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Cell-Penetrating Peptides Transport Therapeutics into Cells

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Abstract

Nearly 30 years ago, certain small, relatively nontoxic peptides were discovered to be capable of traversing the cell membrane. These cell-penetrating peptides, as they are now called, have been shown to not only be capable of crossing the cell membrane themselves but can also carry many different therapeutic agents into cells, including small molecules, plasmid DNA, siRNA, therapeutic proteins, viruses, imaging agents, and other various nanoparticles. Many cell-penetrating peptides have been derived from natural proteins, but several other cell-penetrating peptides have been developed that are either chimeric or completely synthetic. How cell-penetrating peptides are internalized into cells has been a topic of debate, with some peptides seemingly entering cells through an endocytic mechanism and others by directly penetrating the cell membrane. Although the entry mechanism is still not entirely understood, it seems to be dependent on the peptide type, the peptide concentration, the cargo the peptide transports, and the cell type tested. With new intracellular disease targets being discovered, cell-penetrating peptides offer an exciting approach for delivering drugs to these intracellular targets. There are hundreds of cell-penetrating peptides being studied for drug delivery, and ongoing studies are demonstrating their success both *in vitro* and *in vivo*.

Keywords: cell-penetrating peptide, protein transduction domain, drug delivery, internalization, Tat, penetratin

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Abbreviations

	CPP	cell-penetrating peptide
1	HIV-1	human immunodeficiency virus type 1
2	mRNA	messenger RNA
3	NLS	nuclear localization signal
4	pAntp	polypeptide corresponding to the <i>Drosophila</i> antenapedia homeobox protein
5	PTD	protein transduction domain
6	siRNA	short interfering RNA
7	SV40	simian virus 40
8	TAT	<i>trans</i> -activator protein or peptide
9		
10		

1. Introduction

Cells are adept at preventing small molecules, proteins, genetic material, and larger protein complexes from entering in a nonspecific manner. The exterior of the cell is composed of a fluid, phospholipid bilayer that is coated with proteoglycans. The lipid bilayer that makes up the cell membrane has a polar surface with a nonpolar interior. Together, the contrasting nature of the membrane lipid bilayer prevents most molecules from passively diffusing directly through the cell membrane. Instead, cells use a combination of ion channels and carrier proteins to transport important molecules such as ions, sugars, and amino acids across the lipid bilayer. Cells also may use endocytosis or exocytosis to transport material in bulk across the membrane.

While precise control of transport across the cell membrane is generally advantageous for the cell, many potential therapeutic drug targets are located within the interior of the cell and require entry of pharmaceutical molecules in order to be effectively treated. This has long been a challenge in the field of gene therapy (Khalil et al. 2006), where a therapeutic gene must not only be transported across the cell membrane, but must also be transported to the nucleus of the cell where it can be transcribed and translated into a therapeutic protein. Similarly, siRNA must also be transported across the cell membrane to the cell cytosol where it can act on the mRNA of the gene being silenced. Likewise, there are a growing number of intracellular targets for cancer therapy. In some cases, cancer treatment may benefit from the intracellular delivery of a particular protein, such as a cytotoxic protein or a tumor suppressor protein that is not natively expressed in an active state (Beitz et al. 1992, Wu et al. 1993, Woods and Lane 2013, Wang et al. 2014). Cancer therapy may also benefit from delivery of peptides that act as inhibitors. Examples include peptides that act by preventing STAT3 from binding to DNA in tumor cells (Turkson et al. 2001), peptides that inhibit angiogenesis by hindering β protein kinase C

(Stebbins and Mochly-Rosen 2001, Kim et al. 2008), and peptides that induce apoptosis by disrupting the membrane of mitochondria (Javadpour et al. 1996, Ellerby et al. 1999).

Large macromolecular drugs, such as genetic material or proteins, are often designed to be transported into target cells through either non-specific or receptor-mediated endocytosis. In contrast, many small molecule therapeutics are believed to undergo transport through some combination of passive diffusion and carrier-mediated transport. There is currently a very spirited debate on where this balance falls (Kell et al. 2011, Di et al. 2012). Dobson and Kell have argued previously that carrier-mediate transport is the predominant mechanism for drug entry (Dobson and Kell 2008), while Sugano *et al.* made a case for a combination of both passive diffusion and carrier-mediate transport (Sugano et al. 2010).

Regardless of how much each mechanism plays in the transport of the drug, cell entry remains a focus for those working in drug design and discovery. An exciting and relatively new approach to transporting pharmaceutical agents into cells is making use of cell-penetrating peptides (CPPs) (recent reviews include: (Koren and Torchilin 2012, Alhakamy et al. 2013, Copolovici et al. 2014, Wang et al. 2014)). CPPs are relatively short peptides, typically less than 30 amino acids, that have been shown to be relatively nontoxic and capable of traversing the cell membrane. They are typically classified as either polycationic, amphipathic, or hydrophobic. Most importantly, they have been used to facilitate the transport of many different therapeutic agents into cells, including plasmid DNA, siRNA, therapeutic proteins, viruses, imaging agents, and other various nanoparticles.

2. Discovery of Cell-Penetrating Peptides

Some of the first studies that led directly to the discovery of CPPs were published in 1988. Up until that time, transport of proteins and peptides through the cell membrane was generally

1 regarded as difficult given their large molecular weight and hydrophilicity (Sternson 1987).
2 Frankel and Pabo, however, discovered that purified *trans*-activator protein (TAT), from human
3 immunodeficiency virus type 1 (HIV-1), was readily taken up from cell culture medium by
4 HL3T1 cells (Frankel and Pabo 1988). They also showed that uptake of TAT was enhanced
5 when chloroquine was added to the medium, suggesting that uptake was likely occurring through
6 endocytosis. At the same time, Green and Loewenstein independently demonstrated that only a
7 portion of the 86 amino acid TAT protein was necessary for both cellular uptake and enzymatic
8 activity (Green and Loewenstein 1988). By investigating various N-terminal and C-terminal
9 deletion mutants, Green and Loewenstein showed that TAT (37 – 57) entered cells and retained
10 ~ 40 % of the activity of the full-length TAT protein.

11 Nearly 10 years after the initial discovery of the TAT peptide, even smaller TAT peptides
12 were shown to translocate through the cell membrane. In one study, Vivès et al. found that TAT
13 (48 – 60) was able to enter cells and that cellular uptake of the peptide was not inhibited at 4 °C
14 (Vives et al. 1997), implying an alternative to the endocytic mechanism suggested earlier by the
15 findings of Frankel and Pabo. Further, Vivès et al. were able to rule out potocytosis and
16 caveolae-mediated endocytosis by using respective pathway inhibitors. At the same time, Vivès
17 et al. published a second paper in which they showed that the C-terminal end of the TAT peptide
18 could be further truncated to TAT (48 – 57) without significant loss of translocation ability
19 (Vivès et al. 1997). Park et al. followed up this study showing that the peptide could be
20 truncated even more to TAT (49 – 57) (Park et al. 2002).

21 Around the same time as the discovery of TAT, the Prochiantz group identified a 60 amino
22 acid region of the *Drosophila* antennapedia homeobox protein (pAntp) that was capable of
23 penetrating differentiated neurons (Joliot et al. 1991). Joliot et al. were investigating the effects

1 of injecting the DNA-binding peptide on nerve cell differentiation when they discovered that the
2 peptide was able to penetrate differentiated nerve cells (i.e., without injection) and accumulate in
3 the nucleus. While the entry mechanism was unclear, they were able to rule out internalization
4 due to cell damage by comparing the uptake of the pAntp peptide with proteolytic fragments of
5 fluorescent ovalbumin.

6 Prochiantz's group followed this initial report with a study investigating the uptake
7 mechanism and also the minimal sequence required for cell entry (Derossi et al. 1994). Earlier
8 studies had shown that the third α -helix of the pAntp peptide, which consists of three α -helices
9 with a β -turn between the second and third helix, may be especially important in cell entry; there
10 is a high degree of phylogenetic kinship in the third α -helix and studies showed that
11 internalization of the peptide was especially sensitive to mutations in this region (Le Roux et al.
12 1993). Derossi et al. went on to show that truncation of the third α -helix by deletion of the c-
13 terminal glutamate produced a 16 amino acid peptide capable of translocating through cell
14 membranes (Derossi et al. 1994). Further, they were able to show that entry of the peptide was
15 not limited to neuronal cells nor was it dependent on binding a specific transporter. Once again,
16 the mode of cell entry was not clear, but this time Prochiantz's group demonstrated that
17 internalization was energy-independent, occurring at 4 °C and avoiding the endolysosomal
18 uptake pathway.

19 Hundreds of additional CPPs have been discovered in the two decades that have passed
20 since the discovery of TAT and the pAntp peptide. Table 1 provides a short list of some of these
21 peptides, their source, peptide sequence, and examples of the cargo they have been used to
22 transport. CPPs are typically classified by either their physical-chemical properties or by their

1 origin. The peptides listing in Table 1 have been grouped based on their physical-chemical
2 properties: cationic, amphipathic, or hydrophobic.

3 Similar to TAT and the pAntp peptide, many of the CPPs were derived from natural
4 proteins or peptides, including viral proteins, heparin-binding proteins, DNA/RNA-binding
5 proteins, homeoproteins, signal peptides, and antimicrobial peptides. The VP22 peptide, for
6 example, was derived from a herpes virus structural protein (Elliott and O'Hare 1997). The
7 origin of DPV3 was a heparin binding protein (De Coupade et al. 2005). TAT, which comes
8 from the HIV-1 virus, was derived from the RNA-binding, *trans*-activator protein. The origin of
9 the pAntp peptide was a homeoprotein, which is a class of DNA-binding proteins. K-FGF was
10 derived from the signal peptide in Kaposi's sarcoma fibroblast growth factor (Lin et al. 1995),
11 and while none are included in Table 1, there are many known CPPs that were derived from
12 antimicrobial peptides such as Bac7, buforin 2, tachyplesin and melittin (Fennell et al. 1968,
13 Kobayashi et al. 2000, Sadler et al. 2002, Jain et al. 2015).

14 Some CPPs are derived from a combination of natural proteins or peptides and are referred
15 to as chimeric. Transportan and Pep1 are two peptides listed in Table 1 that are chimeric.
16 Transportan was produced by combining the neuropeptide galanin and the wasp venom peptide
17 mastoparan (Pooga et al. 1998). A common approach with chimeric CPPs has been to combine
18 signal peptides with NLS peptides. Pep1 is a result of this approach, where a tryptophan-rich
19 hydrophobic domain that associates with cell membranes has been fused to a SV40 NLS peptide
20 (Morris et al. 2001). MPG is another chimeric peptide produced using this approach. MPG
21 combines the SV40 NLS peptide with a signal peptide taken from HIV glycoprotein 41 (Morris
22 et al. 1997).

Synthetic peptides are the final classification group based on the origin of the CPP. Model amphipathic peptide (MAP) and polyarginine are both listed in Table 1 and were two of the first synthetic peptides developed. These early synthetic peptides were designed to mimic CPPs based on natural proteins. For example, polyarginine was investigated for its cell-penetrating ability because of its similarity to TAT, which also has a large number of arginine residues (Wender et al. 2000, Tünnemann et al. 2008). MAP was meant to be a model peptide that had amphipathic alpha helical secondary structure, which could be used to study if unique structural features were necessary for membrane passage, as was thought to be the case with the pAntp peptide (Oehlke et al. 1996). Since the discovery of these two synthetic peptides, and many more like them, molecular cloning techniques and screening tools such as phage display have enabled high-throughput screening of large peptide libraries. These libraries have yielded additional synthetic CPPs that, unlike polyarginine and MAP, tend to be more hydrophobic and lack amphipathic structure (Kamada et al. 2007).

Lastly, there has been recent interest in developing non-peptide mimics of CPPs. These mimics of CPPs are typically built upon polymer backbones. By modifying the polymer side chains an array of polymers have been synthesized that show robust transduction of cells. While these non-peptide mimics of CPPs are not the focus of this paper, the interested reader is referred to a recent review on the subject (Sgolastra et al. 2013).

3. Mechanism of Cellular Entry

From the beginning there has been considerable interest in exactly how CPPs enter cells. The mechanisms are still not entirely understood, but what we are beginning to recognize is that the mechanisms by which CPPs enter cells seems dependent on not only the type of CPP but also its concentration (Brock 2014), the cargo that it transports, and the cell line on which it is being

1 tested. Arriving at this understanding has not been easy, as early studies provided contradictory
2 findings. As described above, Frankel and Pabo (1988) found that chloroquine enhanced the
3 uptake of Tat peptide, implying that an endocytic mechanism was likely. Vives, Brodin, and
4 Lebleu (1997), however, showed that the uptake of TAT occurred at 4 °C, which implied that
5 endocytosis was unlikely. In addition, the Prochiantz group showed that pAntp was also able to
6 enter cells at 4°C , thereby lending further evidence to an energy-independent, non-endosomal
7 uptake mechanism for CPPs (Derossi et al. 1994). The problem with these studies, and many
8 other early studies, was that they predominantly relied on fluorescence microscopy of fixed cells
9 or flow cytometry to measure uptake of CPPs. In 2002, it was shown that fixation of cells could
10 lead to artifactual redistribution of peptides that had otherwise simply been bound to the cell
11 membrane (Lundberg and Johansson 2002, Richard et al. 2003). Both studies also showed that
12 despite extensive washing, cationic CPPs remained bound to the cell membrane, which could
13 erroneously lead one to conclude from flow cytometry that the CPPs had been internalized.
14 Taking into account the issues with flow cytometry and cell fixation, Richard et al. (2003) were
15 able to show that the uptake of the TAT peptide is indeed inhibited at 4 °C. Endosomal
16 distribution and the kinetics of uptake also helped confirm that TAT, under the conditions
17 described in the study, was internalized in an endocytic manner.

18 Figure 1 summarizes the different routes by which a CPP might enter a cell. While direct
19 translocation across the cell membrane occurs in some cases, it is generally accepted that most
20 CPPs and CPP-cargo complexes enter cells through endocytosis, including: macropinocytosis,
21 clathrin-mediated endocytosis, and caveolae/lipid raft-mediated endocytosis. Some CPPs have
22 been shown to use more than one of these internalization routes and to sometimes use different
23 routes simultaneously. Investigations into the TAT peptide, for example, have shown that TAT

1 is internalized through macropinocytosis (Kaplan et al. 2005), clathrin-mediated endocytosis
2 (Richard et al. 2005), and caveolae-mediated endocytosis (Ferrari et al. 2003).

3 In spite of some of the early errors assessing cellular entry, CPPs have been shown to
4 directly penetrate the cell membrane in a non-endocytic manner, especially at high
5 concentrations of the peptide (Fretz et al. 2007, Kosuge et al. 2008). Many different models
6 have been proposed to explain this observation, a few of which include inverted micelles,
7 transient toroidal pores, and adaptive translocation.

8 Derossi et al. were the first to suggest CPPs may be entering through an inverted micelle
9 (Derossi et al. 1996). They hypothesized that the pAntp was entering cells through either an
10 inverted micelle or through fluid phase pinocytosis. The authors went on to provide ample
11 justification for the inverted micelle model, including an explanation that substitution of the CPP
12 tryptophan residues, which are known to induce formation inverted micelles, abolished cell
13 penetration.

14 The formation of transient toroidal pores, similar to the carpet model (Pouny et al. 1992),
15 has also been suggested as a possible mechanism for direct translocation. Molecular dynamics
16 simulations of the TAT peptide interacting with a lipid bilayer showed that at high
17 concentrations of the TAT peptide transient pores could form in the membrane (Herce and
18 Garcia 2007). The model showed that attraction of the TAT peptide with the phospholipid head
19 groups on both the outer and inner lipid leaflets leads to a thinning of the lipid bilayer and
20 ultimately penetration of the membrane. The lack of observable membrane leakage has led
21 many to discount this mechanism. The transient nature of the pores, however, combined with the
22 cellular membrane repair response may explain why no membrane leakage or cell damage is
23 observed (Palm-Apergi et al. 2009).

Another proposed mechanism for direct translocation of the cell membrane by cationic CPPs is adaptive translocation. The idea that a hydrophilic, charged peptide could directly migrate through the hydrophobic lipid bilayer initially seems improbable. Rothbard et al., however, proposed a mechanism by which they suggested cationic peptides form transient ion pair complexes with negatively charged membrane components; the reduced, yet still positive charge associated with the ion pair complex allows the peptide to adaptively diffuse through the cell membrane, where the driving force for diffusion is the membrane potential (Rothbard et al. 2005). Rothbard et al. supported their proposed model by explaining that reduced membrane potential resulted in reduced cellular uptake of the peptide without any noticeable effect on endocytosis. Further, they showed that increased membrane potential resulted in increased cellular uptake of CPPs.

In addition to the models described here, there may be a newly discovered model that is not entirely understood. A recent study by Hirose et al. found that arginine-rich CPPs conjugated to hydrophobic small molecules directly translocated the cell membrane at sites where the peptides formed unique “particle-like” structures composed of multiple vesicles on the plasma membrane (Hirose et al. 2012). Penetration into the cell occurred through both direct translocation and endocytosis. Further, direct translocation was found to be dependent on the presence of the hydrophobic fluorescent dye Alexa488 that was attached to the peptide.

The importance of the Alexa488 in the study performed by Hirose et al. brings us back to an issue raised earlier; the mechanism used by CPPs to enter cells depends not only on the peptide but also other factors such as the size and physicochemical nature of its cargo (Maiolo et al. 2005). For example, when TAT was fused to a protein, cellular uptake of the complex occurred through caveolae-mediated endocytosis (Fittipaldi et al. 2003). When the peptide was

1 conjugated to a fluorophore, however, uptake occurred through clathrin-mediated endocytosis
2 (Richard et al. 2005). Further, internalization of TAT/fusion protein complexes was largely
3 limited to endocytosis, but TAT/small peptide complexes were internalized through both
4 endocytosis and rapid, direct membrane translocation (Tunnemann et al. 2006).

5   Understanding cellular internalization means not only studying the CPP complex as a
6 whole, but also considering the potential limitations of the different techniques used to study
7 internalization. One concern with many of the methods that have been used to study endocytosis
8 of CPPs is the use of chemical inhibitors. While chemical inhibitors can be used to help
9 understand the role of different endocytic uptake pathways in the internalization of CPPs, they
10 also have a substantial impact on cell viability, thereby significantly affecting cells in ways that
11 may make interpretation of the results difficult. Additionally, the performance of chemical
12 inhibitors can be highly dependent on the cell line used. Holm, Andaloussi, and Langel recently
13 wrote a book chapter describing several methods (including fluorescence microscopy, HPLC,
14 flow cytometry, spectrofluorometry, electron microscopy, and mass spectroscopy) for studying
15 the uptake of CPPs, which can avoid the use of chemical inhibitors (Holm et al. 2011). The
16 authors provide a list of the advantages and drawbacks associated with the different methods and
17 advocate for the use of more than one approach in order to avoid the negative aspects of the
18 different methods.

19 **4. Applications using Cell-Penetrating Peptides**

20   CPPs have been used in a wide number of applications, ranging from simple cell culture
21 transfection to the systemic delivery of therapeutics. The focus here is to highlight a few of the
22 many instances where CPPs have been used to deliver therapeutics, specifically nucleic acids,
23 proteins, small molecules, and imaging agents. Many of the examples of *in vivo* delivery are

1 limited to small animals, and application of CPPs to human therapeutics has yet to prove
2 effective. CPPs enhance the general uptake of a therapeutic but in doing so reduce the
3 specificity. This loss of specificity is not only likely to impact efficacy but also safety. Much of
4 the recent work has focused on using CPPs along with targeting ligands to improve delivery of a
5 therapeutic. As approaches to improve the targeted delivery of biomolecules are combined with
6 CPPs, therapeutics may be delivered more efficiently, thereby greatly improving treatment
7 outcomes.

8 *Nucleic Acid Delivery*

9 One of the first biomedical applications of CPPs was delivery of nucleic acids into cells.
10 As previously mentioned, large hydrophilic molecules, such as nucleic acids, are generally
11 inefficient at passing through the cell membrane. To improve the delivery of nucleic acids,
12 plasmid DNA has been complexed with cationic polymers or liposomes, which condense DNA
13 into particles capable of entering cells through endocytosis. Similar to these polymeric and
14 liposomal materials, cationic CPPs have also been used to condense plasmid DNA into small
15 particles. One of the first studies showing this was by Morris et al., who demonstrated that CPPs
16 could be complexed with oligonucleotides through electrostatic attraction, which resulted in
17 improved gene delivery (Morris et al. 1997). Soon after, Transportan and pAntp were used to
18 deliver peptide nucleic acid (PNA) by covalently linking the CPPs to the PNA (Pooga et al.
19 1998). These developments spurred additional investigations into using CPPs to deliver nucleic
20 acids. Recently, van Asbeck et al. sought to better understand what physicochemical and
21 molecular factors most influence the successful delivery of CPP/siRNA complexes (van Asbeck
22 et al. 2013). The authors discovered that CPP/siRNA complexes with the most negative zeta-
23 potentials in serum were the most resistant to siRNA release over a 20 hour incubation period

1 compared to less negatively charged complexes. They also found that the zeta-potential of
2 CPP/siRNA complexes in serum did not correlate with improved cellular association, which they
3 explained could demonstrate the importance of serum proteins or CPP conformation on the
4 ability of CPP/siRNA complexes to associate with the cell membrane.

5 CPPs have also been coupled recently to other molecules to improve gene delivery after
6 entry into the cell. Favaro et al., for example, investigated combining TAT with a fusion protein
7 partially composed of the intracellular transport protein dynein light chain Rp3 for improving
8 DNA delivery to the nucleus (Favaro et al. 2014). They found that using the CPP and the
9 transport protein improved gene delivery by more than 7-fold compared to the transport protein
10 alone. Similarly, Lindberg et al. functionalized CPPs to not only penetrate the cell membrane
11 but to also facilitate better intracellular trafficking by disrupting endolysosomes (Lindberg et al.
12 2013). They delivered anti-microRNA oligonucleotides into cells by combining the
13 endosomolytic agent trifluoromethylquinoline to the PepFect14 CPP, producing a new CPP that
14 the authors named PepFect15. Compared to the PepFect14 complexes, PepFect15 produced a
15 one-fold improvement in anti-microRNA delivery at the optimum CPP:oligonucleotide molar
16 ratio.

17 CPPs have been used also to enhance existing synthetic gene vectors *in vivo*. Hayashi et al.
18 demonstrated that polyarginine functionalized lipoplexes efficiently silenced genes in a mouse
19 liver (Hayashi et al. 2011). An interesting and unexpected finding in subsequent work was that
20 CPPs seemed to improve the long-term gene expression from vectors that normally only provide
21 for very transient gene expression. One example of this was a TAT-PEI vector that extended the
22 gene expression period from about 1 month to nearly 7 months compared to TAT or PEI alone
23 (Yamano et al. 2014). Another demonstration of this effect by Yamano et al., showed that a

1 TAT-modified liposome could be used to deliver a reporter gene with reasonable levels of
2 expression for up to 7 months (Yamano et al. 2014).

3 One of the main limitations of gene vectors utilizing CPPs is low cell specificity. A
4 popular approach for improving synthetic gene vectors has been to incorporate targeting ligands
5 with CPPs to better control cell-specific attachment. Interestingly, synergism between the CPP
6 and the targeting ligand has often been observed. In a recent study, Fang et al. found that the
7 coupling of a vascular endothelial growth factor receptor-1 targeting peptide to TAT internalized
8 siRNA into cancer cells more readily than the CPP alone (Fang et al. 2013). Improved and/or
9 more selective gene delivery has also been reported with targeting ligands, such as mannose,
10 folate, and RGD (Suk et al. 2006, Jiang et al. 2011, Hu et al. 2014). The same approach has also
11 been applied to the delivery of siRNA using folate and penetratin (Cheng and Saltzman 2011).
12 The authors suggested that the multiple ligand approach increased the avidity for their siRNA
13 nanoparticles toward folate expressing cancer cells.

14 Interestingly, CPPs have also been demonstrated to transport nucleic acids to poorly
15 accessible tissues such as the skin and brain. Recently, Chen et al. used the SPACE peptide to
16 deliver siRNA encapsulated in liposomes topically to the skin (Chen et al. 2014). Other hard to
17 reach tissues, such as the brain, have also become more accessible to therapeutics because of
18 CPPs. TAT-functionalized micelles were used to deliver a dual formulation of siRNA and
19 camptothecin to a glioma through the nasal cavity in mice (Kanazawa et al. 2014). Similarly, a
20 polyarginine CPP and a transferrin targeting ligand improved delivery of plasmid DNA
21 encapsulated in a liposome to the brain (Sharma et al. 2013).

1 In addition to synthetic vectors, CPPs have been used also to improve viral gene delivery
2 vectors. Viral vectors are typically only efficient at gene delivery to cells that have the receptors
3 that a virus needs for cell attachment and entry. CPPs have been combined with viral vectors,
4 however, to widen the range of cell types that viral vectors are able to infect. By forming
5 complexes through electrostatic attraction, several types of CPPs were found to improve the
6 infectivity of an adenovirus gene vector, including cells poorly permissive to infection (Eto et al.
7 2009, Nigatu et al. 2013). Similarly, the ability of adeno-associated virus and baculovirus to
8 deliver genes has also been improved using CPPs (Chen et al. 2011, Liu et al. 2014). Together,
9 these studies demonstrate that viral vectors can be modified to deliver genes to a much wider
10 range of targets, including various cancers and stem cells. In one study, TAT was shown to
11 increase the infectivity of an oncolytic adenovirus, which led to improved survival of tumor
12 bearing mice (Yu et al. 2011). Park et al. recently investigated the effects of oligomeric CPPs on
13 adenoviral gene delivery to transduce stem cells (Park et al. 2010). They found that tetramers of
14 CPPs were better than CPP monomer at delivering adenovirus to mesenchymal stem cells.

15 Some CPPs have been proven also to be effective antimicrobials and capable of penetrating
16 other types of non-mammalian cells. With the prevalence of multidrug resistant bacteria, the
17 development of peptides to either act as antimicrobials or to deliver antimicrobial agents is an
18 area of growing interest. Bai et al. and Rajasekaran et al. delivered PNA into gram-negative
19 bacteria, as well as intracellular bacteria strains using CPPs (Bai et al. 2012, Rajasekaran et al.
20 2013). Both CPP-PNA constructs inhibited microbial growth, demonstrating that gene silencing
21 can be an effective approach to control bacteria. Although many antimicrobial CPPs have been
22 discovered, the mechanism behind their broad penetration ability remains an area of active
23 investigation. In one recent study, Rodriguez et al. found that the membrane potential is a key

factor for cell entry of the model antimicrobial peptide Iztli-1 (Rodriguez Plaza et al. 2014). This provides a basis for further studies to understand why many CPPs can also act as antimicrobial peptides.

Peptides, Proteins, and Enzymes

As with gene delivery, proteins are typically unable to passively enter cells. Because many therapeutic targets are inside of cells, CPPs provide an ideal means for intracellular delivery of the growing number of pharmaceutically relevant proteins (Leader et al. 2008). In fact, early studies on the *in vivo* behavior of CPP conjugates of β -Galactosidase coupled to TAT demonstrated that CPPs can facilitate the delivery of proteins into most cell types (Schwarze et al. 1999). These prior successes have been built upon recently with CPPs transporting proteins into cells for treating specific diseases. TAT has been used to deliver the herpes virus E2 protein to induce apoptosis in cervical cancer cells (Roeder et al. 2004). In another strategy to treat cancer, a polyarginine CPP was coupled with a tumor suppressing p53 protein to inhibit the growth of bladder cancer cells (Araki et al. 2010). An interesting approach to build upon transporting anti-cancer proteins has been to use the tumor environment to modulate the function of CPPs, where by controlling the activity of the CPP, more cancer suppressing protein can be delivered to tumors. Fei et al., for example, constructed a CPP that would be active in the acidic environment of tumor tissue by linking the MAP CPP to a pH-sensitive peptide (Fei et al. 2014). This approach improved the delivery of glutathione S-transferase to tumor cells in mice while reducing delivery of the protein to other tissues.

CPPs have been incorporated also into gene editing strategies for treat disease. Specifically, CPPs are being used to deliver endonucleases into cells as an approach to remove disease-

1 causing genes. In one recent study, a CPP was used to deliver the Cas9 protein and guide RNA
2 for RNA-guided endonuclease gene editing (Ramakrishna et al. 2014). Using this method, gene
3 disruption was observed in several cell types. In another study, Liu et al. used polyarginine to
4 transport transcription activator-like effector endonuclease (TALEN) protein into cells (Liu et al.
5 2014). The authors showed that the TALEN protein was able to specifically disrupt CCR5 and
6 BMPRI1A genes.

7 As described in several earlier examples, CPPs make it possible to transport enzymes into
8 cells, which can lead to treatments for a wide range of diseases. Enzymes that prevent oxidative
9 damage are just one of several types of enzymes that are of interest for intracellular delivery,
10 which can lead to preventative treatments against ischemic injury. For example, TAT was
11 combined with an enzyme called glyoxalase to prevent oxidative damage of neuronal cells (Shin
12 et al. 2014). In another example, antioxidant enzymes catalase and superoxide dismutase were
13 delivered into cells using TAT, which protected the cells from oxidative stress (Kwon et al. 2000,
14 Jin et al. 2001). In an interesting approach to develop a long duration treatment for acute
15 lymphoblastic leukemia, CPPs were used to deliver enzyme into red blood cells (Kwon et al.
16 2009). The enzyme L-asparaginase was delivered into functional red blood cells using the
17 LMWP CPP. This offers an innovative strategy to improve the circulation characteristics of
18 proteins and many other types of molecules.

19 CPPs have been used in several protein delivery applications in which the target cell type
20 was non-mammalian, such as bacteria, fungus, and protozoa. The few brief examples presented
21 here demonstrate the broad penetrating ability of CPPs through cell membranes much different
22 than that used to move proteins into human or mammal cells. Marchione et al., for example,
23 demonstrated that the ZEBRA peptide CPP was able to deliver a green fluorescent protein into a

1 pathogenic fungus (Marchione et al. 2014). This demonstrates the possibility that other types of
2 protein therapeutics may be delivered into fungal cells. Keller et al. used several different types
3 of CPPs to compare the ability of the CPP to deliver a protein (BSA or β Galactosidase) into a
4 protozoa (Keller et al. 2014). The authors noted that this approach could lead to new strategies
5 to study protozoa and inhibit their growth. CPPs have also been used to deliver molecules into
6 other animal cell types, which can serve as important tools for advancing science as well as
7 benefitting human health. For example, Ma et al. used CPPs to deliver antigens into antigen
8 presenting cells to provide fish immunity against bacterial infection (Ma et al. 2014), showing
9 that CPPs can be an effective component in the formulation of novel vaccines.

10 *Small Molecules*

11 Similar to the large biomolecules discussed earlier, small hydrophilic molecules can also
12 face difficulties in traversing the hydrophobic cell membrane. In addition, diseases, conditions
13 such as cancer, can also prevent effective transport of small molecules across the lipid bilayer.
14 CPPs have been shown to be a practical approach in improving the delivery of small molecule
15 cytotoxic drugs into cancer cells, while reducing the exposure of non-diseased tissues. In one
16 study, doxorubicin was coupled with polyarginine and found to inhibit tumor growth *in vivo* with
17 fewer side effects compared to doxorubicin alone (Nakase et al. 2012). The authors noted that
18 the cause behind the accumulation of the drug in the tumor was likely improved by the
19 interaction of the cationic CPP with glycosaminoglycans. Cai et al. used the hydrophobic
20 PFV CPP to increase the delivery of a stealth liposome loaded with doxorubicin into cancer cells
21 (Cai et al. 2014). An interesting result in this study was that the hydrophobic CPP modified
22 liposomes caused a reduction in tumor volume and resulted in fewer side effects compared
23 to an unmodified liposome.

Other strategies to improve small molecule drug delivery have focused on introducing new functionalities to CPPs for greater intracellular control of CPP/drug conjugates. Lelle et al. investigated a CPP that was cross-linked with doxorubicin using a reducible disulfide linker (Lelle et al. 2014). The CPP conjugates were found to be cytotoxic toward cancer cells with release of the doxorubicin into the cell cytosol. Nasrolahi et al. studied the anticancer activity of doxorubicin conjugated to a CPP in drug resistant cancer cells (Nasrolahi Shirazi et al. 2013). The authors observed more antiproliferative activity from the doxorubicin conjugated to the cyclic W(RW)₄ CPP compared to the linear (RW)₄ CPP. Additionally, they found that the cyclic CPP doxorubicin conjugate had improved trafficking to the nucleus and reduced cellular efflux compared to free doxorubicin.

Other types of small molecules have been delivered by CPPs as part of novel technologies ranging from neutron capture cancer therapy to methods for developing new cell preservation protocols. Although the examples presented here are broad, they show that CPPs have wide utility beyond the delivery of well-studied molecules. As part of an innovative cancer treatment, a CPP attached to a boron compound was delivered into malignant glioma cells for boron neutron capture cancer therapy (Michiue et al. 2014). The CPP combined with the boron compound was found to improve the killing of cancer cells compared to the boron compound without peptide. CPPs are also being used as part of innovative strategies to preserve cells without cryopreservation. The preservation agent trehalose was delivered into cells using a newly developed CPP, KRKRWHW, with the overall intent to investigate alternative cell preservation by desiccation (Wei et al. 2014). The authors demonstrate that the novel CPP enters cells and that the inclusion of the preservation agent is non-toxic. Another example of delivery of a small molecule using CPPs is the conjugation of the Tat CPP to a fluorescent label to

1 photosensitize intracellular vesicles (Meerovich et al. 2014). The label was further used as part
2 of a strategy to damage the lipid membrane upon excitation of the label.

3 Novel chemotherapy approaches have focused on limiting the activity of anticancer drug-
4 CPP conjugates to tumor tissue. One approach that is growing in popularity is to make the CPP
5 functional only when an enzyme common to the extracellular environment of tumors is present.
6 Olson et al. demonstrated this with a CPP connected to an anionic polyion by a tumor matrix
7 metalloproteinase cleavable linker, allowing the CPP to be activated in tumor tissue (Olson et al.
8 2009). Xia et al. used this strategy to deliver paclitaxel with an activatable LMWP (ALMWP),
9 protected by a polyanionic peptide that would only become active by tumor metalloproteinases
10 removing the polyanion (Xia et al. 2013). Another approach to producing activatable CPPs has
11 been to use the acidity of the tumor environment to control the peptide activity. Jin et al. made
12 TAT inactive by converting the lysine amino groups of peptide to succinyl amides (Jin et al.
13 2013). The group found that the acidity of tumor tissue can convert the modified peptide back to
14 the active form of TAT, which caused better accumulation of peptide to the tumor.

15 *Imaging*

16 Imaging is of growing importance in the detection and monitoring of disease markers,
17 which can lead to more effective disease management. CPPs have been coupled with
18 fluorophores, contrast agents, quantum dots, and other readily detectable molecules for
19 visualizing the intracellular environment as well as specific cells comprising a tissue. In fact,
20 fluorophores were one of the earliest cargos conjugated to CPPs as a means to study their activity.
21 Since then, CPPs have become important tools in biology for delivering fluorophores and
22 quantum dots for intracellular detection. Earlier studies, such as that by Delehanty et al.,

1 demonstrated that quantum dots could be transported across the lipid bilayer using polyarginine
2 (Delehanty et al. 2006). Subsequent work has utilized CPPs to deliver fluorescent agents into
3 cells for targeted intracellular delivery, which has also proven to be especially useful in cell
4 biology. Liu et al. was recently able to use the HR9 peptide to deliver quantum dots into cells
5 without delivery to a lysosome or other organelles (Liu et al. 2011). In other cell biology
6 applications, oxygen sensitive probes have been coupled to CPPs (Dmitriev et al. 2010). Using
7 these probes allowed for the study of oxygen use inside cells. New fluorescence strategies using
8 CPPs have also been developed for better diagnosis of diseases over-expressing certain
9 intracellular markers, such as the ubiquitin E3 ligase Smurf1 (Suh et al. 2014). Researchers were
10 able to observe Smurf1 binding through a CPP bearing a FITC label, a black hole quencher, and
11 a Smurf1 binding peptide. Changes in peptide confirmation with binding allowed for the group
12 to observe the peptide and protein binding.

13 CPPs have been designed to activate in certain tissue environments, and this has led to
14 several exciting imaging applications. For example, there is great interest in developing CPPs
15 that will activate in tumors, which can in turn improve the detection of cancer cells and allow for
16 real time imaging so that tumors may be removed more effectively. CPP fluorophore conjugates
17 have been designed to become active in tumors through an anionic domain that may be removed
18 by tumor matrix metalloproteinases, effectively labeling tumor cells (Jiang et al. 2004). Efforts
19 to better detect cancer metastasis through MRI and to improve the surgical removal of cancer
20 have used activatable CPPs with fluorescent labels and gadolinium. (Nguyen et al. 2010, Olson
21 et al. 2010). In another approach, an activatable CPP allowed for Savariar et al. to detect tumors
22 *in vivo* (Savariar et al. 2013). In this study, a tumor specific protease disrupts the quenching of a
23 label when the CPP is activated, which results in ratiometric detection of tumor metastasis. This

approach has also been used for detection of thrombin activation in which a CPP was made active by thrombin, allowing the CPP to be internalized in nearby cells, which can lead to better treatment of atherosclerosis and strokes (Whitney et al. 2013). Another exciting approach has been the development of chemically sensitive linkers to produce other novel activatable CPPs. This has led to new possibilities of detecting chemicals through imaging. For example, Weinstain et al., developed a hydrogen peroxide activatable CPP to observe inflammation in the lungs of mice using FRET (Weinstain et al. 2013). This enabled the group to detect oxidative stress both *in vitro* and *in vivo*, which has the potential to be a useful tool to monitor several types of diseases.

5. Conclusions

CPPs offer an exciting potential to transport many different types of therapeutic drugs across the cell membrane and into cellular compartments or the cell cytoplasm where a drug can be most effective. While we do not entirely understand nor can we necessarily predict how a CPP and its cargo will be transported into cells, we are developing the tools and knowledge necessary to harness their ability. Already, there are hundreds of CPPs being explored for the delivery of therapeutic small molecules, peptides, proteins, nucleic acids, and imaging agents. These studies are demonstrating success both *in vitro* and *in vivo*. Successful transition from the laboratory setting to the clinic is only a matter of time.

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Table 1. Examples of some common cell-penetrating peptides and their applications.

Name	Source	Sequence	Cargo	References
Cationic Peptides				
TAT (48–60)	HIV TAT Protein	GRKKRRQRRRPPQ	Gene Vectors, Proteins, Peptides, Label	(Frankel and Pabo 1988, Green and Loewenstein 1988, Vivès et al. 1997)
Penetratin	Antennapedia	RQIKIWFQNRRMKWKK	Gene Vectors, Proteins, Peptides, Label	(Joliot et al. 1991, Derossi et al. 1994, Nigatu et al. 2013, Alves et al. 2014, Nielsen et al. 2014)
Polyarginine	Synthetic	RRRR _n	Gene Vectors, Liposomes, Label	(Wender et al. 2000, Zhang et al. 2006, Tünnemann et al. 2008, Sharma et al. 2013)
Oct4	Oct4 Protein	DVVRVWFCNRRQKGKR	Protein, Label	(Harreither et al. 2014)
WT1-pTj	Wilms Tumor Protein 1	KDCERRFSRSDQLKRHQRRTGVKPFQ	Label	(Massaoka et al. 2014)
DPV3	Heparin Binding Protein	RKKRRRESRKKRRRES	Proteins	(De Coupade et al. 2005)

Amphipathic Peptides

Transportan	Galanin and Mastoparan	GWTLNSAGYLLGKINLKALAALAKKIL	Gene Vector, Label	(Pooga et al. 1998, Fisher et al. 2004)
MAP	Synthetic	KLALKLALKALKAALKLA	Label	(Oehlke et al. 1998)
VP22	Herpes Virus Structural Protein	DAATATRGRSAASRPTERPRAPARSASRPRRPVE	Proteins	(Elliott and O'Hare 1997, Dilber et al. 1999, Zender et al. 2002)
Pep1	SV 40 NLS and a hydrophobic domain	KETWWETWWTEWSQPKKKRKV	Proteins, Gene Vectors	(Morris et al. 2001, Kim et al. 2012, Nigatu et al. 2013)
KW	Synthetic	KRKRWHW	Small Molecule	(Wei et al. 2014)

Hydrophobic Peptides

KFGF	Kaposi Fibroblast Growth Factor	AAVLLPVLLAAP	Peptides, Antibodies	(Lin et al. 1995),(Shin et al. 2005)
FGF12	Fibroblast Growth Factor 12	PIEVCMYREP	Proteins	(Nakayama et al. 2011),(Nakayama et al. 2014)
Integrin β 3 Peptide	Integrin β 3	VTVLALGALAGVGVG	Used for Peptide Sequence	(Liu et al. 1996)

			Identification	
C105Y	α 1-Antitrypsin	PFVYLI	Gene Vector, Peptide, Liposome	(Rhee and Davis 2006, Watkins et al. 2009, Cai et al. 2014)
TP2	Synthetic	PLIYLRLLRGQF	Label	(Marks et al. 2011, He et al. 2013)

Figure 1. Proposed mechanisms for cellular entry of cell-penetrating peptides.