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Therapeutic peptides for chemotherapy: trends and challenges for advanced delivery systems

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Abstract:

The past decades witnessed an increasing interest in peptides as clinical therapeutics. Rightfully considered as a potential alternative for small molecule therapy, these remarkable pharmaceuticals can be structurally fine-tuned to impact properties such as high target affinity, selectivity, low immunogenicity along with satisfactory tissue penetration. Although physicochemical and pharmacokinetic challenges have mitigated, to some extent, the clinical applications of therapeutic peptides, their potential impact on modern healthcare remains encouraging. According to recent reports, there are more than 400 peptides under clinical trials and 60 were already approved for clinical use. As the demand for efficient and safer therapy became high, especially for cancers, peptides have shown some exciting developments not only due to their potent antiproliferative action but also when used as adjuvant therapies, either to decrease side effects with tumor-targeted therapy or to enhance the activity of anticancer drugs via transbarrier delivery. The first part of the present review gives an insight into challenges related to peptide product development. Both molecular and formulation approaches intended to optimize peptide's pharmaceutical properties are covered, and some of their current issues are highlighted. The second part offers a comprehensive overview of the emerging applications of therapeutic peptides in chemotherapy from bioconjugates to nanovectorized therapeutics.

Key words: therapeutic peptides, stability, cancer therapy, drug delivery, active targeting, nanomedicines.

Table of contents

Abbreviations

Aa = Aminoacid

SPPS: solid phase synthesis

PK: Pharmacokinetic

ADME: adsorption, distribution, metabolism, and excretion.

GIT: gastrointestinal tract

PEG: Polyethylene glycol

RES: reticuloendothelial system

GnRH: Gonadotropin-releasing hormone.

LC-MS: Liquid Chromatography-Mass spectrometry

NMR: nuclear magnetic resonance

ABC: accelerated blood clearance

IA-LC-MS/MS: immunoaffinity liquid chromatography tandem mass spectroscopy

MAPKs: mitogen-activated protein kinases family

CPNPs: cyclic peptide nanoparticles

PA: amphiphilic peptide

ACP: anticancer peptide

CPP: Cell penetrating peptide

EPR: enhanced permeation and retention effect

VEGF: Vascular endothelial growth factor.

CSC: cancer stem cells

McAbs: monoclonal antibodies

LP: Liposomes

DTX: docetaxel

DOX: doxorubicin

NPs: nanoparticles

ELISA : Enzyme-linked immunosorbent assay

1. Introduction

Therapeutic peptides represent an attractive class of pharmaceutical compounds, falling structurally between small molecules and proteins, yet biochemically and therapeutically distinct from them.[1,2] They can be differentiated from proteins based on the size and structure. Peptides are conventionally defined as molecules made up of 2 to 50 amino acids, whereas proteins are composed of 50 or more amino acids.[3,4]
As intrinsic signaling molecules for many physiological functions (hormones, neurotransmitters, growth factors, and ion channel ligands), peptides represent an excellent opportunity for drug discovery and development. Indeed, owing to their remarkable biological activity and properties, peptides stand as a good starting point for drug discovery campaign to address novel biological targets that sustain the progression of various diseases.[2] In general, peptides are efficacious ligands that bind to specific cell receptors with high selectivity leading to satisfactory safety, tolerability, and potency profiles for clinical translation.[5,6]

The era of peptides as clinically viable therapeutics began with the discovery and isolation of the 51 amino acid (aa) hormone insulin in 1921, which then became two years later the first commercially available peptide drug that has allowed diabetic patients to attain tight glycemic control.[7] Oxytocin, the first peptide hormone to be made synthetically available in 1954, has also been long exemplified for its clinical efficacy in inducing labor in case of non-progression of parturition.[8] After this breakthrough, progress was slow until 1963, when Bruce Merrifield developed a rapid and easier way to produce peptides by solid phase synthesis (SPPS).[9] Since then, chemical synthesis of peptides has been revolutionized and lots of peptides have been prepared using this approach.[10]

Biopharmaceutical companies have made tremendous progress over time. The global peptide therapeutics market was valued at 21.5 billion in 2016 and is expected to keep growing over the next 10 years.[9,11,12] This steady growth would be sustained in part by the increasing prevalence of cancer and metabolic disorders over the forecast period. Indeed, the demand for efficient and safer drug therapy is very high nowadays, especially due to the growing prevalence of cancers among the population from children to elderly.[13–18] Recent data report that approximately 400 peptide drugs are currently

being evaluated in clinical trials with over 60 already approved worldwide.[12,19]

There are many key factors that contribute to this significant expansion of therapeutic peptides. Indeed, recent advances recorded in fields such as medicinal chemistry, biotechnology and nanotechnology have encouraged pharmaceutical companies to increase their investments in peptide drug discovery.[4,20] First, novel approaches based on rational computational design are now playing a crucial role in the development of peptide drugs that can target almost any protein of interest, including oncogenic proteins.[21–23] Secondly, controlled chemical synthesis tools enabling to play around their shape and structure in order to impact properties such as high target affinity, selectivity, low immunogenicity along with a relatively higher tissue penetration, are now available.[22,24] Lastly, advances in peptides purification processes and analytical characterization have further enlarged the benefits for peptides drug development.[25–28]

Despite all the above assets, peptide drugs still encounter a range of physicochemical and biological barriers that withhold them from unlocking their full therapeutic benefits. In fact, many of them often tend to aggregate and are sometimes poorly water soluble.[29] Pharmacokinetic (PK) issues such as low permeability to cell membrane, metabolic instability, short half-life due to rapid renal clearance are not rare and contribute to the challenge.[30] Some of the potential advantages and disadvantages of peptides as drugs, compared to small molecule drugs, are summarized in Table 1.

Various strategies have been successfully developed to tackle some of these weaknesses and improve therefore peptide drugability. Among them are more traditional peptide design approaches that focus on structural modifications to enhance the overall ADME properties of peptides.[31–34] Elsewhere, peptides have been extensively studied as an important class of components in chemotherapy. The increasing availability of useful information about sequences, structures, and pattern interactions of oncogenic proteins has stimulated a growing interest in the design of peptides that could specifically bind to these untapped targets. Hence, over the last years, researchers have been very much successful in designing anticancer peptide drugs to inhibit various mechanisms which give tumor cells proliferative advantages over healthy cells, including apoptosis, cell cycle, angiogenesis and autophagy.[35–37]

For cancer therapy, however, reaching effective accumulation of anticancer drug doses at the tumor sites requires a range of advanced delivery technologies capable to overcome several tumor microenvironment barriers. These include tumor-targeting peptides and cell-penetrating peptides, as well as peptide drug conjugates and strategies focusing on nanotechnology-based drug delivery systems. Herein, we provide insight into the latest advancements that have been made throughout the development process of therapeutic peptides by highlighting new trends and challenges towards their improved delivery for better cancer treatments.

Table 1. Comparison between peptides and small molecules. Adapted from ref 29

Small molecules	Peptides
● ~80% drug market	● ~ 2% drug market
● Low cost*	● High cost
● Permeable*	● Low permeability
● Stable*	● Limited plasma stability (degradation by proteases)
● Relatively good oral bioavailability	● Short half-life (rapid renal clearance)
● Easy synthesis	● Challenging synthesis
	● Poor oral bioavailability
	● Limited to extracellular targets
	● High binding affinity
	● Excellent target specificity
	● Broad disease targets
	● Low toxicity and immunogenicity
	● Low risk of drug-drug interaction

* Most often

2. Challenges for peptide-based drug

2.1. Barriers to efficient delivery

Although the clinical potential of peptide drugs is immense, scientists involved in the development of these pharmaceuticals are compelled to cope with complex issues in order to promote their translation from bench to bedside in a very effective manner. As stated earlier, most of physicochemical and biological barriers for peptide drugs development are related to their propensity to aggregate**,** susceptibility to acid/basic hydrolysis, oxidation, and other endogenous factors, such as limited membrane permeability, metabolic degradation, uptake by the reticuloendothelial system and fast kidney filtration.

Such poor characteristics of peptides hamper seriously their efficient delivery and impact in health care. i) Oral delivery of peptides is challenging due to substantial biological obstacles such as variable pH across the gastrointestinal tract (GIT), proteolytic enzymes that are highly expressed in GIT and the presence of intestinal epithelial barriers.[38,39] Moreover, first pass metabolism by the liver following oral administration eliminates significant amounts of absorbed peptides[39] ii) The extremely short half-life precludes comfortable parenteral delivery, as daily multiple injections would be required to maintain the therapeutic levels. This can be a deterrent to medication adherence and increases the cost of treatment.[40,41] iii) The aggregation of peptides (amphiphilic peptides tend to associate through hydrophobic interactions) must also be avoided as they can produce unwanted immunogenicity and sometimes lead to lack of selectivity involving interactions with different receptors or targets (poor specific biodistribution). [42,43]

Alternatives non-parenteral routes such as pulmonary, vaginal and nasal routes may impose additional biological barriers to these hard-to-handle drugs. Indeed, despite large surface area and highly vascularized tissue, transmucosal routes have showed limited bioavailability of peptides driven by bad tissue permeability (hydrophilicity), protease activity (macrophages enzymes), and rapid clearance by nasal and respiratory tracts.[44]

From a processing point of view, physicochemical properties such as conformational stability, sensitivity to light, moisture and heat, susceptibility to break down in physical environment should also be carefully considered.[45,46]

2.2. Strategies to improve properties of peptide drugs

Several approaches intended to overcome the shortcomings of peptides have been proposed in the literature. It is then expected that, with the right approach at the development stage, one might find the possibility to improve properties of peptides and achieve thereby suitable delivery.

Those approaches are based on medicinal chemistry as well as formulation strategies. Medicinal chemistry efforts center around changes in the therapeutic agent itself (modification of the chemical structure) to boost its pharmaceutical properties, whereas formulation strategies rely on the use of excipients to enhance physicochemical and some biological properties of therapeutic peptides.

Among chemical strategies, conjugation with specific moieties (Polyethylene glycol (PEG), carbohydrates, fatty acid, …), replacement of naturally occurring L-amino enantiomers by D-amino acid forms, and cyclization have been widely promoted.[47–50] In a very general view, these chemical modification approaches are proved means to achieve (but not limited to) biological half-life extension as well as controlled *in-vivo* metabolism of peptide.[51–53] Figure 1 gives an overview of some of the most used structure modifications to optimize peptides properties.[52]

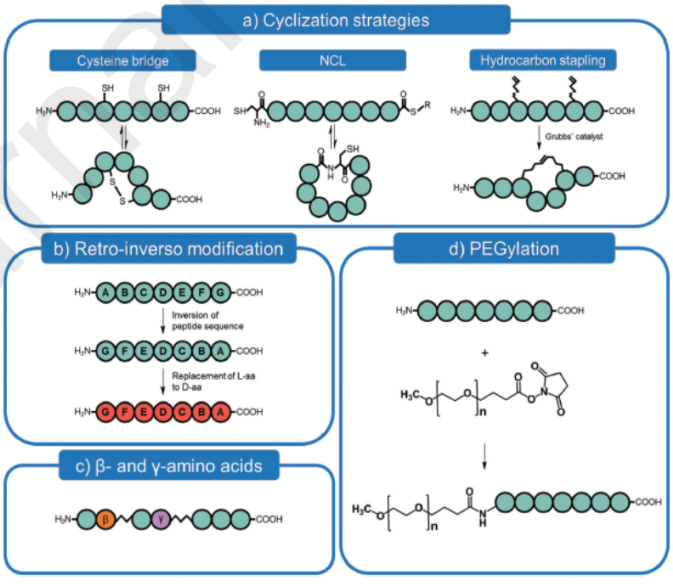

Figure 1. Strategies for optimizing peptides properties. (a) Cystein bridges, native chemical ligation (NCL) and hydrocarbon stapling are approaches to generate cyclic peptides. (b) retro-inversion modification consists of the inversion of the amino acid residues in the original peptide sequence and the subsequent inversion of the chirality of

each individual residue. (c) Incorporation of β-amino acids (2 additional carbons) and ɣ-amino acids (3 additional carbons) in the peptide sequence. (d) PEGylation of peptides at the N-terminus can be achieved using chemical approaches, such as *N*- hydroxysuccinimide (NHS)-based chemistry. aa: amino acid, R: rest group. Figure reproduced with permission from ref 60

Table 2. Examples of chemically modified peptide drugs

Peptide name	Pharmacological class	Modification strategies	Outcomes	Ref.
Oxyntomodulin	Peptide agonists of the glucagon-like peptide 1 (GLP-1) receptor (GLP1R)	Pegylation with different high molecular weight polyethylene glycol (PEG)	Yield resistant analogues against dipeptidyl peptidase IV (DPP-IV) degradation with no significant loss of GLP1R agonist activity.	[54]
SAMP-A4	Antimicrobial peptide	Fatty acid conjugation (hexanoic acid), glycosylation and PEGylation.	Introduction of hydrophobic fatty acids at the N-terminus of SAMP-A4 showed better biostability than hydrophilic glycosylation and PEGylation.	[55]
C34	Inhibitor of HIV-1 fusion	Pegylation with PEG 40kDa	Significant extension of half-life by preventing kidney filtration and proteolysis.	[56]
Peptide YY3−36	Endogenous ligand of the neuropeptide Y2 receptor (Y2R)	PEGylation and lipidation	Half-life extension with different effects on in vitro actions of PYY3−36.	[57]
Insulin	pancreatic hormone	Conjugation with sialic Acid	Improvement of physical stability against biophysical aggregation and fibril formation.	[58]
Linaclotide	Agonist of guanylate cyclase-C (GC-C)	Backbone cyclization	Improvements in gastrointestinal half- life (>8 h vs linaclotide 48 min)	[59]
Glucagon	pancreatic hormone	Site-specific stereo-chemical inversion	Increased aqueous solubility, and resistance to fibrillation	[60]

2.2.1. Chemical strategies (PEGylated Peptides)

PEGylation is now considered to be one of the most advanced and cost effective approaches in modern pharmaceutical industry that furnishes therapeutics with improved pharmacological and pharmaceutical properties, including a couple of successful drug products that have entered the market as reported by Turecek, P. L *et al.*[53] and Mora, J. R *et al*.[61] The effect of PEGylation on peptide PKs includes avoiding recognition by the reticuloendothelial system (RES), reduction of immunogenicity, and control over enzymatic proteolysis and renal clearance, with potentially beneficial changes in

biodistribution.[51,62] Its ultimate benefit is obtained through optimization of several parameters such as polymer size, shape and degree of conjugation without adversely affecting binding and activity of peptides.[53,63]

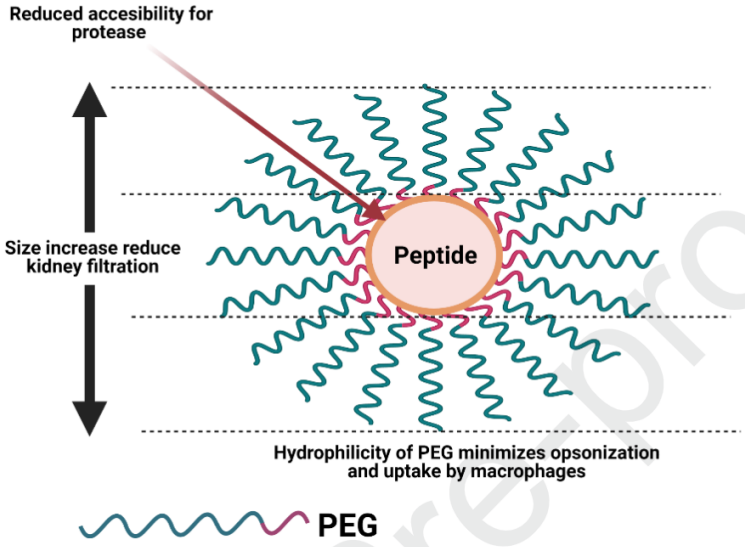

Figure 2. Advantages of peptides PEGylation

However, numerous studies have now shown that PEG polymers are not without shortcomings. More than a decade ago, Webster R *et al* already pointed out some concerns about the disposition of PEG moiety itself, which appears to accumulate in the cytoplasm (cytoplasmic vacuolation), making thereby its complete removal or clearance from the body highly questionable.[64] Years later, Rudmann, D.G *et al*, made similar observations by using immunohistochemical procedure in animal study. In fact, this study revealed that the tissue distribution profile of PEGylated therapeutics is guided largely by the absolute PEG load and PEG molecular weight, as high molecular weight PEGs show slow renal clearance, therefore have a greater potential to accumulate within cells.[65] There are very few published data available addressing the *in vivo* fate and PK profile of PEGylated therapeutics as sensitive and accurate bioanalytical tools may be required for direct quantification of PEG moieties in biological samples.[66] Such studies have proved difficult to implement for the following reasons: i) high MW PEGs (PEG > 10 kDa, the most

used for proteins and peptides PEGylation) are usually mixtures of PEG with randomly varying chain lengths. ii) PEGs polymers display a recalcitrant nature towards ionization, which makes quantitative investigations more challenging even with powerful analytical tools like LC-MS.[66,67]

Khandelwal, P *et al.* developed an alternative nuclear magnetic resonance (NMR) method, enabling the determination of PK parameters of PEGylated pharmaceuticals in preclinical studies. By reason of a single sharp peak obtained for all equivalent methylene protons of PEG polymers, there is an amplification of the signal which makes the method insensitive to polymer heterogeneity.[68] Similarly, Elliott, V.L. *et al.* also suggested interesting analytical methodologies combining gel electrophoresis and NMR spectroscopy to understand the biological fate of a model PEGylated peptide, ^{40K} PEG-insulin, within a rat model. This study showed that PEG moiety remained detectable for several weeks in both serum and urine following intravenous administration of ^{40K} PEG-insulin (4mg/kg), thanks to immunoblotting with an antibody to PEG and NMR analysis. Even more interestingly, the authors provided *in vivo* evidence of conjugate cleavage using western blotting with anti-insulin IgG which indicated that the terminal half-life of the insulin moiety was far shorter than that of the PEG moiety.[69]

Another emerging area of concern with PEGylated pharmaceuticals is the potential induction of anti-PEG antibodies towards PEGylated drugs in some patients leading to an accelerated blood clearance (ABC) upon repeated exposure. Although PEGylation indeed reduces the immunogenicity of the modified molecules, the growing clinical evidence of anti-PEG antibodies has gained much attention lately.[70,71] There are even research works reporting a complete inhibition of therapeutic action of PEGylated drug as a result of PEG-antibodies raised by animals. For instance, Moreno, A. *et al.* investigated how anti-PEG antibodies effect the therapeutic activities of PEGylated modified aptamer.[72] They could demonstrate that anti-PEG antibodies can directly bind to and inhibit anticoagulant aptamer function both *in-vitro* and *in-vivo*.[72] It is noteworthy that considerable effort is being made to bring up analytical methods that would promote proper investigations in this area. Although there is no golden standard method, immunoaffinity liquid chromatography tandem mass spectroscopy (IA-LC-MS/MS) could be more suitable than ELISA for accurate quantification of anti-PEG antibodies.[73–76]

Readers can refer to the review published recently by Hong, L. et al, for extensive details on detection methods that have been developed for pre-screening and quantitative detection of anti-PEG antibodies, including techniques such as western blot, acoustic membrane microparticle technology, enzyme linked immunosorbent assay.[77] Altogether, those shortcomings around PEG polymers are sustaining the current development of new materials that offer an enzymatically and hydrolytically degradable alternatives of PEG polymers in drug delivery and bioconjugation.[78,79]

2.2.2. Formulation strategies

Formulation scientists, on their side, also suggested several effective delivery approaches that help tackle issues such as aggregation and degradations of peptides. Additives like sugars, non-ionic surfactants, cyclodextrins have shown ability to improve both the physical and chemical stability of peptides formulations.[80] W.J. Fang et al, studied the effect of carbohydrates as well as addition of surfactant on the chemical and physical stability of glucagon during freeze-drying and storage in dried formulations. The result showed that trehalose provided superior protection of glucagon secondary structure during freeze-drying than did those with hydroxylethyl starch alone or β-cyclodextrin. Moreover, 0.01% polysorbate 20 or carbohydrate excipients reduced aggregation occurring during freeze-drying and incubation. [81] R. Oliva et al, also presented the encapsulation in sulfobutylether-b-cyclodextrin (SBE-b-CD) has an effective way to improve stability of antimicrobial peptides for their pharmacological applications. [82]

The use of protease inhibitors to reduce degradation of peptides drug has been also examined not only for oral administration, but also for other transmucosal routes.[83]
K.P. Amanchet al, evaluated the effect of protease inhibitors on pulmonary bioavailability of therapeutic peptides with varying molecular weights in the rat. Dry powder formulations of leuprolide (1.2 kD), salmon calcitonin (3.4 kD), human insulin (5.8 kD), human leptin (16.0 kD) were prepared with or without protease inhibitors and then administered intrapulmonary to rats. Protease inhibitors (1 mg/kg) increased the bioavailability of calcitonin by more than 50%. Similarly, the bioavailability of leptin was increased 2.1-fold in the presence of bestatin (an aminopeptidase inhibitor). [84] Microspheres (1-1000 μm)

and complexation within hydrogels have been employed as another strategy for enhancing peptide delivery.[85,86] The major advantages of these systems are their ability to enhance stability of fragile compounds like peptides. It is also possible to achieve controlled release and prolonged residence time at the site of absorption and/or action with those systems, improving therefore the efficacy.[44,87] A 6-month extended-release formulation of leuprolide acetate was achieved by encapsulating the drug in polylactic-co-glycolic acid microspheres. Polylactic-co-glycolic acid is a bulk-eroding polymer, which is characterized by allowing water to permeate throughout the polymer matrix, degrading it over time, and allowing, therefore, the drug to be released in a controlled rate. [88]

Recent advancements in experimental technology have made possible the identification and validation of encouraging intracellular targets that could provide better options for the management of important diseases such as cancer.[37,89,90] With the unveiling of these untapped therapeutic targets structures, researchers are extensively working on the development of suitable peptides-based drug candidates that can serve as novel therapeutic approach for cancer.[91,92] Given that peptides usually cannot cross cell membrane in their native form, the scale of challenge is higher. On top of that, several other limitations are also emerging, especially those related to physical barriers posed by tumor microenvironment that preclude efficient delivery.[93,94] Therefore, for therapeutic peptides, the clinical success is undoubtedly dependent upon the development of advanced delivery platforms enable to overcome those barriers and deliver their payload subsequently at the intracellular compartment of tumor cells. Numerous advanced delivery strategies will be discussed in greater details in the following sections of this review.

3. Peptides in chemotherapy

Traditional chemotherapy has side effects and depress immune responses.[95,96] These drawbacks are often linked to the inability to deliver the correct amount of drug directly to cancer cells without causing undue toxicity. Thus, research and development of novel chemotherapeutic agents with low toxicity to normal human cells is currently one of the

most typical directions in anticancer drug development. Among them, anticancer peptide-based drugs remain a promising group of therapeutics that shows a huge potential for antitumor therapy.[13,17,97–99]

There are hundreds of experimentally verified anticancer peptides in the literature and more comprehensive information about those bioactive peptides can be easily access through search in dedicated databases like cancerPPD [100], omicX [101], to name only a few. Such a prolific research activity results from the fact that many sequences, structures and pattern interactions of oncogenic proteins have become available.[102] Interestingly, studies have also shown that the activation or oncogenic mutation of these proteins often results in deregulated signaling, increased cell proliferation, decreased apoptosis and stimulation of tumor angiogenesis.[103–105] The relevance of this knowledge is that, peptides could now be specifically designed as ligands and bind to those proteins identified as molecular targets, located either on the cell surface or in the intracellular compartment, to eliminate cancer cells.[20,21,96,104,105] Since their emergence as biotherapeutics, peptides have got many interesting pharmaceutical applications for cancer therapy due not only to direct antiproliferative action, but also when used as adjuvant therapies, to control certain side effects or to enhance the activity of anticancer drugs via transbarrier delivery.[16,20,21,106] Therefore, for further discussion in this section, therapeutic peptides will be divided into three distinct categories, namely peptides targeting cellular signaling pathways, tumor homing peptides and cell penetrating peptides (figure 3).[96]

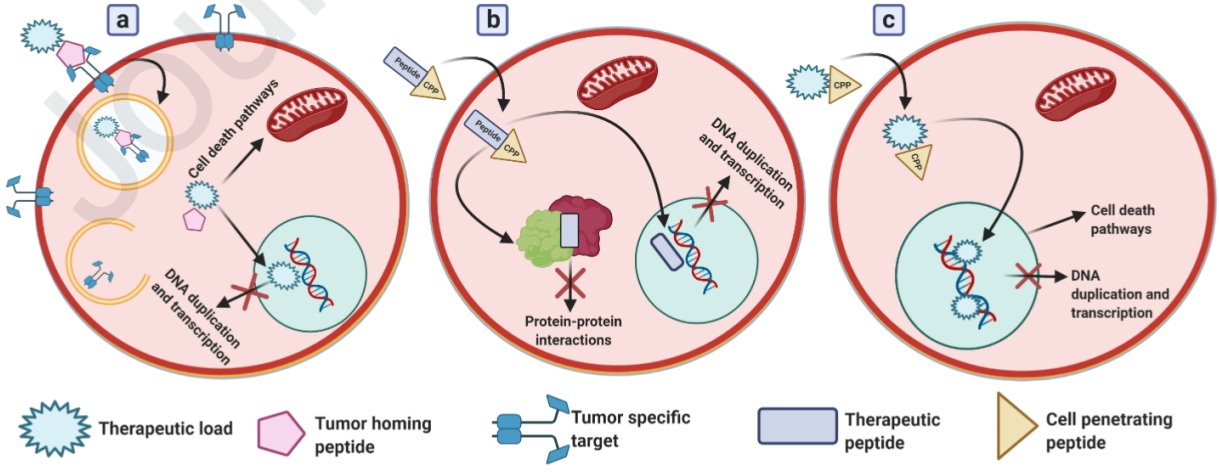

Figure 3. Peptides used for the targeted delivery of therapeutic agents, (a) Tumor homing peptides carrying a therapeutic load bind to the specific receptors on the cell surface and are endocytosed; drugs are released from endosomes inducing cell death or inhibiting DNA duplication; (b) Peptides targeting aberrant cellular signaling pathways are often conjugated with CPP and, after internalization, inhibit protein-protein interaction and DNA transcription, or cause cell death with some other mechanism; (c) Cell penetrating peptides are covalently coupled to various drug carriers and used for the targeted delivery of drugs. Figure adapted from ref 85.

3.1. Peptides targeting cellular signaling pathways

Herein are included peptides that have direct cytotoxic or antiproliferative action. They represent, next to proteins and others macromolecular therapeutics, a second generation of anticancer compounds holding enormous promise and making use of smart approaches to tackle cancer.[13,17,107] It is quite challenging to suggest a suitable classification of anticancer peptides as basically any mechanism that can give tumor cells proliferative advantages over healthy cells seems to be explored in the search for new anticancer peptide drugs. Nevertheless, Wu, D. *et al.* have suggested three groups based on their mechanism of action, namely pro-apoptotic, necrosis-inducing and inhibitory peptides.[99]

Pro-apoptotic and necrosis-inducing peptides are well described in the literature.[104,108,109] Many of them are pore-forming agents that occur naturally in living organisms and perform specific biological activities.[110] They are for instance involved in immune system defense mechanisms as host defense peptides, particularly in the killing of pathogens (e.g. defensins and cathelicidins). From a structural point of view, the majority of them are short, positively charged peptides, and able to form amphipathic structures in non-polar solvents.[111] The presence of the positive charge gives them the possibility to bind to the negatively charged cell or organelle membranes via electrostatic interactions, disrupting their function, and inducing thereby cell death.[111] The distinction between pro-apoptotic and necrosis-inducing peptides resides in the fact that the former causes cell death by disruption of the mitochondrial membrane, which then triggers the release of intrinsic apoptotic-inducing factors, such as cytochrome C, whereas the latter target negatively charged molecules on the cell membrane.[112] Moreover, necrosis induced cell death occurs rapidly and prematurely, in contrast to apoptosis which is a highly controlled and regulated (involves both intrinsic and extrinsic factors) process of programmed cell death.[113]

Many cancer cells have developed mechanisms to protect themselves from apoptosis. They are able to do so notably via overexpression of anti-apoptotic proteins that disrupt the ratio of anti-and-pro-apoptotic proteins, which is known to play an important role in the regulation of cell death.[113] Under-expression of pro-apoptotic proteins may also result into decreased apoptosis of tumor cells.[35] A large diversity of human cancer cells such as prostate, neuroblastoma, kidney, breast cancer[114], acute lymphoblastic leukaemia[115] have been noticed to frequently inactivate apoptosis signalling cascades. Therefore, pharmacological scientists are currently taking the advantages of the increased understanding of proteins (BCL-2 family, p53, inhibitor of apoptosis proteins) and signalling pathways (caspase, death receptors…) that regulate apoptosis to open up novel avenue for the development of several peptides-based cancer therapies targeting apoptosis.[116]

Inhibition of key tumour cell signalling pathways also offer a wide range of possibilities for cancer treatment. So far, several biological targets have been investigated using inhibitory peptides to gain control over tumor angiogenesis, cell cycle regulation, cell migration and gene transcription. In the scientific literature, we found a lot of examples of inhibitory peptides targeting each of these vital processes for several oncotherapy outcomes.[111,112] Significant number of those studies have focused on the design of peptides to inhibit mitogen-activated protein kinases family (MAPKs). Indeed, dysregulation of MAPKs signalling is observed in one-third of all human cancers such as breast, lung, thyroid, adenocarcinoma, bladder, liver and kidney.[117,118] On top of that, recent evidence also indicates that MAPK pathways play key roles in cancer progression and installation of therapeutic resistance.[119] It becomes then clear that developing effective inhibitors that target subfamilies of MAPKs pathways represents a great potential for cancer therapy (Figure 4).[116]

From a delivery standpoint, several strategies have been invoked for these subcellular targeting peptides going from the structural modification of the peptide itself to the use of molecular and nano-based delivery carriers. These delivery systems promote efficient cell uptake through various mechanism including clathrin and caveolin dependent endocytosis, energy independent uptake etc. [120–122] Among them, cell penetrating peptides (CPP) have been widely used. These molecular carriers are able to interact with

cell membrane, via either electrostatic or hydrophobic interaction followed by direct translocation of the CPP or endocytosis, to finally release the cargo in the cytosol. [123,124].

A list of different anticancer peptides recently developed, their mechanism of action, and cell lines on which they have been examined are depicted in Table 3.

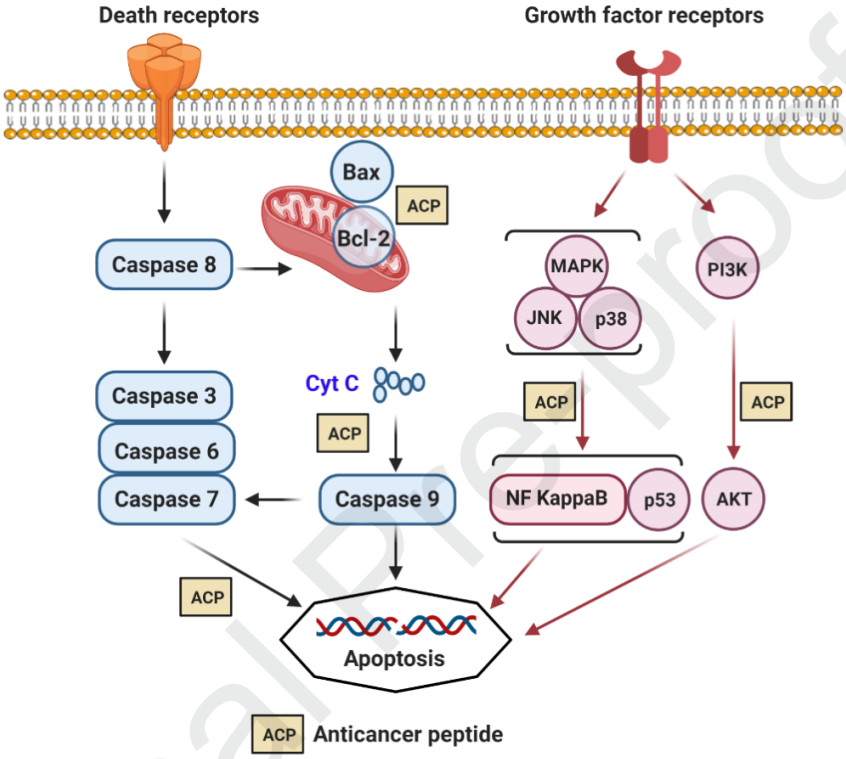

Figure 4. Schematic overview of apoptosis mechanisms for anticancer peptides. Apoptosis occurs via different pathways in cells. Some ACP can alter the ratio of BCl-2/Bax, while some others can activate caspases or trigger Cyt C release. JNK and p38 MAPK pathways and PI3K are also involved in ACP induced apoptosis. Figure inspired from reference [109]

Table 3. Examples of anticancer peptides underdevelopment

Name of the peptide	Molecular mechanism	Tumor cell lines	Validation model	References
Ruviprase (4.4 kDa peptide)	Act on various intrinsic apoptosis pathways.	MCF-7	*In-vitro*	[125]
Kla, RGD−kla	Caspase-3 activation	B16F10	*In-vivo*	[126]
NGR-sIL-24 peptide,	Upregulation, Bax/Bcl-2, cytochrome c release, and cleavage of caspase-3	U937 and A549	*In-vitro*	[127]

2PP7-Pep2-KLAK	Apoptosis through down-regulation of the expression of EZH2	THP-1	*In-vitro*	[115]
Pep5	Activation of ERK1/2	MDA-MB-231	*In-vitro*	[128]
ZXR-1 (FKIGGFIKKLWRSKLA), ZXR-2 (FKIGGFIKKLWRSLLA)	Caspase-3 activation	Hela	*In vitro*	[129]
CM 7 (DQIIANN)	Inhibition of c-Met-mediated signaling	MKN-45	*In-vitro*	[130]

3.2. Tumor homing peptides (cancer targeting peptides)

Cancer antigens that are exposed exclusively on the surface of cancer cells may serve as valuable means for achieving targeted drug-delivery in cancer therapies. Scientists have indeed taken advantage of this discovery to develop effective tumor homing strategies, based on disease-specific molecular recognition agents, acting like guided missiles that specifically recognize unique set of proteins or receptors which are overexpressed on tumor cells.[52] The rational of the targeting concept here is that an anticancer drug or a receptor imaging probe coupled to a ligand (tumor homing motif) would preferentially accumulate in the tumor, resulting in either greater activity and fewer side effects elsewhere in the body or better therapy evaluation, respectively (Figure 5).[16,131]

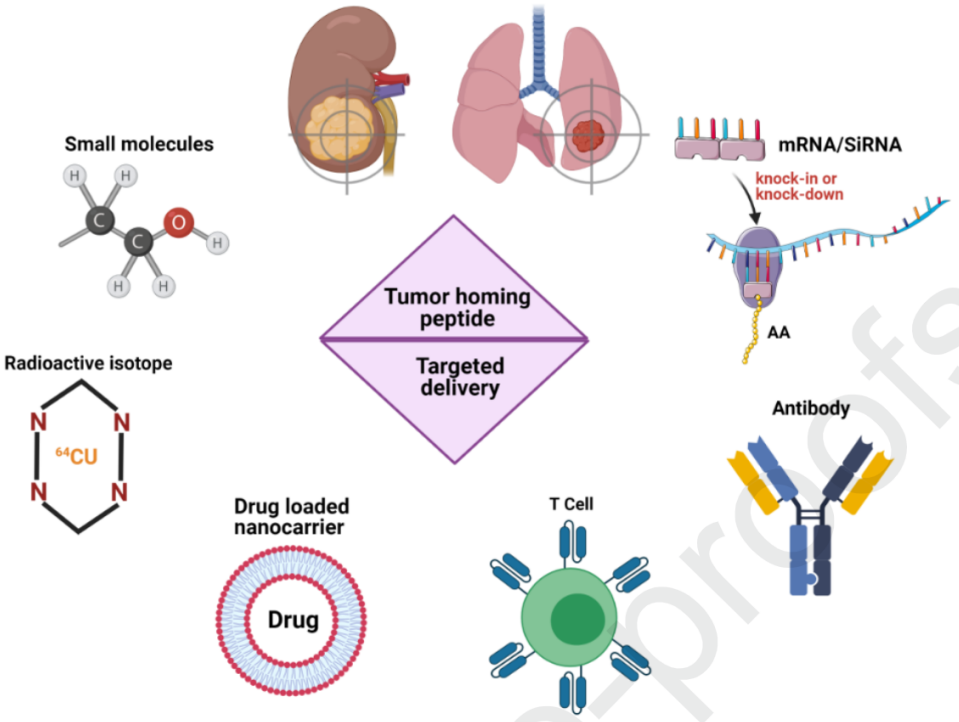

Figure 5. Therapeutic payloads that can be carried by peptides for cancer-targeted therapy and imaging. Ideally, anticancer therapeutics carrying various payloads of drugs can be like guided missiles with the capacity of targeted delivery toward many types of cancers. Cancer targeting peptides can guide various types of therapeutics to attack cancer cells. In addition to small molecules and radioactive isotopes, therapeutics such as miRNA/siRNA, immunotherapies and liposomal drugs are all able to conjugate with cancer-targeting peptides. Figure adapted with permission from ref 16.

Several types of tumor targeting ligands have been investigated including antibodies, antibody fragments, aptamers, peptides, small molecules and others.[132] Antibodies are one of the most widely used in clinics to deliver anticancer drugs specifically to tumor tissue as they may also hold intrinsic therapeutic effect.[133,134] Nevertheless, antibodies-based products suffer from deficiency such as limited stability *in vivo*, slow diffusion into the tumor tissue and high cost of production.[135] Today peptides are preferred over antibodies since they might be readily optimized for high specificity and better tissue penetration, with the capacity of targeting many types of cancer cells. [136,137] Tumor targeting peptides are low molecular weight compounds (<10 kDa), generally shorter than cell penetrating peptides (CPP). They include a wide range of peptidic ligands - linear, cyclic, macrocyclic and cyclotidic peptides identified mainly through phage display technology.[138,139] Their increased popularity result from the fact

that many peptide-binding receptors offer several attractive features namely a tumor - to normal cell expression ratio of around 3:1, which is sufficiently high to ensure the cellular delivery of appropriate amounts of chemotherapeutics.[140] Table 4 summarizes some of the recently studied peptide-binding receptors in the area of targeted tumor therapy.

Table 4. Recent examples of tumor homing peptides

Receptor family	Targeted receptor	Tumor expressing	Targeted peptide ligand	Reference
Integrins	αVβ3, α3β1, FAK, ITIGB3,	Glioblastoma, melanoma, breast, prostate cancer	Ck11, Ga-68-TRAP, GA-68-NOTA-SDM17, RGD peptide, UNC10245092	[141–144]
Epidermal growth factor receptor	ErbB-1, HER 2, ErbB-3.	Breast, colon, kidney, ovarian	HP2 (YDLKEPEH), GE11 (YHWYGYTPQNVI) D4 (LARLLT) P160	[145–149]
Somatostatin receptor	SSTR-2, SSTR-3, SSTR-4	Endocrine tumors, breast, lung, Kidney,	PEN-221, 68Ga-DOTATOC	[150,151]
Gonadotropin-releasing hormone receptor	GnRH-I, GnRH-II	Prostate, ovary, endometrium, urinary bladder	GnRH-I; Glp-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2	[152–154]
Bombesin receptor	BB1 (NMB), BB2 (GRP), BB3	Lung, colon, pancreas, prostate, breast, pancreas	FAM-K-BBN, HYNIC-Asp-[D-Phe13] BBN (7–13)-NHCH2-CH2-CH3.	[155,156]

Some of those peptides may also exhibit multifunctional delivery capabilities owing to their stimuli-driven, self-assembly properties resulting in well-defined nanostructures. These structures have proven to be highly beneficial for cancer treatment. Indeed, over the past few decades, it has been well established that the tumor environment different from the

normal tissue environment. Lower pH, higher temperatures, overexpression of some receptors, hypoxia, and specific expression of some proteases are the main biological features in tumor tissues.[157] Hence, the rational design of self-assembling peptides with noticeable tumor microenvironment responsiveness is well suited for controlled release or targeting of anticancer drugs to tumors sites. [158–160]. Currently, several peptides from diverse sources and chemical structure (β-sheet, α-helix, collagen-like peptides, elastin-like polypeptides, lipidated peptides and peptide amphiphiles) are being designed for this purpose.[159,161–163] They are able to form various supramolecular entities through non covalent and spontaneous interactions (hydrogen bonds, electrostatic, hydrophobic, π-π stacking and so on) which then self-assemble under multiple processing or environmental conditions. Ionic strength, pH, and temperature are the mostly used triggers to initiate the formation of supramolecular nanostructures.[164,165]

Leming Sun *et a*l, for instance, investigated scalable synthesis of cyclic peptide nanoparticles (CPNPs) and nanotubes using three different methods, phase equilibrium, pH-driven, and pH-sensitive methods. The effect of PEG-modification of peptide before and after assembly process on dimensions of self-assembled nanostructures were studied using atomic force microscopy and dynamic light scattering techniques. The results showed that the diameter of PEG modified CPNPs was far smaller than the CPNPs self-assembly from unmodified cyclic peptides with the same cyclic peptide concentration and under similar process conditions, suggesting the reduction of aggregation due to limited hydrophobic interactions.[165] Furthermore, the obtained CPNPs displayed exceptional physicochemical characteristic and tunable functionality such as high loading capacity and stimuli responsive drug delivery behaviour that could be useful in many biomedical scenario including cancer.

There is an increasing number of multifunctional anticancer nano-therapeutics under development which make use of self-assembled structures prepared from bioconjugates constituted of tumor homing peptides attached to a potent chemotherapeutic, and whose results demonstrate significant improvement of efficacy, and by-passing of off-target toxicity.[160,166] Tyson J. Moyer *et al*, described an enhanced binding affinity towards DR5 (a clinically relevant death receptor which initiates caspase-dependent apoptosis) of TRIAL-mimetic peptides after incorporation of an amphiphilic peptide (PA) into their

sequence to generate supramolecular self-assembly. The encapsulation of the chemotherapy drug paclitaxel within nanostructures while self-assembled showed enhanced cytotoxicity of these nanostructures against cells expressing DR5. When tested on an orthotropic xenograft model of breast cancer with MDA-MB-231 cells, these nanostructures with encapsulated paclitaxel inhibited mammary tumor growth and were significantly more effective than paclitaxel alone and saline controls. (Figure 6). [167]

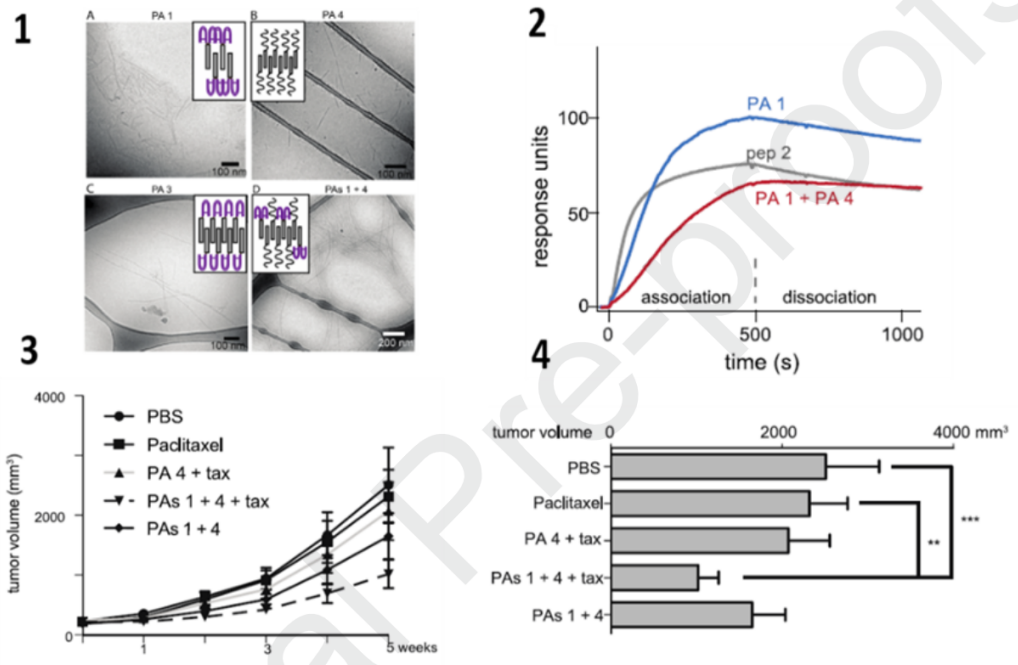

Figure 6. **[1]** Characterization of TRIAL-mimetic and PEG Pas (A-) cryo-TEM of (A) PA 1, (B) PA 4, and PA3 respectively. **[2]** Surface Plasmon resonance curves of PA1 (blue), peptide 2 (gray), and coensembled Pas 1 and 4. In vivo efficacy of PA 1 nanostructures: **[3]** Tumor volume plotted over 5 weeks as the result of treatments that included different combinations of PA 1, PA 4, and paclitaxel. **[4]** Histogram of tumor volumes after 5 weeks shows significant differences in tumor volume for PAs 1 and 4 with paclitaxel treatment. Reproduced from reference 130

3.3. Cell penetrating peptides (CPP)

CPP are a class of diverse peptides, typically with 5-30 amino acids, that unlike most peptides can easily cross the cellular membrane.[168] Investigations on CPP started in 1988 with the discovery of transactivating transcriptional activator protein (TAT), that was noted to be easily taken up by cells in tissue culture.[169] Later on, it appears that CPP were not only able to cross over the cellular plasma membrane via either energy dependent or independent mechanism[170], but they can also take cargos ranging from

classical small molecules to different types of proteins and oligonucleotides along with them, both *in-vitro* and *in-vivo.*[171–173] Importantly, CPPs also have tumor penetrating capabilities that enhance cargo (bioconjugates or anticancer drug loaded NPs) extravasation and spreading in tumor tissue.[173–176] Furthermore, CPP are well suited for imaging functionality because of their better accumulation in desired tissues, which amplified their correlated imaging signals for image-guided diagnosis.[177,178] References[168,179,180] provided extensive details about their discovery, classification and mechanism of transduction.

One of the major challenges with existing conventional chemotherapy is the ineffective penetration of drug molecules into the tumor.[181] Indeed, several conventional chemotherapeutics show limited diffusion from blood vessels into extravascular tumor tissue resulting in poor overall bioavailability.[182] One of the arguments could be that the tumor physical microenvironment is made up of an abnormal extracellular matrix, pericytes, and cancer-associated fibroblasts which create an obstacle to the penetration of numerous anticancer drugs, leading to aberrant drug delivery in tumors.[183,184] This issue causes cancer cells to develop resistance as a result of the accumulation of suboptimum drug concentration inside the tumor.[185] Taxanes, for instance, represent a class of anticancer drugs whose limited tissue penetration has been described as a mechanism for the development of resistance in a range of solid tumors.[186] On the other hand, reaching the intracellular therapeutic level of large biomolecule anticancer drugs such as peptides and proteins, that need to move inside the cell across the membrane to act on their intracellular targets, is also a critical problem to overcome. In this context, the use of CPP could be beneficial as they might spark deep tumor penetration as well as intracellular delivery of both bioconjugates and anticancer loaded nanoparticles. In this way, the efficacy of chemotherapy would be greatly improved.[187–189]

Cuihua *et al.* have recently studied a peptide-peptide co-administration therapy using hybrid peptide kla $(KLAKLAK)_2$, an apoptosis inducing peptide that act by disruption of mitochondrial membranes, attached to HPRP-A1 as a cell penetrating peptide. Kla has a poor eukaryotic cell-penetration ability that required CPP delivery strategy. The result showed a significant apoptosis rate of up to 65% and 45% on MCF-7 and A549 cell lines, respectively.[190] In another very similar study done by the same research group, Kla was

coupled with TAT via caspase-3 cleavage site. When administrated *in vivo* and taken up by mouse melanoma and human breast cancer cells, there was an activation of endogenous caspase-3, which then cleaved the Tat-KLA conjugate leading to subsequent release of the pro-apoptotic peptide $(KLAKLAK)_2$. Additionally, endocytosis pathway of the conjugate was also investigated using various endocytosis inhibitors. The outcome showed that TAT-conjugate was taken up at even faster rate by breast cancer cells via endocytosis. Overall, the two strategies of conjugation showed strong anticancer activity *in-vitro* and low toxicity to normal cells.[187] There is still a growing attention towards the development of CPP-conjugates to improve cancer therapy. Table 5 listed additional research works that focused on the design of CPP – anticancer drug conjugates.

Table 5. Recent examples of CPP-conjugates with various types of anticancer drugs.

CPP	Anticancer drug	Cancer cell line/tumor model	Reference
dNP2	Doxorubicin	Tumor spheroid of Hela	[191]
TATs	Gambogenic acid	EJ Human bladder Carcinoma	[192]
TATs	Kla (proapoptotic peptide)	A 549 (NSCLC)	[187], [193]
BP16, BP308	Chlorambucil	Capan-1, MCF-7, SKMEL-28	[194]
Octa-arginine (R), TAT, LMWP	Paclitaxel	A 549, B16F10 tumor bearing mice	[195]
Cyclic $[W(RW)_4]$, NGR (CKRRMKWKK), Synthetic iRGD (CRGKGPDC)	Doxorubicin	CCRF-CEM, SK-OV-3, HCT-116, HT-1080, MCT-7, mice bearing HepG2 and Huh-7 xenografts	[196], [189], [197]
TLR2	Peptide $D(KLAKLAK)_2$	Murine leukemia model	[198]
Oligoarginine (tetra, hexa, or octopeptide)	Vindoline	HL-60	[199]
SP90-C	HIV-1 Vpr (anticancer protein)	Triple negative MDA-MB-231.	[188]
iRGD peptide	Gemcitabine, Sorafenib	Murine pancreatic cancer model, HT-1080 spheroids	[200], [201]
TATs	Camptothecin	HeLa	[202]

Penetratin(desMet)	Methotrexate	MDA-MB-231	[203]

Given that CPP are hydrophobic in nature, their uptake generally involves a strong binding to membrane lipids.[204] Thus, most CPP will be internalized by all cell types. This lack of selectivity towards cancer cells represents a major drawback for their clinical application. To increase cell type specificity for CPP-mediated delivery, alternative strategy is their conjugation to homing peptides. For instance, iRGD peptide, is able to target tumor tissues, but in opposition to standard RGD peptides, it is also capable to spread much more into extravascular tumor tissue.[205,206] This approach combines the cell-penetrating capacity of CPPs with the ability of homing peptides to recognize specific cells types.[188]

Tetsuya Kadonosono, reported that CPP, also referred to as protein transduction domains (PTD) facilitate the extravasation of fused proteins by binding to neuropilin-1 (NRP1), a vascular endothelial growth factor (VEGF) co-receptor expressed on the surface of endothelial and some tumor cells. They examined the capacity of the amphipathic and cationic CPP/PTDs, PTD-3 and TAT-PTD, respectively, to bind cells *in vitro* and trigger accumulation of their cargos in xenograft tumors *in vivo*. The result shows that tumor accumulation of those fluorescent fused proteins, which was first a consequence of EPR effect, was significantly increased when PTD-3 was used. (Figure 7). [174]

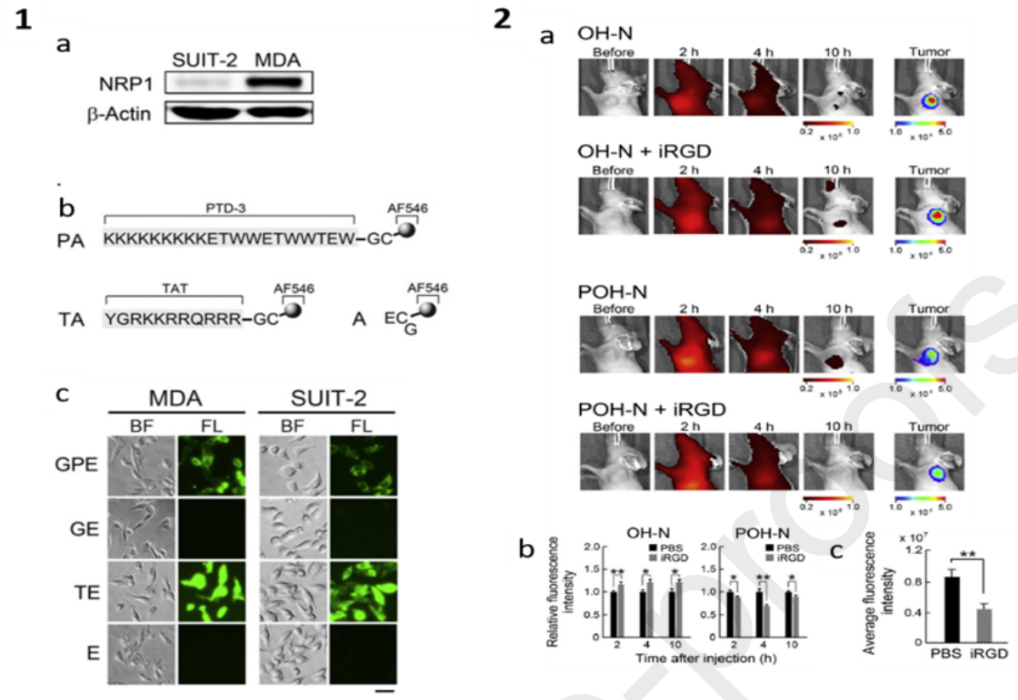

Figure 7. **[1]** Contribution of NPR1 to CPP: PTDs binding to cells. (a) Expression of NPR1 in human SUIT-2 or MDA-MB-231 cells analyzed by western blotting. (b) Schematic diagram of some fluorescent dye-conjugated CPP/PTDs and their controls. (c) Micrographs of MDA- MB-231 or SUIT-2 cells treated with CPP/PTD-fused protein for 1h. **[2]** Effect of iRGD on the delivery of POH-N and TE. **(a)** in vivo imaging of tumor-bearing mice after probe administration. The right panel (tumor) shows bioluminescence images of SUIT-2/HRE-Luc xenograft at 10hr. **(b)** Fluorescence intensity of tumors after probe administration with or without iRGD administration. **(c)** The GFP fluorescence intensity of tumors sections. Reproduced from reference 137.

4. Nanotechnology based chemotherapy with peptides

The application of nanotechnology in the development of advanced therapies for cancer has already shown impressive progress and continue to draw the attention of both academia and industries. The justification of the anticancer nanomedicine approach relies firstly on enhanced permeation and retention effect (EPR) that characterize a few numbers of cancer tissues, and which provides the possibility of passive targeting for nanovectorized therapeutics.[207] Thanks to their unique physical properties (size, shape), ability to load various drugs, and the diversity of their chemical composition including fusogenic materials, nanomedicines have the capability of cytosolic accumulation via endocytosis, providing, therefore, an excellent means for achieving intracellular delivery of cargo macromolecular therapeutics.[208] Inorganic, polymeric and lipid-based nanocarriers are some of their major representatives.[209–211]

Each of them has got specific design features, but also strengths and limitations concerning biomedical utilizations. The current literature largely covers groundbreaking applications of nano-based drug delivery systems for different kinds of anticancer agents such as peptides, siRNA, proteins, and McAbs, even though the majority of them have not yet made their way into clinics.[210,212,213]

In clinical practice, the encapsulation of anticancer peptides (ACP) into nanosized vehicles as a delivery platform has the potential to produce the following benefits: (i) reducing hemolytic toxicity of payloads (for cationic peptides),[170] (ii) overcoming the problem of short circulation time of the payloads by improving serum and metabolic stability while protecting them from being rapidly cleared from the body,[214] (iii) controlled release of the payloads specifically at the tumor site through the design of stimuli-responsive drug delivery systems (e.g., pH, temperature, and magnetically triggered formulations), or via ligand functionalization of nanocarriers, [215] (iv) facilitate efficient intracellular delivery of the cargo to reach subcellular targets thanks to the use of fusogenic and endosomolytic materials, (v) co-delivery of multiple drugs for synergic effects using a single delivery platform.

4.1. Improve hemocompatibility of membranolytic ACP.

The *in vivo* application of membranolytic peptides (cationic amphiphilic peptides) for cancer therapy is hampered by toxicity due to off target interactions, especially with blood cells components. In previous section of this review, we have already reported several studies that explored the possibility to overcome this drawback by covalently binding those membranes active peptides with tumor homing ligands in order to achieve selective delivery. Rather than preparing bioconjugates, many other research works have proposed encapsulation into nanocarriers to address nonspecific cytolytic issues.

Melittin (MLT), for instance, is an important cytolytic peptide that can induce apoptosis by regulating the expression of 3383 genes, and the PI3K/Akt-regulated p53 pathway.[216] However, its clinical applications are severely restricted owing to its nonspecific toxicities like hemolysis.[216] Li, Y. et al., recently reported MLT-loaded zeolitic imidazolate framework-8 (MLT@ZIF-8) nanoparticles, as a delivery platform to improve its biological stability and inhibit the hemolysis bioactivity of MLT. In fact, MLT was loaded into a simple

porous nanoscale system (ZIF-8) obtained using Zn^{2+}, and 2-methylimidazole in aqueous medium. The formed (MLT@ZIF-8) has shown a robust colloidal stability (narrow size distribution of around 160 nm, PDI ≤ 0.3 up to 7 days), and the encapsulated MLT could not hemolyse red blood cells. Moreover, the MLT@ZIF-8 displayed enhanced apoptosis compared to free MLT.[217] In another study, Matthew R et al., took advantages of the membrane-specific interactions of anticancer peptides (ACP) to prepare a new class of peptide-loaded lipid particles, which they termed lipopeptisome. This interesting nano design retained Lasioglossin, a synthetic ACP within a lipid lamellar corona (liposomes, LP) to avoid contact with red blood cells and healthy tissues. The confirmation of ACP integration in LP bilayer was evidenced by confocal microscopy using a fluorescently-labeled lasioglossin. The result has further shown that incorporation of ACP within LP particles did not disrupt or diminish its fusolytic ability, leading to enhanced accumulation of the peptide into cancer cells membranes and potent tumor toxicity profile, with minimal toxicity towards non-cancerous cells and erythrocytes.[218]

4.2. Prolong effect and improve subcellular targeting of ACP

Achieving high antitumor efficacy with chemotherapy also implies that the systemically delivered antitumor product must be able to perform a wide range of delivery cascade from bloodstream circulation, tumor accumulation and penetration, to tumor cell uptake and acid endosomal/lysosome compartment escape.[219,220] From many researchers' perspective nanotechnology is revealed as a crucial resource that could provide technical solutions to cope with these critical delivery challenges (figure 8).[221]

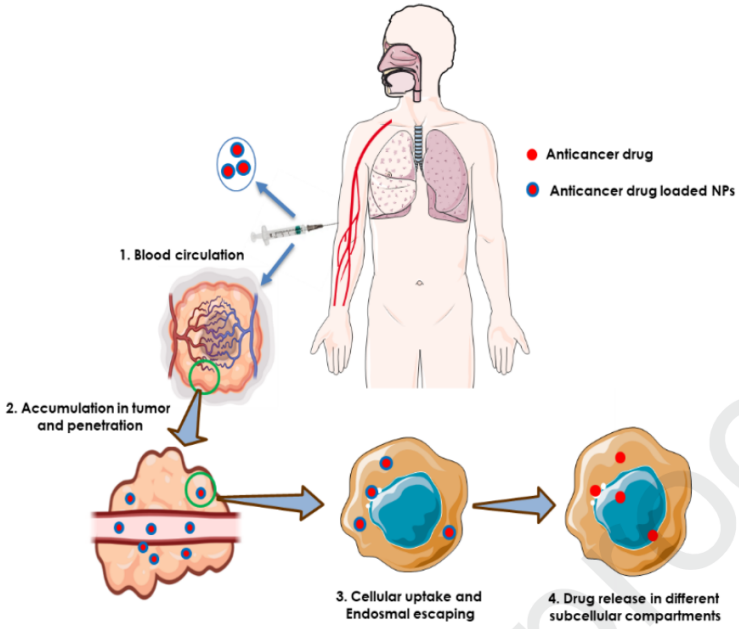

*Figure 8. CAPRIR cascade of systemic cancer drug delivery: extracellular and intracellular steps. (CAPRIR stands for **C**irculation, **A**ccumulation, **P**enetration, **I**nternalization, and **R**elease). Figure Inspired from ref 202*

Ducat, E. et al., evaluated the usefulness of stealth pH sensitive LPs (DOPE: CHEMS: CHOL: DSPE-PEG750) as a suitable carrier combining increased circulation life-time and intracellular capabilities for a therapeutic peptide termed Print 3G. The formulation showed good physicochemical characteristic with Print 3G entrapment efficiency of about 60%. Moreover, confocal and flow cytometry analysis revealed that the encapsulated peptide was able to be delivered into the nucleus of tumorigenic and non-tumorigenic breast cancer cells.[222] Wu, Y. et al., examined the potential of mesoporous silica nanoparticles (MSNs) as a promising delivery vehicle of anticancer peptides. In this study, MSNs decorated with folic acid were loaded with N9 peptide (Bcl-2 pro-apoptotic proteins functional converter). The results showed that the functional MSNs loaded N9 peptide had excellent anticancer efficiency with great selectivity, inducing approximatively 52% of HeLa cells into apoptosis. Moreover, the large pore size of functional MSNs afforded efficient loading capacity of the peptide along with remarkable *in vitro* intracellular delivery capability.[223] Sumeet Kapoor et al., focused on the use of polyhydroxybutyrate (PHB) as a potential nanovehicle for the delivery of anticancer peptides. From PHB^{72K} – PEG^{4K} copolymer that was prepared and characterized by ^{1}H NMR and ^{13}C NMR, the authors

produced nanoparticles encapsulating NuBCP-9 (FSRSLHSLL), an anticancer peptide that acts as a molecular switch to induce a Bcl-2 conformational change, converting it from a cytoprotector to a cytocide. PHB^{72K} – PEG^{4K} nanoparticles showed encapsulation efficiency of 61% and exhibited sustained release of peptide over a period of 26 days at physiological pH. Confocal laser microscopy confirmed efficient cellular uptake and induction of apoptosis by peptide loaded NPs in a time dependent manner. Moreover, *in vivo* intraperitonial administration of 20 mg/kg NuBCP-9/NPs twice a week for three weeks triggered 90% tumor regression in Ehrlich syngeneic mouse model. This study has pretty much shown the potential of PHB^{72K} – PEG^{4K} based nanoformulation as a tool for targeting intracellular proteins.[224]

Protein nanoparticles is another type of drug delivery vehicles that have attracted huge attention owing to their good biocompatibility, efficient cellular uptake, good biodegradation and highly organized structure (virus like particles and caged proteins).[225] We found out recent interesting works where they have been effectively used for delivering anticancer peptides into tumors. Dongmei Wang et al., evaluated the delivery of a proapoptotic peptide KLAK (KLAKLAK)2, by nanocage obtained from heat shock protein (HSP) self-assembly. In this study, it was expected that HSP nanocage could be efficiently internalized into cells and subsequently promote the apoptosis of cancer cells. The results of characterization showed that KLAK loaded HSP nanoparticles have a mean diameter of 40 nm and spherical shape. *In vitro* studies demonstrated efficient cellular uptake and lysosome escape of these nanoparticles. Furthermore, a significantly enhanced antitumor activity was observed in B16F10 melanoma cells, which was associated with the proapoptotic activity of KLAK delivered by HSP nanocages (Figure 9).[226]

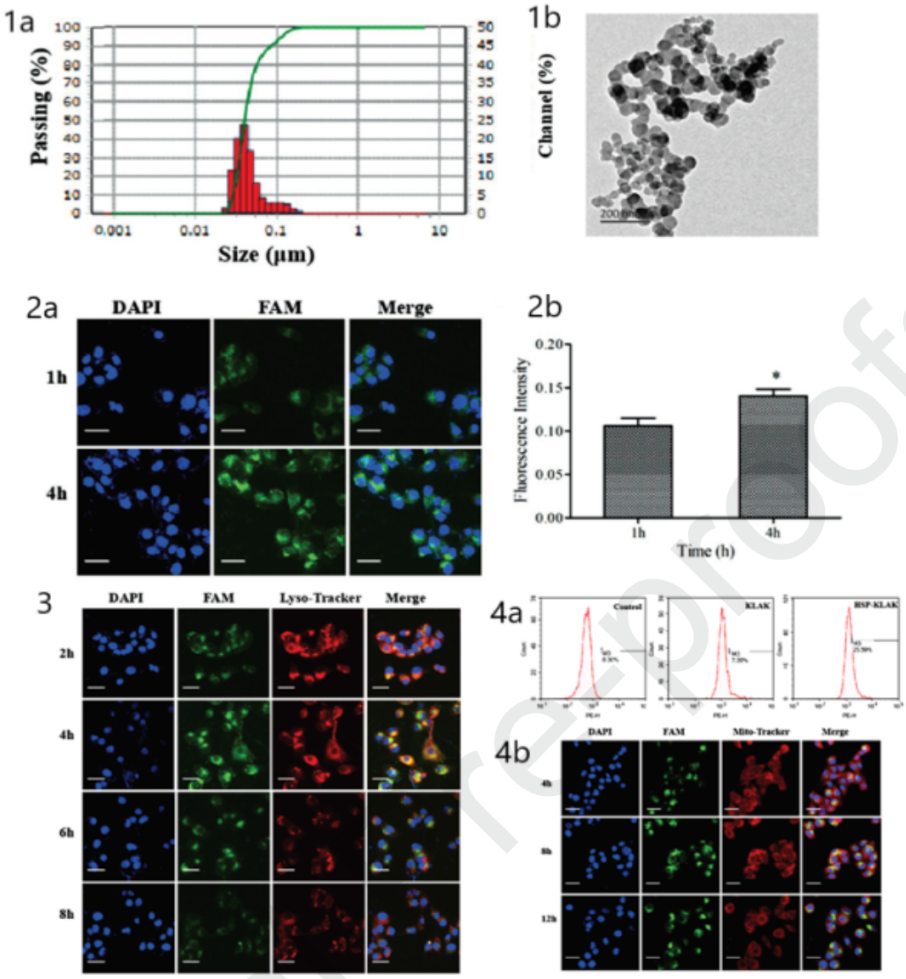

Figure 9. [1] Preparation and characterization of HSP-KLAK NPs, 1a) The particle size determined by dynamic light scattering (DLS). 1b) TEM image of HSP-KLAK. [2] Analysis of HSP-KLAK nanoparticles uptake in B16F10 melanoma cells. [2] Analysis of HSP-KLAK nanoparticles uptake 2a) The uptake of HSP-KLAK nano particles uptake in B16F10 melanoma cells. The uptake of HSP-KLAK nanoparticles was examined in B16F10 cells by CLSM imaging after 1h or 4hr of incubation; 2b) Mean fluorescence intensity analysis of cells treated with FAM-labeled HSP-KLAK nanoparticles. [3] The lysosome escape analysis of HSP-KLAK nanoparticles in B16F10 melanoma. B16F10 cells were treated with FAM-labeled HSP-KLAK nanoparticles (green) for 2,4,6,8 h, and stained with Lyso-Tracker (red), sacle bar:20μm. [4] Antitumoral and pro-apoptotic analysis of HSP-KLAK nanoparticles in melanoma cells. (4a) The pro-apoptotic activity of HSP-KLAK nanoparticles was detected via flow cytometry analysis. 4b) The colocalization of HSP-KLAK nanoparticles with mitochondria after 4, 8, or 12h of treatment using a mitochondria specific probe (Mito-tracker, red). Figured reproduced from ref 176

Ma, B. et al. have gone even way further in this area by using a tetrameric protein scaffold with multiple functionalities for antitumor peptides delivery. The purpose of this innovative design strategy was to antagonize intracellular MDM2/MDMX for p53 activation, after

extending the structure of peptide grafted carrier protein, PMIBcr/Abl, by a C-terminal Arg-repeating hexapeptide (cell penetrating moiety) to facilitate its cellular uptake. The result of confocal suggested that cellular uptake pathway for PMIBcr/Abl-R6 is indeed endocytosis-independent as heparin almost completely blocked it. Moreover, a dose-dependent growth inhibition of HCT116 *p53*+/+ cells was observed with PMIBcr/Abl-R6, while a look into the mechanism of action suggested that PMIBcr/Abl-R6 induces apoptosis of wild type p53-harboring tumor cells by antagonizing MDM2 to activate the p53 signaling pathway. [227] Overall, this protein based delivery strategy provided a clinically viable solution that could be applied for the development of many other peptide therapeutics to target a large variety of intracellular protein-protein interactions responsible for disease initiation and progression, especially cancers.

Mozhi, A et al., reported an interesting delivery approach to achieve intracellular co-delivery of both an anticancer peptide and a classical chemotherapy drug. In this study, an amphiphilic copolymer poly (b-amino esters)-poly (ethylene glycol) was synthesized and conjugated with the dual-targeting proapoptotic peptide CGKRKD(KLAKLAK)2. The conjugate has the ability to self-assemble into a core-shell micellar structure at physiological pH of 7.4. This self-assembly property was subsequently used to encapsulate the anticancer drug docetaxel (DTX). CGKRKD was the functional moiety that target angiogenic blood vessels in tumors and tumor cells, whereby the engineered micelles were efficiently internalized into tumor cells. Once inside the acidic endosomal compartment, the pH-responsive micellar carriers disassembled and released both pharmacological agents. The result further showed that CGKRK efficiently transports D(KLAKLAK)2 towards mitochondria to trigger mitochondria-dependent apoptosis, while DTX affects microtubulin arresting the cancer cell cycle. Thus, the combination of DTX and the therapeutic peptide displayed a synergistic antitumor effect in an MCF-7 cell line.[228]

Table 6. Summary of the ACP targeting subcellular components.

ACP Sequence/name	Subcellular localized targeting	ACP mechanism	Reference
Print 3G	Nucleus	Antagonist of oncoprotein involved in breast cancer.	[222]

NuBCP-9 (N9)	mitochondria	Molecular recognition switch that converts Bcl-2 from protectors to cancer killers.	[91]
NuBCP-9 (FSRSLHSLL)	Intracellular proteins	Induce a Bcl-2 conformational change.	[224]
KLAK (KLAKLAK)2	mitochondria	Proapoptotic activity	[229]
Dodecameric peptide (PMI)	Intracellular proteins	Inhibitor of the p53-MDM2/MDMX interaction	[227]
CGKRKD(KLAKLAK)2	mitochondria	Trigger of mitochondria-dependent apoptosis	[230]

4.3. Nanoparticles functionalization with peptides.

Beyond the fact that peptides are looked at as promising active therapeutic molecules but with many susceptibilities that require advanced delivery strategies to address them, there is another area of flourishing and vigorous research that focuses on the development of peptide-functionalized nanoparticles (NPs) as therapeutic and diagnostic tools for cancer treatment. Indeed, peptides can be engineered and used to decorate the surface of nanoparticles. This surface functionalization accomplishes plenty of functions like increasing cancer cell specificity, enhancing tumor penetrating ability, providing stimuli-responsive shielding, and facilitating endosomal/lysosomal escape of the nanocarriers.[220,231,232] Moreover, it is currently possible to design pharmaceutical nanosystems combining several of these features in one single particle, offering thereby a powerful tool to tackle the tumor's pathological complexity.[233,234] Compared to the previous generations of NPs, the third generation of NPs (ligand mediated NPs) displayed improved PKs and pharmacodynamics of the encapsulated therapeutic load, at least in preclinical stage, although their real benefit remains to be proved in clinical settings.[235]

Among the most extensively studied ligand-mediated NPs for the delivery of drugs and genes to tumours, there are peptide-functionalized liposomes. LP are spherical vesicular lipid based nanocarriers consisting of physiological compounds, mainly phospholipids. They represent one of the most versatile drug delivery platforms (conventional LPs, stealth LPs, pH-sensitive etc.). The typical bilayer structure surrounding an aqueous compartment has made them a suitable carrier for both hydrophilic and hydrophobic active

compounds, which has then led to many successful applications in delivery of anticancer pharmaceuticals such as peptides, siRNA, proteins and McAbs.[236] Liposomes (LP) can be decorated with functional peptides in two ways. Firstly, peptides are grafted on the surface of preformed LPs through post-insertion or post conjugation technique where ligand peptides are added on preformed LPs. The second way is by mixing lipids with peptide and let them self-assemble into vesicles.[237] Most of LPs are sterically stabilized by the PEG polymers of different molecular weights, which also serves as an anchor point for those ligand peptides, facilitating their insertion into the surface of LPs. Shahin, M. et al., prepared ligand peptide (p18-4)-DSPE-PEG conjugated that binds specifically to breast cancer cell line MDA-MB-435 on its surface. The ligand peptide was then coupled to doxorubicin (DOX) loaded LPs through conventional, post insertion technique. Interestingly, the p18-4-DSPE-PEG decoration of LPs by these methods did not have a notable effect on the size of prepared LPs and DOX release, but increased the uptake and cytotoxicity of encapsulated DOX in MDA-MB-435 cells.[238]

Although the functional peptide ligands are grafted onto LPs surface mainly through PEG polymers conjugation, they are still susceptible to being recognized by the immune system that accelerates their clearance. It should be noted that the immune response is modulated by both the physicochemical properties of the peptide ligand and the chemical nature of the coating agent used to achieve stealth character. Longer PEG molecules (e.g. PEG 5000) provide a better surface shielding against protein corona formation (opsonisation), but may mask the peptide molecule, while shorter PEG molecules (e.g. PEG750) may provide sufficient access for the peptide molecule to bind to the target receptor but may reduce the steric barrier.[239,240] Unfortunately, as already discussed in paragraph 2.2.1, PEG polymers can adversely impact targeting efficiency and promote immune reaction of host organisms over repeated dosing. Hence, A. Ranalli et al. studied the use of bio-inspired lipopeptide, whose architecture can be easily modified to allow straightforward conjugation of tumor homing ligands, as an alternative to PEG to evade immune system with functionalized NPs. The result showed that lipopeptide coating limits serum-protein adsorption even upon prolonged incubation in serum physiological conditions, outperforming PEGylated LPs.[241] Ligand peptides characteristics such as sequence structure, charge, and hydrophilicity should also be considered in this regard.

A.K. Nowinski et al. demonstrated that designing peptide systems enriched with negatively charged glutamic acid/lysine portion increases the stealth character of ligand peptides. [244] The external charge of the LPs arising from phospholipids and charged peptides is also critical. Therefore, lipids and peptides that are nearly neutral or negatively charged at physiological pH should be privileged as much as possible to avoid the immune response. Some preclinical studies reporting enhanced tumor targeting and penetration from peptides functionalized liposomes are presented in Table 7.

Table 7. preclinical studies reporting enhanced tumor targeting and penetration from peptides functionalized liposomes

Peptides (structures)	Encapsulated molecule	Therapeutic outcomes	Ref
TD (ACSSSPSKHCG)	vemurafenib	Targeted inhibition of melanoma via the skin. Enhanced delivery of vemurafenib loaded liposomes across the skin with effective inhibition of subcutaneous melanoma in male mice.	[242]
DP7 (VQWRIRVAVIRK)	mRNA vaccine	Transfer of mRNA efficiently into different type of dendritic cells (DC) *in vitro.* Enhanced stimulation of DC maturation ($CD103^+$) both *in vitro* and *in vivo*	[243]
RIPL peptide (IPLVVPLRRRRRRRRC)	docetaxel	Significant inhibition of tumor growth and prolonged survival time in BALB/c nude mice with human ovarian carcinoma (SK-OV-3) cell tumors.	[244]
LinTT1 peptide (AKRGARSTA)	Doxorubicin	Higher interaction with 3D breast cancer spheroids leading to enhanced internalization and uptake of functionalized liposomes in 3D spheroids of MDA-MB-231 cells than bare liposomes.	[245]
TR peptide [c(RGDfK)-AGYLLGHINLHHLAHL (Aib)HHIL-Cys]	paclitaxel	Stronger transport ability across BBB, enhanced killing of glioma cells and brain cancer stem cells (CSCs). Better targeting of glioma, and elimination of brain CSCs and the VM channels in tumor in vivo.	[246]
Pentapeptide (c(RGDyK)	AL3810 (phase II clinical)	Better addressability of blood - brain tumor barrier. Decrease of opsonization with subsequent diminution of uptake by macrophages.	[247]
helical polypeptide p37	siRNA	Higher gene transfection efficiency and lower toxicity. Better delivery efficiency for survivin siRNA (siSuvi) to SK-BR-3 and MCF-7. Efficient growth inhibition of xenograft in mice.	[248]

5. Conclusion and perspectives.

The field of peptides is currently booming with variety of novel applications. Taking full advantage of the remarkable progress recorded recently in chemistry, biotechnology, and nanotechnology, this class of chemical compounds has undergone a significant transformation that creates not only opportunities but also challenges. With the help of modern chemical synthesis tools and in-depth knowledge about protein sequences, structures and interaction interfaces, therapeutic peptides that inhibit protein-protein interactions are being designed and investigated for various untapped targets. Significant number of them are showing interesting *in-vitro* pharmacological activity against various types of cancers. Nevertheless, limited bioavailability, serum stability, blood circulation half-life, cell membrane permeability restricts heavily the clinical translation of many brilliant preclinical findings. Considerable efforts are being made to optimize pharmaceutical properties of therapeutic peptides. While medicinal chemistry strategies such as modification of non-natural amino acids, cyclization, and glycosylation stabilize them against proteases, the low molecular weight still expose them to rapid renal clearance. Molecular strategies like conjugation with macromolecular moieties, especially PEG polymers (PEG > 5 kD) not only prolong the blood circulation time, but could also lower immunogenicity and trigger accumulation in tumor through passive targeting. However, the rising of scientifically sound evidences regarding the clinical safety of PEGylated therapeutics still remains a concern to think about. Recent research activities are forging new applications for peptides as a delivery vehicle, namely tumor homing and cell-penetrating peptides. For instance, potent anticancer agents are coupled with tumor targeting peptides to address nonspecific toxicity. Even more, those tumor homing peptides can now be designed in such a way that they acquire self-assembly properties with impressive delivery capabilities (encapsulation of chemotherapeutic agents, stimuli responsive property etc.). This kind of multifunctional delivery platform might hold the key for treating effectively cancers, but their clinical translation would also bring a lot of practical challenges. CPP drug conjugates represent a straightforward way to impact optimum tumor tissue penetration and achieve intracellular delivery of an anticancer drug but they lack specificity. Steps towards the design of tumor selective CPP is needed to

improve the *in vivo* utility. Nanocarriers also attract many researchers due to the versatility of their chemical composition and structural characteristics which make them exceptional carriers for preferential delivery of therapeutic peptides in tumors. In fact, a lot of clinically valid strategies are under investigation with different types of nanocarriers. In this regard, it is important to anticipate challenges such as scale up production, sterilization, and long term stability to speed up their uses in human. Altogether, we believe that clinically approved products based on anticancer peptides bioconjugates, anticancer peptides loaded nanomedicines, and peptides-mediated nanomedicines will emerge as well as state-of-the-art knowledge concerning their formulation, analytical characterization, biological performance, and industry-scale production.

Author contributions

All authors read and approved the manuscript

Conflict of interest

The authors declare that they have no competing interests.

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Graphical Abstract

Therapeutic peptides for chemotherapy: trends and challenges for advanced delivery systems

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