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CELL PENETRATING PEPTIDES IN PHARMACOLOGICAL ASPECT

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1. INTRODUCTION

Cell-penetrating peptides and proteins (CPPs) are compounds which can penetrate live cells and can transport cargoes and labels into the cell or even into subcellular organelles. Some cells are easy to transduce or to transfect, other cells only difficult or not to transfect. CPPs are very effective in transporting peptides, proteins, hydrophilic bioactive synthetic or natural compounds. Furthermore, they are tools for transporting drugs, including herbal products from classical chine´s medicine. CPPs are also able to transport ribonucleic acids and derivatives of them like peptide nucleic acids or morpholino oligomers. CPPs are also able to penetrate barriers like blood-brain barrier, conjunctiva of eye, skin, epithelial tissue, and intestinal mucosa. As membrane-active peptides some of them can kill microorganisms directly or can transport effective antimicrobial drugs into infected cells.[1]

Cell penetrating peptides (CPP), also known as protein transduction domains, could provide a solution to many of these barriers, with promising clinical implications. The discovery of the ability of Trans-Activator of Transcription (Tat) protein, expressed by human immunodeficiency virus, to transfect cultured cells and induce viral gene expression without a receptor has led to the discovery of novel CPPs effective at delivering various diagnostic or therapeutic cargo across the cell membrane.[2,3]

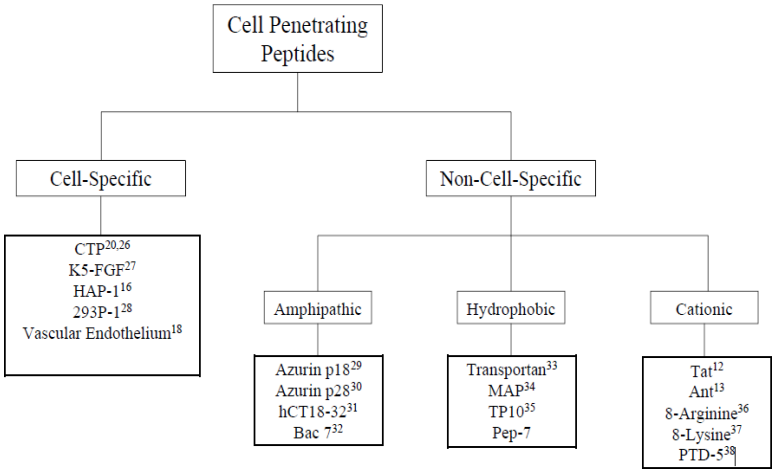
First Cell-penetrating peptides (CPPs) were described about 30 years ago. Till now more than 9,000 publications appeared, and more than 800 peptides are described. The number of yearly publications about CPPs has remained constant at a high level, about 800 in each of the last years. Nevertheless, of the intensive research in this field a highly effective and selective internalization of drugs and imaging labels into live cells, especially into all cell-types, remains till now challenging. Thus, till now no one CPP is approved by Food and Drug Administration (FDA) as a drug. But it seems that the CPPs are more promising and convenient for clinical use than chemical detergents, electrical or mechanical penetration methods, and in certain cases also as viral vectors.[4-8]

The firstly described cell-penetrating peptides were a substance P analog[9], HIV-TAT (47-57) derived from TAT-protein of virus HIV-1[10] and penetratin, a peptide derived from Drosophila antennapedia gene homeobox.[11]

While CPPs from first generation exhibit mostly only a low cell- and tissue-selectivity, the peptides from the newer generation are characterized by distinct selectivity for certain cell types, especially for tumor cells. Aiming to enhance cell- and tissue-selectivity specific properties of tumor cells and tumor environment can be used such as fenestrated capillaries, low pH- value, or hypoxia. Thus, some CPPs form their active CPP-conformation only under these conditions. One of the very important contributions in the field of tissue selectivity was the development of activatable CPPs.[12] In the inactive state these peptides are closed like a Swiss army pocketknife and can be opened by activators e.g., certain tumor-cell specific proteases or under hypoxic conditions. Additionally activation can be performed, for instance with photo-activatable CPPs using an UV- or IR-irradiation focused on target.[13]

1. TYPES OF CELL PENETRATING PEPTIDES

Although there are multiple classification systems, for the purpose of this review, we will classify CPPs into cell- and non-cell-specific types. Depending on their sequence they can be divided into cationic, amphipathic, hydrophobic, and acidic peptides. Cell-specific CPPs can target specific cells and deliver cargoes selectively thereby diminishing doses needed as well as limiting off target side effects. Using cell- specific CPPs would also be advantageous in the process of upscaling the experiments to human trials because less peptide is required due to its specificity.[14]



Many peptides from the new generation are derived from venoms of amphibian, snakes, and insects.

Cationic CPPs HIV-TAT

From TAT-protein of virus HIV-1[10] various sequences were derived and tested for their uptake efficiency. Commonly used is the sequence HIV-TAT (47-57).

Penetratin

Penetratin is a penetrating peptide derived from Drosophila antennapedia gene homeobox.[11]

Protamine

An old protein and the newly developed low- molecular-weight protamine (LMWP) and derivatives. Long time before discovery of CPPs, complexes containing protamine derivatives were used to transport insulin, interferon, glucagon- like peptide, or somatostatin. Because this heterogeneous group of peptides evoked many undesired side e ffects during clinical trials, Byun et al.[15] developed a short-chained low- molecular-weight protamine, abbreviated LMWP. The sequence of this peptide contains a compact region of arginine residues. LMWP helps to target drug resistant breast cancer and enables drug delivery to the brain via intranasal administration.

Crotamine

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.[16] In most publications about application, 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(Δ10–37). This designed peptide was also named nucleolar targeting peptide (NrTP), due to its preferential accumulation in the nucleoli of cells.[17] The ability of crotamine to transport cargoes into actively proliferating cells[18], makes crotamine and its derivatives suitable markers.

Table 1. Selected peptides from the First Generation.

Structural Characterization		
Cationic Peptides		
1	Penetratin	Drosophila antennapedia gene homeobox
2	HIV-TAT(47-57)	TAT-protein of virus HIV-1, sequence 47-57
4	VP22	Herpes simplex virus transcription factor 267-300
5	Oligo-arginines	R(6 – 11)
Amphiphatic Peptides		
5	MPG-peptides	Designed: sequence of HIV-protein gp4 fused to NLS
6	Transportan	Chimeric Galanin/mastoporan 10-mer
	KLA-sequences	
	pVEC	Vascular endothelial cadherin
Hydrophobic Peptides		
7	Caiman crocodylus	Signal sequence of light chain
8	Integrin β3-fragment	Tumor homing
	hCT(9-32)-br	Sequence from human calcitonin
Acidic Peptides		
9	SAPE	Amphipathic negative CPP
10	Poly(glutamic acid)	Negative charged polymer

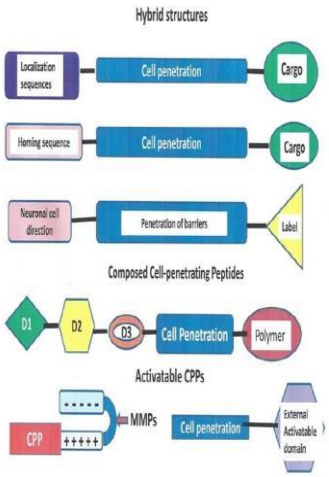


Figure 1. Hybrid or composed structures and activatable CPPs.

D1,D2,D3: domains for endosomal leakage, homing, subcellular localization, tissue selectivity.

Maurocalcine

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.[19] Numerous analogs have been derived by simultaneous internal cysteine replacement with 2-aminobutyric acid (Abu) and sequence truncation.[20] Fragment 1–9, which corresponds to the hydrophobic surface, is called mini- maurocalcine. Gold nanoparticles containing a maurocalcine-analog allow biomedical imaging of cancer cells.

Buforins

Buforins are firstly isolated from Asian toad Bufo bufo gargarizans. Buforin I contains 39 amino acid residues, buforin II is a 21-mer peptide.[21] Both buforins show complete sequence homology with the N-terminal region of histone H2A. It is assumed that they are formed in the stomach of toads by proteolytic cleavage of H2A with pepsin. They are potent antimicrobial peptides; they also exhibit anti-cancer activities.[22] They do not exhibit cytotoxic activity against several eukaryotic cells and are nearly non-hemolytic in respect to human erythrocytes.

These properties make the buforin-peptides attractive for pharmaceutical applications.

Nucleus-Penetrating Peptide Penetrating Peptide CB5005

CB5005 is a rationally designed peptide containing a cell-permeable sequence cascading to a NF-ƙB 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.[23] 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.

Antimicrobial peptide cathelicidin: sC18

Cationic antimicrobial peptides (AMPs) are present in all living organisms. They can inactivate bacterial or viral pathogens by permeabilizing their membranes. The CAMP cathelicidin (CAP18) binds to lipopolysaccharides. Neundorf at coworker[24] found that the peptide fragment sC18 fulfills all requirements for a good CPP: It facilitates internalization of cargo into living cells with high transduction rates and efficiencies. The peptide sC18 shows no cytotoxic effects.

Buffalo cathelicidin family

Members of the newly identified buffalo-derived cathelicidin (CAT) family exhibit preferential binding to multiple tumor cell lines. Additionally, they show higher translocation efficiency than most other CPPs. Therefore, CAT is considered as a novel tumor homing CPP with great potential for selective drug delivery.[25]

Spider toxin Lycosin-I

Similar to venoms 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 and display an unprecedented low selectivity over noncancerous cells. Moreover, the conjugate to gold nanoparticles shows an efcient photothermal e ffect under near infrared irradiation, leading to the killing of cancer cells in vivo.[26]

Amphiphatic Peptides

MPG-Peptides

The French research group in Mont Pellier developed a series of designed peptides for transport of different cargoes via formation of non-covalent complexes.[27,28] The amphipathic basic sequences are derived from a fusion sequence of HIV protein gp41, a hydrophilic lysine-rich nuclear localization sequence, and a spacer.

Hydrophobic CPPs

To the group of hydrophobic CPPs belong such peptides like Kaposis sarcoma fibroblast growth factor[29]**,** integrin β3- fragment[30] or human calcitonin partial sequence 9-32.[31] They are mostly partial sequences of functional proteins e.g. translocation sequences and can e ffectively transport cargoes through interaction with receptor proteins or membrane lipids.

Acidic Sequences Azurin

Azurin is a 128-amino acid-long bacterial protein. It belongs to the cell-penetrating peptides and simultaneously inhibits multiple tumor-promoting pathways.[32,33,34] Azurin preferentially enters cancer cells, where it exerts cytostatic and cytotoxic (apoptotic) effects with no side effects for normal cells.[35] Like other newly developed CPPs azurin can directly kill tumor cells by influencing specific signaling pathways.

For instance, Bernardes et al.[36] reported that azurin exerts anticancer activity by interacting with multiple targets and interfering with multiple steps of tumor progression. In a first in- class, first- in- human clinical trials, the azurin peptide p28 has been shown to display very little toxicity and high antitumor activity in many advanced-stage cancer patients.[37] The C-terminal sequences 96 to 113 show structural similarity to a ligand known as ephrin B2 and bind to the corresponding receptor who's signaling is involved in cancer progression. Thus, azurin and its C-terminal fragments can also contribute to cancer cell growth inhibition.[38] Azurin enhances additionally the sensitivity of tumor cells to various chemotherapeutics.[39]

2. MECHANISM OF CELLULAR UPTAKE OF CELL PENETRATING PEPTIDES

Despite some common properties of CPPs, especially their cationic nature, it is believed that the translocation mechanism is not the same for different families of CPPs. Also, most CPPs utilize two or more pathways depending on the experimental conditions. Here, we have briefly reviewed the two major cellular uptake mechanisms, nonendocytotic or energy- independent pathways and the endocytotic pathways.

a) **Direct Penetration-** Direct penetration via energy independent pathways may include different mechanisms that have been described as inverted micelle formation[40], pore formation[41], the carpet-like model[42] and the membrane thinning model.[43] The first step in all these mechanisms constitutes interaction of the positively charged CPP with negatively charged components of membrane such as heparan sulfate (HS) as well as the phospholipid bilayer. They involve stable or transient destabilization of the membrane associated with folding of the peptide on the lipid membrane.[44,45,46] The subsequent mechanism of internalization depends highly on the peptide concentration, peptide sequence, and lipid composition in each model membrane study.

Generally, direct penetration is most probable at high CPP concentrations and for primary amphiphatic CPPs such as transportan analogues and MPG.[47–49] The "inverted micelle" is one model suggested already at an early stage for the direct penetration of penetratin.[50] In addition to the interaction between the positively charged CPP and negatively charged components of the lipid membrane, interaction between hydrophobic residues such as tryptophan and the hydrophobic part of the membrane is also shown to be involved in this mechanism. Therefore, this mechanism is not probable for the highly cationic CPPs such as TAT(48–60).

Pore formation includes descriptions by the barrel stave model and the toroidal model.[51] In the barrel stave model, helical CPPs form a barrel by which hydrophobic residues are close to the lipid chains, and hydrophilic residues form the central pore. In the toroidal model, lipids bend in a way that the CPP is always close to the headgroup, and both CPP and lipids form a pore. In both mechanisms, pores appear when the concentration of the peptide is more than a certain concentration threshold, which is different for different peptides. In the carpet- like model[52] and membrane thinning model[53], interactions between negatively charged phospholipid and cationic CPPs result in a carpeting and thinning of the membrane, respectively. Subsequent translocation of the CPP is achieved when CPP concentration is above a threshold concentration.

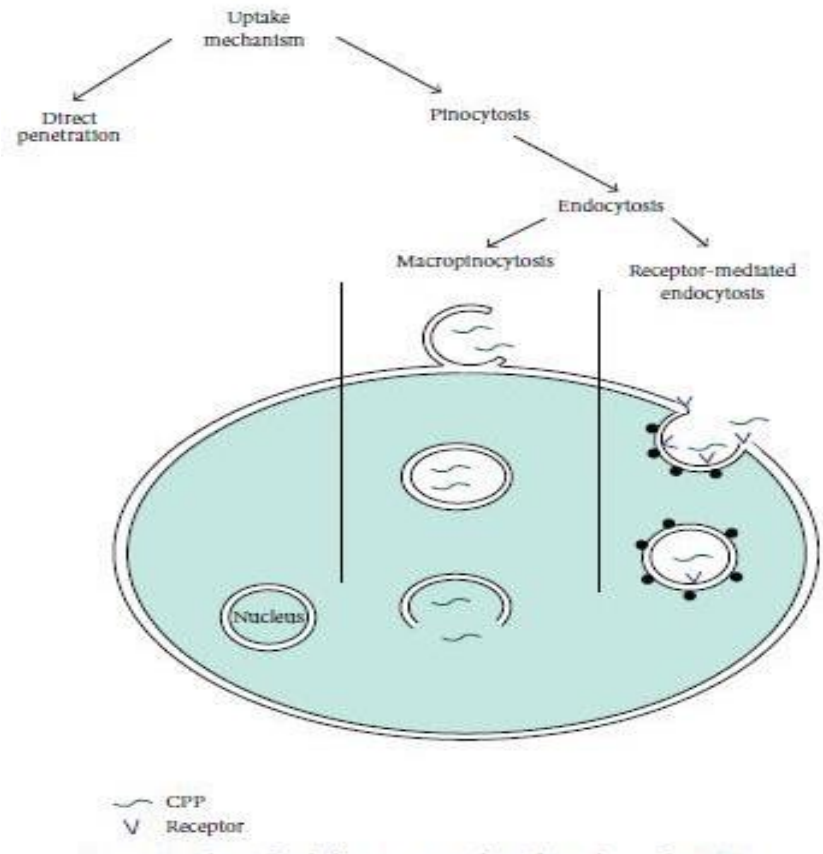


FIGURE 1: Scheme for different suggested uptake pathways for CPPs.

b) **Endocytosis**- Endocytosis consists of several pathways including phagocytosis for uptake of large particles and pinocytosis for solute uptake. Pinocytosis is categorized as macropinocytosis, endocytosis dependent on the coat proteins clathrin or caveolin, or endocytosis independent of clathrin and/or caveolin (Figure 1).[54,55] Macropinocytosis is associated with the inward folding of the outer surface of the plasma membrane, which results in the formation of vesicles called macropinosomes.

Resulting macropinosomes are surrounded by membrane similar to the cell membrane. Dynamin protein is required for membrane invagination. In receptor-mediated endocytosis, clathrin or caveolin pits are involved in the mechanism of uptake. Both clathrin and caveolin proteins cover the intracellular part of the membrane. They are required for invagination of the membrane and help to form the vesicles after binding the extracellular molecule to the membrane receptor. Clathrin-coated vesicles are about a few hundred nanometers in diameter, while caveolin-coated are about 50–80nm in diameter.[54, 55]

Earlier studies had suggested that direct penetration was the uptake mechanism for most CPPs. This conclusion was based on the observation that peptides enter the cell even at 4◦C, therefore, by an energy-independent route. Later studies showed that experimental artifacts were responsible for this conclusion. Using methanol or formaldehyde to fix the investigated cells for confocal microscopy may result in some experimental artifacts.[56,57] Nowadays by using trypsin to remove outside associated peptides and live cell confocal microscopy, one generally avoids this problem.[56] For most CPPs, it is now generally concluded that endocytosis is involved in the translocation mechanism. However it is most likely that different mechanisms operate under different conditions for all CPPs.

Factors Affecting the Mechanism of Cellular Uptake

In the study of the uptake mechanisms, both physicochemical properties of the CPP and the utilized experimental conditions are of importance.

Structure activity relationship (S.A.R) studies are able to recognize the importance of the individual residues in the CPP sequence. They have shown the importance of positive charges, especially arginine residues, in the uptake mechanism as well as hydrophobic alpha helical structures.[58,59,60] It has been shown that most CPPs are rich in arginine residues and that arginine (and in particular, its guanidinuim group) is more favorable than lysine for delivery and CPP activity of the peptides.[61,62,63,64] However, this is not the case considering the high effect of TP10 and some other CPPs lacking arginine in their sequences. The CPP conformation and the length of the CPP sequence are other factors affecting the mechanism of uptake. This is shown by the difference between Pvec and scrambled pVEC in the uptake efficiency. The latter has no uptake, whereas the former efficiently translocates into various cell lines.[60,65] Thermodynamic binding studies have shown that primary and secondary amphiphatic CPPs can directly penetrate through the cell membrane at low micromolar concentrations. However, non-amphiphatic CPPs mainly use endocytosis at low concentrations.[66] CPP conformations including induced alpha helices and beta sheets are also important in explaining the membrane perturbation and subsequent translocation by CPPs.

Contradictory results are often reported which may arise from experimental conditions that differ in important respects. The first important factor is the CPP concentration, which affects the mechanism of CPP entry. Direct penetration is more probable for primary hydrophobic CPPs at high concentrations, whereas endocytosis is the main uptake mechanism at low concentrations. The concentration threshold for direct penetration varies between different CPPs, different cell lines, and the presence of and type of cargo.

It should be emphasized that the presence of the cargo may alter the CPP uptake pathway. Type of the cargo as well as the size and binding methodology have been shown to influence the CPP translocation mechanism. TAT attached to a large cargo is mostly entrapped in the endosomal vesicles; however, it redistributes throughout the cell cytosol when attached to a small cargo.[67] Furthermore, labeling a peptide with different fluorophores may also influence the uptake mechanism, intracellular distribution, and cytotoxicity of the peptide.[68,69] Other experimental factors of importance for the uptake mechanisms are, for example, cell type, temperature, and incubation time.

3. PHARMACOLOGICAL ASPECT

Cell-penetrating and cell-targeting peptides in drug delivery

Unless temporally masked before being exposed at a particular site of action, the use of CPPs to mediate drug delivery into a specific cell type is far to be common in pharmacology. Such exposure can be triggered by the local pH, the presence of specific enzymes or the mechanical release from a scavenging structure, as reviewed in the previous sections. The dispersion of most of the drug-CPP chimeras all over the body due to non-specific interactions with ubiquitous cellular components requires the use of elevated doses of material with potential secondary effects. Despite these drawbacks, some spectacular biological effects have been reported following in vivo delivery of drugs loaded on CPPs, but the delivery of drugs into the right cell through a peptide-recognition motif has not been extensively quantified in terms of efficacy. In most of the studies, the used doses are often simply the dosages required to obtain the expected biological response, and whether such doses are very high is seldom commented in these works.

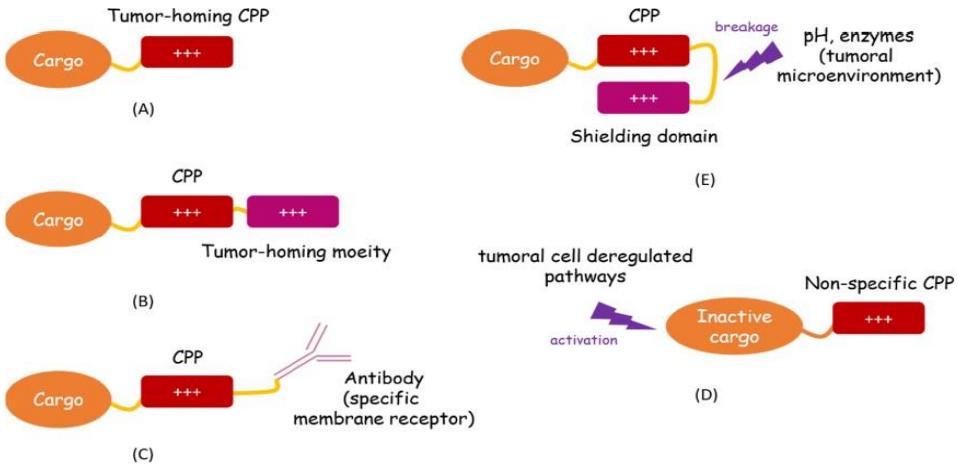
We believe that the future will lie in the development of modular units in which CPPs and CTPs will be assembled and disassembled sequentially. The first part of such a unit could be a polymeric structure coated with a cell-recognition peptide motif in order to concentrate the structure at the target place. Then, this structure could be dismantled by using a localized device, such as hyperthermia or ultrasounds, in order to free the internalized drug- loaded CPP in close vicinity of the target. In this way a drug could be efficiently concentrated at the tumor site and then efficiently delivered into the cancer cells. We expect the peptide-based delivery system of the future to be multi- unit entities, in which each unit will have a very precise function, triggered at the right moment in order to provide a more efficient and/or more tolerated therapeutic delivery strategy. In addition, peptide-based delivery vehicles remain very interesting to develop because of several advantages of peptides for drug delivery purpose.

CPPs as Molecular Carriers in Cancer

In the past 30 years, much effort has been dedicated to finding novel therapies against cancer.

CPPs are receiving more attention thanks to their ability to deliver large cargoes of various nature inside cells. However, there is still no FDA (Food and Drug Administration) approved CPP-conjugated drug, although p28 is listed in two phase I trials (clinicaltrials.gov) for the treatment of solid p53 tumor.[70]

The issues to address before translating CPPs into clinics are the following: the route of administration (oral administration is the best option for pharmaceutical industries); the stability in vivo and the non-specific intracellular uptake. Great progress has been made to improve those parameters. Indeed, many novel CPP-based delivery systems have been developed, introducing chemical and structural modifications or anti-proteases shielding for example. In the search for enhanced specificity, incredible advance has been made. Two main strategies have received particular attention: engineering peptides with a preference for tumoral cells or targeting cancer cell intracellular properties.



For the first strategy, the easiest way is to develop tumor-homing CPPs (Figure 2A). These peptides have a particular affinity for cancer cells or tissues because of physicochemical features of the tumor, or because of the expression of a specific biomarker. CPPs can also be coupled to a moiety that brings specificity towards tumoral cells (Figure 2B) or to an antibody that recognizes a specific marker expressed at the membrane of cancer cells (Figure 2C). Finally, it is possible to use ACPP—that is to say, shielding strategies, in which the CPP function is inactive in physiological conditions but activated in the close neighborhood of tumor, where the microenvironment is different (Figure 2D). For the second approach, CPP can enter any kind of cell but the cargo is only active inside tumoral cells, where molecular pathways are deregulated (Figure 2E). In terms of clinical applications, many factors need to be considered: cost, ease of synthesis, elimination, and immunogenicity, among others. Taking these elements into account, tumor-homing CPPs seems to be the most promising approach. Indeed, they are short and quickly eliminated, with a negligible toxicity. They are less expensive to produce than CPP- antibody conjugates. Moreover, the addition of complex molecules, such as antibodies, liposomes, nanoparticles, or biopolymers, increases the risk of immunogenicity. The same evaluation should be made concerning the cargo. Although coupling with a tumor- homing CPP appears to be a useful tool to reduce the chemotherapeutic agent's toxicity, the risk of off-target effects, as well as development of resistance mechanisms, remain substantial. Application of gene therapy, while very promising in the case of inherited monogenic disorders, seems to be much more complicated in the context of cancer. The abnormalities are polygenic and there is high genetic heterogeneity, not only between tumors in different patients, but also between tumors at different sites within the same individual. On the contrary, targeting protein-protein interactions with CPP-protein conjugates allows for interference with many different pathways which are

common to all tumors, with a limited risk of resistance-mechanism development. Stimulating the immune system with vaccine peptides also holds great promise. This technology allows the immune system cells to recognize tumor antigens more effectively and specifically attack and destroy cancer cells. Ultimately, peptides such as RT53 that combine (1) a tumor- homing property to (2) a specific effect on protein-protein interactions involved in cancer to (3) a specific immune response seem to be the most promising therapeutic strategy.

Administration and Biodistribution

Administration of CPPs, free or conjugated to polymers, dendrimers and nanoparticles can be performed in different ways. The literature describes besides oral application also inhalation, nasal-, intestinal-, parenteral or endothelial-administration as well as intravenous and intraperitoneal injection. Time-dependent distribution studies of CPP and cargo in the whole body, the so-called Pharmacodynamic studies, are a prerequisite for clinical trials and it is recommended to start with these studies in a very early stage of research. Furthermore, for clinical use it is important to avoid toxic reactions in any organ, also outside the target organ. Thus Qiu et al.[71] studied the induction of apoptosis and necrosis by some CPPs on normal human liver cells. The kidney viability due to possible accumulation, also the elimination through and influence on kidneys has to be estimated for candidates which are chosen for clinical application. Acritical view of related publications shows that only few publications contain such recommended studies about biodistribution. While in first two decades in the history of CPPs mainly studies on internalization and transport of cargoes into cell cultures were published in the recent research quite more pharmacodynamic and pharmacokinetic studies on animals are performed.

In general, aiming to enhance concentration at target organ and to avoid toxic side actions on other organs the kind of administration should be chosen, if necessary, topically or by injection.

Selectivity of targeting, especially to tumor tissue, can be enhanced by coupling to polymers or nanoparticles via extravagation through leaky capillaries of this tissue. Thus, for targeting lymphatic tumor is recommended the use of homing sequences. Additionally, selectivity can be enhanced by combination with targeting ligands in response to specific properties of tumor cells or by external photo- or thermally activation.

Application for Imaging Diseased Tissues

CPPs can be also used for imaging of diseased tissues. Detecting primary tumors in a very early stage strongly improves the chances of successful therapy. Thus, structures from Swiss army pocketknife type were used for detecting colon-, breast-, pancreatic cancer, squamous cell carcinoma and thyroid carcinoma. For diagnostic use the CPPs can be coupled to sensitive markers, like NIR- fluorescence markers, NMR-sensitive Gd-complexes, or radionuclide. Imaging with cell-selective and labeled CPPs allows not only detecting tumors, metastases, thrombosis or inflammations but also the complete removal of diseased tissue by guided surgery through visualizing themargin between healthy and diseased tissue.[72]

Preclinical and Clinical Studies

As discussed in the introduction a huge number of publications about CPPs which has appeared in the last two decades are concerned to pharmaceutical tasks, contain animal experiments, and some of them clinical trials, too. In their review Guidotti et al.[73] list 28 clinical trials with corresponding NCT registration. Few of the trials are already in phase 3. It is commonly accepted that the process of developing a drug, from inception through the first experiments followed by animal experiments and clinical testing, can take on average more than twenty years. Thus, on this 30th birthday of the CPPs it is not unexpected that no one compound is approved as drug by the FDA. The huge number of research papers reflect the high promises of a therapeutically use. CPPs open not only new therapeutic possibilities but also can enhance efficiency and selectivity of already existing drugs. Based on their functional properties CPPs can be used for penetration of barriers, for killing of microorganisms and for treatment of diseases through internalization of effective drugs into target tissue.

Antimicrobial activity

Due to their action on biomembranes certain CPPs exert directly antimicrobial activities. They can act against bacteria, fungi, viruses, and protozoa or they can transport antibiotics into infected cells.[74] Results obtained with CPPs are very promising mainly for resistant bacteria and difcult to treat fungi.[75,76] Use of certain CPPs against such viruses as Herpes simplex opens new therapeutic possibilities.[77] Certain CPPs show some potential for drug development even in the fight with such worldwide distributed diseases as malaria or visceral leishmaniasis.

Stroke, inflammation, pain

Aiming to reduce infarct size and to support the recovery different CPPs are used to deliver such compounds into myocardial cells which are influence signal pathways. The internalization of ligands for protein binding domains, inhibitors or activators of protein kinases and other enzymes helps to treat stroke, infarct, and different kinds of pain. Transport of heat shock proteins[78], an inhibitor protein of NFκB[79] and the protein p53 is applied to treat mainly inflammation processes. Thus, delivery of the anti-apoptotic peptide BH4 prevents cardiac ischemia-reperfusion injuries.[80] Inhibiting intracellular kinases by inhibitors which are conjugated to HIV-TAT protect the ischemic heart.[81] CPPs mediated delivery of therapeutic molecules also protects against cerebral ischemia and reduce the pain in the central nervous system.[82]

Muscular disease Duchenne

Duchenne, a genetically based muscular disease, is till now difficult to treat. Successful attempts were undertaken to correct the faulty splicing of RNA from muscle protein dystrophin. Derivatives of nucleic acids such as peptide-nucleicacids (PNAs) and morpholino oligonucleotides (Mos) eithercovalently coupled to CPPs or non-covalently complexed with suitable CPPs open a door to treat this disease. In particular, the application of splice correcting morpholino oligonucleotides (SCONs) aids in the treatment of, Duchenne muscular dystrophy.[83] Very successful trials with most sophisticated CPPs were undertaken by the group from Ü. Langel.[84]

Treatment of Glioblastoma

The ability of certain CPPs to penetrate the blood-brain barrier (BBB)[85,86] allows to undertake preclinical and human clinical trials to treat the glioblastomas. This treatment leads to considerable size reduction of the tumor.[87,88] Furthermore, with help of penetration the BBB also neuropathic pain can be treated. Cationic CPPs are used for delivering anti- inflammatory compounds into cells aiming treatment of rheumatoid arthritis[89,90] Because CPPs are able to penetrate also other barriers like, skin, mucosa and conjunctiva of eye they are able to avoid administration of drugs by painful injections with needles. Their replacement by non-invasive crèmes containing CPPs seems to be very convenient. Even oral applications of CPP-bearing drugs were successfully performed. Many attempts were undertaken to administrate insulin without needles.[91] Not unexpectedly, the number of registered trials for cosmetic use of CPPs, for needleless administration of botulinum toxin, exceeds the number of trials in other application fields.

Therapy of eye diseases

Therapy of eye diseases plays an important role by in vivo experiments with animals and clinical application of CPPs. Eyes are surrounded by conjunctiva, have in front the cornea and inside the lens, the glass body, and the retina. Therapies are required for cornea, lens and especially for retina. Till now many drugs are administrated by often repeated injections. CPPs could allow the administration by dropping conjugates formed from drugs and CPPs. As well as inner cornea, lens and retina are achievable with CPPs.[92] Thus, cataract and macula-degeneration possibly can be treated in future with help of CPPs and without injection into the eyes. It has been shown that CPPs are able to penetrate the ocular barrier without impairing its biological function.[87,88]

Treatment of different kinds of tumors

Very most preclinical and clinical trials were performed for treatment of different kinds of tumors. The aim of cancer therapy is according to Bitler and Schröder to destroy invasive carcinoma without "sacrificing the patient's quality of life".[93]

CPPs help to transport into tumor cells such classical anti-cancer drugs like cis-platin, doxorubicin, paclitaxel, vimentin or compounds of classical chine's medicine. This is important because until now the efcient intracellular delivery of anti-cancer drugs like organometallic compounds is an important focus in clinical research. Since CPPs from first generation only help to increase the intracellular amount of anti-cancer drug in many cell types, tumor-specific CPPs from the newer generation and activatable CPPs deliver the drugs selectively into cancer tissues, such helping to avoid site actions on all types of fast regenerating tissues as mucosa or hairs. From the new generation of CPPs the natively tumour specific peptides like protamine, crotamine, azurin, peptide CB5005, buffalo cathelicidin, spider toxin Lycosin-I, maurocalcine, buforins, and PepFect peptides), are of special interest. For highly specific targeting of cancer cells, protease-activatable LMWP derivatives have been developed.[94] They help to target drug-resistant breast cancer and enables drug delivery to the brain through intranasal administration.[94,95]

From the tumour specific enzymes Azurin and Oncogenase very effective CPPs could be developed, which have been used for selective tumour killing. In summary it can be concluded that tissue selective CPPs can be used for very selective treatment with coupled drugs. Some CPPs act synergistic with chemotherapeutics or enhance sensitivity of tumor tissue to these drugs[96] Preclinical studies were performed besides with covalent conjugates between CPPs and cargoes by the non-covalent strategy, too. Thus Bonnet et al. Internalized inhibitors of cyclin-dependent kinases[97] and siRNA targeting cyclin B1 in mouse tumour models. In particular, the formation of non-covalent complexes of cationic CPPs with highly selective inhibiting RNAs promises significant potential in the fight against cancer diseases.[98]

Gene Therapy

Gene therapy is the transfer of functional and therapeutic genetic material such as plasmid DNA (gene transfer), siRNA, shRNA and miRNA for gene modulation or of nucleases for gene editing. In contrast to the uptake of proteins and drugs, the transfer of native DNA and plasmids is considerably more difficult. In vitro and in animal experiments genes could be successfully internalized and expressed. In contrast to DNA, certain RNA types and oligonucleotide mimics of RNA have been successfully internalized. They are applied for blocking hepatic metastases, the treatment of resistant tumors, muscular dystrophy and infections with resistant bacteria. Using protamine as a component of a multifunctional vector, also He et al.[99] transfected tumor cells with CRISPR-Cas9 plasmid to modulate the properties these cells. Nevertheless, transfection with DNA and plasmids remains crucial formany therapeutic purposes, such as, the treatment of cardiovascular diseases, including those used to avoid of heart

transplantations, and treatment for cystic fibrosis. However, using CPPs in the clinic, the stable integration of genes into the genome remains far from achievable at this stage.

Vaccination with CPPs

The recent situation with SARS-CoV-2 pandemic gives rise to discuss application of CPPs for development of vaccines. The use of CPPs for generating vaccines against cancer was described in various publications.[100-102] Mainly the group of Bolhassani was involved in that research review.[103] They describe for instance in original publications the use of a DNA-vaccine against human papilloma virus (HPV) type 16 induced cancer. Because HPV E7 oncogene is constantly expressed by HPV infected tumor cells and by all pre-cancerous cells, this oncogene is an ideal target for tumor specific immunotherapy. For internalization of DNA into antigen presenting cells MPG-peptides can be used.[98] Internalization of negatively charged DNA or RNA requires an about tenfold molar excess of positively charged CPPs. In the recent pandemic situation vaccines are required for all people in the world. It means that for an RNA-vaccine oligonucleotides must be prepared in an amount of some hundred kilograms but CPPs are necessary in a tenfold higher amount.

Because both compounds, nucleic acids and peptides respectively, have to be prepared and synthesized in high purity for clinical use. It seems to be impossible to generate such an amount in a very short time. The syntheses of few metric tons CPPs require not only highly sophisticated equipment but also chemists with much experience.

Despite of the expected advantages by complex formation with of CPPs compared to lipids in the BioNTech-Pfizer product the application of CPPs for vaccine generation cannot be recommended.

It takes too long time and syntheses of CPPs are too expensive. Nevertheless, after succeeding with the pandemic situation the advantage and disadvantage of RNA for generating vaccines in a pandemic situation must be discussed thoroughly. In contrast to a lipid-complexed RNA- vaccine the preparation of viral vectors is a well-established process and allows an easy up scaling. RNA-CPP-vaccines or RNA- liposome-vaccines can be recommended only in the tumor therapy, because of the significant smaller number of patients. But generally, delivery of gene vaccine (DNA, pDNA or RNA, respectively) with CPPs for anti-cancer therapy remains a challenge for the near future.

CONCLUSION

The CPPs represent a potentially valuable tool for the cellular delivery of important cargo molecules, considering their low toxicity and independence of membrane receptors and cell types. Since the discovery of the two well-known CPPs, the TAT and penetratin peptides, the number of known CPPs has considerably increased and their properties have been elucidated. Numerous preclinical applications for the treatment of certain diseases have been found due to the drug-delivery capabilities of the CPPs. The progressive and continuous application of CPPs shows that they are efficient delivery vectors. Because of the need to ameliorate the drug delivery, a great number of CPP-based applications are still drawing the attention of researchers.

In this review, the current tendency in drug delivery by CPPs is summed up. Conjugation with CPP increases cell-surface affinity and eventual cellular uptake of bioactive molecules.

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