventional transfection agents to improve efficiency (58,122,123).

Gene regulation at the RNA level using siRNA, ribozymes or antisense oligonucleotide analogues, does not require nuclear uptake, however cellular uptake is necessary but is limited by the negatively charged nature of the oligonucleotides. To study the interaction between RNA and RNA binding protein, a PNA conjugated via disulfide linkage to transportan 10 was utilized to promote the cytosolic delivery of the PNA (124). Recently siRNA against vascular endothelial growth factor (VEGF), a multifunctional angiogenic growth factor, was complexed with cholestryl-R9 and shown to enhance tumor regression efficacy of the siRNA both *in vitro* and *in vivo* (125). Thiol containing siRNA against luciferase and GFP transgenes, were conjugated to penetratin and transportan by a disulfide linkage and were shown to efficiently reduce transient and stable expression of the reporter genes in several cell types (126). The disulfide link would be reduced in the cytoplasm to release the bioactive siRNA. It can be concluded that complexing or conjugating oligonucleotides to CPP can achieve significant intracellular delivery with biological activity, however endosomal escape remains as a rate-limiting step (117–121,123,125,126).

Liposomes and Nanoparticles

Pharmaceutical carriers like liposomes and nanoparticles have also been modified with CPPs to increase their uptake (100). Although these carriers provide protection to their payload and improve drug properties such as solubility, the size of the carriers limits their cellular uptake. Unlike proteins and peptide cargo, nanoparticle or liposomes can be modified with a higher amount of CPP per particle. The density of the modification is shown to affect the efficiency of cellular uptake and also the internalization pathway (58,127). Low density of octaarginine on liposomes results in clathrin-mediated endocytosis, whereas a higher density results in macropinocytosis. Liposomes modified with Tat, Antp or octaarginine were tested for their suitability for aerosolization by assessing their efficiency of cellular uptake and toxicity in airway epithelial cells. Contrary to most findings showing oligoarginine as a more efficient CPP compared to Tat, in this study, liposomes modified with Tat were shown to be more efficient than octaarginine modified liposomes, but octaarginine modified liposomes were less toxic than Tat liposomes (58). The efficiency of octaarginine liposomes to deliver siRNA against the human double minute gene 2 (HDM2) was compared to unmodified liposomes and lipofectamine in three lung tumor cell lines. While octaarginine modified liposomes were able to effectively silence the gene and reduce proliferation of cancer cells, they also showed serum stability even after 24 h post incubation (128). Tat modified nanocarriers for gene delivery to the lungs has also been studied. *In vitro* studies showed lower transfection efficiency with Tat functionalized PEG-PEI nanocarriers. However, when tested *in vivo*, a 600% increase in transfection efficiency was observed compared to the control, PEI nanocarriers due to a combination of factor including: increased DNA uptake mediated by CPP, increased DNA condensation and stability (122).

Torchilin *et al.* have applied CPP to their 'SMART' multifunctional, nanocarrier drug delivery system, which contains an external myosin-specific monoclonal antibody modified PEG polymer (for targeting and prolonged circulation) and an internal function consisting of Tat peptide that is exposed only under lower pH conditions (129). An *in vivo* study on the proof-of-concept has not yet been reported. Gold nanoparticles were modified with the Tat peptide for targeting the nucleus and potential application in imaging (130). Ultra small paramagnetic iron oxide nanoparticles (USPIO), used as contrast agents in MRI, were functionalized with the Tat peptide and used to efficiently label CD4 + T cells while maintaining the chemotactic, transmigratory and regulatory functions of the cells (131).

In Vivo Studies

Regardless of controversies on the mechanism of internalization and methodological issues *in vitro*, several attempts for *in vivo* application of CPP have been successful. The first *in vivo* study reported for CPPs was that of the Tat-β galactosidase fusion protein delivered intraperitonially in mice. Consistent with *in vitro* data of a lack of cell specificity, Tat-β-gal fusion protein was shown to localize in almost all tissues including the brain. In addition, the enzymatic activity of the fusion protein was not compromised *in vivo* as shown by X-gal assay (39). The ability to penetrate the brain has sparked interest in examining the therapeutic potential for cerebral ischemia therapy. Reduction in cerebral infarction volume and improved neuorological outcomes in rodent models after cerebral artery occlusion has been shown with Tat-cJNK peptide (132), Tat-NMR2 peptide (133) and Tat-Bcl-xL recombinant protein (134), reinforcing the findings of Tat penetration into the brain. Two other disease areas that have seen considerable applications of CPP *in vivo* are inflammation and cancer. Rodent models of asthma and rheumatoid arthritis are commonly used to study the effect of anti-inflammatory responses. Increased anti-inflammatory effect of Tat-DN-H-Ras or Tat-DN-E85 in mouse models of asthma was shown through the blockade of Ras or PI3 kinase activity, while Tat-I-κB inhibited inflammation in a mouse model of rheumatoid arthritis through inhibition of transcription factor NF-κB (135,136).

For cancer therapy, several *in vitro* studies that tested the potential of CPPs to deliver proteins and peptides based on cell cycle regulation in cancer cells have been extended to *in vivo* tumor models. A peptide derived from the C-terminus of p53 protein (p53C) conjugated to the Tat peptide is one of the examples. Mutations in the p53 protein are noted in most cancer types, and its activity can be restored by the p53C peptide in cancer cells, leading to apoptosis. To stabilize and increase the half-life of the p53C-Tat peptide *in vivo*, Snyder et al. used the retro inverso form of the p53-Tat peptide and demonstrated efficacy by inhibition of tumor growth after intraperitoneal injection, and also prolongation in survival time (more than

200 days survival) of mice bearing B-cell lymphoma compared to untreated mice (35 days survival) (137). In *in vitro* studies it was shown that Smac, a mitochondrial protein that inactivates the inhibitors of apoptosis proteins (IAP), when conjugated to Tat or Antp peptide, sensitizes cells to pro-apoptotic stimuli. Two separate groups tested the feasibility of applying the Smac-CPP peptide with an anticancer agent in *in vivo* tumor models. In one study, intracranial co-administration of the Smac-Tat peptide and TNF-related apoptosis inducing ligand (TRAIL) in a glioblastoma xenograft model resulted in over 100% increase in survival time for treated mice compared to untreated mice (138). In the second study, SmacN7 conjugated to oligoarginine (SmacN7-R8) potentiated the activity of chemotherapeutic agents cisplatin and taxol, resulting in a significantly increased inhibitory effect on the growth of H460 xenografts as compared to cisplatin or taxol alone or a combination of cisplatin and unmodified SmacN7 (139). Other successful applications of tumor suppressor peptide fused to CPP *in vivo* include the Tat-VHL peptide effective against renal cell carcinoma tumor grafted in nude mice (140) and Antp-p16 peptide effective against pancreatic cancer cell tumor grown subcutaneously and intraperitoneally in nude mice (106).

Since CPPs are non-selectively distributed to all tissues *in vivo*, pro-drug approaches that utilize targeting in combination with CPPs need to be crafted in order to increase effectiveness and minimize exposure to non-targeted cells thereby reducing the necessary dose. To selectively deliver tumor-imaging agents to cancer cells *in vivo*, a CPP-fluorophore was linked to a polyanion sequence through a linker that can only be cleaved in the presence of matrix metalloproteinases that are normally over-expressed in tumors. The polyanion sequence was included to reduce the cellular association of the conjugate to cells through electrostatic interaction (140). Another group utilized the hypoxic microenvironment of tumors to include an oxygen dependent domain (ODD) of hypoxia inducible factor (HIF) in the Tat-caspase 3 fusion protein, that stabilizes it in tumor environment but degrades it in normal tissues (141).

So far, almost all of the pre-clinical *in vivo* studies show encouraging results, whether any one of the applications can be translated to success in clinical trial remains to be determined. A few years ago, Cell Gate, a California based company, initiated clinical trials for a topical formulation of cyclosporine A-CPP conjugate based on their successful pre-clinical results.

CONCLUSION AND EXPERT OPINION

From the myriad published studies involving CPPs, it is clear that CPPs have potential to overcome the obstacle of crossing the cell membrane barrier and aid in transporting cargo into and across the cell. Nevertheless, great care has to be taken in designing and interpreting the studies that provide a fundamental understanding in the processes involved in the interaction of the peptides with the cell and their downstream fate. A clear understanding can only facilitate rational and systematic selection and utilization of delivery vehicles with a better rate of success and less waste of resources. Although research in this field has been ongoing for about 15 years, a consensus on the internalization mechanism has not been reached because of methodological reasons. It is puzzling to note how artifactual results from cell fixation can mislead and create more controversies in the field. Differences in the numerous variables of the experimental procedures employed by various groups makes it harder to compare studies and arrive at a conclusion especially on the mechanism of internalization. These variables include: different sources and purity of CPP, label or cargo utilized, concentration of CPP, cell -type, -density and -stage of cell cycle, incubation time and final end point or read-out.

In addition to these variables, we believe that the following two issues must be resolved before CPPs can be used effectively for the design of cytoplasmic delivery of various bioactive molecules.

1. A new classification of CPPs should be established. It is clear more than one type of CPP exists that can deliver macromolecules into the cells. Therefore, it is likely that there are different CPPs for cytosolic or nuclear localization. These CPPs may have different mechanisms of transport, such as membrane transduction- or endocytosis-dependent pathways. A comparison between two CPPs for either mechanistic or carrier properties may be inappropriate if they belong to two different CPP classes. At this stage, the arginine-rich and amphipathic oligopeptides are considered as two major classes of CPP. However, the implications of the classes of CPPs on the mechanism of transport and the intracellular localization need to be further elucidated. Furthermore, the possibility that conjugation of CPP to a macromolecular cargo may alter the class that the CPP falls in should also be considered.
2. The intracellular processing of CPP-cargo conjugates should be fully investigated. At present, the intracellular processing of CPPs and their conjugates is still a largely underdeveloped area. However, it is conceivable that the intracellular processing will determine not only the localization but also the bioactivity of the cargo molecules. It is interesting to compare the evolution of the field in CPP with that of endocytosis. The rapid progress in the field of endocytosis during late 1970s and early 1980s was a result of the elucidation of intracellular routes of different types of receptor-mediated endocytosis (142). Conceivably, a fully delineated pathway of a CPP-cargo conjugate inside the target cells can be used for designing and optimizing the CPP-mediated drug delivery system.

Finally, with the excitement of delivering macromolecules into the cell, little attention is being paid to the physiological significance of membrane transduction processes. Mother Nature has formed such a pathway for a specific reason and deciphering it can help not only in taking advantage of this unique transport pathway in drug delivery, but also understanding the mechanisms behind cellular pathologies and physiology.

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