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Recent advances in the use of cell-penetrating peptides for medical and biological applications[☆]

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ABSTRACT

The selective permeability of the plasma membrane prohibits most exogenous agents from gaining cellular access. Since many therapeutics and reporter molecules must be internalized for activity, crossing the plasma membrane is essential. A very effective class of transporters harnessed for this purpose are cell penetrating peptides (CPPs), a group of short cationic sequences with a remarkable capacity for membrane translocation. Since their discovery in 1988, CPPs have been employed for the delivery of a wide variety of cargo including small molecules, nucleic acids, antibodies and nanoparticles. This review describes recent advances in the use of CPPs for biological and therapeutic applications. In particular, an emphasis is placed on novel systems and insights acquired since 2006. Basic research on CPPs has recently yielded techniques that provide further information on the controversial mechanism of CPP uptake and has also resulted in the development of new model membrane systems to evaluate these mechanisms. In addition, recent use of CPPs for the development of new cellular imaging tools, biosensors, or biomolecular delivery systems have been highlighted. Lastly, novel peptide delivery vectors, designed to tackle some of the drawbacks of CPPs and enhance their versatility, will be described. This review will illustrate the diverse applications for which CPPs have been harnessed and also demonstrate the remarkable advancements these peptides have facilitated in cell biology.

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1. Introduction

The plasma membrane enveloping cells is highly effective in its role as a selectively permeable barrier. While this phospholipid bilayer is essential to cell survival and function, it also presents a major challenge for intracellular delivery of cargo. Since therapeutics, reporter molecules, and imaging agents often require intracellular access to function, identifying strategies for membrane translocation is essential. While compounds can sometimes be chemically modified to improve permeability, this often requires many iterations before translocation is achieved without sacrificing activity [1]. Alternatively, a synthetic transporter can be utilized to promote cellular uptake, an approach that can be employed for a wide range of applications.

While a variety of transporters currently exist for cargo translocation, cell-penetrating peptides (CPPs) have become one of the most popular and efficient techniques for achieving intracellular access. CPPs are typically short cationic sequences and may be derived from natural sources or be synthetically designed constructs [2]. Initially discovered in 1988, Tat, the HIV transactivator of transcription protein, was the first sequence found to be capable of translocating cell membranes and gaining intracellular access [3,4]. Upon further investigation, it was shown that this sequence could be shortened to a few amino acids, referred to as the Tat peptide, without sacrificing translocation capacity [5]. This discovery was then followed by the first use of CPPs as vectors when penetratin was employed for the delivery of a small exogenous peptide in 1994 [6,7]. Since then, the list of available CPPs has grown dramatically and the number continues to increase (Table 1). While the choice of CPP often depends on the application at hand, some of the most commonly used peptides include Tat, polyarginine, penetratin and transportan. These CPPs have successfully delivered proteins [8], nucleic acids [9], small molecule therapeutics [10], quantum dots [11], and MRI contrast agents [12], to name but a few types of cargo. In addition, this highly efficient translocation capacity has been observed in a variety of cell lines with minimal toxicity, overcoming challenges often faced with other delivery methods [13].

This review will highlight the use of CPPs for medical and biological applications, with particular attention paid to advances made since 2006. It will also briefly discuss factors affecting mechanism of peptide uptake and the consequences of these mechanisms on the usage of CPPs. Since CPPs have recently been comprehensively reviewed in the literature [14–17] the purpose of this review will be to emphasize only the most interesting and influential advances in this field and to discuss the impact of these discoveries.

2. Cellular uptake of CPPs: new insights into the route of entry

Although the mechanism of CPP uptake across the plasma membrane has been the subject of numerous studies, a unifying pathway for translocation remains elusive. It has been suggested that various properties of peptides, such as molecule length and charge delocalization, as well as the properties of the associated cargo, such as size and charge, can have a significant impact on the mechanism of peptide uptake [18]. Comparative and systematic studies of CPP panels in multiple cell lines using various incubation conditions and cell stresses have aided in the selection of an optimal CPP for a given application. What has become evident, however, is that a single CPP

may use multiple modes of cellular entry that can depend on the context of the experimental conditions. These modes are broadly categorized into two groups: energy-dependent endocytosis and energy-independent direct translocation across the membrane bilayer (Fig. 1A). Since the former mechanism can result in endosomal sequestration and decreased bioavailability, a full understanding of the impact that peptide and cargo physiochemical properties have on the mechanism of uptake is necessary for the rational design of effective delivery vehicles.

A recent study of the cellular uptake of three CPPs – the antennapedia/penetratin peptide, nona-arginine (R9), and Tat – provided the community with a thorough and comprehensive look at different mechanisms of CPP uptake that may be operative in a single system [19]. Focused on clarifying the endocytic pathway utilized by CPPs, the study provided evidence that a single endocytic mechanism could not be resolved, and that macropinocytosis, clathrin-mediated endocytosis, and caveloae/lipid raft mediated endocytosis all occurred. In addition, the fractional component of a particular type of uptake was dictated by the CPP sequence, as well as the concentration utilized. Interestingly, the two peptides with the most delocalized charge, Tat and R9, shared commonalities of both endocytic as well as direct mechanisms of uptake. At low peptide concentrations, only inhibitors of clathrin-mediated endocytosis did not have an effect on internalization, suggesting that cellular import was mediated by both macropinocytosis and caveloae/lipid raft mediated endocytosis. Perhaps most remarkable is that above a certain concentration threshold, peptide internalization for Tat and R9 ceased to be dependent on endocytic mechanisms. Rather, translocation occurred by direct uptake through specific nucleation zones as determined by time-lapse microscopy. For penetratin, however, higher peptide concentrations were not sufficient to induce direct uptake. Partial inhibition of endocytosis was also necessary to direct access to this pathway. Similarly, for the Tat and R9 peptides, inhibition of macropinocytosis and caveloae/lipid raft-mediated endocytosis augmented direct uptake. However, paradoxically, inhibition of clathrin-mediated endocytosis suppressed this effect.

Table 1
Amino acid sequences of cell penetrating peptide.

Cell-penetrating peptide	Amino acid sequence
Tat _{49–57}	RKKRRQRRR
Polyarginines	RRRRRRRRRR (R ₉)
R ₉ F ₂	RRRRRRRRRF
Decalsine	KKKKKKKKKK (K ₁₀)
Penetratin	RQKIKIVFQNRRRMWKWKK
Transportan	GWTLNSAGYLLKIQNLKALAALAKKIL
HIV-Tat derived PTD4	YARAARQARA
Hepatitis B Virus Translocation Motif (TLM)	PLSSIFSRIGDP
mPrP _{1–28}	MANLGYWLLALFVTMWTDVGLCKKRPKP
POD	GGG(ARKKAAKA) ₄
pVEC	LIIILRRRRIRKQAHHSK
ARF _(1–22)	MVRFLVTLRIRRACGPPRVRV
EB1	LIRLWSHLIHIWFQNRRLKWKKK
Rath	TPWWRLWTKWHHKRRDLPRKPE
CADY	GIWRALWRLRSLSWRLWRA
Histatin 5	DSHAKRHGYKRKFHEKHSHRGY

Cationic residues have been bolded for emphasis.

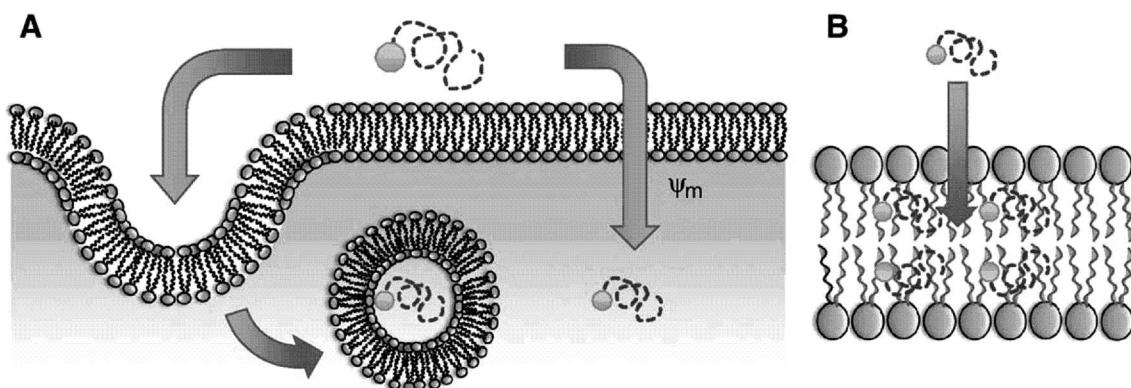


Fig. 1. Translocation of CPP conjugates across the plasma membrane. (A) Translocation of CPPs can occur via endocytic mechanisms or through direct diffusion in an energy independent manner that may be mediated by membrane potential. (B) Novel NMR studies of CPP membrane vesicle interactions indicate that CPPs partition into both leaflets without the presence of an electrical potential across the membrane.

Nevertheless, it is suggested that perturbation of endocytotic mechanisms promotes accumulation of peptide on the membrane for cellular import via direct uptake. This work is very valuable as it compares mechanisms of uptake in a systematic and controlled fashion, and clearly highlights the roles that cargo, cell line, cell density and numerous other factors play in CPP uptake mechanisms. Lastly, the work clearly shows that CPPs can harness alternate mechanisms of uptake and that sometimes these different pathways can operate concurrently.

Another advance in the study of CPP translocation mechanisms was reported by a collaborative team who used single-molecule spectroscopy to monitor cellular uptake [20]. Observation of single-molecule dynamics of CPPs using real-time microscopy allowed for comparisons to be made between molecular transporters that used endocytosis versus those that used direct uptake for membrane translocation. The creation of a novel fluorophore dicyanomethylenedihydrofuran (DCDHF) that exhibits an enhanced quantum yield in the constrained environment of the membrane allowed for the diffusion coefficient and residence times of single peptides within the plasma membrane to be observed. Through this investigation, it was concluded that even at low peptide concentrations (as low as 1 nM), multiple import mechanisms are implicated. This technique is very powerful as it allows researchers to examine mechanisms of uptake in a highly detailed and comparative manner. In addition, its application to other common CPPs will provide invaluable information on how these peptides interact with the plasma membrane at a single molecule level.

As mentioned above, direct uptake is the other dominant mechanism of translocation harnessed by CPPs. Direct uptake is not simply passive diffusion across the membrane – rather it is driven by plasma membrane potential. In order to investigate this mechanism, studies aimed at understanding the interaction of CPPs with the cellular membrane are essential. To characterize these CPP-lipid bilayer interactions, the association of penetratin with POPC/POPG vesicles was studied using solid-state NMR paramagnetic relaxation enhancement experiments [21]. The study determined that in reconstituted vesicles, penetratin inserts into each leaflet of the bilayer at a depth determined by the ratio of peptide to lipid (Fig. 1B). Moreover, the results suggested that an essential feature of the electroporation model of peptide entry, asymmetric membrane binding, does not occur in the system tested. Instead, the authors suggested that membrane translocation is driven by a preferential interaction of cationic residues with the anionic phospholipid head-groups on the internal face of the vesicle [21]. This is the first instance where NMR has been applied to study the interactions of CPPs with membranes and these studies provided information that is not easily obtained with other techniques. Going forward, it might be interesting

to see whether this distribution of penetratin changes in a system with membrane potential.

Although CPP uptake has been the subject of numerous studies, it is clear that further work is necessary to understand the variables affecting modes of internalization. One interesting and untested approach would be to use model yeast organisms, such as *Saccharomyces cerevisiae*, for this purpose. CPPs have been successfully internalized into *S. cerevisiae* [22] and this organism is genetically tractable and versatile. In addition, *S. cerevisiae* has been particularly useful in understanding the mechanisms involved in endocytosis (reviewed in [23]), aiding in the identification of proteins involved in the endocytic process as well as identification of regulation mechanisms governed by ubiquitination. Therefore, this model organism could serve as a powerful tool for understanding the endocytic mechanisms involved in CPP import. In addition, the creation of yeast gene deletion arrays [24,25] has made the prospect of identifying novel factors essential to CPP import a possibility by allowing for testing of CPP uptake in multiple models of endocytosis dysfunction in high throughput.

3. Illuminating cell biology: CPPs in imaging and biosensing applications

The use of CPPs to deliver imaging agents and biosensors has been the focus of many studies, resulting in the generation of agents with improved permeabilities [26–28]. Recently, research aimed at delivering agents across the blood–brain barrier [11], targeting radiolabeled antibodies to intracellular sites [29], and visualizing viral infection in real time [30] have advanced this area, providing novel and powerful tools in the development of new imaging agents.

3.1. Delivering quantum dots across the blood–brain barrier

Crossing the blood–brain barrier, a series of tight junctions between endothelial cells, presents a major hurdle for labeling brain tissue. To penetrate this barrier, Tat was recently harnessed to deliver quantum dots into rat brain tissue [11] (Fig. 2A). A microcatheter was used to administer Tat-conjugated quantum dots intra-arterially at a proximal cervical carotid artery in rats. Tat successfully and rapidly delivered the quantum dots to the brain and at such a high loading that gross fluorescent visualization of the rat brain was possible with a low power hand-held UV lamp. Impressively, this was accomplished without manipulation of the blood–brain barrier and the quantum dots were capable of migration beyond the endothelial cell line of injection to reach brain parenchyma. While these findings are very encouraging, the animals were euthanized prior to gross brain visualization. Since the downstream application of these quantum

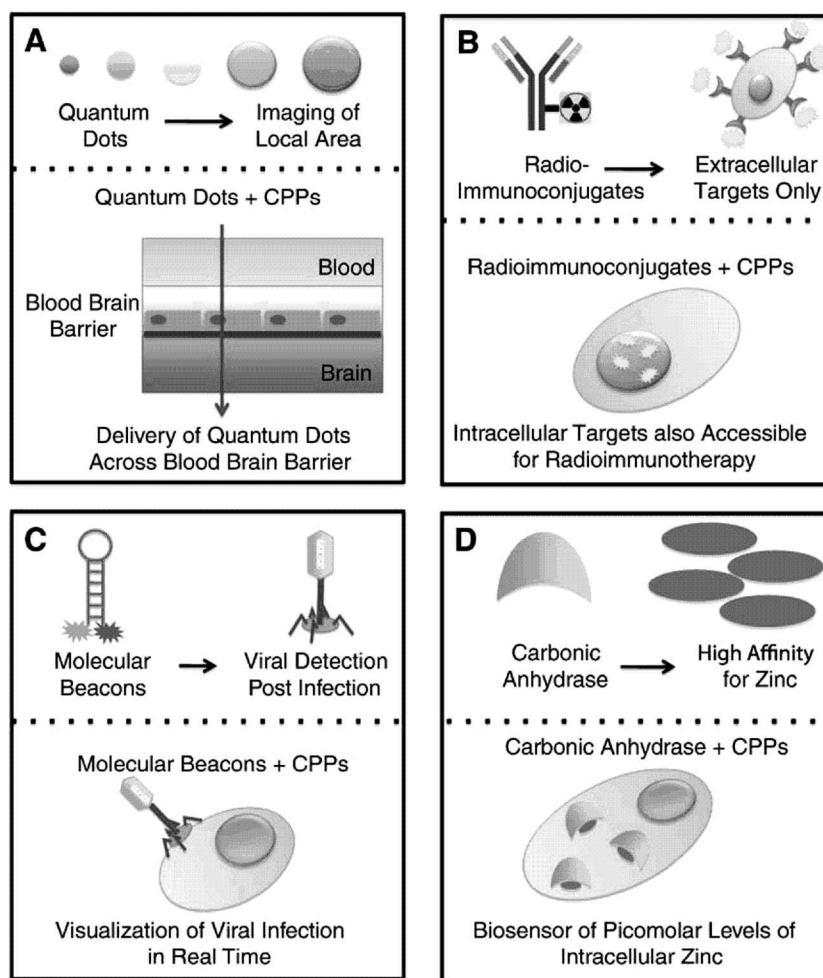


Fig. 2. Overcoming imaging barriers with CPPs. (A) Quantum dots conjugated to CPPs are able to cross the highly impermeable blood-brain barrier. (B) Radioimmunoconjugates can access intracellular molecules upon conjugation to CPPs. (C) Molecular beacons attached to CPPs can be harnessed to visualize viral infection in real time. (D) Carbonic anhydrase, has been conjugated to CPPs to yield a highly sensitive biosensor for measuring intracellular zinc levels.

dots are for tumour visualization of human patients during surgical procedures, it would be interesting to determine if this type of visualization could be conducted in live rats undergoing a similar operation.

3.2. CPPs for the intracellular delivery of antibodies

Large molecules, such as antibodies, are notoriously problematic cargo for delivery vectors. Over a decade ago, the remarkable translocation capacity of Tat was used to successfully deliver antibodies into cells [31]. However, only recently has this type of approach been used to deliver monoclonal antibodies, extensively used for radioimmunotherapy and radioimmunodetection, to intracellular targets (Fig. 2B). Tat was conjugated to an antibody directed against the intracellular cyclin-dependent kinase inhibitor, p21^{WAF-1/CIP-1} and this conjugate was able to traverse cell membranes two-fold more efficiently than unconjugated antibodies [29]. Upon internalization, Tat directed the radioimmunoconjugate to the nucleus and a 5-fold increase in nuclear radioactivity was observed compared to the antibody alone. Importantly, the delivered antibody remained in its functional conformation and was able to interact with the target, effectively inhibiting G₁-S phase cell cycle arrest. This appears to be the first report where conjugation to Tat has resulted in delivery of an intact radiolabeled antibody to the nucleus. The ability to target the conjugate to the nucleus also had an additional, and unexpected, advantage. Since most deiodinases are absent from the nucleus, the targeted immunoconjugates suffered from less intracellular deiodina-

tion. This increased stability is also a novel finding and suggests these types of conjugates may not require the more stringent radio-iodination techniques that are otherwise used. This work should have significant impact, as radioimmunotherapy and detection will no longer be limited to extracellular targets.

3.3. Visualizing viral infection in real time with CPPs

Another imaging-related application of CPPs to emerge recently involves the visualization of real-time viral infection of living cells [32]. It has previously been shown that molecular beacons are able to detect viral RNA in infected cells [30]. These single-stranded molecular beacon oligonucleotides were specifically chosen to target a non-coding region of a viral genome and were labeled with a fluorophore at one end and a quencher at the other. Prior to encountering the virus, these constructs existed in a stem-loop structure with the quencher and fluorophore in close range, resulting in an absence of fluorescence. However, as the viral particles entered cells, the molecular beacons hybridized with the viral genome and changed conformation, separating the quencher and fluorophore. As a result, fluorescence was detected allowing the viral infection to be visualized. While this approach is highly sensitive (detection of a single viral particle was possible), it was only successful in fixed cells permeabilized with Triton to allow uptake of the molecular beacons. In order to deliver these molecular beacons in a less invasive manner, a subsequent study harnessed Tat to deliver the constructs into uncompromised cells

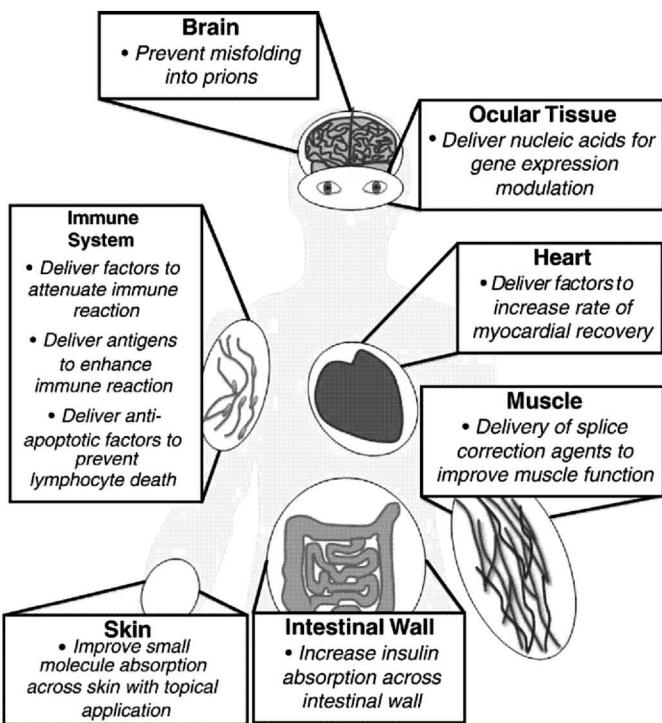


Fig. 3. Harnessing CPPs for delivery of challenging targets. The versatility of CPPs, both in their capacity to deliver a wide variety of cargo and in their application as vectors at a range of sites, is illustrated in this figure. These agents can cross highly selective barriers (such as the intestinal wall and skin) and deliver challenging molecules (such as anionic nucleic acids and large proteins) while retaining the biological activity of the cargo.

(Fig. 2C). This improvement allowed for real time detection of viral replication and infection [32]. Within 2 h of viral introduction, fluorescence could be detected, even with only 1 pfu of virus, a sensitivity and speed previously unattained for enteroviruses. This enabled the direct observation of cell-to-cell spreading of viral infection in real time scale. It was also possible to quantify the viral plaque-forming units using this system and in a much more rapid manner than was previously achievable (2 h versus the 48 h incubation period for plaque assays). Therefore, by harnessing CPPs, a sensitive, rapid and real-time system for monitoring viral infection was developed.

3.4. Harnessing CPPs to deliver intracellular biosensors

In addition to delivering imaging agents, CPPs can also be used to deliver light-emitting biosensors. Zinc, a cofactor for many enzymes, transcription factors and immune system proteins, is considered to be the second most abundant trace element in the human body [33–36]. While it is essential for the functionality of many enzymes, it is also toxic in certain circumstances, such as during seizures and ischemic insult [37–41]. Therefore, methods that facilitate monitoring of zinc levels, distribution of zinc in the body, or incorporation of zinc into proteins are of great interest. However, since various agents such as proteins, glutathione, and histidine bind most cellular zinc, only a very minor fraction remains free and available for detection [42,43]. Therefore, most studies to date have been limited to measuring free zinc levels in cell lines that are enriched in zinc, rather than in ordinary resting cell lines [44–46]. In an effort to gain a better understanding of zinc levels in traditional cells, a ratiometric fluorescent zinc biosensor was recently developed [47]. Human carbonic anhydrase was used as a sensor transducer and its fusion to Tat allowed the construct to be efficiently internalized without the need for cell membrane manipulation (Fig. 2D). The signal was quantitative and shown to be able to detect zinc levels as

low as 5–10 pM in the nucleus and cytoplasm of cells. This highly sensitive and novel biosensor can now be applied to the study of zinc levels in other commonly used cell lines and can also be used to study the role of zinc in cell biology.

4. Cellular trojan horses: CPPs in bioactive cargo delivery

Small molecules, proteins and nucleic acids are all capable of modulating cellular function and producing therapeutic effects. However, these molecules may exhibit attenuated activities *in vivo* because cell impermeability impedes the activity they display *in vitro*. Often, this is due to their large size or anionic character. Therefore, CPPs have been harnessed to translocate these agents across the cell membrane. This section will focus on recent applications where CPPs have been used to deliver bioactive cargo for biological or therapeutic goals. Fig. 3 summarizes the utility of CPPs for the delivery of various bioactive molecules to challenging target areas.

4.1. Small molecule delivery

One of the most powerful capabilities of CPPs is their capacity to deliver a wide variety of compounds and macromolecules into the cytosol in an active form. Numerous small molecule chemotherapeutics, such as Taxol [48], cyclosporine A [10], and methotrexate [49] have shown improved activity when conjugated to a CPP. Remarkably, even though a CPP-drug conjugate may show less activity in a purified biochemical system, highly efficient cellular uptake can overcome this shortfall. It has been demonstrated that although a CPP-methotrexate conjugate experiences a 20-fold loss in potency than the drug alone, it is a highly efficient cytotoxin of a methotrexate-resistant cell line [49]. These studies demonstrate that CPP conjugates can effectively increase the intracellular concentrations of bioactive small molecules and that this can counteract decreases in drug activity that result from conjugation.

4.2. Harnessing CPPs for delivery of peptides, peptoids and proteins

Biologically-active macromolecules often have physicochemical properties that limit intracellular accumulation. Harnessing CPPs as molecular transporters allows these molecules to be delivered intracellularly and at sufficient levels to have a biological effect. A pertinent example is the utilization of CPPs for the delivery of peptides and proteins to modulate intracellular processes.

CPP-mediated alteration of transcription factor activity was recently described involving a rationally designed peptide inhibitor of the transcription factor STAT-6 that bound only to the phosphorylated (active) form of the protein to prevent dimerization and activity [50]. To achieve intracellular delivery, the inhibitory peptide was conjugated to the HIV-Tat-derived PTD4 CPP. Internalization of the conjugate exerted a dominant negative effect and was discovered to attenuate ovalbumin-induced inflammatory responses and mucus production when the conjugate was delivered to the upper airway of mice. As STAT-6 activity is linked to allergic disease [51], this indicates that the STAT-6 inhibitory peptide could be a promising novel treatment for allergic rhinitis and asthma.

Another example of CPP-mediated modulation of the immune response is seen in recent work to overcome bacterial sepsis. The pathogenic mechanism of sepsis has been shown to be due to the extensive apoptotic loss of lymphocytes and dendritic cells, resulting in immunosuppression and eventually death [52,53]. Since therapies that inhibit apoptosis have been shown to improve survival [54,55], CPPs were used for the delivery of anti-apoptotic proteins as a treatment for sepsis [56]. Bcl-x_L and its BH4 domain were conjugated to Tat and these conjugates were administered to mice suffering from *Escherichia coli*-induced lymphocyte apoptosis. It was observed that *in vivo* administration of the conjugate decreased sepsis-induced

lymphocyte apoptosis, and a hypothesis was presented suggesting that the Tat-BH4 protective mechanisms might be post-transcriptional, perhaps involving a protein–protein interaction. This work is the first to harness CPPs to deliver anti-apoptotic proteins to combat sepsis-induced apoptosis. The potency of this system indicates that it is a highly effective method to decrease immune system depletion and improve survival following sepsis.

Inhibition of apoptosis could also be beneficial for the treatment of neurodegenerative disorders, cancer and ischemic injuries [57]. While many anti-apoptotic agents target caspase activity [58,59], cellular compensatory pathways decrease the efficacy of these compounds [60]. Instead, the ability to inhibit apoptosis upstream of caspase activation could be more beneficial. One such target is the apoptotic protease-activating factor (Apaf-1), a component of the apoptosome-holoenzyme [61]. A peptoid inhibitor for Apaf-1 had been previously designed but the ligand suffered poor cell membrane permeability, and therefore cellular efficacy, despite structural modifications [62]. Recently, the group further modified the peptoid inhibitor by conjugating the ligand to Tat and penetratin [63]. Both CPPs increased cellular uptake but the penetratin conjugate was found to be more effective at inhibiting apoptosis [63] presumably due to observed toxicity of the Tat conjugate. CD spectroscopy of peptides in differing membrane-mimetic environments demonstrated that the attached peptoid cargo could have an effect on the conformation behavior of CPPs, especially in the case of the Tat conjugate. As specific membrane interactions are an essential step in translocation [64], the authors surmised that differing lipid-peptide interactions of Tat and the Tat-peptoid conjugate resulted in membrane damage from the latter. This study is another example of harnessing CPPs for improved intracellular delivery where the CPP does not interfere with the efficacy of the cargo molecule. However, it does point out the possible effects of cargos on CPP conformation and highlights the necessity of selection of an appropriate carrier molecule for each application.

Another therapeutically relevant advance in the CPP field involved the VP22-directed delivery of the GATA4 transcription factor to combat myocardial injury [65]. VP22 is a herpes simplex virus protein with cell-penetrating properties [66]. It was found that co-culture of fibroblasts expressing GATA4-VP22 with mesenchymal stem cells (MSC) activated expression of GATA4-inducible genes in MSCs. Moreover, the intracellular delivery of GATA4-VP22 was shown to have a beneficial effect after myocardial infarction in Lewis rats as indicated by improved ventricular remodeling and increased cardiac function. To fully evaluate the therapeutic potential of this system, the activity of recombinant purified protein may need to be assessed. Addition of purified components will identify potential toxicity associated with over-dosage, as GATA4-VP22 can be carefully titrated into the *in vivo* system. This construct could serve as a novel therapeutic to improve myocardial function following injury.

Another area where CPPs can contribute to pharmacological applications is through their delivery of antigens. Vaccines are designed to stimulate an immune reaction in response to an antigen and the greater the antigen exposure, the stronger the response. With this rationale in mind, a model antigen, ovalbumin, was conjugated to the translocation motif of the Hepatitis B virus [67]. An augmented immune response with a higher titer of anti-ovalbumin antibodies was noted compared to ovalbumin alone. In addition, the authors report that this conjugation resulted in an improvement of both cellular and humoral immune system responses. Therefore, harnessing CPPs to more efficiently deliver antigens intracellularly could dramatically improve vaccine efficacy.

In addition to CPPs delivering therapeutically relevant cargo, some CPPs themselves have biological activity. Prion diseases are fatal neurodegenerative disorders that include bovine spongiform encephalopathy (BSE) and Creutzfeldt-Jakob disease (CJD) in humans [68]. In these diseases, neurodegeneration is caused by a misfolded prion protein (PrP^{Sc}) thought to be infectious and able to convert normal

protein isoforms, PrP^{C} , into the misfolded version. Recently, a peptide composed of the first 28 amino acids of the normal isoform PrP^{C} was discovered to exhibit cell-penetrating properties [69]. In addition, these PrP^{C} -CPPs also possessed a region for specific interaction with the PrP^{Sc} to prevent any further PrP^{C} to PrP^{Sc} conversion. Further work to maximize the therapeutic potential of this peptide may be achieved through optimization of its cell-penetrating ability. Indeed, this construct shows strong promise as potential therapeutic agent for the future.

For some applications, a very high intracellular level of cargo may be required, a level unattainable even by CPPs when cells are in a normal physiological environment. A recent study has shown that CPP-directed delivery can be enhanced by exposing cells to low voltage electrical pulses [70]. Conjugation of a peptide inhibitor of glycogen synthase kinase-3 to Tat showed an increase in uptake by an order of magnitude when electrical pulses were administered with the conjugate. The group hypothesized that the electrical pulses caused the CPP-cargo to adsorb more efficiently on the cell surface and therefore, uptake was enhanced. This method of increasing cargo uptake will be highly beneficial, especially for applications where the amount of CPP used cannot be increased for fear of excess toxicity. Importantly, the authors showed that these low voltage electrical pulses do not cause cellular toxicity and do not induce apoptosis. This technique can also be used to selectively deliver cargo to a particular region of interest, simply by controlling the site of pulse administration.

4.3. Delivery of nucleic acids and siRNA

The delivery of anionic biomolecules, such as nucleic acids, has been very challenging. Unassisted uptake of nucleic acids occurs only at very low levels and vector-assisted delivery of these molecules often results in endosomal entrapment or degradation by nucleases [71,72]. In the past, two approaches have been taken to circumvent these roadblocks: engineering endosomal escape [73–75], which will be discussed later in this review, and harnessing neutral nucleic acid analogs, such as peptide nucleic acid (PNA) [76,77]. The latter are nucleobase derivatives with a peptide backbone, a structure that results in an uncharged molecule resistant to cellular degradation. However, despite this modification, PNA retains the complementary base pairing functionality of natural nucleic acids.

One other such neutral mimic, phosphorodiamidate morpholino oligomers (PMOs) have been used successfully for the attenuation of nonsense mutations through exon removal [78]. CPPs have recently been harnessed for PMO delivery into mouse models of Duchenne-muscular dystrophy [79,80]. These mice possess a nonsense mutation in the dystrophin gene at exon 23, mimicking the dystrophinopathies in humans. The conjugate was administered by intraperitoneal injection and an enhancement of splice correction at the nonsense mutation was noted. In addition, sustained dystrophin expression was obtained and muscular architecture was improved.

CPPs have also aided the delivery of small interfering RNA (siRNA) used in the modulation of gene expression [81]. Previous reports have highlighted the use of Tat for efficient delivery of siRNA to cells for gene silencing [9]. A recent report described a novel CPP, a “peptide for ocular delivery” (POD), that is capable of delivering large and small molecule cargo into ocular tissues [82]. POD successfully delivered siRNA into human embryonic retinal cells and a >50% gene silencing effect was observed. POD also delivered plasmid DNA to cells achieving >50% transgene expression. Furthermore, topical administration of a POD-dye conjugate to the cornea in mice resulted in efficient distribution into neighboring ocular tissue and rapid cellular uptake. Further studies are necessary to determine if POD-nucleic acid conjugates, administered via topical administration to the cornea, would also be able to achieve ocular tissue distribution and rapid cellular uptake. Nevertheless, this work demonstrates the potential utility of CPPs to enhance nucleic acid delivery to ocular tissue.

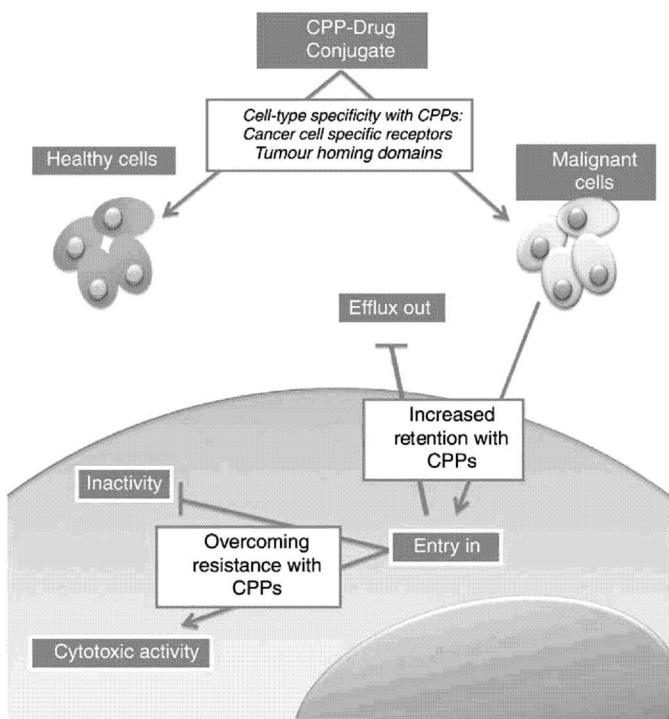


Fig. 4. CPPs improve drug efficacy via diverse pathways. Combining CPPs with tumour homing domains allows drugs to be delivered selectively to cancer cells and at concentrations needed for efficacy. Conjugation of CPPs to drug molecules also allows for increased retention in tumours by decreasing efflux out of the cell. CPPs can also be used to overcome drug resistance by increasing intracellular drug concentrations in tumour cells.

4.4. Increasing absorption with CPPs

CPPs not only aid with intracellular delivery, but can also be harnessed to improve absorption across skin and intestinal barriers. Both these barriers are notoriously difficult to translocate, so improvements in this area could be of great therapeutic value.

An example of CPP-enhanced absorption involved CPP-cyclosporine A conjugates where the peptide successfully directed dermal absorption with a simple topical application [10]. The absorption was much more efficient than with unconjugated cyclosporine A. In addition, an *in vivo* reduction in inflammation was also noted with the CPP-cyclosporine A conjugate but not with unconjugated drug. This finding may be of significant therapeutic value as drugs can now be administered topically and directly to the skin region of interest rather than administered systemically.

The intestinal barrier is another formidable challenge to delivery and as such, insulin dosing for diabetes treatment has typically been accomplished through subcutaneous injections. A set of recent studies, however, have focused on a novel approach for the efficient intestinal adsorption of this hormone [83–85]. In these studies, it was determined that co-administration of various CPPs with insulin enhanced intestinal uptake, compared to the absence of absorption noted with insulin alone. Furthermore, the co-administration increased plasma insulin levels and reduced blood glucose levels, suggesting that the insulin remained functional. A particularly impressive aspect of the results reported was that covalent attachment of the CPP to insulin was not necessary for this effect – a simple co-administration would suffice. This finding simplifies drug preparation and reduces the likelihood that the CPP will hinder the biological effect of insulin. These studies demonstrate how CPPs can be used for efficient delivery of bioactive molecules for biomedical applications.

5. CPPs in cancer therapy

CPPs have become invaluable tools in the search for more effective cancer therapies. These peptides have been utilized in many innovative applications, both to deliver chemotherapeutic drugs to cells [86] as well as to deliver pro-apoptotic proteins [87]. This section will highlight some of the recent developments in this field and will attempt to illustrate the breadth of cancer-related applications for which CPPs have been harnessed.

5.1. Modifying anticancer agents with CPPs to achieve cancer-cell selectivity

While CPPs can efficiently deliver chemotherapeutic agents to cells, conventional CPPs, like Tat, are unable to do so with specificity (Fig. 4). This would result in both tumour and healthy cells receiving drug, an undesirable situation for anti-cancer treatment. To rectify this inherent lack of specificity, a Tat-derived CPP was conjugated an anti-Her-2/neu peptide mimetic, AHNP [86]. This mimetic binds selectively to ErbB2[88], an epidermal growth factor receptor over-expressed in 30% of breast cancers [89]. This newly designed cancer cell-specific transporter was then harnessed for therapeutic purposes. A STAT3 inhibiting peptide (STAT3BP), known to block the activity of STAT3 transcription factors [90], was added to this construct. This target was chosen because STAT3 has been shown to be constitutively activated in 50% of breast carcinomas and is implicated in poor prognosis [91,92]. The presence of the cancer-targeting transporter allowed the therapeutic STAT3BP to be delivered specifically to cancer cells but not untransformed cells and this specificity was achieved both *in vitro* and *in vivo*. In addition, STAT3BP activity was not affected by conjugation to Tat-AHNP and the cargo was able to disrupt STAT3 activation, induce apoptosis and inhibit tumour growth *in vivo*.

Another strategy to engineer tumour specificity into CPP constructs relies on tumour homing domains [93,94]. A PEGA homing domain, previously shown to accumulate in breast tumour vasculature [95], was conjugated to the pVEC CPP [96]. The homing domain was inherently cell impermeable but upon attachment to the CPP, efficient and selective uptake into breast tumour blood vessels was observed in mice. Next, a chemotherapeutic agent, chlorambucil, was conjugated to this construct and found to be four times more effective against cancer cell lines than drug administered alone. Furthermore, the construct was not trapped in lysosomes, allowing the majority of drug to be available for prevention of cell proliferation. This is the first report where the combination of a homing domain with a CPP allowed for selective and efficient intracellular delivery of an anti-cancer drug.

5.2. Harnessing CPPs for improved retention of therapeutics in tumours

Radioimmunotherapy, the use of an antibody labeled with a radionuclide to deliver cytotoxic radiation to tumour cells, is a highly specific cancer treatment relying on the tumour targeting ability of the antibody [97]. Unfortunately, single-chain FVs (scFVs) antibody fragments undergo slow accumulation in tumours and are prone to rapid elimination from circulation [98,99]. Since both these factors limit efficacy, CPPs were harnessed to direct scFV uptake out of circulation and promote rapid uptake into tumours [100] (Fig. 4). Penetratin was injected with scFVs and tumour uptake and retention was investigated [100]. Improved tumour retention and a more homogenous distribution of the antibody fragment was achieved upon CPP administration. In addition, the CPP did not alter tumour specificity and the conjugate continued to localize selectively to transformed sites rather than non-target tissues. Interestingly, the CPP did not require covalent attachment to the antibody for these improvements to be noted. This feature of the system is highly advantageous and will simplify the implementation of such an approach. This appears to be the first use of CPPs to deliver such scFVs and the significantly improved retention will no doubt increase their therapeutic potential.

5.3. Cytotoxic CPPs

The p14ARF protein is a potent tumour suppressor that is often deregulated in cancers [101]. Previous work on this protein had identified a 22-amino acid peptide derived from the N-terminal of p14ARF, which was capable of mimicking the function of the intact p14ARF protein [102,103]. A closer look at this sequence showed that the peptides had a 20% arginine content. Since CPPs are also often arginine-rich sequences, this peptide was tested for its ability to traverse cell membranes [87]. Indeed, it was found to be highly cell permeable, with uptake rivaling that of the Transportan 10 CPP. In addition, it was also shown to be capable of delivering functional splice correcting PNA into cells. This pARF14 peptide retained its cytotoxic bioactivity with a 45% decrease in proliferation observed upon incubation with cells. This is the first report where a peptide, derived from a larger protein, was found to possess both anti-proliferative and cell penetrating capacities.

5.4. CPPs combating chemotherapeutic resistance

One of the most common mechanisms of multidrug resistance is over-expression of drug efflux pumps by tumour cells [104,105]. Furthermore, these pumps often cause resistance to a range of chemotherapeutic agents, severely limiting treatment options [106]. Since these energy-dependent membrane proteins are designed to pump out drugs that enter cells passively, Dubikovskaya et al. sought to test whether compounds that enter cells through other pathways are effluxed less efficiently [48] (Fig. 4). CPPs, known to promote cargo delivery through mechanisms other than passive uptake, were harnessed to test this hypothesis. Octaarginines were conjugated to Taxol, a widely used chemotherapeutic agent, via a bioactivatable disulfide linker. Upon cellular uptake, the high intracellular glutathione

concentration caused disulfide cleavage and the CPP was released from Taxol preventing the peptide from interfering with drug activity. The conjugates were more active than Taxol alone, both in cell lines and animal models. Impressively, activity was even observed against Taxol-resistant cells, both *in vitro* and *in vivo*. In addition, when delivered into mice via intra-peritoneal injection, the conjugate remained near the site of injection, probably due to rapid uptake and adherence of arginine residues to the cell membrane. This localization increased the concentration of cargo in the tumour vicinity and also reduced the risk of systemic toxicity. Furthermore, since the cargo was not active until the linker was cleaved, the drug was released in a sustained manner and the bolus effect observed with free drug administration was not observed. This report describes a system where CPPs are attached to drugs via releasable linkers to combat drug resistance. Moreover, it appears that this approach is generalizable to other drugs where resistance has been encountered and also suggests that the strategy could be extended to antimicrobial and anti-parasitic treatment where drug resistance due to efflux is commonly encountered.

6. Novel CPPs: covering new cellular territory

While CPPs have impressive transporting abilities, they exhibit a limited range of intracellular localization profiles, and often remain sequestered in endosomes. This limitation restricts their use in applications where efficient transport to the cytoplasm or particular cellular organelles is required. Recently, strides have been made in this area, with CPPs being modified to improve endosomal escape, to deliver cargo to specific sub-cellular organelles, and to deliver especially challenging anionic cargo. This section will highlight some of these achievements and discuss the role these novel CPPs can play in future applications.

6.1. Improving CPP escape from endosomal entrapment

As mentioned above, an undesirable consequence of endocytic uptake for many CPPs is the subsequent vesicular entrapment. A number of chemical agents, such as chloroquine [107], calcium [108] and sucrose [109], have been used to promote escape but their inability to be used in an *in vivo* setting limits their applicability (Fig. 5). Since this entrapment severely limits the activity of translocated cargo, several groups have been working towards enhancing the escape capacity of CPPs.

To tackle this challenge, Tat was conjugated to polyhistidines (Tat-10H) so that the histidine imidazole group (pK_a 6.0) could act as a proton sponge in acidic endosomes (pH 5–6.5) [73] (Fig. 5). Upon entrapment, protonation of the histidine residues would result in osmotic swelling, endosomal membrane disruption, organellar lysis and release of cargo. This modified CPP was used for transfection of plasmid DNA encoding the luciferase reporter gene. By assaying luciferase levels, it was determined that an impressive 7000-fold increase in transfection efficiency could be achieved over the original Tat peptide. This level of gene expression is comparable to that of PEI 25 kDa, the “gold standard” for non-viral vectors. Since the plasmid DNA cargo must gain nuclear access for luciferase biosynthesis, these results suggest that the Tat-10H was able to induce greater endosomolysis than the original Tat peptide and this difference was responsible for the observed increase in luciferase levels.

The proton sponge effect was also harnessed for the delivery of short interfering RNA (siRNA) [74]. Penetratin was modified by incorporating histidine residues, not just to form a proton sponge, but to also promote formulation of an α -helix upon protonation in endosomes [74] (Fig. 5). The resulting endosomolytic peptide (EB1) was then co-incubated with siRNA to allow non-covalent CPP-siRNA complexes to form. The siRNA remained functional and this report is the first rational CPP modification where protonation results in formation of a secondary structure for endosomolysis.

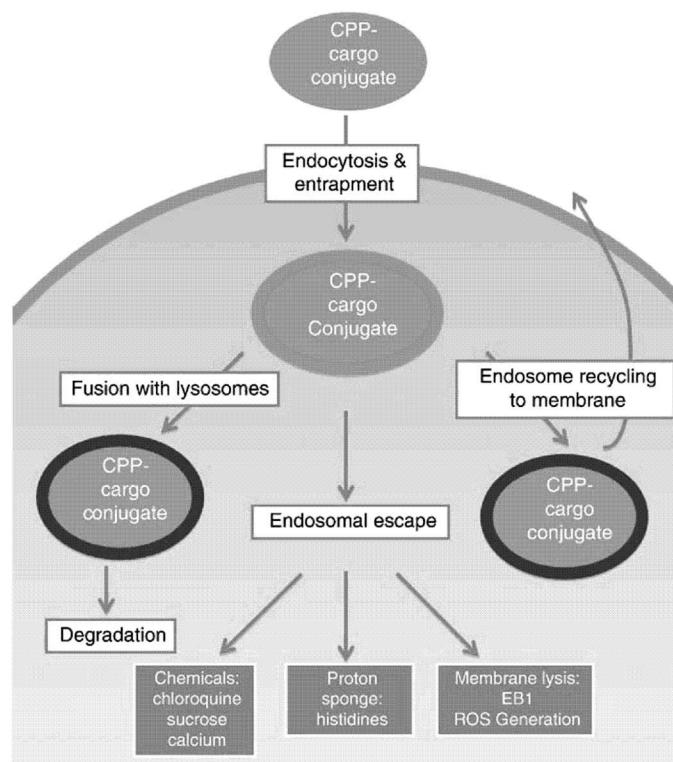


Fig. 5. Improving endosomal escape to achieve increased biological activity. Chemical agents, such as chloroquine and sucrose, can increase endosomal escape of CPPs. Inclusion of histidine residues in the CPP sequence allows for the formation of a proton sponge and consequently endosomal disruption. CPPs can also be designed to promote endosomal membrane lysis via formation of an alpha-helix or ROS generation.

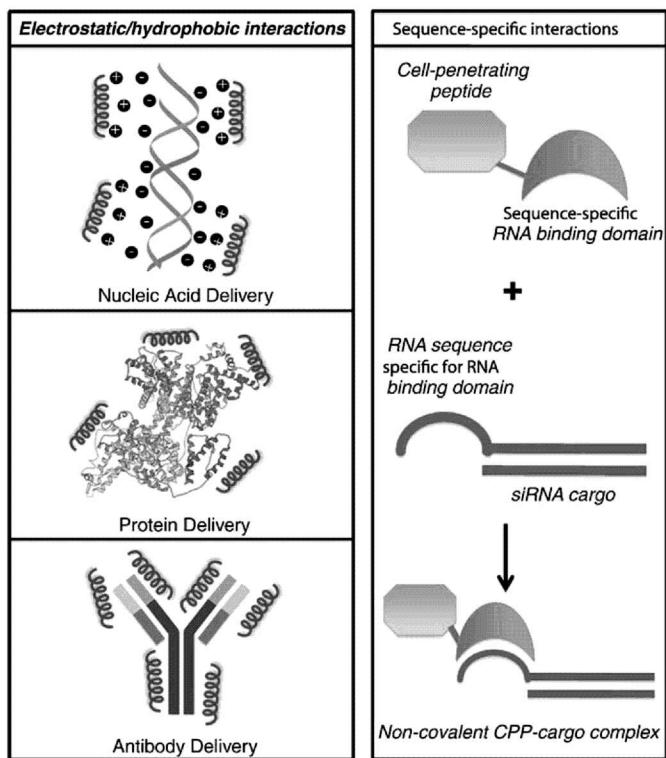


Fig. 6. Use of non-covalent complexation between CPPs and cargo. Electrostatic and hydrophobic forces can be harnessed to complex CPPs to a range of cargos such as nucleic acids, proteins and antibodies. Sequence-specific interactions between an RNA binding domain and its target sequence can also be harnessed for non-covalent complexation.

Another method for endosomal escape has been developed relying on incorporation of 6-aminohexanoic acid (Ahx) into the CPP. It is thought that this amino acid imparts flexibility to the peptide and also decreases interaction of the CPP with negatively charged heparan sulfates on the cell surface [75]. This weaker interaction allows the CPP and attached cargo to escape more efficiently from endosomes than CPPs lacking Ahx. Recently, a polyarginine CPP was modified to test this approach by interspersing the arginine amino acids with Ahx residues [109] (Fig. 5). The CPP was conjugated to PMO and tested for splice correction functionality. Increased activity and endosomal escape was observed compared to CPPs lacking the Ahx residue, such as Tat and R₉F₂.

Photochemical internalization, the use of photochemical treatment to induce endosomal escape, has been used in a number of applications [110–112]. In one such study, PNAs were conjugated to CPPs and internalized but activity was limited by endosomal entrapment [113]. To promote escape, PCI was harnessed. Cells were co-incubated with CPP-PNA and the photosensitizer AlPc_{2a} and irradiated with red light. AlPc_{2a}, a membrane soluble compound, produces reactive oxygen species (ROS) upon photostimulation (Fig. 5). ROS causes damage to endosomal membranes and consequently, cargo is allowed to escape [114]. A dramatic increase in bioactivity by two orders of magnitude was observed upon light stimulation. While being limited to tissues where radiation can be administered, photochemically-triggered endosomal escape could be a very therapeutically relevant technique since it is not as toxic to cells as other endosomal escape agents. Also, since the light stimulation can be directed at a region of interest, this method allows for site selectivity.

6.2. Non-covalent CPPs

Most CPPs are attached to their cargo via a covalent bond. However, recently, several CPPs have been identified which are

capable of non-covalent cargo interactions and, furthermore, these sequences have successfully delivered some of the most challenging cargo with high efficiency.

The assembly of cargo/CPP complexes via non-covalent interactions is advantageous because it simplifies conjugation protocols. In addition, different cargo can be delivered simply by mixing the two compounds, eliminating the need to optimize individual synthesis schemes. Lastly, non-covalent interactions between the carrier and cargo decrease the likelihood that the CPP will interfere with the bioactivity of the payload. For these reasons, non-covalent CPPs are advantageous over traditional sequences.

One such CPP is the recently identified Rath peptide, derived from the avian infectious bursal disease virus [115]. This sequence adopts a dominant β-structure, unusual for CPPs, which are typically either unstructured or α-helical (Fig. 6). In addition, this single sequence is capable of non-covalent interaction and delivery of both proteins and nucleic acids. The delivery is rapid (30 min for an oligonucleotide and 1 h for an antibody) and temperature independent, suggesting a non-endocytic mechanism of uptake. This is favourable as it avoids endosomal entrapment and improves bioavailability. Lastly, the peptide is capable of delivering large cargo to primary cells, a more challenging target than immortalized cell lines. These impressive characteristics make this novel CPP highly applicable for delivery of a wide variety of cargo.

Another interesting and novel CPP, CADY, was recently described [116]. This 20-amino acid amphipathic peptide combines both cationic arginine and aromatic tryptophan residues into its design. In the cell membrane, this sequence folds into a helical conformation with the cationic residues on one face and the aromatic groups on the other. CADY forms stable complexes with siRNA via electrostatic interactions and can deliver this anionic cargo into a variety of cell lines (Fig. 6). This CPP can also translocate siRNA into suspension and primary cell lines, notoriously difficult targets. CADY appears to harness a non-endocytic mechanism of uptake for membrane translocation, a feature that reduces endosomal entrapment and increases bioavailability of cargo. This is the first description of an amphipathic peptide capable of non-covalent complex formation and delivery of siRNA. This highly efficient CPP will serve as a very useful technology for gene silencing of a wide variety of targets.

In addition to forming electrostatic CPP-cargo complexes, non-covalent assemblies can also be formed through sequence-specific interactions. In an elegant system recently developed, Tat was fused to an RNA binding domain that specifically recognizes a short RNA sequence [117] (Fig. 6). The siRNA cargo was then designed to include this RNA sequence. This allowed the cargo to form complexes with the CPP via non-covalent, but highly specific interactions. This association was sufficient for the CPP to direct internalization of the siRNA cargo and endocytic uptake was observed. In addition, since a large portion of the cargo was sequestered in endosomes and biologically unavailable, a 10 s photostimulation of the fluorophore was used to induce cargo release and obtain siRNA mediated gene silencing. Since the region exposed to light stimulation can be controlled, a region-specific silencing was achieved simply by shining light on only a subset of cells despite siRNA delivery into all cells. Furthermore, the toxicity observed when this method was used was considerably lower than that observed with traditional transfection reagents.

6.3. Multivalent CPPs

CPP activity can also be improved through multivalency, an approach where conjugates contain more than one CPP per molecule. The tetramerization domain of p53 was harnessed to promote self-assembly of multivalent molecules with four CPPs per delivery vector [118]. The impact of multivalency on cellular uptake and nuclear localization was assessed using deacarginine, decalysine and Tat. The tetrameric CPPs displayed a 10- to 100-fold enhancement in cellular

uptake relative to their monomeric counterparts and these assemblies were able to deliver plasmid DNA more efficiently into cells, yielding up to 1000-fold increases in activity, compared to the monomers.

The concept of multivalent CPPs has been further improved via the oligomerization of synthetic amino acids capable of zinc (II) coordination [119]. Monomers, dimers, tetramers and octamers of Tyr-ZnDPA, a tyrosine derivative with an appended 2,2-dipicolylamine unit for zinc complexation were designed. These CPPs entered cells via endocytosis at higher levels than octaarginine. This suggests that these CPPs are beneficial over other CPP sequences, not only because of superior uptake, but also because the delivery system could be selectively activated in zinc-rich tissues.

6.4. Organelle-specific CPPs

Targeting CPPs and their cargo to specific sub-cellular organelles has received little attention upto this point, with most of the work focused primarily on passage across the plasma membrane, rather than organellar membranes. Most CPPs localize to the nucleus or cytoplasm, and the ability to target these peptides to other cellular compartments could have important biological and medical applications. This would be especially beneficial if the cargo must be in a particular location to function. In order to address this problem, our laboratory has developed CPPs that are also mitochondria-penetrating peptides (MPPs) [120]. By introducing lipophilic residues and establishing a balance between cationic character and hydrophobicity, these peptides were able to efficiently gain mitochondrial access. In addition to designing these highly efficient MPPs, specific thresholds of charge and lipophilicity required for mitochondrial translocation were identified. These chemical thresholds will be very useful in the design of future applications requiring mitochondrial access. MPPs rival CPPs in their uptake across the plasma membrane, and these novel peptides are now being harnessed for delivery of various bioactive cargos.

One area where the ability to access one sub-cellular compartment over another has been shown to be beneficial is in the study of cellular responses to ROS. Since MPPs are capable of mitochondrial access and CPPs are capable of nuclear access, a singlet oxygen-sensitizer was attached to each of these types of peptides and the cellular responses to ROS generation in each of these organelles was monitored [121]. A higher level of cell death was noted when ROS generation occurred in the nucleus compared to the mitochondria. In addition, different cell survival pathways were activated depending on the site of oxidative stress. This type of analysis demonstrates the utility of CPPs capable of specific sub-cellular localization within the cell.

Another recently discovered CPP that could be harnessed for the selective delivery of cargo to the mitochondria is the human antimicrobial peptide Histatin 5. This antimicrobial peptide is found in human saliva and has been shown to target fungal and protozoan mitochondria. Its anti-protozoan activity is thought to be due to its ability to accumulate within the mitochondria and inhibit F₁F₀-ATPase [122]. This causes a decrease in mitochondrial membrane potential, a reduction in respiration and eventually bioenergetic collapse of the parasite. Since Histatin 5 is able to translocate cell membranes it can be conjugated to another leishmanicidal agent and delivery into the parasitic mitochondria would produce a single molecule with dual antimicrobial activity.

7. Conclusion

This review provides ample evidence that CPPs have been very useful in a wide variety of biological applications. They have successfully delivered drugs to combat resistance, translocated immunogenic compounds to improve immune system responses and delivered imaging agents for the real-time monitoring of viral replication. In addition to their delivery capacity, CPPs themselves can have biological activity, such as anti-prion effects or cytotoxicity. This

field is rapidly expanding and many groups are working towards developing new CPPs or improving existing peptides. This work will no doubt result in the development of novel technologies and applications with CPPs at the crux of their function.

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