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Breaking in and Busting out: Cell-penetrating Peptides and the Endosomal Escape Problem

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Review

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Abstract: Cell-penetrating peptides (CPPs) have long held great promise for the manipulation of living cells for therapeutic and research purposes. They allow a wide array of biomolecules from large, oligomeric proteins to nucleic acids and small molecules to rapidly and efficiently traverse cytoplasmic membranes. With few exceptions, if a molecule can be associated with a CPP, it can be delivered into a cell. However, a growing realization in the field is that CPP-cargo fusions largely remain trapped in endosomes and are eventually targeted for degradation or recycling rather than released into the cytoplasm or trafficked to a desired subcellular destination. This 'endosomal escape problem' has confounded efforts to develop CPP-based delivery methods for drugs, enzymes, plasmids, etc. This review provides a brief history of CPP research and discusses current issues in the field with a primary focus on the endosomal escape problem, for which several promising potential solutions have been developed. Are we on the verge of developing technologies to deliver therapeutics such as siRNA, CRISPR/Cas complexes and others that are currently failing because of an inability to get into cells, or are we just chasing after another promising but unworkable technology? We make the case for optimism.

Keywords: cell-penetrating peptides; endocytosis; endosomal escape; protein transduction domains; TAT.

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Introduction

Nearly 30 years ago, HIV researchers Frankel and Green (1, 2) independently stumbled upon a phenomenon that ultimately gave rise to a potentially transformative technology: the HIV protein transactivator of transcription (TAT) could readily pass through the plasma membrane of uninfected mammalian cells. Further, and most importantly, TAT retained its normal functionality and subcellular localization post-entry as it readily entered the nucleus and promoted gene transcription. This surprising ability to traverse the cellular membrane was later mapped to a stretch of 12 basic amino acids and this penetrative property could be conferred onto other proteins to which that sequence was fused (3, 4). A new area of intense scientific research was thus born: the delivery of bioactive molecules into mammalian cells via direct penetration of the plasma membrane.

Since Frankel's and Green's discoveries, a large number of peptides that are rapidly internalized and enable transport of macromolecular cargos into mammalian cells *in vitro* and *in vivo* have been discovered or designed. These peptides came to be known as cell-penetrating peptides (CPPs, also frequently referred to as protein transduction domains, or PTDs). The term CPP now refers to a broad grouping of non-homologous short peptides, the majority of which are hydrophilic and cationic in nature, though amphiphilic, anionic and hydrophobic CPPs have been reported. A database of more than 1600 CPPs is described by Agrawal et al. (5). Their unifying property is the ability to penetrate the plasma membrane for delivery of cargo into cells, from peptides and proteins, both small and large, to various nucleic acids, including DNA, RNA, small interfering RNAs (siRNAs) and peptide-nucleic acids (PNAs) (6–9).

Thirty years later, CPP-based technologies have largely failed. Frankel and Pabo (1) first reported that cellular entry of TAT was likely via an endosomal-independent mechanism as introduction of the endosomal-blocking agent chloroquine enhanced TAT uptake into cells. However, much of the subsequent data supporting spontaneous membrane translocation were found to be

the result of experimental artifacts. It was soon realized that majority of CPPs became trapped in endosomes following delivery into cells and, as a result, any associated cargo became trapped as well (10, 11). It is likely the case that, owing to endosomal entrapment, the vast majority of CPP cargos are eventually targeted to either the lysosome for degradation or back to the plasma membrane for recycling and subsequent ejection from the cell. We have dubbed this the 'endosomal escape problem'.

In spite of setbacks, CPPs still have significant therapeutic and investigative potential. Currently, more than 25 CPP clinical trials are underway, including a Phase III (12, 13). To successfully transition CPP-based technology into therapeutic delivery systems, several considerations need to be made. This review focuses on fundamental questions and the challenges faced by the field in this endeavor: How do CPPs get into the cell? Once inside, how is cargo released from the endosome into the cytosol? How do we ensure biological activity and correct subcellular localization of cargo following endosomal release? And finally, how can we ensure specificity in cellular targeting?

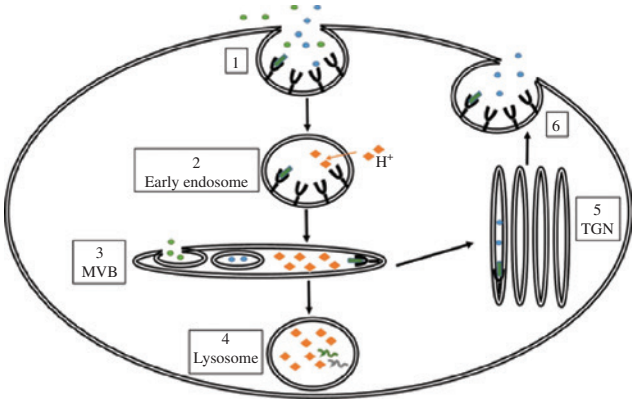

Figure 1: An overview of endocytosis.
Endosome formation may be receptor (clathrin/caveolin) or non-receptor mediated (macropinocytosis). Following invagination (clathrin/cavaeolin) or evagination (macropinocytosis) of the plasma membrane, an early endosome is formed (1). As the endosome travels into the cell, it transitions to an early endosome and becomes increasingly acidified by pumping in cytosolic hydrogen ions into the vesicular lumen (2). As the early endosome matures, it begins to sort cargo by the formation of multiple smaller intraluminal vesicles (ILVs) (3). Some of these vesicles may re-fuse with the endosomal membrane and deliver contents directly into the cytosol, called 'backfusion' while others will be destined for either the lysosome (4) or the trans-Golgi network (TGN) (5). Cargo delivered to the lysosome will be degraded while cargo delivered to the TGN will be recycled back to the plasma membrane. Some receptors can bypass the TGN and be directly recycled back to the plasma membrane from the early endosome (6).

Breaking in: mechanisms of cellular entry

Currently, the field recognizes endocytosis to be the primary mode of CPP cellular entry and subsequent endosomal escape, the rate-limiting step for the effectiveness of CPPs to deliver cargo into living cells. However, there is still much controversy on how CPPs enter cells. In this section of the review, we will provide a brief history of 30 years of mechanistic insight on CPP cellular entry with a primary focus on endocytosis.

A brief glance at endocytic pathways

Endocytosis is a broad term encompassing a variety of pathways utilized by the cell to bring outside molecules in for a multitude of purposes – receptor and lipid recycling, inhibition of signal transduction, uptake of solutes/nutrients and destruction of foreign/unwanted materials. The unifying property of endocytic pathways is, simply put, engagement of the plasma membrane for the formation of an intracellular membrane-bound organelle which will then transport the molecules to some destination within the cell. It is a tightly coordinated, energy-dependent process requiring extensive cellular adaptors and cytoskeletal rearrangements. As illustrated in Figure 1, following organelle formation, an endocytic vesicle undergoes a pH-dependent maturation process as it transitions from the early sorting endosome (pH ~6–6.5) into the late endosome or multivesicular body (MVB; pH ~5). The acidification process is dependent on ionic gradients, requiring calcium, sodium and potassium efflux as well as hydrogen and chloride influx. An endosome has three potential fates – it may be targeted to the lysosome from the MVB for destruction of its intraluminal contents or its contents may be recycled back to the plasma membrane either directly from the sorting endosome or after routing from the MVB to the trans-Golgi network. Alternatively, some endosomal contents such as nutrients are needed by the cell and, thus, must be released into the cytosol. This latter process is mediated by the formation of intraluminal vesicles (ILVs) inside the endosome, essentially the formation of an exosome within the endosome. The ILV then undergoes a process known as 'backfusion' where it fuses with the endosomal membrane and its contents are released into the cytosol.

Endocytosis can further be broken down into phagocytosis and pinocytosis depending on whether entry is

fluid phase or not. We will limit our discussion to pinocytic pathways most commonly engaged by CPPs – macropinocytosis, clathrin-dependent and caveolin-dependent endocytosis and lipid-raft endocytosis. A full description of these pathways is outside the scope of this review; however, a brief description of these pathways is provided below:

1. Macropinocytosis is defined as a receptor-independent and coat-independent process. First described by Warren Lewis in 1931, this is a form of cellular '*pino*' (drinking) '*cytosis*' (14). Macropinocytosis is classically thought to be a non-specific mechanism to bring solutes/nutrients into the cell via large endocytic vesicles. However, more recent studies point to a need for stimulation at the plasma membrane via the presence of extracellular growth factors [reviewed in Ref. (15)]. Further, different cell types may utilize this process in different ways given their particular needs. For example, some cells use this process to obtain nutrients or to recycle portions of their membranes while immune cells utilize macropinocytosis for the regulation of antigen presentation.
2. Clathrin-dependent endocytosis is a receptor-driven process that results in the formation of a 'coated' vesicle. The trimeric coat protein, clathrin, for which this pathway is defined, was the first coat protein to be isolated from cellular membrane-bound vesicles in 1976 by Pearse (16). During endocytosis, three-footed triskelion subunits assemble via adaptor proteins at cholesterol-deficient regions of the plasma membrane, forming into a lattice work to create a highly ordered 'caged' structure which is internalized. This pathway is considered ubiquitous across all cell types and is utilized in a variety of ways; transferrins, low-density lipoproteins, hormones and neurotransmitters (during reuptake) are a few examples of molecules taken up by this pathway.
3. Caveolin-mediated entry shares both similarities and distinctions from clathrin-mediated entry. Caveolins are also coat proteins that form tight associations with cholesterol present in the plasma membrane. Unlike the trimeric clathrins, following recruitment to the plasma membrane, caveolins form a distinct 'U' shape, with both N- and C-termini pointing toward the cytoplasm. The resulting invaginations resemble cave-like structures called caveolae, for which they were originally named (17). Not surprisingly, caveolins are found localized to cholesterol-rich lipid rafts. Many growth factors utilize this pathway, as do some pathogens. Further, caveolin-dependent endocytosis is important in transendothelial transport. Unlike the more ubiquitous mechanism of clathrin-dependent endocytosis, caveolae formation is impacted by many cellular factors such as cell type as well as cell cycle progression. Further, some cells express caveolins at low levels or not at all.
4. Lipid raft endocytosis is a non-receptor-mediated, concentration-dependent form of endocytosis occurring at cholesterol-enriched lipid rafts in the plasma membrane but does not rely on caveolin coat formation. In this form of endocytosis, glycosylphosphatidylinositol-anchored proteins (GPI-AP) group into distinct microdomains, invaginate and form GPI-enriched intracellular vesicles. This pathway is primarily used as a constitutive means of bringing in extracellular fluids through lipid-raft-mediated pathways based in membrane-molecule interactions and has been described for SV40-virions, vitamins, GPI-binding proteins, MHC-class I, IL-2 and IgE. Even though these pathways show significant variability for the requirement of local mediators and in cargo fate (recycling, degradation, intracellular release), they share commonalities in that they are receptor-mediated and proceed via an absorptive fluid-phase mechanism [reviewed in Ref. (18)].

Breaking in via endocytosis: the case and conundrum

Individual CPPs may engage one or more of the forms of pinocytosis defined above. With regard to TAT, different groups have reported cellular uptake by all of these endocytic mechanisms (19–25). Further, some CPPs such as the well-studied arginine-rich, 16-residue peptide corresponding to the third helix of the *Drosophila melanogaster* transcription factor Antennapedia homeodomain (Antp; penetratin) may enter via direct penetration (26). The debate is ongoing; CPP entry mechanism(s) is a heavily explored area with minimal consensus. Much of this likely owes to differences in laboratories, cell types, CPP constructs and experimental conditions.

Frankel and Pabo (1) demonstrated in their seminal paper that lysotrophic agents enhanced instead of blocking full-length TAT uptake into cells. This gave rise to the direct penetrance hypothesis that was rapidly embraced by many in the field. Three years later, Mann and Frankel (27) provided data that full-length TAT likely entered via an undefined absorptive-phase endocytic pathway. Namely, they found that uptake was significantly reduced when performed at 4°C (energy independent) compared

with 37°C. Interestingly, this was a cell-dependent effect as cervical cancer-derived cells (HeLa) showed a striking difference in energy-dependent uptake while temperature reduction had a notably smaller, though similar, impact on a T cell-derived cell line (27). This was the first illustration of a cell-type effect on TAT uptake and these findings hinted at complexities with which we are still coming to grips, including the importance of cell-specific differences in CPP uptake.

The 1990s saw a flurry of reports of direct penetrance by CPPs. However, many of these observations were later found to be artifactual (10). In particular, Lundberg et al. (10) demonstrated that intracellular redistribution and dispersed cytoplasmic and nuclear distribution of CPPs resulted from cellular fixation, whereas live cell imaging experiments indicated a punctate (likely endosomal) distribution. More recent work utilizing live cell imaging has shown evidence for the potential of non-endocytic uptake of some CPP constructs, but these data are not without constraints on the concentration of the CPP and nature of the cargo (19, 28).

By the onset of the 21st century, over a decade from the initial reports of TAT's ability to traverse the plasma membrane, multiple conflicting reports were published in support of one endocytic pathway over another engaged by TAT as well as those of other newly discovered CPPs, as outlined in Table 1. In 2007, Duchardt et al. (19) designed a series of experiments to clarify contradictory data on the use of endocytic pathways by CPPs that plagued the field. They studied concentration-dependent uptake mechanisms of three well-characterized cationic CPPs (penetratin, TAT and R9) employing specific endosomal pathway inhibitors and tracers. It was demonstrated that all three CPPs engaged multiple forms of endocytosis (clathrin-dependent, caveolin-dependent and macropinocytosis) and that the concentration of the CPP played a role in determining the endocytic pathway that was used. While these data are seemingly at odds with previous work, many proteins, receptors and viruses make use of all of these forms of endocytosis. Further, while all of these pathways exhibit specificity, there exists a large degree of functional redundancy and crosstalk among them.

Ten years later much remains unresolved concerning how CPPs enter a cell. Even though endocytosis gained the limelight as the primary means of entry, direct penetrance still remains a distinct possibility and an active area of interest in the field. Determinants dictating CPP utilization of one endocytic pathway over another, or bypassing the endosome entirely, are thought to be highly contextual and dependent on the nature of the CPP, concentration, receptor availability, media conditions and cell type. A

Table 1: Experimental evidence for the use of different endocytic pathways by TAT in different cell lines.

Endocytic pathway	Cell line	Endocytic pathway inhibitors[a]/tracers	Methodology
Macropinocytosis	Tex.loxp.EG (T-cells)	β-Cyclodextrin; nystatin; EIPA; cytochalasin D (23)	Flow cytometry, live cell imaging (23)
	Cos-7 (fibroblasts)	EIPA; cytochalasin D/RFP-labeled caveolin (23)	Live cell imaging (23)
	CHO	Dyamin knockdown/transferrin (23)	Immunohistochemistry (23)
	K562 (lymphoblasts)	ATP-depletion; EIPA (29)	Flow cytometry (29)
Clathrin-dependent	HeLa	Chlorpromazine; potassium reduction/transferrin (30)	Flow cytometry (30)
		Chlorpromazine (effective at high concentrations of TAT – 40 μm); EIPA; MβCD (19)	Flow cytometry, live cell imaging (19)
	Jurkat T-cells	4°C Incubation; chlorpromazine; filipin; Eps15, dynamin and intersectin dominant-negative mutants (25)	Radioactivity labeling, immunohistochemistry (25)
	CHO	Potassium reduction, nystatin/transferrin (30)	Flow cytometry (30)
Caveolin-dependent	HeLa	4°C Incubation/cholera toxin, transferrin, CFP-labeled caveolin (20)	Live cell imaging (20)
		Cytochalasin D, MβCD, 4°C incubation/transferrin, EEA-1, cholera toxin (24)	Live cell imaging, immunohistochemistry, reporter assays, flow cytometry (24)
	Cos-1 (fibroblasts)	Cytochalasin D, MβCD, 4°C incubation/caveloin-1 (24)	Flow cytometry, immunohistochemistry (24)
Lipid raft	Jurkat T-cells	Cytochalasin D, MβCD, 4°C incubation (24)	Flow cytometry, immunohistochemistry (24)
Undefined	PBM-MOs	ATP-depletion, 4°C incubation (30)	Flow cytometry (30)
	HUVEC	ATP-depletion, 4°C incubation (30)	Flow cytometry (30)

[a]Inhibitors: macropinocytosis – EIPA; clathrin – cytochalasin D, chlorpromazine, potassium reduction; caveolin – flipin, β-cyclodextrin, nystatin; lipid raft-β-cyclodextrin, nystatin, potassium reduction.

better understanding of the factors influencing one form of uptake over another in different target cells and tissues will be paramount in optimizing targeting, uptake and endosomal escape strategies to develop CPPs for effective cargo delivery *in vivo* and *in vitro*.

A receptor for CPPs?

Regardless if cellular entry is direct or endosomal, the CPP must initially come into contact with the plasma membrane to facilitate uptake. Thus, requirements for cellular entry are a primary question in the field. Currently, CPP-membrane interaction is thought to be governed by either non-specific electrostatic interactions or via ligand-receptor binding. Given that TAT, the prototypical CPP, is derived from a HIV protein, much of the work in this arena has been based on HIV research and has utilized either TAT or TAT-homologous arginine-rich CPPs.

Early work on HIV entry mechanisms revealed that full-length TAT could bind, among other molecules, ubiquitously expressed cellular proteoglycans (namely heparan sulfate proteoglycans, or HSPGs), integrins and chemokine (C-X-C motif) receptor 4 (CXCR4), though in the latter case it serves as an antagonist (31–36). All of these molecules readily activate endocytic pathways in both clathrin-dependent and -independent mechanisms. In particular, binding to HSPGs and integrins was mapped to the basic domain of TAT, which also confers its cell-penetrating properties (31). In 1993, Vogel et al. (34) reported that while the basic domain of TAT could readily bind integrin $\alpha v\beta 5$, antibodies blocking this interaction had no effect on TAT uptake into cells. Since these observations, HSPGs have received prominent attention as potential TAT receptors (29, 30, 33, 37–39).

Interaction between TAT's basic domain (its CPP sequence) and heparan sulfates (HSs) was originally elucidated by Rusnati et al. (40). These observations were expanded upon in 2001 by Tyagi et al. (33) who provided evidence for the need for cell surface proteoglycans for cellular internalization of TAT constructs, as cells defective in glycosaminoglycan (GAG) synthesis showed reduced TAT uptake. Mounting evidence for the roles of HSPGs in uptake and endocytosis of arginine-rich CPPs such as TAT have provided a new framework for the study of receptor-mediated entry of CPPs (29, 30, 33, 37–40). With respect to cationic CPPs, interactions with negatively charged GAGs comprising HSPGs are thought to concentrate these CPPs at the plasma membrane to facilitate endosomal uptake.

Expanding upon the HSPG-CPP interaction hypothesis, Letoha et al. (29) in 2010 and Kawaguchi et al. (37) in 2016 identified the ubiquitous HSPG syndecan 4 as a potential receptor for the classical arginine-rich CPPs. Letoha et al. (29) reported that syndecan 4 enhanced the uptake of TAT, penetratin and the bioengineered octoarginine peptide R8 via an energy-dependent endocytic mechanism they attributed to macropinocytosis. This is not surprising as syndecan 4 is known to activate multiple downstream small GTPases involved in different endocytic pathways including macropinocytosis, clathrin-dependent and caveolin-dependent endocytosis as well as lipid raft-dependent endocytosis (41, 42). A major limitation to this work is the use of a cell-based overexpression system in lymphoblasts, which normally do not express syndecans. There is some doubt as to whether a cell that does not normally express this molecule will recreate normal syndecan-mediated cellular uptake pathways following transfection.

Kawaguchi et al. (37) later performed a screen for binding partners of R8 in HeLa cells. They identified 17 potential R8 binding partners, seven of which are proteoglycans and two of which are core components of the extracellular matrix (ECM). They confirmed that syndecan 4 facilitated cellular entry of R8 in a concentration-dependent manner, as siRNA-mediated knockdown of syndecan 4 impacted uptake at a low concentration (1 μM), but only had little effect at a high concentration (10 μM) (37). In contrast to previous observations, R8 appeared to predominantly utilize a clathrin-dependent endocytic pathway instead of micropinocytosis though this may be a by-product of experimental conditions (22).

The other recently identified candidate receptor, CXCR4, is highly expressed on migratory cells such as immune and cancerous cells. CXCR4 was originally identified as a key receptor for HIV type X4 and works in concert with co-receptor CD4 and cell-surface HSPGs to facilitate viral entry into target cells (35, 36). In 2012, Tanaka et al. (43) provided evidence for the use of CXCR4 followed by subsequent macropinocytosis by the arginine-rich CPP R12. In support of CXCR4 and syndecan 4 serving as receptors for arginine-rich CPPs, CXCR4 is normally complexed with syndecan 4 and that association promotes binding of its natural ligand, stromal cell-derived factor 1 (SDF-1) (44). At odds with this, however, Tanaka et al. found that CXCR4 facilitated uptake of R12 but not the other arginine-rich CPPs, TAT or R8, the latter being the proposed receptor of syndecan 4 (37, 43). The failure of TAT to bind CXCR4 likely owes to the fact that binding to CXCR4 has been mapped to TAT's central 'chemokine-like' domain though direct analysis

of its basic domain binding to CXCR4 has not been performed to our knowledge. Finally, CXCR4 is found in low abundance in normal, non-migratory, healthy cells and tissues; so, while CXCR4 may facilitate the uptake of at least one CPP, R12, as noted by the authors, it is likely not the sole receptor.

Identifying receptors for different CPPs will be paramount in untangling mechanisms of entry and may lead to the development of more efficient cargo-delivering CPPs. However, if CPPs can readily utilize more ubiquitously expressed molecules such as HSPGs, this may reduce the therapeutic value without effective targeting strategies. Further, failure to control endocytic pathway engagement means a variety of fates may await any given molecule every time it is introduced.

Busting out: escape from the endosome

The field has increasingly recognized that the principal roadblock to the development of therapeutics is cargo entrapment in the endocytic pathway (13, 45). Almost all described CPP technologies are reliant on covalent crosslinking or non-specific hydrophobic interactions (46). In our opinion, it is here that CPPs currently fail as a workable technology. If cellular entry is receptor mediated, it could well be that the high affinity of the CPP for its receptor is in part due to low off rates and hence trapping of its linked cargo in the endosomes may essentially be a kinetic problem. In this section, we focus on some of the more promising approaches that have been developed to facilitate escape from the endosome.

Promising tricks for facilitating endosomal escape

The most promising 'tricks' to overcome entrapment include the use of endosomolytic agents, reversible covalent binding and reversible high-affinity non-covalent binding. The Pellois group has developed an endosomolytic agent to promote release of cargo via endosomal leakage. Their dimerized disulfide-linked TAT (dfTAT) can destabilize endosomes for the delivery of co-incubated cargo (47, 48). This methodology is particularly attractive as it allows for introduction of cargo, even multiple cargos, without direct interaction with TAT. Further, utilization of disulfide bonds should enhance stability for systemic delivery.

Another promising method is the use of endosomal disruption agents based on antimicrobial peptides (49). Using a CPP that also incorporated two membrane-disrupting antimicrobial peptides, investigators were able to overcome endosomal entrapment, hypothesizing that membrane disruption occurred during vesicular trafficking as the CPP reached the 'critical membrane disrupting concentration', thus effecting endosomal escape. A follow-on study demonstrated the utility of Salomone et al.'s (50) design as a transfection agent with ionically linked nucleic acids and CPPs. Other similar efforts to selectively disrupt endosomal membranes include engineering of 'endosomal escape domains' (EEDs) (51) and use of pH-sensitive peptides (23).

Several groups have developed novel reversible strategies that may have advantages over endosomolytic agents in toxicity, specificity and simplicity. Among the reversible strategies, the most common is the use of thiol coupling. The reducing environment of both the endosome and the cytoplasm should be an effective means to reduce disulfides and uncouple cargo from CPP. Further, as with the dfTAT model, the use of thiol coupling should provide stability and protect the cargo/CPP during systemic delivery. Another approach described by Rossi et al. (52) utilizes an interesting photocleavable linkage to deliver cleaved peptides to the cytosol. While this may overcome entrapment, this strategy is seemingly impractical for many applications as it would be difficult to get light to many places within patients. The potential of this strategy as a research tool, however, may be high.

Models that utilize non-covalent CPP-cargo linkages can overcome problems associated with covalently bound cargo. Our group has recently described a novel CPP-adaptor method that exploits normal ionic gradients to free cargo following endosomal entry (53). We associated CPPs with their cargos via a reversible coupling between a CPP-containing calmodulin and cargo that contains a calmodulin binding site (CBS). Our initial findings, described in Ref. (53), showed that TAT-fused calmodulin (TAT-CaM) bound CBS-containing cargos with nanomolar affinity in the presence of calcium but not at all in its absence. In that study, three model cargos and three distinct cell lines were used, demonstrating general, efficient and rapid delivery of cargo to the cytoplasm at a much lower dose (1 μM) than necessary using other means. Further, when calcium was removed from bound TAT-CaM-cargo complexes, very rapid dissociation ensued. As illustrated in Figure 2, the high calcium concentrations of the extracellular environment ensures high-affinity CPP-cargo binding. Following entry into the cell, the extracellular calcium is rapidly lost as the endosome becomes increasingly acidified (54). We

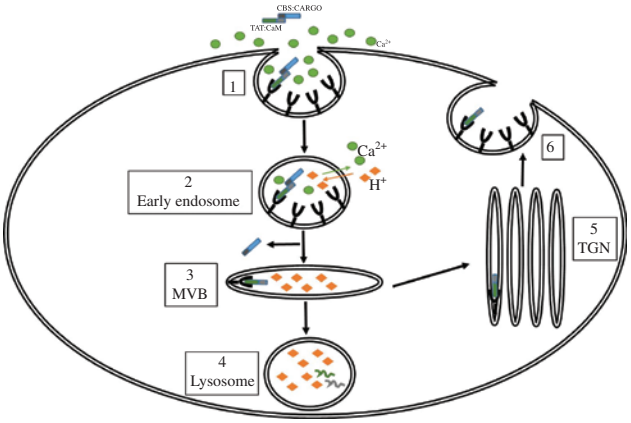

Figure 2: Proposed model for TAT:CaM-mediated intracellular delivery of CBS:CARGO.
TAT fused with a calmodulin (TAT:CaM) will readily associate with cargo containing a calmodulin-binding site (CBS:CARGO) in the extracellular environment owing to high levels of calcium (1). Following binding of TAT:CaM to a receptor, an endosome will form containing the TAT:CaM-CBS:CARGO as well as high levels of extracellular calcium. As the endosome matures, calcium will be pumped out of the intraluminal space while hydrogen ions are brought in (2). This shift in calcium concentrations within the endosome will cause the CBS:CARGO to disassociate from TAT:CaM, allowing for release of the cargo into the cytosol, presumably through ILVs, even if TAT:CaM remains tightly bound to its receptor (3). TAT:CaM itself may have multiple possible fates following formation of the late endosome. It may be sent to the lysosome for degradation if it retains its ability to bind its receptor in increasingly acidic conditions (4) or sent to the TGN (5) for recycling back to the plasma membrane (6).

hypothesize that as calcium levels drop, the CPP adaptor releases its cargo from the early endosome prior to formation of the more acidic late endosome.

Considerable attention has been given to the design of new, more efficient, delivery mechanisms. The use of non-covalent association of CPPs with their cargo is, in our opinion, a key strategy to enhance bioavailability of biologically active molecules. By non-covalently coupling the cargo from the CPP, problems with endosomal entrapment and the potential for a loss of biological activity are easily surmountable. Finally, these methodologies typically require reduced and more therapeutically feasible concentrations of protein for the desired effect.

Subcellular localization: targeting freed cargo to specific intracellular compartments

Once cargo has been delivered into the cell and successfully escaped the endosome, how does the cargo get targeted to a particular location within the cell? Much of our understating of subcellular localization of proteins is derived from the basic knowledge of signal peptides that facilitate localization of proteins to intracellular compartments. Within the endomembrane system, such delivery is achieved through sorting of localization signals and subsequent vesicular-mediated targeted delivery. However, many subcellular compartments can directly import cytosolic proteins provided they contain a specific targeting sequence. Such examples include mitochondrial import; receptor-mediated nuclear import export; cotranslational entry into the ER and import of cytosolic proteins, such as catalase, into peroxisomes post-budding off the ER membrane.

Protein replacement therapy aims to deliver a fully functional protein to replace one that has been mutated, lost or is underexpressed. Under ideal circumstances, a protein is successfully delivered into the cell, freed from the endosome and behaves exactly like a wild-type endogenous protein. In the past decade, several groups have been successful in this endeavor. A group from the Hebrew University-Hadassah Medical School, led by Lorberboum-Galski et al. (55), has developed and successfully delivered functional proteins to the mitochondria *in vitro* to restore defects in the electron-transport chain and acetyl Co-A production (56) associated with life-threatening mitochondrial diseases. Other groups have also demonstrated the ability to deliver functional copies of proteins into cells to alleviate dysfunctional proteins associated with mitochondrial and lysosomal diseases. For example, in 2012, Honda et al. (57) were able to deliver functional subunits of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex to the cytosol to restore its activity *ex vivo* in neutrophils from patients suffering from chronic granulomatous disease (CGD), a disease characterized by improper reactive oxygen species (ROS) production resulting from defective NADPH complexes. While conceptually simple, these approaches are not without significant consideration to ensure the protein will behave as it should within the cell. The protein must be produced in highly purified, properly folded conformation, have its native subcellular localization signal accessible to subcellular import machinery (in the case of non-cytosolic proteins), show normal dynamics of stability and activity, be expressed at normal endogenous levels, engage with binding partners and, in many cases, be able to undergo post-translational modification.

Delivery of therapeutic molecules into the cell often requires artificial means for intracellular localization of the molecule to the compartment of interest. The predominant approach for targeting CPP-bound cargo calls for the fusion of canonical signal sequences to cargo proteins, or even to the CPP tag itself [reviewed in Ref. (58)].

Using this methodology, cargos have been delivered to the nucleus (59, 60), the nucleolus (61), the lysosome (62), the peroxisome (59), the mitochondria (59, 63, 64) and the endoplasmic reticulum (59, 65), to name a few. Drawbacks to this approach include decreased uptake of tagged CPPs (65) and potential endosomal entrapment en route to the intended compartment. The underlying mechanism via which some of these CPP-cargo complexes gain entry into specific subcellular compartments is less well understood. Within the endomembrane system, it is tempting to speculate that one might be able to exploit endosomal entry for cargo targeting though ensuring proper sorting within the late endosome may present challenges.

Targeting CPPs to specific cell types

The specific delivery of cargo to a particular target cell or tissue via CPP-mediated methods remains the Holy Grail of pharmacological work in this field [reviewed in Ref. (66)]. The central problem is that of specificity: given that CPP uptake is thought to occur via a general mechanism with a ubiquitous cellular receptor such as HSPGs or membrane apparatus, it is extremely facile to deliver CPP-tagged cargo proteins systemically. The technical challenge, albeit not insurmountable, is to deliver them to a particular site, tissue or organ system.

Initial work in this area centered on localized injection of CPP-tagged cargo proteins. While these approaches yield the desired effect of delivery of CPP cargo directly to tumor cells, they are only effective for solid, localized tumors or easily isolated or discovered target regions.

Other strategies involve exploiting the biochemical nature of target cells/tissues. So-called activatable cell-penetrating peptides (ACPPs) pair technical innovation with an improved working knowledge of the affected cellular biochemistry and have been successful with some degree of target specificity by several groups [reviewed in Ref. (67)]. These ACPPs are activatable in a stimulus-dependent manner and are caged or masked until they are in the vicinity of their target. This methodology employs an anionic inhibitor to shield cationic CPPs until they are near cells or tissues whose extracellular environment contains a product capable of cleaving the anionic inhibitor, thus freeing the CPP at a target site. This methodology was successfully used in 2004 to target tumor cells by Jiang et al. (68) who used a proteolytic cleavage site targeted by metalloproteases secreted from tumor cells. These results were further confirmed in two reports in 2009 by Olson et al. and Aguilera et al. of the Tsien group (69, 70). However, due to endosomal entrapment (70) and significant off-target uptake by other tissues *in vivo* (71), this technology has developed primarily as a means to target tumors for imaging purposes, which in and of itself has significant value.

More recent studies have utilized pH-, photo- and hydrogen peroxide-sensitive CPP linkers to target desired tissues. In 2014, Weinstain et al. (72) described a hydrogen peroxide-sensitive linker that was targeted at lung tissues following induction of lipopolysaccharides (LPS)-mediated inflammation in a murine model. Given that infection results in high levels of ROS, the constructs would ideally only be cleaved in regions undergoing an inflammatory response (perhaps cleverly harnessing the endosomal entrapment problem in a good way, withholding cargos from off-target cell cytoplasms and targeting them for destruction!). In 2016, Yang et al. (73) successfully delivered siRNA in nanoparticles to tumors by exploiting lower pH conditions present in those tumors. However, that cleavage of the linker relied on both low pH conditions and exposure to near IR light confers substantial accessibility limitations. While these studies, and others, yield clever and promising targeting mechanisms, they still lack the degree of specificity that may be needed for widespread therapeutic application as the potential for off-target uptake by healthy cells remains a significant concern.

One means of overcoming off-target effects utilizing stimulus-dependent methodology is to deliver an ACPP that can only be cleaved within targeted cells, provided they contain the product needed for cleavage. This so-called 'Trojan Horse' strategy was first employed by Vocero-Abkani et al. (74) in 1999 when the TAT peptide was used to deliver procaspase-3 to HIV-infected cells. Here, the proteolytic site was replaced by the proteolytic site for HIV-1 protease. Upon uptake of the cargo into HIV-infected cells, caspase-3 is processed, killing the infected cells (74). And while this methodology may have significant therapeutic potential, it is certainly limited to specific diseases and lacks broader applications.

Perhaps most promisingly, recent biopanning strategies have identified a number of short peptides that confer cell-type-specific delivery of cargo proteins via CPP-mediated transduction. Zahid et al. (75) used this approach to identify a cardiac-specific peptide, termed cardiac-targeting peptide (CTP), which facilitated cargo uptake specifically to cardiomyocytes following systemic introduction. More recently, a cancer-specific CPP was designed by Lim et al. (76). This CPP, called BR2, showed enhanced selectivity and nearly a 70% overall increase in cellular uptake in cancer cell lines in comparison with normal cells.

When moving from bench to bedside, enhancing specificity and decreasing off-target effects will be

paramount. Considerable attention needs to be given to the nature and goal of the treatment and optimization of the CPP construct to serve these purposes. What has become increasingly clear is that a one-size-fits-all model will not be useful in this endeavor. As argued above, CPPs that bind ubiquitously expressed membrane proteins will have little promise as to specificity without a coincident secondary targeting scheme, such as a nanoparticle. However, with significant advances in understanding CPP-cell interactions and the biochemical nature of afflicted cells of interest, designer therapeutic CPPs are clearly on the horizon.

Outlook: cause for optimism

CPPs hold the immense promise of rapid, efficient, nontoxic delivery of biomolecules into living cells and thus represent great hope for development of enabling technologies for delivering therapeutics now stymied by poor cellular entry. They may also confer other advantages such as fine control of dosing as compared to transfection or other disruptive delivery method. Technical problems with CPP delivery, while significant, may soon be solved with rather simple solutions that dissociate cargo from CPP via spontaneously cleavable or non-covalent linkages, opening the door to new generations of therapeutics. Prospects for even further enhanced utility by cell-specific targeting and increased ease of coupling, etc. may make them even more profoundly effective.

Highlights

- CPPs can readily enter a variety of cells utilizing multiple forms of endocytosis and, potentially, via direct penetrance.
- CPP-based technologies are one of the most promising means for delivery of a wide variety of cargo (proteins, peptides, DNA, siRNAs) into living cells.
- Endosomal entrapment of CPP-conjugated cargo is a limiting factor for the effectiveness of CPPs for protein delivery.
- Current approaches to achieve endosomal escape of CPP-associated cargo are destabilization of endosomes, the use of cleavable linkers and noncovalent attachments.
- Biological CPPs likely enter cells via ubiquitous mechanisms; however, designer CPPs are effective at targeting specific cells and tissues.

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References

1. Frankel AD, Pabo CO. Cellular uptake of the tat protein from human immunodeficiency virus. Cell 1988; 55: 1189–93.
2. Green M, Lowenstein PM. Autonomous functional domains of chemically synthesized human immunodeficiency virus Tat trans-activator protein. Cell 1988; 55: 1179–88.
3. Fawell S, Seery J, Daikh Y, Moore C, Chen LL, Pepinsky B, Barsoum J. Tat-mediated delivery of heterologous proteins into cells. Proc Natl Acad Sci USA 1994; 91: 664–8.
4. Vives E, Charneau P, Van Rietschoten J, Rochat H, Bahraoui E. Effects of the Tat basic domain on human immunodeficiency virus type 1 transactivation, using chemically synthesized Tat protein and Tat peptides. J Virol 1994; 68: 3343–53.
5. Agrawal P, Bhalla S, Usmani SS, Singh S, Chaudhary K, Raghava GPS, Gautam A. CPPsite 2.0: a repository of experimentally validated cell-penetrating peptides. Nucleic Acids Res 2016; 44: D1098–103.
6. Furuhata M, Kawakami H, Toma K, Hattori Y, Maitani Y. Intracellular delivery of proteins in complexes with oligoarginine-modified liposomes and the effect of oligoarginine length. Bioconjug Chem 2006;17:935–42.
7. Henriques ST, Costa J, Castanho MA. Translocation of beta-galactosidase mediated by the cell-penetrating peptide of pep-1 into lipid vesicles and human HeLa cells is driven by membrane electrostatic potential. Biochemistry 2005; 44: 10189–98.
8. Montrose K, Yang Y, Sun X, Wiles S, Krissansen GW. Xentry, a new class of cell-penetrating peptide uniquely equipped for delivery of drugs. Sci Rep 2013; 3: 1661.
9. Presente A, Dowdy SF. PTD/CPP peptide-mediated delivery of siRNAs. Curr Pharm Design 2013; 19: 2943–7.
10. Lundberg M, Wikström S, Johansson M. Cell surface adherence and endocytosis of protein transduction domains. Mol Ther 2003; 8: 143–50.
11. Richard JP, Melikov K, Vives E, Ramos C, Verbeure B, Gait MJ, Chernomordik LV, Lebleu B. Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. J Biol Chem 2003; 278: 585–90.
12. Glogau R, Blitzer A, Brandt F, Kane M, Monheit GD, Waugh JM. Results of a randomized, double-blind, placebo-controlled study to evaluate the efficacy and safety of a botulinum toxin type A topical gel for the treatment of moderate-to-severe lateral canthal lines. J Drugs Dermatol 2012; 11: 38–45.
13. Lonn P, Dowdy SF. Cationic PTD/CPP-mediated macromolecular delivery: charging into the cell. Expert Opin Drug Deliv 2015; 26: 1–10.
14. Lewis WH. Pinocytosis. Bull John Hopkins Hosp 1931; 49: 17–27.
15. Lim JP, Gleeson PA. Macropinocytosis: an endocytic pathway for internalising large gulps. Immunol Cell Biol 2011; 89: 836–43.

16. Pearse B. Clathrin: a unique protein associated with intracellular transfer of membrane by coated vesicles. Proc Natl Acad Sci USA 1976; 73: 1255–9.
17. Yamada E. The fine structure of the gall bladder epithelium of the mouse. J Biophys Biochem Cytol 1955; 1: 445.
18. Mayor S, Parton RG, Donaldson JG. Clathrin-independent pathways of endocytosis. Cold Spring Harb Perspect Biol 2014; 6: a016758.
19. Duchardt F, Fotin-Mleczek M, Schwarz H, Fischer R, Brock R. A comprehensive model for the cellular uptake of cationic cell-penetrating peptides. Traffic 2007; 8: 848–66.
20. Ferrari A, Pellegrini V, Arcangeli C, Fittipaldi A, Giacca M, Beltram F. Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time. Mol Ther 2003; 8: 284–94.
21. Kaplan IM, Wadia JS, Dowdy SF. Cationic TAT peptide transduction domain enters cells by macropinocytosis. J Control Release 2005; 102: 247–53.
22. Nakase I, Niwa M, Takeuchi T, Sonomura K, Kawabata N, Koike Y, Takehashi M, Tanaka S, Ueda K, Simpson JC, Jones AT, Sugiura Y, Futaki S. Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. Mol Ther 2004; 10: 1011–22.
23. Wadia JS, Stan RV, Dowdy SF. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. Nat Med 2004; 10: 310–5.
24. Fittipaldi A, Ferrari A, Zoppé M, Arcangeli C, Pellegrini V, Beltram F, Giacca M. Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins. J Biol Chem 2003; 278: 34141–9.
25. Vendeville A, Rayne F, Bonhoure A, Bettache N, Montcourrier P, Beaumelle B. HIV-1 Tat enters T cells using coated pits before translocating from acidified endosomes and eliciting biological responses. Mol Biol Cell 2004; 15: 2347–60.
26. Derossi D, Joliot AH, Chassaing G, Prochiantz A. The third helix of the Antennapedia homeodomain translocates through biological membranes. J Biol Chem 1994; 269: 10444–50.
27. Mann DA, Frankel AD. Endocytosis and targeting of exogenous HIV-1 Tat protein. EMBO J 1991; 10: 1733.
28. Jiao C-Y, Delaroche D, Burlina F, Alves ID, Chassaing G, Sagan S. Translocation and endocytosis for cell-penetrating peptide internalization. J Biol Chem 2009; 284: 33957–65.
29. Letoha T, Keller-Pintér A, Kusz E, Kolozsi C, Bozsó Z, Tóth G, Vizler C, Oláh Z, Szilák L. Cell-penetrating peptide exploited syndecans. Biochim Biophys Acta 2010; 1798: 2258–65.
30. Richard JP, Melikov K, Brooks H, Prevot P, Lebleu B, Chernomordik LV. Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors. J Biol Chem 2005; 280: 15300–6.
31. Chang HC, Samaniego F, Nair BC, Buonaguro L, Ensoli B. HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region. AIDS 1997; 11: 1421–31.
32. Rusnati M, Coltrini D, Oreste P, Zoppetti G, Albini A, Noonan D, d'Adda di Fagagna F, Giacca M, Presta M. Interaction of HIV-1 Tat protein with heparin. Role of the backbone structure, sulfation, and size. J Biol Chem 1997; 272: 11313–20.
33. Tyagi M, Rusnati M, Presta M, Giacca M. Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans. J Biol Chem 2001; 276: 3254–61.
34. Vogel BE, Lee S-J, Hildebrand A, Craig W, Pierschbacher MD, Wong-Staal F, Ruoslahti E. A novel integrin specificity exemplified by binding of the αvβ5 integrin to the basic domain of the HIV Tat protein and vitronectin. J Cell Biol 1993; 121: 461–8.
35. Xiao H, Neuveut C, Tiffany HL, Benkirane M, Rich EA, Murphy PM, Jeang K-T. Selective CXCR4 antagonism by Tat: implications for *in vivo* expansion of coreceptor use by HIV-1. Proc Natl Acad Sci USA 2000; 97: 11466–71.
36. Ghezzi S, Noonan DM, Aluigi MG, Vallanti G, Cota M, Benelli R, Morini M, Reeves JD, Vicenzi E, Poli G, Albini A. Inhibition of CXCR4-dependent HIV-1 infection by extracellular HIV-1 Tat. Biochem Biophys Res Commun 2000; 270: 992–6.
37. Kawaguchi Y, Takeuchi T, Kuwata K, Chiba J, Hatanaka Y, Nakase I, Futaki S. Syndecan-4 is a receptor for clathrin-mediated endocytosis of arginine-rich cell-penetrating peptides. Bioconjug Chem 2016; 27: 1119–30.
38. Kosuge M, Takeuchi T, Nakase I, Jones AT, Futaki S. Cellular internalization and distribution of arginine-rich peptides as a function of extracellular peptide concentration, serum, and plasma membrane associated proteoglycans. Bioconjug Chem 2008; 19: 656–64.
39. Console S, Marty C, García-Echeverría C, Schwendener R, Ballmer-Hofer K. Antennapedia and HIV TAT 'protein transduction domains' promote endocytosis of high Mr cargo upon binding to cell surface glycosaminoglycans. J Biol Chem 2003; 278: 35109–14.
40. Rusnati M, Tulipano G, Urbinati C, Tanghetti E, Giuliani R, Giacca M, Ciomei M, Corallini A, Presta M. The basic domain in HIV-1 Tat protein as a target for polysulfonated heparin-mimicking extracellular Tat antagonists. J Biol Chem 1998; 273: 16027–37.
41. Brooks R, Williamson RC, Bass MD. Syndecan-4 independently regulates multiple small GTPases to promote fibroblast migration during wound healing. Small GTPases 2012; 3: 73–9.
42. Christianson HC, Belting M. Heparan sulfate proteoglycan as a cell-surface endocytosis receptor. Matrix Biol 2014; 35: 51–5.
43. Tanaka G, Nakase I, Fukuda Y, Masuda R, Oishi S, Shimura K, Kawaguchi Y, Takatani-Nakase T, Langel U, Gräslund A, Okawa K, Matsuoka M, Fujii N, Hatanaka Y, Futaki S. CXCR4 stimulates macropinocytosis: implications for cellular uptake of arginine-rich cell-penetrating peptides and HIV. Chem Biol 2012; 19: 1437–46.
44. Hamon M, Mbemba E, Charnaux N, Slimani H, Brule S, Saffar L, Vassy R, Prost C, Lievre N, Starzec A, Gattegno L. A syndecan-4/CXCR4 complex expressed on human primary lymphocytes and macrophages and HeLa cell line binds the CXC chemokine stromal cell–derived factor-1 (SDF-1). Glycobiology 2004; 14: 311–23.
45. Erazo-Oliveras A, Muthukrishnan N, Baker R, Wang T-Y, Pellois J. Improving the endosomal escape of cell-penetrating peptides and their cargos: strategies and challenges. Pharmaceuticals 2012; 5: 1177–209.
46. Koren E, Torchilin VP. Cell-penetrating peptides: breaking through to the other side. Trends Mol Med 2012; 18: 385–93.
47. Erazo-Oliveras A, Najjar K, Dayani L, Wang TY, Johnson GA, Pellois JP. Protein delivery into live cells by incubation with an endosomolytic agent. Nat Methods 2014; 11: 861–7.
48. Najjar K, Erazo-Oliveras A, Pellois J. Delivery of proteins, peptides or cell-impermeable small molecules into live cells

by incubation with the endosomolytic reagent dfTAT. J Vis Exp 2015; 103. doi: 10.3791/53175.
49. Salomone F, Cardarelli F, Di Luca M, Boccardi C, Niforsi R, Bardi G, Di Bari L, Serresi M, Beltram F. A novel chimeric cell-penetrating peptide with membrane-disruptive properties for efficient endosomal escape. J Control Release 2012; 163: 293–303.
50. Salomone F, Cardarelli F, SIgnore G, Boccardi C, Beltram F. *In vitro* efficient transfection by CM18-Tat11 hybrid peptide: a new tool for gene-delivery applications. PLoS One 2013; 8: e70108.
51. Lonn P, Kacsinta AD, Cui XS, Hamil AS, Kaulich M, Gogoi K, Dowdy SF. Enhancing endosomal escape for intracellular delivery of macromolecular biologic therapeutics. Sci Rep 2016; 6: 32301.
52. Baccile JA, Morrell MA, Falotico RM, Milliken BT, Drew DL, Rossi FM. Modular synthesis of photocleavable peptides using click chemistry. Tetrahedron Lett 2012; 53: 1933–5.
53. Salerno JC, Ngwa VM, Nowak SJ, Chrestensen CA, Healey AN, McMurry JL. Novel cell penetrating peptides effect intracellular delivery and endosomal escape of desired protein cargos. J Cell Sci 2016; 129: 893–7.
54. Gerasimenko JV, Tepikin AV, Petersen OH, Gerasimenko OV. Calcium uptake via endocytosis with rapid release from acidifying endosomes. Curr Biol 1998; 8: 1335–8.
55. Rapoport M, Saada A, Elpeleg O, Lorberboum-Galski H. TAT-mediated delivery of LAD restores pyruvate dehydrogenase complex activity in the mitochondria of patients with LAD deficiency. Mol Ther 2008; 16: 691–7.
56. Marcus D, Lichtenstein M, Saada A, Lorberboum-Galski H. Replacement of the C6ORF66 assembly factor (NDUFAF4) restores complex I activity in patient cells. Mol Med 2013; 19: 124–34.
57. Honda F, Hane Y, Toma T, Yachie A, Kim E-S, Lee S-K, Takagi M, Mizutani S, Morio T. Transducible form of p47 phox and p67 phox compensate for defective NADPH oxidase activity in neutrophils of patients with chronic granulomatous disease. Biochem Biophys Res Comm 2012; 417: 162–8.
58. Cerrato CP, Künnapuu K, Langel Ü. Cell-penetrating peptides with intracellular organelle targeting. Expert Opin Drug Deliv 2017; 14: 245–55.
59. Ngwa VM, Axford DS, Healey AN, Nowak SJ, Chrestensen CA, McMurry JL. A versatile cell-penetrating peptide-adaptor system for efficient delivery of molecular cargos to subcellular destinations. PLoS One 2017; 12: e0178648.
60. Wang H-Y, Chen J-X, Sun Y-X, Deng J-Z, Li C, Zhang X-Z, Zhuo RX. Construction of cell penetrating peptide vectors with N-terminal stearylated nuclear localization signal for targeted delivery of DNA into the cell nuclei. J Control Rel 2011; 155: 26–33.
61. Herce HD, Garcia AE, Cardoso MC. Fundamental molecular mechanism for the cellular uptake of guanidinium-rich molecules. J Am Chem Soc 2014; 136: 17459–67.
62. Dekiwadia CD, Lawrie AC, Fecondo JV. Peptide-mediated cell penetration and targeted delivery of gold nanoparticles into lysosomes. J Pep Sci 2012; 18: 527–34.
63. Cerrato CP, Pirisinu M, Vlachos EN, Langel Ü. Novel cell-penetrating peptide targeting mitochondria. FASEB J 2015; 29: 4589–99.
64. Marcus D, Lichtenstein M, Cohen N, Hadad R, Erlich-Hadad T, Greif H, Lorberboum-Galski H. Heterologous mitochondrial targeting sequences can deliver functional proteins into mitochondria. Int J Biochem Cell Biol 2016; 81: 48–56.
65. Zhang J, Sun A, Xu R, Tao X, Dong Y, Lv X, Wei D. Cell-penetrating and endoplasmic reticulum-locating TAT-IL-24-KDEL fusion protein induces tumor apoptosis. J Cell Phys 2016; 231: 84–93.
66. Vivès E, Schmidt J, Pèlegrin A. Cell-penetrating and cell-targeting peptides in drug delivery. Biochim Biophys Acta 2008; 1786: 126–38.
67. MacEwan SR, Chilkoti A. Harnessing the power of cell-penetrating peptides: activatable carriers for targeting systemic delivery of cancer therapeutics and imaging agents. Wiley Interdiscip Rev Nanomed Nanobiotechnol 2013; 5: 31–48.
68. Jiang T, Olson ES, Nguyen QT, Roy M, Jennings PA, Tsien RY. Tumor imaging by means of proteolytic activation of cell-penetrating peptides. Proc Natl Acad Sci USA 2004; 101: 17867–72.
69. Aguilera TA, Olson ES, Timmers MM, Jiang T, Tsien RY. Systemic *in vivo* distribution of activatable cell penetrating peptides is superior to that of cell penetrating peptides. Integr Biol (Cambr) 2009; 1: 371–81.
70. Olson ES, Aguilera TA, Jiang T, Ellies LG, Nguyen QT, Wong EH, Gross LA, Tsien RY. In vivo characterization of activatable cell penetrating peptides for targeting protease activity in cancer. Integr Biol (Cambr) 2009; 1: 382–93.
71. van Duijnhoven SM, Robillard MS, Nicolay K, Grüll H. Tumor targeting of MMP-2/9 activatable cell-penetrating imaging probes is caused by tumor-independent activation. J Nucl Med 2011; 52: 279–86.
72. Weinstain R, Savariar EN, Felsen CN, Tsien RY. *In vivo* targeting of hydrogen peroxide by activatable cell-penetrating peptides. J Am Chem Soc 2014; 136: 874–7.
73. Yang Y, Xie X, Yang Y, Li Z, Yu F, Gong W, Li Y, Zhang H, Wang Z, Mei X. Polymer nanoparticles modified with photo-and pH-dual-responsive polypeptides for enhanced and targeted cancer therapy. Mol Pharm 2016; 13: 1508–19.
74. Vocero-Akbani AM, Vander Heyden N, Lissy NA, Ratner L, Dowdy SF. Killing HIV-infected cells by transduction with an HIV protease-activated caspase-3 protein. Nat Med 1999; 5: 29–33.
75. Zahid M, Phillips BE, Albers SM, Giannoukakis N, Watkins SC, Robbins PD. Identification of a cardiac specific protein transduction domain by in vivo biopanning using a M13 phage peptide display library in mice. PLoS One 2010; 5: e12252.
76. Lim KJ, Sung BH, Shin JR, Lee YW, Yang KS, Kim SC. A cancer specific cell-penetrating peptide, BR2, for the efficient delivery of an scFv into cancer cells. PLoS One 2013; 8: e66084.