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Influence of protein transduction domains on intracellular delivery of macromolecules

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As the plasma membrane and blood–brain barrier selectively restrict the entry of most compounds into cells to < 500 Da, delivering macromolecules into cells was, until recently, little more than a goal. However, with significant effort to capitalise on therapeutic targets available in the post-genomic era, novel approaches for delivering therapeutic macromolecules are being rapidly developed. The discovery of small cationic peptides, termed peptide/protein transduction domains or cell-penetrating peptides, which cross biological membranes, has emerged as a venerable Trojan horse to transport large, biologically active molecules, such as peptides, proteins and oligonucleotides, into mammalian cells *in vitro*, as well as in preclinical models and clinical trials *in vivo*. This review discusses the implications of peptide/protein transduction domain-mediated delivery of macromolecules and their possible uses as important primary drug delivery agents.

Keywords: Antp, cell-penetrating peptide, macromolecular therapeutic, peptide/protein transduction domain, Poly arginine, transactivating transcriptional activator

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1. Introduction

The most frequently used cellular macromolecular transporters contain short cationic peptides sequences of 8 – 16 amino acids in length, termed peptide/protein transduction domains (PTDs) or cell-penetrating peptides (CPPs) [1,2]. Protein transduction was originally observed in 1988 after full-length HIV transactivating transcriptional activator (TAT) protein was shown to enter mammalian cells, leading to transcriptional activation from an HIV long-terminal repeat promoter construct [3,4]. Since the initial discovery of TAT-mediated transduction, other novel transduction domains have been identified within several other proteins, including the third α-helix of the antennapedia homeotic transcription factor (Antp) that makes contact with DNA [5-7], and synthetic peptide carriers, such as polylysine and polyarginine [8-10] (**Table 1**). Although there seems to be little-to-no homology between the primary and secondary structures of these PTDs, the rate of cellular uptake has been found to strongly correlate with the number of basic residues present. This observation suggests that the presence of a common internalisation mechanism is likely to be dependent on an interaction between the positively charged side groups of the basic residues on PTDs, primarily arginine residues, and negatively charged acidic cell surface molecules (probably glycans, carboxylic acids, sulfates and phosphates) [11,12].

Although different PTDs show similar characteristics of cellular uptake, PTDs vary in their efficacy for transporting protein cargo into cells. Fusion proteins created with the TAT (amino acids 47 – 57) PTD have shown markedly better cellular uptake than similar fusions using the 16 amino acid sequence from the Antp PTD. In contrast, retro-inverso forms of TAT or homopolymers of arginine (8 × arginine) seem to increase cellular uptake several-fold [11,12]. Moreover, the Antp PTD

Table 1. Commonly used protein transduction domains/cell-penetrating peptides.

PTD	Sequence	Residue length	Properties
TAT(47-57)	YGRKKRRQRRR	11	Arginine rich with 2^0 amphipathicity
Antp(43-58)	RQIKIWFQNRRMKWKK	16	Arginine rich with 2^0 amphipathicity
Poly Arg^8	RRRRRRRR	8	
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	27	Lysine-rich, amphipathic helical

efficiently transduces into cells when associated with chemically synthesised peptides; however, the efficiency seems to decrease with the incorporation of larger molecular weight protein cargoes [13,14]. Both TAT and Antp have been used extensively as transporters for relatively small cargo, such as small molecules, peptides and oligonucleotides [5,6,15-17]. However, TAT has also been successfully used to deliver much larger molecules, including full-length proteins [18], 45-nm iron beads [19], λ-phage particles [20] and 200-nm liposomes containing complexed plasmid DNA [21,22], into cells. Transduced cargo has been shown to carry out intracellular functions ranging from cytoskeletal reorganisation and targeted recombination of genomic DNA [18] to protein–protein interactions (reviewed in [23-25]). Moreover, TAT-mediated delivery is undergoing testing in several clinical trials. The outcomes of these clinical trials should guide the future direction of the field of PTD-mediated delivery.

2. Mechanism of TAT-mediated protein transduction

PTDs represent novel agents to overcome the bioavailability delivery problem to transport macromolecular cargo across the plasma membrane and into the cell. Although this approach was first observed in 1988, only recently has the mechanism of this miraculous feat been understood.

2.1 TAT-cell-surface interactions and internalisation by macropinocytosis

Full use of PTDs for macromolecular therapeutic cargo requires an understanding of the molecular basis for transduction into cells. This information can then be used to enhance delivery efficiency. Interactions with cell-surface proteins, receptors and other structural components of the outer cell membrane are likely to be critical for cellular uptake. Due to the requisite cationic nature of TAT, Antp and 8 × arginine PTDs, cell-surface proteoglycans (e.g., heparan sulfate), are thought to be instrumental for cationic PTD uptake [26]. Consistent with this observation, heparan was shown not only to bind TAT [27-29], but to also compete with TAT binding to the outer cell membrane and internalisation [26]. Indeed, cells deficient in their ability to sulfonate heparan proteoglycans do not effectively internalise TAT peptides [26], suggesting that the proportional size of heparan chains and the cationic nature of the PTD are both important for the outer membrane interactions and internalisation. However, these observations need to be investigated in other cell types, as other glycans are also likely to be involved here.

Cationic PTDs have been shown to transduce into most, if not all, mammalian cell types in culture. This polygamous nature of PTDs may result from the global expression of heparan sulfate chains. However, it is still unknown how or which signals stimulate PTD internalisation on binding to heparan sulfate or other moieties on the outer cell membrane. Initial attempts to observe PTD intracellular uptake used visual observations of PTD-treated and fixed cells, showing artifactually wide distribution of TAT and other PTD-conjugated molecules throughout the cytoplasm and nucleus [30,31]. However, confocal fluorescence imaging of live cells revealed that the vast majority of PTDs are present within vesicles inside the cytoplasm and vesicle escape seems to be the rate-limiting step for macromolecular cargo delivery into cells.

To circumvent the visualisation problem and begin to dissect the requirements for delivery of macromolecular cargo into the cytoplasm and nucleus of cells, Wadia *et al.* devised a 'hand-in-fire' phenotypic assay using a TAT-fusion protein with the bacteriophage Cre DNA recombinase [16]. Reporter cells were used that contain a stable integration of a eGFP reporter gene proceeded by a transcriptional STOP DNA segment flanked by LoxP recombination sites (recognised by Cre). This prevents eGFP expression until the Lox-STOP-Lox DNA segment is removed by Cre. Thus, expression of eGFP requires TAT-Cre to enter the cytoplasm, be transported to the nucleus and finding the single, integrated reporter gene. With this phenotypic assay in hand, Wadia *et al.* showed that TAT-Cre transduction, release and recombination of DNA occurred in as little as 15 min. As predicted, addition of heparan sulfate inhibited TAT-Cre transduction and gene recombination, and TAT-Cre was present in endosomal vesicles.

Wadia *et al.* used the TAT-Cre phenotypic assay to demonstrate that TAT-conjugated proteins and peptides enter mammalian cells by a lipid raft-dependent, receptor-independent, specialised form of fluid phase endocytosis, termed macropinocytosis [16] (**Figure 1**). Macropinocytosis [32] is an actin-dependent endocytic pathway that induces cellular uptake

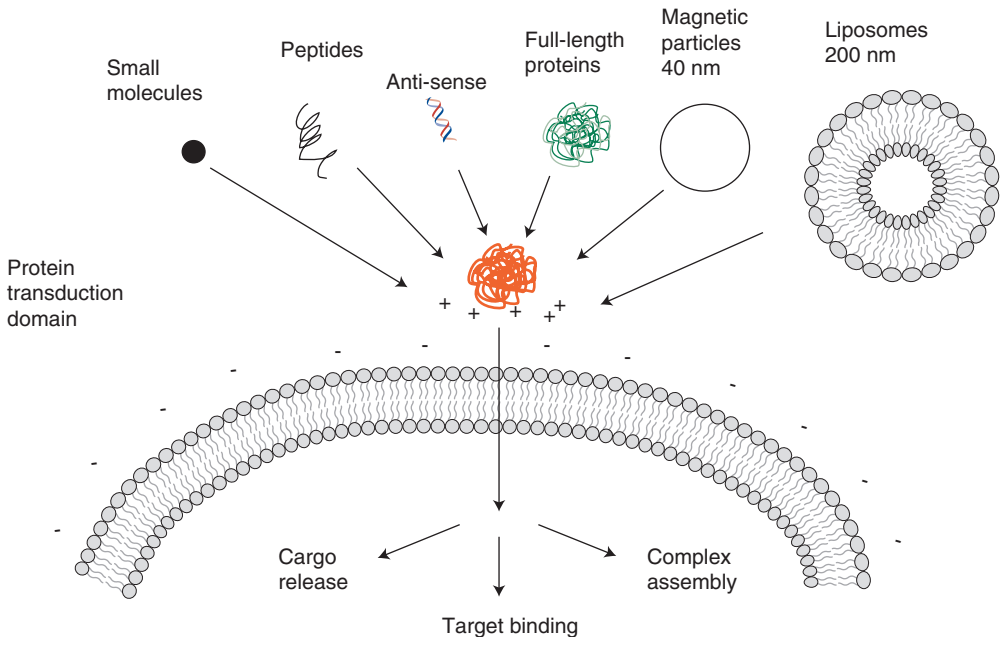

Figure 1. Protein transduction into cells. Cationic PTDs carry cargo into cells by a receptor-independent, fluid-phase macropinocytosis, a specialised form of endocytosis. Delivered classes of cargo molecules represent a wide range of sizes and biophysical properties from small molecules to peptides to proteins to PNA to DNA to phage particles to magnetic nanoparticles and liposomes.
PTD: Protein transduction domain.

of non-selective bulk fluid (**Figure 2**). Although macropinocytosis is thought to be receptor independent, it is activated by cellular growth factors and cytokines (reviewed in [17,33]). Consistent with this notion, inhibitors of macropinocytosis, such as amiloride and 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), blocked transduction, but dynamin-dominant negative, which blocks clathrin- and caveolin-mediated endocytosis, had no effect [16,34,35] (**Figure 3**). Although macropinosomes are considered inherently leaky vesicles compared with other types of endosomes [36,37], most TAT proteins remained trapped within these intracellular compartments up to 24 h post-treatment. Importantly, macropinosomes also do not seem to traffic to lysosomes [38,39], suggesting that these vesicles may serve as an intracellular reservoir that essentially protects the PTD-cargo.

2.2 Escape from macropinosome vesicles

The transfer of PTD-conjugated cargo from macropinosomes to the cytosol is likely to be the rate-liming step of PTD-mediated transduction. As < 1% of PTD-bound molecules are released, liberating this vesicular reservoir will greatly improve therapeutic delivery efficiency. However, important questions remain regarding the molecular details of understanding the nature of how macromolecules are released from macropinosome vesicles, with numerous groups investigating various strategies to enhance escape. Little is known about how vesicular transport and release of macromolecules occurs; however, the relative pH of endocytic vesicles has been suggested to affect the ability of PTDs and their cargo to be released into the cytoplasm (**Figure 3**). Recent reports have shown that acidification of vesicles containing PTD-fusion peptides and nanoparticles [40-42] induce an increase in escape. Wadia *et al.* took advantage of this pH drop and introduced a pH-sensitive membrane, destabilising, fusogenic peptide domain from influenza virus haemagglutinin (HA2), fused to the TAT peptide (TAT–HA2), that dramatically increased the efficiency of TAT-cargo escape into the cytoplasm [16].

To understand the potential requirement for a pH dependency in vesicle escape, Magzoub *et al.* performed a series of elegant biophysical experiments on large unilamellar vesicles combined with a pH gradient generating compound [43]. In this study, the authors used two cationic PTDs derived from Antp and the N terminus of the bovine unprocessed prion protein (bPrPp, amino acids 1 – 30). When the PTD-containing endosomes were acidified by creating a transmembrane gradient across the large unilamellar vesicles using the nigericin ionophore, there were significant amounts of both Antp and PrPp PTD peptide escape compared with low/no escape from non-nigericin-treated control vesicles [43]. More recently, Lee and Tannock used chloroquine and omeprazole to enhance the release of the basic pH anticancer drug doxorubicin [44]. Unfortunately, many of the agents that alter the pH of endosomes, such as chloroquine, affect most, if not all, vesicles and, hence, are extremely cytotoxic at the effective concentration. In contrast, the TAT–HA2 approach discussed above targets escape from macropinosomes that contain only PTD-cargo and results in a cytotoxic free form of enhanced escape [16]. Alternatively, photochemical transduction enhancers have been used to enhance the destruction of

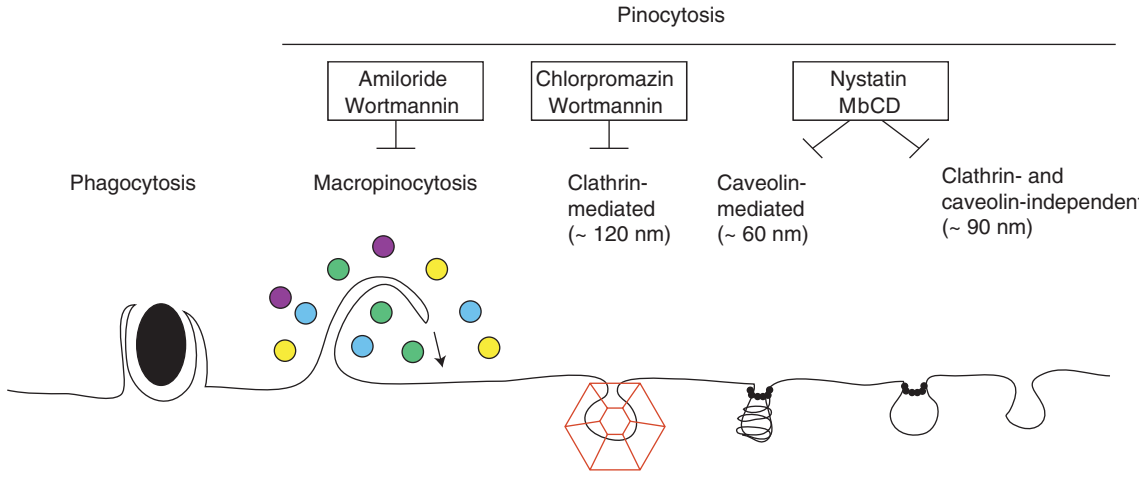

Figure 2. Endocytosis-mediated intracellular entry of macromolecules. Macromolecules may enter the cell in a variety of energy-dependent methods. The exact signalling cascades that facilitate each are still under dispute.
Adapted from [63].

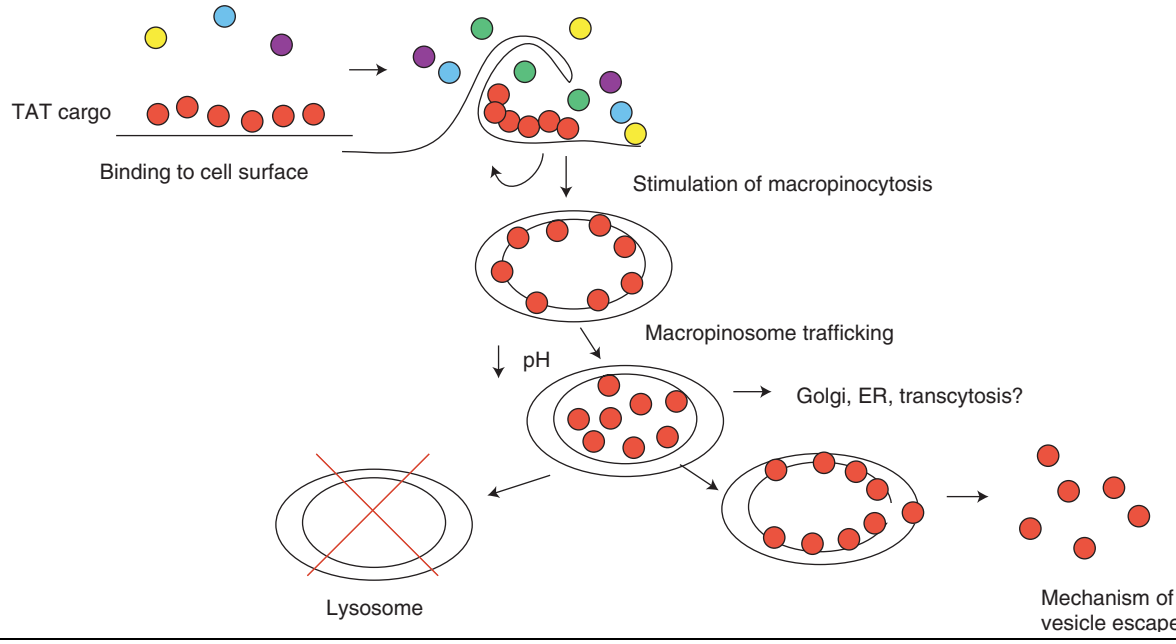

Figure 3. Mechanism of TAT-mediated transduction into cells via macropinocytosis. TAT-conjugated cargo has been shown to enter a variety of different cell types via a mechanism known as macropinocytosis. Once TAT-enclosed macropinosomes enter the cytoplasm, low pH stimulates endosomal release of TAT-conjugated macromolecules.
ER: Endoplasmic reticulum; TAT: Transactivating transcriptional activator.

PTD-containing vesicles, albeit with limited potential for tissue penetration [45].

3. Peptide/protein transduction domains as therapeutic delivery agents

The delivery of peptides and proteins that modulate the intracellular signalling cascades has powerful therapeutic potential. Although PTDs have shown a wide range of uses in manipulating the biology of cultured mammalian cells [46], *in vivo* systemic delivery of biologically active compounds has great therapeutic potential. Reporter PTD-fusion proteins, such as TAT-β-galactosidase, have been shown to distribute throughout most, if not all, tissues of mouse models [15]. In addition, administration of TAT-conjugated peptides have been shown to manipulate tumour burden, as well as heart function in preclinical models [47]. As an example, Chen *et al.* reported a therapeutic benefit of the δPKC inhibitor peptide, TAT-δV1-1, toward significant infarct size reduction in an *in vivo* porcine model of acute myocardial infarction [48].

3.1 Peptide/protein transduction domains and cancer therapeutics

One of the most impressive features of PTD-mediated delivery of macromolecular therapeutics is its tremendous versatility. A broad spectrum of cargo molecules have been successfully delivered that represent a wide range of sizes and biophysical properties, including small molecules, peptides, protein functional domains, full-length proteins, oligonucleotides, phage particles, magnetic nanoparticles and liposomes. In the context of cancer therapy, transduction overcomes several problems encountered with traditional chemotherapeutics. Conventional chemotherapies for cancer tend to show low specificity for the target and largely affect normal cells, as well as tumour cells. Furthermore, these agents are often transported out of cells by the P-glycoprotein pump (MDR1), resulting in resistant tumour cells [17]. In contrast, transduction as a delivery modality is independent of MDR1 and also allows for size-independent development of novel agents that specifically target cancer cells. Several groups have demonstrated the ability to manipulate tumour biology in preclinical models with transducible peptides [17], including blocking Cdk2/cyclin A activity [49], sequester MDM2 [50] or induction of apoptosis by mitochondrial disruption [51].

The von Hippel-Lindau (VHL) tumour suppressor gene normally functions in renal cell growth by binding to the cytoplasmic domain of the insulin-like growth factor 1 receptor (IGF-1) and interrupting IGF-1 signalling [52]. Mutations in the IGF-1 binding domain of VHL lead to unregulated IGF-1 receptor signalling and renal cell growth [52,53]. Intraperitoneal administration of TAT-VHL peptide slowed the growth of subcutaneous renal cell carcinoma tumours in nude mice, primarily through inhibition of cell proliferation rather than by induction of apoptosis. TAT-VHL peptide treatment also reduced tumour invasion into the underlying tissue. This study also provides strong immunohistochemical evidence that the TAT-VHL peptide was homogeneously delivered to the tumours after intraperitoneal injection.

Another report found that intraperitoneal delivery of an Antp-p16 fusion peptide moderately inhibited the growth of pancreatic carcinoma cells growing as intraperitoneal and subcutaneous tumours in nude mice [54]. Although p16 functions primarily as an inhibitor of cell-cycle progression, the authors found that Antp-p16 peptide slowed tumour growth by inducing apoptosis of cancer cells *in vivo*. Importantly, both these detailed studies [53,54] failed to find indications of cytotoxicity to normal cells and tissues in the preclinical models, suggesting that the effective dose was capable of specifically targeting the cancer cells and not the surrounding normal cells. If this proves to be a general rule, cell-penetrating peptides may prove to be a powerful tool in the design of anticancer agents, which target tumours and spares normal tissue.

The p53 DNA damage sensor tumour suppressor gene is mutated in > 50% of all human malignancies, leading to a loss of p53 function. Because p53 plays an important role in cellular apoptotic responses, p53 mutations lead to increased tumour resistance to therapy and unregulated cancer cell growth [55]. Due to continuous DNA damage in cancer cells, p53 reactivation has been postulated to result in cancer-cell-specific apoptosis. Indeed, introduction of an Antp-conjugated peptide derived from the C-terminal domain of p53 effectively induced cancer-cell-specific apoptosis; however, normal cells were unaffected [56,57]. More recently, Snyder *et al.* fused a modified p53 C-terminal fragment (containing D-amino acids to increase peptide half-life) to TAT [58]. Treatment of mice with the TAT-p53C′ peptide bearing metastatic peritoneal cancer resulted in a 6-fold increase in longevity in one model system, and 50% cure in another. Yet other examples of the use of PTDs in cancer therapeutic development involve targeting the hypoxic nature of solid tumour microenvironments with inhibitors to hypoxia-inducing factor-α [59] and inhibitors of the merlin protein necessary for Schwannoma tumours that occur sporadically in patients with neurofibromatosis [60].

3.2 Disulfide linkages to enhance intracellular delivery of peptide/protein transduction domains

So far, most published studies using cationic PTDs have used covalently conjugated PTD-cargo fusion proteins produced either from bacterial expression systems and purified, or as synthetic peptides [17]. Although this is an effective means of testing the validity of the PTD-conjugated molecule on the cellular signalling process of interest, the influence of the PTD on the intracellular localisation and biological activity of the macromolecule being delivered must be carefully studied. To overcome the potential limitations of a peptidyl linkage, several groups have employed the use of PTDs attached to their cargo via labile and cleavable cysteine disulfide bonds. For example, Stein *et al.* showed that when an antibody against tetanus toxin was conjugated to TAT via a labile disulfide bond, activity was significantly higher than when irreversibly covalently conjugated directly to TAT as a fusion protein [61]. In addition, *ex vivo* and *in vivo* models of acute myocardial infarction found that a disulfide linked inhibitory peptide against δPKC translocation (δV1-1) delivered into rat and pigs had an IC_{50} of 50 pM, whereas irreversible covalent conjugation was ineffective [14,48]. Therefore, either cargos that are reversibly disulfide conjugated to PTDs escape macropinosomes more efficiently, or PTD interferes with cargo function once inside the cell.

Other non-covalent PTD-cargo attachments (e.g., thiazolidine, thioester, amide, oxmine, hydrazine and thiolmalmeide linkages) have also been described [62]. **Figure 4** demonstrates some common methods of PTD-cargo conjugation. Clear studies to discern between these two possibilities need to be performed and evaluated.

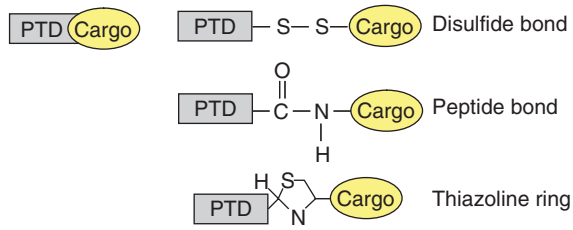

Figure 4. Common PTD–cargo attachment models.

4. Conclusion

Over the last decade, numerous studies have revealed the utility of PTDs for modulating cellular signalling processes in culture, in living organisms and, recently, in human clinical trials. Collectively, these studies have shown that PTDs have great therapeutic potential in numerous areas to expand our repertoire of experimental therapeutics with a decidedly crisper specificity than small-molecule therapeutics. The use of PTD-delivered macromolecular cargos have shown great promise in preclinical animal models of heart disease, stroke, cancer and other diseases responsible for significant numbers of human suffering, morbidity and death. Several PTD-conjugated peptides and macromolecules have now advanced into clinical trials. Although more studies exploring the pharmacodynamic and pharmacokinetic properties of PTD-macromolecules are clearly needed, the potential benefit of delivering PTD-conjugates to virtually every cell in the human body represents a novel and substantial area for future drug targeting and development.

5. Expert opinion

Although transduction with cationic peptide domains dates to before the identification of HIV1 TAT's ability to transduce across the cell membrane (in 1988) [3,4], for the most part, the field of macromolecular delivery by PTDs/CPPs has just emerged from its rather lengthy nascent beginnings and is now only approaching early childhood. Indeed, what was only recently considered macromolecular heresy by many, is now being tested in multiple clinical trials. Moreover, the lack of a size restraint has and will continue to allow the development of very creative approaches to solve therapeutic problems. However, there remains many unanswered questions that need to be resolved in the next 5 – 10 years to both fully harness the potential of this approach for delivery of experimental therapeutics, and to understand the potential pitfalls as soon as possible (and find paths around them).

The path ahead will require the extensive improvement of escape from macropinocytotic vesicles, to harness a controlled release approach and to target specific cell types via receptors/cell surface molecules. This will clearly require identification of the genes that are involved in macropinocytosis uptake at the cell membrane and trafficking of the vesicles within the cytoplasm. We know of only a single validated gene, namely actin, that is involved in macropinocytosis. This is even more surprising when one considers that this cellular process was initially described > 50 years ago. Moreover, improvements in promoting transcytosis, to begin to tap into delivery of macromolecular cargo in the CNS, has the potential to open entire areas of the body to therapeutic intervention. Thus, understanding the mechanism of transduction, its cellular requirement and the genetic basis is absolutely needed to move forward on a firm footing.

The use of cationic PTDs/CPPs is but one of a limited handful of approaches that are proven in preclinical models to deliver macromolecular therapeutics. The approach has great potential to take advantage of RNAi selectivity, peptide/enzymatic amplification, and intracellular antibody specificity for treatment of disease. However, it is still very early days. We must avoid the self-inflicted hyperbole of past promising approaches that ultimately failed and move transduction delivery forward on solid scientific ground. Indeed, compared with the known pros/cons of small molecule therapeutics, we know only a modicum of what is required to successfully bring this approach to bear on human disease. Yet, given the potential to introduce macromolecular therapeutics into cells, the next 10 years should prove very interesting, if not truly exciting.

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