

**REVIEW**

# New generation of cell-penetrating peptides: Functionality and potential clinical application

Siegmond Reissmann<sup>1</sup>  | Margarita P. Filatova<sup>2</sup>

<sup>1</sup>Faculty of Biological Sciences, Institute of Biochemistry and Biophysics, Friedrich Schiller University, Dornburger Str. 25, Jena, Thuringia, 07743, Germany

<sup>2</sup>Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia

**Correspondence**

Siegmond Reissmann, Faculty of Biological Sciences, Institute of Biochemistry and Biophysics, Friedrich Schiller University, Dornburger Str. 25, 07743 Jena, Germany.  
Email: siegmond.reissmann@uni-jena.de

Cell-penetrating peptides (CPPs) can transport various cargoes through membranes of live cells. Since the first generations of CPPs suffered from insufficient cell and tissue selectivity, stability against proteases, and escape from endosomes, a new generation of peptides, with optimized properties, was developed. These are either derived from natural sources or created through the combination of multivalent structures. The second method allows achieving high internalization efficiency, high cell and tissue selectivity, and release from endosomes via hybrid structures, combining sequences for endosomal release, homing sequences, and sequences for activation at the target tissue and for local delivery of cargoes. CPPs with innate tumor selectivity include azurin, crotamine, maurocalcine, lycosin-I, buffalo cathelicidin, and peptide CB5005. Some of them can penetrate the membranes of live cells and influence intracellular signaling pathways, thereby exerting cytotoxic effects against tumor cells. To obtain multilayer penetration and stabilization against proteolytic degradation, as well as for better handling, CPPs are often conjugated to nanoparticles. A special problem for tumor treatment is the efficiency of drug transport through three-dimensional cell cultures. Therefore, the capability of CPPs to deliver the drug even to the innermost tissues is of crucial importance. Notably, the ability of certain CPPs to penetrate barriers such as skin, the blood-brain barrier (BBB), and cornea or conjunctiva of eyes enabled the replacement of dangerous and painful injections with soothing sprays, creams, and drops. However, it is difficult to rank the efficacy of CPPs because transport efficiency and tissue selectivity depend not only on the CPP itself but also on the target tissue or organ, as well as on the cargo and method of CPP-cargo coupling. Therefore, the present review describes some examples of new-generation CPPs and aims to provide advice on how to find or create the right CPP for a given task.

**KEYWORDS**

cell-penetrating peptides, endosomal escape, new generation of CPPs, penetration of barriers, selectivity for cancer cells, stabilization against proteolytic degradation, venom- and enzyme-derived CPPs

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2021 The Authors. *Journal of Peptide Science* published by European Peptide Society and John Wiley & Sons Ltd.

## 1 | INTRODUCTION

In the preceding review written by one of us and published 6 years ago in the same journal,<sup>1</sup> the structures, functions, and mechanisms of action of CPPs were described, together with their history. The present review is based on this previous article but is focused on newly developed CPPs, their functional characteristics, and their potential use in preclinical and clinical studies.

Despite intensive research in this field for the last 30 years, the development of effective CPPs for selective internalization of drugs or imaging labels in tissues and organs still remains challenging. In fact, to date, no CPP or composition containing CPPs has been approved by the Food and Drug Administration (FDA). Moreover, despite worldwide efforts to produce vaccines against the novel coronavirus SARS-CoV-2, until recently no CPP has ever been used for vaccine development. This lack of results is in strong contrast to the promising properties of CPPs as nonviral transport vectors. In the following sections, we will also provide possible explanations for this unexpected outcome.

Nevertheless, CPPs are undoubtedly more promising and convenient for clinical use than chemical detergents or polymers, electrical and mechanical penetration methods or viral vectors. These methods can be taken into consideration only for *in vitro* use, for instance, in studies conducted on isolated cells, cell cultures, tissues, and, only in some cases for animal studies. However, they are not suitable for clinical applications, with very few exceptions.

On the other hand, CPPs can penetrate the membranes of live cells and deliver different types of cargoes to cells or even directly to subcellular organelles; therefore, they can be used as biochemical

tools in cellular studies or as drug transporters. In particular, signal transduction inhibitors that influence intracellular signaling pathways require effective means for delivery to target tissue or target cells.

However, the first-generation CPPs not only had low cell and tissue selectivity but were mainly transported into intracellular vesicles, that is, endosomes. In contrast to some other problems remaining till now, cell and tissue selectivity could be greatly enhanced in the newest generation of CPPs. Additionally, most peptides from the new generation can escape entrapment in endosomes thanks to the insertion of special sequences or to their conformational properties, which can trigger their release from endosomes.

The new generation of CPPs mainly consists of peptides that fulfill the following requirements: transport with sufficient efficiency, high selectivity, and escape from endosomes. Figure 1 summarizes the used mechanisms for enhancing tissue- and cell-selectivity. These newly developed peptides mostly derive from natural sources; indeed, they consist of partial sequences of proteins from venoms of snakes, scorpions, and spiders or they are either fragments of enzymes or fusogenic and penetrating cell surface components. Notably, many of these new-generation CPPs exhibit a pronounced intrinsic selectivity for tumor tissues. The selective transport of cargoes into an organ seems to be quite more difficult as selective transport into a cell line or tissue. The group of Brock has critically reviewed the current status of renal targeting drugs. They found that drugs also accumulate in the liver and that renal targeting goes mainly with an increase in kidney-to-liver ratio.<sup>2</sup>

Moreover, to introduce or enhance target selectivity, activatable CPPs can be rationally designed. Their activation can be triggered by

### Tissue-selectivity

- **Release of CPPs from vessels by homing sequences** (tumor homing sequences)

### Tumor-selectivity

- **Release of polymer-bound CPP and cargo from leaky vessels**
- **Innate selectivity for tumors**
- **Activatable cell-penetrating peptides (ACCPs):**  
Protease-activatable CPPs (activation by MMPs)  
Low pH-value-activatable CPPs (pHLIPs)  
Hypoxia-activatable CPPs

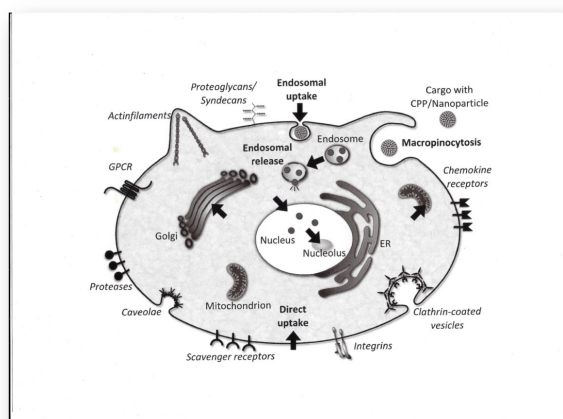
### Ligands for tumor-specific receptors

- Receptor ligands (HER 2)
- Receptor antibodies

### Cell-selectivity

#### Uptake of different CPPs occurs by different mechanisms

Different cell types contain binding receptors in different frequency of occurrence, resulting in preference to structural types of CPPs.



**FIGURE 1** Cell- and tissue-selectivity of internalization into live cells

cell-specific proteases, for example, tumor-specific proteases such as matrix metalloproteinases,<sup>3,4</sup> fibroblast activation protein (FAP),<sup>5</sup> lower pH values than those found in healthy tissues,<sup>6,7</sup> or under hypoxic tissue conditions.<sup>8,9</sup> Furthermore, cancer selectivity can be enhanced by fusion with homing sequences,<sup>10</sup> which guide the translocation of the CPP with its cargo from blood vessels to the target tissue.

In addition, certain newly developed CPPs can directly kill tumor cells by influencing specific signaling pathways. For instance, Bernardes et al.<sup>11</sup> reported that azurin exerts anticancer activity by interacting with multiple targets and interfering with multiple steps of tumor progression.

With the aim of improving pharmacokinetic parameters and ease of handling, some CPPs were coupled to polymers, dendrimers, liposomes, and nanoparticles.<sup>2,5,12–17</sup> These complex structures permit more precise delivery of CPPs to target cells by coupling them with additional affinity ligands, such as selective antibodies,<sup>5</sup> ligands for membrane receptors,<sup>18,19</sup> or other ligand-binding proteins.<sup>20</sup>

CPPs can also be used for imaging of diseased tissues. Indeed, to highlight tumors or inflamed tissues, marker molecules must be conjugated. For instance, fluorescent labels can be used as markers,<sup>21</sup> especially those emitting in the near-infrared (NIR) region,<sup>22,23</sup> together with metal-chelating complexes for sensitive NMR imaging<sup>24</sup> and radionuclides.<sup>25</sup> Therefore, imaging with fluorescence-labeled and tissue-specific CPPs can be very helpful for visualizing the margin between healthy and diseased tissues during surgery.<sup>4</sup>

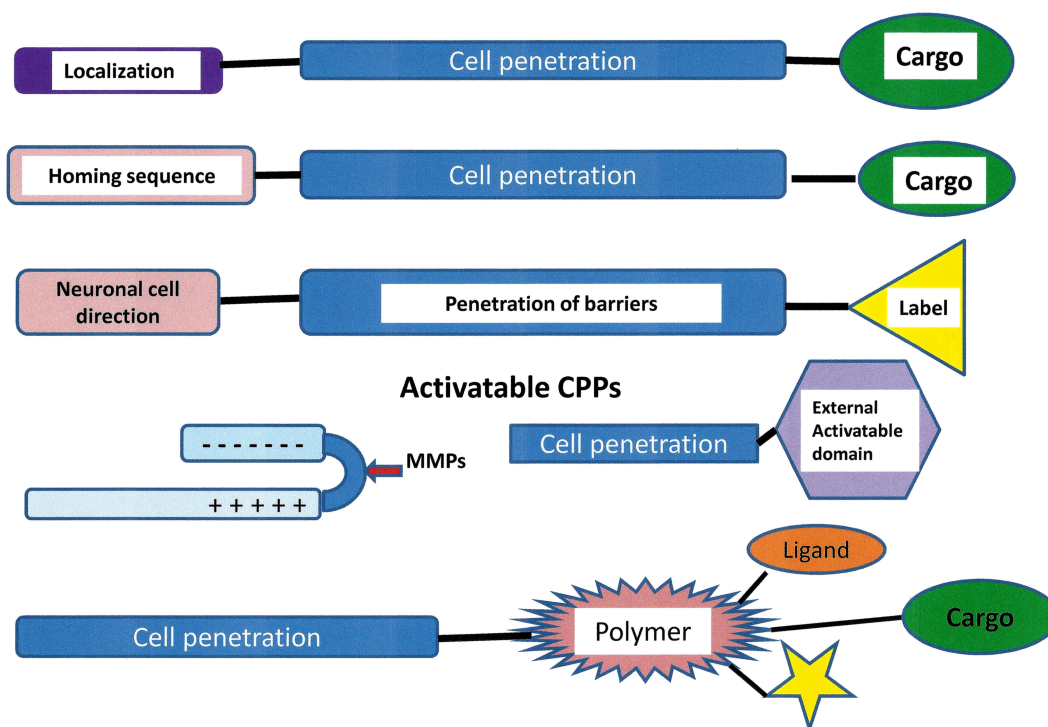
Gene therapy is normally based on the principle that exogenous DNA can adjust the availability of deficient or altered gene products to normal physiological levels. However, despite the many attempts undertaken in many laboratories from different countries, therapy

with DNA and plasmid DNA (pDNA) remains a challenge. On the one hand, it is difficult to deliver exogenous DNA or pDNA into target tissues of patients with the help of CPPs. On the other hand, it seems to be easier to influence intracellular de novo protein synthesis via transfection of homeobox proteins and CRISPR-Cas9 ribonucleotide complexes<sup>26</sup> or through internalization of oligonucleotides<sup>27</sup> and their pseudo-derivatives,<sup>28</sup> which are methods also belonging to gene therapy.

In order to enhance the stability of CPPs against proteolytic degradation and thereby make them more suitable for therapeutic applications, different techniques have been used, such as insertion of nonproteinogenic amino acids or structural different components, cyclization, or conjugation to polymers or nanoparticles, as described more in detail later in the chapter “Stabilization against proteolytic degradation.”

Most tumors consist of different cell types, extracellular matrix, and macrophages; nevertheless, despite the different internalization efficiencies of CPPs for certain cell types, efficient CPPs must transport the cargo into the center of a three-dimensional composed tissue.<sup>29</sup> Furthermore, it has become increasingly important to find or create CPPs that can penetrate barriers such as the blood-brain barrier (BBB), cornea or conjunctiva of the eye, skin, epithelial tissue, and intestinal mucosa. Such barriers consist of many layers of different cells. Therefore, crossing these barriers requires the ability to perform transcellular migration.

Altogether, these requirements cannot be fulfilled by any single molecule, but they necessitate the development of multifunctional composite molecules or nano-carriers. Some of these basic composite structures are shown in Figure 2.



**FIGURE 2** Composed structures of CPPs

## 2 | NEW CELL-PENETRATING PEPTIDES—“NEW GENERATION”

At the time the aforementioned review was published, certain new peptides had been developed for an enhanced transport efficacy, escape from endosomes, and specificity towards target cells. Over the past few years, the transduction and transfection of difficult-to-transfect cells have emerged as a novel challenge to be overcome. This problem is relevant for clinical applications. In fact, hard-to-transfect human cells are, *inter alia*, stem cells, primary cells (e.g., human fibroblasts, human vein endothelial cells, hematopoietic cells, and hippocampal and cortical neurons), mast cells, natural killer (NK) cells, and macrophages. Nevertheless, only a few authors have focused their research on hard-to-transfect cells. However, we detected a large difference between the efficiency of internalization of peptides and proteins, on the one hand, and of linear and cyclic DNA, on the other hand. In fact, by our experience, not only small-sized proteins but also large proteins could be easily transported with CPPs, even into difficult-to-transfect cells. For example, noncovalent complexes formed from amphipathic CPPs and proteins could be easily transported into such difficult-to-transfect cells such as Kasumi-1 and *Leishmania* cells.<sup>30–32</sup> However, we were unable to deliver plasmids into the same cells even when approaching the problem with a variety of different CPPs.<sup>33</sup> Because immune cells are also hardly to transfect cells it seems reasonable that other transport vectors are preferred for the development of vaccines against the novel coronavirus SARS-CoV-2. This fact contrasts with the results of preclinical vaccination studies with CPPs described by the very informative, interesting, and intelligent review from Bolhassanis group.<sup>34</sup>

In our former review, 92 different CPPs were listed, classified, and subclassified according to different perspectives. Depending on their sequence, CPPs can be divided into basic, amphipathic, neutral, hydrophobic, and acidic peptides. The shortest transporter consists of five amino acids whereas the longest transporters are hybrid fusion proteins of more than 100 amino acids.

Among first- and new-generation CPPs are many lysine- and arginine-rich cationic peptides. Cationic CPPs such as HIV-TAT are usually unsuited for forming noncovalent complexes with proteins, as they require, in most cases, covalent conjugation to marker molecules, proteins, or drugs. On the other hand, their positive charges favor their interaction with negatively charged components of the cell membrane and plasma. However, upon interaction with blood plasma, these CPPs are prone to rapid elimination by the reticuloendothelial system.<sup>34,35</sup> Neutral and acidic CPPs have also been intensively studied. Moreover, amphipathic peptides such as MPG-type peptides<sup>36</sup> have been applied in different preclinical and clinical studies. Intensively studied acidic CPPs derive from fragments of the bacterial enzyme cupredoxin, mainly the p18 and p28 fragments of azurin. They exhibit a distinct affinity for tumor cells and have great potential for clinical application.<sup>37</sup> The following chapter presents and describes the properties and applications of some new-generation CPPs, which were selected from the literature of the past few years. Please acknowledge that this selection is also based on our personal point of view.

## 3 | CATIONIC CPPS

### 3.1 | Protamines

This family of heterogeneous polycationic peptides is characterized by a high content (~67%) of arginine residues. These basic peptides have an average molecular weight of approximately 4500 Da and can be isolated from fish or human sperm. Protamines are zinc finger proteins that may play a role in the stabilization of sperm chromatin and in the inhibition of transcription. Indeed, their DNA-binding ability is markedly enhanced by zinc ions. Thus, they may play a role as regulatory factors during sperm chromatin condensation.<sup>38</sup> Long time before the discovery of CPPs, protamines were used for pharmaceutical compositions. For example, insulin or insulin derivatives can form complexes with protamine and Zn ions, thereby producing formulations that can be administered to patients with type I and also with type II diabetes mellitus. Moreover, complexes containing protamines were also used for the release of interferon alpha, glucagon-like peptide 1, and somatostatin from pharmaceutical compositions.

*Low-molecular-weight protamine: VSRRRRRRGRRRR*<sup>39,40</sup>

Because this heterogeneous group of peptides evoked many undesired side effects during clinical trials, Byun et al.<sup>39</sup> developed a short-chained low-molecular-weight protamine, abbreviated LMWP. The sequence of this peptide contains a compact assembly of arginine residues and resembles, to some degree, HIV-TAT (47–57). This short fragment is used in clinical treatment as a substitute for heparin antidote.<sup>39</sup> It effectively triggers the transduction of peptides and proteins into live cells. Furthermore, the conjugation of insulin to LMWP enables its transport through epithelial cell membranes and the intestinal mucosal layer.<sup>41</sup> In addition, because of the compact region of positively charged arginine residues, LMWP can function as an efficient gene carrier<sup>42</sup> and has been used for siRNA delivery into cancer cells.<sup>43</sup> Moreover, LMWP helps to target drug-resistant breast cancer<sup>44</sup> and enables drug delivery to the brain through intranasal administration.<sup>45</sup> For highly specific targeting of cancer cells, protease-activatable LMWP derivatives have been developed.<sup>46</sup> Surprisingly, LMWP can also be applied to the mineralization of human dental pulp stem cells.<sup>47</sup>

### 3.2 | Crotamine

Crotamine: 42 amino acid residues with three disulfide bridges: C<sub>4</sub>-C<sub>36</sub>, C<sub>11</sub>-C<sub>30</sub>, and C<sub>18</sub>-C<sub>37</sub> YKQCHKKGGHCFPKEKICLPSSDFGKMDCRWRWKCKKGGSG.<sup>48,49</sup>

4-Ser-crotamine(Δ10–37): YKQSHKKGKGGSG-amide.<sup>50</sup>

Cys-[4-Ser-crotamine(Δ10–37)]: C-YKQSHKKGKGGSG-amide.<sup>23</sup>

Crotamine is the peptide component of the venom from the South American rattlesnake, *Crotalus durissus terrificus*. Its amino acid sequence contains 42 residues, linked by three disulfide bridges.<sup>48,49</sup> It is a defensin-like cationic peptide that possesses anticancer,

antimicrobial, and antifungal properties and thus is potentially useful for diagnostics and therapeutics.<sup>51</sup> Campairo et al.<sup>52</sup> confirmed the efficacy of orally administered crotamine as an antitumor agent, which showed several positive metabolic effects in treated animals. In another study, the authors confirmed the long-term safety of internalization of full-length crotamine into renal proximal tubular epithelial cells.<sup>53</sup> In contrast to the postulated low toxicity, Silvestrini et al.<sup>54</sup> showed that intradermal application of crotamine induces inflammatory and immunological changes. In particular, the systemic acute inflammatory responses to crotamine are similar to those triggered by histamine and thus limit the therapeutic use of this peptide in its original form.<sup>54</sup> Notably, crotamine exhibits significant DNA-binding properties in vitro.<sup>55</sup> Furthermore, its cell-penetrating and antitumoral activities are combined with inhibitory properties on voltage-gated potassium channels.<sup>56</sup>

In most publications, a structurally minimized sequence was described with a deletion from positions 10 to 37, thus combining residues 1–9 with residues 38–42 to form 4-Ser-crotamine( $\Delta$ 10–37). This peptide was also named nucleolar targeting peptide (NrTP), due to its preferential accumulation in the nucleoli of cells.<sup>50,57,58</sup> Its internalization involves binding to heparan sulfate proteoglycans,<sup>48</sup> followed by endocytosis and escape from vesicles into the cytosol. NrTPs can be successfully internalized into all cell types, except erythrocytes.<sup>58</sup> In addition, as a positively charged peptide, it can stimulate the uptake of DNA and plasmids into cells through the formation of electrostatic complexes.<sup>48</sup> Furthermore, the ability of crotamine to transport cargoes both in vitro and in vivo, in particular into actively proliferating cells,<sup>59</sup> makes crotamine and its derivatives suitable markers of actively proliferating cells, such as cancer cells. For instance, very recently Tansi et al.<sup>23</sup> found that a fluorescent derivative of Cys-[4-Ser-crotamine( $\Delta$ 10–37)] could reveal tumor microheterogeneity by preferential internalization into proliferating breast cancer cells, whereas it was homogeneously distributed in fibrosarcoma, colon cancer, and noncancerous endothelial cells. Thus, this peptide can be used to determine the level of heterogeneity within tumors. In the used spliced analog, cysteine at position 4 was replaced by serine to unambiguously couple labels and cargoes to an additional N-terminal cysteine. The suitability of crotamine for diagnostics and tumor therapy has been studied in various models.<sup>51,60–62</sup> In addition to cell-penetrating and selective antifungal and antitumoral properties,<sup>63</sup> crotamine exhibits potent anti-plasmodial activity,<sup>64</sup> which can help in the treatment of malaria.

### 3.3 | Maurocalcine

33-mer basic peptide with three disulfide bridges: C<sub>3</sub>–C<sub>17</sub>, C<sub>10</sub>–C<sub>21</sub>, and C<sub>16</sub>–C<sub>32</sub> GDCLPHLKLCKENKDCSSKCKRRGTNIEKRCR.<sup>65</sup>

Minimaurocalcine: GDCLPHLKL.<sup>66</sup>

Cys-(3-Abu-minimaurocalcine): C-GD (Abu)LPHLKL.<sup>23</sup>

MCA-UF 14–25 KD (Abu)-(Abu)SKK (Abu)KRRG-amide.<sup>67</sup>

Maurocalcine was first isolated from the Tunisian scorpion *Scorpio maurus palmatus*. Full-length maurocalcine is a 33-mer basic peptide cross-linked by three disulfide bridges.<sup>65</sup> Numerous analogs have

been derived by simultaneous internal cysteine replacement with 2-aminobutyric acid (Abu) and sequence truncation.<sup>66,67</sup> Fragment 1–9, which corresponds to the hydrophobic surface, is called minimaurocalcine. Protonation of the histidine residue renders the cell penetration activity pH sensitive, making the peptide a suitable tool for specific targeting of cancer cells within acidic tumor environments.<sup>66</sup> Interestingly, maurocalcine potently and reversibly modifies the channel-gating behavior of the type 1 ryanodine receptor.<sup>65</sup> It binds to disialoganglioside GD3 at the cell surface. Maurocalcine was tested as a drug carrier to achieve targeted internalization.<sup>68</sup> Notably, D-maurocalcine, an analog with all amino acids in the D configuration, combines efficient cell penetration with proteolytic stability.<sup>69</sup> Perret et al.<sup>70</sup> showed that disulfide bridges stabilize the peptide against proteolytic degradation in mouse blood. In addition, gold nanoparticles containing a maurocalcine analog can be delivered to different cell lines, allowing biomedical imaging.<sup>71</sup> Finally, internalization studies performed by Tansi et al.<sup>23</sup> demonstrated a preference of Cys-(3-Abu-mini-maurocalcine) for tumor cells but with a diminished transport efficiency and cell selectivity than the spliced crotamine analog.

### 3.4 | The spider toxin Lycosin-I

Ac-KGWFKAMKSIKFIKAKEKLKEHL-amide.<sup>35,72</sup>

Similar to toxins from snakes and scorpions, toxins from spiders contain peptides with cell-penetrating properties and high affinity to cancer cells. For example, lycosin-I, a toxin from the spider *Lycosa singoriensis*, interacts selectively with breast and prostate cancer cells. When conjugated to spherical gold nanoparticles, lycosin-I exhibits selective intracellular translocation towards cancer cells but also displays an unprecedented low selectivity over noncancerous cells. Moreover, this conjugate shows an efficient photothermal effect under NIR irradiation, leading to the killing of cancer cells in vivo.<sup>73</sup>

### 3.5 | Tumor homing CPPs from the buffalo cathelicidin family

BuCATHL4C abbreviated CAT: RIRFPWPWRWPWRRVRG.<sup>74</sup>

Cathelicidin is an antimicrobial peptide. In particular, members of the newly identified buffalo-derived cathelicidin family exhibit preferential binding to multiple tumor cell lines. Additionally, they show higher translocation efficiency than most other CPPs. Therefore, CAT is considered a novel tumor homing CPP with great potential for selective drug delivery.<sup>74</sup>

### 3.6 | The nucleus-penetrating peptide CB5005

CB5005: KLKLALALAVQRKRQKLMF.<sup>75</sup>

CB5005 is a rationally designed peptide containing a cell-permeable sequence cascading to a NF- $\kappa$ B nuclear localization

sequence. This peptide can penetrate the brain, owing to its unique affinity for brain endothelial cells, accumulate at the tumor site, and infiltrate deeply into tumor spheroids.<sup>75</sup> Interestingly, CB5005 not only penetrates cells but also enters their nuclei, thereby displaying some potential in the treatment of glioblastomas. Indeed, CB5005 functions simultaneously as a CPP and as a tumor growth inhibitor. In particular, when conjugated with doxorubicin, it exerted a synergistic effect in the treatment of glioma.<sup>75</sup>

### 3.7 | The cyclic peptide TD-34

Cyclo (ACSSKSKKHCG) with a disulfide bridge between C<sub>2</sub> and C<sub>10</sub>.<sup>76</sup>

The cyclic peptide TD-34 is another rationally designed CPP, developed for transdermal cargo delivery through Caco-2 cells. It has been stabilized against proteolytic cleavage by cyclization and is very effective in transdermal transport of cargoes such as insulin.<sup>76</sup> In particular, TD-34 was found to enhance the delivery of insulin across Caco-2 cell monolayers.<sup>77</sup>

## 4 | AMPHIPHILIC PEPTIDES

### 4.1 | Designed peptides of the PepFect series

PepFect 14: Stearyl-AGYLLGKLLLOOLAAAALOOIL-NH<sub>2</sub>.<sup>78,79</sup>

PepFect 32: Stearyl-LLOOLAAAALOOILLFFYGGSRGKRNNFK TEEY-NH<sub>2</sub>.<sup>80</sup>

O: nonproteinogenic amino acid ornithine.

Ülo Langel and his research group made many important contributions to the development of effective and selective CPPs.<sup>78</sup> In particular, this group created a series of PepFect peptides, derived from the N-terminal sequence of galanin, for three purposes: first, to transport different types of ribonucleic acids and mimics; secondly, to ameliorate tissue- or barrier-specific internalization; and third, to enhance proteolytic stability.

Interestingly, peptides belonging to this series show minimal toxic side effects. Therefore, for instance, a noncovalent complex of PepFect 14 with siRNA was used for targeted gene silencing in human stem cells,<sup>79</sup> allowing to study gene function especially when hypomorphic knockdown is needed. Moreover, PepFect 14 can deliver mRNA in cancer cell spheroids, as confirmed by efficient mRNA transport into primary ovarian cancer cell explants.<sup>80</sup> This peptide could also transport mRNA mimics such as splice-switching oligonucleotides into muscle cells and thus proves useful for the treatment of Duchenne muscular dystrophy.<sup>81</sup> Moreover, a PepFect 32-pDNA complex exhibited high translocation and transcytosis through the BBB, thereby displaying high transfection efficiency to glioma cells in an in vitro model.<sup>82</sup>

Commonly, these peptides penetrate cells by binding class A scavenger receptors, which facilitate the internalization of complexes consisting of PepFect peptides and nucleic acids.<sup>83</sup> However, to

further enhance uptake selectivity, an additional receptor was exploited for transcytosis of PepFect 32 through the BBB and its uptake in glioma cells. In fact, PepFect 32 was covalently bound to the targeting ligand angiopep-2, which interacts with the low-density lipoprotein related protein (LRP1) receptor. This construct could deliver siRNA in human glioblastoma cells with higher efficiency than the parent peptide PepFect 14.<sup>82</sup> Furthermore, the Langels group could significantly improve, through the use of peptides from the PepFect family, the efficiency of telomerase-targeting cancer therapy. Indeed, they could significantly lower the necessary concentration of a commercial telomerase inhibitor.<sup>84</sup>

## 5 | ACIDIC SEQUENCES

### 5.1 | Azurin

Azurin is a 128-amino acid-long bacterial protein with cell-penetrating activity that simultaneously inhibits multiple tumor-promoting pathways.<sup>85,86</sup> It belongs to the family of monomeric copper-containing enzymes. In addition to azurin and some variants from other microorganisms, other bacterial products, such as pseudoazurin, plastocyanin, mavicyanin, and rusticyanin, have been investigated for their cell-penetrating properties.<sup>87,88</sup> Azurin preferentially enters cancer cells, where it exerts cytostatic and cytotoxic (apoptotic) effects with no side effects for normal cells.<sup>89</sup> In general, certain bacterial peptides and proteins, including anticancer antibiotics, represent a promising group of potential anticancer drugs.<sup>90,91</sup> Interestingly, even the bacterium *Pseudomonas aeruginosa* itself was used to treat cancer, in the form of stents and catheters containing biofilms or in membrane-encased devices.<sup>88</sup>

#### 5.1.1 | Highly effective and selective partial sequences of azurin

Azurin p18: LSTAADMQGVVTDMGASG.<sup>89</sup>

Azurin p28: LSTAADMQGVVTDMGASGLDKDYLPDD.<sup>89,92</sup>

Azurin C-terminal fragment 88-113: GSGEKDSVTFDVSCLKKEGE QYMFCT.<sup>93</sup>

Azurin C-terminal fragment 96-113: TFDVSKLKEGEQYMFCT.<sup>93,94</sup>

Cell-penetrating partial sequences of azurin derive from the enzyme cupredoxin secreted by *P. aeruginosa*.<sup>85,86</sup> For example, the fragment p18 is a cell-penetrating peptide whose sequence consists of residue 50 to 67 of azurin.<sup>89</sup> This fragment represents the putative transduction domain of the protein, responsible for penetration into cancer cells. Azurins can enter neoplastic cells from lung cancer, breast cancer, and melanoma, but they cannot enter noncancerous cell lines.<sup>86</sup> On the other hand, peptide p28, the longest azurin fragment, going from residue 50 to 77, is less hydrophobic and proteolytically more stable than its shorter counterpart. This fragment acts as an inhibitor of p53 ubiquitination and thus exerts anti-proliferative activity,<sup>92,95</sup> especially by inducing apoptosis of human cancer cells.

Notably, in first-in-class, first-in-human phase I clinical trials, p28 has been shown to display very little toxicity and high antitumor activity in many advanced-stage cancer patients.<sup>96,97</sup> For well-controlled clinical treatment, a method was developed to determine the blood concentration of p28.<sup>98</sup> The azurin fragment p28 contains six aspartic acid residues and only one positively charged lysine residue; thus, it belongs to the class of anionic CPPs. Fragment p18 is less negatively charged, as it contains two aspartic acid residues.

On the other hand, the C-terminal sequence of azurin, from residue 96 to 113, shows structural similarity to a ligand known as ephrin-B2 and can bind the corresponding receptor, which is involved in cancer progression. Thus, azurin, its fragment p28, and its C-terminal fragments contribute to cancer growth inhibition.<sup>93</sup> Furthermore, when conjugated to nicotine, azurin acts as a radiosensitizer, enhancing the efficacy of radiation treatment of lung cancer and other cancers in which cells overexpress ephrin receptors.<sup>93</sup> Azurin also enhances the sensitivity of tumor cells to various chemotherapeutics<sup>94</sup> by hijacking intracellular signaling networks.<sup>11</sup> Moreover, Metha et al.<sup>99</sup> found that p28 counteracts angiogenesis and tumor growth by inhibiting the phosphorylation of VEGFR2, FAK, and AKT. According to Bernardes et al.,<sup>11</sup> azurin also exerts anticancer activity by interacting with multiple targets and interfering with multiple steps of tumor progression. For instance, treatment with azurin p28 triggered cell cycle arrest in human breast cancer cells.<sup>100</sup> A very promising finding is that peptide p28 and an azurin-like protein called Laz, derived from *Neisseria meningitidis*, exhibit high cytotoxicity towards glioblastoma cells, allowing the development of novel therapeutic approaches.<sup>101</sup> Moreover, after intensive studies, the group of Das Gupta patented some azurin fragments with low toxicity for their use in cancer prevention. However, in contrast to this group, Puni et al.,<sup>102</sup> found that azurin can induce apoptosis in macrophages in a caspase-dependent manner. Very recently Sasidharan et al.<sup>103</sup> proposed to use azurin and its fragment p28, as anti-SARS-CoV-2 inhibitor. Peptide p28 exhibited a strong affinity to the spike protein and ACE-2 receptor. Thus, this peptide has a potential as therapeutic against SARS-CoV-2, mainly for patients which do not respond to vaccines.

The mechanism of cell penetration of these anionic azurin peptides has been investigated by Taylor<sup>89</sup> and Yamada et al.,<sup>104</sup> together with their preferential uptake by cancer cells and the domains required for their translocation. Taylor et al. postulated that the mechanism of cellular penetration and cancer cell selectivity of both azurin fragments is unique relative to most other CPPs.<sup>89</sup> Indeed, by combining inhibitors of discrete steps of the internalization process, these authors suggested that azurin p18 and p28 penetrate human cancer cells via a receptor-mediated endocytic process. However, azurin peptides cannot interact with cell surface glycosaminoglycans like cationic CPPs. Conversely, they preferentially penetrate cancer cells via caveosome-mediated but also caveosome-independent pathways. The azurin fragments p18 and p28 can also penetrate the plasma membrane directly and reach late endosomes, lysosomes, and the Golgi apparatus. These findings are consistent, to some degree, with studies on the membrane transport of another class of anionic peptides, type SAP(E) proline-rich peptides. Indeed, the interaction with

the negatively charged cell surface is not required for the internalization of this family of peptides.<sup>105</sup> However, these explanations are not sufficient to elucidate similarities and differences in the uptake pathways of different CPP types, nor to understand the specific pathway for the uptake of azurin fragments.

On the other hand, pinpointing the conformational requirements for the uptake of cationic, amphipathic, or anionic CPPs seems slightly more difficult. Indeed, according to Frederic Heitz and Gill Divita,<sup>36</sup> conformational versatility seems to be crucial for efficient cellular uptake. In our opinion, the penetration of the plasma membrane requires a distinct conformational flexibility, including the ability to assume a helical conformation when crossing the lipid bilayer. In contrast to this assumption, conformationally constrained cyclic CPPs exhibit high internalization efficiency. Furthermore, we must consider that CPPs and cargoes can form nanoparticles of different sizes and zeta potentials and that such nanoparticles exhibiting CPPs at their surface can penetrate membranes.

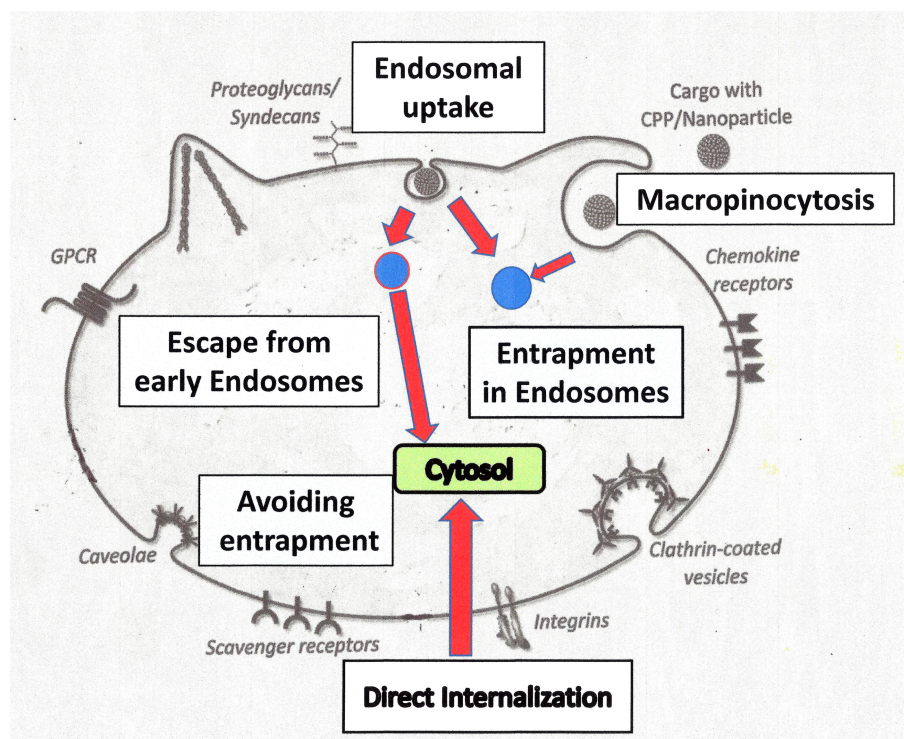
## 6 | CELL-PENETRATING PROTEINS WITH SPECIFIC FUNCTIONAL PROPERTIES

### 6.1 | Avoiding endosomal entrapment or triggering endosomal escape

In addition to their low cell and tissue selectivity, the entrapment in endosomes is a major drawback of the use of CPPs from the first generations. Therefore, many attempts have been made to circumvent this obstacle. On the one hand, auxiliary compounds such as chloroquine or even irradiation-activatable compounds can be added to induce the release of CPPs from endosomes. For instance, in lipid-based nanocarriers, the use of cationic lipids facilitates endosomal escape. On the other hand, transport peptides can display partial sequences to avoid entrapment or to promote their release into the cytosol.<sup>106</sup> Figure 3 shows the different ways of internalization into live cells, the endosomal uptake with entrapment in endosomes and release from endosomes and the direct internalization, too.

#### 6.1.1 | Composed peptides with endosomal leakage domains

Endosomal leakage domains such as CM18 can be fused to the sequence of penetrating peptides, to obtain, for example, CM18-HIV-TAT(47–57) and CM18-PTD-4. The sequence of CM18 derives from the hydrophilic N-terminal sequence (1–7) of cecropin A, combined with the N-terminal sequence (2–12) of melittin. Interestingly, even upon fusion with HIV-TAT(47–57), this conjugate retains its structural and functional characteristics: it assumes a typical  $\alpha$ -helical secondary structure in hydrophobic environments, preserves the ability to cross intracellular membranes, enables the translocation of membrane-impermeable molecules, and shows no detectable cytotoxicity.<sup>107</sup>



**FIGURE 3** Endocytosis and transport into cytosol

CM 18-HIV-TAT(47–57)

YGRKKRRQRRR- KWYLFKKIGAVLKYLTTG.<sup>107</sup>

This hybrid peptide is applicable as a delivery vector for plasmid DNA. For instance, Salomone et al.<sup>107</sup> used this vector for successful delivery of the plasmid pCMV-GLuc 2 into HeLa and CHO-K1 cells.

(His)<sub>6</sub>-CM18-PTD4

His-His-His-His-His-His-KWYLFKKIGAVLKYLTTG-YRAAARQARA.<sup>26</sup>

This peptide is composed of a histidine-rich domain fused to the endosomolytic peptide CM18 (cursive) and the protein transduction domain PTD4 (bold). Within a short co-incubation time, it can form noncovalent complexes with very large functionally active proteins. This rationally designed peptide promotes the delivery of cargo proteins into difficult-to-transfect cells. For example, it can deliver CRISPR-Cas9 and -Cpf1 ribonucleoproteins into human T-cell-derived Jurkat cells and primary NK cells. Thus, it could enable the use of the very promising CRISPR recombinant proteins for therapeutic approaches, such as gene editing. This designed hybrid peptide can also efficiently deliver functional proteins with gene-regulating activity, such as homeobox B4.<sup>26</sup>

### 6.1.2 | Single peptides with alternating chirality of amino acid residues

In contrast to ligands for hormone receptors, CPPs seem to be less sensitive to changes in their amino acid configuration. For example, in the sequence of HIV-TAT, and also in oligo-arginine peptides the L-arginine residues can be replaced by D-arginine residues without loss of transport activity. Also, in azurin p28 D-amino acids could be

inserted in different positions. Firstly, Lättig-Tünemann et al.<sup>108</sup> have replaced in some linear cationic and also one cyclic peptide either all L-amino acids by their D-configuration or used alternating chiralities. These replacements leads in most cases to increased cellular uptake and to localization in nucleus.

Oligo-arginine: rRrRrRRR.<sup>109</sup>

Cys-(HIV-TAT(47–56;r,R): C-YGRrRrRQRr-amide.<sup>23</sup>

Azurin fragment p28 with D-amino acids at different positions.<sup>110</sup>

The HIV-TAT(47–57) peptide is derived from the cell transduction domain of the naturally existing transactivator of transcription (TAT) protein of the human immunodeficiency virus type I (HIV I).<sup>111</sup> The use of HIV-TAT fragments for delivery of therapeutic agents and nanoparticles into cells and tumors has been repeatedly validated.<sup>15,112</sup> Moreover, some HIV-TAT fragments and oligoarginine peptides with alternating chirality of arginine residues can release the cargo directly into the cytosol without being entrapped in endosomes.<sup>108,109,113</sup> However, we must consider that peptides with alternating chirality of arginine residues assume a different conformation, which influences solubility and enhances adhesion to surfaces.<sup>23</sup> Indeed, D-amino acid replacement in the azurin p28 peptide can result in a decreased intracellular uptake although its preferential uptake into cancer cells is not altered.<sup>109</sup> Finally, conjugation of CPPs to special dendrimers, or to N-terminal HA2-subunit of the influenza virus, also increases endosomal release.<sup>114</sup>

### 6.1.3 | Cyclic peptides

Cyclic (HIV-TAT), cyclic (HIV-TAT) with alternating chirality of amino acids,



cyclic (RRRRRRRRR),<sup>108,115,116</sup>

Cyclo(F-naphthylalanine-RRRRQ) and analogs.<sup>117,118</sup>

In some studies, it could be shown that cyclization of CPPs stimulates not only the cellular uptake but also the release from endosomes or triggers a nonendosomal uptake mechanism.<sup>115,116</sup> Thus Pei's group proposed for a cyclic cationic heptapeptide escape from early endosomes by vesicle budding,<sup>117</sup> whereas Hackenberger's group assumes a nonendocytotic pathway for the investigated cyclic peptide.<sup>115</sup>

### 6.1.4 | Peptides with intrinsic endosomal release: Bindin fragments

B18: LGLLLRHLRHHSNLLANI.<sup>119</sup>

B55<sup>120</sup>:

KAVLGATKIDLPVDINDPYDLGGLLRHLRHHSNLLANIGDPAVREQVL-SAMQEEE.

The bindin fragments B18 and B55 are partial sequences of a surface protein of sea urchin sperm. B18 represents the amino acid sequence 103–120 and B55 the sequence 83–137. Bindin triggers the attachment of sperm to glycoconjugate receptors on the egg surface and the fusion of egg and sperm plasma membranes.<sup>121</sup>

At first, research on bindin peptides focused only on the interaction between sperm and egg, whereas they raised a renewed interest for the penetration of human cells. B18 exhibits many similarities to viral fusion proteins. Zink-ions promote formation of the active helical conformation. B18 represents the “minimal membrane-binding and fusogenic motif.”<sup>120</sup> Both peptides, B18 and B55, can self-assemble in lipid bilayers and transport biomacromolecules. Thus, an enhanced green fluorescent protein (EGFP)-fused B55 peptide was developed to promote the intracellular uptake of coadministered biomacromolecules such as dextrans, monoclonal single-chain antibodies, and even pDNA into human cell lines.<sup>120,122</sup> Moreover, both peptides can perform not only endosomal escape but also nuclear translocation.<sup>120</sup>

### 6.2 | Transporters for thermally targeted cargo delivery

CPP fusion proteins with elastin-like peptides (ELPs) (Val-Pro-Gly Xaa-Gly)<sub>n</sub><sup>123</sup>

Xaa: guest residue, any proteinogenic amino acid except Pro

Elastin-like peptides (ELPs) are bioengineered and biocompatible proteins that, when conjugated to CPPs, can act as carriers for delivery of therapeutic peptides with temperature- and pH-dependent properties.<sup>124</sup> Their basic structure derives from a sequence repeat (Val-Pro-Gly-Xaa-Gly) found in the mammalian elastin protein. ELPs undergo temperature-dependent inverse phase transition<sup>125</sup> and accumulate in tumors upon exposure to externally focused heat (40–42°C).<sup>123</sup> For example, ELPs conjugated to antibody fragments against the epidermal growth factor receptor

(EGFR) through self-assembly of nanoparticles can mediate NIR-light-induced cell killing. Therefore, ELPs are considered, together with CPPs, potential carriers for thermally targeted delivery of therapeutic peptides.<sup>126</sup>

### 6.3 | Specific transport into neuronal cells

#### 6.3.1 | RDP KSVRTWNEIIPSKGCLRVGGRCHPHV NGGRRRRRRRRRRR.<sup>127</sup>

The recombinant fusion product RDP consists of 39 amino acids derived from the rabies virus glycoprotein (RVG) fused with a non-arginine residue at the C terminus. RVG is the only known protein component that interacts specifically with neuronal cells. The nerve-binding region of the new peptide RDP was partially taken from the residues 189–214 and 330–357 of the glycoprotein RVG, which guide viral entry into neuronal cells. Thus, the protein RDP enables the delivery of fused proteins into neuronal cells, such as the glial cell-derived neurotrophic factor (GDNF). Notably, this fusion protein exhibits a neuroprotective effect in Parkinson's disease animal models. The uptake of these RDP fusion proteins seems to occur through GABA receptors.<sup>127</sup>

#### 6.3.2 | Tumor-homing peptide LyP-1: CGNKRTRGC linear or with S-S- bridge between N<sup>1</sup> and N<sup>9</sup><sup>128</sup>

The tumor-homing peptide LyP-1 recognizes neuropilin receptors expressed in glioma tumor tissues. Its linear form displays enhanced penetrating properties compared to the cyclic peptide. However, both forms can interact with cells of breast cancer, prostate cancer, tumor vasculature, and glioma. Moreover, they promote the penetration of drugs into these tumors, even when non-covalently bound.

### 6.4 | Stabilization against proteolytic degradation

Since peptides can be degraded by many different proteases in the skin, blood, intestinal tract, organs, and tissues, CPPs should be stabilized against enzymatic degradation. For this reason, their N- and C-terminus or peptide backbone can be modified by fatty acylation or amidylation,<sup>78,79,129</sup> and certain amino acid residues in their sequence can be replaced by residues in D configuration<sup>23,109,110</sup> or by non-proteinogenic amino acids and moieties.<sup>78,79,129,130</sup> For example, peptides can be cyclized through the formation of disulfide or lactam bridges,<sup>48,49,65,76,130,131</sup> or even by modification or cyclization of the peptide backbone.<sup>132–135</sup> Qian et al.<sup>117</sup> tested even the oral bioavailability of an applied cyclic hexapeptide by studying time dependent plasma concentrations after oral administration. In many cases,

coupling to polymers enhances the body circulation time sufficiently for navigation to the target tissue and drug delivery.<sup>12–17,133,136,137</sup> A very simple and intelligent example is the use of the guanidine-rich carrier D-sorbitol-3-(guanido moiety)<sub>8</sub>, which is proteolytically stable and more efficient in the internalization of cargoes than octa-arginine.<sup>138</sup> In our opinion, this stable and efficient compound also provides a potential method for reducing the costs of clinical application of CPPs.

## 7 | CONCLUSIONS AND PROSPECTS

Cell-penetrating peptides are promising agents for disease diagnosis and therapy because they can drive cell- and tissue-specific internalization of diagnostic labels and therapeutics into live target cells. To positively identify the most effective CPPs for clinical trials, it is recommended to use cellular 3D models. Moreover, the transduction domains of proteins from venoms, viral proteins, such as RVG, and bacterial proteins, such as azurin, can be exploited to transfect cells selectively. Thus, peptides from the new generation are either derived from these natural sources or designed as hybrid peptides with domains of different functions. These peptides exhibit intrinsic or activatable cell and tissue selectivity and preferences for cargo types.

The necessity of combining various properties in one molecule, such as high transport efficiency, effective escape from or release from intracellular vesicles, enhanced proteolytic stability, and easy handling, has led to the development of complex hybrid structures. For instance, fusion proteins allow the combination of sequences performing different functions; alternatively, efficient CPPs can be coupled to multifunctional nanoparticles. An advantage of conjugation to nanoparticles is the improvement of tissue selectivity, which can be achieved through coupling with ligands for target-specific binding proteins, such as tumor-specific receptors. In addition, together with topical administration, local thermal activation or irradiation of these carriers can be used for highly selective diagnosis and therapy. A realistic goal for the next future is the improvement of classical drugs through their CPP-mediated delivery into specific tissues and organs.

Detection of tumors and small metastases requires not only highly selective CPPs or conjugates, but also their coupling with highly sensitive labels and the use of highly sensitive detection methods such as NIR-fluorescence scanning and NMR tomography. Therefore, broadening the clinical use of CPPs requires the development not only of highly sensitive labels but also of new and more sensitive detectors, such as human-body NIR-fluorescence scanners.

Furthermore, owing to their ability to mediate the transport of antibiotics and antimicrobial oligonucleotides into infected cells, CPPs can help to treat infections of multidrug-resistant bacteria. In addition, since CPPs can penetrate barriers, their use can allow to replace repeated injections with various kinds of painless, safe, and needleless drug administration. Finally, some CPPs are promising drug transporters for the treatment of glioblastomas, other neurodegenerative diseases, and eye diseases.

However, more effective CPPs need to be developed for in order to better manipulate hardly to transfect cells, such as stem cells, macrophages, and antigen-presenting cells, especially at the time of the pandemic coronavirus disease COVID-19.

On the other hand, the development of CPPs as transport vehicles for drugs suffers from a lack of theoretical knowledge for predicting the most suitable CPPs for the chosen cargo and target tissue. In fact, prediction of sequences with optimized functions is only possible for structurally homogenous groups of CPPs, cargoes, and tissues. Therefore, manifold experimental trials are necessary for optimizing the combinations of CPPs, cargoes, and target tissues or organs.

Future research should focus on diseases for which other therapies have failed so far. Furthermore, CPPs can be applied for the improvement of classical drugs by tissue-specific delivery into cells or cell organelles and for the transportation of drugs through barriers such as the skin, the BBB, and the cornea, lens, and conjunctiva of eyes. Based on the current preclinical and clinical trials, the most promising fields for clinical application of CPPs seem to be the treatment of infarct, neurodegenerative processes, muscle dystrophy, inflammation, thrombosis, eye diseases, and different kinds of cancer: brain, cervix, breast, ovarian, prostate, melanoma, thyroidal, and pancreatic cancer.

For an improved stability against proteases, better handling, and especially for an enhanced tissue selectivity, certain CPPs can be conjugated to polymers or nanoparticles. However, to conjugate the right CPPs to the right polymer or nanoparticle, biodistribution and pharmacodynamic studies should be performed for CPPs, nanoparticles, and conjugates. In summary, CPPs should be further improved in order to develop self-navigating, biocompatible particles for drug delivery into targeted tissues.

These complex requirements, together with the expected high cost of CPPs, may explain why after about 30 years from the discovery of the first CPP, none of them has been approved by the FDA. Indeed, the use of CPPs as vectors for vaccination suffers not only from high cost but also from the necessity of transducing or transfecting immune cells, which are hardly to transfect cells.

## ACKNOWLEDGEMENTS

Open access funding enabled and organized by Projekt DEAL.

## AUTHOR CONTRIBUTIONS

Siegmund Reissmann and Margarita P. Filatova contributed equally to this work.

## ORCID

Siegmund Reissmann  <https://orcid.org/0000-0002-1083-5672>

## REFERENCES

1. Reissmann S. Cell-penetration: scope and limitations by application of cell-penetrating peptides. *J Pep Sci.* 2014;20(10):760-784.
2. Van Asbeck AH, Dieker J, Boswinkel M, van der Vlag J, Brock R. Kidney targeted therapies: a quantitative perspective. *J Control Rel.* 2020;328:762-775.

3. Savanier EN, Felsen CN, Nashi N, et al. Real-time in vivo molecular detection of primary tumors and metastases with ratiometric activatable cell-penetrating peptides. *Cancer Res.* 2013;73:855-864.
4. Metildi CA, Felsen CN, Savariar EN, et al. Ratiometric activatable cell-penetrating peptides label pancreatic cancer, enabling fluorescence-guided surgery, which reduces metastases and recurrence in orthotopic mouse models. *Ann Surg Oncol.* 2015;22(6):2082-2087.
5. Tansi FL, Ruger R, Bohm C, et al. Potential of activatable FAP-targeting immunoliposomes in intraoperative imaging of spontaneous metastases. *Biomaterials.* 2016;88:70-82.
6. Andreev OA, Engelman DM, Reshetnyak YK. pH-sensitive membrane peptides (pHLIPs) as a novel class of delivery agents. *Mol Membrane Biol.* 2010;27(7):341-352.
7. Fei L, Yap L-P, Conti PS, Shen W-C, Zaro JL. Tumor targeting of a cell-penetrating peptide by fusing with a pH-sensitive histidine-glutamate co-oligopeptide. *Biomaterials.* 2014;35(13):4082-4087.
8. Splith K, Bergmann R, Pietzsch J, Neundorf I. Specific targeting of hypoxic tumor tissue with nitroimidazole-peptide conjugates. *Chem-MedChem.* 2012;7(1):57-61.
9. Trentin D, Hall H, Wechsler S, Hubbell JA. Peptide-matrix-mediated gene transfer of an oxygen-insensitive hypoxia-inducible factor-1 $\alpha$  variant for local induction of angiogenesis. *Proc Natl Acad Sci U S A.* 2006;103(8):2506-2511.
10. Diao Y, Han W, Zhao H, et al. Designed synthetic analogs of the  $\alpha$ -helical peptide temporin-La with improved antitumor efficacies via charge modification and incorporation of the integrin  $\alpha$ v $\beta$ 3 homing domain. *J Pep Sci.* 2011;18:476-486.
11. Bernardes N, Ribeiro AS, Abreu S, et al. The bacterial protein azurin impairs invasion of FAK/Src signaling in P-cadherin-overexpressing breast cancer cell models. *PLoS One.* 2013;8(7):e69023.
12. Ndeboko B, Lemamy GJ, Nielsen PE, Cova L. Therapeutic potential of cell penetrating peptides (CPPs) and cationic polymers for chronic hepatitis B. *Int J Mol Sci.* 2015;16(12):28230-28241.
13. Park S, Ej J, Lee J, Rhim T, Lee SK, Lee KY. Preparation and characterization of nonarginine-modified chitosan nanoparticles for siRNA delivery. *Carbohydr Polym.* 2013;92(1):57-62.
14. Alibolandi M, Alabdollah F, Sadeghi F, et al. Dextran-b-poly (lactide-co-glycolide) polymersome for oral delivery of insulin: *in vitro* and *in vivo* evaluation. *J Control Rel.* 2016;227:58-70.
15. Deshpande P, Jhaveri A, Pattni B, Biswas S, Torchilin V. Transferrin and octaarginine modified dual-functional liposomes with improved cancer cell targeting and enhanced intracellular delivery for the treatment of ovarian cancer. *Drug Deliv.* 2018;25(1):517-532.
16. Rajala A, Wang Y, Zhu Y, et al. Nanoparticle-associated targeted delivery of eye-specific genes to eyes significantly improves the vision of blind mice *in vivo*. *Nano Lett.* 2014;14(9):5257-5263.
17. Liu X, Liu C, Zhang W, Xie C, Wei G, Lu W. Oligoarginine-modified biodegradable nanoparticles improve the intestinal absorption of insulin. *Int J Pharm.* 2013;448(1):159-167.
18. Calce E, Monfregola L, Saviano M, De Luca S. HER2-mediated anticancer drug delivery: strategies to prepare targeting ligands highly specific for the receptor. *Cur Med Chem.* 2015;22(21):2525-2538.
19. Manosroi J, Lohcharoenkai W, Goetz F, Werner RG, Manosroi W, Manosroi A. Novel application of polioviral capsid: development of a potent and prolonged oral calcitonin using polioviral binding ligand and Tat peptide. *Drug Dev Ind Pharm.* 2014;40(8):1092-1100.
20. Zhao Y, He Z, Gao H, et al. Fine tuning of core-shell structure of hyaluronic acid cell penetrating peptides/siRNA nanoparticles for enhanced gene delivery to macrophages in antiatherosclerotic therapy. *Biomacromolecules.* 2018;19(7):2944-2956.
21. Olson ES, Whitney MA, Friedman B, et al. *In vivo* fluorescence imaging of atherosclerotic plaques with activatable cell-penetrating peptides targeting thrombin activity. *Integrative Biol.* 2012;4(6):595-605.
22. Tansi F, Kallweit E, Kaether C, et al. Internalization of near-infrared fluorescently labeled cell-penetrating peptide and of proteins into human fibrosarcoma cell line HT-1080. *J Cell Biochem.* 2015;116:1223-1231.
23. Tansi FL, Filatova MP, Korojev DO, et al. New generation CPPs show distinct selectivity for cancer and non-cancer cells. *J Cell Biochem.* 2019;120(4):6528-6541.
24. Malone CD, Olson ES, Mattrey RF, Jiang T, Tsien RY, Nguyen QT. Tumor detection at 3 tesla with an activatable cell penetrating peptide dendrimer (ACPPD-Gd), at T1 magnetic resonance (MR) molecular imaging agent. *PLoS One.* 2015;10(9). e0137104/1-e0137104/15
25. Duijnhoven SMJ, Robillard MS, Nicolay K, Gruell H. Development of radiolabeled membrane type-1 matrix metalloproteinase activatable cell-penetrating peptide imaging probes. *Molecules.* 2015;20(7):12076-12092.
26. Del Guidice T, Lepetit-Stoffaes JP, Boerdeleau LJ, et al. Membrane permeabilizing amphiphilic peptide delivers recombinant transcription factor and CRISPR-Cas9/Cpf1 ribonucleoproteins in hard to modify cells. *PLoS One.* 2018;13(4):e0195558.
27. Srimanee A, Arvanitidou M, Kim K, Hallbrink M, Langel . Cell-penetrating peptides for siRNA delivery to glioblastomas. *Peptides.* 2018;104:62-69.
28. Moulton HM, Wu B, Jearawiriyapaisarn N, Sazani P, Lu QL, Kole R. Peptide-morpholino conjugate: a promising therapeutic for Duchenne muscular dystrophy. *Annals New York Acad Sci.* 2009;1175 (Oligonucleotide Therapeutics): 55-60.
29. Van den Brand D, Veelken C, Massuger L, Brock R. Penetration in 3D tumor spheroids and explants: adding a further dimension to the structure-activity relationship of cell-penetrating peptides. *Biochim Biophys Acta (Biomembranes).* 2018;1860(6):1342-1349.
30. Keller AA, Maess MB, Schnoor N, Scheiding B, Lorkowski S. Transfecting macrophages. In: Rousselet, ed. *Macrophages: Methods and Protocols, Methods in Molecular Biology.* New York, Dordrecht, Heidelberg, London: Human Press, Springer; 2018;1784:187-195.
31. Mussbach F, Franke M, Zoch A, Schaefer B, Reissmann S. Transduction of peptides and proteins into live cells by cell penetrating peptides. *J Cell Biochem.* 2011;112(12):3824-3833.
32. Keller AA, Mussbach F, Breitling R, et al. Relationships between cargo, cell penetrating peptides and cell type for uptake of non-covalent complexes into live cells. *Pharmaceuticals.* 2013;6(2):184-203.
33. Keller A-A, Scheiding B, Breitling R, et al. Transduction and transfection of difficult-to-transfect cells: systematic attempts for the transfection of protozoa Leishmania. *J Cell Biochem.* 2019;120:14-27.
34. Kardani K, Milani A, Shabani SH, Bolhassani A. Cell penetrating peptides: the potent multi-cargo intracellular carriers. *Expert Opin Drug Deliv.* 2019;16(11):1227-1258.
35. Zhang P, Ma J, Yan Y, et al. Arginine modification of lycosin-I to improve inhibitory activity against cancer cells. *Org Biomol Chem.* 2017;15(44):9379-9388.
36. Deshayes S, Konate K, Aldrian G, Grombetz L, Heitz F, Divita G. Structural polymorphism of non-covalent peptide-based delivery systems: highway to cellular uptake. *Biochim Biophys Acta.* 1798;2010:2304-2314.
37. Gao M, Zhou J, Su Z, Huang Y. Bacterial cupredoxin azurin hijacks cellular signaling networks: protein-protein interactions and cancer therapy. *Protein Sci.* 2017;26(12):2334-2341.
38. Vighi E, Montanari M, Ruozi B, Tosi G, Magli A, Leo E. Nuclear localization of cationic solid lipid nanoparticles containing protamine as transfection promotor. *Europ J Pharm Biopharm.* 2010;76(3):384-393.

39. Byun Y, Singh VK, Yang VC. Low molecular weight protamine: a potential non-toxic heparin antagonist. *Thromb Res.* 1999;94(1):53-61.
40. He H, Ye J, Liu E, Liang Q, Liu Q, Yang VC. Low molecular weight protamine (LMWP): a nontoxic protamine substitute and effective cell-penetrating peptide. *J Control Rel.* 2014;193:63-73.
41. He H, Sheng J, David AE, et al. The use of low molecular weight protamine chemical chimera to enhance monomeric insulin intestinal absorption. *Biomaterials.* 2013;34(31):7733-7743.
42. Caoduro C, Kacem R, Boukari K, et al. Carbon nanotube-protamine hybrid: evaluation of DNA cell penetration. *Carbon.* 2016;96:742-752.
43. Suh JS, Lee HJ, Nam H, et al. Control of cancer stem cell like population by intracellular target identification followed by treatment with peptide-siRNA complex. *Biochem Biophys Res Comm.* 2017;491(3):827-833.
44. Wang H, Zhao Y, Wang H, et al. Low-molecular-weight protamine-modified PLGA nanoparticles for overcoming drug-resistant breast cancer. *J Control Rel.* 2014;192:47-56.
45. Xia H, Gao X, Gu G, et al. Low molecular weight protamine-functionalized nanoparticles for drug delivery to the brain after intranasal administration. *Biomaterials.* 2011;32(36):9888-9898.
46. Xia H, Gu G, Hu Q, et al. Activatable cell penetrating peptide-conjugated nanoparticles with enhanced permeability for site-specific targeting delivery of anti-cancer drug. *Bioconjug Chem.* 2013;24(3):419-430.
47. Suh JS, Kim KS, Lee JY, Choi YJ, Lee JY, Park YJ. A cell permeable fusion protein for the mineralization of human dental pulp stem cells. *J Dental Res.* 2012;91(1):90-96.
48. Nascimento FD, Hayashi MAF, Kerkis A, et al. Crotonamine mediates gene delivery into cells through the binding to heparin sulphate proteoglycans. *J Biol Chem.* 2007;282(29):21349-21360.
49. Radis-Baptista G, Kerkis I. Crotonamine, a small basic polypeptide myotoxin from rattlesnake venom with cell-penetrating properties. *Curr Pharmaceutical Design.* 2011;17(38):4351-4361.
50. Radis-Baptista G, de la Torre BG, Andreu D. A novel cell-penetrating peptide sequence derived by structural minimization of a snake toxin exhibits preferential nucleolar localization. *J Med Chem.* 2008;51(22):7041-7044.
51. Chan JY-W, Zhou H, Kwan YW, Chan SW, Radis-Baptista G, Lee SM-G. Evaluation of zebrafish model of the toxicity of rhodamine B-conjugated crotonamine, a peptide potentially useful for diagnostics and therapeutics. *J Biochem Mol Toxicol.* 2017;31(11):e21964. <https://doi.org/10.1002/jbt.21964>
52. Campairo JD, Marinovic MP, Carapeto FC, et al. Oral treatment with rattlesnake native polypeptide crotonamine efficiently inhibits the tumor growth with no potential toxicity for the host animal and with suggestive positive effects on animal metabolic profile. *Amino Acids.* 2018;50(2):267-278.
53. Campairo JD, Dam W, Monte GG, et al. Long term safety of targeted internalization of cell penetrating peptide crotonamine into renal proximal tubular epithelial cells *in vivo*. *Sci Rep.* 2019;9(1):1-13.
54. Silvestrini AVP, de Macedo LH, de Andrade TAM, Mendes MF, Pigoso AA, Mazzi MV. Intradermal application of crotonamine induces inflammatory and immunological changes *in vivo*. *Toxins (Basel).* 2019;11(1):39. <https://doi.org/10.3390/toxins11010039>
55. Karpel RL. DNA binding proteins and drug delivery vehicles: tales of elephants and snakes. *Curr Prot Pept Sci.* 2015;16(8):718-726.
56. Peigneur SO, Orts DJB, de Silva ARP, et al. Crotonamine pharmacology revisited: novel insights based on the inhibition of K<sup>v</sup> channels. *Mol Pharmacol.* 2012;82(1):90-96.
57. Rodrigues M, de la Torre BG, Radis-Baptista G, Santos NC, Andreu D. Efficient cellular delivery of  $\beta$ -galactosidase mediated by NrTPs, a new family of cell-penetrating peptides. *Bioconjug Chem.* 2011;22(11):2339-2344.
58. Rodrigues M, Andreu D, Santos NC. Uptake and cellular distribution of nuclear targeting peptides (NrTPs) in different cell types. *Biopolymers.* 2015;104(2):101-109.
59. Nascimento FD, Sancey L, Pereira A, et al. The natural cell-penetrating peptide crotonamine targets tumor tissue *in vivo* and triggers a lethal calcium-dependent pathway in cultured cells. *Mol Pharm.* 2012;9(2):211-221.
60. Hernandez-Oliveira e Silva S, Rostelato-Ferreira S, Rocha e Silva TAA, et al. Beneficial effect of crotonamine in the treatment of myasthenic rats. *Muscle Nerve.* 2013;47(4):591-593.
61. Pereira A, Kerkis A, Hayashi MAF, et al. Crotonamine toxicity and efficacy in mouse models of melanoma. *Exp Opin Investig Drugs.* 2011;20(9):1189-1200.
62. Kerkis I, Silva FDS, Pereira A, Kerkis A, Radis-Baptista G. Biological versatility of crotonamine: a cationic peptide from the venom of South American rattlesnake. *Exp Opin Investig Drugs.* 2010;19(12):1515-1525.
63. Dal Mas C, Pinheiro DA, Campeiro JD, et al. Biophysical and biological properties of small linear peptides derived from crotonamine, a cationic antimicrobial/antitumor toxin with cell penetrating and cargo delivery abilities. *Biochim Biophys Acta (Biomembranes).* 2017;1859(12):2340-2349.
64. El Chamy MS, Dai Mas C, Oliveira EB, et al. Inhibition of malaria parasite plasmodium falciparum development by crotonamine, a cell penetrating peptide from the snake venom. *Peptides.* 2016;78:11-16.
65. Fajloun Z, Kharrat R, Chen I, et al. Chemical synthesis and characterization of maurocalcine, a scorpion toxin that activates Ca<sup>2+</sup> release channel/ryanodine receptors. *FEBS Lett.* 2000;469(2-3):179-185.
66. Tisseyre C, Bahemberae E, Dardevet L, Sabatier J-M, Ronjat M, De Waard M. Cell-penetration properties of a high efficient mini-maurocalcine peptide. *Pharmaceuticals.* 2013;6(3):320-339.
67. Poillot C, Bichraoui H, Bahemberae E, et al. Small efficient cell-penetrating peptides derived from scorpion toxin maurocalcine. *J Biol Chem.* 2012;287(21):17331-17342.
68. Aroui S, Ram N, Appaix F, et al. Maurocalcine as a non-toxic drug carrier overcomes doxorubicin resistance in the cancer cell line MDA-MB 231. *Pharm Res.* 2009;26(4):836-845.
69. Poillot C, Dridi K, Bichraoui H, et al. D-Maurocalcine, a pharmaceutically inert efficient cell-penetrating peptide analogue. *J Biol Chem.* 2010;285(44):34168-34180.
70. Perret P, Ahmadi M, Riou L, et al. Biodistribution, stability, and blood distribution of cell penetrating peptide maurocalcine. *Int J Mol Sci.* 2015;16(11):27730-27740.
71. Khomehchian S, Nikkhal M, Madani R, Hosseinkhani S. Enhanced and selective permeability of gold nanoparticles functionalized with cell penetrating peptide derived from maurocalcine animal toxin. *J Biomedical Materials.* 2016;104(11):2692-2700.
72. Liu Z, Deng M, Xiang J, et al. A novel spider peptide toxin suppresses tumor growth through dual signaling pathways. *Curr Mol Med.* 2012;12(10):1350-1360.
73. Tan H, Huang Y, Xu J, et al. Spider peptide lycosin-I functionalized gold nanoparticles for *in vivo* tumor targeting and therapy. *Theranostics.* 2017;7(12):3168-3178.
74. Xu Y-Y, Cao X-W, Fu L-Y, Zhang T-Z, Wang F-J, Zhao J. Screening and characterization of a novel high-efficiency tumor-homing cell-penetrating peptide from the buffalo cathelicidin family. *J Pep Sci.* 2019;25(9):e3201.
75. Zhang L, Zhang Y, Tai L, et al. Functionalized cell nucleus-penetrating peptide combined with doxorubicin for synergistic treatment of glioma. *Acta Biomater.* 2016;42:90-101.
76. Chang M, Li X, Sun Y, et al. Effect of cationic cyclopeptides on transdermal and transmembrane delivery of insulin. *Mol Pharm.* 2013;10(3):951-957.

77. Chang M, Li X, Sun Y, et al. A potential mechanism of cationic cyclopeptide for enhancing insulin delivery across Caco-2 cell monolayers. *Biol Pharmaceut Bull.* 2013;36(10):1602-1607.
78. Regberg J, Shrimanee A, Erlandsson M, et al. Rational design of a series of novel amphipathic cell-penetrating peptides. *Int J Pharmaceutics.* 2014;464(1-2):111-116.
79. Ervin E-H, Pook M, Teino I, Kasuk V, Trei A, Pooga M, Meimers T. Targeted gene silencing in human embryonic stem cells using cell-penetrating peptide PepFect 14. *Stem Cell Res Therapy.* 2019;10(43). <https://doi.org/10.1186/s13287-019-1144-x>
80. van den Brand D, Gorris MAJ, van Asbeck AH, et al. Peptide-mediated delivery of therapeutic mRNA in ovarian cancer. *Europ J Pharm Biopharm.* 2019;141:180-190.
81. Ezzat K, Andaloussi SE, Zagloul EM, et al. PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and solid formulation. *Nucleic Acids Res.* 2011;39(12):5284-5298.
82. Shrimanee A, Arvanitidou M, Kim K, Hällbrink M, Langel Ü. Cell-penetrating peptides for siRNA delivery to glioblastomas. *Peptides.* 2018;104:62-69.
83. Juks C, Lorents A, Arukuusk P, Langel Ü, Pooga M. Cell-penetrating peptides recruit type A scavenger receptors to the plasma membrane for cellular delivery of nucleic acids. *FASEB j.* 2017;31(3):975-988.
84. Alarcon M, Eriksson J, Langel Ü. Novel efficient cell-penetrating, peptide-mediated strategy for enhancing telomerase inhibitor oligonucleotides. *Nucleic Acid Therapeutics.* 2015;25(6):306-310.
85. Chakrabarty AM, Bernardes N, Fialho AM. Bacterial proteins and peptides in cancer therapy: today and tomorrow. *Bioengineered.* 2014;5(4):234-242.
86. Chakrabarty AM. Bacterial azurin in potential cancer therapy. *Cell Cycle.* 2016;15(13):1665-1666.
87. Perez-Henarejos SA, Alcaraz LA, Donaire A. Blue copper proteins: a rigid machine for efficient electron transfer, a flexible device for metal uptake. *Archives Biochem Biophys.* 2015;584:134-148.
88. Bernardes N, Chakrabarty AM, Fialho AM. Engineering of bacterial strains and their products for cancer therapy. *Appl Microbiol Biotechnol.* 2013;97(12):5189-5199.
89. Taylor BN, Mehta RR, Yamada T, et al. Noncationic peptides from Azurin preferentially enter cancer cells. *Cancer Res.* 2009;69(2):537-546.
90. Karpinsky TM, Adamczak A. Anticancer activity of bacterial proteins and peptides. *Pharmaceutics.* 2018;10(2):54. <https://doi.org/10.3390/pharmaceutics10020054>
91. Fialho AM, Chakrabarty AM. Recent patents in bacterial proteins as potential anti-cancer agents. *Recent Pat Anticancer Drug Discov.* 2007;2(3):224-234.
92. Jia L, Gorman GS, Coward LU, et al. Preclinical pharmacokinetics, metabolism, and toxicity of azurin p-28 (NSC745104) a peptide inhibitor of p53 ubiquitination. *Cancer Chemothe Pharmacol.* 2011;68(2):513-524.
93. Chaudhari A, Mahfouz M, Fialho AM, et al. Cupredoxin-cancer interrelationship: azurin binding with EphB2, interference in EphB2 tyrosine phosphorylation, and inhibition of cancer growth. *Biochemistry.* 2007;46(7):1799-1810.
94. Micewicz ED, Jung C-L, Schaeue D, Luong H, McBride WH, Ruchala P. Small azurin derived peptide targets ephrin receptors for radiotherapy. *Int J Pept Res Ther.* 2011;17(3):247-257.
95. Yamada T, Mehta RR, Lekmine F, et al. A peptide fragment of azurin induces a p53-mediated cell cycle arrest in human breast cancer cells. *Mol Cancer Ther.* 2009;8(10):2947-2958.
96. Warso MA, Richards JM, Mehta D, et al. A first-in-class, first-in-human, phase I trial of Azurin p28, a non-HDM2-mediated peptide inhibitor of p53 ubiquitination in patients with advanced solid tumors. *Brit J Cancer.* 2013;108(5):1061-1070.
97. Fialho AM, Bernardes N, Chakrabarty AM. Exploring the anticancer potential of the bacterial protein azurin. *Microbiology.* 2016;2(3):292-303.
98. Gorman GS, Coward LU, Freeman L, Noker PE, Beattle CW, Jia L. A novel and rapid LC/MS/MS assay for bioanalysis of azurin p28 in serum and its pharmacokinetics in mice. *J Pharm Biomed Anal.* 2010;53(4):991-996.
99. Mehta RR, Yamada T, Taylor BN, et al. A cell penetrating peptide derived from azurin inhibits angiogenesis and tumor growth by inhibiting phosphorylation of VEGFR-2, FAK and Akt. *Angiogenesis.* 2011;14(3):355-369.
100. Yamada T, Mehta RR, Lekmine F, et al. A peptide fragment of azurin induces a p53-mediated cell cycle arrest in human breast cancer cells. *Mol Cancer Ther.* 2009;18(8):2947-2958.
101. Fialho AM, Salunkhe P, Manna S, Mahall S, Chakrabarty AM. Glioblastoma multiforme: novel therapeutic approaches. *Neurology.* 2012;e642345. <https://doi.org/10.5402/2012/642345>
102. Puni V, Sharma R, Zaborina O, Chakrabarty AM. Energy-generating enzymes of *Burkholderia cepacia* and their interaction with macrophages. *J Bacteriol.* 2003;185(10):3167-3178.
103. Sasidharan S, Selvary C, Singh SK, et al. Bacterial protein azurin and derived peptides as potential anti-SARS-CoV-2 agents: insights from molecular docking and molecular dynamic simulations. *J Biomol Struct Dyn.* 2020. <https://doi.org/10.1080/07391102.2020.1787864>
104. Yamada T, Fialho AM, Punj V, Bratescu L, Das Gupta TK, Chakrabarty AM. Internalization of bacterial redox protein azurin in mammalian cells: entry domain and specificity. *Cell Microbiol.* 2005;7(10):1418-1431.
105. Martin I, Teixido M, Giralt E. Design, synthesis and characterization of a new anionic cell-penetrating peptide: SAP(E). *ChemBiochem.* 2011;12(6):896-903.
106. LeCher J, Nowak SJ, McMurry JL. Breaking in and busting out: cell-penetrating peptides and the endosomal escape problem. *Biomol Concepts.* 2017;8(3-4):131-141.
107. Salomone F, Cardarelli F, Signore G, Boccardi C, Beltram F. In vitro efficient transfection by CM<sub>18</sub>-TAT<sub>11</sub> hybrid peptide: a new tool for gene delivery applications. *PLoS One.* 2013;8(7):e70108.
108. Lättig-Tünnemann G, Prinz M, Hoffmann D, et al. Backbone rigidity and static presentation of guanidinium groups increases cellular uptake of arginine-rich cell-penetrating peptides. *Nature Comm.* 2011;2:1459/1-1459/6.
109. Ma Y, Gong C, Ma Y, et al. Direct cytosolic delivery of cargoes *in vivo* by a chimera consisting of D- and L-arginine residues. *J Control Rel.* 2012;162(2):286-294.
110. Yamada T, Signorelli S, Cannistraro S, Beattle CW, Bizzari AR. Chirality switching with an anionic cell-penetrating peptide inhibits translocation without affecting preferential entry. *Mol Pharm.* 2015;12(1):140-149.
111. Frankel A, Pabo C. Cellular uptake of the tat protein from human immunodeficiency virus. *Cell.* 1988;55(6):1189-1193.
112. Torchilin VP. Tat peptide-mediated intracellular delivery of pharmaceutical nanocarriers. *Adv Drug Deliv Rev.* 2008;60(4-5):548-558.
113. Jerath G, Goyal R, Trivedi V, Santhoshkumar TR, Ramakrishnan v syndiotactic peptides for targeted delivery. *Acta Biomaterialia.* 2019;87:130-139.
114. Erazo-Oliveras A, Muthukrishnan N, Baker R, Wang T-Y, Pellois J-P. Improving endosomal escape of cell-penetrating peptides and their cargos: strategies and challenges. *Pharmaceutics.* 2012;5(11):1177-1209.
115. Schneider AFL, Wallabregue ALD, Franz L, Hackenberger CPR. Targeted subcellular protein delivery using cleavable cyclic cell-penetrating peptides. *Bioconjug Chem.* 2019;30(2):400-404.

116. Nischan N, Herce HD, Natale F, et al. Covalent attachment of cyclic TAT peptides to GFP results in protein delivery into live cells with immediate bioavailability. *Angew Chem Int Ed*. 2015;54(6):1950-1953.
117. Qian Z, Martyna A, Hard RL, et al. Discovery and mechanism of highly efficient cyclic cell-penetrating peptides. *Biochemistry*. 2016; 55(18):2601-2612.
118. Qian Z, LaRochelle JR, Jiang B, et al. Early endosomal escape of a cyclic cell-penetrating peptide allows effective cytosolic cargo delivery. *Biochemistry*. 2014;53(24):4034-4046.
119. Rocha S, Pereira MC, Coelho MAN, Möhwald H, Brezesinski G. Adsorption of fusigenic peptide B18 onto solid surfaces: insights into the mechanism of peptide assembly. *Langmuir*. 2007;23(9): 5022-5028.
120. Nikura K, Horisava K, Doi N. A fusigenic peptide from sea urchin fertilization protein promotes intracellular delivery of biomacromolecules by facilitating endosomal escape. *J Control Rel*. 2015;212:85-93.
121. Lopez A, Miraglia SJ, Glabe CG. Structure/function analysis of the sea urchin sperm adhesive protein bindin. *Develop Biol*. 1993;156(1):24-33.
122. Nikura K, Horisava K, Doi N. Endosomal escape efficiency of fusigenic B18 and B55 peptides fused with EGFR single chain Fv as estimated by nuclear translocation. *J Biochem*. 2016;159(1):123-132.
123. Bidwell GL, Raucher D. Application of thermally responsive polypeptides directed against c-Myc transcriptional function of cancer therapy. *Mol Cancer Ther*. 2005;4(7):1076-1085.
124. Bidwell GL, Raucher D. Cell-penetrating elastin-like polypeptides for therapeutic peptide delivery. *Adv Drug Del Rev*. 2010;62(15): 1486-1496.
125. Krukau A, Brovchenko I, Geiger A. Temperature-induced conformational transition of a model elastin-like peptide GVG (VPGVG)<sub>3</sub> in water. *Biomacromolecules*. 2007;8(7):2196-2202.
126. Pille J, van Lith SAM, van Hest JCM, Leenders WPJ. Self-assembling VHH-elastin-like peptides for photodynamic nanomedicine. *Biomacromolecules*. 2017;18(4):1302-1310.
127. Fu A, Zhao Z, Gao F, Zhang M. Cellular uptake mechanism and therapeutic utility of a novel peptide in targeted delivery of proteins into neuronal cells. *Pharm Res*. 2013;30(8):2108-2117.
128. Ciobanasu C, Dragomir I, Apetrei A. The penetrating properties of tumor homing peptide Lyp-1 in model lipid membranes. *J Pept Sci*. 2019;25:e3145.
129. Oh D, Nasrolahi Shirazi A, Northup K, et al. Enhanced cellular uptake of short polyarginine peptides through fatty acylation and cyclization. *Mol Pharm*. 2014;11(8):2845-2854.
130. Jerath G, Goyal R, Trivedi V, Santhoshkumar TR, Ramakrishnan V. Conformational constrained peptides for drug delivery. *J Pep Sci*. 2020:e3244.
131. Horn M, Reichart F, Natividad-Tietz S, Diaz D, Neundorf I. Tuning the properties of novel short cell-penetrating peptide by intramolecular cyclization with a triazole bridge. *Chem Commun*. 2016;52(11): 2261-2264.
132. Reichart F, Horn M, Neundorf I. Cyclization of cell-penetrating peptide via click-chemistry increases proteolytic resistance and improves delivery. *J Pept Sci*. 2016;22(6):421-426.
133. Park SE, Sajid MI, Parang K, Tiwari RK. Cyclic cell-penetrating peptides as efficient intracellular drug delivery tools. *Mol Pharm*. 2019; 16(9):3727-3743.
134. Jing X, Yang M, Kasimova MR, et al. Membrane adsorption and binding, cellular uptake and cytotoxicity of cell-penetrating peptidomimetics with  $\alpha$ -peptide/ $\beta$ -peptoid backbone: effects of hydrogen bonding and  $\alpha$ -chirality in the  $\beta$ -peptoid residues. *Biochim Biophys Acta (Biomembranes)*. 2012;1818(11): 2660-2668.
135. Wallbrecher R, Depre L, Verdumen WPR, et al. Exploration of the design principles of a cell-penetrating bicyclic peptide scaffold. *Bioconjug Chem*. 2014;25(5):955-964.
136. Silva S, Almeida AJ, Vale N. Combination of cell-penetrating peptides with nanoparticles for therapeutic application: A review. *Biomolecules*. 2019;9:22/1-22/24. <https://doi.org/10.3390/biom9010022>
137. Desai P, Patiolla RR, Singh M. Interaction of nanoparticles and cell-penetrating peptides with skin for transdermal delivery. *Mol Membrane Biol*. 2010;27(7):247-259.
138. Im J, Das S, Dongjun J, et al. Intracellular and transdermal protein delivery mediated by non-covalent interactions with a synthetic guanidine-rich molecular carrier. *Int J Pharmaceutics (Amsterdam)*. 2017;528(1-2):646-654.

## AUTHOR BIOGRAPHIES

**Siegmond Reissmann** was born in 1940 in Chemnitz, Germany, and graduated from the Friedrich-Schiller University Jena: diploma in Chemistry 1963, PhD in 1968, and habilitation in 1977. He is a Professor in Biochemistry since 1985 and was Director of the Institute of Biochemistry and Biophysics from 1985 to 2005. After retirement, he was a Scientific Advisor of the Jena Bioscience GmbH and gave lectures in Biochemistry at the universities in Jena and Chemnitz. His research interest includes syntheses and conformational studies on peptides, SAR studies and investigations on the molecular action mechanisms of the peptide hormone bradykinin and bradykinin-potentiating peptides, and studies on internalization of various cargos into different live cells with cell-penetrating peptides. He has studied abroad with Y. A. Ovchinnikov, V. T. Ivanov, M. P. Filatova (Moscow), K. Blaha and I. Fric (Prague), K. Medzihradsky (Budapest), P. Fromageot (Paris), J. M. Stewart (Denver), and V. Ruby (Tucson).

**Margarita Filatova** was born in 1939 in Moscow USSR. She was a 1958–1962 student of Moscow University, Faculty of Chemistry. She got her diploma in Chemistry 1962 and her PhD in 1968. She worked from 1962 to 1974 and from 2004 till now as Senior Researcher in the Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry (Russian Academy of Sciences). From 1974 to 2000, she became a laboratory leader in the Institute of Biological and Medical Chemistry. Her research of interest includes syntheses and conformational studies on peptides, SAR studies on the peptide hormone bradykinin and on bradykinin-potentiating peptides, combined with investigations on the molecular action mechanisms of both peptides. Based on her research on bradykinin-potentiating peptides, she was involved in developing inhibitors of the angiotensin-converting enzyme (ACE). In last years, her interest is focused on syntheses of cell-penetrating peptides and studies on cell penetration. She has studied abroad with Prof. S. Reissmann (Germany) and Prof. G. R. Nikiforovich (USA).

**How to cite this article:** Reissmann S, Filatova MP. New generation of cell-penetrating peptides: Functionality and potential clinical application. *J Pep Sci*. 2021;27:e3300. <https://doi.org/10.1002/psc.3300>