The current role of cell-penetrating peptides (CPPs) in cancer therapy

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Abstract
Cell-penetrating peptides (CPPs) are an inhomogenic class of peptides with the ability to translocate in cells and to carry attached cargos with them inside. Owing to this striking ability the further development and application of CPP-based delivery strategies have steadily emerged during the past years. The following review aims to summarize some of these recent concepts and to higlight the current role of CPPs in cancer therapy.

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1. Introduction

Cancer is still one of the most threatening diseases with the most cases of death worldwide. Not only is the early diagnosis of cancer of high importance, but also an effective therapeutic strategy. This is realized by means of surgical excision of the affected tissue often combined with radiation therapy and chemotherapy. Several drugs are commonly used for this purpose; however, upcoming resistances and the need for a more personalized treatment strategy still let researchers develop novel anti-cancer compounds. Although several of such new compounds show excellent activity profiles, often their further development is hampered by an only poor pharmacokinetic profile. Often this is based on their occasionally poor bioavailability due to limited cellular uptake. In fact, overcoming the cell's plasma membrane and reaching the intracellular target site resemble the major hurdles for an efficacious therapeutic agent. The plasma membrane surrounds all living cells and acts as a protective barrier that controls the in- and outflow of compounds within the environmental media. Drug molecules usually must overcome this barrier to reach their site of action. Once inside the cell, they act via different mechanisms, as e.g. the selective interruption or activation of a signal transduction pathway, or by direct interaction with the DNA in the nucleus. Beside the use of small molecule drugs, the current trend involves the use of macromolecules as anti-cancer agents, such as proteins, monoclonal antibodies, nucleic acids and nanoparticles, and the combination thereof. Despite the numerous advantages, the biodistribution and translocation of these hydrophilic macromolecular drugs is still a big challenge, prevented by the low permeability due to the intrinsic characteristics of the biological membranes. In the course of time multiple approaches have been developed, such as the use of liposomes, microinjection, electroporation or also viruses and bacteria that are used particularly for gene transfer. Although through all these systems it is possible to import macromolecules into

living cells, each of them presents a series of limitations that preclude their use in *in vivo* studies, in particular for possible therapeutic applications in clinic. The greatest obstacles can be summarized in their low internalization efficiency, complexity of manipulation, demand for expensive equipment, difficulty of release into the cytosol, and sometimes in cell toxicity and immunogenicity.

Recently, the direct transfer of macromolecules into cells has been obtained by so-called "cell-penetrating peptides" (CPP), a class of short peptides rich in basic amino acids, characterized by exceptional translocation properties across cell membranes. [1, 2] Usually, CPPs consist of less than 35 amino acids, hold a positive net charge, and possess the ability to translocate across the plasma membrane. Thereby, CPPs can carry with them associated ligands, from small chemical molecules to nano-sized particles and large fragments of DNA, into the cell interior. The most studied CPP is the domain of the protein TAT (transactivating regulatory protein) of human immunodeficiency virus type 1 (HIV-1). The first evidence emerged in two articles published in the same issue of *Cell* in 1988 [3, 4], which underlined the possibility for the protein TAT to enter mammalian cells when simply added to the culture medium. A domain between the amino acids 47 and 57, having the sequence YGRKKRRQRRR, is the region of the protein responsible for the translocation. Some other examples found in nature, in addition to the TAT peptide, are presented by penetratin, a transcription factor from Drosophila [5, 6], VP22 from virus Herpes simplex [7] and pVEC, a peptide of 18 amino acids derived from the cadherin of murine vascular endothelium [8]. It is interesting to note that these peptides have very different amino acid sequences and secondary structures while the mechanism of transfer within the cells seems to be similar. Trying to change these residues it was understood that the arginine (R) plays a fundamental role. Indeed same results in cell internalization were obtained with synthetic oligopeptides consisting in homoarginine, highlighting in particular maximum efficiency with R8/9. [9, 10] Since the discovery of CPPs, intensive research has been carried out on the underlying entry mechanism in order to be able to totally exploit their transport properties but till now, in the literature, conflicting data are shown [11, 12]. It cannot be excluded that different internalization mechanisms are used concomitantly; furthermore, the permeation properties can vary in relation to the type of the associated cargo molecule and in respect to different cell types used. Despite this process of translocation remains unresolved, the effectiveness of the method is unequivocal, which promises to open new frontiers for research, holding great potential as *in vitro* and *in vivo* delivery vehicles.

However, although this strategy is very elegant and works well from a theoretical point of view, it has its difficulties and pitfalls in practice. First of all, it is important to choose a suitable conjugation method for each molecule that has to be carried by the CPP. Furthermore, the CPP to cargo ratio and the employment of peculiar linker systems are of

relevance. Moreover, CPPs are normally not specific and they are consequently taken up in a variety of certain cells and tissues, leading to increased toxicity and side effects. That is why new strategies are now being developed in order to enhance their selectivity, and it is essential to understand which method is the best in every distinct context. Finally, there are different classes of cell-penetrating peptides, each of which works better for certain types of cells and it is important to choose the optimal sequence for the envisaged goals. [13] In particular, different sequences (exhibiting cationic or amphipathic characteristics) imply also distinct properties regarding internalization efficiency but also toxicity. [14]

The community seems to evolve really vast, but still many details have to be taken in consideration for planning an optimal strategy when using CPPs. In this review, we summarize some of the innovative approaches that have been studied in order to go beyond the limitations with CPP application. In particular we focus on the selective delivery of drugs in the field of anti-cancer therapy, with specific emphasis on very recently published papers. We will first describe the different strategies that have been followed for synthesizing CPP-cargo conjugates, focusing our attention on covalent binding and fusion techniques. The cargoes described herein include small molecule drugs, peptides and proteins. Also non-covalent complexes and some applications for intracellular delivery of nanoparticles will be shortly depicted. In addition, we will cover strategies to obtain a targeted delivery towards cancer tissues. The last part of this review will deal with recently obtained advances of CPPs, to show how this new technique is actually reaching an increasing success in the treatment or diagnosis of cancer. Also drawbacks connected with the use of CPPs will be considered and discussed.

2. Different strategies for the synthesis of CPP-cargo constructs

Depending on the type of molecule that has to be transported inside the cell, there are various methods used to allow the conjugation between the carrier moiety and the cargo of the drug delivery system. These different conjugation procedures involve a distinct synthetic pathway and may have impact on the route of administration, cell entry mechanisms, distribution inside the cell, and on other different effects on the cellular level. In addition, based on the therapeutic question and nature of the target, which the drug must act on, the choice of conjugation way plays a very important role. [15] This paragraph will contemplate in particular the formation of conjugates by covalent binding between CPPs and small molecule drugs, proteins and peptides. But also fusion techniques are described, which allow the synthesis of constructs including proteins. Other methods comprehending non-covalent complexes and nanoparticles constructs are finally shortly mentioned.

2.1 Covalent CPP-cargo conjugates

Most CPP-cargo conjugates synthesized so far, particularly that one including small molecule drugs, are characterized by covalent bonding, including stable as well as cleavable linkages. However, this kind of conjugation method can be achieved by chemical reactions with or without the employment of such linker molecules. Thus, disulphide bond formation, thioether formation or amide bonding were often utilized for connecting small molecule drugs to CPPs, [16] but also for coupling proteins and peptides, peptide nucleic acids (PNAs) and morpholino oligonucleotides to CPPs (for examples see Tabel 1). [17]

Table 1: Examples of covalently connected CPP-cargo constructs. For more information refer to main text.

Name	Sequence	Cargo	Connection via	Ref
Oligoarginine	RRRRRRRRRRR	BSH	disulfide bond	[18]
	RRRRRRRR	Taxol	disulfide bond	[19]
	RRRRRRRR	Doxorubicin	thioether bond	[20]
	RRRRRRRR	Doxorubicin	disulfide bond/oxime linkage	[21]
	RRRRRRRR	inhibitors of CyclinE/A-CDK	amide bond	[22]
	RRRRRRRRR	APT_{STAT3}	peptide bond	[23]
Tumor homing CPP	RLYMRYYSPTTRRYG	Taxol	ester bond	[24]
Maurocalcine	GDCLPHLKLCKENKDCCSKKCKRRGTN IEKRCR	Pt chelator	amide bond	[25]
sC18	GLRKFRLRKFRNKIKEK	Cymantrene complexes	amide bond	[26]
Low molecular weight protamine	VSRRRRRRGGRRRR	L-asparaginase	disulfide bond	[27]
Penetratin	CRQIKIWFQNRRMKWKK	KLA	disulfide bond	[28]
		LP4	peptide bond	[29]
FHV	RRRRNRTRRNRRRVR	p53 C-terminal domain	peptide bond	[30]
p53 C-terminal domain analogue with CPP characteristics	GKKHRSTSQGKKSKL			[31]
TAT	YARVRRRGPRR	PLHSpT	peptide bond	[32]
		peptides inhibiting autophosphorylati	peptide bond	[33]

		on of EGFR		
transportan 10 (TP10)	AGYLLGKINLKALAALAKKIL	$SRC1_{LXXLL}$	peptide bond	[34]

Disulfide linkage is one of the most widely used methods for linking small molecule drugs to CPPs. For example, in the context of boron neutron capture therapy, mercaptoundecahydrododecaborate (BSH) was fused to the CPP R11 by a disulfide bond in order to allow cell penetration. The resultant conjugate was localized in the nuclei of glioma cells and showed a higher biological effect compared with the group treated with pure BSH, which stayed outside the cell. On the other hand, the compound could not be detected in the normal brain area. [18] In another study by Wender et al. R8 was conjugated to the drug taxol using again a disulfide linkage that is cleaved in the reducing environment of the cytosol, releasing there the free drug. This conjugate, in the treatment of human ovarian carcinoma, possessed comparable cytotoxicity and a better activity than the active drug alone, avoiding the efflux pump resistance. [19] Also peptides were coupled via disulphide linkage to CPPs. For example He et al, coupled the CPP *"low molecular weight protamine"* to L-asparaginase by using the bifunctional cross-linker 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide (SPDP) leading to the formation of a disulfide bridge. This compound was then encapsulated into red blood cells for the treatment of acute lymphoblastic leukaemia, interrupting L-asparagine supply in malignant cells. [20] Another example in which the conjugation was done by formation of a disulfide bridge between two cystein residues incorporated in two peptidic moieties was presented by Alves et al. To facilitate cellular uptake of the apoptotic peptide KLA it was conjugated to the CPP penetratin. The construct had a cytotoxic effect against cancer cell lines, including multidrug resistant cells, but not towards healthy ones, showing a selective effect *in vitro*, probably owing to differences in membrane composition. The mechanism of action of this conjugate was directed against mitochondria, in particular damaging their metabolic activity, and it could bypass the apoptosis resistance becoming an optimal alternative of synergistic strategy together with traditional chemotherapy. [21]

Nakase et al. used the CPP R8 again for conjugation with doxorubicin in order to prove the accumulation of the system in tumours. In this case the authors first prepared a doxorubicin-maleimide compound that was then coupled with R8 [$(D\text{-}Arg)_8$-Gly-Cys-amide], leading to the formation of a thioether bond. [22]

The use of heterobifunctional crosslinkers was investigated by Lelle et al, who exploited a novel linking method containing a thiol and an aminooxy group. The CPPs used had two distinct sequences (R8 and a proline-rich amphipathic peptide), while the final conjugate

contained both a cleavable disulfide bond that can be reduced inside the cell, and a stable oxime linkage to bind the drug doxorubicin. [23]

Some other strategies like amide and ester coupling have also been applied. An example is the conjugation of taxol to a tumor homing CPP by a succinic acid linker, as described by Tian et al. Taxol has excellent self-assembly properties that permit the formation of a nanospherical construct. The taxol molecules are released by ester bond hydrolysis and can then exert the activity inside the cell. This system could be used for the co-delivery of other therapeutic molecules to cancer cells, like for example doxorubicin, in order to exhibit a synergistic effect. [24]

During the last years there emerged a steady increasing interest in the use of metal-containing drug molecules. One of the most prominent example is cisplatin that is frequently used as anti-cancer drug in combination therapy, and one of the best therapeutics against glioblastoma. Notably, cisplatin and related compounds are often characterized by their high toxicity and several side effects. Moreover, problems with solubility and water stability of such metal-containing compounds often limit the application of also novel developed compounds that show interesting new activity spectra. These drawbacks can be significantly reduced by the attachment to a CPP, as it has been shown in the work by Aroui et al. They synthesized a novel platinum-maurocalcine conjugate by using an amide bond linkage. The conjugate showed a higher activity in U87 cells than cisplatin itself by targeting the intracellular redox system at lower doses and inhibiting the activity of ERK and AKT cascades. These additional activities could be essential in the treatment of resistant cancer cells. [25] Recently, we synthesized several cymantrene-peptide conjugates by coupling functionalized cymantrene complexes via amide bonds to the CPP. [26, 27] Cymantrenes are cyclopentadienyl manganese tricarbonyl metal complexes that exert cytostatic effects when in combination to CPPs. [28, 29] The CPP used in this study was the sC18 peptide, previously developed in our group. [30] In some cases, a cathepsin B sensitive cleavage site was also introduced between the peptide and the metal complex. [31] Cathepsin B is known to be over-expressed in several cancer cells, and thus, the selective release of the drug inside the cells can be enhanced. In fact, our study demonstrated high activity of these CPP-conjugates even again drug-resistant cells. [31] Amide bond formation was also used for for the conjugation of the CPP R8 with novel inhibitors of cyclinE/A-CDK (cyclin-dependent kinases) in order to analyze the activity of the constructs against cancer cells. In fact, the conjugation via an amide bond of this potent inhibitor resulted in an accumulation of tumor suppressor p27, blockage of cell cycle progression and cell survival. Furthermore, the presence of the CPP allowed the inhibitor to easily permeate the cells reaching a promising activity in several tumor cell lines. [32]

In another study presented by Ueda et al. in 2012, a multifunctional D-isomer peptide for the treatment of glioblastoma multiform (GBM) was designed. This was composed by a CPP (FHV, derived from flock house virus), a penetration accelerating sequence (Pas, derived from the retro sequence peptide of the cathepsin D cleavable sequence) and the C-terminus domain of p53, a biologically active tumor suppressor protein. The whole peptide was prepared by traditional solid phase peptide synthesis in line. As shown by *in vivo* studies it could inhibit the growth of GICs (glioma-initiating cells) and glioma cell lines with no effect on normal cells. [33] In the same year, p53 being frequently mutated in various human cancer types, was studied by Suhorutsenko et al, too. The research group synthesized short p53 protein analogues, starting from their C-terminal domain, and varying them by using CPP prediction algorithms. After modification with stearic acid to increase the transfection efficiency, they observed an increase in cellular uptake *in vitro* and certain selectivity in apoptotic activity against p53-mutant cells. [34] Not only had the protein p53 attracted the attention of scientists involved in the search for new possible appealing targets in tumor therapy. Plk 1 (Polo-like kinase 1), for example, plays key roles in regulating cell cycle events and is over-expressed in many cancer cell lines. For instance Plk 1 is essential during mitosis and in the maintenance of genomic stability. Its inhibition by specific phosphopeptide sequences has been proposed as an interesting strategy to inhibit tumor growth. In this framework, Kim et al. synthesized by solid phase peptide synthesis a new delivery system by conjugating PLHSpT, the minimal sequence necessary for the binding, to TAT peptide with the purpose of increasing the cell membrane penetration. *In vitro* studies showed inhibited cancer cell proliferation by blocking mitosis but also inducing apoptosis. [35] In addition to these examples, also the steroid receptor coactivator-1 (SRC-1) could be included in this group of proteins over-expressed in cancer, especially in breast cancer cells. This cofactor is characterized by the presence of a recognition motif LXXLL that is directly responsible for the binding to nuclear receptors. In this view, Tints et al. synthesized one of these sequences, able to bind estrogen receptors, and conjugated it to a cell-penetrating peptide transportan 10 (TP10), as an effective vehicle for the delivery of the active peptide to cellular targets. *In vitro* studies revealed high cytotoxicity in breast cancer cells with the induction of apoptosis. Importantly this effect was not affected by the estrogen receptors status, so that ER-negative breast cancer cells could be also treated by this strategy. [36] With this in mind, many other representative peptides exhibiting selectivity to attractive tumor targets could be found; among all the cases, we mention STAT3-binding peptides [37], the voltage-dependent anion channel 1 (VDAC1) - based peptides [38] and oligopeptides inhibiting autophosphorylation of EGFR [39]. All these active peptides against tumor disease have a hydrophilic sequence and cannot easily permeate the cell membrane. In these circumstances, a covalently

conjugated cell-penetrating peptide represents an efficient carrier to enhance the cellular uptake and to obtain a potent anti-tumor activity. Within all of these studies dealing with cancer active peptides, the peptides were often simply attached to the CPP sequence by using solid phase peptide synthesis obtaining a final product without any inconvenient intermediate purification procedure.

Table 2: Examples of fusion proteins including a CPP. For more information refer to main text.

CPP name	Sequence	Cargo	Ref
HBHAc	KKAAPAKKAAAKKAPAKKAAAKK	arginine deiminase	[40]
BR2	RAGLQFPVGRLLRRLLR	scFv Ab against mutated K-ras	[41]
TAT	GRKKRRQRRRPQ	Gelonin toxin	[42]
		apoptotic protein BID	[43]

One other important strategy for the delivery of proteins by the help of CPPs is the formation of fusion proteins generated by recombinant expression (see Table 2). As promising anticancer treatment, Yeh et al. reinvented the already known arginine depletion strategy trying to overcome the arginine deiminase (ADI) resistance in MDA-MB-231 cells. A pH-sensitive CPP-based fusion protein delivery system, which is able to carry ADI inside the cells, was constructed. The CPP HBHAc was incorporated with the pH-sensitive peptide HE and fused to ADI achieving tumor selective delivery in the mildly acidic tumor microenvironment of breast cancer cells. [40] In 2013, Lim et al. designed a new CPP starting from the sequence of the anticancer peptide, buforin IIb. This CPP, BR2, can efficiently enter cancer cells by endocytosis thanks to the interaction with negatively charged gangliosides on the outer cell surface. Notably it shows no toxicity to normal cells. The ability of efficient drug delivery was proven by fusion to a single-chain variable fragment (scFv) antibody directed towards a mutated K-ras. The experiments were conducted with HCT116 cells causing a high level of apoptosis. This could be a useful and innovative drug delivery system with a high selectivity toward cancer cells. [41] Antibody targeting strategy and genetically engineered fusion technique were also employed by Shin et al, who proposed a new method to fight colorectal cancer by fusing to the sequence of TAT a molecule of so called gelonine, a very potent toxin that inhibits protein synthesis, but with an extremely poor cellular uptake. In order to obtain selectivity for this compound, a heparin conjugated anti-carcinoembryonic antigen (CEA) monoclonal antibody was associated *via* reversible electrostatic interaction. In this way, this CPP-fused chimeric protein was evaluated and showed a significant therapeutic efficacy against colorectal cancer therapy with a reduced toxicity to healthy tissues. [42] Additionally, in a recent study by Orzechowska et al, cells were sensitized to cytotoxic drugs by delivery of the apoptotic protein BID (BH3-interacting domain death agonist) fused to the CPP TAT. This method gave good results in prostate and non-small human lung cancer cells

providing a possible tool to improve the efficiency of therapeutic agents against this cancer cell types. [43]

2.2 Generation of non-covalent CPP-cargo complexes

Covalent linking methods are sometimes limited by the concern that the synthetic covalent bond between CPP and the active moiety may alter the biological activity of the latter. This is the reason why many systems are often planned as non-covalent complexes, where all the entities are independent but at the same time connected to each other. Nowadays, this strategy is often performed with cell-penetrating peptides applied in gene therapy, e.g. for the delivery of genes, antisense oligodeoxynucleotides (ODNs), or small interfering RNA (siRNA). [44, 45] The negatively charged nucleic acids can in fact be easily complexed by electrostatic interaction with the often positively charged CPP, forming a stable complex. Frequently an excess of peptides is used that not only protects the nucleic acids from degradation but also helps to improve distribution, targeting and penetration of the nucleic acid in cells or tissues. Examples of recent works show how the systematically degradation of siRNA molecules can be avoided and their intracellular delivery promoted. [46-48] Non-covalent complexes have been also used for the delivery of small molecule drugs, even if the covalent conjugation is prevalently employed (see above). Li et al. described the formation of a complex between the active molecule doxorubicin and a particular CPP called CADY-1 that is a self-assembled peptide. This stable complex led to a longer blood residence time of the construct and better permeability of the drug with the subsequent improvement in therapeutic index. [49] Cyclic CPPs are known to be less susceptible to degradation and in a work by Mandal et al. cyclic cell-penetrating nuclear-targeting sequences were complexed with doxorubicin leading to efficient and targeted molecular transport. [50] Also we have recently investigated the impact of cyclization for the activity of CPPs. A shorter version of the CPP sC18 was cyclized using copper (I) - catalyzed alkyne - azide click reaction. The cyclized peptide exhibited increased proteolytic resistance and cytosolic cellular distribution. However, when complexed with plasmid DNA encoding for the enhanced green fluorescent protein (EGFP) the cyclized version demonstrated highly improved complexation and uptake of the plasmid in contrast to the linear CPP that was not able to transfect the used cancer cells at all. [51] In another study, such cyclized CPPs containing a triazole were used to complex the drug daunorubicin in breast cancer MCF-7 cells. Also in this case the cyclization improved the transport efficiency of the herein used CPP. [52]

2.3 Generation of multimodal nanoparticles for anti-cancer therapy

Nanoparticles are increasingly being studied as multimodal platforms at the same time for the possibility of grafting bioactive molecules, as a diagnostic tool (fluorescence, magnetism) or

therapeutic treatment (energy production). To get a deeper view into this emerging field and the use of NPs in anti-cancer therapy and diagnosis, the readers should refer to these excellent recent reviews. [53-56] However, the inability to pass through the lipid membranes of cells greatly limits their *in vitro* and *in vivo* use. To bypass this pitfall, CPPs can be set on their surface to facilitate and accelerate the cellular uptake, and to reduce possible cytotoxic effects. Moreover, owing to their size several other ligands or functionalities can be fixed on the same nanoparticle. Vehicles as polymeric nanoparticles or liposomes are typically used to develop a controlled release system. This approach can be used to improve the distribution, the absorption and the targeting of molecules which otherwise would be quickly eliminated or would not be able to reach the target tissue. [57]

Among the simplest polymeric systems on the market, the classic example is the combination of liposomes with doxorubicin. Since the application of doxorubicin as anti-cancer drug may cause cardiac toxicity problems, researchers try to find solutions to circumvent these side effects. By releasing the drug more slowly, a lower dose can be used and global toxicity can be reduced. These systems, however, after a certain time become ineffective because doxorubicin is a substrate for Pgp (glycoprotein P, an efflux pump) and the affected cells become resistant. [58] Beside the addition of Pgp inhibitors, [59] the use of CPPs can be essential to overcome this resistance. In this context, mesoporous silica nanoparticles derivatized with an activatable CPP polyarginine and doxorubicin were synthesized by Liu et al. and the activity was efficiently demonstrated *in vivo* proving no side effects and tumor growth inhibition. [60] In another study conducted by Wang et al. against multi drug resistance, *low molecular weight protamine* was used as CPP connected with poly(lactic-co-glycolic acid) (PLGA) nanoparticles additionally loaded with doxorubicin. The presented data suggest that this system could actually act against upcoming resistance by various mechanisms, like enhanced cellular uptake, accumulation in nuclei and diminished efflux. [61] *Low molecular weight protamine* with MMP2 cleavage site was also connected to paclitaxel-loaded PEG-co-PCL nanoparticles for targeted glioblastoma therapy inducing enhanced selectivity, cytotoxicity and cellular uptake in C6 glioma cells. [62]

3. Different strategies for targeted CPP-cargo delivery

One of the main problems when using CPPs is their lacking target specificity. Avoiding unspecific uptake is mandatory to limit and exclude loading of healthy cells with CPP-drug conjugates. During the last years, different strategies have been described to obtain more selective CPPs in order to circumvent pathological changes in particular tissues provoked by the unspecific distribution of CPP-cargo conjugates. To circumvent such unspecific CPP uptake, also masking of the positive charges of the CPPs might be necessary and can be

realized by the formation of so called activatable cell-penetrating peptides (ACPPs). Here, the CPPs are often fused to a polyanionic sequence, pH-sensitive polyethylene glycol (PEG) chains, or proteins.

3.1 Active delivery strategies

Changes in the local environment typically seen in cancer tissues can be used to actively deliver CPPs to the tumor tissue avoiding cellular uptake to normal cells. The following conditions may count to this, as reduced pH, presence of over-expressed metalloproteinases, and the accumulation of particular receptors on cell surfaces. However, also external triggers like heat, ultrasound and magnetic field can be used for a targeted drug uptake. Systems that combine both concepts of active and passive addressing of tumors are more and more popular, and in the following paragraphs some of these methods are taken into account.

A possible strategy that is often followed by researchers in order to obtain a selective CPP that can target tumor tissue without involving normal cells is the insertion of particular cleavage sites to the sequence of the CPP. These can be cleaved e.g. by metalloproteinases like MMP-2/-9, which play an important role in angiogenesis and metastasis of tumors, and are frequently over-expressed in cancer tissues. Another possibility is to make advantage of pH change in cancer tissue, which is normally characterized by mildly acidic conditions, differently from the neutral pH of the rest of the cells. For instance, an MMP-2 cleavage site was introduced by Li et al. between a CPP and a polyanionic peptide in order to block the penetration in normal tissue building an activatable pro-form. The ACPP was conjugated to protoporphyrin IX, a light-sensitive molecule, therefore utilized as therapy against different forms of cancer by photodynamic therapy. After cleavage and activation of the CPP in cancer tissue, this photosensitizer could be introduced inside the cells generating by irradiation reactive oxygen species. Tumor size was decreased without any systemic toxicity. [63] Many other examples of similar ACPPs, containing a metalloproteinase cleavage site, describe the conjugation to different cargoes like methotrexate [64] and hTERT siRNA [65]. Moreover, this method can be also be used in order to control drug delivery and precisely track drug release in living cells. For example, Cheng et al. designed a novel drug delivery system made of three different components, in particular a fluorophor, a functionalized CPP with a cleavable site for metalloproteinase MMP-2 and the active drug doxorubicin. In the cancer tissue the structure is cleaved and the drug can easily pass through the cell membrane thanks to the activity of the CPP. Meanwhile the fluorophor will self-aggregate because of hydrophobic interactions, and turn on yellow fluorescence. By means of that, they could observe real time *in vivo* delivery of the drug. [66] Similarly, Savarian et al. projected an ACPP in order to evaluate the presence of metastases by means of Cy5 that is quenched by Cy7 till the linker between the two fluorophors is cut by MMP2 and 9 in tumor tissues. The

fluorescence emission is increased and the presence of the tumor and corresponding metastases can be easily detected. [67] The same ratiometric activatable CPP system was also used by Hauff et al. in order to improve tumor identification. [68]

Not only metalloproteinases can be involved in the selective cleavage of CPPs in cancer tissue as was shown in the work by Liu et al, where TAT-liposomes loaded with doxorubicin were activated by the endoprotease legumain. This is a lysosomal cystein protein, whose expression directly corresponds to the malignancy of the tumor itself. Furthermore, the legumain, normally present in the cellular plasma, moves to the cell surface if conditions like starvation or hypoxia occur. The CPP TAT loses some of the permeation ability when conjugated to the legumain cleavage site, but this capacity is restored when in contact with this enzyme so that the CPP can enter efficiently and selectively in tumor cells but not in normal cells. [69]

As already mentioned, also the difference in environmental pH between normal and tumor tissue can be exploited to favour the selective therapeutic delivery. In fact, the tumor tissue is characterized by a slightly lower pH and many acid-labile systems have been designed in the last years. [70] In this work by Fei et al, a $(HE)_{10}$ peptide was combined to the CPP MAP to mask the positive charges till reaching the tumor tissue. At this point, the lower pH would in fact protonate the histidine residues and allows the CPP to express its positive charges and the ability to pass over the cell membrane. [71]

Another approach is to combine CPPs with receptor targeting moieties. Indeed, a great number of receptors are over-expressed in tumor tissue, and cells and can be targeted with extremely diverse ligands. Regarding their size as well as chemical structure these molecules are characterized by a very high heterogeneity. For instance, drug conjugates of the glycoprotein transferrin enable an efficient accumulation of drugs in cancer cells. Li et al. synthesized lipid nanoparticles for the delivery of siRNA loaded with the CPP R8 and the targeting ligand transferrin [72], showing excellent gene silencing activity *in vitro* and *in vivo*. Transferrin can also be targeted by receptor-targeting sequences directly located within the CPP sequence, as showed by Youn et al. in a work about neuro-targeted siRNA delivery. [73] Folic acid receptor is overexpressed in a variety of malignant cells. Vitamin folic acid can bind to this receptor with a high affinity and thus makes it an attractive target for the targeted drug delivery in tumors. Gao et al. used a combination of folate targeting and tumor microenvironment-sensitive polypeptides (with the presence of metalloproteinases cleavage sites) to deliver docetaxel loaded nanoparticles to tumor cells. The enhanced cellular uptake was caused by both the folate receptor and MMP2 overexpression in tumor tissue. [74]

On the other hand, another possible approach could be to address tumoral endothelial cells by targeting receptors, such as integrins. Integrins are one of the major families of transmembrane cell adhesion receptors; they are overexpressed on the surface of cancer cells

and they are involved in tumor angiogenesis, progression and metastasis. In particular, they can be selectively targeted with cyclic and linear RGD peptide sequences. By inhibiting the endothelial cell proliferation, vital nutrients and oxygen will be no longer adequately provided from the tumoral blood vessel system. In this way, the tumor growth and the formation of metastases would be suppressed. Many researchers are working in this field, and the interested in synthesizing different variables of RGD increased more and more since the development of *cilengitide* by Kessler et al. that failed in the phase III in the clinical development. [75] Chen et al. developed albumin-based nanoparticles composed of a CPP moiety, a targeting moiety (cRGD) and the active drug doxorubicin with pH dependent self-assembly behaviour. After coming in contact with the endosomal environment the drug could be easily released and accumulated inside the nuclei. [76] Liposomes were also loaded with paclitaxel and selectively targeted to tumor cells by a multifunctional CPP with a targeting moiety cyclic RGD showing selectivity to integrin receptors. This strategy was then applied in glioma cells inducing the strongest inhibition and apoptosis. [77] The targeting of integrin $\alpha_v\beta_3$ by the ligand cRGD was connected by Crisp et al. to the use of MMP2 cleavage site in the CPP sequence to deliver the chemotherapeutic monomethylauristatin E. [78] Moreover, a dual targeting strategy was used to deliver paclitaxel loaded liposomes. The two targeting ligands were selective towards integrin and neuropilin I receptors, having a synergistic action and increasing the selectivity for glioma cells. [79]

NGR was also used as a ligand for the delivery of doxorubicin by thermosensitive pegylated liposomes. In this work the drug delivery system was selectively targeted to tumor cells by the double action of NGR and thermosensitive liposomes that hinder the action of the CPPs till reaching the tumor tissue where the temperature is a bit higher. [80]

As another example of targeting ligands a breast tumor homing cell penetrating peptide was also used for the selective delivery of the drug (-)-epigallocatechin-3-gallate. Silica nanoparticles were used as vectors in this case. [81]

Since already used in prostate cancer detection, the two receptor prostate-specific antigen (PSA) and prostate specific membrane antigen (PSMA) can be utilized in the targeted delivery of anticancer drugs, in this case for the delivery of siRNA. Xiang et al. designed liposomes exposing an activatable CPP, which can be activated by PSA cleavage in the tumor tissue, and a folate moiety, selective to PSMA receptors. When the folate moiety attracts the liposome to the cancer cell surface, the CPP is activated and the system is taken up. [82]

During the last years, photosensitive approaches have gained increasing interest among researchers. In particular Yang et al. published some interesting work about this subject. siRNA molecules were delivered by cationic liposomes bearing an NGR peptide as targeting ligand and a CPP, shielded by photolabile groups able to neutralize its positive charges. In this study, they used NIR illumination, because of its characteristics of deep tissue

penetration and being less harmful to cells. After light treatment, the CPP was exposed, and its functionality restored allowing cellular penetration. [83] The year after, the same group published another work in the same direction but this time adding a pH-responsive polypeptide. The de-shielding of the CPP occurred in this case through the double action of intrinsic lowered pH in tumor tissue and external NIR illumination. [84] The same research group synthesized thermal and magnetic dual-responsive liposomes for siRNA delivery, too. In particular, magnetic fluid Fe_3O_4 was combined with thermosensitive lipids; the liposomes would accumulate at the tumor site by a magnetic force, replaced then by an alternating current magnetic field that induced the iron nanoparticles to produce heat. By this heat the thermos-sensitive lipids could undergo to a gel to liquid phase transition and the CPP-siRNA conjugate could pass inside the cell. Arriving in the cytosol the disulfide bond between CPP and nucleic acid would be reduced and the siRNA could silence the corresponding mRNA in the cytosol. [85] Hyperthermia was also employed by Ryu et al. in an experiment consisting in the synthesis of a construct based on an elastin-like polypeptide (ELP), a CPP named Bac and the C-terminal domain of the p21 peptide. Upon external application of localized mild hyperthermia, the ELP aggregates and accumulates in tumor tissue. [86]

3.2 Taking advantage of the enhanced permeability and retention effect

Typically, tumors are characterized by strongly increased angiogenesis, which means that the new formation of blood vessels around the malignant tissue increases in order to satisfy the enhanced nutrient requirement of the cancer cells. However, these vascular systems significantly differ from the healthy one since the endothelium has a series of defects that make it permeable. Furthermore, the pressure of the interstitial tissue fluid of tumors is increased, with the result that the efficiency of small drug molecules is dramatically reduced, since these are easily eliminated. Nevertheless, precisely these two factors provide the solution approach for a possible targeted addressing of tumors. In fact, the principle of passive addressing of tumors with nanotherapeutics that are able to passively and selectively accumulate in the permeable tumor tissue has become the gold standard in our time and is often used with the term established by Matsumura and Maeda *"enhanced permeability and retention (EPR)" effect*. [87]

For instance**,** PEG has the ability to shelter complexes that consist of CPPs and active molecules until they reach the tumor tissue, thereby prolonging their half-life in the blood circulation and avoiding unwanted metabolism. In addition, PEG induced steric hindrance may favour the accumulation in tumor tissue by the above-mentioned EPR effect. In a work by Veiman et al. the MMP cleavage site was introduced between a CPP molecule, complexed with a plasmid DNA, and a PEG molecule. As soon as the nanoparticles passively accumulated in the tumor environment through the EPR effect, the metalloproteases would

cleave the substrate and the PEG would finally allow the CPP to come in contact with the cell surface. Then the CPP would penetrate inside, while transporting the gene inside the cell interior. [88] Recently, Wang et al. followed the same strategy for the delivery of siRNA targeting Plk1 (polo-like kinase 1) mRNA. [89] Also the work of Zhu et al. went in the same direction: here the nanoparticles were composed by self-assembling PEG and the active drug was paclitaxel, but the high tumor accumulation of the system was, here again, the result of the combined EPR effect with the up-regulated MMP2 in the tumor. [90] Koren et al, on the contrary, investigated PEGylated liposomes containing doxorubicin, TAT and a targeting ligand mAb. The carried PEG molecules are characterized by different lengths and were conjugated by a pH-sensitive hydrazone bond. When liposomes accumulated in tumor tissue by the EPR effect and mAb active targeting, the mildly acidic environment led to the cleavage of the degradable bond and exposition of the CPP to the cell surface, enhancing cellular uptake of the small molecule drug. [91]

In summary all these examples show that particularly during the last years a multimodal approach is more and used. Combination of a delivery unit, with a cytotoxic payload and a targeting sequence may be the right way to support a successful and efficient fight against tumoral cells.

4. Concluding remarks

One can observe that many recent publications are including *in vivo* experiments with CPPs, both for imaging or therapeutic applications. This fact highlights the increasing interest in developing peptide-based delivery vectors. Anyway, still many problems are connected with this kind of strategy. It is important to understand all the disadvantages and side effects in order to overcome them and synthesize a new effective drug delivery system that is active also *in vivo*. In fact, although many CPPs are being tested, only one CPP, called p28, has reached phase I in clinical trial in the context of cancer treatment, in particular against solid tumors expressing p53, as well as for CNS malignancies. [92]

Problems connected with the use of CPPs include upcoming immunogenicity, since the sequences are novel to the organism to which they are being administered, as well as cytotoxicity, caused by the perturbation of plasma membrane dynamics. [93] Both side effects are deeply related to the particular sequences; for this reason, one cannot talk about a general problem of this class of peptides, since each CPP is defined by a distinct amino acid composition. Many studies have been recently done to establish a possible action on the immune system and a consequent immunological reaction in the organism, but no general immune response was observed. About toxicity one can state that in general cationic CPPs are less toxic than amphipathic CPPs, even if *in vivo* studies show positive results about the safety at the employed doses. Nevertheless, it is important to always analyze both

immunogenicity and toxicity since, as already mentioned, every CPP is different from the others and their effect can also change in the presence of a cargo.

Another issue is the lack of selectivity, as already described in the previous paragraphs. Many are the strategies adopted but sometimes the penetrating activity of the CPP is so strong that the targeting ability of the specific targeting moieties used can be completely hidden, and no positive result in selectivity to cancer tissue would be gained. [94] Together with the use of targeting molecules, the ability of CPPs should be shielded by using pro-drug strategies based on electrostatic interactions or PEG systems that could block penetration by steric hindrance. [95]

Blood stability is also a very important attribute that a drug should possess in order to reach the target without being destroyed by blood proteases before arriving to the tissue. [96] This obstacle can be circumvented applying different shielding strategies in order to protect the structure of the CPPs till reaching the desired tissue, utilizing for instance more stable D-amino acid configurations, [97] backbone cyclization [51, 52, 98] or, finally, backbone stabilization through β- and γ-peptoids inside the sequence. [2] In a recent study, Shen et al. tried to overcome all the problems connected with the systemic administration of CPPs, proposing a more favourable cell-based platform for local peptide or protein production within the target tissue. To improve the intercellular transport, they designed a new CPP based on a triple repeat of modified TAT and a secretory signal peptide, with improved transduction activity and secretion efficacy. This is still a developing method but it can lead to a big improvement in cell-based delivery of CPPs precluding degradation by proteases in the blood, metabolism and too early excretion. [99]

Endosomal escape is also a decisive concern, since for many CPPs the main entry pathways proceeds via endocytotic mechanisms. In fact, the CPP construct must be taken up by cells, but more importantly, cargoes have to be released and to reach their extra-endosomal targets in the cytosol or in the nucleus. This could be made by using endosomolytic sequences or fusogenic compounds. [17, 100, 101] It is also true that, even if the majority of the active molecules remain inside the lysosomes, the small quantity that succeeds in escaping and get to the cytosol can be sufficient for the biological activity. [102]

Specific attention should be also paid on small molecule drug delivery with the aim to overcome the problem of multidrug resistance (MDR) that develops in tumor cells after repeating exposures to the same drugs. MDR can often be attributed to the up-regulation of efflux pumps, particularly active with lipophilic drugs inserted in the membrane. CPPs could help in this sense by changing the solubility properties of the drugs, favouring their entrance in the cytosol and releasing them by different mechanisms. [103] [104] [16] Such CPP-conjugates were also designed to promote some particular routes of administration. In the context of small molecule drugs delivery, docetaxel cyclodextrin inclusion-loaded PLGA

nanoparticles were administrated with CPPs to enhance its oral bioavailability. Bu et al. demonstrated how this system displayed the maximal cytotoxicity against breast cancer MCF-7 cells, enhancing absorption and bioavailability of the drug itself as a promising oral delivery carrier. [105] Oral administration by new formulation approaches has been also studied in recent works even if this field is still growing and many more experiments have to be done. [106] [107] Transdermal delivery capability could be also enhanced by cell-penetrating peptides. The proapoptotic peptide KLA, for example, was delivered by Gautam et al. to different cancer cells *in vitro* and into the skin *in vivo* by a new CPP IMT-P8. After internalization, the construct, containing a compartment-specific localization sequence, could localize to mitochondria causing cell death thanks to the peptide ability of disrupting the mitochondrial membrane. These results suggest that this could actually be used as topical delivery vehicle in dermal diseases. [108]

In general, cell-penetrating peptides offer a number of distinctive merits and can be involved in many strategies for the delivery of anticancer active drugs, the latter being easily conjugated in many different ways *via* either chemical or genetic engineering method without affecting their intrinsic activity. Furthermore, they can efficiently transport attached cargos into almost all types of cells and this property makes cell-penetrating peptides a very eclectic element in the design of new drug delivery systems. CPPs will be used to revolutionize drug design and development by providing better bioavailability of the traditional chemotherapeutics at a much earlier stage of drug development, facilitating effective transition from preclinical to clinical phase in drug development. The research is still increasing and many pitfalls are being overcome and solved by new strategies; so, very soon conjugation of old and new active drugs to cell-penetrating peptides will become a more widely established clinical modality for the treatment of those malignancies for which there currently are no good treatment options.

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