Review

Cell-Penetrating Peptide-Mediated Protein Delivery: From Mechanistic Principles to Therapeutic and Research Applications

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Abstract: Delivering functional proteins across biological membranes remains a major challenge in both drug therapy and experimental research. Cell-penetrating peptides (CPPs) offer a promising solution due to their intrinsic ability to translocate across cell membranes without causing membrane disruption. In this review, we focus on the delivery of protein-based biologics and first summarize the key mechanisms underlying CPP-mediated protein delivery, including the molecular modes of CPP-cargo (CPP and cargo protein association) protein binding and the associated transmembrane translocation pathways. Next, we highlight recent advances in the applications of CPP-mediated protein delivery. In the context of pharmaceutical development, CPPs have been employed to deliver nanobodies and antigen-based vaccines, with ongoing studies exploring their therapeutic potential against various diseases. In basic research, CPPs enable the intracellular delivery of target proteins for nuclear magnetic resonance (NMR) spectroscopy and fluorescence imaging, thereby facilitating the structural characterization of proteins or real-time tracking of their dynamic behaviors in cellular environment.

Keywords: cell-penetrating peptides; protein delivery; nanobody delivery; vaccine delivery; in-cell NMR; live-cell imaging

1. Introduction

Protein delivery is of critical importance in both pharmaceutical development and experimental research. In the pharmaceutical domain, protein-based macromolecular drugs have witnessed rapid advancement in recent years owing to their ability to directly interact with target proteins, thereby offering high specificity, potent bioactivity, and low toxicity. These attributes effectively address the limitations of conventional small-molecule drugs, such as short half-life, severe adverse effects, and slow onset of action [1,2]. In experimental research, investigating protein conformation within the intracellular environment yields more physiologically relevant results, as the crowded interior of cells characterized by a high total concentration of macromolecules can significantly influence protein conformational stability and folding pathways [3,4]. Nevertheless, membrane lipids constitute a context-dependent membrane-permeability barrier, limit most proteins from independently crossing the membrane and gaining access to the intracellular space into the cell. Consequently, effective strategies for intracellular protein delivery are urgently required to overcome this limitation.

Current intracellular protein delivery strategies can be broadly categorized into physical, chemical, and biological approaches. Physical methods rely on the formation of transient pores on the cell membrane to facilitate protein entry [5–7]. Chemical methods involve modifying the target membrane to confer transmembrane capability, including small molecules, polymer modifications, and encapsulation by nanocarriers such as liposomes [8–10]. Biological methods exploit the invasive properties of engineered bacteria or virus proteins as delivery vectors to introduce proteins into cells [11,12]. Among chemical methods, lipid nanoparticles (LNPs) improve drug stability and extend circulation time but suffer from low endosomal escape, currently mainly serving nucleic acid delivery [13]. Certain CPPs possess direct transmembrane or endosomal escape-promoting ability, enhance cytosolic delivery, and offer flexible design and low immunogenicity, which are suitable for small size protein delivery. Moreover, they can modify LNPs to achieve synergistic targeted delivery [14,15]. Figure 1 summarizes the current application scenarios of CPPs.

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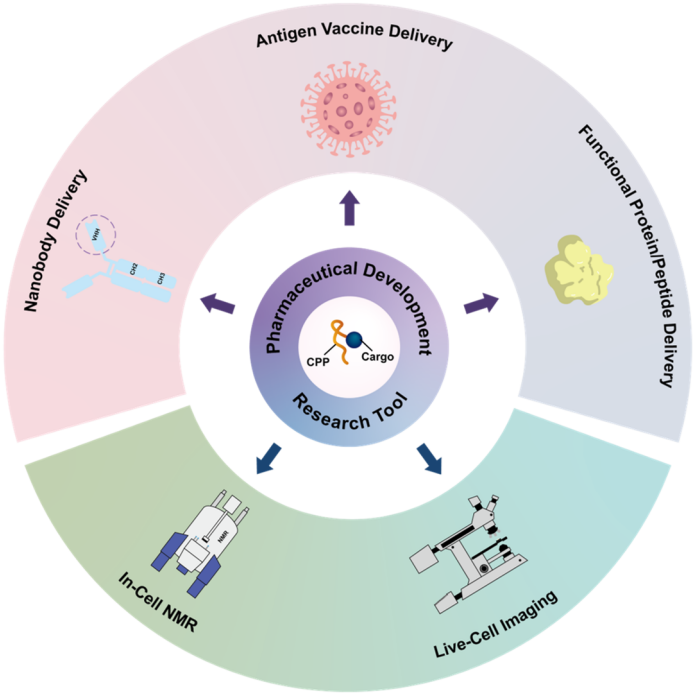

Figure 1. CPPs currently facilitate pharmaceutical delivery (nanobodies, antigen vaccines, and other functional proteins/peptides) and functional protein characterization (in-cell NMR, live-cell imaging).

CPPs are short peptides, typically 5–30 amino acids in length, that can facilitate transport across cellular membranes. They can bind cargoes such as peptides, proteins, and nucleic acids that cannot cross the cell membrane independently, and deliver them into cells. Frankel and Pabo first observed that cells can take up the purified protein when measuring the activity of the HIV-1 TAT protein in 1988 [16]. This observation initiates research in this field. In 1991, the group of Prochiantz found that Penetratin, a homologous domain protein of the Drosophila antennapedia protein, can also effectively cross membranes and enter cells [17]. In 1998, the group of Langel designed the first chimeric peptide carrier, Transportan, for delivering peptide nucleic acids (PNAs) and proposed the concept of CPP [18]. In its early days, CPPs also bore another name: protein transduction domain (PTD), which emphasized their functional aspect. The group of Dowdy combined beta-galactosidase protein with the TAT PTD and successfully crossed the blood-brain barrier (BBB) [19]. Since then, researchers have continuously discovered or synthesized new CPP sequences and applied them to the intracellular delivery of various cargoes.

Previous reviews have broadly covered CPP applications in delivering nucleic acids, proteins, and other cargos, with protein delivery primarily targeting specific diseases such as cancer. Our review classifies deliveries by cargo type and summarizes the mechanisms and application scenarios of CPP-mediated delivery of nanobodies, antigen vaccines, and metabolic peptides, aiming to extend CPP delivery to other disease therapies and metabolic regulation. Furthermore, CPP use in studying protein structure and function within live cells has received comparatively limited focused attention. We therefore review CPP-mediated functional protein delivery for in-cell NMR and live-cell imaging, offering guidance for investigating metabolic mechanisms involving proteins.

2. Mechanism of CPP-Mediated Protein Delivery

To deliver proteins into cells, the binding mode between CPPs and proteins, along with the CPP translocation mechanism, can affect delivery efficiency and protein functions [20]. The binding mode of CPP to protein falls into two types: covalent conjugation and non-covalent complexation [21]. Covalent conjugation links the CPP and cargo protein through a covalent bond, common methods include expressing them as a fusion protein [22–25], forming a disulfide bond through oxidation of two cysteine thiol groups [26], or linking via click chemistry copper-catalyzed azide-alkyne cycloaddition (CuAAC) [27,28]. Among these, fusion protein expression provides the most stable linkage and avoids chemical crosslinkers, thus offering excellent biocompatibility. However, its irreversible connection means the fused CPP might interfere with the delivered protein's function by affecting its structure. The disulfide bond linkage is reversible; the highly reducing intracellular environment, such as glutathione (GSH), can reduce the disulfide bond, enabling controlled and site-specific release of the cargo protein. The click chemistry method is selective and allows precise conjugation of CPP to specific sites on the protein, thereby

maximizing preservation of protein activity. Covalent coupling ensures high conjugation efficiency between CPP and cargo protein, guaranteeing that sufficient cargo molecules reach the cytoplasm, making it suitable for applications requiring high delivery efficiency, such as drug delivery. Nevertheless, these methods involve complex modification processes, high technical demands, and preparation costs. For non-covalent complexation, CPP and the cargo protein form a complex through natural forces such as electrostatic attraction and hydrophobic interactions [20]. Experimentally, co-incubation usually produces this complex [29,30]. Unlike covalent conjugation approaches, non-covalent complexation involves fewer chemical modifications to the protein structure and exerts less influence on protein activity, allowing researchers to study the structure and function of the cargo protein itself. Furthermore, this method features simpler and faster operations, making it an ideal choice for high-throughput screening of numerous CPP sequences or protein cargoes. However, because non-covalent binding is less stable, the complex may dissociate before reaching the cytoplasm, resulting in low delivery efficiency.

The transmembrane mechanisms of CPP remain to be elucidated, existing models include energy-dependent endocytosis and energy-independent direct membrane penetration two pathways [31]. At low concentrations, most researchers agree that cells internalize CPPs and their cargo generally through vesicular encapsulation [32], which encompasses macropinocytosis [33,34], clathrin-mediated endocytosis (CME) [35,36], caveolin-mediated endocytosis [35,37,38], and clathrin/caveolin-independent endocytosis [39,40]. The endocytic pathway accommodates a broad range of cargoes and operates under mild conditions, but it requires the cargo to overcome the endosomal escape bottleneck [41]. Increasing concentrations enhance CPP-plasma membrane interactions, eventually leading to transient membrane perturbation or pore formation. This process allows the CPP-cargo complex to diffuse directly into the cytoplasm [42,43]. The membrane potential-driven model represents one mainstream direct translocation mechanism: CPP first electrostatically adsorb to the membrane surface, then locally amplify the membrane potential to induce pore formation, and finally traverse the membrane pore in a chain-like conformation [44]. Other direct translocation hypotheses include the inverted micelle model [45], vesicle budding and collapse (VBC) [46], and the carpet model [47]. These pathways achieve rapid intracellular cargo delivery, bypass the limitations of endocytic capacity and lysosomal degradation, and thus offer higher cytoplasmic delivery efficiency. However, these methods typically restrict the size of the delivered cargo and may impose potential cytotoxic effects. Notably, CPP-cargo uptake mechanism and efficiency also depend strongly on CPP and cargo characteristics, cell type, and environmental conditions [48]. For example, in the case of HeLa cells, long chain CPPs usually show higher uptake than shorter peptides, and positive charges on CPP-cargo complexes facilitate uptake [49]. Amphipathic α-helical structure also enhances penetration efficiency [49]. Moreover, delivery efficiency declines with increasing cargo size, as R9 alone cannot mediate a 31-residue polyglycine chain transmembrane, and efficient transport of large protein cargoes may require multiple CPP molecules acting cooperatively [50].

When CPP-cargo enters cells via endocytosis, it must undergo endosomal escape to reach the cytoplasm; otherwise, the endocytic pathway sequesters it in vesicles and delivers it to lysosomes for degradation, precludes cargo protein access to action sites, lowering actual uptake below apparent. Currently, the overall endosomal escape efficiency of CPPs remains low, representing a major bottleneck for achieving efficient intracellular delivery [51]. To address this issue, researchers have attempted to modify CPPs based on previously proposed endosomal escape models to improve delivery efficiency. The proton sponge effect represents one strategy for CPP endosomal escape. After cellular uptake of CPP-cargo into endosomes, the acidic endosomal environment protonates weakly basic groups such as amines on the CPP. To maintain endosomal pH, vacuolar ATPases continuously pump in more protons, simultaneously triggering chloride ion influx to preserve electroneutrality. This increase in endosomal osmotic pressure drives substantial water influx, ultimately causing endosome rupture and releasing CPP-cargo into the cytoplasm [52]. Study has shown that introducing histidine residues enhances endosomal buffering capacity and promotes endosomal escape [53]. VBC constitutes another common CPP endosomal escape strategy. Under acidic conditions, CPPs bind to the endosomal membrane, forming CPP-enriched lipid domains that induce local membrane curvature changes and generate tension. This tension drives spontaneous budding of small vesicles from the endosomal membrane, which then rapidly disassemble to release CPP-cargo into the cytoplasm [54]. Research indicates that CPPs with constrained structures such as α-helices or cyclization, or those containing amphipathic arginine residues and hydrophobic groups, achieve higher escape efficiency. Additional endosomal escape mechanisms include virus-mimicking membrane fusion, pore formation, and local membrane perturbation [55]. Based on these mechanisms, combining CPP with endosome escape peptide (EEP) or modifying CPP with EEP can further enhance endosomal escape efficiency and improve cytosolic delivery [56,57].

Figure 2 summarized the binding modes of CPP to cargo protein, together with the possible models of transmembrane pathway mentioned above.

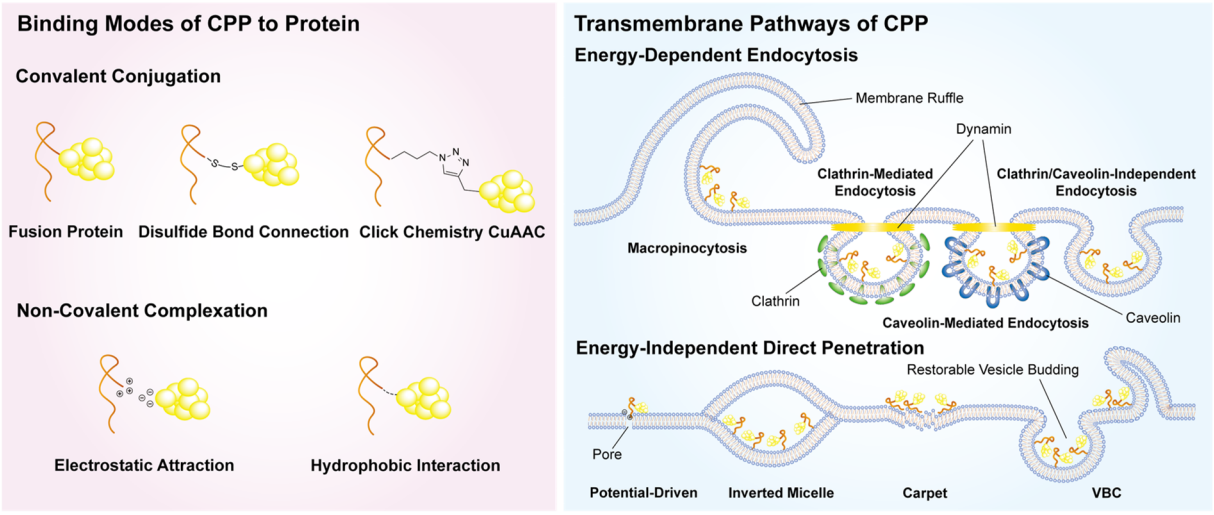

Figure 2. CPP binds with cargo protein via covalent (fusion expression, disulfide bonding, click chemistry) or non-covalent (electrostatic, hydrophobic) strategies, and penetrates membrane through energy-dependent endocytosis (macropinocytosis, clathrin-mediated, caveolin-mediated, clathrin/caveolin-independent) or energy-independent direct penetration (potential-driven, inverted micelle, carpet, VBC).

3. Therapeutic Applications of CPP

Preclinical studies and limited early clinical-development experience suggest that CPPs effectively deliver various types of pharmaceuticals, including nanobodies and vaccines, thereby enhancing their therapeutic efficacy. Consequently, CPP-mediated protein delivery therapy exhibits promising potential for clinical application, covering multiple fields such as oncology, neurological diseases, tissue repair, and immunotherapy.

3.1. Nanobody Delivery

Nanobodies comprise a class of single-domain antibody fragments derived from heavy chain antibodies of camelid animals. With a molecular weight of only approximately 15 kDa, they exhibit strong specificity, tight binding affinity, low immunogenicity, and high stability [58–61]. These properties endow nanobodies with promising clinical therapeutic potential across various fields, including autoimmune diseases [62], oncology [63,64], infectious diseases [65], and neurodegenerative disorders [66,67]. However, the application of nanobodies still faces several challenges, particularly their limited transmembrane capability, which restricts their targets to extracellular domains. To enhance the intracellular delivery efficiency of nanobodies, researchers have employed CPPs to bind with nanobodies, thereby forming transbodies. Figure 3 illustrates the difference between nanobody and conventional antibody, and summarizes the CPP-mediated nanobody delivery strategies and applications.

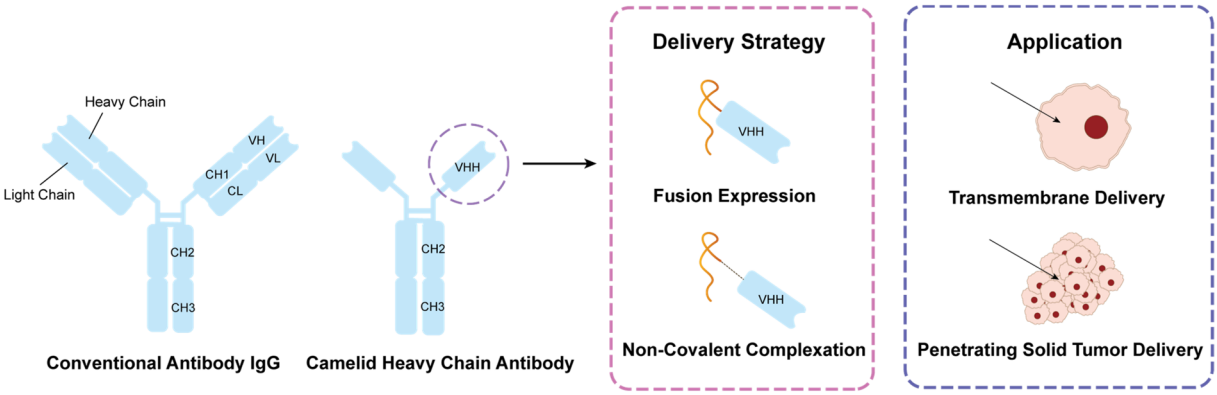

Figure 3. Nanobodies are smaller than conventional antibodies and can be coupled to CPPs via fusion expression or non-covalent complexation, enabling both cytosolic delivery and solid-tumor penetration in certain models.

In existing studies, the conjugation of CPPs with nanobodies mainly relies on *in vitro* expression of fusion proteins via covalent bonding. In 2011, Thanongsaksrikul et al. [68] proposed an innovative concept of constructing a "transbody" by fusing a CPP with an anti-botulinum neurotoxin nanobody according to their previous work. They expect that these transbodies can penetrate nerve cells and provide a novel targeted agent for

treating botulism and addressing the limitation of conventional antitoxins that cannot act on intracellular toxins. Subsequently, Glab-ampai et al. [69] fused a selected NS4B-specific nanobody with CPP Penetratin and validated its anti-hepatitis C virus activity in Huh7 cells through *in vitro* experiments. Their results showed that certain transbodies efficiently entered cells, significantly suppressed viral replication, and restored the innate immune response of host cells. In the anti-tumor field, Tabtimmai et al. [70] fused CPP R9 with an EGFR-targeting nanobody for non-small cell lung cancer therapy. *In Vitro* experiments demonstrated that the resulting transbodies can effectively enter A549 cells, significantly reduced tumor cell migration, and hold promise for mitigating the off-target toxicity and drug resistance defects of TKIs. They also performed biocompatibility assessments, confirming low non-specific cytotoxicity of the transbody toward cell lines such as HepG2 and MCF-7. GPX4 represents another promising anti-cancer target, particularly for overcoming tumor drug resistance. Li et al. [71] developed a transbody that fuses a GPX4-targeting nanobody and CPP R10, and further incorporated an auxiliary peptide, TNB-R10, to enhance delivery efficiency, thereby inducing ferroptosis for the treatment of drug-resistant cancer. Using a zebrafish model, they indicated that the R10-fused nanobody 12E possesses ferroptosis-inducing ability and exerts minimal impact on zebrafish development. In summary, fusing CPPs with nanobodies enables effective intracellular delivery of nanobodies. Some selected transbodies exhibit acceptable therapeutic potential and preliminary safety profile *in vitro* and *in vivo*, and show potential for application in related pharmaceutical development for treating various diseases such as toxin poisoning, viral infections, and cancer.

More recently, a study has also demonstrated that non-covalently complexed CPP enable transmembrane delivery of nanobodies. Chen et al. [72] developed an eye drop for treating pathological retinal and choroidal neovascularization. They prepared the anti-VEGF nanobody LQ015 and CPP Penetratin separately and mixed them at a specific molar ratio before use. The two components form a complex through electrostatic interactions, and Penetratin then helps LQ015 penetrate ocular surface barriers such as the cornea, conjunctiva, and sclera, ultimately reaching the retina-choroid complex to achieve non-invasive drug delivery. This method allows flexible adjustment of the molar ratio of the two components to optimize the balance between drug permeability and safety.

Transbodies serve not only in treating human diseases but also in treating other mammalian diseases. Antiviral research targeting conserved non-structural proteins of viruses via the TAT-nanobody fusion strategy has achieved progress in important animal viruses, including porcine reproductive and respiratory syndrome virus (PRRSV) and bovine viral diarrhea virus (BVDV). Wang et al. [73] focused on PRRSV treatment. They constructed a fusion protein combining CPP TAT and nanobody Nb6 that targets RNA polymerase Nsp9. *In Vitro* experiments indicate that this fusion protein efficiently penetrates the membrane in MARC-145 cells and porcine alveolar macrophages (PAMs) and inhibits the replication of multiple PRRSV strains. For BVDV treatment, researchers have studied several non-structural proteins essential for virus replication, including NS3, NS5A, NS5B, and NS4B. Ma et al. [74] fused the NS5B-targeting nanobody Nb1 with TAT. Dong et al. [75] fused TAT with Nb7, which targets BVDV NS5A, a viral RNA replication regulatory protein. These two transbodies can both effectively enter MDBK cells and inhibit viral replication. Zhao et al. [76] reported a nanobody, Nb91, that targets a membrane-anchored protein of BVDV replication complex, NS4B. Using TAT for delivery, they further used Bend cells, the natural target cells of BVDV *in vivo*, and this nanobody inhibits the replication of both cytopathic and noncytopathic BVDV strains. Currently, research on transbody therapy for mammalian viral diseases remains primarily at the *in vitro* stage. Researchers target different components of the viral replication machinery such as enzymes, regulatory factors, and replication complexes to identify optimal therapeutic strategies. In the future, these transbodies should undergo *in vivo* testing for efficacy and toxicity to advance their clinical development.

CPPs also serve as key components in autophagy-targeted protein degradation technologies based on nanobodies. In this approach, a lysosome-targeting module binds to a nanobody, guiding the nanobody-captured target protein to the lysosome for degradation via chemically induced proximity (CIP). Sun et al. [77] bound two small molecule-nanobody conjugate inducers of proximity (SNACIPs) respectively with CPP cR10* through disulfide bond, and achieved rapid and reversible regulation of protein localization, signal transduction, ferroptosis, and other biological processes via reversible small molecule-induced dimerization, and successfully targeted the ligand-inaccessible TPX2 protein *in vitro* and *in vivo* to inhibit tumor growth. He et al. [78] fused nanobodies with the autophagy protein LC3B and also binding them to cR10* via a disulfide bond, they achieved autophagic degradation of intracellular soluble proteins, organelles, and aggregated proteins in *in vitro* experiments using HeLa and OVCAR3 cells. Yang et al. [79] further advanced this delivery platform from a general tool toward a therapeutic strategy. By fusing a KRAS nanobody with the tumor-targeting CPP BR2 and the lysosome-binding motif CTM, degrader TKD achieves selective lysosomal degradation of multiple KRAS mutants, exhibits potent anti-tumor activity *in vitro* and *in vivo*, and shows synergistic effects with immunotherapy such as PD-1 antibody and cetuximab. Compared with cR10*, BR2 exhibits tumor cell selectivity, thereby improving the targeting and degradability *in vivo*. These studies show an evolution in application scenarios: from research tools for dynamically

regulating intracellular processes, to modular degradation tools, and further to candidate pharmaceuticals against KRAS-mutant cancers, and demonstrate the broad applicability and therapeutic potential of CPP-mediated nanobody delivery platforms for targeting previously undruggable proteins.

CPPs not only facilitate intracellular delivery but also prove useful for penetrating solid tumors. Camps et al. [80] fused the anti-EGFR nanobody 7D12 with several CPPs, including R9, Penetratin, TAT, and hLF, and evaluated their performance in 2D tumor cell lines, 3D tumor spheroids, and *in vivo* biodistribution studies. Their results demonstrated that CPPs enhance membrane binding, endocytosis, and three-dimensional penetration; however, they also lead to non-specific distribution. Future research should therefore balance the effects of CPP and the targeting nanobody.

CPPs enable nanobody pharmaceuticals to adopt various administration forms, including oral and inhalation intake. Jing et al. [81] loaded anti-TNF-α nanobody VHHm3F and antimicrobial peptide LL37 into milk extracellular vesicles (EVs) using TAT as a mediator. In the C57BL/6 mouse model, the incorporation of TAT enables efficient encapsulation of nanobodies inside EVs and protects them from degradation in the gastrointestinal tract. Recently, Franz et al. [82] covalently conjugated CPP R10 to anti-CFTR nanobody NB1 via disulfide bonds for the treatment of cystic fibrosis. CPP additives assist the conjugates to directly penetrate cell membranes into the cytoplasm, eliminating the need for EVs or genetic manipulation. In CF bronchial epithelial cells and patient-derived primary airway cultures, this transbody efficiently restores the maturation, membrane localization, and chloride channel function of CFTR. Compared to traditional injection, CPPs enable flexible and diverse administration modalities for nanobody pharmaceuticals. These modalities hold promise for enabling patients to self-medicate at home, thereby improving treatment convenience.

In summary, both covalent conjugation and non-covalent mixing enable CPPs to achieve intracellular delivery of nanobody. Preclinical studies *in vitro* and *in vivo* have demonstrated that these transbodies exhibit therapeutic potential against various tumors and viral infections, while also providing preliminary experimental evidence for their biocompatibility. However, research on the transmembrane mechanisms of transbodies remains limited. Different CPP-nanobody conjugates may employ distinct transmembrane mechanisms. Elucidating these mechanisms will help further improve drug delivery efficiency and guide the determination of administration routes and dosages, which holds potential influence for the clinical translation of such pharmaceuticals.

3.2. Vaccine Delivery

Protein-based vaccines provide antigen fragments and activate immune system. Their cost-effectiveness and straightforward production render them highly promising for disease treatment applications [83,84]. Most protein-based vaccines rely on dendritic cell (DC) recognition and phagocytosis, which promotes DC maturation and antigen cross-presentation, thereby enhancing $CD8^+$ cytotoxic T lymphocyte (CTL)-mediated immune responses. This mechanism is depicted in Figure 4. However, current studies indicate that these vaccines generally exhibit weak immunogenicity, partly because DCs more readily phagocytose larger particles [85–89]. Employing CPPs to deliver peptide antigens into DCs represents a common strategy to improve the immune activation capability of protein-based vaccines, especially in the fields of cancer treatment and HIV therapy.

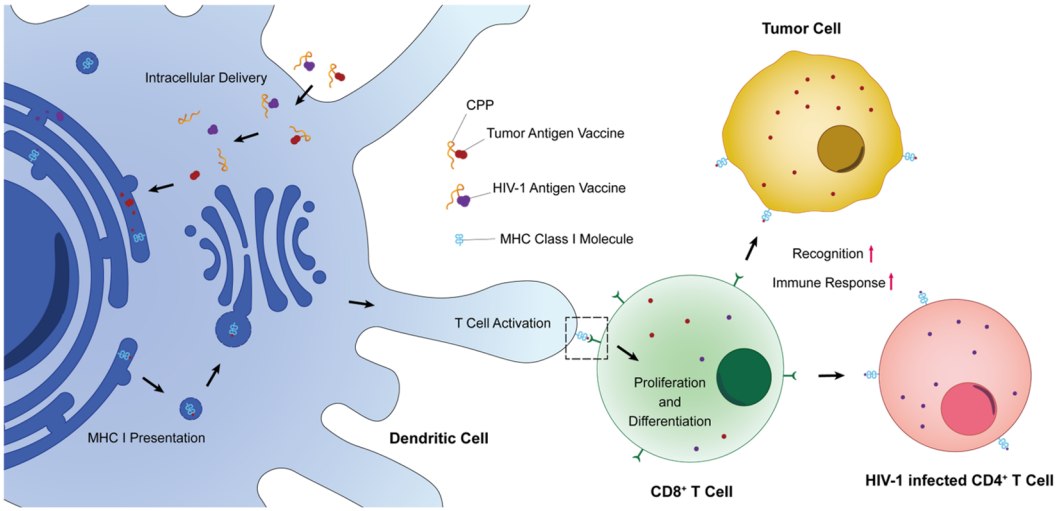

Figure 4. CPP delivers antigen into DCs for MHC I presentation to $CD8^+$ T cells, enhancing immune responses. This strategy currently targets cancer and HIV-1 therapy.

3.2.1. Cancer Therapy

Both covalent and non-covalent conjugation of CPPs with target antigens enable the construction of CPP-mediated cancer vaccines. Covalent conjugation methods include expressing fusion proteins, click chemistry, disulfide bonding, and SpyTag/SpyCatcher assembly. Shahbazi et al. [90] fused CPP hPP10 with the HPV16 E7 protein and achieved a delivery efficiency of 63.66% in HEK-293T cells with low cytotoxicity. Backlund et al. [91] compared eight CPPs: Penetratin, TAT, Arg8, MPG, Bpep, DPV6, pVEC, and TP10, and identified Penetratin and MPG as the most effective. Furthermore, different CPP-antigen conjugation methods (click chemistry and direct fusion protein expression) did not significantly affect the immune outcome. Mechanistically, CPPs not only facilitate transmembrane antigen transport but also improve serum stability and lymphatic accumulation, thereby extending the duration of antigen presentation. They further discovered that CPP-antigen conjugates do not act by directly crossing the membrane into the cytoplasm; instead, cells internalize them via endocytosis, after which Batf3-dependent conventional type 1 dendritic cells (cDC1) cross-present the antigen and activate the immune response. The above approaches lack specific targeting to DCs and rely solely on passive DC uptake. To address this, Wylie et al. [92] conjugated XCL1 to the CPP-B16 melanoma antigen through disulfide bond to enhance targeting to $XCR1^+$ cross-presenting DCs. They found that introducing furin/cathepsin B cleavage sites, which allows XCL1 to dissociate from the CPP-antigen within endosomes, is necessary for the antigen to undergo CPP-mediated endosomal escape and to induce immune responses. The vaccine developed through this strategy has now entered preliminary clinical trials. For non-covalent conjugation, Shahbazi et al. [90] formed complexes by co-incubating the HPV E7 protein with three CPPs respectively: Pep-1, Cady-2, and p28. All these complexes showed efficient delivery and low cytotoxicity in HEK-293T cells, with P28 giving the highest delivery efficiency. Applying the P28-based vaccine to tumor-bearing mouse models effectively induced a Th1-type immune response and led to tumor elimination, demonstrating potential for clinical translation. Thus, both covalent and non-covalent CPP-antigen conjugations can achieve effective vaccine delivery. The targeting of vaccine molecules to DCs and the mechanism of antigen release are key factors that influence delivery efficiency and will shape the future development of therapeutic cancer vaccines.

Based on CPP-mediated delivery, adding adjuvants can further enhance vaccine therapeutic efficacy. The toll-like receptor 9 (TLR9) agonist CpG represents one commonly used adjuvant. Wu et al. [93] conjugated a non-endosomal transmembrane CPP CLIP6 to the model antigen ovalbumin (OVA) to target B16-OVA tumors. Their experiments demonstrated that adding CpG further triggers DC maturation, enhances antigen cross-presentation, and activates $CD8^+$ T cells. Shi et al. [94] covalently linked CPP R8 to three distinct antigens. In aqueous solution, each conjugate self-assembles with CpG via electrostatic attraction to form nanoparticles. This method also significantly inhibits tumor growth and offers the advantage of requiring no external carrier. Heat shock proteins (HSPs) constitute another adjuvant type that has found application in HPV treatment. Bolhassani et al. [95] fused CPP hPP10 with the E7 protein as the antigen and compared the adjuvant effects of Hsp20 and Hsp27 from the small heat shock protein family (sHSPs). Their results showed that hPP10 enhances protein delivery efficiency and immunogenicity, while Hsp27 potentiates the Th1-type immune response; their combination achieves both prophylactic and therapeutic effects. Furthermore, Belnoue et al. [96] established a modular self-adjuvanting cancer vaccine platform KISIMA. This platform comprises a ZEBRA-derived CPP, a multiantigenic domain (Mad), and a self-adjuvanting TLR2/4 agonist (TLRag), where the CPP and TLRag act synergistically to initiate effective multiantigen T cell responses. *In Vivo* experiments in mice evaluated all the above vaccines for their prophylactic and therapeutic antitumor efficacy. The results showed that adding an adjuvant enhanced vaccine-mediated inhibition of tumor growth to a certain extent. Furthermore, for vaccines using TLR agonists as adjuvants, combining them with anti-PD-1 therapy further potentiated the therapeutic effect.

3.2.2. HIV-1 Therapy

The CPP-mediated antigen delivery strategy also offers a promising approach for developing HIV-1 vaccines. The Nef protein, a multifunctional accessory protein crucial for viral replication and pathogenesis, frequently serves as a target for anti-HIV vaccines [97]. On this basis, to address the high variability of HIV, researchers screened and combined other epitopes that are highly conserved across different HIV strains, thereby enabling the vaccine to potentially cover multiple HIV strains and induce a broad-spectrum immune response. Namazi et al. [98] designed a recombinant peptide containing immunogenic conserved epitopes from the HIV-1 Nef, TAT, Gp160, and P24 proteins, and mixed it with the CPP LDP-NLS or CyLoP-1 at various molar ratios to achieve non-covalent delivery into HEK-293T cells. Their experimental results demonstrated that CPPs, especially CyLoP-1, form complexes with the recombinant peptide, achieve effective intracellular delivery, and exhibit no significant cytotoxicity; the positive charge and small particle size of these complexes play critical roles in this process. Sabaghzadeh et al. [99] designed

and expressed a fusion protein, LDP12-Nef-MPER-V3, where LDP12 acts as a CPP, and Nef, MPER, and V3 represent three target antigens. *In Vitro* HEK-293T cell experiments confirmed that CPPs in the form of a fusion protein also deliver antigens into cells efficiently. The above studies remain at the basic research stage prior to vaccine development. They have only verified the CPP delivery feasibility and cytotoxicity through *in vitro* experiments. Future work will further test their immunogenicity using *in vivo* mouse models and evaluate the anti-HIV efficacy of the constructed vaccines.

Adding adjuvants to HIV-1 vaccines further enhances immunogenicity on the basis of CPP delivery. A member of the sHSP family, Hsp20, has proven to be an effective adjuvant. Rostami et al. [100] generated a recombinant Hsp20-Nef protein and incubated it with the CPP M918 to form nanocomplexes. *In Vitro* HEK-293T cell experiments showed that stable complexes form and enter cells; incorporating Hsp20 leads to a smaller particle size and a higher positive charge of the nanocomplexes, potentially benefiting both delivery and immune response elicitation. Subsequently, Rostami et al. [101] also used the Hsp-20Nef fusion protein to form nanoparticles with CPPs via non-covalent complexation. They extended the study to *in vivo* BALB/c mouse model, and compared the efficacy of M918 and Penetratin. Both CPPs achieved effective delivery of the antigen vaccine with low cytotoxicity, but M918 performed better in inducing Th1-type immune responses, holding higher application potential.

Overall, researchers have applied various CPPs in preclinical studies of therapeutic vaccine delivery. The results show that different CPPs may achieve optimal delivery efficiency for different vaccines. Moreover, for both cancer vaccines and HIV vaccines, adding adjuvants further enhances the immune response beyond CPP-mediated delivery. In addition, some studies have found that CPP-antigen complexes might enter cDC1 via endocytosis and undergo cross-presentation to induce immune responses, providing guidance for the design and optimization of CPPs. However, current vaccine delivery largely relies on passive cellular uptake, and the potential impact on normal tissues *in vivo* requires further investigation. Future vaccine designs could consider constructing targeted CPPs to improve delivery efficiency and biosafety.

3.3. Functional Protein/Peptide Delivery

Functional proteins and peptides play an important role in metabolic regulation, enabling precise and flexible adaptation to internal and external environmental changes while ensuring that energy and substrate production and utilization align accurately with the real-time demands of organisms [102]. These proteins and peptides therefore feature prominently in the treatment of metabolic diseases, but their efficient, targeted intracellular delivery remains a substantial challenge. CPPs offer a powerful strategy to circumvent physiological barriers such as cellular membranes, enhance drug absorption at the site of action, and prolong its duration, thereby addressing the persistent issues of low bioavailability and insufficient targeting specificity [103–105].

3.3.1. Diabetes Therapy

CPPs can serve as carriers for insulin delivery in diabetes therapy. Rehmani et al. [106] constructed a trifunctional peptide GET containing R8 and formed nanocomplexes with insulin via electrostatic interactions. This approach enhanced transepithelial transport efficiency by 22-fold *in vitro* compared with free insulin and successfully reduced blood glucose in diabetic mice through oral administration. Diedrichsen et al. [107] found that branched modifications of Penetratin and Penetramax significantly improved intestinal insulin delivery, with the dimeric Penetramax achieving 5.8-fold higher bioavailability than insulin alone, positioning it as a promising candidate for oral insulin delivery. Beyond the oral route, Yin et al. [108] non-covalently mixed CPP FPS with insulin and administered the mixture via nasal inhalation in mice, which promoted absorption across the nasal mucosa and potentiated the hypoglycemic effect. In addition to direct insulin delivery, some studies have employed CPPs to assist GLP-1 delivery for glycemic control. Sun et al. [109] covalently linked FPS to a GLP-1 analog through solid-phase synthesis. In mouse models, this conjugate markedly enhanced the glucose-lowering effect after intranasal administration, with rapid onset and sustained action, and achieved efficacy approximately comparable to that of intramuscular injection. Furthermore, Oh et al. [110] demonstrated *in vitro* that CPP NCAM1-PrP could enter pancreatic β-cells and inhibit islet amyloid polypeptide (IAPP) oligomerization, thereby delaying β-cell failure.

In summary, CPPs, whether through covalent or non-covalent conjugation, can improve the delivery efficiency of protein therapeutics for diabetes, with applications in both direct and indirect glycemic regulation as well as in protecting pancreatic islet cells. At the same time, CPPs enable non-invasive administration routes such as oral and nasal delivery, which potentially improve patient compliance over conventional injections, although in some experiments the glucose-lowering effect did not reach the level achieved by subcutaneous injection.

3.3.2. Body Weight Regulation

CPPs have also found application in delivering drugs for body weight regulation. Khafagy et al. [111] non-covalently mixed Penetratin with leptin and administered the formulation intranasally to healthy mice. Penetratin significantly enhanced the systemic absorption of leptin, and repeated administration over 28 consecutive days effectively suppressed appetite, reduced triglycerides, and slowed body weight gain. In the context of obesity therapy, Lee [112] fused the CPP aMTD with the SOCS3 SH2 domain and achieved enhanced leptin signaling in mouse models, resulting in a 14.9% reduction in body weight after 14 days of repeated dosing. Beyond leptin delivery, CPPs also serve as carriers for AMP-activated protein kinase (AMPK) modulators, thereby controlling body weight through regulating adipocyte differentiation. Shen et al. [113] used TAT-modified liposomes to achieve visceral adipose-targeted delivery of the PDBSN peptide, which activated the AMPK pathway in mice, inhibited precursor cell differentiation in visceral fat, and improved glucolipid metabolism. Ji et al. [114] linked Penetratin to the AMPK-CIDEA interfering peptide (ACIP) via a linker. In a mouse cachexia model, this construct inhibited aberrant AMPK degradation and significantly ameliorated body weight and fat loss, with intraperitoneal injection emerging as the optimal route of administration.

In summary, current CPP-mediated delivery systems for weight-regulating agents primarily operate through the leptin and AMPK pathways, and can exert effects in both preventive and therapeutic settings.

3.3.3. Organelle Metabolic Disorders Treatment

The onset of metabolic diseases such as diabetes and obesity often coincides with endoplasmic reticulum (ER) stress and mitochondrial dysfunction, and these organellar metabolic disturbances in turn exacerbate the disease, creating a vicious cycle [115]. In this context, developing pharmaceuticals that modulate the metabolism of organelles such as the ER and mitochondria represents another important facet of metabolic disease therapy. Modified CPPs can achieve specific organelle targeting, thereby delivering drugs precisely to their sites of action to exert therapeutic effects [116]. Hilan et al. [117] combined R9 with a GRP78-targeting peptide and assembled the construct with siRNA into nanoparticles. These nanoparticles can enter DU145 cells via endocytosis, localize to GRP78, silence it, induce robust ER stress, and activate pro-apoptotic signaling. Kang et al. [118] attached three different CPPs to the surface of drug-loaded vesicles via an MEL-maleimide linker. Their results showed that these carriers possess endosomal escape capability, and the Penetratin-modified carrier exhibited superior ER-targeting ability over TAT- and R7-modified counterparts. Baile et al. [119] expressed TAT as a fusion with the mitochondrial protein frataxin (FXN) containing a mitochondrial targeting sequence (MTS), this fusion protein can penetrate the cell membrane, enter mitochondria, and release mature, functional FXN, thereby alleviating FXN deficiency in Friedreich's ataxia (FRDA) patients. Raghav et al. [120] enhanced the cardioprotective effect of TAFAZZIN gene therapy by fusing Penetratin to the mitochondrial acyltransferase TAFAZZIN protein, although the underlying mechanism remains unclear. Zabit et al. [121] constructed a fusion protein comprising TAT, MTS, and mPiC (a key carrier on the inner mitochondrial membrane). *In Vitro* experiments demonstrated that this protein can localize to the inner mitochondrial membrane and largely restore Pi and Cu transport.

In summary, exploiting the intrinsic properties of CPPs or combining them with ER- or mitochondria-targeting peptides enables targeted protein delivery; exogenous proteins undergo release, recognition, and processing within the organellar microenvironment and subsequently exert their functions, and some of these agents have already entered clinical trials or show potential for clinical translation.

Overall, preclinical studies indicate that CPPs can achieve functional protein/peptide delivery for metabolic regulation, while the optimal CPP depends on the cargo type, formulation, delivery route, and intended metabolic pathway. However, the mechanisms of cellular uptake and intracellular trafficking, as well as off-target distribution and safety in normal tissues, remain insufficiently characterized *in vivo*. Future studies should therefore prioritize targeted CPP designs and systematic *in vivo* evaluation to improve delivery efficiency, tissue selectivity, and biosafety.

Table 1 categorizes and summarizes the cargo proteins delivered by selected commonly used CPPs in the field of disease therapy, as well as the corresponding coupling strategies, targets, and experiment systems, as mentioned in this section.

Table 1. Summary of some effective CPP-mediated protein delivery systems mentioned in Section 3.

CPP Name	CPP Sequence	Cargo Type	Coupling Strategy	Delivery Target	Experiment System	Reference
Penetratin	RQIKIWFQNRRMKWKK	Anti-HCV nanobody	Fusion expression	Intracellular	Huh7 cells	[69]
		TAFAZZIN protein		Cardiac tissue	Mouse model	[120]
		Anti-EGFR nanobody	Sortase A-mediated transpeptidation	2D tumor cell lines and 3D tumor spheroids	A431 and SKOV3 cells Mouse model	[80]
		Anti-VEGFA nanobody	Non-covalent complexation (physical mixing)	Ocular barrier penetration	Rabbit, mouse, and monkey models	[72]
		HIV-1 Nef protein		Intracellular	HEK-293T cells Mouse model	[101]
		Insulin		Transepithelial permeation	Caco-2 cells Mouse model	[107]
		Leptin		Nasal mucosal permeation and brain delivery	Mouse model	[111]
		ACIP	Propynylglycine linker	Abdominal adipose tissue	Mouse model	[114]
TAT	YGRKKRRQRRR	Anti-PRRSV nanobody	Fusion expression	Intracellular	MARC-145 cells, PAMs	[73]
		Anti-BVDV nanobody			MDBK and Bend cells	[74–76]
		Anti-TNF-α nanobody		Colon epithelial permeation	Mouse model	[81]
		FXN		Mitochondrial matrix	FRDA patient	[119]
		MTS and mPiC		Mitochondrial inner membrane	HeLa, HEK293 and HepG2 cells	[121]
		Anti-EGFR nanobody	Sortase A-mediated transpeptidation	2D tumor cell lines and 3D tumor spheroids	A431 and SKOV3 cells Mouse model	[80]
		Tumor-associated antigen	Click chemistry/Fusion expression	Lymph nodes and cDC1 cells	Mouse model	[91]
		Liposome-encapsulated PDBSN peptide	Non-covalent complexation (physical mixing)	Visceral fat	Mouse model	[113]
R8	RRRRRRRR	Tumor-associated antigen	Click chemistry/Fusion expression	Lymph nodes and cDC1 cells	Mouse model	[91]
			Solid-phase synthesis			[94]
		Insulin	Non-covalent com-plexation (physical mixing)	Transepithelial permeation	Mouse model	[106]
R9	RRRRRRRRR	Anti-EGFR nanobody	Fusion expression	Intracellular	A549 cells	[70]
			Sortase A-mediated transpeptidation	2D tumor cell lines and 3D tumor spheroids	A431 and SKOV3 cells Mouse model	[80]
R10	RRRRRRRRRR	Anti-GPX4 nanobody	Fusion expression	Intracellular	Zebrafish model	[71]
		CFTR-binding nanobody	Thiol-reaction		CFBE41o-cells, primary CF airway epithelia	[82]
cR10*	Cyclo-(RRRRRRRRRR)	SNACIP	Thiol-reaction	Intracellular	HeLa cells	[77]
		Autophagy-targeting nanobody chimera			HeLa and OVCAR3 cells	[78]
hPP10	KIPLPRFKLKCIFCKKRKRKRKR	HPV16 E7 protein	Fusion expression	Intracellular	HEK-293T cells Mouse model	[90,95]
p28	LSTAADMQGVVTDGMASGLDKDYLKPDD	HPV16 E7 protein	Non-covalent complexation (physical mixing)	Intracellular	HEK-293T cells Mouse model	[90]
M918	MVTVLFRRLRIRRACGPPRVRV	HIV-1 Nef protein	Non-covalent complexation (physical mixing)	Intracellular	HEK-293T cells Mouse model	[100,101]

3.4. Translational Challenges and Safety Considerations

In preclinical studies, CPPs have shown potential for drug delivery, but currently few CPP-mediated drug delivery system has received market approval. The main challenges include potential cytotoxicity, lack of tissue selectivity, risk of immunogenicity, and poor *in vivo* stability.

CPPs can induce cytotoxicity through various mechanisms, the most common of which involves interactions with cell membranes during penetration, leading to membrane disorganization and potentially disrupting the cell membrane and causing cell death. Ji et al. [122] showed that CPP TP10 disrupts the cell membrane of Jurkat cells and induces necrosis. Henriques et al. [123] found that CPP Pep-1 causes membrane disintegration through a detergent-like mechanism. However, these cytotoxic effects are concentration-dependent; at lower concentrations, CPPs can still cross membranes effectively without causing irreversible cell damage. Different CPPs exhibit different toxicity thresholds [124]. None of the preclinical pharmaceuticals discussed earlier in this section showed obvious cytotoxicity, and most CPP-mediated protein-based pharmaceuticals function effectively at safe concentrations. Furthermore, CPP cytotoxicity may also depend on peptide length, with longer peptide chains likely being more toxic. Ghaemi et al. [125] compared arginine-rich CPPs of different lengths and observed higher cytotoxicity for longer peptides. Fasoli et al. [126] fused the cell-penetrating antimicrobial peptide CM_{18} with CPP TAT_{11}; the originally low-toxicity CPP then caused detergent-like irreversible membrane disintegration. They attributed this change to a shift in the membrane interaction mechanism of the chimeric peptide from a toroidal pore model to a carpet model. Attaching a cargo protein via fusion to a CPP exerts a similar length-dependent effect. Cardozo et al. [124] found that TAT alone exhibits low toxicity, but after conjugating a cargo peptide, it becomes significantly toxic at high concentrations. Therefore, they recommend using the shortest possible peptide sequences and pursuing the highest affinity for the target to ensure safety. In addition to disrupting the plasma membrane, some studies indicate that certain CPPs can also damage membrane-containing organelles such as mitochondria [122,127]. Moreover, increasing CPP concentrations may activate intracellular stress signaling pathways, leading to apoptosis. In summary, regardless of the pathway through which a CPP induces cytotoxicity, limiting its working concentration or optimizing its sequence can effectively maintain cytotoxicity within a low range and improve the biosafety of the pharmaceutical.

CPPs efficiently cross various membrane systems due to their positive charge and amphipathic nature. This penetrating ability generally lacks selectivity, allowing them to enter both target and normal cells [128]. To reduce non-specific effects on normal tissues during the clinical translation of CPP-mediated protein drug delivery, researchers introduce responsive residues or make targeted modifications to the CPP sequence based on disease microenvironment features such as pH changes, altered enzyme activity, or endocytic mechanism [129–131]. These strategies keep CPPs in an inactive state in normal tissues and activate their delivery function only upon reaching the diseased site. Alternatively, researchers use external triggers such as specific wavelengths of light by coupling CPPs with photolabile protecting groups, thereby achieving controlled release of the CPP-cargo complex.

In clinical translation, the immunogenicity of CPPs also warrants consideration. Although CPPs are generally considered to have low immunogenicity, and none of the preclinical studies described above detected obvious immunogenicity, a Phase I study published in 2016 in patients with advanced malignancies reported that patients developed histamine class I infusion-related reactions (itching urticaria) when the intravenous dose of DTS-108 (a pharmaceutical that uses human-derived CPP DPV1047 to deliver small molecule drug SN-38) reached a certain level [132]. Current strategies to reduce drug immunogenicity include shielding antigenic epitopes via PEGylation or encapsulating the pharmaceutical within nanocarriers, thereby preventing immune cells from contacting and recognizing the drug molecules [133,134]. Alternatively, replacing L-amino acids with D-amino acids in the sequence or using non-natural amino acids reduces the drug's ability to activate immune responses after cross-presentation [135]. These approaches also protect drug molecules from protease recognition and degradation, thereby improving their *in vivo* stability and prolonging their duration of action.

Currently, most CPP-mediated protein-based pharmaceutical delivery systems remain at the preclinical research stage, but they have achieved significant advances in areas such as cancer therapy. Some of these pharmaceuticals have entered clinical trials or been approved by FDA and are summarized in Table 2.

Table 2. Pharmaceutical formulations containing CPP in clinical stages or FDA-approved (clinicaltrials.gov, accessed on 12 August 2026).

Pharmaceutical Type	Pharmaceutical Name	CPP Type	Cargo Type	Clinical Stage	ClinicalTrials.gov ID	Indication	Reference
CPP-mediated delivery	DaxibotulinumtoxinA (DAXI)	RTP004	BoNTA	FDA-approved	/	Aesthetic treatment of moderate-to-severe glabellar lines	[136]
	Nomalbofusp (CTI-1601)	TAT	MTS and FXN	Phase II completed in 2023	NCT05579691	FRDA	[119]
	Lucicebtide (ST101)	Penetratin	Active sequence targeting C/EBPβ	Phase I/II active	NCT04478279	Refractory solid tumors with varying histologies	[137]
	KISIMA-01	Z12	TLR-peptide agonist and modulable multi-antigenic domain	Phase Ib active	NCT04046445	IV colorectal cancer	[138]
Active CPPs/stapled peptides	Sulanemadlin	ALRN-6924 (Stapled peptide)	Intrinsic ALRN-8714 (Active metabolite) targeting MDM2 and MDMX	Phase Ib terminated in 2022	NCT04022876	Prevention of chemotherapy-induced side effects	[139]
				Phase I/II completed in 2020	NCT02264613	Advanced solid tumors or lymphomas	
	p28	p28	Intrinsic p53 targeting	Phase I completed in 2011	NCT00914914	Advanced solid tumors	[140]
				Phase I completed in 2015	NCT01975116	Recurrent or progressive central nervous system tumors	

4. Research Applications of CPP

Structural studies of functional proteins underpin understanding of life processes, health maintenance, and metabolic disease intervention, and guide pharmaceutical design. To examine proteins in a more native intracellular environment and obtain physiologically relevant structural and functional information, researchers often introduce these molecules into live cells for direct observation [141]. Combining CPP-mediated protein delivery with several technologies such as NMR and microscopic imaging, allows characterization within live cells. Figure 5 summarizes the binding strategies and characterization technologies employed for CPP-mediated delivery of functional proteins into live cells for research purposes.

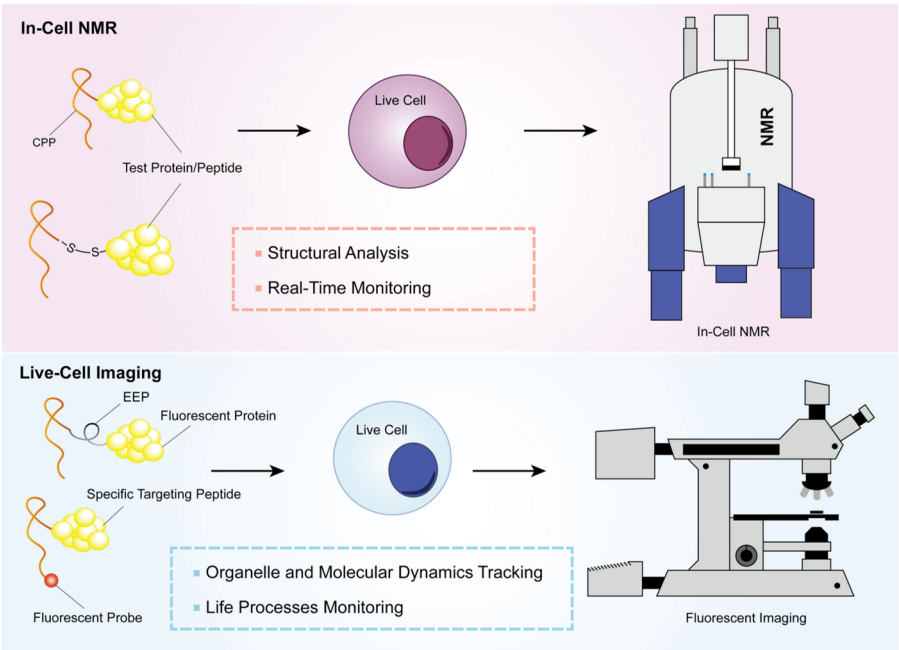

Figure 5. For in-cell NMR, CPP mainly conjugates with functional proteins through fusion expression or disulfide bond, enabling structural resolution and real-time monitoring of proteins in their near-native intracellular conditions. For live-cell imaging, CPP combines with EEP and fluorescent probe to deliver cargo into cells for real-time tracking of cellular life processes.

4.1. In-Cell NMR

To observe the structural dynamic changes of biological macromolecules and their interactions with other cellular components, Serber and Dötsch proposed in-cell NMR spectroscopy, a nondestructive technique for investigating macromolecular structures [142]. Commonly used experimental system construction approaches fall into two types: intracellular protein overexpression [143,144] and delivery of exogenously purified proteins via microinjection [145,146], electroporation [147,148], or CPPs [149].

Protein overexpression has reached maturity in prokaryotic cells such as *Escherichia coli*, but its application in eukaryotic cells remains limited. Overexpression elevates protein concentrations far beyond physiological levels, may causing proteins to aggregate and thereby hindering the observation of properly folded proteins by NMR spectroscopy [150]. In contrast, delivering exogenously purified proteins enables us to control protein concentration within an optimal range, which improves the resolution of NMR signals [147]. Furthermore, because this approach labels proteins *in vitro* after purification, it bypasses the constraints of cellular metabolic processes and thus offers greater universality across diverse cell types.

Among exogenous purified protein delivery approaches, microinjection and electroporation inflict partial physical damage on the cell membrane, which sometimes leads to cell death [151,152]. Seeking a more efficient and milder method, Inomata et al. [149] in 2009 first employed CPP TAT as a carrier to deliver target protein cargo into cells. They also achieved high-resolution multi-dimensional NMR spectroscopy in living human cells for the first time, thereby opening a new chapter in exogenous protein delivery for in-cell NMR.

In protein delivery for in-cell NMR, most reported CPPs adopt a covalent bonding strategy. Two experimental approaches enable this method: fusing the CPP sequence with the target protein sequence for expression [149,153,154], or chemically linking them after purifying the target protein [102,103]. Subsequently, researchers incubate the isotope-labeled CPP-cargo complex with cells in the presence of an auxiliary agent [149]. After translocation, cytoplasmic enzymes cleave the protein and release the cargo for NMR analysis [153]. Recent years have seen this approach applied to study various functional proteins, as follows.

Researchers utilize CPPs to deliver proteins associated with neurodegenerative diseases for NMR investigations. Zigoneanu et al. [153] covalently fused the TAT-derived cytoplasmic transduction peptide (CTP) YGR_2AR_6 to the N-terminus of α-synuclein, and studied its membrane-binding behavior in Parkinson's disease via ^{19}F NMR in CHO-K1 cells. They found that adding CTP alters the membrane-binding mode of α-synuclein, but whether its secondary structure is affected remains to be further investigated. Danielsson et al. [155] covalently linked the amyotrophic lateral sclerosis (ALS)-associated protein superoxide dismutase (SOD1) to CPP TAT or R8 via a disulfide bond, delivered the construct into HeLa cells, and employed ^{19}F HMQC spectroscopy to detect changes in protein structure. Their study demonstrated that the reducing environment inside cells reduces the disulfide bond, releasing free SOD1, and that CPP delivery does not affect the folding or function of the released SOD1.

CPP-mediated in-cell NMR also enables the study of tumor-related protein mechanisms. For example, Dowarha et al. [156] investigated how the S100A1 protein affects p53-MDM2 complex formation by fusing a 17-residue peptide derived from S100A1 with TAT to perform HSQC-NMR competitive binding experiments with MDM2 and p53. Using circular dichroism (CD) spectroscopy, they confirmed that adding TAT did not significantly alter the conformation of the S100A1-derived peptide.

Besides, CPP-mediated in-cell NMR also applies to real-time monitoring of cellular health status. Hembram et al. [154] employed human calbindin D9k (P47M + C80) as a model protein, and incorporated it into HeLa cells after binding through disulfide bond with TAT. By recording ^{1}H-^{15}N SOFAST-HMQC spectra at defined time intervals, they obtained the variations in intracellular Ca^{2+} concentration.

In summary, CPPs, especially TAT, can successfully deliver functional proteins into cytoplasm for applications including ^{19}F and ^{15}N in-cell NMR. Both fusion protein linkage and disulfide bond conjugation can release the target protein with the help of intracellular environment. Several experiments have explored how CPPs affect the structure and function of cargo proteins. The results generally show that the CPP-cargo complex may influence the structure and function of cargo, but shows no significant effect after its release. In some cases, protein conformation remains unchanged even when conjugated to the CPP. Therefore, under normal circumstances, researchers can use CPP-mediated in-cell NMR to observe the structure and function of target proteins under intracellular environment. However, different CPP-cargo conjugations, especially those expressed as fusion proteins, may produce different effects, which future work will require case-by-case analysis.

4.2. Live-Cell Imaging

Optical imaging has emerged as a key tool in experimental research [157]. Using microscopes, researchers can observe the internal and surface structures, dynamic processes, and molecular events of cells, enabling them

to understand the mechanisms of life activities at the molecular level. Unlike fixed-cell imaging, live-cell imaging offers the advantage of capturing bioprocesses in real time and allows observation of cellular events closer to their native state *in vivo* [158].

For fluorescent live-cell imaging, researchers can introduce fluorescent labels via intracellular expression of fluorescent proteins [159–162] or by applying exogenous small-molecule fluorescent probes [163,164]. Endogenous fluorescent protein fusion expression provides high targeting specificity and excellent biocompatibility, but not all target proteins allow stable fusion constructs. In contrast, exogenous fluorescent probes typically offer better fluorescence brightness and photostability, but lack of specific structure-targeted labeling ability and good cell permeability [165,166]. To combining these advantages of both, researchers conjugate CPP to fluorescent molecule [167]. CPP-cargo complexes mostly enter cells via endocytosis and subsequently localize to the endosome/lysosome system. Consequently, endosome escape constitutes a key rate-limiting step in CPP-mediated cytoplasmic delivery of cargos [168–170]. Therefore, researchers additionally add EEPs to further enhance the delivery efficiency of target fluorescent proteins. Liou et al. [170] found that the red fluorescent protein (RFP) fused with both CPP R9 and the hemagglutinin-2-derived fusion peptide (HA2, an EEP) exhibited higher cytoplasmic fluorescence intensity than RFP fused with R9 alone, confirming that HA2 can promote the transduction efficiency of CPP. Mohammed et al. [171] tested another EEP, the Pseudomonas aeruginosa exotoxin A translocation domain (ETA), and fused it with CPP R10 to deliver enhanced green fluorescent protein (EGFP) into the cytoplasm. The results showed that ETA increased the cytoplasmic delivery efficiency by about 40 times. Moreover, some fusion peptides can combine the functions of both CPP and EEP. For example, Niikura et al. [172] constructed a fusion peptide B55 derived from sea urchin fertilization protein binding protein. B55 can bind to the cell membrane, facilitates efficient endocytosis of EGFP without requiring CPP fusion, and directly mediate endosome escape.

In recent years, the field of molecular imaging has witnessed a growing combination between small-molecule probes and specific-targeting proteins. Researchers have integrated peptides with specific targeting capabilities and fluorophores possessing high fluorescence quantum yields, and then delivered these conjugates into cells using CPPs. Li et al. [173] coupled a heptapeptide sequence KKVAVVR derived from the proline-rich region of the tau protein with CPP $(rR)_3R_2$, FITC fluorescent group, and an amino caproic acid (Ahx) protecting group to construct a microtubule-targeted fluorescent probe. *In Vitro* experiments confirmed that this tau-derived heptapeptide successfully enters cells and specifically binds to microtubule proteins and aggregated microtubules. However, insufficient affinity resulted in unclear fluorescence labeling. In another study, Li et al. [174] created a cell-junction-targeting probe by conjugating a ZO-1 recognition unit (RASSRPRPDDLEI) with CPP $(rR)_3R_2$, FITC fluorophore, and Ahx protecting group. Experiments demonstrated that this probe can transmembrane into cells and bind to the target protein ZO-1, though the imaging clarity still requires improvement. Collectively, these two studies validate that small peptides derived from specific binding proteins, when conjugated with small-molecule fluorophores and delivered via CPP, can achieve labeling and imaging of subcellular structures. Nonetheless, both probes face a common bottleneck of insufficient binding affinity, which hinders high-quality imaging. Future strategies such as amino acid site-directed mutagenesis, CPP optimization, and fluorophore modification can enhance probe binding affinity and intracellular localization accuracy, thereby promoting their broader application.

Coupling nanobody and fluorescent molecule with CPP offers an alternative strategy for designing fluorescent labels in live cells. Schneider et al. [175] covalently coupled a fluorescent group and the light stabilizer Trolox to nanobodies via expressed protein ligation (EPL) technology, and achieved efficient intracellular delivery by binding through disulfide bond to the CPP R10. These nanobodies can specifically label endogenous targets including GFP, mCherry-tagged proteins, nuclear fibronectin, and proliferating cell nuclear antigen (PCNA) in live cells, thereby breaking through the limitations of traditional fluorescent probes and enabling dual-color super-resolution imaging as well as long-term dynamic monitoring.

In summary, CPPs delivering fluorescent proteins via the endocytic pathway for intracellular imaging face the challenge of endosomal escape. Introducing an EEP sequence into the CPP effectively promotes endosomal escape and improves cytosolic delivery efficiency. To observe specific biological processes, researchers employ solid-phase synthesis to conjugate CPPs, fluorescent probes, and peptides with specific targets, thereby constructing targeted fluorescent probes. Tight binding of the probe to the functional protein of interest enhances imaging quality, but may also introduce potential toxicity and interfere with biological processes. Therefore, future studies should continue to place significant emphasis on optimizing probe affinity.

5. Conclusion and Prospect

CPPs can shuttle exogenous proteins across the cell membrane barrier, establishing themselves as a promising platform for both pharmaceutical development and research applications. For pharmaceutical development, CPPs can deliver nanobodies and their derivatives into cells, thereby overcoming the inability of conventional antibody pharmaceuticals to enter the cytoplasm. This strategy extends the application of nanobodies from targeting extracellular membrane proteins to intracellular protein targets. CPPs can also deliver antigen-based vaccines to DCs, subsequently activating the $CD8^+$ CTL-mediated immune pathway. Adjuvants further improve the delivery efficiency. The introduction of CPPs enhances antigen immunogenicity, providing a new method for immunotherapy of refractory diseases such as cancer and chronic infections. Furthermore, CPPs promise to enable the intracellular delivery of functional proteins/peptides to ameliorate metabolic diseases such as diabetes and obesity. Incorporating a targeting unit to CPP holds promise for targeted drug delivery to organelles, including ER and mitochondria, and for modulating their metabolic disorders. To aid research, CPPs deliver proteins for in-cell NMR and live-cell imaging, thereby enabling the intracellular visualization of protein structure and functional tracking under near physiological conditions within live cells.

Current research on CPP-mediated drug delivery often compares the effects of drugs with or without CPP at the cellular or animal level. Researchers typically quantify fluorescence intensity to evaluate CPP delivery efficiency, and use staining or other methods to measure cell metabolic activity for cytotoxicity assessment. Nevertheless, neither cellular nor animal models completely mimic the immune and metabolic microenvironment of humans. As the application of CPPs for protein delivery moves from basic research toward clinical translation and industrialization, the toxicity and safety in humans of CPPs may emerge as future focal points, which researchers have developed smart activatable peptides to enable targeted delivery without affecting normal tissues *in vivo*. Moreover, AI-driven intelligent design facilitates more rapid screening and optimization of CPP sequences [176,177]. By employing AI-assisted design and optimization of CPP sequences, we can reduce the number of candidate peptides with application prospects, cuts down the number of wet experiments, and improves screening efficiency while lowering costs. At the same time, Generative AI may expand CPP design beyond naturally occurring sequences and known peptide variants. It can generate novel peptides from scratch that do not exist in the training dataset, thereby broadening the boundaries of CPP sequences.

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