Advanced Drug Delivery Reviews 180 (2022) 114044

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/adr

Cell-penetrating peptides in protein mimicry and cancer therapeutics

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ARTICLE INFO

Article history:
Received 1 July 2021
Revised 29 October 2021
Accepted 4 November 2021
Available online 10 November 2021

Keywords:
Cell-penetrating peptides
Protein mimics
Cancer therapy and diagnostics
Intracellular signaling
Cancer cell targeting

ABSTRACT

Extensive research has been undertaken in the pursuit of anticancer therapeutics. Many anticancer drugs require specificity of delivery to cancer cells, whilst sparing healthy tissue. Cell-penetrating peptides (CPPs), now well established as facilitators of intracellular delivery, have in recent years advanced to incorporate target specificity and thus possess great potential for the targeted delivery of anticancer cargoes. Though none have yet been approved for clinical use, this novel technology has already entered clinical trials. In this review we present CPPs, discuss their classification, mechanisms of cargo internalization and highlight strategies for conjugation to anticancer moieties including their incorporation into therapeutic proteins. As the mainstay of this review, strategies to build specificity into tumor targeting CPP constructs through exploitation of the tumor microenvironment and the use of tumor homing peptides are discussed, whilst acknowledging the extensive contribution made by CPP constructs to target specific protein–protein interactions integral to intracellular signaling pathways associated with tumor cell survival and progression. Finally, antibody/antigen CPP conjugates and their potential roles in cancer immunotherapy and diagnostics are considered. In summary, this review aims to harness the potential of CPP-aided drug delivery for future cancer therapies and diagnostics whilst highlighting some of the most recent achievements in selective delivery of anticancer drugs, including cytostatic drugs, to a range of tumor cells both *in vitro* and *in vivo*.

Contents

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https://doi.org/10.1016/j.addr.2021.114044
0169-409X/

1. Introduction

Despite extensive research and generations of therapeutic modalities, cancer still represents a global health burden that was estimated to have caused ten million deaths in 2018 [1]. Cancer is usually treated by a combination of surgery, chemotherapy and radiotherapy, all of which are accompanied by serious side effects. An additional problem is the development of drug resistance by cancer cells [2]. A serious obstacle in the treatment of this disease is a low permeability of the cell membrane to therapeutics. All these call for more effective drug delivery systems. One hope for improved control of the disease is the use of the cell-penetrating peptides (CPPs) that can aid in the targeted delivery of anticancer drugs to tumor tissue whilst normal healthy tissue remains unharmed. In this review we present different CPPs and their potential use in cancer treatment. Although the discovery and use of CPPs spans more than 30 years, they have yet to enter clinical practice. Altogether, around 30 CPPs are in clinical trials, but only a few have entered Phase III; for oncological treatments only one has reached Phase I clinical trials and no CPP or CPP-drug construct has been approved by the FDA for cancer thus far [3]. In this review we present recent research and the enormous potential offered by CPPs in the treatment of cancer.

2. What is a cell-penetrating peptide (CPP)?

The short definition of a CPP is: CPPs are up to 40 amino acids long peptides, with the ability to gain access to the cell interior by means of different mechanisms, and/or with the capacity to promote intracellular effects by themselves or by the delivered bioactive cargoes. Almost the same definition appeared 20 years ago in the first book on CPPs [4] and in many papers on CPPs thereafter [5]. A great number of CPPs have been identified in the past 30 years. Analyses of their structures and properties indicate that the only common feature of CPPs is their ability to cross biological membranes and carry cargos inside cells. The name cell-penetrating peptide and its abbreviation CPP appeared first in 1998 [6] and was soon generally accepted. Other names for CPPs found in the literature include; protein transduction domains (for CPPs generated from protein sequences that enable protein to enter the cell), membrane transduction peptides, Trojan horse peptides, bioportides (intrinsically bioactive CPPs designed to target and modulate intracellular protein–protein interactions without the use of a cargo) and some others.

In the year 1988 a paper was published describing the first transfer of a functional protein (horseradish peroxidase) into the cell by using a protein named trans activator of transcription (Tat-protein), the latter being derived from the human immunodeficiency virus [7]. It soon became evident that effective intracellular translocation did not require the complete Tat-protein. The minimal domain of the Tat-protein needed for cellular-internalization of the cargo comprised of 11, mainly cationic, amino acids [8]. In this way the first CPP simply called Tat was born, followed by a 16 amino acid long peptide from the homeobox Antennapedia transcription factor of *Drosophila melanogaster* [9], later called penetratin. From there on, the number of CPPs increased enormously, the precise number is still unknown. The freely accessible database of CPPs (https://webs.iiitd.edu.in/raghava/cppsite/) now (fall 2021) contains 1855 entries with the following information: peptide sequence, nature of the peptide, chemical modifications, experimental validation techniques, structure of the peptide and types of cargo delivered.

2.1. Classification of CPPs

Traditionally, the classification of CPPs is based on their physicochemical properties, their origins, or other characteristic features [10]. Physicochemical properties of CPPs arise from CPP structure. In this respect CPPs are usually divided into three groups: cationic, amphipathic and hydrophobic. Being composed of mostly positively charged amino acids, mainly Arg (R) and Lys (K), cationic CPPs are positively charged at physiological pH. Examples of cationic CPPs are Tat (GRKKRRQRRRPQ, [8], and poly-arginine $(Arg)_{8\text{-}10}$ (cf. Arg_8 RRRRRRR) [11]. Amphipathic CPPs are constructed of a suitable combination of hydrophilic and hydrophobic amino acids, where amphipathicity is due to the sequence or is achieved after folding of the CPP into an α-helix with a hydrophilic and a hydrophobic face: an example is MAP, KLALKLALKALKAALKLA, [12]. Hydrophobic CPPs are made of hydrophobic amino acids but some hydrophilic amino acids must also be included, otherwise the CPP would become insoluble in bodily fluids and be retained in the hydrophobic core of the lipid bilayer when passing through the cell membrane. An example is Pep-7, a synthetic hydrophobic α-helix, SDLWEMMMVSLACQY, [13]. It should be stressed here that some anionic peptides have also been shown to penetrate into cells [14].

In accordance with their origins, CPPs are usually also divided into three groups: protein-derived, chimeric and synthetic. Protein-derived CPPs represent part of the natural protein structure that can transfer across the membrane. The oldest example is Tat. Chimeric CPPs are composed of two peptides from different origins. An example is Transportan (abbreviated TP, GWTLNSAGYLLGKINLKALAKISIL [6] which is composed of part of the hormone galanin followed by the sequence of wasp venom mastoparan. Synthetic CPPs are artificially designed, an example is MAP (see sequence above).

Another level of classification is of particular clinical importance. In this classification, CPPs are divided into cell-specific and non-cell-specific peptides. As their names clearly indicate, cell-specific CPPs are able to deliver cargos into specific cells, whilst non-cell-specific CPPs are not selective.

Because of the great number of very different CPPs, all levels of CPP classification are very broad and usually give overlapping results. In order to classify CPPs more precisely, taking into account as many properties as possible, a new approach was adopted. Classes of CPPs were grouped into pairs to cover eight levels of

classification. In each level, two classes of CPPs with the opposite properties were presented. This approach was recently published [15] and is as follows.

1. Protein-derived vs. designed
2. Classified by physico-chemical properties vs. classified by structural properties
3. Predicted vs. random
4. Linear vs. cyclic
5. Protein mimics vs. cargo delivery vectors
6. Nonspecific vs. targeted
7. "Direct" translocators vs. endocytosis enhancers
8. "Non-toxic" vs. antimicrobial

Clearly, many CPPs can simultaneously belong to several subclasses more details in [15]. This classification system is open for additional discussions and changes.

2.2. Cell internalization of CPPs (with cargo)

There are two main routes of CPPs with or without cargo by which they can transfer through cell and organelle membranes: direct penetration or one of several endocytic mechanisms. However, detailed mechanisms of CPP internalization are still not clear. It seems that direct internalization is related to the destabilization of the membrane and is particularly true for the cationic CPPs that include lysine or arginine in their structure, for instance polyarginines [16,17]. Destabilization of the membrane can be initiated by the accumulation of positively charged CPPs on the negatively charged membrane surface, by attracting water molecules that bind to charged amino acids of CPPs in the hydrophobic core of the lipid bilayer [18], or by inducing transfer pores in the membrane [19]. Curvature of the membrane and the depletion of the amount of cholesterol in the membrane could further destabilize the lipid bilayer thereby assisting the peptide to traverse the membrane. For penetratin it was [20] proposed that spontaneous direct translocation of the membrane occurs through formation of an inverted micelle. Later [21] it was suggested that this mechanism could also be utilized by several other cationic CPPs.

Another internalization mechanism, which seems to be the main route of entry for many CPPs with or without cargo, encompasses different mechanisms of energy-dependent endocytosis, including clathrin- and caveolin-mediated endocytosis, other receptor-dependent endocytotic mechanisms, macropinocytosis, and possibly other endocytic routes [22]. It seems that for cancer tissue the most important route of internalization is macropinocytosis [23]. CPP (and cargo) normally accumulate in endosomal vesicles which further mature into lysosomes where CPP and cargo are usually degraded. Therefore, fast endosomal escape is a prerequisite for the effective delivery of a cargo into the cytosol. Different strategies were developed to enhance endosomal escape of CPP and cargo so as to achieve more effective delivery. A review of these strategies was recently published [24]. It should be stressed here that many CPPs in the delivery of their cargo seem to be able to use both direct and endocytic routes of internalization. It was suggested for instance that Tat and penetratin can cross the membrane passively only at low concentrations while at higher concentrations they use both direct and endocytic routes [25]; interestingly, another group has obtained entirely opposite results [26].

3. Strategies for CPP conjugation to anticancer cargos

Anticancer molecules or complexes should enter the tumor cells in order to act effectively. Internalization of anticancer drugs can be markedly improved by coupling to a CPP.

3.1. Cell-penetrating sequences in protein structures

Concerning proteins as anticancer drugs, some possess intrinsic cell-penetrant properties, the so-called shuttling proteins. In some cases, cellular penetration of proteins can be induced or enhanced by incorporating a cell-penetration promoting sequence into the structure of the protein. In addition to the Tat-protein, several other proteins with transducing capabilities have been identified, for instance, ANTP [27], HoxB4 [28], the herpes simplex virus type 1 VP22 transcription factor [29,30], OCT 4 that can enter the cell and influence its programming [31], engrailed-2 (En-2), that is able to penetrate cells and function as a transcription factor [32],

Engrailed 1, EN1 [33], Knotted-1 [34], Otx2 [35], Pax4 involved in vertebrate organogenesis [36], the protein NeuroD/BETA2 [37], protein–ligand complex neocarzinostatin, NCS [38], Omomyc mini-protein, a Myc inhibitor [39], human papillomavirus minor capsid protein L2 [40], to name but a few.

Finding CPP sequences can be a matter of reasonable guesswork, or it can be generated by computerized prediction algorithms [41–43]. Successful incorporation of CPP sequences into proteins to promote their effective penetration into cells has been achieved in some cases. It is also of paramount importance not to disturb protein structure and function when introducing cell-penetrant sequences. Therefore, it is usual practice to include cell-penetrating sequences into open loops [44] of anticancer proteins or as flanking CPP sequences at the C- or N-termini of proteins. Moreover, when CPPs are incorporated into protein loops, the propensity for cellular penetration is enhanced when the CPP is constrained by the protein into a cyclic structure [45,46]. Genetic engineering is clearly of great use, permitting the expression of such constructs in one step. However, in most cases, anticancer molecules are coupled to CPPs.

3.2. Coupling of anticancer molecules to CPPs

Cargos can be conjugated to CPPs in different ways – by covalent coupling or by forming a complex, using multiple weak interactions [47]. When covalent attachment of a cargo to a CPP is employed, the cargo can be attached directly to the CPP or via a suitable spacer where the cargo is tethered to the CPP through a cleavable bond, such as hydrazine or disulfide [48]. It has long been known that inclusion of cargos usually decreases the rate and efficiency of translocation, an observation that is dependent upon the nature of the cargo and its dimensions [49]. However, in some cases it was observed that penetration efficiency can be increased if the CPP is designed to self-assemble into spherical micelles thus locally increasing its local interface density [50] or when the flexible spacer is replaced by a constrained cyclic spacer [51]. Spacers can also contribute to site-specific intracellular delivery of the cargo and thus ensure selectivity towards cancer tissue [52]. Selectivity or targeting is discussed in the next section in more detail.

Oligonucleotides can also be covalently attached to a CPP, either to a 3′ or 5′ site via a suitable linker, which may or may not be cleavable – a recent review of these attachments to siRNA is available [53]. When a cargo is non-covalently conjugated to a CPP, the conjugation is governed by multiple electrostatic and hydrophobic interactions between the CPP and its cargo. An interesting example is to couple a modified negatively charged leucine zipper to the CPP and a modified positively charged leucine zipper to the cargo molecule, or vice versa; zippers can electrostatically bind to each other thus coupling the CPP to the cargo [54]. Non-covalent coupling of CPPs to cargos usually results in the formation of complex nanoparticles – for a comprehensive review see [55].

Extensive research has recently focused on decorating liposomes and nanoparticles with CPPs, thus enhancing their penetrative propensity and propounding their use as viable delivery

vehicles. Several reviews on this topic have been published, here we list only four [56–59]. Although these constructs and CPPs in general have not been accepted for clinical use, their potential clinical applications are under intensive testing and discussion. Three of the most recent reviews are listed here [60–62]. Of equal importance, is the establishment of the precise intracellular *loci* of the delivered cargo. Several methods have been developed to locate the delivered cargo within subcellular compartments and tissues. A recent review of these methods has been published by [63].

The reticuloendothelial system (RES) plays a pivotal role in host defence through clearance of foreign material in the circulation and tissues. Thus, a concern and challenge to the therapeutic utility of cationic CPP constructs is premature removal by immune cells of the RES, leading to a reduced half-life and lack of efficacy *in vivo* and this is particularly exemplified by the use of large cationic liposomal constructs used for the delivery of oligonucleotides [64]. Moreover, it has been suggested that the employment of neutral or acidic CPPs may lead to a more favourable *in vivo* pharmacokinetic outcome [65].

Conversely, the inclusion of CPP technology has been shown to improve the therapeutic potential of cationic liposomes and their use in cancer therapy. Nakamura et al. [66] demonstrated the superiority of R8-modified liposomes in vaccinology. The inclusion of octaarginine (R8) into OVA containing liposomes enhanced antigen presentation and antigen-specific cytotoxic lymphocyte responses compared to cationic liposomes and pH-sensitive liposomes, whilst significantly suppressing tumour growth *in vivo*. Clearly, premature elimination of cationic CPP constructs by the RES poses a future challenge for translation into the clinical setting, whilst further clarification of the role of RES in *in vivo* CPP metabolism is required.

Antibody delivery and immune detection in cancer, Section 4.3., provides further evidence, including *in vivo* experiments, of CPP constructs, as opposed to being destroyed by the immune system, being used to enhance tumor-specific immune responses.

4. Strategies for targeting different tumor cells

Initially it seemed that CPPs, frequently coupled to fluorescent dyes, could enter non-selectively into practically all genres of cell lines, which was regarded as a great achievement. However, this unique feature was undesirable when considering CPPs as drug delivery molecules. In fighting numerous disease states, specific delivery into cells, tissues, organs and organelles is mandatory. Therefore, much effort has focused on the improvement of CPPs to specifically target diseased tissue. The most studied disease is cancer. To ensure their survival and progression, tumor cells create a unique microenvironment within their surrounding milieu which is typified by a decreased pH, hypoxia or exclusive expression of specific proteins on the outer surface of tumor cells. These distinctive features were used to invent strategies to develop targeted CPPs that internalize into specific tumor cells whilst sparing healthy tissue. Universal systematic presentation of such strategies has not been generally accepted. An additional problem is that the targeting strategies are overlapping, see Fig. 1.

4.1. pH sensitive methods

It is known that cancer cells require a high amount of energy frequently supplied by an increased level of anaerobic glycolysis. This is accompanied by a high production of lactate and protons. Therefore, the surrounding tumor microenvironment is usually acidic, which assists in tumor growth, metastasis and drug resistance. This can be used to target tumor tissue by pH-responsive peptides which can be coupled to a CPP and an anticancer cargo or to a nanocarrier [67]. Of particular interest are so called pH low insertion peptides (pHLIPs), introduced and presented by Han and coauthors [68], see Fig. 1B. Depending on the pH of their environment they can exist in different states. At lower pH conditions, their acidic amino acid residues bind protons losing their negative charge, thereby enabling pHLIPs to penetrate deeper into the membrane, which subsequently promotes their cell internalization.

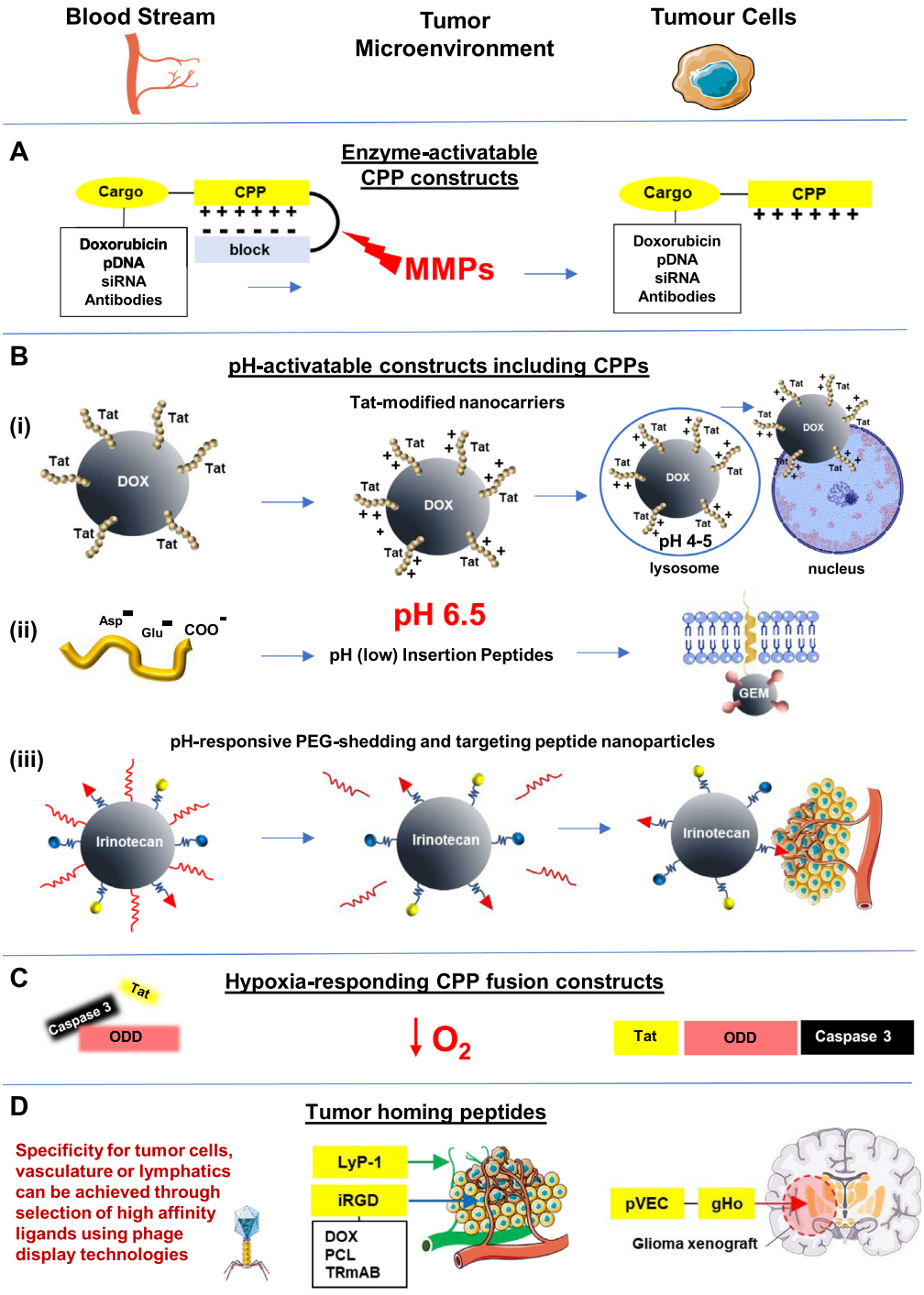
4.2. Hypoxia responding approaches

Hypoxia is another characteristic of the tumor microenvironment that has been used for targeting cancer tissue. Treating tissue with Tat fused to a fragment of the oxygen-dependent degradation domain of hypoxia-inducible factor-1α protein, showed that such fusion constructs are stable in hypoxic environments and can penetrate cancer cells, whilst degradation occurs in the local microenvironment of normal cells, thus rendering the latter unaffected [69]. This approach is frequently combined with others and can be used with a pH sensitive CPP, for instance [70], see Fig. 1C.

4.3. Enzyme-triggering activation

The development of activable CPPs (aCPPs) has been another approach used for target-specific delivery, particularly to cancer tissue, see Fig. 1A. Membranes of tumor cells are extracellularly coated with different enzymes that are not expressed by healthy

Fig. 1. Building specificity into tumor targeting CPP constructs. **(A)** Enzyme activatable CPP constructs exploit the expression of matrix metalloproteases (MMPs) within the tumor microenvironment. Penetrative ability determined by cationic charge is shielded outside of the tumor microenvironment by anionic residues. Cleavage of an MMP-specific linker liberates the CPP and cargo and penetrative propensity is restored. **(B)** pH-activatable constructs including CPPs. **(i)** Amidization of the Lys residues of Tat-functionalized PEGylated micelles carrying doxorubicin (DOX) renders the construct penetratively inert in the blood stream. On reaching the low pH of the tumor microenvironment, cationic charge is restored and Tat becomes functionalized. Functionalization is further restored in the acidic conditions of lysosomes enabling escape and nuclear delivery [233]. **(ii)** The penetrative propensity of pH (low) Insertion Peptides (pHLIPs) is lost owing to the negative charges provided by Asp, Glu and the C-terminus. Protonation of these residues within the low pH of the tumor microenvironment renders pHLIPs more hydrophobic to assist intracellular cargo delivery through formation of a transmembrane helix within the lipid bilayer of the plasma membrane [234]. pHLIP-modified gemcitabine (GEM)-coated magnetic nanoparticles have demonstrated potential for future targeting of pancreatic cancer [68]. **(iii)** pH-responsive and PEG-shedding targeting peptide nanoparticles are multivalent constructs showing potential for the specific delivery of Irinotecan to colon cancer. Demonstrating low toxicity in the blood and non-tumor cells, PEG shedding in the acidic tumor microenvironment exposes a CPP (yellow), a targeting peptide specific for angiogenic neovasculature (red) and mitochondria-targeting peptide (blue) [67]. **(C)** The Tat-ODD (oxygen-dependent degradation domain)-caspase 3 fusion protein degrades in the microenvironment of normal cells but stabilizes in the hypoxic conditions of the tumor microenvironment [69]. **(D)** Specificity for tumor cells, vasculature or lymphatics can be achieved through selection of high affinity ligands using phage display technologies. There is a wealth of studies regarding the utility of tumor homing peptides to facilitate therapeutic cargo delivery. A mere fraction is represented above. Of particular significance to this review are tumor homing peptides which demonstrate cell penetrating properties. Derived from *in vivo* phage display, LyP-1 accumulates specifically within tumor lymphatics (designated as green) whilst sparing normal lymphatic tissue [235]. Similarly, the 9 amino- acid cyclic analogue of the tumor vasculature integrin binding RGD peptide, the tumor penetrating peptide iRGD, distributes more extensively into extravascular tumor tissue and increases tumor vascular and tissue permeability to co-administered therapeutics such as doxorubicin (DOX), paclitaxel (PAC) and trastuzumab (TRmAB) [87,236]. The glioma targeting delivery vector gHoPe2, consisting of the glioma targeting peptide gHo and the CPP pVEC, crosses the BBB to selectively accumulate within xenograft tumor tissue [106]. Servier Medical Art: https://smart.servier.com was used in the construction of this figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)


Blood Stream
Tumor Microenvironment
Tumour Cells
A
Enzyme-activatable CPP constructs
Cargo
CPP
block
Doxorubicin pDNA siRNA Antibodies
MMPs
B
pH-activatable constructs including CPPs
(i)
Tat-modified nanocarriers
Tat
DOX
pH 4-5
lysosome
nucleus
(ii)
Asp Glu COO
pH 6.5
pH (low) Insertion Peptides
GEM
(iii)
pH-responsive PEG-shedding and targeting peptide nanoparticles
Irinotecan
C
Hypoxia-responding CPP fusion constructs
Caspase 3
ODD
O2
Tat
ODD
Caspase 3
D
Tumor homing peptides
Specificity for tumor cells, vasculature or lymphatics can be achieved through selection of high affinity ligands using phage display technologies
LyP-1
iRGD
DOX
PCL
TRmAB
pVEC
gHo
Glioma xenograft

tissue. One family of such enzymes are matrix metalloproteases, which are endopeptidases capable of degrading protein structures in the extracellular matrix and are important for tumor invasion and metastasis. The term aCPPs was first used by the R.Tsien's group [71]. They blocked positively charged polyarginine CPPs through intramolecular shielding with a negatively charged peptide domain. The CPP and shielding domain are connected by a suitable peptide linker that can be cleaved by metalloproteases. In this way, the cell-penetrating ability of the CPP is reactivated by protons in the tumor cell microenvironment and the aCPP can deliver the anticancer cargo exclusively into tumor cells, whilst the construct is unable to penetrate normal cells. This approach, in which the circulating CPP is not active in the bloodstream, but becomes activated at the intended site of cargo delivery, may also be classed as a prodrug mechanism. Furthermore, this technology has demonstrated potential utility for the detection of tumor cells and the delineation of the margin between tumor and normal tissue [72], besides its great potential for specific delivery of antitumor therapeutics [73]. Such tumor-oriented carriers could be further optimized, for instance with photosensitive CPPs that are activated after illumination by UV-light [74]. aCPPs have been used for the selective delivery of drugs into tumor cells and as such doxorubicin [75,76], pDNA [48,77,78], siRNA [79] and antibodies [80] have been delivered. This strategy has already proven successful for tumor imaging, more than 15 years ago [71] and more recently, for tumor gene therapy delivering plasmid DNA [81] and for the inhibition of tumor growth [82].

4.4. Tumor homing ligands

Tumor targeting ligands require a suitable targeting molecule on the surface of the cancer cell and a corresponding high affinity ligand which specifically binds to the targeted molecule. In general, affinity targeting is aimed towards recognition of cancer cell receptors by antibodies or their fragments, proteins, peptides, carbohydrates, aptamers or small receptor ligands that are attached to CPPs, see Fig. 1D. The interactions between receptors and CPP bound ligands could be applied to targeting cancer tissue since cancer cells express a different repertoire of receptors to that of normal tissue. An important step in this direction has been the synthesis of cancer cell penetrating peptides and specific tumor homing peptides [83–88]. Moreover, several classical cell surface receptors [89–93], specific localization sequences, for instance NLS [94–96], or mitochondrial SS-peptides [97,98] and mtCPP1 [99], can be used for targeting.

A number of tumor tissue homing peptides have been identified. Some examples are iRGD (CRGDKGPDC) that incorporates an integrin-binding RGD motif for recognition of the tumor endothelium [100], the CPP YTA2 which is coupled to cytostatic methotrexate (MTX) [101] and the cyclic peptide cCPGPEGAGC (PEGA) which becomes penetrant when bound to pVEC and functions as a breast tumor homing peptide in mice [102]. More complex approaches include nanoparticles and anticancer agent-loaded liposomes. Examples include silver nanoparticles (AgNPs) decorated with either the cancer cell penetrating peptide RPARPAR which is recognized by the tumor-cell receptor NRP1, or the cancer cell homing peptide GKRK, recognized by the tumor-cell receptor p32 [103]. These constructs were used to target PPC1 prostate cancer and melanoma cells *in vitro*. More complex liposomal delivery systems include, liposomes loaded with doxorubicin, dually labelled and surface-anchored with the CCK8 tumor targeting peptide and the CPP R8 [104].

Other tissue specific targeting systems can be used to target tumor cells. The blood–brain-barrier (BBB) is particularly difficult to penetrate, but several brain targeting homing peptides have been developed [105] and glioma targeting CPPs have been developed through the construction of chimeric peptides consisting of glioma targeting sequences and CPPs, for instance the homing peptide gHo and the CPP pVec, resulting in the tumor-targeted delivery vector gHoPe2 [106]. A further example includes the transferrin receptor-targeting peptide linked to myristoylated transportan (myr-TP-Tf) [107]. This construct encapsulates siRNA and carries it across the BBB into the brain to enter not only glioma cells, but also murine neurons and astrocytes, whereupon it induces gene silencing. The amphipathic helical peptide NF55 has recently been used *in vivo* [108,109] and condenses plasmid DNA (pDNA) into stable nanoparticles which enter different tumor mice models including intracranial glioblastoma; pDNA can be constructed in such a way as to reduce tumor growth. For the improved survival of mice bearing glioma, a new nanoparticle delivery system (AsTNP) was constructed by coupling the AS1411 aptamer, which targets glioma and the TGN peptide, which promotes passage through the BBB [110]. Besides brain tissue, cardiac tissue can also be targeted by coupling a 30-nucleotide long RNA fragment (RNA Apt30) to a CPP [91]. This construct, called phospholamban, can transfer RNA into rat cardiomyocytes; by choosing suitable RNA it can further improve calcium ion transients and myocyte contraction.

It is obvious that several reports on finding novel selective delivery vectors with selectivity towards cancer cells lack the appropriate controls of uptake by "normal" cells; likely such controls are not easily available. We suggest that the vector (CPP) selectivity is best described in the reports describing the *in vivo* delivery to tumor cells. In Table 1, we exemplify few available reports on *in vivo* tumor delivery by CPP assistance, specifically on nuclear and mitochondrial specificity. Additionally, a few examples are available of different CPP-assisted plasmid expressions in tumors *in vivo* and are exemplified by some of our own reports [81,111,112]. Every one of such reports is also an example of the nuclear delivery of the plasmid.

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Targeting tumor cells is one of the challenges for development of CPP-based therapeutic and diagnostic strategies, especially *in vivo*. We believe that the described approaches have successfully laid the foundations for targeted delivery systems with potential clinical utility once the final hurdles have been surmounted.

5. Modulating protein–protein interaction by CPPs that internalize proteins, mimicking peptides or oligonucleotides, or through internalizing shuttling proteins

There is no doubt that protein–protein interactions play an important role in cancer. In this review we use a broad definition of mimicry that includes promoting and preventing any protein–protein interaction in cancer cells that disturbs cancer development or contributes to tumor reduction by the action of small peptides or organic molecules that are carried into the cancer cell via CPPs or full-length shuttling proteins.

The structures of many proteins have now been resolved owing to the sequencing of the human genome. Furthermore, it is now possible to fully characterize the interactions between many proteins and their domains. Proteins bind into complexes specifically with well-defined surfaces but also non-specifically through weak

Table 1
Selected examples of nuclear and mitochondrial CPP targeting of tumor cells *in vivo*.

Peptide	Description
Nuclear targeting	
buforin IIb	buforin IIb accumulated in nuclei, induce mitochondria-dependent apoptosis with *in vivo* tumor suppression activity [237]
NLS-VP22	fusion of TmHU protein, NLS and VP22 transfected an immunogenic DNA vaccine in mice [238]
PF14	*in vivo* delivery of pDNA and tumor-specific gene induction by PF14 functionalized with PEG and a MMP substrate [112]
Tat, penetratin and R11, NLS	CPP- and NLS-fused with APIM-consensus peptide targeted proliferating cell nuclear antigen, essential for DNA replication and repair in multiple myeloma cell lines and primary cancer cells [239]
ETWW	showed cellular and nuclear localization using an endocytic pathway and binding at the major groove of nuclear DNA in a spheroid model and *in vivo* [240]
NLS NPY	$Tb1^{59}$-NPY modified with a fatty acid, a cathepsin B-cleavable linker, NLS and a DOTA chelator, in nude mice bearing MCF-7 xenograft showed tumor-specific uptake [241]
Mitochondrial targeting	
MTD-DS43	mitochondrial targeting domain of Noxa protein, MTD, fused to DS43 showed anti-tumoral activity in mice with CT26 colon carcinoma [242]
TPP	tumor penetrating peptide from oligomerization domain of Hsp70 was shown to be taken up via endosomes followed by co-localization with mitochondrial membranes in tumors expressing Hsp70 [243]
iRGD	procytotoxic peptide, m(KLA)-iRGD, internalized tumor cells, yielding mitochondrial-induced apoptosis and in tumor-bearing mice, a significant reduction in tumor volume and inhibition of metastasis [244]
chol-FRFK/D	nano-formulation of antimycin A, targeted to mitochondria and lung cancer by liposomes modified with Chol-FRFK/D [245]
Tat-PCC	Tat modified propionyl-CoA carboxylase, $\alpha_6\beta_6$ PCC, was delivered into mitochondria *in vitro*, *in situ*, and *in vivo* [246]
CAMP-hMT1A	CAMP, conjugated with human metallothionein 1A, hMT1A localized to mitochondria in a mouse model of PD, rescued movement impairment and dopaminergic neuronal degeneration [247]
Pep-1	whole mitochondria, conjugated with Pep-1 (P-Mito) showed decrease of transplant-induced growth inhibition, suggesting antitumor potential [248]
Mito-ff	Mito-ff, a mitochondria penetrating tripeptide showed mitochondrial disruption both *in vitro* and *in vivo* [249]
R8	R8-MTS ($ALD5^{MTS}$), conjugated to doxorubicin, showed mitochondrial disruption and inhibition of tumor growth in 4 T1-bearing mice *in vivo* [250]
Tat	Tat-conjugated short peptides from calpain-1 C2-like domain inhibited mitochondrial calpain-1 and prevented neurodegenerative diseases of the eye [251]
MPP (CFxrFxKFxrFxK)	concurrent impairment of nucleus and mitochondria was shown to express anti-tumor and anti-metastatic effects by using MPP-Dox-loaded HPMA copolymer, inhibiting lung tumor metastasis by damaging mitochondria/nucleus of breast cancer 4 T1 cells *in vivo* [252]

bonds [113,114]. On the basis of this knowledge, it is possible to affect protein–protein interactions by designing peptides and short proteins that mimic the interactions between both intracellular and extracellular proteins, thus modifying cellular function and dynamics [115]. These intervening structures, usually peptides, can arise from the sequences of natural proteins and their stability can be enhanced by suitable modifications. Mimetic peptides usually bind to partner proteins and in this way block protein–protein interactions; they are mostly inhibitors of interactions but in rather rare cases they can also be activators of the partner protein [116]. They are often detected by combinatorial screening methods, for instance by phage display, synthetic peptide libraries or *in silico* molecular modeling [117]. A challenging problem is replication of the correct active fold, a matter of protein dynamics and induced fit processes. The vast majority of protein mimics (small organic molecules or peptides) known today have been designed to inhibit the activity of oncogenes or activate tumor suppressors [117]. If mimicking peptides are not able to penetrate cells, their bioavailability to the cell interior is often facilitated by conjugation to CPPs.

5.1. Protein mimics with CPP properties

It is desirable for a mimetic peptide to include in its structure (sequence) the ability to bind to its protein partner, whilst possessing effective CPP translocation properties. The term "bioportide" was pioneered by the Howl group, to characterize CPPs with bioactivity and distinguish them from the more classical biologically inert vectors [118]. It was shown that positively charged protein helices can be frequently used as a starting point to obtain bioportides with mimetic properties [119]. Examples are the peptides Cyt c^{86-101} and Cyt c^{77-101}, representing helical domains of Cytochrome *c* and mimicking its function [120]. These protein mimicking CPPs could be of potential clinical utility in cancer therapy.

A number of peptides with CPP properties have recently been identified which affect the development and propagation of cancer tissue, see Table 2. Earlier examples are peptides from the tumor suppressor protein p14ARF which bind to HDM2 [121], e.g. ARF^{1-22} [48]; SERPINA5, a protein C inhibitor (PCI) [122–124]; a cyclic CPP constructed from a component of the histone H3 tail [125].

To inhibit the growth of cancer cells, inhibition of the p53/HDM2 interaction has been used [126], exemplified by a helix-loop-helix cyclic peptide that was constructed by combining a p53 epitope in one helix and the R6 peptide with CPP properties in the adjacent helix; a vascin (an amyloid peptide, based on an amyloidogenic fragment of vascular VEGFR2 [127]; p28 entering cancer cells, binding to p53 and restoring its functionality [128]; reactivation of p53 achieved by short peptide inhibitors of MDM2 and MDMX proteins with cell-penetrant properties [129]; peptide inhibitors of p53-MDM2/MDMX and beta-catenin-Bcl9 interactions *in vitro* and *in vivo* [130]; the LinkTer peptide, derived from the intrinsically disordered region (IDR) of p53 (289–322) [131].

Additional examples include the following (Table 2) : stapled peptide with high affinity for the estrogen receptor (R4K1) [132]; a CPP encompassing the PCNA (proliferating cell nuclear antigen) interacting motif APIM [133]; NEMO/IKKβ interaction inhibition by bicyclic peptides [134]; helical sulfono-γ-AApeptides that mimic the α-helical domain of BCL9 (B-cell CLL/lymphoma 9 protein) [135]; vinylphosphonites were used for the inhibition of the interaction between BCL9 and beta-catenin [136]; $E4orf4_{64-95}$, named large adenodiaphorin or $LadD_{64-95}$, inhibited the survival of human U87G glioblastoma cells [137]; cTI (tachyplesin-I), a host defense peptide from the horseshoe crab *Tachypleus tridentatus* [138]; Anionic and proteolytically stable roseltide (rT7 [139]; the Ser/Thr kinase CK2 targeting using CK2β-mimicking cyclic peptides to modify the CK2α/CK2β interaction [140]; Mut3DPT-PP2A/SET peptide blocked the interaction between the Ser/Thr phosphatase PP2A and its oncogenic inhibitory binding partner SET [141];

Table 2
Selected protein mimics with CPP properties, relevant for cancer. The presented protein fragments demonstrate CPP properties as well as mimic/inhibit the source protein.

Protein source	Description
Cyt c	Cyt c^{86-101} and Cyt c^{77-101}, helical domains of Cytochrome *c* mimicking its function [120]
p14ARF	ARF^{1-22} restricts the proliferation of different cancer cells by mimicking the p14ARF protein [48].
p53-binding protein 2	CDB3 stabilized p53 following cellular internalization *in vitro* [253], potentially useful as a cancer treatment
PCI	PCI^{1-11} and PCI^{1-18} are effective CPPs which can transport the entire protein into cells [124]
histone H3 tail	cyclic CPP containing three-times methylated Lys and Tat, internalizes into cells and binds to histone demethylase PHF8 causing its subsequent inhibition [125]
p53	inhibition of p53/HDM2 interaction with a helix-loop-helix cyclic peptide combining a p53 epitope and R6 peptide with CPP properties [126]
VEGFR2	vascin, amyloidogenic fragment of vascular VEGFR2, inhibited VEGFR2-dependent tumor growth in a tumor mouse model [127]
p53	p28 binds to p53 and restores its functionality [128]
inhibitors of MDM2 and MDMX	stapled peptides with high affinity for the estrogen receptor suppressed estrogen positive breast cancer [129]
NFκ**B**	NEMO/IKKβ interaction was inhibited by bicyclic peptides in which one ring represented a motif for binding to the NFκB modulator (NEMO) whilst the other ring displayed motives pertaining to various CPPs, inhibiting the proliferation of ovarian cancer cells [134]
peptide inhibitors of p53-MDM2/MDMX and Bcl9 interactions	blocked the interactions between p53 and MDM2/MDMX in the cytoplasm and between β-catenin and Bcl9 in the nucleus, yielding potent inhibition of tumor growth and metastasis *in vitro* and *in vivo* [130]
BCL9	the mimic the α-helical domain of BCL9 (B-cell CLL/lymphoma 9 protein) disrupted β-catenin/BCL9 interactions after entering cancer cells [135]
E4orf4	$E4orf4_{64-95}$ (large adenodiaphorin or $LadD_{64-95}$) inhibited survival of human U87G glioblastoma cells [137]
κ-casein	RL2 (121 aa) showed cellular uptake into human cancer cells and the consequential antitumor activity [254]
omomyc	omomyc (91 aa) enters cells and nuclei as omomyc/MYC and omomyc/MAX heterodimers and promotes displacement of oncogenic MYC from its genomic location, yielding the transcriptional reprogramming of cancer cells [255]
tachyplesin-I	cTI, a host defense peptide from the horseshoe crab *Tachypleus tridentatus* showed anticancer properties and cellular uptake in melanoma cells [138]
roseltide (rT7)	rT7 exhibited cellular penetration and inhibition of proteasomal activities, suggesting the potential in both anti-cancer or anti-inflammatory therapies [139]
CK2 (Ser/Thr kinase)	CK2β-mimicking cyclic peptides modify the CK2α/CK2β interaction showing cytotoxicity toward cancerous HeLa cells [140]
p53	LinkTer peptide, p53(289–322) target a partner IDR from the anti-apoptotic iASPP proteinand promoted apoptosisin p53-WT human cancer cells [131]
Mut3DPT-PP2A/SET peptide	the peptide blocked the interaction between the Ser/Thr phosphatase PP2A and its oncogenic inhibitory binding partner SET and reduced tumor size *in vivo* using breast UNLP-C3H/S tumor cells grafted into mice [141]
APIM, bacterial DNA sliding β-clamp	APIM-derived peptides, ATX-101, demonstrated both antibacterial and anti-mutagenic activities [256]
Grb7 (growth factor receptor bound protein 7)	Myristoylated and non-myristoylated peptides based on the calmodulin binding site of Grb7 inhibited proliferation, migration and invasiveness of A431 tumor cells [142]
protein kinase B (PKB)	$FCHO1^{560-571}$, from the PKB substrate motif of human FCH domain only 1, following penetration, inhibited proliferation via PKB/ERK/SMAD4 pathways in K^{RAS}-mutated A549 lung cancer cells and suppressed tumor growth and decreased the size and weight of tumors in A549-xenograft mice [143]
Ras protein	sC18 showed cellular uptake and interaction with intracellular prenyltransferases as well as modulating downstream signaling of Ras proteins, particularly K-Ras-4B, in pancreatic cancer cells [144]

peptides based on the calmodulin binding site of Grb7 (growth factor receptor bound protein 7) [142]; $FCHO1^{560-571}$ from the PKB substrate motif of human FCH domain only 1 [143]; sC18, bearing a C-terminal CaaX prenylation motif based on Ras sequences [144].

A number of proteins have the ability to "travel" along DNA [145,146], most of them by sliding on the DNA major groove, which is contacted and recognized by stable α-helical protein structures, 20–60 amino acids long. These sequences can be identified and separately synthesized, representing sliding protein mimics. Sliding proteins affect DNA metabolism and gene regulation [145,146]. Sliding molecules (sledges) [147,148] are defined as "small basic molecules such as peptides that bind and slide along DNA and can translocate cargo, for example a protein molecule, along DNA". This definition can also include cationic CPPs, such as Tat [149] which also travel along DNA. More than 10 000 sled motifs have been predicted using both human and mouse proteomes [149]. It is expected that many CPPs have DNA sliding abilities, a feature that can be used for many different applications, including cancer treatment.

The discovery that short fragments in protein sequences could be used as protein mimics provided a breakthrough in the development of novel pharmacological strategies Our understanding today is that it is easy to find CPP sequences in natural proteins, which can be facilitated using currently available prediction algorithms. However, it is more difficult to locate amino acid sequences which possess the dual/overlapping pharmacophores for both penetration and protein mimicry. such prediction algorithms were to be developed, cell penetrating protein mimics with high target specificity could soon become a new reality for pharmacology and drug development. The above examples clearly suggest that such an approach is indeed possible, particularly when targeting some of the most poignant intracellular signaling pathways integral to cancer.

5.2. Delivery of protein mimics as cargos by CPPs

As discussed in several reviews [117,118], peptides have been utilized to interfere with protein–protein interactions in cancer cells. To gain highly effective access to the interior of cancer cells, they have been fused to CPPs, most commonly to Tat, penetratin and different polyarginines such as R11, and R9 [117,118]. In most cases, mimicking peptides are the inhibitors of protein–protein interactions and they are used to decrease tumor cell progression. In the following section of this review, additional and more recent examples will be presented, see Table 3.

Multiple examples of CPP-conjugated protein mimics include many CPPs in combination with many short peptide mimics of the targeted interface between proteins integral to cancer intracellular signaling pathways, constructs that ultimately lead to cancer cell death. To name a few examples from Table 3, the following mimics can be selected : DOCK2-specific inhibitor [150]; sequence derived from the BRC2 tumor suppressor protein [151]; inhibitory

Table 3
Selected examples of delivery of protein mimics or inhibitors as cargos by CPPs.

Protein mimic or target	CPP	Description
DOCK2-specific inhibitor	CPP	CPP-DOCK2-specific inhibitor of DOCK2/Rac interaction inhibited lymphocytes in the human MINO cell line [150]
derived from BRC2 tumor suppressor protein	R9	R9-coupled BRC2 tumor suppressor protein-derived peptide (BRC4) reduced DNA damage promoted by the RAD51 protein, demonstrating potential in treatment of cancer [151]
inhibitory peptide of OGT	penetratin, Tat	CPP-inhibitory peptide of OGT (O-GlcNAc transferase) inhibited OGT resulting in a decrease in breast cancer cell proliferation [152]
CtBPs, transcriptional co-repressor, carboxy-terminal binding protein	Tat, Pep-1	inhibitor of CtBPs fused with CPP entered tumor cells, bound to CtBPs and prevented interaction with partner proteins [153]
NF-κB inhibition	CB5005	CPP-PEGylated liposomes showed NF-κB inhibition, delivered doxorubicin into intracranial glioblastoma in animal models, and synergised the killing of glioma cells compared to doxorubicin-loaded liposomes alone [257]
annexin A1	CPP	CPP-ANXA1$^{20-30}$ and -ANXA1$^{28-30}$ (annexin A1-derived) blocked binding of annexin to the receptor tyrosine kinase EphA2, targeting its degradation with a consequential suppression in the growth of gastric cancer and colon cancer cells, *in vitro* and in mice [154]
SMAC (second mitochondria-derived activator of caspases)	penetratin, Tat	CPP-SMAC protein-derived peptides [155,156] showed antitumor activity
CIP1 (a cyclin-dependent kinase inhibitor)	P21	p21WAF1/CIP1 showed antitumor activity [157]
ErbB2 targeting peptide	Tat	anErbB2 targeting peptide showed antitumor activity [158]
proapoptotic peptide	Tat	P15 (a proapoptotic peptide) showed antitumor activity [159],
p53	Tat	peptide derived from the C-terminal of p53 showed antitumor activity [160]
NKp44	R11, NLS	R11-NLS-pep8 decreased viability of different cancer cell lines and decreased tumor growth *in vivo* [161]
TRAIL, a protein related to TNF	R8	R8-TRAIL hindered tumor growth *in vitro* and *in vivo* [162]
Inhibition of phosphodiesterase 8A/C-Raf complex	CPP	PPL-008 was used to inhibited the complex in a melanoma cell line and in a melanoma xenograft mouse model, showing tumor tissue labeling [163]
transcription factors	CPP, NLS	interfering peptide blocked the interaction between two transcription factors involved in the Hippo signaling pathway TEAD and YAP, showed an anti-tumoral effect in xenograft models of breast cancer [164]
anticancer peptide domain	iRGD	iRGD-HPRP-A1 showed tumor-targeting and tumor-penetrating activities by binding to the neuropilin-1 receptor, killing cancer cells by disrupting the cell membrane and inducing apoptosis *in vitro* and *in vivo* [165]
MDM2	cHLH	cHLH-p53-R binds MDM2, showing toxicity towards cancer cells by its ability to selectively target, cross, and disrupt cancer cell membranes [166]
HKII and the mitochondrial channel, VDAC1	Pal-pHK-pKV	Pal-pHK-pKV-HKII (N-terminus of hexokinase-II) enhanced apoptosis by perturbing the interaction between HKII and VDAC1 showed mitochondrial colocalization in human non-small cell lung cancer A549 cells, accompanied by substantial cell death [258]
ASH2L	Tat	Tat- ASH2L peptides, bind and inhibit DPY30 activity, inhibiting the growth of MLL-rearranged leukemia and other MYC-dependent hematologic cancer cells [167]
ASF1 (conserved H3-H4 histone chaperone) peptide inhibitors	Tat	Tat-ip3 and -ip4 (ASF1 peptide inhibitors) showed uptake into U2OS cells, inhibition of the ASF1-histone interaction and inhibition of tumor growth in mouse allografts [168]
Grb7, adapter protein in cancers which binds HER2	penetratin-modified bicyclic peptides	peptides targeting the SH2 domain of Grb7, showed bioactivity as inhibitors of Grb7 [169]
tumor suppressor connexin43	Tat	Tat-Cx43$^{266-283}$ mimic the effect of connexin43 and inhibits the proto-oncogene c-Src, intraperitoneal delivery decreased the invasion of intracranial tumors generated by GL261 mouse glioma cells in immunocompetent mice [170]
Src-inhibitory sequence of the gap junction protein connexin43	Tat, pVEC and penetratin	Tat-Cx43$^{266-283}$ inhibited the activity of oncogenic Src and lead to disruption of EGF and FGF-2 signaling and down-regulation of β-catenin during gliomagenesis [178]
TX-101 (proliferating cell nuclear antigen PCNA motif)	APIM	APIM -TX-101 increased the anti-cancer activity of EGFR/HER2/VEGFR inhibition *in vitro* and *in vivo* [259]
PCNA$^{126-133}$, mimicking proliferating cell nuclear antigen	R9	R9-caPeptide disrupted PCNA-protein interactions in pancreatic cancer cells, causing lethal DNA damage [175]
Cdc42-binding peptide	R9	R9-Cdc42-binding peptide modulated specificity for small GTPases in Ras-driven cancer cell models [171]
derived from inner centromere protein INCENP, targeting survivin	R9	R9-INC peptides inhibited cell growth of pancreatic cancer MIA PaCa-2 and breast cancer MDA-MB-231 cells [172]
tyrosinase-related protein 2-derived peptides	Tat	Tat-Trp2$^{180-188}$ activated melanoma-specific immunity [173]
pro-apoptotic peptide sIL-24	NGR	NGR-sIL-24 showed binding and cellular uptake of sIL-24 in U937 and A549 cancer cells accompanied by inhibition of cell growth [174]
lipid-mimicking peptide Pm45	RGD	RGD-Pm45 intercalated into lipid bilayers of erythrocyte vesicles and killed integrin αvβ3-expressing MDA-MB-231 breast cancer cells *in vitro* and enhanced the therapeutic effects of encapsulated doxorubicin in an MDA-MB-231 mouse xenograft model [260]
inhibitor of the MDM2/p53 interaction	Tat	Tat-S100A1inhibited cell proliferation and induced cell cycle arrest at the G2/M phase [176]
Inhibitor of the activation of NFκB	SynB1	SynB1-ELP1-p50 (NLS-derived peptide that inhibits the activation of NFκB) showed inhibition of proliferation and induction of apoptosis in breast cancer cells [177]
c-myc inhibitor peptide H1	PNDD, short form of the Pseudomonas Exotoxin w. Tat, pVEC, penetratin	PNDD-mediated delivery of the c-myc inhibitor peptide H1, attenuaing cell proliferation and inducing cell death in various tumor cell lines [261]

peptide of OGT (TPVC(S-propyl-UDP)TA) [152]; inhibitor of CtBPs (transcriptional co-repressors, carboxy-terminal binding protein) [153]; CPP-conjugated annexin A1-derived ANXA1$^{20-30}$ and ANXA1$^{28-30}$ [154].

Most of the mimicking peptides are derived from inhibitory proteins, but some activating mimicks are also known. Therefore, activation and inhibitory effects can be combined. However, to achieve entrance into the cells mimicking peptides or proteins are combined with CPPs, most often with penetratin or Tat. Older examples are typified by the SMAC protein-derived peptides (SMAC; second mitochondria-derived activator of caspases) attached to Penetratin [155] or Tat [156], as well as p21WAF1/CIP1 (a cyclin-dependent kinase inhibitor) [157], an ErbB2 targeting peptide [158], P15 (a proapoptotic peptide) [159], and a peptide derived from the C-terminal of p53 [160], all fused to the CPP Tat, bar the latter peptide mimic which uitilized multiple CPPs. All of these constructs showed antitumor activity, for some of them this was also demonstrated *in vivo*.

More recent examples include : R11-NLS-pep8 (EASALV-CIRLVTSSKPRTVA, derived from NKp44) [161]; TRAIL, a protein related to TNF [162]; inhibitor of phosphodiesterase 8A - C-Raf complex [163]; a chimeric tri-functional peptide composed of Mut7 DPT, NLS and an interfering peptide [164]; anticancer peptide domain HPRP-A1 [165]; peptide cHLH-p53-R with high affinity for MDM2, [166]; Tat-conjugated peptides, derived from ASH2L which bind DPY30 [167]; Tat-modified ip3 and ip4 (ASF1 peptide inhibitors) [168]; penetratin-modified bicyclic peptides targeting the SH2 domain of Grb7 [169]; Tat-Cx43$^{266-283}$ mimicking the effect of the tumor suppressor connexin43 and inhibit the proto-oncogene c-Src [170]; R9-modified 16-mer cyclic Cdc42-binding peptide [171]; R9-linked INC peptides (inner centromere protein INCENP-derived) [172]; tyrosinase-related protein 2-derived Trp2$^{180-188}$ [173]; pro-apoptotic peptide sIL-24 with an NGR (Asp-Gly-Arg) motif as a CD13-targeting component [174]; R9-caPeptide (PCNA$^{126-133}$, mimicking proliferating cell nuclear antigen) [175]; Tat-S100A1, designed to inhibit the MDM2/p53 protein–protein interaction [176]; thermally responsive SynB1-ELP1 that inhibits the activation of NFκB [177]; Tat-Cx43$^{266-283}$, the Src-inhibitory sequence of the gap junction protein connexin43 [178].

The conjugation of CPPs with protein-derived mimics was a natural step in development of cell-penetrating influencers of protein–protein interactions. If the short protein-derived active mimic cannot be turned into a CPP (cf. above), it has been natural to connect it with a known CPP. Above it has been shown that this strategy has been successful *in vitro* and *in vivo*.

5.3. Antibody delivery and immune detection in cancer

Several examples are available where the immune response of tumor cells is studied after treatment of cells or animals with different CPPs or CPP/cargo conjugates. Multiple CPPs (TP10, PepFects, Tat, stearyl-$(RxR)_4$) were found to be nontoxic and nonimmunogenic *in vitro* and *in vivo* [179]. For example, the Tat-OVA protein induced antigen specific cytotoxic lymphocytes [180], penetratin-OVA induced in vivo an immune response against OVA expressing tumor cells [181,182], the Tat-Her2/neu (human epidermal growth factor receptor 2) induced tumor-specific T-cell responses in an *in vivo* murine breast tumor model [183], pirarubicin coinjected with iRGD significantly improved immune status of breast cancer bearing mice [184]. This suggests opportunities for the use of CPP systems in immune-based detection and imaging of cancer mechanisms. Additionally, CPP-antigen conjugates have been used in CPP based vaccinations in vivo as well as for cancer immunotherapy [185–193].

The CPP-protein **conjugation by fusion** seems to be a fruitful strategy for the creation of cell transducing proteins, several attempts can be found for antibody and antigen delivery [194–198]. Examples include several antibody or antibody fragment delivery systems, e.g. Tat-SpA (staphylococcal protein A) fusion protein, mixed with Fl-IgG was observed intracellularly [199,200], 9D11-Tat-antibody targeting hepatitis B virus X protein significantly suppressed viral transcription, replication, and protein production both *in vitro* and *in vivo* [201], Pep-1-, PEPth-antibody targeting cancer cells reached the cytosol of LS174T cells [202], R9-HuscFvs transbodies entered enterovirus-infected cells [203], CPP-transbodies, -HuscFvs showed anti-HIV-1 activity [204], CPP (R9, penetratin, Tat and hLF)-EGFR-binding nanobody 7D12 induced endocytosis [205]. Additionally, CPP-"flashbodies" (antibody probe connected with antigen-dependent fluorescence intensity) showed cell internalization [206].

Chemical conjugation of CPPs with Abs or their fragments, yielding cellular or tumor uptake has been reported, e.g. Tat(44–57)-Cys-scFv(L19) [207], transportan-GFP and -ABs, [208], Cys-THIOMAB antibody-drug-penetratin in mouse tumor xenografts [209], mAbs (matuzumab, trastuzumab and ADC Kadcyla®) conjugated to tetrameric CPPs showed internalization into A431 cells and in mice bearing A431 xenografts [210], cR10-TAMRA, -anti-GFP nanobody GBP1, -NLS-mCherry, functional enzymes, nanobodies and full-length immunoglobulin-G antibodies showed cytosolic/nuclear delivery in HeLa Kyoto cells via endocytosis and endosomal escape or by directly transducing the membrane [211]. AB fragment (RAD51, a protein involved in homologous recombination DNA repair), inhibited RAD51 ssDNA binding *in vitro* when fused to iPTD [212].

Biotinyl-CPP conjugates between avidin- and biotin (or avidin- and biotin-modified Abs), have been widely used in studies of CPP mechanisms and detection and a few examples are highlighted below, most carried out with different cancer cell lines. Intracellular N-ε13-biotinyl-transportan was visualized by indirect immunofluorescence after permeabilization of Bowes' cells with methanol and adding of streptavidin-FITC or streptavidin-Texas red; the role of non-endocytotic uptake was suggested [6]. Later, the biotinyl-transportan was used for visualization of cellular delivery and localization of anti-biotin monoclonal antibodies [208]. An Nα-Biotinyl-TP10/Fl-streptavidin complex was used in the report on photo-induced endosomal escape of CPP-SA [213]. Complexed with avidin biotinyl-penetratin, -Tat, transportan, and -pVEC were shown to use the endocytotic, clathrin-dependent and independent mechanism of the protein cellular transduction [214]. Deleted sentence

Complexation of CPPs/Abs has been reported in but a few cases**.** CPPs from the spider M−lycotoxin with encapsulated antibodies (IgGs) showed cellular uptake by inducing macropinocytosis [215]. A sorbitol-based molecular carrier with 8 guanidine units (Sor-G8) was used for intracellular and transdermal delivery of proteins (GFP, albumin, concanavalin A, and IgG) though after complexing, was more efficient than R8 complexes [216]. [216]. CPP mimic-Abs for protein kinase C theta showed delivery into primary human CD4 T cells in a humanized mouse model [217]. However, R6/bevacizumab, administered using eye drops had no effect on increasing desired biological stability and increased aggregation [218].

Targeting proteins with CPPs been achieved by conjugated Abs. Antibody targeted triggered electrically modified prodrug type strategy, ATTEMPTS [219] uses "non-covalent conjugation of targeting antibodies linked to heparin and CPP linked to the protein drug", which releases the CPP-drug complex for specific internalization into specific antibody-targeted tumor cells [219], This stategy has demonstrated potential for colorectal cancer therapy [220], and for the treatment of acute lymphoblastic leukemia.

6. Improving chemotherapy by coupling chemotherapeutics to CPPs

Chemotherapy is used to suppress tumor growth and the metastatic proliferation of cancer. The cytostatic action of different agents has greatly improved survival prognosis. However, this is marred by many undesirable side effects owing to the inherent lack of tumor cell specificity of chemotherapeutics and their subsequent deleterious effects on healthy tissue. Selective delivery of chemotherapeutics into cancer cells is a long-term goal for which delivery by tumor targeting CPPs could be of great benefit. Since no CPP has yet to be approved for clinical use, we shall only present experimental results with the most frequently used cytostatic agents, doxorubicin, methotrexate, and paclitaxel.

6.1. Doxorubicin (DOX)

Doxorubicin is commonly used as a cytostatic but it generally induces tumor cell resistance. To avoid this, DOX was simultaneously coupled to penetratin and Tat [221]. The constructs were shown to be internalized by several drug sensitive and drug resistant cancer cells with increased cytotoxicity. The same group has further observed that mitochondrial disruption is the main mechanism in DOX-induced apoptotic signaling, however DOX–CPPs are probably able to trigger additional apoptotic pathways independent of mitochondrial events [222]. Another group investigated the influence of different CPPs (Tat, penetratin and mastoparan), on the transport of DOX encapsulating transferrin (Tf) liposomes across the brain endothelial barrier, *in vitro* and *in vivo* [223]. They observed efficient translocation of anticancer drug-loaded Tf-CPP liposomes across the brain endothelial barrier, demonstrating that CPP-mediated transport across the restrictive blood–brain barrier is indeed possible. Recently, such combined delivery of DOX with different nanocarriers has undergone extensive study. In one study, reversibly crosslinked hybrid micelles (RCMs) were engineered based on the CPP Tat, tocopherol PEG -succinate and PEG-*b*-poly (aspartic-lipoic acid). RCMs loaded with DOX behaved as pH sensitive drug delivery vectors and DOX successfully accumulated in cancer cells [224]. Another recent example is the construction of Asp-Gly-Arg peptide (NGR)-modified nanobubbles (NBs) containing CPP-decorated DOX and CPP-decorated c-myc-siRNA [225]. By this procedure encapsulation efficiencies of DOX exceeded 80%. Release of DOX could be triggered by ultrasound since above 80% DOX was released from NBs after sonication while less than 5% DOX was discharged in the absence of ultrasound.

6.2. Methotrexate (MTX)

One of the first demonstrations that CPPs can successfully be used to deliver cytostatics into a drug resistant cell model was by Lindgren and co-workers[101]. They constructed two different CPPs, YTA2 and YTA4, and monitored the intracellular delivery of MTX to MTX-resistant breast cancer cells MDA-MB-231 by fluorescence microscopy and quantitative fluorometry. It was shown that MTX-CPP constructs entered and destroyed cells more efficiently than MTX alone, despite MTX resistance. The same group later coupled MTX to a matrix metalloproteinase-activatable tumor targeting CPP, named NoPe, a modification of YTA4. Following intravenous injection, they showed selective accumulation of the cargo in tumor tissue of MDA-MB-231 tumor bearing mice [226]. Other groups have selectively delivered MTX or its modified structure to cancer tissue and have used CPPs such as polyarginine or penetratin [227] or the selective cell penetrating peptide (RLWMRWYSPRTRAYGC) coupled to functionalized polymersomes (construct abbreviated SCPP-PS) for the targeting of human lung cancer *in vivo* [228], in addition to utilizing numerous other CPPs [229].

6.3. Paclitaxel (PTX)

PTX is a first-line chemotherapeutic that has proven efficacy against many solid tumors. However, it exhibits several application problems due to its poor solubility, low membrane permeability and lack of cancer cell selectivity. To minimize these problems, a "smart" peptide-drug conjugate was constructed, by linking PTX with a multifunctional peptide consisting of a tumor targeting peptide (TTP) and a CPP [230]. This novel construct, TTP-CPP-PTX, was called LTP-1. It could deliver PTX into LHRH receptor-overexpressing MCF-7 cancer cells, demonstrating an approximately two-fold higher degree of cellular uptake compared to PTX alone. LTP-1-induced cytotoxicity was also enhanced, giving an IC_{50} of 3.8 nM compared to 6.6 nM for PTX alone. Encouragingly, LTP-1 exhibited less cytotoxicity towards normal cells and an ability to overcome PTX-resistance. In another study, the tumor homing CPP tLyP-1 was fused to the N-terminal of human H chain ferritin (HFtn) and PTX molecules were encapsulated into an HFtn nanocage [231]. These nanoparticles were tested for cytotoxicity against MDA-MB-231 and SMMC-7721 tumor cells *in vitro* and *in vivo* (in mice). It was demonstrated that the tLyP-1-HFtn-PTX nanoparticles selectively accumulated within and penetrated tumor regions. Verified by the breast cancer cell model in BABL/c nude mice, tLyP-1-HFtn-PTX displayed higher *in vivo* therapeutic efficacy with lower systemic toxicity. Another group [232] first used a dual-functional polyarginine CPP R7 for delivery of PTX but because of the unstable noncovalent bond between R7 and PTX, a novel positive CPP carrier of P9 was developed to improve CPP-PTX affinity via a double-proline hairpin tail. This new construct, as it was shown, can quickly translocate into the HeLa cells within 1 min and exhibits no noticeable cytotoxicity, eventually resulting in a significant reduction in tumor cell viability. The construct was also tested *in vivo*, demonstrating that the P9-PTX complex dramatically inhibits tumor growth.

Collectively, these experiments endorse coupling of chemotherapeutic agents to CPPs in order to achieve a more effective delivery of cytostatic drugs to tumor cells, with fewer side effects and reduced damage to normal healthy tissue.

7. Conclusions

Peptides hold great potential for cancer therapy and diagnostics. CPPs have been used extensively as delivery vectors for intracellular and *trans*-barrier delivery of multiple bioactive cargos, including protein mimics (short, natural protein-derived peptides) which can either be conjugated to CPPs or used independently owing to intrinsic cellular translocation properties. Such "permeable" protein mimics are usually designed to either enhance or inhibit certain defined routes of intracellular signaling. Specific targeting of different cancer types has been achieved with a remarkably high number of examples, such as using tumor-homing sequences, or triggering pro-drug activation by pH, hypoxia or different enzymes. This review clearly summarizes suggestions for the future applications of biobarrier-passing and peptide-based strategies in cancer therapy and diagnostics.

Funding

This research was funded by the Swedish Research Council, Estonian Ministry of Education and Research (IUT20-26) and by the EU (2014–2020.4.01.15–0013).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

None.

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