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Review Article

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A Review on the Cell-Penetrating Peptides

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Abstract
The therapeutic potential of biomacromolecules in treating various diseases is undeniable; however, their application is significantly limited by the inherent barrier function of the cell membrane. Cell-penetrating peptides (CPPs), a class of small peptides comprising fewer than 30 amino acids, have garnered considerable attention due to their exceptional ability to traverse cell membranes. These peptides ease the intracellular delivery of various macromolecules, such as proteins, polypeptides, and nucleic acids, thereby overcoming the limitations imposed by the plasma membrane. CPPs exhibit versatile translocation properties, allowing them to penetrate cells independently and serve as delivery vehicles for therapeutic agents or CPP/cargo complexes. This unique capability has positioned them as promising candidates in treating and diagnosing diseases, including cancer. Their ability to transport biologically active molecules across the plasma membrane enhances the efficacy of therapeutic interventions that would otherwise face significant challenges due to poor cellular uptake. This review explores the classification of CPPs based on their structure and properties, shedding light on the mechanisms that enable their efficient membrane penetration. It also touches on various therapeutic applications of CPPs, emphasizing their potential to revolutionize drug delivery systems. Despite their promise, the clinical utility of CPPs is hindered by challenges such as stability, specificity, and potential cytotoxicity. Addressing these limitations is crucial to unlocking the full potential of CPPs as innovative delivery tools in modern medicine.

Keywords:
cell-penetrating peptides; detection; therapeutic potential; cancer therapy

1. Introduction

Polypeptides, proteins, and genetic biomacromolecules have demonstrated significant potential in the treatment of various diseases. Despite their efficacy, the large molecular size and low lipophilicity hinder their ability to traverse cell membranes efficiently. This limitation restricts the therapeutic applications of these biomacromolecules, particularly in the medical field. Several techniques, including liposome-assisted delivery, viral vectors, electroporation, and microinjection, have been employed to assist their transport into cells. However, these methods often suffer from drawbacks such as low delivery efficiency, cytotoxic effects, and compromised cellular health. Poor targeting specificity has further limited their large-scale clinical use. As a result, the development of safe and efficient transmembrane delivery systems for biomacromolecules remains a critical challenge. Among the emerging solutions, cell-penetrating peptides (CPPs) have shown promise in addressing these issues.

One of the earliest discoveries in this field of CPP was the transactivator of transcription (TAT) protein derived from the human immunodeficiency virus (HIV), which was identified by Green et al. in 1988 to possess the ability to cross cell membranes [1]. Subsequent studies, such as those conducted by Brigati et al. in 2003, established that the amino acid residues located at positions 47–57 of the TAT protein are crucial for transmembrane activity [2,3]. CPPs, also known as protein transduction domains (PTDs) or membrane transduction peptides (MTPs), are relatively small peptides consisting of 5–30 amino acid residues. These peptides have a unique capacity to cross cellular membranes independently and can also facilitate the transport of biomacromolecules through

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covalent or non-covalent interactions. Particularly, CPPs show remarkable versatility, as they can penetrate a wide variety of cell types and, when administered in vivo, are even capable of crossing the blood-brain barrier (BBB) [2]. Till date, numerous naturally occurring and synthetically engineered CPPs have been identified. Examples include the TAT protein from HIV, the ANTP protein derived from the Drosophila transcription factor, VP22 from herpes simplex virus type 1 (HSV-1), and synthetic peptides such as polyarginine and polylysine [2]. These peptides have garnered significant interest due to their broad range of transmembrane targets and their potential applications in therapeutic delivery. This review explains the classification, structure, and therapeutic potential of CPPs, highlighting their diverse roles in modern medicine. It also examines their shortcomings and the challenges associated with their clinical application. Despite their immense potential, CPPs face limitations such as stability concerns, off-target effects, and potential toxicity, which must be addressed to fully realize their therapeutic value.

2. Structural Characteristics and Classification of Cell-Penetrating Peptides

CPPs encompass a wide variety of types and are categorized based on factors such as their origin, mechanisms of uptake, biological applications, and physical properties. Despite these classifications, there is no universal consensus regarding a standardized grouping system. Typically, CPPs are short peptides consisting of 10 to 30 amino acids, often enriched with positively charged residues like lysine and arginine. These cationic amino acids contribute to their overall positive charge, allowing interactions with the negatively charged phospholipid bilayer of cell membranes. Prominent examples of CPPs include TAT, penetratin, and transportan. CPPs are often grouped into three categories based on their chemical and physical characteristics: cationic, amphiphilic, and hydrophobic. Mainly cationic and amphiphilic CPPs account for approximately 85% of the total identified CPPs, while hydrophobic peptides constitute only about 15% [4]. Among these, cationic CPPs have been the most extensively studied due to their efficient interaction with cellular membranes. Figure 1 below shows the different types of CPPs available.

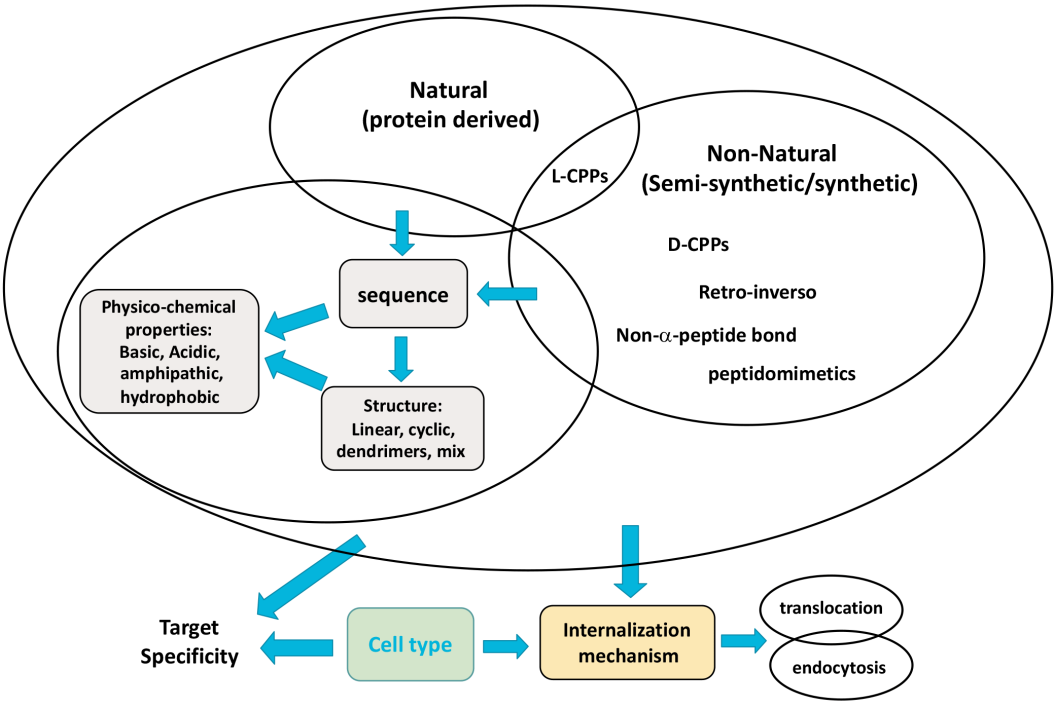

Figure 1: Classification and Properties of Cell-Penetrating Peptides (CPPs). Adapted from [5]. https://doi.org/10.1002/cmdc.202300236.

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2.1. Cationic CPPs

Cationic CPPs, such as TAT, penetratin, polyarginine, P22N, DPV3, and DPV6, are composed of peptide sequences that are rich in arginine, lysine, and histidine. The guanidinium group present in arginine plays a crucial role in forming hydrogen bonds with the negatively charged phosphate groups on cell membranes. This interaction facilitates CPP entry into cells under physiological pH conditions. These peptides often interact electrostatically with glycoproteins on the cell surface and are internalized independently of receptor-mediated mechanisms. The efficiency of cationic CPPs is influenced by the number and spatial arrangement of positively charged arginine residues within their structure [6]. Most cationic CPPs contain more than five positively charged amino acids, which are critical for their functionality [7]. Among these, polyarginine sequences show the highest efficiency for cell entry, making them particularly promising for therapeutic applications [8]. Studies by Chu et al. (2015) showed that the internalisation potential of oligoarginine increases with peptide length, with R8 to R10 being the optimal range for delivery applications [9]. Experimental analyses of oligoarginines ranging from three to twelve residues revealed that efficient membrane perforation requires at least eight arginine residues, with activity progressively increasing as the number of residues increases [2]. Although lysine shares a positive charge with arginine, it lacks the guanidinium group, resulting in comparatively lower membrane permeability when used in isolation [10]. Research by Futaki et al. (2001) further underlined that cationic CPPs with at least eight positively charged residues achieve superior membrane-penetrating efficacy [11].

Other amino acids also play critical roles in CPP functionality. For example, substituting tryptophan at position 14 with phenylalanine in penetratin results in a loss of membrane permeability [12]. Nuclear localization signal (NLS) sequences represent a subset of cationic CPPs. These are short peptides enriched in arginine, lysine, and proline that make possible the transport to nuclear pore complexes. NLS peptides are further classified as mono- or bi-partite based on whether they contain one or two clusters of basic amino acids. An example of a monotype NLS is PKKKRKV from Simian virus 40 (SV40), while bitype sequences are exemplified by nucleoplasmin. The smallest known NLS sequence with membrane-permeating ability is KRPAATKKAGQAKKKL [2]. However, due to their lower charge (fewer than eight positive residues), NLS peptides are not inherently effective CPPs [13]. When conjugated with hydrophobic sequences, NLS peptides can form amphiphilic CPPs with enhanced properties. Cationic CPPs are being actively explored for their versatility in biomedical applications, including drug delivery, gene therapy, and molecular imaging.

2.2. Amphiphilic CPPs

Amphiphilic CPPs are a group characterized by their ability to possess both hydrophilic and hydrophobic properties. They are further subdivided into four categories: primary amphipathic, secondary α-helical amphipathic, β-sheet amphipathic, and proline-rich amphipathic CPPs [14]. Primary amphipathic CPPs are either derived from natural protein sequences or designed by combining hydrophobic peptide domains with nuclear localization signals (NLSs). For instance, MPG (GLAFLGFLGAAGSTMGAWSQPKKKRKV) and Pep-1 (KETWWETWWTEWSQPKKKR KV) are synthetic examples of this. Both are based on the NLS sequence PKKKRKV from Simian Virus 40 (SV40) but incorporate hydrophobic domains to enhance their amphipathic nature. In MPG, the hydrophobic domain is derived from a fusion sequence, while in Pep-1, it is represented by the peptide segment KETWWETWWTEW. These hydrophobic sequences are joined to the NLS through a short linker peptide, WSQP [15]. Alternatively, some primary amphipathic CPPs originate from naturally occurring proteins. Examples include pVEC, ARF (1-22), and BPrPr (1-28), which demonstrate similar structural and functional features but are directly sourced from protein sequences rather than synthetic design. Secondary α-helical amphipathic CPPs function by interacting with the cell membrane through their α-helical conformation. These peptides possess distinct hydrophilic and hydrophobic regions distributed across different faces of the helix, allowing effective membrane association and penetration. A representative example is MAP (KLALKLALKALKAALKLA), which demonstrates significant transmembrane activity through this mechanism [2]. The functionality of β-sheet amphipathic CPPs is closely tied to their ability to form β-sheet structures. The alignment of amino acid residues in pleated β-sheets is essential for their interaction with and entry into cell membranes. Peptides unable to establish this configuration lose their membrane-permeating capabilities, highlighting the critical role of β-sheet formation in their mechanism of action [16].

Proline-rich amphipathic CPPs are characterized by their high proline content, which facilitates the adoption of a polyproline II (PPII) helical structure in aqueous environments. Unlike the typical right-handed α-helix with 3.6 residues per turn, PPII helices are left-handed and consist of approximately three residues per turn. Examples of proline-rich amphipathic CPPs include the bovine an-

timicrobial peptide Bac7 and synthetic peptides such as (PPR)n, where "n" can vary between 3 and 6 [2]. These unique structural features contribute to their efficient cellular uptake and potential therapeutic applications.

2.3. Hydrophobic CPPs

Hydrophobic CPPs are composed predominantly of nonpolar amino acid residues and possess a net positive charge of less than 20%. Their functionality relies on the presence of hydrophobic motifs or specific chemical groups essential for traversing the lipid bilayer. Despite being less extensively studied than other CPP classes, hydrophobic CPPs have notable examples, such as fibroblast growth factor-derived peptides like K-FGF and F-GF12, which are associated with Kaposi's Sarcoma [17]. These peptides represent an alternative strategy for intracellular delivery, particularly in hydrophobic environments where traditional CPPs might face limitations.

3. Membrane Penetration Mechanism of Cell-Penetrating Peptides

Even though CPPs have emerged as a research hotspot in recent years, their membrane-penetrating mechanism is still contemplated. On the internalisation methods of several CPPs or CPP/cargoes, there appears to be unanimity [18,19]. At the moment, there are primarily two mechanisms of CPPs transmembrane: direct transmembrane (energy-independent pathway) and energy-dependent endocytosis, depending on whether or not energy is needed during internalisation [20]. The specific mode of entry into cells is linked to the physical and chemical properties of CPPs, the size, charge, and type of cargo transported, the accumulation of CPPs and cargo complexes, the medicated tissues and cell types, and so on [21] (Figure 2).

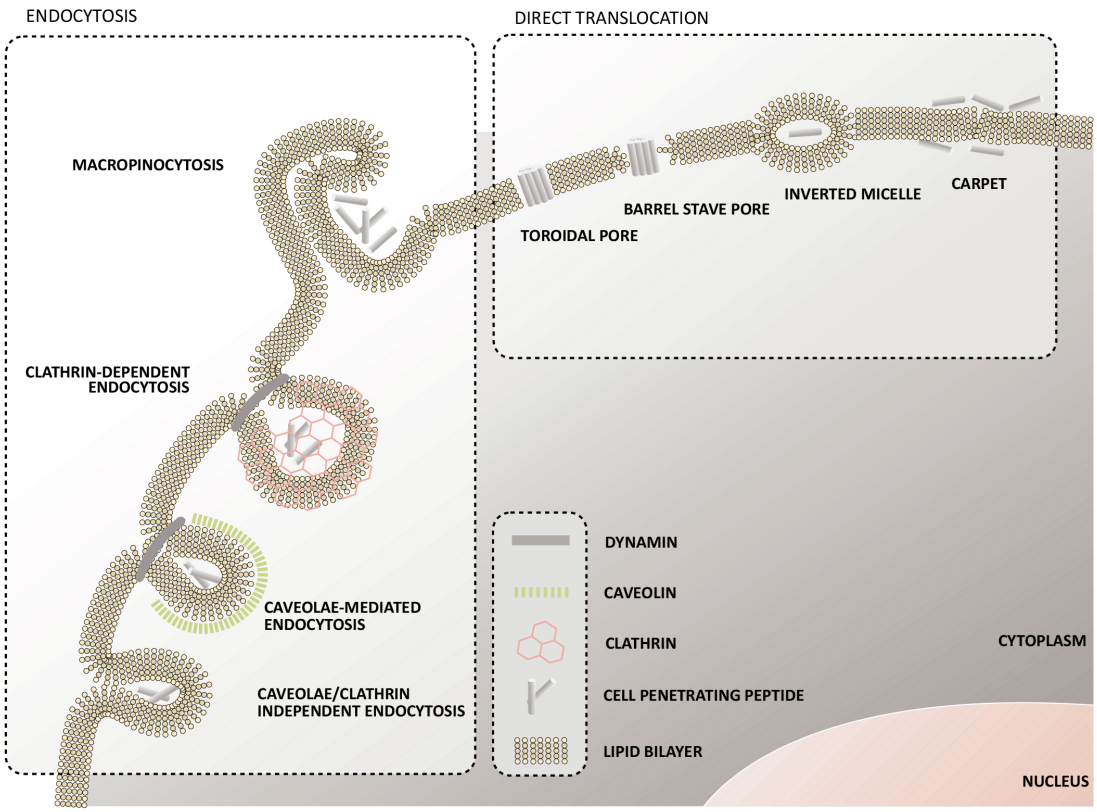

Figure 2: Mechanisms of Cellular Uptake of CPPs. This figure shows the two primary pathways by which CPPs enter cells: endocytosis and direct translocation. Endocytic routes include macropinocytosis, clathrin-dependent, caveolae-mediated, and clathrin/caveolin-independent mechanisms, often requiring vesicular trafficking. Direct translocation involves pore formation (barrel stave, toroidal) or membrane disruption via inverted micelle and carpet models. Figure adapted from [22]. https://doi.org/10.3390/ph3040961.

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3.1. Direct Membrane Penetration

Direct membrane penetration is an energy-free pathway, with proposed mechanisms including inverted micelle mode [23], carpet mode [24], perforation mode, i.e., "Barrel-Stave" model [25], and plasma membrane sparse mode [21]. Each one of these modes necessitate cationic CPPs to first engage with anionic components on the plasma membrane, such as phospholipid bilayers, causing modifications in plasma membrane integrity, and the successive internalisation process is dependent on the type and concentration of CPPs, the quantity of cargo, etc. attributes, cell lines treated, and storage conditions, etc. [2]. While these mechanisms can describe some facets of CPP transmembrane transport, none can establish a comprehensive internalisation pathway relevant to all types of CPPs. When nuclear magnetic resonance (NMR) was used to investigate the interaction between penetratin and phospholipid membranes, the inverted micelle model was proposed [26]. The cationic CPPs first interact with the anions on the phospholipid membrane, and then the phospholipid bilayer rearranges and shuttles due to the interaction between the CPPs' hydrophobic amino acid residues and the phospholipid membrane, and finally, the CPPs are wrapped [27]. The micelle, entombed within the inverted micelle, moves to the other side of the lipid bilayer membrane and explicitly discharges CPP into the cytoplasm [2]. The mechanism cannot work for CPPs without hydrophobic amino acid residues such as TAT and oligoarginine, since this model assumes hydrophobic amino acid residues are required to form flipped micelles. Carpet structures have been employed to characterize not only the translocation mechanism of some antimicrobial peptides but also the cytotoxicity of CPPs at high concentrations. Throughout this mode, cationic CPPs carpet the surface of the negatively charged plasma membrane like on flooring, and then the hydrophobic amino acid residues engage with the plasma membrane's hydrophobic core because the basic amino acid residues in the CPPs face the plasma membrane surface. When CPP concentrations reach a certain level, the phospholipid membrane becomes regionally compromised, permitting CPPs to penetrate the cell [28,29]. The sequence, also known as the barrel stave sequence, is a mechanism for antimicrobial peptide internalisation in bacteria. The α –helical structure of the amphiphilic CPPs is required for the formation of the pore-type channel. CPPs are grouped to the cell surface and implanted in parallel structures into the cell membrane to establish a barrel-like conduit [18]. The hydrophobic area of the α-helix adheres to the membrane lipid's outer surface to construct the conduit, while the hydrophilic region binds to the phospholipid's hydrophilic head, forming the conduit's central core and facilitating CPP transmembrane translocation and internalisation. The CPPs are delivered into the cytoplasm [19]. The "plasma membrane thinning" impact was first used to explain magainin's mechanism of action [30]. After CPPs form a carpet structure, the anomaly of the interaction between the outer leaflet charges induces the rearrangement of negatively charged membrane lipids and the thinning of the plasma membrane in this model. CPP interaction or aggregation on the plasma membrane surface lowers the surface tension of the plasma membrane's local surface, allowing CPPs to insert into the plasma membrane and penetrate the cell [2]. However, since no plasma membrane permeability was observed, this mechanism is highly unlikely to be plausible. It is possible to explain why the cell membrane does not appear permeable by considering the transient properties of plasma membrane pores combined with the repair response of the cell membrane [31].

3.2. Endocytic Pathway

The involvement of endocytic pathways in CPP internalisation has been a central focus in understanding the mechanisms of CPP-mediated membrane penetration. Initial studies suggested that CPP uptake occurred through a non-receptor-mediated, non-transporter-dependent, and energy-independent direct internalisation process. Over time, however, four distinct endocytic pathways have been identified: clathrin-mediated endocytosis [32], caveolin-mediated endocytosis [33], macropinocytosis [34], and clathrin- and caveolin-independent endocytosis [35]. Early research by Vives et al. (1997) demonstrated that TAT could enter cells at 4°C or under energy-depleted conditions, supporting the idea of energy-independent internalisation and excluding the involvement of endocytosis [36]. However, subsequent studies revealed limitations in these early methods, such as the use of methanol or formaldehyde for cell fixation, which could redistribute CPPs on the cell surface, thereby obscuring the true internalisation process [37]. Cationic CPPs also exhibit strong membrane affinity, complicating the distinction between membrane-bound and internalized peptides when analyzed by flow cytometry. This limitation often led to overestimation of internalisation rates [2]. To address these concerns, Richard et al. (2003) utilized more precise techniques, confirming through flow cytometry and cell fixation that TAT internalisation occurs via endocytosis [38]. Similarly, Wadia et al. (2004) observed co-localization of TAT-Cre with the endocytic marker FM4-64, providing direct evidence for an endocytic mech-

anism underlying TAT-mediated transport of exogenous molecules [34].

While some CPPs can bypass membranes through direct translocation, the majority rely on endocytic processes for internalisation. This mode of entry, however, poses challenges for cargo release, as CPP-bound materials often remain sequestered in endosomes and are subsequently degraded in lysosomes unless they successfully escape the endosomal compartment [39,40]. In receptor-mediated endocytosis, CPPs or their cargo bind to membrane receptors, inducing curvature through interaction with epsin proteins. Clathrin and hetero-tetrameric adaptor proteins (AP-2) assemble to form clathrin-coated pits, which mature into vesicles encapsulating the CPP-cargo complex [35,41]. The Caveolin-mediated endocytosis pathway involves CPP binding to receptors located in lipid rafts—hydrophobic membrane regions enriched in sphingomyelin and cholesterol. The cavin-1 and caveolin proteins assist vesicle formation and endosome generation, mediating cargo internalisation [42,43]. The preference for caveolin-mediated pathways may vary based on the size and properties of the associated cargo [44].

When CPPs are bound to larger macromolecular cargos (e.g., >30 kDa), they often employ macropinocytosis, an actin-dependent endocytic pathway characterized by membrane ruffling and vesicle formation. This process is initiated by CPP interactions with membrane proteoglycans, which activate rac proteins. These proteins, in turn, trigger F-actin rearrangements that also assist vesicle formation and cargo uptake [35,45]. Unlike other pathways, macropinocytosis is receptor-independent and often lipid raft-dependent, making it particularly suited for CPPs carrying large payloads [2,46]. In specialized cells like macrophages, CPP uptake can occur through an alternative mechanism that does not involve clathrin or caveolin. Instead, CPPs are opsonized and recognized by cell surface receptors such as Fc receptors. Actin activation drives the internalisation of CPP-cargo complexes into membrane-coated vesicles [47]. Regardless of the endocytic pathway utilized, CPPs and their cargos must escape endosomes to avoid lysosomal degradation and exert their biological effects. This escape is facilitated by processes such as pH gradient alterations, vesicle accumulation, and CPP interaction with charged endosomal membranes. These interactions can lead to increased membrane rigidity and eventual rupture, enabling the release of CPPs and their associated cargos into the cytoplasm [6,7]. The choice of endocytic pathway for CPP internalisation is influenced by the peptide's physicochemical properties and the characteristics of the bound cargo, including size, charge, and hydrophobicity. For example, TAT has been shown to use different pathways—lipid raft-mediated endocytosis for protein cargos and clathrin-mediated endocytosis for smaller fluorescent molecules.

4. Factors Affecting the Cellular Uptake Mechanism

Although various CPPs share a lot of commonalities, their absorption methods may differ greatly. This results in inconsistent findings, mostly due to the numerous variables that influence the cellular absorption and translocation process. The physicochemical characteristics, concentration of the peptide and its payload, and features of the plasma membrane, including its lipid and protein composition, may be broadly categorised as variables that affect the absorption pathways of cell-penetrating peptides. Due to variations in experimental settings, conflicting data about the internalisation process of CPPs frequently occur. The CPP concentration is the primary critical element. It has been seen in several instances that the administered concentration significantly influences the uptake mechanism. The net charge of the peptides is another consideration, particularly the positive charges that result from arginine residues [48]. The majority of CPPs include arginine residues, which are better for the transport and absorption of CPP than lysine (in particular, the guanidinium group of arginine) [48]. Amphipathicity is another element that has been linked to absorption. Whereas nonamphipathic CPPs need endocytosis, primary and secondary amphipathic peptides can directly cross the cell membrane at low concentrations [49]. The internalisation process may also be affected by the experiment's temperature. The temperature dependency of R8 translocation through plasma membranes has been seen by Fretz et al., 2007 [50]. At 4 °C, diffuse signals from the fluorescently tagged peptide are more pronounced in the cytoplasm, which is often an indication of a direct translocation across the membrane, they have discovered [50]. At 37 °C, however, both diffuse and punctate signals were seen, suggesting that an endocytic process may be activated at higher temperatures [50]. The absorption route can also be significantly influenced by the cargo molecules coupled to CPPs.

4.1. Role of Cargo Molecules in CCP Uptake

The cargo coupled to the CPP is frequently a key internalisation component. The absorption of TAT conjugated to peptides and globular proteins in live cells was compared by Tünnemann et al., 2006 [51]. They discovered that the uptake process was significantly influenced by the size of the payload fused to TAT. While the TAT-peptide conjugates were dispersed widely throughout the cell, the

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bigger complexes comprising proteins were visible inside vesicular structures [51]. This suggests that a separate absorption method results from the size of the cargo, which naturally determines the size of the total complex. The likelihood of the complex being taken up by direct translocation increases with decreasing size. Endocytosis, however, predominates at larger dimensions. This is comparable to the situation where the presence of a cargo molecule chooses between two alternative endocytosis methods when comparing the uptake mechanism of unconjugated TAT versus TAT fused to a cargo [41]. Whereas its conjugated counterpart is more likely to adopt CvME, unconjugated TAT favours CME for cell entry [48]. Cargo impact can also affect how other arginine-rich peptides are taken up. The effects of cargo molecules on the absorption of R7 and R7W were examined by Maiolo et al., 2005 [52]. In the cytoplasm of the tested cells, the peptides alone displayed diffuse signals. The diffuse signal from endocytic vesicles was significantly reduced following fusing with cargo peptides, but the punctate signal barely changed.

4.2. Role of Concentration in CCP Uptake

Since multiple uptake routes can be stimulated by CPP concentration, it is a crucial component. It is thought that endocytosis often takes place at low peptide concentrations and that direct penetration takes over at greater concentrations. Fretz et al., 2007 [50] evaluated the dependency on concentration while examining the impact of temperature on the absorption of R8. Vesicular staining in the cytoplasm was seen at lower peptide concentrations, indicating endocytic absorption [48]. Vesicular and diffuse labelling was seen at greater concentrations, suggesting that direct penetration and endocytosis may take place concurrently [53]. The concentration-dependent absorption of R9 and TAT has also been investigated [53]. In addition, these peptides exhibit substantial cytosolic labelling at higher concentrations and mostly vesicular signals at lower concentrations. Yet things become more difficult since endocytic inhibitors had only a minimal impact on the absorption of R9 and TAT at low concentrations (<5 uM), but clathrin inhibitors appear to have a significant impact at higher concentrations [48]. In addition to the quick, nonendocytic absorption via nucleation zones, it was shown that the peptides are, to some extent, taken up by vesicular structures at greater concentrations [54].

4.3. Role of the Type of Cells Involved in CCP Uptake

The CPP uptake process is greatly influenced by the cell lines that were employed in the research. The nature of the extracellular matrix and the plasma membrane, in particular, can have a significant impact on CPP uptake. It is well known that the extracellular matrix's negatively charged GAGs and the positively charged CPPs have their first interaction during internalisation. Hällbrink et al., (2004) looked at the impact of the peptide-to-cell ratio on the absorption of cells into Chinese hamster ovary (CHO) cells [55]. To be more precise, they were interested in how the absorption would alter if the number of peptides, rather than the concentration, were raised while maintaining the same number of cells. They also looked at how cell number and confluence affected uptake. They demonstrated that increasing the internal peptide concentration more effectively than increasing the exterior peptide concentration required double the incubation volume at a certain number of cells. Moreover, applying a constant peptide concentration to various cell densities showed that the uptake decreased with increased confluence. The variable membrane composition or the various endocytosis patterns of the developing cells may be the reason for this. Another hypothesis is that when confluence rises, membrane access diminishes [48]. These findings offer an illustration of how experimental variables in cell culture might affect uptake effectiveness. A thorough analysis of the absorption of 22 distinct CPPs in four different cell lines was conducted by Mueller et al., 2008 [56]. They investigated the internalisation of several of the most well-known CPPs, including penetratin, TAT, transportan, Pep-1, MPG, MAP, R7, and R9. They employed Cos-7, HEK293, HeLa, and MDCK as representative cell lines. According to their behaviour, the data allowed for the classification of CPPs into three groups: high (penetratin, transportan, MAP), medium (TAT, Pep-1, MPG), and low cellular uptake [48]. The results demonstrate that some of the peptides are taken up by cells in a cell-dependent manner, which is interesting. For instance, Cos-7 cells, which resemble fibroblasts and were isolated from monkey kidney tissue, took up MPG preferentially [48]. This could be because the virus that was utilised to create the Cos-7 cell line, SV-40 big T antigen, includes an NLS that was generated from MPG [57].

Research by Gronewold et al. (2017) showed how cell lines affected the absorption of a CPP with potential anticancer action [58]. The antimicrobial peptide CAP18's C-terminal domain was used to create sC18, the CPP. In addition to HEK293, a noncancer cell line, its absorption was examined in the cancer cell lines HeLa, PC-3,

HCT-15, and MCF-7 [58]. Interestingly, nuclear accumulation and a diffuse fluorescent signal with punctate distribution were seen in the cytoplasm of all cancer cell types. However only a punctate distribution and little to no peptide in the nucleus were found in the non-cancer cell line.

5. Detection of CPPs by Fluorescence

Fluorescence is a widely utilised method for locating and investigating CPPs. CPPs should be conjugated to the appropriate fluorophore molecules to use fluorescence procedures on them. The fluorophore moiety often has a covalent link attached to CPP. In all fluorescence techniques, CPP linked to a fluorophore is first incubated with cells, vesicles, or other target structures to allow its penetration into the cell. The fluorophore in the target structure is excited by absorbing light after an appropriate amount of time has passed. The equipment detects the light that the fluorophore produces when it transitions back to its ground state. A certain frequency of light is emitted by each fluorophore. Depending on the molar extinction coefficient and quantum yield, the fluorophore's efficiency, the excitation light's intensity, and the fluorophore's efficiency determine how much light is emitted. However, the fluorophore's neighbourhood can affect how much light it emits, and from this information, it is possible to infer how the fluorophore interacts with other molecules like proteins, peptides, or lipids. The validation of the effective delivery of cargo by identifying the right physiological impact of the cargo in the cell is the best technique to identify the internalisation of the CPP-cargo construct into the cells, as well as into its target component (organelle) [59]. Another strategy is to co-localize the fluorescence light that the CPP-delivered payload emits with the light that certain target markers (molecules, organelles, cell types, tissues, or organs) emit or absorb. CPP assesses possible cytotoxicity about the construct concentration to ensure safe distribution. The precursor of interleukin 1 alpha that is armed with a nuclear localization sequence (NLS) and connected to green fluorescent protein (GFP) has been demonstrated to possess CPP abilities using fluorescence microscopy and flow cytometry [59]. It delivers GFP into the nuclei of Jurkat and HeLa cells. It was also shown that, following intraperitoneal treatment, it is capable of delivering proteins into the cells of many mouse organs, including the spleen, liver, and intestine [60]. Even at 100 uM concentration of the construct, no cytotoxicity was seen. It has been proved that the whole Antennapedia homeodomain protein may preferentially enter the cells by macropinocytosis using the same combination of fluorescent techniques and low temperature [61]. He et al., 2013 originally discovered unique CPPs using a mixture of fluorescence techniques [62]. These CPPs were chosen utilising synthetic membranes and named spontaneous membrane-translocating peptides (SMPTs).

Tetramethylrhodamine (TAMRA) and Alexa Fluor 546 dye were able to enter the cytoplasm of several cells via these peptides when endocytosis was blocked [59]. In contrast to unconjugated TAMRA, which was quickly eliminated when administered through injection to mice, SMTPs transported TAMRA to several organs, where it could be identified even two hours after treatment. They hypothesized that SMTPs may convey a wide variety of additional polar chemicals into cells. Although the internalisation process of the CPP prototype TAT, which was the first CPP found, is not fully understood, it is currently being thoroughly researched. The action of TAT as a CPP is usually associated with the activity of other CPPs, and it also acts as a model and reference CPP [59,63]. Fluorescence spectroscopy, flow cytometry, and confocal imaging were used to investigate the cell uptake of fluorescein isothiocyanate (FAM) tagged TAT [64]. It was discovered that the TAT-FAM construct may enter cells without using endocytosis through two entrance pathways with various energy requirements. A lot of positively charged amino acids make up CPPs like TAT, which are known for their propensity to interact with negatively charged biological structures [59]. To increase their availability and blood circulation time, fewer cationic CPPs are required. The usage of membrane-interacting proteins as the source of the prospective membrane-transferring peptides was used to solve this issue. According to Kim et al., (2015), many hydrophobic CPPs may be generated from the sequence of annexin [63]. These CPPs outperformed the CPP prototype TAT as cargo-delivery agents, as demonstrated by fluorescence flow cytometry and other techniques. These peptides exhibited negligible immunogenicity, extremely mild cytotoxicity, and high serum stability.

6. Detection of CPPs by Radioactivity

CPPs can be labelled with various markers for cell imaging in addition to fluorophores. Labelling radioactive material is one strategy. Radioactive detection of CPPs has several advantages over other detection methods. It is highly sensitive, allowing for the detection of very small amounts of CPPs, and can be used to track the movement of CPPs within cells or tissues. It is also relatively easy to perform and does not require specialized equipment. Cells are typically initially treated with labelled CPP in a normal experiment [59]. To remove the labelled CPP

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that has been adsorbed on the membrane's exterior, cells are next rinsed with a solution that includes a suitably diluted acid [59]. The amount of internalised CPP is then estimated after the radioactivity counter determines the radioactivity. The kinetics of CPP internalisation may be observed if the incubation of the cells is stopped at various time points. TP labelled with 125I is one of the original examples of radioactively labelled CPP [65]. To observe the dynamics of the tagged TP internalisation, the amount of internalised TP inside Bowes cells could be calculated as a function of time [59]. The difficulty of this approach in distinguishing between intact radioactive CPP and its radioactive by-products after the cleavage in the cell is a disadvantage. Using 125I to mark the four cationic CPPs TAT, octa- and nona-arginines, and MAP and monitor their uptake into CHO cells is a more contemporary example [66]. To examine the internalisation of the construct in HeLa and breast cancer cells, other proteins labelled with 125I that are involved in the metabolism of arginine can be fused to CPPs [67]. The results of the first of these two investigations showed that whereas TAT and poly-arginines were primarily kept in the cytosol, MAP was able to cross the nuclear membrane [59]. It should be noted that the presence of Tyr in the peptide structure is a requirement for labelling with 125I; if this is not the case, Tyr may be added to extend the peptide's sequence, but its potential impact on CPP behaviour should be investigated independently [59].

This was accomplished in the work carried out by Zaro et al., 2009 using poly-arginines [66]. In recent radioactivity experiments after CPP internalisation, 68Ga [68] and 64Cu were also utilised as nuclides [69]. A study that indicates other nuclides that might be utilised for the radioactive labelling of CPPs, including 99mTc, 111In, and 177Lu, was recently published by Gharibkandi et al., 2020 [70]. This analysis does not particularly address CPPs, but is generally focused on the radioactive labelling of peptides. There are some limitations to the use of radioactivity for CPP detection. It requires careful handling and disposal of radioactive materials, and there are concerns about the potential health risks associated with radiation exposure. Besides, some CPPs may not be compatible with radioactive labeling due to their chemical properties.

7. Application of Cell-Penetrating Peptides as Transport Vehicles

In addition to neurological disorders, asthma, local ischemia, diabetes, and cancer, the CPP-dependent drug delivery system has been utilized to treat a wide range of diseases. As an effective delivery tool, CPPs have been used to introduce cytotoxic drugs into tumor cells to induce apoptosis in tumor cells.

7.1. CPPs Facilitate Transmembrane Transport of Small Molecule Drugs

Because of their small size and lipophilicity, small molecule anticancer drugs can dissipate into tumor cells efficiently. Multidrug resistance (MDR) develops when tumor cells are subjected to the same drug continuously. To address this issue, some researchers have attempted to combine these drugs with CPPs to facilitate the entry of these small-molecule drugs into cells [71]. Dubikovskaya et al. formed an R8-taxol covalent compound by disulfide bonding an octamer arginine R8 to the anticancer drug paclitaxel [71]. The results revealed that the R8-taxol covalent compound exhibited a similar effect to paclitaxel alone in taxol-sensitive tumor models. However, in taxol-resistant tumor models, R8-taxol covalent drugs were more likely to induce tumor cell apoptosis compared to paclitaxel alone [2]. There are numerous advantages to combining small-molecule drugs and CPPs. It can increase drug water solubility and improve drug utilisation rate, in addition to overcoming multidrug resistance. Lee et al., (2011) used chemical methods to combine doxorubicin, TAT, and polymerized chitosan backbones to generate chitosan/doxorubicin/TAT chimaeras that were compatible with TAT-free chimaeras to enhance the cytotoxicity and targeted transport of anticancer drugs [72]. This chimaera has more efficient cell internalisation than doxorubicin or chitosan/doxorubicin, and it can change the distribution of doxorubicin in the organism, enhance tumor localization, and thus considerably constrain tumor growth [72].

7.2. CPPs Facilitate Transmembrane Transport of Peptides and Proteins

Numerous cancers can be caused by mutations in tumor suppressor genes [73,74]. To reinstate the activity of these proteins and accomplish the goal of cancer treatments, some researchers attempt to insert full-length proteins or polypeptides about tumor suppressor genes into cancer cells. To treat mice with advanced peritoneal cancer metastasis, Snyder et al. created a TAT-p53 chimeric peptide and administered it intraperitoneally. The average survival time of the mice in the experimental group was more than 6 times that of the control group, which had a mean survival time of 10 days [75]. Hosotani et al., 2002 covalently joined a short peptide made up of 20 amino acid residues of the p16 protein to penetratin by a disulfide bond to reinstate the functioning of the p16 protein, and they then tested its effectiveness in an animal model

of pancreatic cancers [76]. According to the findings (tumor in treatment group: 79 ± 17 mg; tumor in control group: 149 ± 12 mg), the complex could greatly slow the growth of cancers [76]. Apoptosis induction of tumor cells was attempted by certain researchers, utilizing various therapeutic techniques. As a protein of mitochondrial origin, Second Mitochondrial-Derived Activator of Caspase (SMAC) is crucial for the regulation of apoptosis [77]. SMAC can deactivate apoptosis-inhibiting proteins when it is released from mitochondria, hence increasing apoptosis. The 7 amino acid residues at the amino terminals of SMAC and TAT were combined to create a chimeric peptide, which was identified by Fulda et al. Research conducted in vitro revealed that while this chimeric peptide was ineffective at stimulating tumor cell apoptosis, it did make tumor cells more susceptible to apoptosis effectors such tumor necrosis factor-related ligand that induces apoptosis (TNF-related apoptosis-inducing ligand, TRAIL) [78]. The conjunction of TRAIL and SMAC-TAT peptide can greatly slow the formation of tumors in a mouse model of human glioma xenografts, and when 0.6 or 2ug of TRAIL is employed, tumor cells can be eliminated [78]. The ribosome-inactivating protein (RIP) generated from plants known as gelonin can efficiently limit protein translation when its half maximum inhibitory concentration (IC50) is at the picomolar level [2]. Poor membrane permeability makes it difficult to treat tumors and frequently fails [79]. A chemical combination of gelonin and TAT or LMWP was used by Park et al. to overcome this obstacle [80]. In mouse colon cancer cells, the gelonin chimeric peptide was shown to have strong antitumor activity when it was injected. At a dose of 100 μg, the chimeric peptide can completely inhibit tumor growth [80].

7.3. CPPs Facilitate Transmembrane Transport of Genes

For the treatment of cancer, genes, antisense oligonucleotide strands, and small interfering RNA (siRNAs) may be useful tools [81]. A particularly alluring therapeutic approach, RNA interference technology is extremely selective in targeting genes and has a special tumor suppressor impact. Drugs containing siRNA have a minimal ability to penetrate cells due to their enormous size, strong charge, and size. CPPs have drawn a lot of attention due to their low toxicity in endeavors to facilitate siRNA entry into cells using cationic liposomes, cationic polymers, and CPPs. In a covalent or non-covalent manner, siRNA can attach to CPPs. Even though covalent bonding is the best type of binding, tight complexes and polymers are simple to produce because of the strong charge interaction between the two [2]. It is more difficult to cross the cell membrane than CPP when combined with another molecule in a 1:1 covalent manner [2]. Cationic CPPs' charge can be easily neutralised by negatively charged siRNA, rendering both siRNA and CPPs ineffective for their intended biological purposes. Researchers have decided to combine siRNA with CPPs in a non-covalent form due to the several disadvantages of covalent binding. They are mostly focused on the viability of siRNA-CPP chimeric peptide research in vitro, however, animal research is also ongoing. In the angiogenesis requisite for tumor growth, vascular endothelial growth factor (VEGF) plays a significant role.

The VEGF gene can be silenced or VEGF receptors blocked to treat malignancies. A cholesterol/R9/siVEGF combination was created by Kim et al., 2006 using R9 chimaera as a carrier and anti-VEGF siRNA (siVEGF) to drastically reduce tumor development and angiogenesis [82]. Cyclin B is a mitotic cycle protein that is necessary for cell division, but tumor cells manifest uncontrolled expression of this protein [2]. To reach therapeutic goals, several researchers try to use RNA interference technologies. Cromez et al. formed an anti-cyclinB1 siRNA complex by combining anti-cyclinB1 siRNA with the shortened version of MPG (MPG-8), which can significantly inhibit cell proliferation by inhibiting the expression levels of cyclinB1 and causing cell cycle arrest [83].

7.4. CPPs-Facilitated Transmembrane Transport of Nanoparticles

Cell-penetrating peptides have been extensively used in the transmembrane transport of numerous nanoparticles, including magnetic nanoparticles, lipid nanoparticles, gold nanoparticles, micelles, and quantum dots, even though the transmembrane mechanism of these molecules is not fully understood. TAT and cross-linked iron oxide particles (CLIOs) were coupled by Josephson et al., (1999) to create a TAT/CLIO complex with an average particle size of 41 nm [84]. The efficiency of the transmembrane was higher than CLIO without TAT. It is advantageous for MRI or magnetic sorting since it is approximately 100 times higher and can mark cells effectively [84]. The transmembrane efficiency of TAT/CLIO increased nonlinearly as the ratio of TAT/CLIO increased, and when the ratio reached 15, the highest transmembrane efficiency was found [2].

The complex created when liposomes and CPPs combine may efficiently enter a variety of cells, including mouse heart muscle cells, mouse lung cancer cells, and human breast cancer cells, and the effectiveness of liposomes' membrane penetration is related to the density of

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CPPs [85]. The penetratin/liposome complex has a higher rate of membrane penetration than the TAT/liposome complex and reaches its maximum absorption in under one hour. Researchers are very interested in employing CPP/nanoparticle complexes to transport diverse anti-cancer medicines since CPPs can successfully facilitate the transmembrane transport of different nanoparticles. Paclitaxel, a drug that can effectively enter malignant glioma cells and suppress tumor cell proliferation, was loaded into lipid nanocapsules with CPP modifications by Balzeau et al., 2013 [86].

7.5. CPPs' role in Cancer Therapy

One challenge in treating cancer was that tumor microenvironment or other obstacles prevented medication transport to tumor cells, particularly in cases of duodenal and brain gliomas. CPPs provided a fresh viewpoint on how to get through a semi-permeable hydrophobic barrier and achieve excellent drug delivery in tissue and subcellular architecture. The majority of CPPs reacted with the high-density anionic charge on cell membranes and possessed positive side chains. Polyarginines of various lengths were often employed for medication delivery [35]. The CPPs' cationic charge density was a significant factor in determining the feasibility of the cargos' translocation. CPPs can be used to improve the specificity and selectivity of chemotherapy drugs by delivering them selectively to cancer cells while sparing normal cells. This approach not only reduces the side effects associated with traditional chemotherapy but also enhances the efficacy of the drugs by increasing their bioavailability and reducing the development of drug resistance. Extracellular vesicles altered with the hexadecanoyl-arginine (R16) peptide revealed moderately good anti-cancer efficacy [87]. Proteins were typically transferred via CPPs by covalent bonding. Table 1 shows some of the CPPs used in cancer treatment.

Table 1: Current list of CPP for cancer.

CPP	Type	Cancer Types	Function	Mechanism	Clinical Trial ID
PEP-010	Cationic	Breast cancer	Restores apoptotic pathways	Disrupts the caspase-9/PP2A interaction, activating caspase-dependent apoptosis	NCT04733027 (Phase I)
ATX-101	Cationic	Multiple myeloma/Sarcoma	Reaches the cell nucleus to enhance damage repair, cellular stress response, and the efficacy of several anticancer agents	Disrupts the PCNA/APIM-containing protein interaction	NCT05116683 (Phase II), NCT04814875 (Phase I/II), NCT01462786 (Phase I)
AVB-620	Cationic	Breast cancer	Real-time tumor visualization during surgery	CPP conjugated with fluorophores Cy5/Cy7 for FRET, targeted to human breast cancer cells due to their MMP overexpression	NCT02391194 (Phase I), NCT03113825 (Phase II)
Z12 and ZEBRA-Derived CPPs	Cationic	Broad spectrum, including aggressive brain cancers	Components of cancer vaccines	Promotes immune responses against tumors when conjugated with multi-epitopic antigens	NCT04046445 (Phase I)
pVEC and PEGA	Cationic	Breast cancer	Targeted drug delivery vector	Selective non-endocytic translocating mechanism (pVEC) by targeting molecular markers on tumor cells when conjugated with homing peptides (PEGA)	
Pep-1	Cationic	Broad spectrum	Targeted macromolecular carrier and drug delivery vector	Highly selective non-endocytic translocation through cancer cell membranes is primarily due to the high presence of acidic components	
MAP	Cationic	Broad spectrum	Bifunctional CPP that disrupts cancer cell membranes	Selective strong electrostatic interactions with negatively charged phospholipids	

p28	Cationic	Multiple cancer types, including glioblastoma and hepatocellular carcinoma	Promotes cell-cycle arrest and apoptosis in tumor cells	Interacts with wild-type and mutant p53 proteins, inhibiting their ubiquitination and regulating their levels	NCT00914914 (Phase I), NCT01975116 (Phase I), NCT05359861 (Phase II), NCT06102525 (Phase I)
SAP and SAP(E)	Proline-rich amphipathic	Broad spectrum	Targeted drug delivery vector with minimal toxicity	Specific electrostatic interactions with negatively charged membrane components (SAP); internalisation of aggregates in a non-clathrin or caveolin-mediated endocytosis (SAP(E))	
Bac1-24	Proline-rich amphipathic	Broad spectrum, particularly solid tumors	Targeted delivery agent of therapeutic proteins and peptides	Hydrophobic domains and specific electrostatic interactions with negatively charged phospholipids	
BIM-SAHB$_A$	Stapled peptide	Hematologic cancers	Restores apoptosis in resistant cancer cells	Blocks the anti-apoptotic sequestration of BAX/BAK BH3 helices, mimicking the BH3 death domain	
SAHB$_D$	Stapled peptide	Cancers where MCL-1 overexpression is a critical survival factor (myeloma, acute myeloid leukemia, melanoma, etc.)	Restores apoptosis in resistant cancer cells	Inhibits the MCL-1 anti-apoptotic activity, disrupting its interaction with pro-apoptotic proteins	
ALRN-6924	Stapled peptide	Broad spectrum, including breast cancer and acute myeloid leukemia	Restores p53 function, reactivating apoptosis	Binds strongly to MDM2 and MDMX, inhibiting the p53 suppression	NCT02264613 (Phase I/II), NCT04022876 (Phase I), NCT03654716 (Phase I), NCT05622058 (Phase)
P1pal-7	Pepducin	Breast, lung, and ovarian cancer	Reduces tumor growth and slows cancer progression. Anti-angiogenic agent	Interacts with PAR1, inhibiting its activation	
EN1-iPeps	Homeodomain-derived	Breast cancer	Triggers a selective apoptosis response	Inhibits the EN1 transcription factor in tumor cells where it is overexpressed	
Vectocell®/ DPVs	HS Binding CPP	Broad spectrum	Targeted drug delivery agent (from small compounds to macromolecules)	Caveolae-mediated endocytosis of DPVs-Glycosaminoglycan clusters	
CPPecp	HS Binding CPP	Tumors with high HS expression, including colon cancer	Inhibits cancer cell migration and angiogenesis	Binding to overexpressed heparan sulfate on the surface of cancer cells	
Melittin and derivatives	Derived from animal venoms and toxins	Broad spectrum	Drug delivery vector and apoptosis inducersin tumor-associated macrophages	An amphipathicα-helix structure enables interactions with the membrane, allowing the internalisation of conjugated pro-apoptotic peptides	

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Lycosin-I and R-lycosin-I	Derived from animal venoms and toxins	Broad spectrum	Induces apoptosis in cancer cells and inhibits cell proliferation	Activates the mitochondrial death pathway and upregulates p27	
Pardaxins	Derived from animal venoms and toxins	Broad spectrum, including aggressive cancers such as ovarian cancer and oral squamous cell carcinoma	Apoptosis inductor	Generation of ROS and mitochondrial membrane depolarization	
BT1718	Cyclic	Solid and refractory tumors	Selective release of cytotoxic agents	Binds to overexpressed MT1-MMP in tumors releasingDM1, a cytotoxic payload	NCT03486730 (Phase I/II)
^{177}Lu-DOTA^{0}-Tyr^{3}-Octreotate (Lutathera®)	Cyclic	SSTR2-positive neuroendocrine tumors	Selective delivery of cytotoxic agents	This radioconjugate utilizes the somatostatin analog TATE to target SSTR2-positive neuroendocrine tumors, delivering a cytotoxic dose ofβradiation	NCT02125474 (Phase II), NCT02236910 (Phase II), NCT03325816 (Phase I/Phase II)

Adapted from: [88]. https://doi.org/10.3390/ijms26010002.

Unfortunately, because of changed biological activity and/or steric hindrance, covalent CPPs technology was not the most efficient method to transfer macromolecules [89]. Delivering oligonucleotides seems to be mostly accomplished by electrostatic adsorption. The linear structure of CPPs typically does not achieve highly satisfying oligonucleotide transfection efficiency due to low charge number, which leads to poor complexation and organizational volatility of nano-carriers [35]. To create a unique bioreducible cationic network employing R9 as a vector, Yoo et al., (2017) synthesised a branched R9 using disulfide bonds [90]. Branching structures enable effective electrostatic adsorption of pDNA or siRNA. In vitro, B-mR9 demonstrated strong intracellular trafficking and biocompatibility. The EPR impact of B-mR9 also demonstrated a targeted effect on the tumor that lasted for 48 hours. In the NCI-H460 harbouring BALB/c nude mice model, B-mR9/siVEGF significantly suppressed tumor development by 56.5% when compared to control, and the therapeutic effectiveness was greater than PEI25k and R9 vector [35].

A fresh method to create a platform for gene delivery was offered by the cationic network formed from CPPs. ULK1 siRNA and the AMPK activator narciclasine have been delivered together in a pH-sensitive and biocompatible micelle system by Tai W., Gao X., (2017) to successfully prevent hepatocellular cancer in preclinical trials by controlling programmed cell death [89]. Subsequent studies confirmed that CPPs were to be the revolutionary siRNA oligonucleotide paradigm. Clinical uses for CPPs were restricted to positive charges that caused systemic and off-target toxicity. In MCF-7 cells, CsA (Cyclosporin A), with electronic neutral, a new highly hydrophobic cyclic CPP, outperformed both pentapeptide VPT (VPTLQ) and PFV by a factor of several times and conventional neutral CPPs by a large margin [35]. By administering a membrane-impenetrable pro-apoptotic peptide (PAD), the effectiveness and toxicity of cyclosporin A were compared to TAT. The uptake of PDA was increased 2.2–4.7-fold in the tumor cell lines examined by CsA when CsA was conjugated to PAD, and cellular uptake of CsA-PAD was often higher than TAT-PAD. Depending on the cell type, the cytotoxicity of CsA-PAD was comparable to or greater than TAT-PAD in four distinct tumor cell lines, but it was much more potent than PAD. CsA-PAD showed an equivalent anti-tumor effect to TAT-PAD in xenografted MCF-7 nude mouse models, but with less systemic toxicity [35]. Although the correct tissue distribution of electroneutral CPPs has to be further evaluated, cationic CPPs likely have more potential application value in vivo than electroneutral CPPs [91]. Hyaluronic acid (HA), a high-affinity ligand for tumor surface-specific overexpressed marker CD44, was one such polyanionic substance employed to coat nanoparticles to decrease toxicity and non-target of positive CPPs [35]. To administer 10-HCPT against hepatocellular carcinoma, Zhao et al., (2018) created a multifunctional liposome modified with TAT and HA (HA/CPPs-10-HCPT-NPs) [92]. Low-intensity focused ultrasound was employed to precisely regulate drug release to tumor tissue. In the multicellular tumor spheroid model, the liposome penetration depth was enhanced 2.76-fold following TAT modification. HA-coated nano-carrier was a useful and promising method for CPPs administration in vivo [35]. Liposome-coupled application of HA and CPPs with the assistance of ultrasound had a considerably greater tumor inhibition against hepatic carcinoma than other groups [92]. Figure 3 below shows the types of cancer that were tested with CPPs.

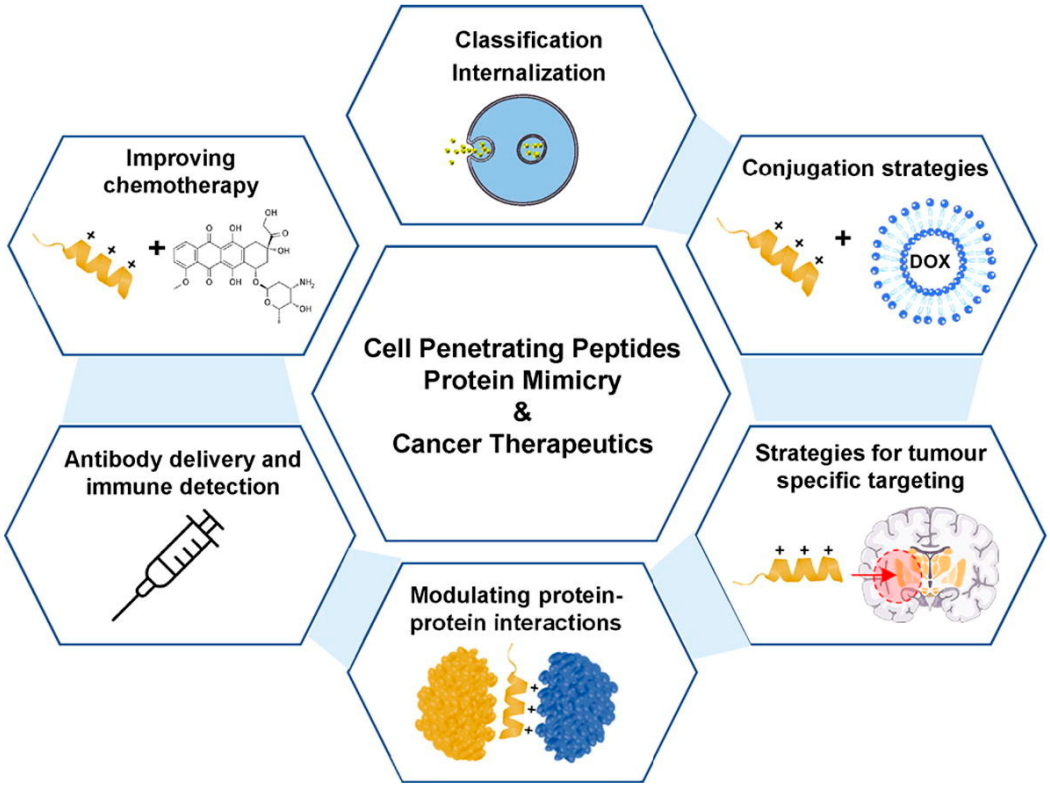

Figure 3: Therapeutic applications of CPPs in cancer treatment. This figure illustrates applications of CPPs in cancer therapy, centering on their role in protein mimicry to allow various therapeutic interventions. CPP has an enhanced chemotherapeutic efficacy as it improves intracellular drug delivery and pharmacokinetics, and also allows the modulation of protein–protein interactions critical for disrupting oncogenic signaling pathways. CPPs can also act as vehicles for antibody delivery and immune detection, as well as for conjugation strategies such as linking with drugs to promote targeted cytotoxicity. Figure adapted from [93]. https://doi.org/10.1016/j.addr.2021.114044.

Poor prognosis, particularly for duodenal cancer, gliomas, and lymph metastases, was a significant obstacle in cancer treatment. Refractory cancers have a limited capacity to be treated since delivery methods couldn't get medications to the treatment site due to the complicated tumor microenvironment. CPPs may serve as the molecular motor for tumor-deepening cargo. To create mixed micelles for siRNA systemic administration, two tandem peptides (pTP-PEG-iRGD and pTP-iRGD) were synthesised by Lo et al., 2018 to solve the target and tumor stroma penetration problems in PDAC [94]. It could successfully get beyond PDAC's delivery obstacles to penetrate tumors in three-dimensional organoids and models of native tumors. Moreover, the mixed micelle complexed siRNA greatly slowed the development of the tumor [94]. To treat gliomas, CPPs may cause cargos to pass the blood–brain barrier (BBB). To transport siRNA against glioma, Liu et al., 2018, created an anionic random-coiled polypeptide (PLG) coated CPPs (PVBLG-8) micelle [95]. To create a stable structure in serum and turn the micelle's surface potential negative, PLG became entangled with the PVBLG/8/siRNA complex [95]. Also, micelle could carry out PVBLG-8's cell penetrating activity in response to low pH in the tumor extracellular microenvironment. CPPs were paired with glioma-homing peptides to precisely translocate siRNA to increase the target limit of CPPs in glioma applications [35,95]. To maximise tumor-specific targeting and gene knockdown impact, the bonding form of two CPPs (PF14, PF28) with targeting moieties via either covalent conjugation or non-covalent complex was adjusted [35]. Lymph nodes nearby were the site of the first tumor metastasis and eventually extended across the entire body, making lymph metastasis an important channel of tumor spread [35]. Because of the blood-lymph barrier, current medicines that target lymphatic metastasis by intravenous injection have limited non-target and low penetration capability. After being administered intravenously for a possible anti-metastasis treatment, a R9 modified cabazitaxel nanoparticle (R9-CN) with a 13 nm size and a little positive charge was shown to have a conspicuous lymph target and deep penetration impact [35]. R9-CN's fluorescence signal persisted in primary tumor locations for at least 24 hours at a high intensity [96]. In a breast cancer lymphatic metasta-

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sis model, R9-CN significantly reduced the tumor growth rate by 1.4-fold and demonstrated a 63.3% inhibition rate of lung metastasis compared to CN. Deep lymphatic penetration made CPP-modified nanoparticles an excellent anti-metastasis platform [96].

7.6. Use of CPPs in the Treatment of Inflammatory Conditions

The stratum corneum and mucosa are the primary delivery-related challenges with transdermal administration, which has high compliance and is an efficient method of local delivery of anti-inflammatory medicines. Polyarginine peptides are frequently used in transdermal medication administration because of their ability to penetrate skin. To treat rat paw edema, Gao et al., (2019) created lornoxicam-loaded lipid gels that were R11 modified (LN-NLC-R11) [97]. Rat paw edema was eliminated, and LN-NLC-R11 dramatically reduced the generation of inflammatory cytokines as compared to NLC in vivo [97]. The distance between CPPs and nanoparticles will affect the effectiveness of cellular internalisation because of steric hindrance.

Due to steric hindrance, the separation between CPPs and nanoparticles will affect the effectiveness of cellular internalisation. To reduce pulmonary inflammation, CPPs modified gene carriers R9Gn-chitosan/siMIF (n = 0, 4, 10) were developed [35]. R9Gn-chitosan/in siMIF's vivo cell uptake, gene silencing effectiveness, and anti-inflammatory efficacy were all enhanced by lengthening the Gn controlled spacer arm [35]. In a mouse model of particulate matter-induced airway inflammation, R9G10-chitosan/siMIF dramatically decreased inflammation and goblet cell hyperplasia of lung tissue compared to R9-chitosan/siMIF [98]. Similar to this, intranasal injection of phospholipase D1 (PLD1) conjugated with TAT enhanced the anti-asthmatic action [99]. Epidermal hyperplasia is a prevalent condition with a significant immune cell infiltration known as psoriasis. A significant contributor to the pathophysiology of psoriasis is signal transducer and activator of transcription 3 (STAT3). The high-affinity peptide designed to inhibit STAT3 is called APTstat3. R9 (APTstat3-9R) altered APTstat3 to increase stratum corneum penetration [35]. After topical intradermal therapy, APTstat3-9R showed positive efficacy in reducing localised psoriasis-like skin irritation [35]. APTstat3-9R, however, had minimal skin penetration after transcutaneous injection because the stratum corneum barrier inhibited it. APTstat3-9R complexed with DMPC/DHPC to create discoidal lipid nanoparticles (DLNPs) with great colloidal stability (20 nm size) and minimal side effects, improving transdermal administration [35]. In a mouse model of psoriasis, DLNPs were able to pass through the stratum corneum because of their lipophilicity and then pass through the openings between epidermal layers to reach the dermal layers [100]. The imiquimod-induced psoriatic mouse model's skin edema and epidermal hyperplasia were successfully decreased by DLNPs [100].

8. Restrictions on the Use of CPPs

Being one of the most effective methods for transfection in many cell types, CPPs have been employed more and more over the past 30 years in the diagnosis and treatment of various diseases. To improve the treatment result, CPPs have proved essential in obtaining therapeutic concentrations in cells and tissues that are challenging to target. Its success is based on both its flexibility and its potent transmembrane delivery feature [35]. They can easily be enhanced, changed, and synthesised.

CPPs are a two-edged sword, though, since they may have serious negative consequences for several reasons. FDA has not yet authorised any CPP-conjugated medications, and numerous clinical trials have been stopped. One of the major limitations of CPPs is their poor specificity for target cells and tissues. CPPs can penetrate virtually any cell membrane, including healthy cells, which can result in off-target effects and unwanted toxicity. This lack of selectivity can be particularly problematic for cancer treatment, where the goal is to target cancer cells specifically while sparing healthy cells. To overcome this limitation, several strategies have been developed to enhance the specificity of CPPs, such as conjugating them with targeting moieties, such as antibodies or aptamers, that recognize cancer-specific markers or receptors. The quick consent from blood has a downside in that enzymatic breakdown might cause a therapeutic payload to deteriorate in circulation before reaching the therapeutic location. Because of its polypeptide nature, CPPs will enhance the likelihood that patients may have an unfavourable immunological reaction, which might both lower the efficacy of the medicine and result in an unpleasant immune stress response [101]. To increase exposure and decrease the immunogenicity of treatments, it can be administered in high doses and at regular intervals; however, doing so causes toxicity. The alternative method is to provide treatments subcutaneously, which can lessen the immune system's tendency to produce anti-drug antibodies [102]. CPPs can be internalised by practically all types of cells due to treatments being absorbed by normal tissues - off-target by cells. However, it should be noted that anything might become cytotoxic at a high dosage, and many concerns need to be solved before being formally administered to the patients [35]. CPPs are foreign molecules that can trig-

ger immune responses, leading to inflammation and other adverse effects. They can accumulate in certain organs, such as the liver and kidneys, which can cause toxicity and organ damage over time [35]. To address these issues, researchers have focused on developing CPPs with reduced immunogenicity and toxicity profiles, as well as improving their pharmacokinetic properties to enhance their clearance from the body. The majority of studies revealed minimal toxicity of CPPs. CPPs' cytotoxicity is a major basis of apprehension [103]. As a typical CPP, the MAP, for instance, shares structural similarities with antimicrobial lytic peptides that impact microorganisms by disrupting their plasma membranes [104]. In addition to these limitations, CPPs also face several challenges in molecular diagnostics and cancer treatment. One of the challenges is the efficient delivery of CPP-cargo conjugates to their intended targets. CPPs are often used to deliver cargoes, such as nucleic acids, to specific cell types, such as cancer cells, where they can regulate gene expression or induce cell death. However, the effective delivery of these conjugates to their intended targets remains a significant challenge due to the complex and heterogeneous nature of tumors and the surrounding extracellular matrix. Another challenge is the development of CPP-based therapeutics that can overcome drug resistance mechanisms. Cancer cells can develop resistance to chemotherapy drugs and other targeted therapies, which can limit their effectiveness. To address this issue, researchers have focused on developing CPP-based therapeutics that can bypass drug resistance mechanisms and enhance the efficacy of existing therapies. MAPs can cause the leaking of protons, proteins, metal ions, etc., due to their amphipathic action in the presence of artificial micelles, which leads to cell death from plasma membrane injury [35,105]. The ability of cationic CPPs to attach to glycosaminoglycans is well acknowledged, however, it is unclear if CPPs can interact with certain membrane receptors [106]. Because of a reduced local concentration, the efficacy of CPP-conjugated therapies can be decreased in tissues with extensive tissue distribution [35]. As a result, it is critical to target certain cells as much as possible while disregarding normal cells. Another major issue is the transport of CPPs and CPP/cargo complexes to the cytosol from endosomes before lysosomal destruction [107]. It is thought that medications that remain inside endosomes are unable to perform biologically. To hasten the release of the carrier from the endosome into the cytosol, CPPs should be designed in a way that efficiently promotes endosomal escape [35]. To achieve effective therapy, the most crucial ways for CPP delivery are not only translocated to the target tissue or organ but also targeted into certain organelles in the cell, such as the nucleus and mitochondria, thereby ensuring that the therapeutic cargo reaches its precise intracellular site of action [108]. To summarise, it is needed urgently to develop optimal CPPs with low toxicity, high efficiency, and specificity to fulfil the clinical use of CPPs and to solve therapeutic problems related to CPPs. Despite these challenges and limitations, CPPs have the potential to revolutionize molecular diagnostics and cancer treatment. Their ability to penetrate cell membranes and deliver various types of cargoes to cells has opened up new avenues for the development of targeted therapies and precision medicine. In the future, further research is needed to address the challenges and limitations of CPPs and to fully realize their clinical potential.

9. Future Prospects

In clinical trials, evaluating the efficacy of a therapeutic agent is paramount; however, several other critical factors must be considered for successful drug development. These factors include large-scale production feasibility, physicochemical properties, stability, toxicity, pharmacokinetics, formulation strategies, and the chosen route of administration. For CPPs, despite their ability to efficiently transport biomolecules across cellular membranes, challenges such as poor specificity, limited stability, and rapid clearance from the body restrict their clinical applicability [109]. To improve their pharmacological efficacy, various strategies have been investigated to overcome these limitations.

Cationic CPPs have demonstrated an ability to improve the intracellular delivery of large hydrophilic biomolecules, including peptide nucleic acids and oligonucleotides. However, their uptake primarily occurs via endocytic pathways, necessitating effective mechanisms to promote endosomal escape to maximize bioavailability. Promising approaches involve conjugating lipid moieties, particularly fatty acids, to cationic peptides, which can enhance their biological activity. The efficacy of these lipid modifications depends on the length of the fatty acid chain, with longer chains (ranging from C8 to C16) showing progressively increased effects [109]. However, this enhancement comes at a cost, as cytotoxicity also rises with increasing fatty acid chain length. Extensive in vitro investigations have indicated that CPPs generally exhibit low toxicity and minimal immunogenicity. However, their immunogenic potential varies depending on physicochemical characteristics such as molecular size, charge distribution, amino acid composition, structural morphology, and the specific type of conjugated cargo [109]. These attributes collectively influence both membrane integrity and the probability of eliciting an immune response. So more studies are required to fully

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understand and mitigate any immunological risks associated with CPPs. The fusion or conjugation of CPPs with therapeutic cargoes, such as drugs or vaccines, has been shown to mitigate their inherent toxicity. Nonetheless, precise dose optimization remains a crucial aspect of research in the development of CPP-based therapeutics.

One of the most significant barriers to the clinical application of CPPs is their vulnerability to enzymatic degradation in biological environments. Upon exposure to serum or cellular conditions, CPPs are readily degraded by proteases present in bodily fluids such as blood, gastric and intestinal secretions, extracellular matrix, and intracellular compartments. This rapid degradation severely limits their systemic half-life, thereby reducing therapeutic efficacy. The reticuloendothelial system, particularly in the liver and spleen, actively eliminates positively charged molecules, further restricting the circulation time of peptide-based therapeutics [109]. Addressing these challenges requires strategic chemical modifications that hinder proteolytic recognition and degradation, thereby prolonging the half-life and enhancing cellular uptake. Traditional approaches to improve peptide stability include modifications at the peptide termini. N-terminal acetylation and C-terminal amidation are commonly employed to shield the peptide backbone from enzymatic cleavage. A more advanced and effective strategy involves stereochemical modifications, such as incorporating D-amino acids or non-natural amino acids [109]. These modifications disrupt enzymatic recognition, significantly extending the intracellular retention of CPPs. However, while such alterations can enhance proteolytic resistance, excessive modifications can introduce undesirable toxic effects. For instance, a study comparing two CPP derivatives found that while one retained low toxicity, a fully modified counterpart containing only D-amino acids exhibited severe toxicity in vivo.

An alternative approach to improve CPP stability involves the physical shielding of peptides using hydrophilic polymers, such as polyethylene glycol (PEG). PEGylation can protect CPPs from enzymatic degradation, enhance their metabolic stability, prolong circulation time, and reduce immunogenicity [109]. Despite these advantages, PEGylation also presents a major drawback: it diminishes CPP interactions with cell membranes, ultimately reducing cellular uptake efficiency. To overcome this limitation, precise control over polymer size and conjugation density is required. Researchers have thus explored strategies to ease the detachment of PEG near the target site to restore CPP functionality. However, concerns regarding PEG stability, particularly its susceptibility to oxidative degradation and limited excretion, have prompted investigations into alternative polymers. Polyglycerols, including linear, dendritic, and hyperbranched variants, have emerged as promising substitutes due to their superior resistance to oxidation and thermal stress. Comparative studies have shown that cyclic CPPs exhibit superior internalisation efficiency and stability over their linear counterparts. This enhanced performance is attributed to their increased affinity for target receptors, which facilitates more effective cellular entry. Unlike linear peptides, cyclic CPPs display greater rigidity, reducing entropy loss and allowing tighter interactions with phospholipids in fluid membranes [109]. This property is particularly advantageous for endosomal escape, as higher membrane affinity leads to more efficient transduction. Research has further demonstrated that specific modifications, such as peptide suturing via covalent cross-linking of amino acid side chains, can significantly enhance metabolic stability and membrane permeability by stabilizing the peptide's conformation.

10. Conclusions

Various biomacromolecules, including polypeptides, proteins, and nucleic acids, can be transported into cells by CPPs through the cell membrane, which transcends the limitations of conventional drug administration, and opens up new possibilities for the study of drug carriers and producing positive outcomes. There has been advancement, particularly in the areas of tumor therapy, cardiovascular illness, nervous system disease, and vaccine research, but several issues still need to be resolved before CPPs can truly be used in clinical practice. One is that proteolysis and quick liver and kidney clearance cause CPPs to have a short plasma half-life when transporting proteins or peptides, which leads to poor CPP stabilization. It may be essential to double-double embed CPPs and medications into macromolecular carriers (such as liposomes or biopolymers) to solve this issue. Another option is to link hydrophilic PEG. The second is the lack of tissue and cell type specificity when CPPs help transmembrane transport, which prevents the drug from building up in the target site and results in relatively significant damage to healthy tissues and cells. To get around this issue, several researchers have attempted to construct several modified CPPs that cover up their membrane-penetrating properties before reaching the target site and take advantage of alterations in the tumor microenvironment once they have arrived there. To achieve anti-tumor targeted therapy, CPPs' transmembrane transport function can be restored through, for instance, changes in pH value, enzyme type and activity, or the application of external stimuli. The study of the CPPs delivery system is currently becoming increas-

ingly in-depth. A new concept for the entry of exogenous biomolecules into cells is presented by the transduction technology facilitated by cell-penetrating peptides, which offers promise for the treatment of disorders. As research progresses, the number of cell-penetrating peptides and their analogues is expected to increase, along with further investigation into their mechanisms of membrane penetration.

Abbreviations

CPP	Cell-Penetrating Peptides
TAT	Trans-activator of Transcription
BBB	Blood-Brain Barrier
HIV	Human Immunodeficiency Virus
PTDs	Protein Transduction Domains
NLS	Nuclear Localization Signal
SV40	Simian Virus 40
NMR	Nuclear Magnetic Resonance
PPII	Polyproline II
FGF	Fibroblast Growth Factor
CME	Clathrin-Mediated Endocytosis
CvME	Caveolin-Mediated Endocytosis
AP-2	Adaptor Protein Complex 2
FM4-64	(a fluorescent endocytic marker; typically referred to by its trade name)
CHO	Chinese Hamster Ovary
HEK293	Human Embryonic Kidney 293
MDCK	Madin-Darby Canine Kidney
TP	Transportan
125I	Iodine-125
68Ga	Gallium-68
64Cu	Copper-64
99mTc	Technetium-99m
111In	Indium-111
177Lu	Lutetium-177
GFP	Green Fluorescent Protein
GAGs	Glycosaminoglycans
F-actin	Filamentous Actin
FAM	Fluorescein Isothiocyanate
TAMRA	Tetramethylrhodamine
MDR	Multidrug Resistance
R8	(Arginine) Octamer (8 arginine residues)
R8-taxol	A conjugate of R8 with Taxol (paclitaxel)
R9	(Arginine) Nonamer (9 arginine residues)
p53	Tumor Protein 53
p16	p16 Protein (a cyclin-dependent kinase inhibitor)
SMAC	Second Mitochondrial-Derived Activator of Caspase
TRAIL	TNF-Related Apoptosis-Inducing Ligand
RIP	Ribosome Inactivating Protein
LMWP	Low Molecular Weight Protamine
siRNA	Small Interfering RNA
VEGF	Vascular Endothelial Growth Factor
MPG-8	(A shortened version of MPG; specific peptide variant)
CLIO	Cross-Linked Iron Oxide particles
MRI	Magnetic Resonance Imaging
HA	Hyaluronic Acid
10-HCPT	10-Hydroxycamptothecin
NPs	Nanoparticles
PDAC	Pancreatic Ductal Adenocarcinoma
PLG	(An anionic random-coiled polypeptide; designated as PLG)
PVBLG-8	(A specific cell-penetrating peptide)
PF14	(A specific CPP variant)
PF28	(A specific CPP variant)
CsA	Cyclosporin A
PAD	Pro-Apoptotic Peptide
EPR	Enhanced Permeability and Retention
pDNA	Plasmid DNA
AMPK	AMP-Activated Protein Kinase
ULK1	UNC-51 Like Autophagy Activating Kinase 1
iRGD	Internalizing RGD (Arg-Gly-Asp) peptide
PEG	Polyethylene Glycol
R9-CN	R9-modified Cabazitaxel Nanoparticle
CN	Cabazitaxel Nanoparticle
R16	(Hexadeca-Arginine; 16 arginine residues)
MCF-7	Michigan Cancer Foundation-7 (breast cancer cell line)
R11	An 11-mer arginine peptide used for modification
LN-NLC-R11	Lornoxicam-Loaded Nanostructured Lipid Carrier modified with R11
R9Gn-chitosan/siMIF	A gene carrier composed of R9Gn-chitosan complexed with siRNA targeting MIF (Macrophage Migration Inhibitory Factor)
R9G10-chitosan/siMIF	A variant of the gene carrier where the spacer consists of 10 glycine residues (R9G10) Complexed with siRNA targeting MIF
PLD1	Phospholipase D1
STAT3	Signal Transducer and Activator of Transcription 3
APTstat3-9R	A high-affinity peptide designed to inhibit STAT3 that is modified with 9 arginine residues
DMPC	1,2-Dimyristoyl-sn-glycero-3-phosphocholine
DHPC	Dihexanoylphosphatidylcholine
DLNPs	Discoidal Lipid Nanoparticles
FDA	U.S. Food and Drug Administration
MAP	Model Amphipathic Peptide

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