CPPs differ from most other peptides with respect to specific features that reflect various mechanisms used to enter the cell.

Cell-penetrating peptides: classes, origin, and current landscape

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With more than ten new FDA approvals since 2001, peptides are emerging as an important therapeutic alternative to small molecules. However, unlike small molecules, peptides on the market today are limited to extracellular targets. By contrast, cell-penetrating peptides (CPPs) can target intracellular proteins and also carry other cargoes (e.g. other peptides, small molecules or proteins) into the cell, thus offering great potential as future therapeutics. In this review I present a classification scheme for CPPs based on their physical–chemical properties and origin, and I provide a general framework for understanding and discovering new CPPs.

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Since 2001 more than ten new peptide therapeutics have entered the market, with four reaching global sales over US$1 billion in 2008: Copaxone (US$3.18 billion), Lupron (US$2.12 billion), Zoladex (US$1.14 billion) and Sandostatin (US$1.12 billion). In addition, many new peptides have entered clinical studies, with an average of 16.8 per year between 2000 and 2008, significantly above the 9.6 per year in the 1990s and the 4.6 per year in the 1980s [1].

However, none of the peptides on the market today targets intracellular proteins, thus limiting the potential therapeutic space. An estimate suggests that only 10% of the druggable genome can be targeted by traditional rule-of-five small molecules [2], thereby leaving a large number of targets still untapped. Although many new compounds have been discovered to target protein–protein interactions (PPI), few have made significant progress in clinical trials. For example, Alzhemed failed in Phase III (http://www.bellushealth.com/en/newsroom/?rkey=1508262369&view=96347-2), while the most advanced PPI inhibitors to date are SAR1118 (Phase III), Obatoclax and Navitoclax (both in Phase II) [3].

Cell-penetrating peptides (CPPs) are a class of diverse peptides, typically with 5–30 amino acids, that unlike most peptides can cross the cellular membrane. Since the discovery of the first CPP more than 20 years ago, CPPs have been used for a variety of applications [4]. CPPs can act as vectors for siRNA [5] nucleotides, small molecules, proteins, and for other peptides, both *in vitro* and *in vivo* [6,7]. Importantly, not only can a CPP be used to carry a functional peptide inside the cell, but it can also incorporate a functional motif [8,9].

A few CPPs are currently in clinical trials, including AZX100 [Capstone Therapeutics (http://www.capstonethx.com/); keloid scarring; Phase II], RT001 [ReVance Therapeutics (http://www.revance.com/); wrinkling, skin; Phase II], KAI-9803 [KAI Pharmaceuticals (http://www.kaipharma.com/); myocardial infarction, Phase II] and XG-102 [Auris Medical (http://www.aurismedical.com/); hearing loss; Phase II]. DTS-108 (Diatos SA; cancer) is in preclinical studies.

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CPPs present a great variety in terms of amino acid composition and 3D structure, with examples of cationic, anionic, and neutral sequences showing varying degrees of hydrophobicity and polarity. Although specific groups of CPPs are related by high sequence identity and common structural features, in general CPPs have no sequence homology. This structural diversity results in different modes of uptake, and different levels of uptake depending on the cell line, and other conditions. Furthermore, the type of cargo carried by a CPP, which can be covalently or non-covalently attached to the CPP, can also affect profoundly mode and levels of uptake.

Endocytosis and direct translocation through the cellular membrane are the major mechanisms used by CPPs to gain entry into the cell [10]. Endocytosis occurs by various mechanisms, which can be divided into clathrin-dependent endocytosis (CDE) and clathrin-independent (CDI). In CDE, the cytoplasmic domains of plasma membrane proteins are recognized by adaptor proteins and packaged into clathrin-coated vesicles that are brought into the cell. CDI can come in many forms, such as macropinocytosis and caveolae and/or lipid raft-mediated endocytosis [11]. All these different pathways have been reported to be involved in the uptake of CPPs [12].

Cells internalize and recycle the equivalent of their cell surface one to five times per hour [13]. This continuous internalization process should enable peptides with strong affinity for the cell membrane to enter the cell through at least some endocytic pathway. Alternatively, peptides with the right balance of physical–chemical properties might be able to cross the cell membrane directly, as similar to small molecules. However, the cell membrane is not a homogeneous double layer: some regions are denser; others are more fluid, owing to different lipid composition and lipid density [14,15]. Lipid composition, density and dynamics depend on the cell-type, the specific region of the membrane, and a variety of signaling pathways [16–18]. This heterogeneity results in different levels and modes of uptake depending on the conditions used for testing CPPs [19].

The mode of uptake of many cationic CPPs varies depending on the CPP concentration. Above a concentration threshold, rapid cytosolic uptake is observed, suggesting direct translocation, while at lower concentrations the uptake is primarily endocytic. This behavior has been documented for several cationic CPPs, including R9, Penetratin™, Tat-derived peptides, lactoferrin and $S4_{13}$-PV_{rev} [20–25]. The concentration threshold varies significantly depending on the CPP, but it is generally in the low micromolar range. However, studies on Penetratin in CHO-K1 cells have shown that translocation occurs only below 2 μM [26], thereby suggesting that the cell-type (and thus the membrane composition) affects the balance between different internalization pathways.

The first step for cellular entry of many cationic CPPs is the formation of electrostatic interactions with cell surface glycosaminoglycans (GAGs) [27]. This interaction induces clustering of GAGs at the cell surface and triggers activation of intracellular signals, actin remodeling and cell entry through a variety of internalization pathways ranging from direct translocation to endocytosis. Direct translocation of cationic CPPs might be mediated by acid sphingomyelinase activation, followed by a change in the lipid composition of the cell membrane [28].

Cell-based activity of peptides carried through cationic CPPs often require extracellular concentrations well above 10 μM [29–31], thus reinforcing the notion that certain endocytic pathways do not lead to therapeutically useful concentrations in the cytosol, unless the peptide can access some endosomal escape routes. Entrapment of the peptide in endosomes leads to quick degradation, with the inability to reach cytosolic targets. Because concentrations higher than 10 μM might be difficult to reach for therapeutic applications, this is a major limitation for many CPPs.

Like peptides in general, CPPs suffer other shortcomings as pharmaceutical products: typical short duration of action and lack of oral bioavailability. However, medicinal chemistry and formulation efforts can address both [32]. Short duration of action is caused by proteolysis and rapid renal clearance: proteolysis can be tackled through unnatural amino acids and conformational stabilization of the 3D structure, whereas renal clearance can be addressed by reducing the amount of free peptide in the plasma with various methods such as ‘depot’ formation in the site of injection and association with carrier proteins [33]. To circumvent the lack of oral bioavailability novel routes of administration can be used, including intranasal, inhalation and injectable depot formulations [33].

Physical–chemical properties-based classification

Even though CPPs have a great sequence variety, it is possible to identify three major classes: cationic, amphipathic and hydrophobic. Figure 1 presents a broad overview of the current CPPs landscape. The data presented contain more than 100 diverse CPPs (corresponding to the CPPs shown in Tables 1–6) collected from publications and patents, excluding mutants around the same peptides. Most of the CPPs in this set have a net positive charge (83%); anionic CPPs do not form a class of their own and they are assigned to different classes on a case-by-case basis; amphipathic CPPs, which comprise both cationic and anionic peptides, form the largest class (44%); only 15% of the peptides are classified as hydrophobic.

In this review a CPP is considered cationic if it contains a stretch of positive charges that is essential for uptake, and if the 3D arrangement does not lead to formation of an amphipathic helix. As shown in Fig. 1, the net average charge of cationic and hydrophobic CPPs has some degree of overlap, with some cationic and hydrophobic CPPs both having an average net charge close to +0.2. This is because the set includes a few long CPPs, such as the cationic Fushi-tarazu, which contain a relatively shorter stretch of positive charges essential for uptake, and a few short CPPs, such as the hydrophobic BIP (Bax-inhibiting peptides) pentapeptides, which contain a single positively charged residue. On the basis of these considerations, CPPs with the same average net charge are classified differently.

Cationic CPPs

The first CPP discovered was cationic and was derived from the HIV-1 protein Tat (RKKRRQRRR) [34]. Studies on arginine-based peptides (from R_3 to R_{12}) have shown that the minimal sequence for cellular uptake is octaarginine (R_8), and that increasing the number of arginines increases the level of uptake [20]. Polylysine, in comparison, has a much poorer uptake profile [20].

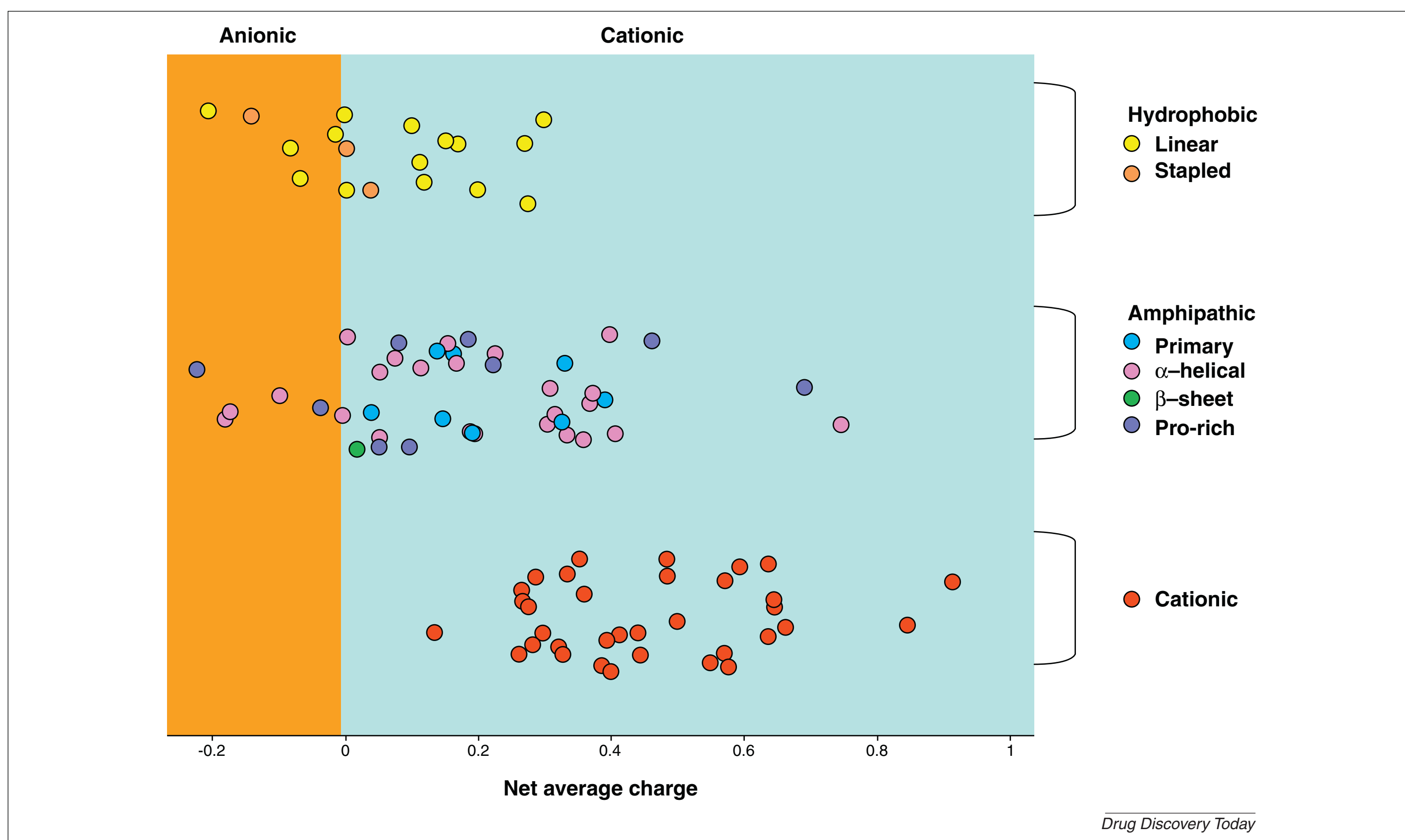

FIGURE 1

Distribution of CPPs by net average charge and class. Anionic CPPs can be classified as hydrophobic or amphipathic CPPs. By contrast, many cationic CPPs are highly charged peptides, without any amphipathic arrangement or hydrophobic character. *Abbreviation:* CPPs: cell-penetrating peptides.

Studies suggest that at least eight positive charges are needed for efficient uptake of several other cationic CPPs [35]. Although charged residues have a crucial role in the uptake of cationic CPPs, other residues can also be crucial. For example, uptake of Penetratin (RQIKIWFQNRRMKWKK), is abolished by mutation of W_{14} to F [36].

A special case of cationic CPPs are nuclear localization sequences (NLSs). NLSs are short peptides based on lysine-, arginine- or proline-rich motifs that can be transported to the nucleus through the nuclear pore complex, which is a multimeric complex containing 50–100 different proteins. NLSs can be further divided into monopartite and bipartite signals, which consist, respectively, of one or two clusters of four or more basic amino acids. For example, nucleoplasmin is a bipartite NLS with minimal sequence KRPAATKKAGQAKKKL, whereas the NLS derived from simian virus 40 (SV40) PKKKRKV is a monopartite NLS. Examples of NLSs include NF-Kb (VQRKRQKLMP), TFIIE-beta (SKKKKTKV), Oct-6 (GRKRKKRT), HATF-3 (ERKKRRRE), and SDC3 (FKKFRKF) [37], among others. Because the number of charges in most NLSs is well below eight, most NLSs are not good CPPs [38], but they can be covalently attached to a hydrophobic peptide sequence to obtain an amphipathic CPP with a good uptake profile.

Amphipathic CPPs

Primary amphipathic

Several primary amphipathic CPPs are chimeric peptides obtained by covalently attaching a hydrophobic domain for efficient targeting to cell membranes to a NLS. For example, MPG (GLAFLGFLGAAGSTMGAWSQPKKKRKV) and Pep-1 (KETWWETWWTEWSQPKKRKV) are both based on the SV40 NLS PKKRKV. The hydrophobic domain of MPG was derived from the fusion sequence of the HIV glycoprotein 41 (GALFLGFLGAAGSTMGA), while that of Pep-1 corresponds to a tryptophan-rich cluster (KETWWETWWTEW), which has high affinity for membranes. Both in MPG and Pep-1 the hydrophobic domain is separated from the NLS through a linker (WSQP).

Other primary amphipathic CPPs are fully derived from natural proteins, such as pVEC, ARF(1–22), and BPrPr(1–28). pVEC contains 13 cytosolic and 5 transmembrane residues from VE-cadherin, whereas ARF(1–22) is derived from the N-terminal domain of the tumor suppressor p14ARF protein. BPrPr(1–28) and the related MPrPr(1–30) are based on the prion protein signal peptide followed by the KKRPKP motif, which corresponds to the N-terminus of the prion protein, once the signal peptide is cleaved. Although the KKRPKP motif is essential for uptake and is sensitive to single point mutations, it does not confer cell penetration when combined with mPrPr(23–50) [39], thus suggesting that the properties of the signal peptide are also important for uptake.

Secondary amphipathic α-helical CPPs

A common structural motif in many peptides and proteins that bind to membranes is the amphipathic α-helix, in which hydrophilic and hydrophobic amino acids are grouped in separate faces of the helix [40]. Secondary amphipathic α-helical CPPs have a highly hydrophobic patch on one face, whereas the other face can

TABLE 1

CPPs derived from heparan-, RNA- and DNA-binding proteins

Cationic		Refs
Heparan binding proteins		
RKKRRRESRKKRRRES	DPV3	[77]
GRPRESGKKRKRKRLKP	DPV6	[77]
GKRKKKGKLGKKRDP	DPV7	[77]
GKRKKKGKLGKKRPRSR	DPV7b	[77]
RKKRRRESRRARRSPRHL	DPV3/10	[77]
SRRARRSPRESGKKRKRKR	DPV10/6	[77]
VKRGLKLRHVRPRVTRMDV	DPV1047	[77]
SRRARRSPRHLGSG	DPV10	[77]
LRRERQSRLRRERQSR	DPV15	[77]
GAYDLRRRERQSRLRRRERQSR	DPV15b	[77]
RNA binding proteins		
RKKRRQRRR	HIV-1 Tat	[34]
RRRRNRTRRNRRRVR	FHV coat	[35,97]
TRQARRNRRRRWRERQR	HIV-1 Rev	[35,97]
TRRQRTRRARRNR	HTLV-II Rex	[35,97]
KMTRAQRRAAARRNRWTAR	BMV Gag	[35,97]
NAKTRRHERRRKLAIER	P22 N	[35]
MDAQTRRRERRAEKQAQWKAAN	λN(1–22)	[35]
TAKTRYKARRAELIAERR	φ21N(12–29)	[35]
TRRNKRNRIQEQLNRK	Yeast PrP6	[35]
DNA binding proteins		
PRRRRSSSRPVRRRRRPRVSRRRRRRGGRRRR	Protamine 1	[98]
Leucine zipper		
RIKAERKRMRNRIAASKSRKRKLERIAR	Human cJun	[35,97]
KRRIRRERNKMAAAKSRNRRRELTDT	Human cFos	[35,97]
Transcription factors		
KRARNTEAARRSRARKLQRMKQ	Yeast GCN4	[35]
Homeoproteins		
RQIKIWFQNRRMKWKK	Penetratin	[75,99]
RVIRVWFQNKRCKDKK	Islet-1	[100]
SKRTRQTYTRYQTLELEKEFHFNRYITRRRRIDI-ANALSLSERQIKIWFQNRRMKSKKDR	Fushi-tarazu	[101]
SQIKIWFQNKRAKIKK	Engrailed-2	[99,101]
RQVTIWFQNRRVKEKK	HoxA-13	[99]
KQINNWFINQRKRHWK	Knotted-1	[99]
RHIKIWFQNRRMKWKK	PDX-1	[102]

be cationic, anionic, or polar. The amphipathicity of a peptide can be visualized through the helical wheel (Fig. 2).

Even though most amphipathic CPPs are cationic, evidence suggests that membrane translocation is a consequence of amphiphilicity and not of positive charges. For example, studies on the model amphipathic peptide (MAP) [41] (KLALKLALKALKAALKLA) have shown that substituting lysines with other polar residues maintains uptake if amphipathicity is conserved: the neutral MAP17 (QLALQLALQALQAALQLA) [42] and the anionic MAP12 (LKTLTETLKELTKTLTEL) [43] are both cell-penetrating.

TABLE 2

CPPs derived from signal peptides

		Refs
Amphipathic (I): signal peptide + NLS		
MGLGLHLLVLAAALQGAKKKRKV	Ig(v)	[83]
MVKSKIGSWILVLFVAMWSDVGLCKKRPKP	BPrPp(1–30)	[103]
MANLGYWLLALFVTMWTDVGLCKKRPKP	MPrPp(1–28)	[104]
AAVLLPVLLAAPVQRKRQKLP	K-FGF + NLS	[105]
Hydrophobic: signal peptide alone		
AAVLLPVLLAAP	K-FGF	[105]

TABLE 3

CPPs derived from antimicrobial peptides

		Refs
Pro-rich		
RRIRPRPPRLPRPRPRPLPFPRPG	Bac7	[53]
VDKGSYLPRPTPPRPIYNRN	Pyrrhocoricin	[106]
Amphipathic		
KCFQWQRNMRKVRGPPVSCIKR	Human lactoferrin (19–40)	[22]
TRSSRAGLQWPVGRVHRLLRK	Buforin 2	[107]
GIGAVLKVLTTGLPALISWIKRKRQQ	Melittin	
GIGKWLHSAKKFGKAFVGEIMNS	Magainin 2	[107]
LLGDFFRKSKEKIGKEFKRIVQRIK-DFLRNLVPRTESC	LL-37	[108]
RGGRLSYSRRRFSTSTGR	SynB1	[109,110]
YKQCHKKGGKKGSG	Crotamine	[111]
ALWKTLLKKVLKAPKKKRKV	S4$_{13}$-PV$_{rev}$	[112]
HARIKPTFRRLKWKYKGKFW	L-2	[113]

TABLE 4

CPPs derived from viral proteins

		Refs
Unknown structure		
TKRRITPKDVIDVRSVTTEINT	Inv3	[85]
Amphipathic		
RQGAARVTSWLGRQLRIAGKRLEGRSK	E^{rns}	[86]
NAATATRGRSAASRPTQRPRAPARSASRPRRPVQ	VP22	[114]
RHSRIGIIQQRRTRNG	HIV-1 VPR 77–92	[115]
KLIKGRTPIKFGKADCDRPPKHSQNGMGK	Ribotoxin2 L3 loop	[86]
PLSSIFSRIGDP	PreS2-TLM	[45]
Amphipathic (β-sheet)		
DPKGDPKGVTVTVTVTVTGKGDPKPD	VT5	[52]

Furthermore, there are many other examples of anionic amphipathic CPPs: GALA (WEAALAEALAEALAEHLAEALAEALEALAA) [44], designed based on another cationic CPP, KALA; p28, a peptide derived from azurin [8]. On the basis of the Protein Data Bank structure of azurin (3N2J), p28 is a helical peptide with a stretch of hydrophobic amino acids clustered on one side of the

TABLE 5

CPPs derived from various natural proteins

		Refs
Cationic		
RRIPNRRPRR	HRSV	[87]
RLRWR	AIP6	[116]
Amphipathic (I)		
MVRRFLVTLRIRRACGPPRVRV	ARF(1–22)	[9]
MVTVLFRRLRIRRACGPPRVRV	M918	[117]
LLIILRRRIRKQAHAHSK	pVEC	[118]
Amphipathic (helical)		
LSTAADMQGVVTDGMASG	Azurin p18	[8]
LSTAADMQGVVTDGMASGLDKDYLKPDD	Azurin p28	[8]
KFHTFPQTAIGVGAP	hCT18–32	[119]
Hydrophobic		
VPTLK (PMLKE, VPALR, VSALK, IPALK)	Bip	[60,61]
PFVYLI	C105Y	[63]
PIEVCMYREP	FGF12	[67]

TABLE 6

Designed CPPs and CPPs derived from peptide libraries

Designed		**Refs**
Cationic		
R8, R9, R10, R12	Polyarginine	[20]
Amphipathic (cationic I)		
KETWWETWWTEWSQPKKRKV	Pep-1	[120]
GLAFLGFLGAAGSTMGAWSQPKKKRKV	MPG	[121]
Amphipathic (cationic II)		
GWTLNSAGYLLGKINLKALAALAKKIL	Transportan	[122]
AGYLLGHINLHHLAHLAibHHIL	TH	[123]
KLALKALKALKAALKLA	MAP	[41]
RRWWRRWRR	W/R	[124]
GLWRALWRLLRSLWRLLWRA	CADY	[125]
LIRLWSHLIHIWFQNRRLKWKKK	EB-1	[126]
Amphipathic (anionic II)		
WEAALAEALAEALAEHLAEALAEALEALAA	GALA	[44]
LKTLTETLKELTKTLTEL	MAP12	[43]
Amphipathic (zero-charge II)		
QLALQLALQALQAALQLA	MAP17	[42]
Amphipathic (Proline-rich)		
(PPR)3, (PPR)4, (PPR)5, (PPR)6	(PPR)n	[54]
(PRR)3, (PRR)4, (PRR)5, (PRR)6	(PRR)n	[54]
GPSQPTYPGDDAPVRDLIRFYRDLQRYLNVVTRHRY	aPP4R1	[55]
GPSQPTYPGDDAPVRDLIRFYRDLRRYLNVVTRHRY	aPP5R1	[55]
GPSQPTYPGDDAPVRDLRRFYRDLRRYLNVVTRHRY	aPP6R1	[55]
$G(P_LXX)_NP_I$	PoliProline-based	[56]
VRLPPPVRLPPPVRLPPP	SAP	[58]
VELPPPVELPPPVELPPP	SAP(E)	[58]
Peptide libraries		
Support-vector machine model		
FKIYDKKVRTRVVKH	SVM1	[96]
RASKRDGSWVKKLHRILE	SVM2	[96]
KGTYKKKLMRIPLKGT	SVM3	[96]
LYKKGPAKKGRPPLRGWFH	SVM4	[96]
HSPIIPLGTRFVCHGVT	SVM5	[96]
YTAIAWVKAFIRKLRK	YTA2	[127]
IAWVKAFIRKLRKGPLG	YTA4	[127]
Plasmid display		
Amphipathic		
RLSGMNEVLSFRWL	SG3	[66]
Phage display		
Hydrophobic		
SDLWEMMMVSLACQY	Pep-7	[65]
VTWTPQAWFQWV		[91]
GSPWGLQHHPPRT	439a	[89]
GPFHFYQFLFPPV	435b	[89]
TSPLNIHNGQKL	HN-1	[128]
Other		
CAYHRLRRC		[90]
Phylomer library		
Cationic		
RCGRASRCRVRWMRRRRI	BEN_1079	
Other		
PYSRPHVQLWYPNRESCRSLIRSLGP	BEN_0805	
Peptide arrays		
Hydrophobic		
PLILLRLLRGQF	Pept1	[64]
PLIYLRLLRGQF	Pept2	[64]
KLWMRWYSPTTRRYG	IVV-14	[92]

helix. Owing to a lack of published structure-activity relationships data on p28, it is unclear whether the uptake of p28 is driven by amphipathicity, and what determines its preferential uptake on cancer cells. An example of amphipathic CPP with a net charge of zero is PreS2 [45], represented through a helical wheel in Fig. 2.

The net charge distribution of amphipathic CPPs in Fig. 2 highlights the broad spectrum of charges seen within this class of CPPs.

It is unclear whether a minimal length is required for the uptake of amphipathic CPPs. Although studies on MAP [42] originally suggested that a minimum of four helix turns is essential, shorter amphipathic CPPs are known today (e.g. PreS2). The degree of hydrophobicity of different amino acids should be investigated further to understand the uptake of amphipathic CPPs. In addition, the amphipathic moment could be used to identify amphipathic peptides. The mean amphipathic moment can be calculated from the formula [46]:

$$<\mu H> = \frac{\sqrt{\left[\sum_{n=1}^{N} H_n \sin(n\delta)\right]^2 + \left[\sum_{n=1}^{N} H_n \cos(n\delta)\right]^2}}{N}$$

where H is the hydrophobicity of the nth amino acid, $n\delta$ is the angle separating side chains along the backbone with $\delta = 100^\circ$ for an α-helix, and N is the number of amino acids. Unfortunately, many different hydrophobic scales have been proposed [47–50], with the same amino acid having a completely different ranking, therefore it is difficult to predict whether any given amphipathic helix would be cell-penetrating. A comparison of amphipathic moments obtained from these different scales with experimental levels of uptake of amphipathic peptides (CPPs and non-CPPs) might help to identify hydrophobic scales that are most useful for predicting CPPs.

Although many CPPs are amphipathic, amphipathic peptides in general are not CPPs. This follows from the observation that uptake of amphipathic CPPs can be severely diminished by single point mutations and deletion, even though the sequence maintains some degree of amphipathicity, as observed for mutants of transportan [51] and MAP [42]. These examples confirm the importance of further quantitative studies to assess the degree of hydrophobicity of amphipathic peptides.

β-Sheet amphipathic CPPs

An amphipathic β-sheet peptide is based on one hydrophobic and one hydrophilic stretch of amino acids exposed to the solvent. Cellular uptake studies on VT5 (DPKGDPKGVTVTVTVTVTGKGDPKPD) suggest that its ability to form β-sheets is essential for uptake, given that analogs where a few residues where mutated using D-amino acids had no propensity to adopt a β-sheet conformation and had extremely poor uptake [52].

Proline-rich amphipathic CPPs

Proline has many unique features among the 20 natural amino acids: it is very rigid because of its pyrrolidine ring; its tertiary nitrogen (in the peptide structure) cannot accept H-bonds like the secondary nitrogen of the other amino acids; if it is high abundant in a peptide structure, in pure water it generates a well-defined secondary structure, polyproline II (PPII). PPII is a left-handed extended helix of 3.0 residues per turn, as opposed to the 3.6 residues of a standard right-handed alpha helix.

Several Pro-rich CPPs have been reported. These include bactenecin-7 (Bac7) [53], synthetically derived peptides $(PPR)_n$ and $(PRR)_n$ (where n = 3, 4, 5, and 6) [54], arginine-rich peptides based on the PPII helix of the avian pancreatic polypeptide [55], and

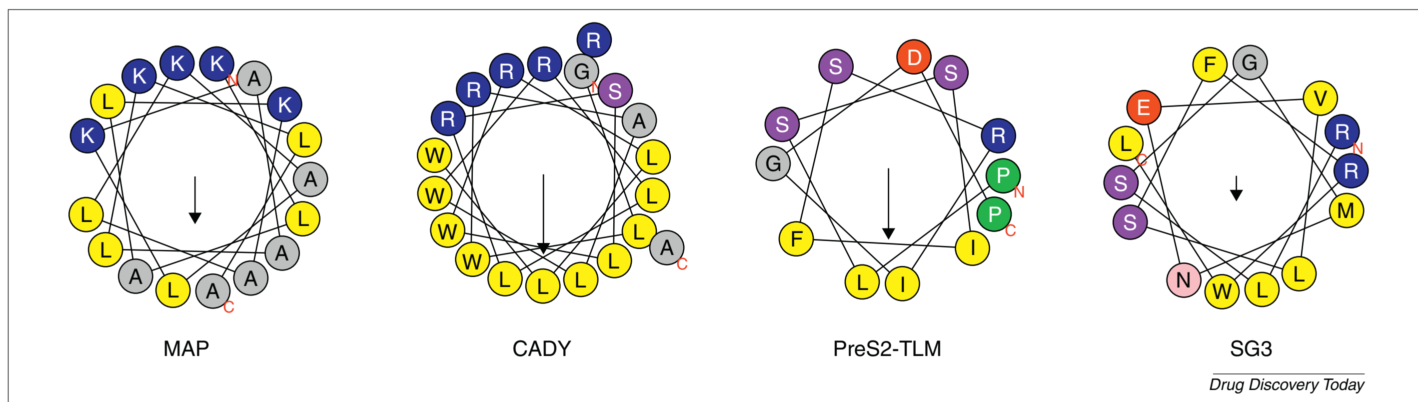

FIGURE 2

The helical wheel representation highlights peptide amphipathicity, with longer vector indicating a larger amphipathic moment. Amino acids are colored by amino acid class (yellow: hydrophobic; grey apolar; purple: polar; blue: positively charged; red: negatively charged; green: proline).

synthetically derivatized polyproline-helix based peptides [56,57] with various R-groups attached to the pyrrolidine ring. An additional example is the CPP SAP, based on the $(VRLPPP)_3$ sequence, which adopts the PPII amphipathic structure [58]. A mutant of SAP with an net negative charge $(VELPPP)_3$ is also a CPP [58]. SAP was derived from the natural sequence of N-terminal domain of γ-zein, which contains a Pro-rich repetitive domain based on the $(VHLPPP)_8$ sequence, responsible for directing γ-zein to the endoplasmatic reticulum [59].

Hydrophobic CPPs

In this article I consider hydrophobic peptides that either: contain only apolar residues; have a low net charge (less than 20% of the sequence); or have a hydrophobic motif or chemical group that is crucial for uptake regardless of the rest of the sequence. Compared with cationic and amphipathic peptides, only a few hydrophobic CPPs have been discovered, however this might be a mere reflection of an historical bias towards cationic and amphipathic CPPs, which were discovered first. Indeed, it appears that random library-based methods have unveiled quite a few new hydrophobic CPPs (Table 6). In this review, hydrophobic CPPs are further divided into peptides based on natural amino acids and chemically modified peptides, which include stapled peptides, prenylated peptides, and pepducins.

Linear hydrophobic peptides based on natural amino acids

This subclass includes anionic and cationic BIP pentapeptides [60–62]. Examples include PMLKE, VPALR, VSALK, IPALK, IPMLK, VPTLQ, QLPVM, ELPVM, VPTLE, VPALK, VSLKK, VSGKK, KLGVM, KLPVT, VPMIK, VPMLK and VPTLK. Scrambling the sequence of these peptides does not significantly affect cellular uptake [61], contrary to what is observed for most amphipathic and cationic CPPs. The lack of sensitivity to sequence scrambling has also been reported for other hydrophobic peptides, such as PFVYLI, which is the minimal cell-penetrating sequence of the longer peptide C105Y (CSIPPEVKFNKPFVYLI) [63].

Recent research suggests that some hydrophobic CPPs can translocate directly through membranes. Marks *et al.* [64] described a series of 18 peptides that spontaneously translocate across synthetic lipid membranes by screening a library of 24,000 randomly selected sequences. Out of the 18 peptides, which were also all found to enter living cells, ten contained either PLIL-XXXXX-GQF or PLIY-XXXXX-GQF and another ten contained XXXX-LRLLR-GQF. The fixed C-terminal -GQF sequence was present in all library members. The library was rationally designed to generate peptides where each position corresponds to specific subsets of amino acids, chosen based on an amino acid preference model from a statistical analysis of known CPPs. CPPs that translocate cell membranes directly could be especially advantageous, because they would be immediately available in the cytosol and the risk of endosomal entrapment and degradation would be eliminated.

Recent work using phage and plasmid display has uncovered several atypical, non-amphipathic, poorly charged peptides that tentatively can be grouped with hydrophobic CPPs. These include Pep-7 (SDLWEMMMVSLACQY) [65], SG3 (RLSGMNEVLSFRWL) [66], and FGF (PIEVCMYREP) [67]. They contain 60%, 57%, and 60%, respectively, of apolar residues, and a net charge between −2 (Pep-7) and +1 (SG3). Further studies would be needed to clarify whether hydrophobicity or other factors drive the uptake of these CPPs.

Stapled peptides

A novel class of hydrophobic CPPs are stapled peptides, obtained by ring-closing olefin metathesis [68]. The stapling confers cell-penetration, and increases peptide helicity by rigidifying the peptide structure. Several publications [69,70] have shown that the stapling increases helicity to varying degrees depending on the peptide sequence and on the position of the staple. Although the stapling does not guarantee that the peptide will be cell penetrating, most stapled peptides are cell-penetrating. Some studies [69,70] have shown that replacing negatively charged residues with neutral or positively charged residues restores uptake of non-cell-penetrating stapled peptides, however cell-penetrating stapled peptides with a net negative charge were later identified [71]. Taken together, these data suggest that the staple itself, rather than high helicity and specific residues, contributes the most to cellular uptake, possibly by conferring high affinity to cellular membranes.

Prenylated peptides

The addition of either a farnesyl (C_{15}) or geranylgeranyl (C_{20}) isoprenoid moiety, known as prenylation, has been reported to

give peptides inherent cell-penetrating ability through an ATP-independent, non-endocytic pathway [72]. Although more work is needed to fully understand the role of prenylation on cellular uptake, current studies suggest that uptake is independent of the specific sequence, as is the case for stapled peptides.

Pepducins

Pepducins are a class of N-terminally lipidated peptides that can cross the cell membrane and bind to the cytosolic region of a variety of transmembrane proteins (GPCRs, MMPs, among others) [73]. The N-terminal lipidation consists of either palmitoyl or other fatty acids. Two pepducins, x1/2pal-i3 and x1/2LCA-i1, have entered preclinical development. Contrary to CPPs, pepducins remain anchored to the cell membrane, and are not released in the cytosol, thus focusing their application to transmembrane receptors.

The role of peptide 3D structure on cellular uptake

The role of peptide secondary structure in cell-penetration remains elusive. First, secondary structure depends on the medium [74]. Peptides can adopt completely different conformations depending on whether they are in water, near the membrane interface, inside the membrane, or bound to a protein. Second, the importance of the secondary structure depends on the mode of uptake and on the peptide class (cationic, amphipathic or hydrophobic). α-helical and β-strand CPPs can be sensitive to mutations that disrupt their 3D structure. For example, levels of uptake change profoundly upon disruption of the disulfide bonds that help to maintain the amphipathic CPP lactoferrin in a helical conformation [22]. Furthermore, studies on the β-sheet peptide VT5 revealed that D-mutations that abolished the β-sheet formation also reduced uptake significantly [52].

By contrast, cationic CPPs such as Tat are more likely to adopt a coiled conformation and it appears that their 3D structure is less crucial. Mutants of Penetratin [AntpHD(43–58)] with one or three prolines ([Pro^{50}]AntpHD(43–58) and [Pro^{45},Pro^{50},Lys^{54},Pro^{55}]AntpHD(43–58)), which disrupt the helical structure of Penetratin observed in bicellar solution by NMR, maintained similar level of uptake as Penetratin both at 4 or 37°C [75]. However, these mutants did not accumulate in the nuclei as much as the parent peptide. A study by Eiríksdóttir *et al.* [74] in which the structures of ten well-known CPPs (including Tat, R9, Penetratin, among others) were investigated by circular dichroism in different media, highlighted that these CPPs are random coils in water, but they become structured or partially structured in small unilamellar vesicles (SUV) prepared from negatively charged phospholipids (DOPG). Neutral (zwitterionic) phospholipids (DOPC) or a mixture of DOPC, sphingomyelin and cholesterol (40/40/20) did not induce formation of an α-helix or β-sheet. Many hydrophobic CPPs appear insensitive to sequence scrambling. This suggests that amino acid composition, rather than the conformation, drives uptake for these peptides.

Origin-based classification

The discovery of the first CPP, the cationic peptide Tat, was followed by the identification of a cationic and partially amphipathic CPP, Penetratin, from the homeodomain of Antennapedia. Mutation studies demonstrated that positive charges and amphipathicity are important features for cell penetration, and these characteristics were carefully considered to design new synthetic CPPs. Concurrently, researchers also looked for these features within domains of natural proteins. Presently, new CPPs without classic features, such as amphipathicity and positive charges are emerging with the advent of high-throughput methods for peptide synthesis.

CPPs derived from natural proteins or peptides

Natural protein motifs are a rich source of CPPs, but establishing a link between a CPP and its role in the protein from which it is derived presents considerable challenges. Proteins might be able to enter the cell through a variety of motifs which, if isolated, would not be cell-penetrating. For example, certain plasma membrane proteins are ubiquinated at the level of their cytosolic tail to control their subsequent endocytosis. Many transmembrane proteins contain specific and diverse recognition motifs (such as YXXφ, where φ is a generic hydrophobic residue, II, [FY]XNPX[YF], among others) in their cytoplasmic interface that are crucial for endocytosis by forming complexes with other proteins, such as adaptors [76]. In addition, naturally occurring proteins might contain sequences which would be cell penetrating if isolated, but may not confer cell-penetration to the full length protein.

In this section CPPs are classified by protein or peptide group. In some cases the cell-penetrating ability of the CPPs is directly linked to the function of the protein or peptide from which it is derived, but this is not always the case, and the role of the CPP domain in the full-length protein might be unknown.

Heparin-binding proteins

Heparin is a member of the glycosaminoglycan (GAG) family of carbohydrates, which includes also the closely related heparan sulfate. For many cationic CPPs, binding to GAGs is an essential step before uptake, and for this reason proteins with domains that bind to GAGs can be a source of CPPs. For example, a family of cationic CPPs named Vectocell® (Table 1) were discovered from human heparin binding proteins [77], such as superoxide dismutase (DPV3 and DPV3/10), epidermal-like growth factors (DPV7–DPV7b), platelet derived growth factor (DPV6), intestinal mucin (DPV10/6), apolipoprotein B (DPV1047), and CAP 37 (DPV15–DPV15b). In analogy with cationic CPPs, not only can heparin-binding proteins bind to glycosaminoglycans, but they are also naturally endocytosed [78,79]. This suggests that the discovery of novel CPPs can be promoted by biological data pointing to proteins that naturally internalize into the cell, and also by identifying proteins with specificity for certain types of cells.

DNA and/or RNA-binding proteins

Because the DNA and RNA backbone is rich with negatively charged phosphates, many DNA and RNA binding proteins contain highly cationic motifs. Probably because this same feature is also important for uptake through binding with glycosaminoglycans, several CPPs are derived from DNA and/or RNA binding proteins. CPPs derived from RNA binding proteins include Tat, Rev, and FHC coat, among others. The Tat motif that is essential for cellular uptake is the same motif that is needed for RNA binding. Other peptides were discovered from the DNA binding

segments of leucine zipper proteins, such as cancer related proteins c-Fos and c-Jun, and the yeast transcription factor GCN4 (Table 1). The ability of certain cationic CPPs to reach the nucleus and bind DNA raises important safety concerns for this class of peptides [80].

Homeoproteins

A special class of DNA binding proteins are homeoproteins, which form a large family of transcription factors containing a conserved DNA-binding motif called homeodomain [81]. Penetratin corresponds to the third helix of the homeodomain of *Drosophila* Antennapedia. Strikingly, many other homeodomains are cell-penetrating, including (but not only) the homeodomains of Islet-1 (pIsl), Engrailed, Fushi-tarazu, Hoxa-5 and PDX-1 (Table 1). Contrary to the cationic motifs of RNA and DNA proteins, which are important for RNA/DNA interaction, the homeodomain is crucial to regulate the subcellular localization of homeoproteins.

Signal peptides

Signal peptides [82] are short peptides of 5–30 amino acids at the N-terminus of secreted proteins and their function is to target the nascent protein for secretion or to specific organelles for further processing. Signal peptides are degraded once they reach the targeted location. Although the targeting function of signal peptides and the cell-penetrating ability of CPPs are based on different mechanisms, most signal peptides contain a hydrophobic central region that might confer affinity for cell membranes.

With a high hydrophobic character, certain signal peptides can cross the cell membrane using a mechanism that is endocytosis- and receptor-independent. Examples of CPPs derived from signal peptides include Ig(v), BPrPr(1–28), MPrPr(1–30) and the peptide derived from Kaposi's fibroblast growth factor (K-FGF) signal peptide (Table 2). Conjugation of signal peptides to NLSs has led to new CPPs. For example, Ig(v) is a chimeric CPP obtained by adding an NLS to the signal peptide of *caiman crocodylus* Ig(v), [83] and the K-FGF signal peptide can translocate membranes either alone or if conjugated to an NLS.

Antimicrobial peptides

Antimicrobial peptides (AMPs) are a group of peptides, many of which were discovered from toxin venoms, with the ability to kill a diverse spectrum of microorganisms. Higher organisms produce AMPs on epithelial surfaces or in endothelial and phagocytic cells as a defense mechanisms against infections. As shown in Table 3, most AMPs are amphipathic peptides (both primary or secondary α-helical and β-strand) with a net positive charge and several hydrophobic residues. Even though many AMPs are lytic to the cell membrane, some are not and can enter eukaryotic membranes [84]. Some AMPs are characterized by a large number of prolines, a feature common to other non-AMP derived CPPs.

Viral proteins

Many viral proteins have developed mechanisms to gain cell entry, and several CPPs were derived from motifs found in viral proteins. In addition to the viral-derived peptides Tat and Rev, which were classified under RNA-binding protein peptides, other examples are presented in Table 4. Inv3 (TKRRITPKDVIDVRSVTTEINT) was recently identified from a *Mycobacterium tubercolosis* membrane protein called *Mycobacterium* cell entry protein (Mce1A 130–151) [85]. With four positively charged residues and three negatively charged residues, Inv3 has a net charge of just +1, and is not amphipathic. Mutation studies on this peptide suggest that positive charges are crucial but negative charges are not: mutations of R led to loss of uptake, while mutations of D or E to K did not have a significant effect. Several viral-protein derived CPPs are based on an amphipathic α-helix, such as Erns from the pestivirus envelope glycoprotein [86], the ribotoxin 2 L3 loop peptide [86]. PreS2-TLM, and a peptide from the human respiratory syncytial virus [87].

Designed CPPs and CPPs derived from large peptide libraries

While the majority of designed peptides have a characteristic amphipathic structure (Table 5), peptides discovered from high-throughput screening on large peptide libraries are much more diverse, with many having a larger number of hydrophobic, rather than cationic amino acids, and no clear preference for amphipathic structures.

CPPs from large combinatorial peptide libraries

Randomized peptide libraries can be obtained through DNA-encoded peptide libraries and thus enable the generation of billions of peptides. Several methods are available to link peptides to their encoding DNA, such as phage display, plasmid display, micro-organism surface display and ribosome display [88]. Alternatively, peptide arrays can be used for high-throughput synthesis of tens of thousands peptides. Several CPPs have been discovered from such libraries, such as SG3 [66] (plasmid display), 439a and 435b [89] (phage display), CAYHRLRRC [90] (selective for leukemia/lymphoma cells; phage display), and VTWTPQAWFQWV [91] (selective for U87MG glioblastoma cells; phage display). These peptides are mainly hydrophobic, as opposed to most peptides described so far. Using a peptide array method over an *in vitro* virus (IVV) library a new amphipathic CPPs was discovered (KLWMRWYSPTTRRYG) [92].

Phylomers

Phylomers are libraries of natural peptides encoded by natural genes of diverse bacterial genome [93]. Compared with random peptide sequences obtained by phage display, phylomer libraries contain millions of peptides with subdomains that already evolved to maintain some structural stability. New CPPs obtained from this approach have also been reported, some cationic, others amphipathic with a net negative charge, and others mainly hydrophobic. Examples include peptides BEN_1079 and BEN_0805 reported in Table 6 (http://www.phylogica.com/media/TechnicalPresentations/PhylogicaTechJune11.pdf).

CPPs prediction from natural proteins and random libraries

Only a few prediction models for CPPs have been proposed, including that by Hansen *et al.* [94] based on Sandberg z descriptors for amino acids [95], and that by Sanders *et al.* based on biochemical properties of peptides (such as amino acid composition, peptide length and net charge) [96]. Peptides discovered through these methods, which are reported in Table 5 have a varied amino acid composition.

Concluding remarks

With a great sequence variety and large differences in terms of physical chemical properties, CPPs can be linear, cyclical, cationic, anionic, hydrophobic, hydrophilic, amphipathic, non-amphipathic, random coiled, α-helical, or β-sheets. However, CPPs differ from most other peptides with respect to specific features that reflect various mechanisms used to enter the cell.

Highly cationic CPPs (i.e. CPPs with at least eight positive charges) can interact with GAGs and enter the cell through endocytic pathways. Above a certain concentration threshold they can also translocate the membrane directly. Generally, highly cationic CPPs do not have specific 3D-structural requirements for uptake.

By contrast, secondary amphipathic CPPs need to be partially helical, at least near the membrane interface, thus exposing the hydrophobic face to the membrane and the hydrophilic face to the solvent. The hydrophilic face of secondary amphipathic CPPs can tolerate a variety of residues, cationic, anionic, polar, but not all amphipathic peptides are cell-penetrating. The main requirement for uptake among amphipathic CPPs remains to be determined. Possibilities include a certain amino acid composition or amino acid repeat in the hydrophobic or hydrophilic face, as well as ability to adopt a sufficiently helical structure.

Novel hydrophobic CPPs, with low net charge and no amphipathic arrangement, are emerging. Some contain a sequence of hydrophobic amino acids, others (stapled peptides, prenylated peptides, pepducins) have chemical modifications based on hydrophobic chains.

The origin of a CPP can provide clues to its mechanism of entry. For example, several cationic CPPs were discovered from heparin binding proteins. Many amphipathic CPPs were either designed or obtained from naturally occurring amphipathic peptides (especially AMPs); AMPs evolved to enter a variety of microbial membranes. Signal peptides are a rich source of hydrophobic CPPs, given their innate ability to target a nascent protein to a specific organelle in the cell.

The diversity of CPPs is advantageous for drug discovery. Because many filters are applied in each step of the drug discovery process, starting with an arsenal of diverse CPPs increases the chances that at least one will progress. CPPs, like small molecules, must undergo a large panel of assays to assess toxicity, tissue distribution, cell selectivity, solubility, plasma stability, among others. By mapping how different classes of CPPs behave relative to these parameters, it will be crucial to determine whether a problem is systemic to a given class of CPPs, or whether it can be overcome through chemical modifications that do not alter uptake.

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