Cell-Penetrating Peptides: Design, Synthesis, and Applications

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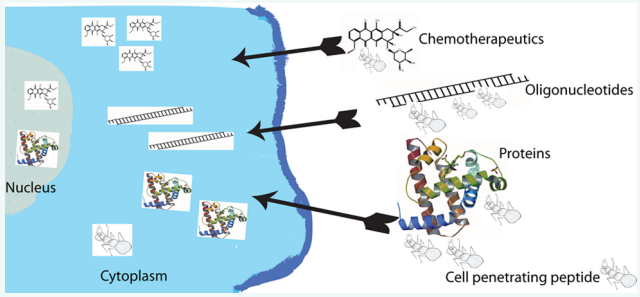
ABSTRACT The intrinsic property of cell-penetrating peptides (CPPs) to deliver therapeutic molecules (nucleic acids, drugs, imaging agents) to cells and tissues in a nontoxic manner has indicated that they may be potential components of future drugs and disease diagnostic agents. These versatile peptides are simple to synthesize, functionalize, and characterize yet are able to deliver covalently or noncovalently conjugated bioactive cargos (from small chemical drugs to large plasmid DNA) inside cells, primarily *via* endocytosis, in order to obtain high levels of gene expression, gene silencing, or tumor targeting. Typically, CPPs are often passive and nonselective yet must be functionalized or chemically modified to create effective delivery vectors that succeed in targeting specific cells or tissues. Furthermore, the design of clinically effective systemic delivery systems requires the same amount of attention to detail in both design of the delivered cargo and the cell-penetrating peptide used to deliver it.



KEYWORDS: cell-penetrating peptides · delivery agents · uptake mechanism · nanoparticles · gene therapy · nucleic acids

The hydrophobic nature of cellular membranes protects cells from an influx of exogenous molecules, including bioactive molecules such as peptides, proteins, and oligonucleotides. Several strategies have been developed to deliver therapeutic agents across cellular membranes. These include microinjection, electroporation, and liposome and viral-based vectors. However, these methods have various drawbacks, including low efficiency, high toxicity, penurious bioavailability, and poor specificity. An alternative strategy to traverse the impermeable phospholipid bilayer of the cell membrane emerged from two unexpected findings. In 1988, the HIV TAT transactivating factor was discovered,[1,2] and a few years later, the *Drosophila* Antennapedia transcription factor[3] proteins were shown to be able to translocate cell membranes and enter cells. These discoveries were followed by the revelation that short sequences of these proteins exerted membrane-crossing properties, including the 16-mer peptide derived from the third helix homeodomain of Antennapedia (later named penetratin) and the 11-mer derived from TAT protein. TAT and penetratin served as the foundation for the development of a new type of molecular vector able to promote the delivery of a variety of cargos: cell-penetrating peptides (CPPs). In recent years, a vast number of interdisciplinary studies report numerous applications for CPPs in the delivery of various cargo such as nucleic acids, polymers, liposomes, nanoparticles, and low molecular weight drugs.[4] The main characteristics of the CPPs are low cytotoxicity, their ability to be taken up by a variety of cell types, dose-dependent efficiency, and no restriction with respect to the size or type of cargo.[5]

In general, CPPs are relatively short peptides that consist of less than 40 amino acids and are able to enter cells by means of various mechanisms, including endocytosis, and are further able to assist in the intracellular delivery of covalently or noncovalently conjugated bioactive cargos.[6] Sequences of common CPPs are provided in Table 1.

Design. CPPs can be divided into different groups based on their distinct characteristics. Another way is to classify them

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based on their origin. For example, the first CPPs were truncated versions of full length proteins such as penetratin[19] and TAT.[2,18] Later it was found that combining parts of several proteins can lead to new, chimeric sequences that can also have cell-penetrating properties.[9,20] As more CPPs emerged, different research groups developed purely synthetic sequences that are also able to penetrate cells; in some cases, these are even more effective penetrators then protein-derived CPP sequences. These synthetic peptides have been developed using prediction programs, rational design strategies, or even trial and error.

The common virtue of all CPPs is that they are able to efficiently pass through cell membranes while carrying a wide variety of cargos inside cells. Interestingly, CPP sequences are known to vary considerably as seen by examining Table 1. There are, however, several similarities between the structural nature of these short peptides. Almost every CPP sequence involves positively charged amino acids. In fact, a chain of arginines forms one of the most widely used CPPs.[21] The membranolytic properties of a given CPP can also be governed by its secondary structure, specifically helicity. It has been shown that peptides with an α-helical region can more efficiently enter cells.

Initially, CPPs were composed of only natural amino acids. Recently, research groups have found it necessary to include nonprimary and even unnatural amino acids[22] and other modifications in order to improve existing CPPs or to create new ones. For example, when lysine residues are replaced with ornithine residues, the peptide becomes more resistant to cellular degradation.[23,24] Cargo delivery efficiency can also be improved by changing the structure of the peptides. For example, peptides have been modified into dendrimers,[25−31] cyclic peptides,[32] and the often used strategy of modifying to the side chains of CPPs, as in the case of TP10 derivatives[33−35] developed by our group and depicted in Table 2. Each modification must be carefully designed to avoid problems that could lead to low synthesis yields, poor solubility, aggregation, or toxicity.

VOCABULARY: **Cell-penetrating peptides (CPPs)** - peptides that contain fewer than 40 amino acids and are able to enter cells by means of various mechanisms, primarily endocytosis, and also are able to facilitate the intracellular delivery of covalently or noncovalently conjugated bioactive cargos (including nucleic acids and low molecular weight drugs) in a nontoxic manner; **Endocytosis** - an energy-dependent process of engulfing molecules carried out by several pathways, including both phagocytosis, which is used to uptake large objects such as other cells, viruses, or bacteria, and pinocytosis (typically one of the macropinocytosis, clathrin- and caveolae-independent endocytosis, or clathrin- and caveolae-mediated endocytosis), which is used for solute uptake followed by endosomal escape; **Prediction of CPPs** - a technique used to discover new classes of CPPs through the use of structure−activity studies and the selection of their descriptors, with the latter being the most challenging aspect of achieving good results. To enhance successfully delivery of either CPPs or CPP−cargo systems into cells and tissues, several strategies can be employed. These include changing the secondary structure (*e.g.*, designing an amphipathic CPP), the hydrophobicity (*e.g.*, a stearoyl group is present in the peptide chain), or charge (*e.g.*, the addition or subtraction of positively or negatively charged amino acids in the sequence); **Gene therapy** - considers the delivery of genetic material into cells, *via* the use of viruses and potentially by CPPs to treat degenerative diseases, various cancers, neurodegenerative diseases, and viral infections;

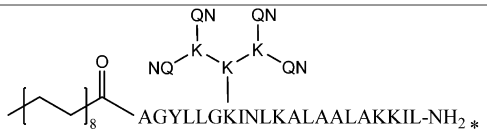
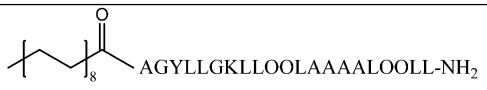
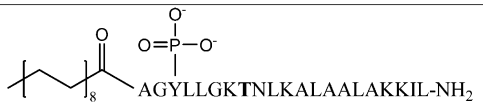
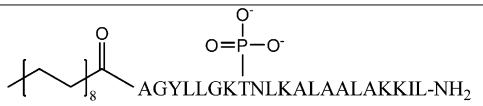
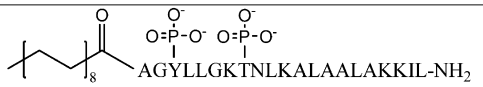
The need for further modifications arises when CPPs are used to deliver larger biomolecules (nucleic acids, proteins) inside cells. For example, *in vivo* delivery requires a longer drug circulation time, which in turn requires a more stable complex to form between the CPP and its cargo. One strategy to achieve this is to add different hydrophilic groups to an already cell-permeable peptide.[35]

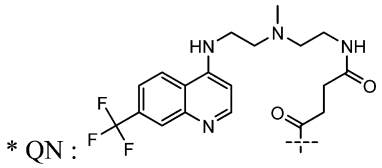
TABLE 1. Sequences of Common CPPs

name	sequence	origin	refs
TAT (48−60)	GRKKRRQRRRPPQ	human immunodeficiency virus type 1 (HIV-1) TAT	1,2
penetratin	RQIKIWFQNRRMKWKK	Drosophila Antennapedia homeodomain	3
MAP	KLALKLALKALKAALKLA[a]	amphipathic model peptide	7
transportan/TP10	GWTLNS/AGYLLGKINLKALAALAKKIL[a]	galanin-Lys-mastoparan	8,9
VP22	NAKTRRHERRRKLAIER	herpes simplex virus	10
polyarginine	R_n,[a] $n = 8,9$	positively charged sequence	11
MPG	GALFLGFLGAAGSTMGA[b]	a hydrophobic domain from the fusion sequence of HIV gp41 and NLS of SV40 T-antigen	12
Pep-1	KETWWETWWTEWSQPKKKRKV[b]	NLS from Simian Virus 40 large T antigen and reverse transcriptase of HIV-1	13
pVEC	LLIILRRRIRKQAHAHSK[a]	VE-cadherin	14
YTA2	YTAIAWVKAFIRKLRK[a]	MMP cleavage site as seeding sequence	15
YTA4	IAWVKAFIRKLRKGPLG[a]	MMP cleavage site as seeding sequence	15
M918	MVTVLFRRLRIRRACGPPRVRV[a]	the tumor suppressor protein p14ARF	16
CADY	GLWRALWRLLRSLWRLLWRA[b]	derived from PPTG1 peptide, W and charged amino acids	17

[a] C-terminal amide. [b] C-terminal cysteamide.

TABLE 2. Examples of Transportan 10 Modifications That Increase Cargo Delivery Efficiency

Name	Sequence of the peptide	Reference
Stearoylated-TP10, PepFect 3		36
PepFect 6		34
PepFect 14		23
NickFect 1		35
NickFect 2		35
NickFect 3		35



When using CPPs as a delivery system, one must consider that drug delivery must often be highly specific. This has also become a major issue in the design of CPPs because they tend to enter cells nonselectively. The use of a specific drug alleviates this problem, but there are also ways to improve the delivery vector. For example, cell-specific peptide sequences have been identified and included within to target breast cancer cells.[37] With these promising results, it is possible that CPPs could one day emerge as tissue-specific transport vectors for a variety of bioactive molecules.[38,39]

Prediction. Predicting the effectiveness of CPPs is not yet a routine task. Currently, this task is obfuscated because the exact mechanism of cellular entry is still debated. Although endocytosis is widely believed to be the main contributor, especially if CPPs are coupled to a cargo, direct penetration[40,41] and even receptor-mediated uptake[42,43] have also been reported for specific CPPs. Resolving these discrepancies is made difficult because there is no generally accepted system for testing the entry of CPPs, and differences in the experimental approach and setup can lead to contradictory results. For example, the cellular entry mechanism of the widely used CPP TAT was initially reported to be dependent on endocytosis almost as frequently as direct penetration.[44] After years of research, the evidence has inclined more toward endocytosis-mediated uptake routes, especially when TAT is coupled to cargos.

Another factor that must be taken into account when predicting CPPs is the natural environment of their action. The general CPP structure and sequence is important; however, cell penetration takes place inside cellular membranes. Therefore, the chemical environment on and within a specific membrane should be considered, including the amount of cell surface proteoglycans and the magnitude or presence of a membrane potential. Conversely, CPPs have been shown to enter yeast,[45,46] bacterial,[47,48] and even plant cells,[49] in

addition to mammalian cells. This might indicate that the peptide sequence has more influence on cellular entry than the cell membrane composition itself.

Successful prediction of effectiveness of CPP entry can, however, be achieved with the aid of structure–activity studies whose success largely depends on the choice of descriptors to employ. First, although effective CPPs are known to possess a positive charge, it has been shown that arginine residues contribute to cellular internalization more than lysine residues. This is probably because of the extra hydrogen bond donation by the guanidinium group. Another important descriptor is the hydrophobicity of a given CPP. When the hydrophobic interactions between the CPP and the cellular membrane are too high, no internalization occurs. Optimal hydrophobicity can lead to vastly improved uptake. For example, different research groups have made wide use of stearoylation and myristoylation of various CPPs.[36,50–56] The third important factor in CPP prediction is the length of the peptide. Usually, CPPs consist of between 15 and 25 amino acids; however, oligoarginines and even pentapeptides have been shown to enter cells. When these factors are taken into account, together with physical properties such as solubility and aggregation, the prediction algorithm becomes rather complicated. Also, it is not known exactly which parts of a given CPP are actually responsible for membrane translocation because some fragments within a sequence may only serve as cargo.

Despite all of these complications, predicting the effectiveness of CPPs is possible. The prediction methods employed can be divided into two classes: the so-called educated guess method, which consists of trial and error, and a completely predictive approach, which relies on a choice of descriptors.

The trial and error method has produced over one-half of all the known CPP sequences. These findings largely originate from studying protein sequences. A segment with more positive charges and a suitable length is selected and then tested for its cell penetration properties *in vitro*. This work is laborious because several partially overlapping sequences have to be chemically synthesized and tested.

A computational approach can be applied by incorporating z-descriptors.[57] Using Sandberg's expanded z-scales, Hällbrink/Hansen *et al.* created an algorithm that finds potential CPPs within protein sequences.[58,59] To build the z-scales, many variables were collected for each amino acid. One downside of this implementation is that the program only accounts for the sum of descriptors and not the actual order of the amino acids. Despite this, this method has yielded several novel CPPs. Improving this approach could involve employing more z-descriptors or additional scales. One recent modification of this approach is the use of basic biochemical properties of peptides instead of z-scores and coupling them with a machine learning technique such as a support vector machine.[60] This method can be applied to screen a large number of candidate CPPs, and interestingly, 100% of the CPPs predicted using this approach were shown to be penetrating. On the other hand, one peptide that was predicted to be nonpenetrating displayed CPP properties, which exemplifies the complexity of predicting cell-penetrating peptides. A few examples of relevant bioactive CPPs obtained with the aid of quantitative structure–activity relationship algorithms include a series of peptides derived from tumor suppressor protein p53 that are able to both translocate several cancer cell lines and induce apoptosis in MDA-MB-231 cells[61] and peptide p28 that is derived from azurin and increases post-translational p53 levels, thereby inhibiting cancer cell growth.[62]

Another method to successfully predict the effectiveness of CPPs with 80–100% accuracy involves combining a computational model based on artificial neural networks (ANNs) with principle component analysis (PCA).[63] The ANN modeling approach was employed because the experimental data of CPPs are essentially binary—the peptide either is or is not able to translocate into cells. PCA was used as a molecular feature selection tool for the ANN inputs and thus enabled the selection of the best molecular descriptors.

In future models, the biological efficacy of CPPs should be included; however, implementing this requires a well-described and universal protocol so that the results can be readily comparable between different research groups.

Structure of CPPs and CPP–Cargo Complexes. The cellular internalization properties of cell-penetrating peptides depend highly on their secondary structure, more specifically on the structure adopted after interaction with the cellular plasma membrane. This makes some CPPs, such as TP10 and MAP, highly membrane-permeable but also renders them cytotoxic at higher concentrations. The overall secondary structure depends on the charge, hydrogen bonds, and helical properties of the peptide. Several attempts have been made to identify CPP structures in different settings. Popular methods to measure the formation of α-helices and β-sheets in peptides are nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy. Investigations made by NMR spectroscopy are more laborious to perform; however, the structure of CPPs has been observed inside cells using the live-cell NMR technique.[64] The environmental medium in which the structures are measured can give divergent outcomes. For example, when using cellular growth media, the results are more in accordance with naturally occurring conditions within living cells, but the measurement procedures and interpretation of the data can become technically difficult. Therefore, most NMR experiments

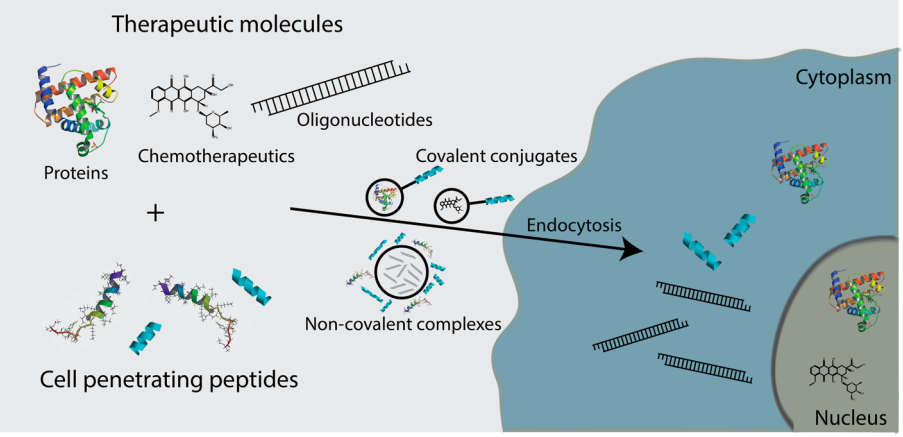


Figure 1. Intracellular delivery of CPP–cargo complexes.

are carried out in water or other subtle buffers. Because interaction with the cellular membrane is a crucial factor in the CPP internalization processes, several research groups have determined the secondary structure of CPPs in model membranes. This can easily be achieved using CD methods combined with phospholipids and unilamellar vesicles and results in a method that characterizes the structure of CPPs in an environment that closely corresponds to a normal cell membrane.[65] Nevertheless, these results must be interpreted carefully. The membrane constituents can vary greatly between different cell types, and in order to draw conclusions on the actual membrane interaction mechanisms, the model system should be as similar to the biological experiment settings as possible. Another method employed to characterize membrane interactions is molecular dynamics. This approach uses data obtained from CD spectra accompanied by thermodynamic data obtained from the analysis of CPP interactions with model membranes. Molecular dynamic simulations are carried out to determine electrostatic interactions, the orientation, and structure of the peptide and peptide–membrane interactions. These results can be used to create a mechanistic translocation model from the initial insertion into the cell membrane to cytosolic entry.[66−68]

Although the interaction of CPPs with cellular membranes often determines their uptake efficiency, cell-penetrating peptides are first and foremost delivery vectors. This means that they must efficiently deliver various cargo molecules to their designated location, whether it is in the cytoplasm or nucleus. The type of CPP employed can have substantive effects on the cargo delivery efficacy. In general, when coupled to larger molecules (*e.g.*, proteins, oligonucleotides (ONs), small interfering RNA (siRNA), micro-RNA (miRNA), plasmid DNA (pDNA), *etc.*), CPPs transport their cargo inside cells using endocytosis. The process consists of (a) complexation between the cargo and peptide, (b) interaction with the cellular plasma membrane followed by endosomal uptake, (c) endosomal escape, and (d) cytoplasmic or nuclear localization (Figure 1). The extent of these steps is determined by the nature of the CPP and its cargo.

Complexes between CPPs and cargo can be formed through a covalent bond or noncovalently. Examples of covalent conjugation are neutral cargo (phosphorodiamidate morpholino oligomers (PMO), peptide nucleic acids (PNA), small drug molecules) complexes which can be coupled to CPPs *via* a disulfide bond, an amide bond, or other specific linkers. Noncovalent complexes are formed *via* electrostatic and/or hydrophobic interactions between negatively charged cargo molecules, such as nucleic acids (siRNA, pDNA, *etc.*), and a positively charged CPP. The structure of these particles can be difficult to determine because the particles can aggregate and form very complex secondary and tertiary structures. A number of common methods used to characterize these nanoparticles include gel electrophoresis, dynamic light scattering (DLS), zeta-potential, and electron microscopy. One limitation of DLS and zeta-potential measurements, as with the use of NMR spectroscopy, is the media used during the measurement. Although results should be obtained in an environment as close to the natural environment of the cell as possible, this is often not possible due to technical limitations. For example, particles tend to aggregate in the presence of serum proteins, thus use of DLS can provide size distributions and polydispersity index values that are unreliable.

The second step in the internalization of CPP–cargo nanoparticles is membrane permeation. When peptides are coupled to larger biomolecules, for example, oligonucleotides, CD and NMR spectra become very difficult to analyze. One approach is to determine the binding affinity and thermodynamic parameters of the complex using isothermal titration calorimetry (ITC) or competitive binding assays. Again, these studies are difficult to carry out under physiological conditions.

Considering that the main mechanism of uptake is endocytosis, CPP−cargo complexes need to escape from the endosomal vesicles in order to induce a biological effect. In general, this is the most difficult step to verify because it can be composed of several processes. There are numerous theories to explain why CPP−cargo complexes escape from endosomes. One line of thinking is that hydrophobic and electrostatic interactions occur between the endosomal membrane and the nanoparticles, which leads to disruption of the endosomes. Another, the proton sponge theory, proposes that endosomes burst due to osmotic pressure.[69] If the complexes remain trapped inside the endosomes, they can be subjected to lysosomal degradation which abolishes the biological effect of the cargo. This process can be modeled using molecular dynamics which take into account the structural aspects of the complex, dynamics of the complex formation, and binding free energies between the complex and the endosomal membranes.[70]

After escaping the endosomes, the CPP−cargo complex needs to dissociate so that the cargo is able to promote its intended biological effect. For this, the nanoparticles must be designed such that they exhibit optimal binding affinity upon reaching the cytosol, after which the cargo is released. Some molecules, such as oligonucleotides and plasmid DNA, need to reach the nucleus in order to be effective. Nuclear localization can be achieved using nuclear localization signals[71] and by facilitating nuclear pore complexes. Further modifications in the structure of existing CPPs and/or the design of new CPPs are required in order to achieve suitable nanocomplexes for *in vivo* applications. For further reference, the reader is pointed to an in-depth model of peptide-based nanoparticle formation and internalization developed by Divita *et al.*[72]

Cellular Uptake Mechanisms of CPPs. Although numerous studies report on the uptake mechanisms that transport CPPs across the plasma membrane, the exact pathways through which CPPs enter cells have not been absolutely resolved.[73,74] Early experiments were often conducted using microscopy or flow cytometry on fixed cells. Despite many similarities among CPPs, the mechanism of their uptake has been seen to vary considerably. The use of different experimental conditions can also affect the cellular uptake and translocation mechanism of CPPs, as evidenced by contradictory observations. For example, contradictory results are obtained through the use of different concentrations, cell types, incubation time, and various physiochemical parameters of the CPPs, including hydrophobicity and net charge.[75−79] Therefore, it is likely that CPPs without any cargo can be taken up by cells *via* multiple pathways, including direct penetration of the plasma membrane and endocytic uptake mediated by clathrin, caveolae, and/or other molecules, depending on the nature of the peptide/cell interaction.

Role of Endocytosis. Endocytosis, including phagocytosis and pinocytosis,[80,81] is a highly regulated process of internalization of solutes and fluids from the extracellular matrix into cells. Phagocytosis is a complex process used to uptake large particles such as other cells, viruses, or bacteria, while pinocytosis is mainly used to uptake smaller particles. Endocytosis occurs by the action of various pathways which can be classified into caveolae and/or lipid-raft-mediated endocytosis,[82] macropinocytosis,[83] cholesterol-dependent clathrin-mediated endocytosis,[84] or caveolae- and clathrin-independent endocytosis (Figure 2). The main differences between the energy-independent pathways and endocytosis lie in the membrane permeation and release steps.

Early mechanistic studies concurred that the cellular uptake of CPPs followed a non-endocytic pathway. These studies, however, showed that experimental artifacts could affect the results.[79] For instance, the fixation of cells with methanol/formaldehyde could redistribute the CPPs bound on the cell surface but not influence their internalization. Following this observation, the majority of the research groups concurred that the involvement of one or more pathways of endocytosis are taking part in the intracellular uptake of CPPs or CPP−cargo complexes, and that this depends not only on the nature of the CPP but also on the type of cargo attached to it.[85]

Generally, both lone CPPs and CPP−cargo complexes can translocate through the cell membrane *via* direct translocation or *via* endocytosis. In non-endocytotic pathways, CPPs localize directly in the cytoplasm after traversing the plasma membrane, whereas during endocytosis, CPPs may or may not be released into the cytosol and can even end up in intracellular vesicular compartments.[24] It is commonly accepted that certain CPP−cargo complexes penetrate the plasma membrane directly and facilitate the intracytosolic delivery of the cargo.[41,86] It has also been proposed that some CPPs, especially TAT, Antennapedia, Poly-Arg, transportan, MPG, and Pep-1, can even pass through the plasma membrane *via* at least one energy-independent pathway.[87] The importance of the presence of membrane-associated proteoglycans on both uptake and macropinocytosis has also been reported.[83] Macropinocytosis does not operate in cells passively; it is activated only when specific stimuli (*e.g.*, growth factors or viruses) are applied. Membrane-associated proteoglycans serve as one of the primary receptors that induce macropinocytosis.

In some cell types, macropinocytosis may be one of the preferred pathways for cellular uptake of arginine-rich peptides and larger CPP conjugates. Although a general scheme for CPP uptake mechanisms remains elusive, there is a consensus that the initial contact between the CPPs and proteoglycans on the cell surface take place through electrostatic interactions, after

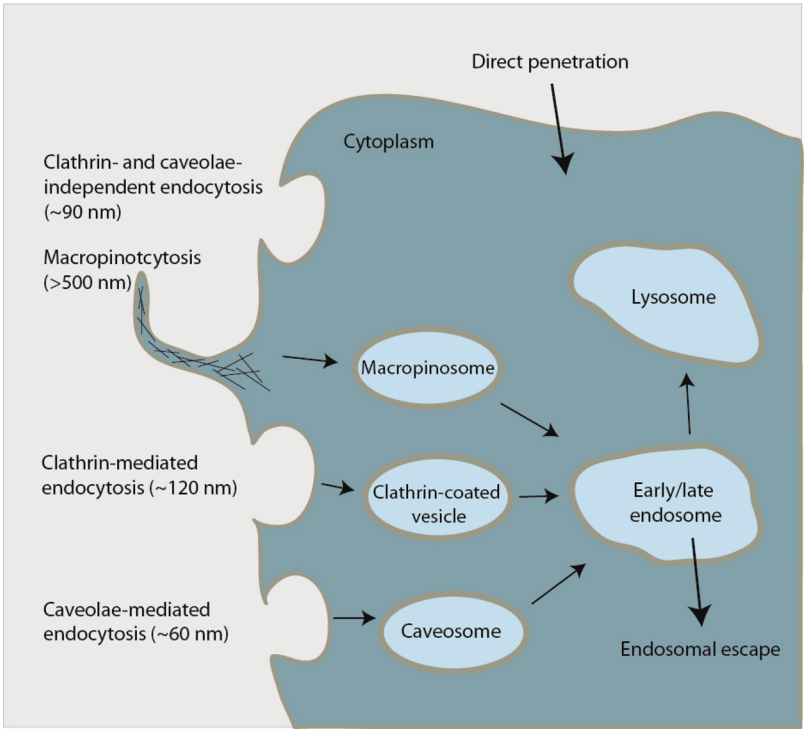


Figure 2. Uptake and released mechanisms of cell-penetrating peptides.

which the cellular uptake pathways are driven by several parameters including (i) the nature and secondary structure of the CPP; (ii) the ability to interact with cell surface and membrane lipid components; (iii) the nature, type, and active concentration of the cargo; and (iv) the cell type and membrane composition.

Role of Glycosaminoglycans. Generally, as most CPPs are positively charged, the first interaction partners they encounter at the cell surface are negatively charged carbohydrates (mainly glycosaminoglycans (GAGs)) and negatively charged lipids. CPPs can bind electrostatically *via* various other cellular polyanions at the cell surface such as negatively charged proteins, inositol phospholipids, N-linked GAGs (*e.g.*, sialic acid), and O-linked GAGs (*e.g.*, heparan sulfate, HS), followed by remodeling of the actin network and selective activation of the small GTPase RhoA or Rac1 but also of RNA, DNA, and tubulin that are involved in endocytotic events and cell signaling.[88] There are known mammalian transmembrane syndecans, and the glycosylphosphoinositide-linked glypicans, which are members of cell surface heparan sulfate proteoglycan families. These can bind a large number of extracellular ligands, thus modulating the action by enhancing formation of their receptor-signaling complexes. Glypicans are covalently linked to a phosphatidylinositol in the outer leaflet of the cell membrane and their glypican ectodomains (extracellular domain) are presumably compact and globular proteins due to their characteristic 14 conserved cysteine residues that are expressed predominantly in the central nervous system.[89] The N-terminal, divergent extracellular domains contain three glycosaminoglycan attachment sites for heparan sulfate near the N-terminus and may bear chondroitin sulfate at a juxtamembrane region.[90,91] The cytoplasmic domains of syndecans interact with a number of proteins, including actin-binding proteins and F-actin. These interactions can be regulated by phosphorylation of an invariant serine residue and three invariant tyrosine residues. There is evidence that cationic CPPs (penetratin, octaarginine, and TAT) utilize syndecan-4 to bind and mediate transport through the plasma membrane into cells.[92] It was reported that heparan sulfate proteoglycans could be responsible for the internalization of the full-length TAT protein.[93] This process was also confirmed to be important for $(R)_8$.[94] Melikov and co-authors have shown that arginine-rich CPPs and arginine-rich CPP−cargo conjugates interact with heparan sulfate proteoglycans with a lower affinity than TAT protein.[95] On the other hand, recent studies have reported that proteoglycans may actually be less important for CPP uptake than was previously thought.[96,97]

Ligands that bind to proteoglycans can be internalized through an endocytotic pathway. For example, both the TAT protein and TAT peptide were shown to bind strongly to heparin, a sulfated GAG that mimics the heparan sulfate proteoglycans. It was also demonstrated that, in addition to heparin and dextran, another sulfated GAG inhibits the cellular uptake of TAT peptide.[98] The internalization efficiency of a given CPP

may vary from one cell type to another according to the membrane carbohydrate composition, such as that found between tumor and normal cells.[99] However, cell surface proteoglycans appear to promote the uptake of arginine-rich cell-penetrating peptides. For transportan−protein complexes, the interaction with the cell surface proteoglycans is highly probable.[100] Prior to cellular uptake, membrane-associated proteoglycans, including heparan or chondroitin sulfate proteoglycans, play a role in the accumulation of arginine-rich CPPs and their NAP conjugates on cell surfaces *via* an interaction between the guanidino function of arginine and sulfates in the proteoglycans.[94,101] Recent reports have shown that scavenger receptors, a family of cell surface glycoproteins, are also involved in the uptake of negatively charged CPP−cargo complexes.[42,102] Indeed, the balance between the internalization pathways of a given CPP, pinocytosis *versus* membrane translocation, is influenced by the carbohydrate composition of plasma cell membranes. For example, mutant CHO cells, lacking the GAGs chondroitin and HS or only HS, were used in different studies. At 37 °C, the internalization efficiency of a fluorescent penetratin, TAT and oligoarginine, was considerably reduced in cells lacking all types of GAGs.[103,104] At 4 °C, when all endocytotic pathways are slowed, the quantities of the three CPPs were similar in wild-type and GAG-deficient cells.[105] The internalization of penetratin was also significantly inhibited in cells lacking only HSs, while that of TAT was inhibited[106] or not,[103] depending on the respective study. All of these studies demonstrate that GAG plays a major role in the internalization of some CPPs and that the endocytotic pathway is strongly dependent on the presence of GAGs on the cell surface.

Role of Negatively Charged Lipids. Lipids are also not distributed symmetrically across the two leaflets of the membrane bilayer of healthy cells. Despite this, a very small portion of negatively charged lipids is found in the outer leaflet. One can hypothesize that peptides are able to recruit negatively charged lipids to create negatively charged nanodomains on the surface of the cell. As an example, the fraction of anionic lipids (*e.g.*, POPG and POPS) is considerably smaller in eukaryotic cells (10%) than in bacterial cells (up to 50%). Primary amphipathic CPPs can bind with strong affinity to both neutral and anionic lipid membranes, which suggests that their membrane interaction is dominated by hydrophobic interactions. The presence of anionic lipids, which localize mainly on the cytosolic side of the cell membrane, does not affect their membrane affinity. Upon membrane binding, the surface tension is reduced, which also indicates their insertion into the membrane. Primary amphipathic CPPs generally penetrate deeper into the hydrophobic core than other CPPs[75] but do not span the bilayer in a pore-like manner. Instead, they display a tendency to self-associate in the headgroup region, which could be relevant for the various models that propose direct translocation of CPPs.[107−109] The presence of a transmembrane potential promotes the insertion of CPPs into membrane with reports of pore formation. However, it has been established that transportan, TP10, and MPG lead to membrane perturbations (leakage) already at submicromolar concentrations, with a mechanism analogous to the behavior of the cationic antimicrobial peptide mellitin.[110] In contrast, a high concentration of anionic lipids in the membrane reduces their insertion into the lipid bilayer, so that membrane lysis is most pronounced for charge-neutral lipid membranes and with a high phosphatidylethanolamine content.[111] Although it is likely that electrostatic bonds play a key role in the interaction of CPPs with lipids, other unidentified lipids from the outer membrane leaflet might be important interaction partners for internalization. As an example, it has been shown that the presence of zwitterionic dioleoylphosphatidylethanolamine in an egg phosphatidylcholine bilayer increased the affinity of penetratin from 28 to 0.5 μM, which is similar to the affinity of penetratin for a pure negatively charged egg polyglutamate bilayer (0.3 μM).[112] These results highlight that a given CPP can interact in cell membranes that not only contain negatively charged lipids but also with cone-shaped lipids that are prone to inducing negative curvature in cell membranes, an observation that is relevant for the direct membrane translocation of peptides associated with these membrane nanodomains.[113]

Proposed Models for Cellular Internalization of CPPs. An early mechanism suggested for the translocation of CPPs was termed the inverted micelle model,[19] and those for antimicrobial peptides were termed the carpet[114] and the pore formation model.[115] The inverted micelle model of internalization was proposed by Alain Prochiantz's group, based on NMR studies of the interaction between penetratin and phospholipid membranes.[19] Although this model can explain the translocation for some CPPs, for example, penetratin,[116] it is not sufficient to explain the uptake of either TAT or polyarginine peptides, which do not contain the hydrophobic amino acids necessary for the translocation process.

In the carpet model, the internalization starts with the peptide binding with negatively charged phospholipids, and following this, the rotation of the peptide leads to interactions between the hydrophobic residues of the peptide and the hydrophobic core of the membrane. Lastly, a small disruption occurs in the lipid packing, which permits the internalization of the peptide.

The final proposed model for cellular translocation is the pore formation (barrel-stave) model, which results from the formation of bundles by amphipathic α-helical peptides. The pores form when the outwardly

facing hydrophobic residues interact with the lipid membrane and the inwardly facing hydrophilic surfaces form a pore when the concentration of the peptide is higher than a certain concentration threshold, which is different for different peptides.[117,118]

It is also possible that peptides may enter the cell by an unknown biological mechanism that works through interactions with other cell surface components, especially when the peptides exist in excessive concentrations. However, it is still a matter of debate which cell surface molecules act as potential electrostatic binding partners for CPPs and how they trigger or mediate their biological uptake.

Uptake Pathways of CPPs and CPP–Cargo Complexes. While the mechanism of CPP-mediated cell entry has been the subject of many studies, there is still no consensus on the mechanisms of translocation. More work is needed to provide clear insight into the mechanisms of cellular uptake and internalization of CPP–cargo complexes. Most likely, a combination of different model systems and techniques is required to study the various mechanisms of cellular entry. However, there is a general consensus that most cell-penetrating peptides or CPP–cargos are taken up by various types of endocytosis. It is suggested that the entry of a CPP–cargo complex into the cell could be influenced by a variety of factors, including the nature of the conjugated cargo (type, size, charge) differences in the physicochemical properties of CPPs (such as molecule length, charge delocalization, hydrophobicity, and other physicochemical parameters), the cell line being utilized, and the concentration of CPP. Cargos are generally covalently linked to a CPP through a disulfide bond; however, the noncovalent strategy has proven to be very efficient.[72,119]

Depending on the size of the cargo, several internalization routes may act simultaneously. For example, for both TAT and Poly-Arg CPPs, both endocytosis and direct entry mechanisms are involved in the cellular membrane permeation process. TAT has been shown to enter cells *via* lipid-raft-mediated endocytosis, caveolae-mediated endocytosis, and/or macropinocytosis when conjugated with large molecules (proteins, quantum dots) and through clathrin-dependent endocytosis when the cargo was a fluorophore.[120] Small cargos, such as peptides, enter slowly by endocytosis and rapidly by transduction *via* an unknown mechanism that provides direct access to the cytosol and depends on the membrane potential.

To improve the efficiency of transfection, Futaki and co-workers evaluated the effect of introducing a hydrophobic moiety (stearoyl) to arginine-rich CPPs. The interaction of these hydrophobic moieties with each other or with nucleobases in the plasmid/CPP complexes may enhance compaction of the pDNA and lead to self-assembling particle formation that will favor the delivery of nucleic acid pharmaceuticals both *in vitro* and *in vivo*.[51]

The initial step of internalization of the nanoparticles may be driven by electrostatic forces between positively charged cationic CPPs and the negatively charged cell surface membrane. Recently, it was reported that the uptake of negatively charged CPP–cargo complexes is mediated by scavenger receptors.[42] Nevertheless, Saleh *et al.* hypothesize that very large CPP/ON nanoparticles may hinder uptake and shift the endocytic pathway toward macropinocytosis.[121] The characteristics of CPP–cargo complexes are largely dependent on the exact ratio of CPP to cargo in the formulation, termed the molar ratio. Four key parameters are thought to affect the transfection efficiency of CPP–cargo complexes: size, charge, stability, and cell association. These parameters affect endosomal uptake of the CPP–cargo complexes, the main route of internalization for macromolecular complexes.[122]

Furthermore, labeling a peptide with different fluorophores may influence both the uptake mechanism and intracellular distribution. Using live-cell imaging of HeLa cells, labeled cell-penetrating peptides were seen to colocalize with transferrin, a glycoprotein marker for endocytosis.[123] However, other studies with fluorescently labeled polyarginine conjugates and fusion proteins show that these do not colocalize with transferrin.[98] It has also been shown that TAT–GFP fusion proteins are taken up by HeLa and Jurkat cell lines *via* lipid-raft-dependent caveolar endocytosis.[124] Thoren and co-authors described both cytoplasmic and nuclear uptake of fluorescein-labeled 1–10 μM R7W into PC-12 and V79 cells *via* a mechanism that is not inhibited by ATP depletion and is even promoted at 4 °C. In contrast, penetratin was internalized in both cell types in an ATP- and temperature-dependent manner.[125]

Role of Concentration. The mode of uptake for many cationic CPPs also varies depending on the CPP concentration. In general, direct penetration most likely occurs at high CPP concentrations (>10 μM) as is often the case for primary amphipathic CPPs such as transportan analogues and mastoparan. When the CPP concentration is increased to a certain threshold, it follows a carpet-like model in which interactions between the cationic CPPs and negatively charged extracellular matrix result in a carpeting and thinning of the membrane, respectively.[77] Endocytosis is believed to be the most common uptake mechanism at low CPP concentrations. Translocation is the internalization pathway activated at low micromolar concentrations of penetratin, while GAG-dependent endocytosis, a cooperative and saturable process, is activated at higher micromolar concentrations.[105] At low peptide concentrations, cellular permeation is achieved by endocytosis, and above a certain concentration threshold, peptide internalization for TAT and R9 occurs by direct uptake.[74] Taken together at low micromolar concentrations, CPPs shuttle from membrane carbohydrates

to membrane lipids and *vice versa*. Higher peptide concentrations potentially lead to GAG clustering, while peptides can still bind to the lipids.

Direct Penetration. The ongoing debate continues over the roles of direct and energy-independent uptake of CPP-mediated cargo delivery. Different energy-independent cell entry pathways for CPPs and CPP−cargos have been suggested, such as the (1) inverted micelle model, (2) pore formation model, (3) carpet model, and (4) membrane thinning model.

Several studies confirm that direct penetration can also be involved in the uptake of several CPP−cargo complexes. For example, arginine-rich peptides, such as TAT, deliver covalently linked protein cargo effectively into the cytosol when cells are pretreated with pyrenebutyrate, suggesting a process of direct penetration.

Other peptides, such as MPG, Pep-1, penetratin, and CADY also seem to translocate into cells *via* direct penetration mechanisms.[17,126,127] Recently, correlations between the cell surface binding and cellular internalization of penetratin show improved uptake of a penetratin analogue with increased number of arginines and can be explained by its enhanced cell surface adsorption.[112,128] In an elegant study, cationic (R6) and amphipathic (MAP) CPPs were incorporated into polyplexes and evaluated for siRNA delivery. The results demonstrated that MAP is both more efficient and a more suitable CPP for siRNA delivery than R6.[129]

A novel series of cell-penetrating peptides derived from an endogenous neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) and its native PACAP isoforms (PACAP27 and PACAP38) efficiently delivered a large and nonpermeable molecule, streptavidin, into cells. An inactive modified fragment of PACAP38, [Arg17]PACAP(11−38), with preserved cell-penetrating physicochemical properties, was also synthesized and successfully used for the intracellular delivery of various cargos such as small molecules, peptides, proteins, and polynucleotides. Studies on the uptake mechanism demonstrate that direct translocation, caveolae-dependent endocytosis, and macropinocytosis were all involved in the internalization of [Arg17]PACAP(11−38).[130,131]

Taken together, the presence of a cargo influences the internalization pathway, the intracellular trafficking, and localization of the conjugate. It is also obvious that conjugating a cargo to a CPP changes the chemical profile of the peptide which alters its interaction with all molecular partners on the cell surface. Thus, the efficiency of the internalization pathway, together with the intracellular trafficking and final intracellular localization of the CPP−cargo complex, relies on the overall size, charge, and hydropathy of the conjugate.

The primary goal is still to obtain the most suitable peptides that are fully controlled in terms of delivery to the correct location while achieving the highest biological efficacy. The greatest challenge will be designing of peptides that have specificity for specific GAGs, lipids, or membrane proteins and thus have the ability to select one internalization pathway over another to specifically deliver the cargo to cells or tissues and thereby promote its biological activity.

CPP Uptake Mechanism Studies. Because the cellular uptake of CPPs is dependent on a variety of factors, including temperature, incubation time, cell type, cargo type and size, and linkage type and size, comparison between different experiments is difficult. This has compounded the controversy surrounding uptake mechanisms. Because it is probable that different endocytic routes are involved in CPP uptake, one needs to interfere with these pathways by using various endocytosis inhibitors or by lowering the temperature to elucidate the uptake mechanism of CPPs. In some examples, chlorpromazine and sucrose are used to inhibit clathrin-mediated endocytosis (CME). In CME, a specific coat protein, clathrin, which contains light and heavy chains, is required to self-assemble on the intracellular face of the cell membrane after which clathrin-coated pits form. Chlorpromazine, a cationic amphipathic drug, triggers the depletion of clathrin and an AP2 adaptor protein complex from the cell membrane and leads to their artificial assembly on endosomal membranes. Due to its amphipathic structure, chlorpromazine is able to insert itself into the plasma membrane and thereby change its fluidity. It can also inhibit phospholipase C which is important for actin dynamics and macropinocytosis. Both of these factors could give rise to misinterpretations.[132] Wortmannin is an inhibitor of PI3K. PI3K-generated lipid mediators are highly needed in the reorganization of the actin cytoskeleton because these molecules regulate nucleation, elongation, and bundling of actin filaments. The fact that oligoarginine internalizes less when incubated with the macropinocytosis inhibitor 5-(*N*-ethyl-*N*-isopropylamiloride) proves that at least a fraction of the peptide is internalized *via* macropinocytosis.[18] However, the uptake of TAT and $(R)_8$ in K562 cells suggests that macropinocytosis is not the only entry mechanism for these peptides. Another study demonstrates that TAT uptake in HeLa cells in the presence of chlorpromazine, a known inhibitor of clathrin-mediated endocytotic pathway, results in 50% inhibition of peptide uptake, while incubation in a potassium-free buffer results in a 40% decrease, thus indicating the involvement of a clathrin-dependent pathway.[133] In addition, studies on the uptake of these peptides in HeLa cells confirmed that the presence of caveolae is not required for cellular internalization.[134] In the case of the treatment of cells with nocodazole and cytochalasin D, the peptide traffic from early to late endosomal structures was inhibited, which demonstrates a cytoskeletal requirement for lysosomal delivery.[135] Treatment of the cells with different

TABLE 3. Endocytosis Inhibitors Used To Study the Uptake Pathways of CPPs

inhibitor or treatment condition	affected pathway	inhibition mechanism
chlorpromazine and sucrose	clathrin-mediated endocytosis (CME)	clathrin/AP2 depletion from plasma membrane to endosomal membranes/dispersion of clathrin from plasma membrane
wortmannin	macropinocytosis and CME	phosphatidylinositol 3-kinase inhibitor
5-(*N*-ethyl-*N*-isopropylamiloride)	macropinocytosis	sodium-proton exchange inhibitor
nocodazole	caveolae-mediated endocytosis	inhibit, respectively, the polymerization of actin and microtubule cytoskeleton
cytochalasin D	macropinocytosis	blocking of actin polymerization, disassembly of actin cytoskeleton
4 °C	endocytosis	CPP uptake inhibition
chloroquine	endosomal escape	promotes endosomal escape

endocytosis inhibitors efficiently suppresses the cellular uptake of pVEC. This effect is more pronounced for wortmannin, which indicates the presence of a clathrin-dependent endocytotic pathway.[136] However, uptake at lower temperatures confirms the presence of non-endocytotic pathways in the pVEC uptake mechanism. A conjugate of pVEC with avidin translocates through the membrane by using clathrin-dependent endocytosis, but the presence of another mechanism is also most likely to occur under different conditions.[14] The uptake of M918 seems to depend on both macropinocytosis and clathrin-mediated endocytosis (CME), and the predominant cellular entry route of TP10 is also consistent with CME.[137] The kinetics of cellular uptake has also been shown to depend on the size of the cargo attached to the CPP. From recent results, it is obvious that endocytosis inhibitors affect both the first-order rate constant and the total uptake level of CPP conjugates. The endocytosis inhibitors affect the uptake of M918 peptide yet have no influence on the uptake of pVEC. In the case of TP10, the uptake is lowered and the rate constant is increased in the presence of chlorpromazine, wortmannin, and sucrose, while cytochalasin D slightly increases the overall uptake but has no effect on the uptake rate. These data suggest that different competing uptake mechanisms with different efficacies may be involved in the simultaneous uptake of different CPPs.[138]

Recently, a new antimicrobial peptide−CPP chimera was designed and chemically synthesized. This chimera is composed of a Cecropin-A/melittin hybrid moiety directly fused to the arginine-rich motif of HIV-1 TAT protein (CM18−TAT11) and exhibits substantially higher uptake in HeLa cells compared with naked CM18. The difference in cellular uptake of the peptides was explained by a higher vesicle-loading ability of CM18−TAT11 compared to CM18 rather than on an increase in the overall number of vesicles during the endocytosis process (Table 3).[139]

Thermodynamic Studies. The binding process between a peptide and a cell membrane (and the associated physical constants) can be determined using different methods. Isothermal titration calorimetry is a very powerful technique which allows for the determination of K_d, stoichiometries, and binding enthalpies (ΔH). All biological membranes contain, as a basic structural unit, a lipid bilayer. Usually, a very large number of different proteins are embedded within or adsorbed onto the lipid bilayer. These components interact with each other and result in a membrane that is not uniform at the molecular level. Binding experiments show that penetratin, TAT, and oligoarginines bind to model membranes containing negatively charged lipids, and their affinity increases with the concentration of anionic lipids (penetratin,[112,140−144] TAT,[145−147] oligoarginines[145,148−150]).

However, a key question is also to examine whether the peptides are able to translocate across protein-free lipid membranes. In order to investigate these processes, two different types of lipid model systems are employed: large unilamellar vesicles (LUVs) and giant unilamellar vesicles (GUVs). It has been shown that penetratin, R7W, TATP59W, and TATLysP59W exhibit different uptake characteristics in live cells.[125] Membrane interaction studies for penetratin, R7W, TATP59W, and TATLysP59W showed that these peptides exhibit a strong affinity for LUVs containing zwitterionic and anionic lipids and that the binding constants decrease in the order penetratin > R7W > TATP59W > TATLysP59W. In contrast, these peptides rapidly traverse the membranes of GUVs.[145] Also, at elevated peptide to lipid molar ratios (P/L), penetratin is able to induce vesicle aggregation.[151] In a later study, R7W, TATP59W, and TATLysP59W peptides induced vesicle aggregation even more extensively than penetratin. However, there is no clear correlation between the peptide charge and the P/L ratio under which aggregation occurs. The finding that the TAT peptides induce aggregation of DOPC/DOPG (60:40) vesicles at lower P/L compared with R7W is consistent with their higher charge. R7W induced stronger aggregation at P/L 1:100 compared with penetratin-induced aggregation at a P/L ratio of 1:45.[152] Dynamic light scattering experiments revealed that kinetically and thermodynamically stable CPP−heparin (*e.g.*, penetratin, TAT) clusters with diameters around 100 nm were formed.[153,154]

Thermodynamic measurements were used to study the mechanism of transfer of antimicrobial, cytolytic, and amphipathic peptides in model membranes by insertion of the peptide into the lipid bilayer from the surface-associated state. The transport of three TP10 derivative peptides, from water to the POPC

membrane interface and from the interface to the hydrophobic bilayer interior, was assessed using stopped-flow fluorescence. The secondary structure of the peptides was established by circular dicroism. Generally, the Gibbs energy of insertion, $\Delta G^{\circ}_{\text{oct-if}}$, is given by $\Delta G^{\circ}_{\text{oct-if}} = \Delta G^{\circ}_{\text{oct}} - \Delta G^{\circ}_{\text{if}}$, where $\Delta G^{\circ}_{\text{oct}}$ is the Gibbs energy of transfer from water to octanol and $\Delta G^{\circ}_{\text{if}}$ is the Gibbs energy of binding to the interface. The peptides adopted an α-helical structure upon binding on the lipid bilayer−water interface. $\Delta G^{\circ}_{\text{oct-if}}$ is ≤20 kcal/mol for TP10 derivatives, which indicates that the peptides can translocate across the bilayer.[155] These values correlate well with the results obtained by Ladokhin and White.[156] The transfer of TP10 from the membrane surface to its hydrophobic core corresponds to $\Delta G^{\circ} = +19.8$ kcal/mol, which is close to the activation energy ($\Delta G^{\ddagger}$) for bilayer insertion of TP10 from a surface-associated state.[157]

Polycationic peptides, such as the TAT peptide and nona-arginine (acetyl-R_9-amide), which cannot form α-helices, transfer to the interface and to octanol. These peptides have very unfavorable energetic values ($\Delta G^{\circ}_{\text{oct}}$ and $\Delta G^{\circ}_{\text{if}}$); however, the Gibbs energy of insertion, $\Delta G^{\circ}_{\text{oct-if}}$, is less than 20 kcal/mol. The highly cationic peptides bind tightly to the anionic lipid membrane which leads to the formation of peptide−lipid salt bridges that allow the translocation across the bilayer.[158] On the other hand, the binding mechanism of CPPs to different GAGs was extensively studied by ITC.[148,153] Heparan sulfate (HS) and chondroitin sulfate B are found at the cell surface, whereas heparin (HI) is not a cell surface GAG but is structurally related to HS. The K_d values varied between low micromolar and high nanomolar concentrations. For the most well-studied peptides (TAT, penetratin, and oligoarginine), GAG binding was always enthalpically favored, which can be explained by favorable hydrogen bond formation. The values of the dislocation constant K_d of TAT peptide binding to heparin, as measured with isothermal titration calorimetry, vary from 0.37 to 1.5 μM. Interestingly, fluorescein-modified TAT has a K_d 10 times higher than unlabeled TAT,[123] indicating that cargo may greatly influence the affinity and specificity of binding to cell surface proteoglycans.[95]

The interaction of a TAT protein transduction domain (TAT-PTD) with the anionic lipid membrane has been evaluated by surface activity and monolayer penetration studies, NMR, and ITC. This study demonstrated that the binding of TAT-PTD can be characterized by both a multisite binding model and an electrostatic attraction/chemical partition model. The binding of TAT-PTD to both lipid membranes and GAGs is an exothermic process, and the values of exothermic binding enthalpy, ΔH°, are less negative for lipids than for glycosaminoglycans. The total free energy of binding for TAT-PTD in equilibrium with lipid vesicles containing 25% anionic lipid is $\Delta G^{\circ} = -5.2$ kcal/mol. It is assumed that TAT-PTD only binds to the outside of the vesicle, and the apparent binding constant, K_{app}, was found to decrease from 1×10^4 to 2.8×10^3 M^{-1}.[147] Membrane binding data for penetratin show that this peptide does not translocate through the phospholipid bilayer and remains on the outer leaflet. The value of the apparent binding constant was found to be $K_{app} = 1.3 \times 10^4$ M^{-1}.[143] These results prove that penetratin binds better than TAT-PTD to lipid membranes due to conformational changes, that membrane binding of penetratin induces a helix formation, and that hydrophobic residues present in penetratin can intercalate between the lipids.

Bioactivity. Although CPPs are generally designed for noninvasive cargo transport, they can also possess specific bioactivity analogous to antimicrobial peptides which are discussed under applications. There is, however, no terminology to distinguish between inherently bioactive CPPs and CPPs that are fused with bioactive sequences. This arises from the fact that it is difficult to determine which part of a CPP sequence is responsible for cellular internalization and which part actually conveys the bioactivity. A similar problem was discussed earlier in the section discussing the prediction of CPP effectiveness.

One of the first bioactive CPPs found is a 22 amino acid long peptide derived from the N-terminal part of the tumor suppressor protein p14ARF, and it showed pro-apoptotic activity. It was demonstrated that it induces apoptosis and, surprisingly, translocates into cells *via* endocytosis.[159] Since then, several apoptotic cell-penetrating peptides have been developed. By modifying the apoptotic BH3 domain of a Bcl-2 family protein using a stapling technique, we confined its structure into an α-helical conformation.[160] This domain is known to induce apoptosis, and when its secondary structure is altered, it becomes cell-permeable, which transforms it into a bioactive CPP. Recently, human cytochrome *c* was analyzed using QSAR, and two sequences that induce tumor cell apoptosis were also shown to have cell-penetrating properties.[161] It has also been reported that a phosphopeptide mimetic of the small heat shock-like protein HP20 is able to both enter cells and inhibit MK2.[162] Caution must be taken when identifying bioactive CPPs because a similar construct, where a CPP was conjugated to an MK2 inhibitor sequence,[163] should not be considered a bioactive CPP, as the two domains can be easily distinguished. Recently, Howl *et al.* introduced two intrinsically bioactive CPP sequences, termed bioportides, that are able to regulate a cAMP-dependent process and display potent antiangiogenic activity *in vivo*.[38] In another study, two peptides with penetrating properties were obtained from studies that utilized quantitative structure−activity relationship algorithms. These were found to induce specific dose-dependent apoptosis in MDA-MB-231 cells.[61]

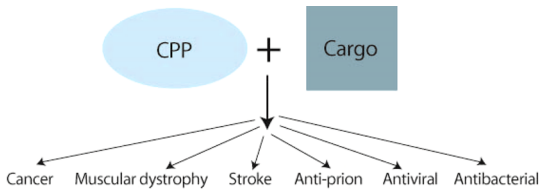


Figure 3. Therapeutic applications of CPP−cargo complexes.

Another important aspect of CPP bioactivity is their possible cytotoxicity. Although this property is generally not associated with CPPs, everything can become toxic once it reaches a certain dose threshold. Because of their efficient membrane disruption properties, both TP10 and MAP can cause membrane leakage as is often the case with antimicrobial peptides. Membrane toxicity can also arise when forming a CPP−cargo complex, and therefore, cytotoxicity assays must be carried out for all CPP applications.

Applications. The ability to introduce drugs (*e.g.*, insulin[164−167]) and other active biomolecules into cells led to investigating the potential use of CPPs as therapeutics. A large number of preclinical studies have reported on the successful applications of complexes of CPPs attached to therapeutic cargos in cancer, muscular dystrophy, cardiology, antiprion diseases, and both viral and bacterial infections (Figure 3).

Cancer Treatment. The systemic treatment of cancer with cytotoxic drugs has been improved for some uncommon tumors such as childhood cancers, lymphomas, and testicular cancers; however, in more common cancers, the improvements are less spectacular, except the combined chemotherapy and endocrine therapy in breast and colorectal cancer. Because treatments with anticancer drugs are sometimes toxic and expensive, new strategies to develop modalities to cure cancer are needed. Anticancer drugs affect cell growth, vascularization, and metastatic spread by different pathways: by preventing effective DNA replication, by damaging the mechanisms of cell division, or by blocking the paths implicated in cell growth that are activated by signals, growth factors, or hormones.[168]

Therapeutics that make use of CPPs have been reported in numerous studies over the last two decades. Properly developed CPPs and their conjugates with therapeutics offer a very promising pathway to deliver lower concentrations of toxic drugs to critical tissues such as tumors, heart, *etc*. Small chemotherapeutic drugs have also been delivered by CPPs (doxorubicin, methotrexate, cyclosporine A, paclitaxel).[169]

In order to inhibit solid tumor growth and to improve patient survival, tumor suppressor proteins can be introduced into cancer cells *in vivo*. For example, the treatment of preclinical terminal peritoneal carcinomatosis and peritoneal lymphoma models with the transducible D-isomer of a retro-inverso peptide, RI-TAT-p53C′, resulted in significant increases in the lifespan and even generated disease-free animals.[170] The growth of bladder cancer cells expressing mutant p53 was inhibited by applying a treatment with J82 and T24 fusion proteins with a D-isomer of cell-penetrating peptides of the p53 C-terminus connected with a retro-inverso version of hemagglutinin-2 protein (HA2).[171] The survival time of animals was considerably extended by using a single dose of d11R-p53C′-riHA2 for an animal model of the peritoneal metastasis of bladder cancer. The C-terminus sequences from p53 protein were used to obtain constructs for two other applications against breast cancer[61] and glioblastoma multiforme.[172] Two stearoylated peptides efficiently induced apoptosis in p53 mutant MDA-MB 231 cells but not in MCF-7 cells that express wild-type p53, thus demonstrating their selectivity.[61] Two constructs derived from the D-isomer of a cell-penetrating peptide (flock house virus, FHV), with sections from a penetration accelerating sequence (Pas: FFLIPKG), and C-terminus of p53 (p53CO) induced the cell death of glioma-initiating cells by preventing the fusion of autophagosomes with lysosomes. dPasFHV-p53C0 was effectively transduced into human glioma-initiating cells in both intracranial and subcutaneous mice models.[172]

Activatable CPPs (ACPPs) are *in vivo* targeting agents which contain a polycationic CPP linked through a specific proteolytically cleavable linker (succinoyl or 6-aminohexanoyl) to a neutralizing polyanionic part in a hairpin-structure-based conformation.[173] The biodistribution of Cy5-labeled ACPPs was established after injection in the tail vein of mice bearing HT-1080 tumors. The enhanced ability of the ACPP compared with other CPPs to reach targeted tissues was demonstrated to be due to their cleavage by disease-associated proteases (MMP-2/9), in cancer and cancer metastases.[174,175] To improve the usefulness of ACPPs that display considerable mortality in mice and have a high background concentration in some tissues, especially cartilage and kidney, their polycationic C-termini were conjugated to dendrimers to create ACPPDs. Furthermore, these ACPPDs have been used for *in vivo* visualization of metalloproteinase activity by using MRI imaging and fluorescence, labeled with Cy5, gadolinium, or both, into both mice bearing HT-1080 xenografts and in the PyMT breast cancer model.[176] This strategy was used for MRI and for dual modality imaging. Furthermore, ACPPs are predicted to become highly selective vectors to deliver macromolecular cargos.

One drawback in the use of chemotherapy is the evolution of drug resistance within the cell population. CPP−drug constructs have great potential to increase the solubility, biodistribution, and pharmacokinetic profiles of currently approved chemotherapeutic drugs. CPP−Dox conjugates (CPPs: TAT, penetratin, maurocalcine analogue isolated from a Tunisian scorpion, Dox: doxorubicin, chemotherapeutic drug) were

demonstrated to exhibit a higher apoptotic efficiency compared with free Dox in the MDA-MB 231 cell line due to the different apoptotic pathways utilized by the CPP construct.[177] Thereafter, TAT and penetratin were covalently bound to Dox and then efficiently delivered into five cell lines which exhibit different chemosensitive properties compared with using Dox alone.[178] Oligoarginines were linked to Taxol *via* a disulfide linker, and these conjugates were shown to be able to overcome resistance to Taxol in Taxol-resistant ovarian carcinoma cells, in animal models of ovarian cancer and in *ex vivo* ovarian cancer patient samples.[179,180] Cationic peptides were able to deliver Dox *in vitro* and *in vivo*, and the amphipathic peptide CADY-1 was able to form noncovalently stable complexes with Dox, which were then efficiently internalized into breast cancer cells (MCF-7) and increased the survival and antitumor activity on xenograft mice compared with free Dox.[181]

The complex formed by a new fluorescently labeled cell-penetrating homochiral cyclic peptide [W_5R_4K] and Dox was observed to efficiently internalize and localize into the nucleus of ovarian adenocarcinoma (SK-OV-3) cells.[32] A noncovalent complex of cyclic peptide $[WR]_4$ and Dox, formed *via* intermolecular interactions, was studied using ITC and shown to be taken up by SK-OV-3 cells in an endocytosis-independent pathway. In an elegant study, Nakase *et al.* demonstrated the efficiency of decreasing tumor proliferation using 4 mg/kg of a $(R)_8$–Dox compound and found no significant loss of bodyweight in tumor-xenografted mice, typically observed using 6 mg/kg of free Dox.[182]

Muscular Dystrophy Treatment. Duchenne muscular dystrophy (DMD) is caused by either deletions or mutations in the dystrophin gene, which disrupts the open reading frame and creates premature termination of translation, thus reducing dystrophin production. A milder form, Becker muscular dystrophy (BMD), is caused by deletions in the DMD gene, which maintains the open reading frame, which results in the production of internally shortened but partially functional dystrophin. Severe DMD can be converted into a milder BMD phenotype by using antisense oligonucleotides that mediate targeted exon exclusion, which restores functionality of the reading frame. In a clinical trial for DMD intramuscular injections of exon-skipping, splice-switching oligonucleotides efficiently restored dystrophin production.[183]

The most common models to study DMD are primary X-linked muscular dystrophy (mdx) myoblast cultures and mdx mouse, which carry a nonsense mutation in exon 23 of the DMD gene that prevents translation of the dystrophin protein. Modified oligonucleotides, PMOs targeted to a donor splice site of intron 23 or other splicing elements in exon 23, force skipping of the exon, which leads to internally deleted dystrophin production. PMOs are uncharged antisense molecules that inhibit gene expression in a sequence-dependent manner by preventing translation or by interfering with pre-mRNA splicing. CPPs conjugated to PMOs and the evaluation of their activity has been tested in different animal models for DMD and has recently been reviewed.[184]

A chimeric peptide–PMO, B-MSP-PMO (B, arginine-rich peptide; MSP, muscle-specific pentapeptide), restored dystrofin protein expression in multiple peripheric muscle groups in *mdx* mice.[185] All striated muscles are affected by DMD, and 30% of deaths in DMD patients are caused by heart failure. Therefore, the induction of dystrophin expression in cardiac muscle is critical for DMD treatment.[186] Using the same arginine-rich peptide B covalently attached to PMO, the efficient PPMO-mediated exon-skipping therapy in DMD patients was demonstrated for the first time to improve treatment for cardiac hypertrophy and diastolic dysfunction.[187] Stearoyl-$(RxR)_4$ mediated the delivery of oligonucleotides in both *in vitro* and *in vivo* models for DMD.[53,188] A stable solid formulation of a chemically modified TP10-based CPP, PepFect14, and a splice-correcting oligonucleotide that is highly soluble in water was demonstrated to have efficient activity in mdx mouse myotubes, a model for DMD.[23]

Stroke Treatment. Strokes occur when blood flow to the brain stops. Within minutes, brain cells begin to die. There are two kinds of stroke. Ischemic stroke is caused by a blood clot that blocks or plugs a blood vessel in the brain, while hemorrhagic stroke is caused by a blood vessel that breaks and bleeds into the brain.

Several studies focus on both the *in vitro* and *in vivo* pharmacological activity of CPP-conjugated compounds.[189] Cao *et al.* produced a biologically active Bcl-xL fusion protein containing an 11 amino acid CPP from TAT protein and a hemagglutinin (HA) tag that allowed *in vitro* and *in vivo* delivery into neurons.[190] An i.p. injection of 9 mg/kg was efficient for a reduction in infarct sizes of up to 40%, 72 h after focal ischemia without altering cortical blood flow, body temperature, or other physiological parameters during or after ischemia. *In vivo* delivery of this protein across the blood–brain barrier occurred within 2–4 h in a murine model of transient focal ischemia.

Delivery of a peptide inhibitor, selective for protein kinase C (δV1-1), conjugated through a disulfide bond to TAT, led to effective protection against cerebral ischemic reperfusion damage in an *in vitro* hippocampal slice model and in *in vivo* rat transient focal ischemia.[191] The D-retro-inverso form of the peptide-specific inhibitor of c-Jun N-terminal protein kinase D-JNKI-1 bonded to TAT, thereby preventing excitotoxicity *in vitro* and reduced *in vivo* brain damage.[192–195] Ifediba *et al.* recently demonstrated the *in vitro* usefulness of optically labeled polyarginine peptides with myristic acid (MPAP)[196] complexed with

siRNA for the treatment of ischemic stroke.[197] Internalization of MPAP−siRNA complexes was evaluated by both confocal microscopy and flow cytometry *in vitro* in cell types implicated in ischemic stroke pathology: cortical neurons, astrocytes, and bEnd.3 brain endothelial cells. Further *in vivo* studies are needed to investigate the delivery of siRNA in the treatment of cerebral ischemia.

Antiprion Treatment. Prion diseases or spongiform encephalopathies belong to a group of progressive conditions that affect the nervous system in humans and animals. In people, prion diseases impair brain function, induce memory and personality changes, cause a decline in intellectual function (dementia), and are more responsible for problems with movement that worsen over time. The signs and symptoms of these conditions typically begin in adulthood, and these disorders lead to death within a few months to several years.

Familial prion diseases of humans include classic Creutzfeldt−Jakob disease (CJD), Gerstmann−Sträussler−Scheinker syndrome (GSS), and fatal insomnia (FI). These conditions form a spectrum of diseases with overlapping signs and symptoms. These diseases are characterized by the accumulation of an abnormal isoform of the prion protein (PrP^{Sc}) in amyloid deposits.[198] Endogenous cellular prion protein (PrP^{C}) expression is an essential requirement for these diseases because mice lacking expression of PrP^{c} cannot be infected with prion disease and neurons lacking PrP^{c} expression cannot be killed by the toxicity of pathogenic scrapie isoform PrP^{Sc}. PrP^{Sc} differs from PrP^{C} on the basis of conformation and the ability to form fibrils *in vitro*. Functional features of PrP^{C} are exhibited in two parts of its N-terminus: (a) region 1−23 which is very hydrophobic and is involved in the entry into endoplasmic reticulum (ER), and (b) the basic part 23−30 which resembles a nuclear localization signal.[199]

PrP^{C} is able to bind to PrP^{Sc} and the peptide PrP106−126 at the same region of the PrP^{C} molecule. Interaction with this region (amino acid residues 112−119) inhibits the uptake of copper into cells and prevents the superoxide-dismutase-like activity of the recombinant protein, which shows that PrP^{Sc} and PrP106−126 directly inhibit the function of PrP^{C}.[198]

Peptides derived from the human prion protein, hPrP19−30 and hPrP100−111, were found to interact specifically with PrP^{Sc} without any significant binding to Pr^{PC}.[200] In addition, prion-protein-derived (PrP-CPPs) peptides were developed that contain the N-terminus 1−22 in mouse mPrP-CPPs and 1−24 in bovine bPrP coupled with sequences 23−28 and 25−30. All peptides tested, including mPrP19−30, influenced PrP^{C} protein levels in GT1-1 cells but had less influence in PrP^{Sc} levels in scrapie infected mouse hypothalmic cell lines (ScGT1-1).[201] The internalization of unprocessed bPrP, with the hydrophobic sequence 1−24 and the basic region 25−30, was demonstrated to be driven into CHO cells *via* macropinocytosis.[202] The lipid-raft-dependent macropinocytosis mechanism was shown to occur in PrP^{Sc}-infected N2a neuroblastoma cells.[203]

Antiviral Applications. Viruses, such as HIV-1, hepatitis B and C, influenza viruses H1N1, H5N1, and severe acute respiratory syndrome (SARS) coronavirus, act on millions of people every year. New or alternative antiviral therapies are required to decrease the risk of global pandemics and to find treatments for acute viral infections. In a recent review, Delcroix and Riley exhaustively described the application of known CPPs to deliver antisense agents into virus-infected cells and animal models. The most widely used antiviral cargos conjugated to CPPs are PMOs.[204] Peptide-conjugated PMO (PPMOs) are water-soluble, nuclease-resistant, and act as steric-blocking antisense agents due to the formation of stable duplexes with complementary RNA. The peptide can be attached at either the 3′ or 5′ end of the morpholino oligomer.[205] Effective cell-penetrating peptides all contained arginine residues: $(RXR)_4B$-, $(RXR)_4XB$-, $R_5F_2R_4C$-, R_9F_2C-, $(RB)_8B$-, and $(RX)_{n=2-8}B$-, with the dash at the C-terminus of the peptide representing the link to the morpholino oligo (R = L-arginine, B = β-alanine, X = 6-aminohexanoic acid, F = L-phenylalanine, C = L-cysteine).

Non-retroviral RNA virus infections are usually dose-dependent and highly specific on the nature of the CPP and PPMO used in *in vitro* and *in vivo* experimental models. These conjugates act by reducing viral replication and significantly increase survival in mice experimentally infected with poliovirus, coxsackievirus B2 and B3, dengue virus, West Nile virus, Japanese and St. Louis encephalitis virus, Venezuelan equine encephalitis virus, equine encephalitis virus, respiratory syncytial virus, Ebola virus, SARS coronavirus, mouse hepatitis virus, porcine reproductive and respiratory syndrome virus, human rhinovirus 14, foot and mouth disease virus, measles virus, and the influenza A virus.[204] The toxicity of PPMOs, their mechanism of action, pharmacologic properties, and the generation and characterization of resistant viruses were all estimated in these studies. Efficient PPMO target sites in viral RNA are composed of regions of highly conserved sequences which is demonstrated to have vital influence in the preinitiation or initiation of translation or in long-range RNA−RNA interactions implied in viral RNA synthesis.[206]

PPMOs were also tested against DNA viruses. Kaposi's sarcoma-associated herpes virus (KSHV) is associated with Kaposi's sarcoma and primary effusion lymphoma.[207] It was demonstrated that KSHV replication and transcription activator (RTA) and latency-associated nuclear antigen (LANA) play key roles in activating KSHV lytic replication and in maintaining KSHV latency, respectively. In one report, the $R_5F_2R_4C$

peptide was covalently conjugated to the 5′-terminus of PMOs and was successfully utilized to suppress both protein expression of KSHV replication and RTA and LANA in BCBL-1 lymphocytes.[208] In another report, PMOs were covalently conjugated to the 5′-terminus of $R_5F_2R_4C$ and $(RXR)_4XB$ peptides and then applied to target the viral IL-6, an early lytic gene.[209] $(RXR)_4XB$ conjugated to PMO was shown to target HSV-1 mRNAs of immediate-early genes, thereby inhibiting viral replication in both cell cultures and in the eyes of mice.[209]

A new cationic lipid peptide, Deca-$(Arg)_8$, was demonstrated to inhibit duck hepatitis B virus (DHBV) replication.[210] This peptide led to a decrease in HBV secretion in HepG2.2.15 cells without targeting the viral polymerase. Moreover, TAT-conjugated PNA successfully inhibited replication of the severe acute respiratory syndrome coronavirus (SARS-CoV) by targeting the −1 PRF signal.[211]

While developing a treatment against both Ebola virus and Marburg virus infections using PMOs, Iversen *et al.* demonstrated that inhibition of the expression of some viral genes is more important than others.[212] A combination of two viral targets led to both higher flexibility and efficacy. They utilized strategic PMO chemistry to find the best candidates for targeting the VP24 gene of Ebola virus and the NP gene of the Marburg virus: (a) single PMOs, (b) PPMOs in which the peptide is an arginine-rich sequence, and (c) PMO*plus* which contains 2−5 positive charges in the linkages between bases. The optimal therapeutic agents were found to be the single agents PMO*plus*, AVI-7537, which targets the VP24 gene of the Ebola virus, and AVI-7288, which targets the NP gene of the Marburg virus. PMO*plus* demonstrated elevated efficiencies compared to those of PMO and higher tolerability compared to that of PPMO.[212]

Antibacterial Applications. Antimicrobial peptides (AMPs) have been identified in different organisms ranging from prokaryotes to eukaryotes. It has been shown that AMPs have bactericidal and/or bacteriostatic effects against many strains of bacteria, and some even display antifungal activity. AMPs are expected to be useful for both food and pharmaceutical applications because they are effective against multi-drug-resistant bacteria but show no toxicity toward eukaryotic cells due to differences in the composition of membrane lipids and differential lipid recruitment by peptides.[213] There are CPPs (TP-10,[214] pVEC,[215−218] TAT,[219−222] Pep-1,[223−225] MAP,[218] penetratin,[218,221,226] ε-poly-L-lysines,[220,227] histones[228]) which function as antimicrobial peptides, depending on the composition of the membrane and the concentration of the peptide. AMPs are cationic peptides which better associate with the anionic membranes of bacteria than the neutral membranes of eukaryotes.[158,229]

A recent study reported on the inhibitory activity of CPPs against infections of the yeast *Malassezia sympodialis*.[230] From 21 peptides that were tested, six CPPs (penetratin, $(R)_9$, TAT, Histatin5, pVec, and scrambled pVec) reduced the viability of *M. sympodialis* by more than 50% already at a concentration of 0.1 $\mu mol \cdot L^{-1}$.

The cationic antimicrobial peptide thanatin was isolated from the hemipteran insect *Podisus maculiventris*. Thanatin is active against both bacteria and fungi and displays low cytotoxicity. S-Thanatin, which substitutes threonine at position 15 with serine, shows higher activity against this Gram-positive bacteria and displays lower toxicity and better tolerance toward both cations and pH conditions (neutral and slightly basic media). The mechanism of S-thanatin against bacteria includes (i) electrostatic and hydrophobic interaction with lipopolysaccharides (LPS) and phospholipids followed by the attachment to the hydrophilic surface of the lipid membrane like "carpet" (limiting step); (ii) membrane destabilization and distortion; (iii) insertion into the cytoplasmic membrane leading to membrane permeabilization and depolarization; (iv) tattering of the membrane and leakage of cytoplasmic material accompanied by a loss in viability.[231]

Two mastoparans (peptides from the venoms of the wasps *Vespula lewisii* and *Vespa xanthoptera*) have similar mechanisms of interaction with the lipid bilayer as TP10 (a 21-residue, chimeric construct obtained by linking the six residues of the neuropeptide galanin, through an extra lysine residue, to the 14 residues of mastoparan from *Vespula lewisii*).[232−234] Both mastoparans investigated are much less active than TP10 with regards to peptide-induced carboxyfluorescein efflux; however, they are more sensitive to the effect of anionic lipids.[233] The high antimicrobial activity of proline-rich peptides, namely, apidaecins (wild-type and mutants), against bacteria was discovered by using measurements of minimal inhibitory concentrations, confocal laser microscopy, and flow cytometry.[235,236]

CONCLUSIONS

Cell-penetrating peptides can efficiently traverse the plasma membrane of both cells and tissues and are successfully used as delivery vectors for therapeutic molecules. Due to their positive charge, CPPs can condense nucleic acids or they can be covalently conjugated to active biomolecules (nucleic acids, peptides, proteins, chemotherapeutic drugs, *etc.*). After efficient cellular internalization, CPPs are able to release their cargo into the cytosol in order to promote the desired biological effect. When coupled to a cargo, CPPs use endocytosis as the main cellular translocation mechanism. There are several ways to increase the uptake and stability of CPP−cargo complexes *in vivo*, for example, chemical modifications of the structure of known CPPs or rational design of novel CPPs. Further investigations into the structure and mechanisms of

uptake of CPP−cargo complexes will be required to evaluate CPPs as potential delivery tools for biomolecules in *in vitro* and *in vivo* models. Prior to use, CPPs must undergo both pharmacological and toxicological studies *in vivo*. Considering evidence from numerous studies, CPPs have the potential to become a universal tool to carry therapeutic molecules across cellular membranes without a risk of toxicity or inflammatory reactions.

Conflict of Interest: The authors declare no competing financial interest.

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