Development of Anticancer Agents with Peptide-Based Drugs

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Peptides are increasingly being developed for use as therapeutics to treat many ailments, including cancer. Therapeutic peptides have the advantages of target specificity and low toxicity. The anticancer effects of a peptide can be the direct result of the peptide binding its intended target, or the peptide may be conjugated to a chemotherapy drug or radionuclide and used to target the agent to cancer cells. Peptides can be targeted to proteins on the cell surface, where the peptide–protein interaction can initiate internalization of the complex, or the peptide can be designed to directly cross the cell membrane. Peptides can induce cell death by numerous mechanisms including membrane disruption and subsequent necrosis, apoptosis, tumor angiogenesis inhibition, immune regulation, disruption of cell signaling pathways, cell cycle regulation, DNA repair pathways, or cell death pathways.

drug delivery peptide therapeutic covalent-based peptide inhibitors PCNA

1. Introduction

Rational drug design involves structural and functional studies to identify and disrupt targets important in cellular maintenance. Different approaches for drug development can include the use of small molecules, antibodies, short DNA aptamers, or peptides. Since the discovery of insulin in 1921, peptide drugs have been developed to treat a wide range of diseases that include cancer, immunological diseases, metabolic disorders, viral infections, cardiovascular diseases, and osteoporosis.

Modern biological research including large-scale genome sequencing and functional genomic studies greatly improved our understanding of malignancy. However, the advancement in our scientific knowledge has not yet been effectively translated into better cancer treatment. The majority of modern drug development efforts focus on a small group of 3000 druggable protein targets consisting of kinases and other enzymes, G protein-coupled receptors, ion channels, and nuclear hormone receptors [1]. This approach excludes more than 85% of the genome and is inadequate to the objective of finding a cure for all cancers. The advancement in peptide technology over the past decades is changing the drug discovery landscape. There are approximately 80 peptide drugs already in the market, more than 150 peptides in the clinical development stage, and another 400–600 peptides at the preclinical trial stage [2].

While the number of peptide drugs entering the market has increased significantly in the past decades, efficient delivery has limited their development. Pharmacologically active peptides are hard to formulate as drug products,

as compared to small-molecules, due to the various challenges in administration and delivery of therapeutic peptides into cancer cells and tumor sites [2][3]. Typically, peptides drugs exhibit shorter circulation half-lives, lower cell permeability, and typically higher rates of enzymatic degradation. The oral delivery of peptides can have limitations, due to a number of factors that include enzymatic degradation and low absorption arising from metabolism by digestive enzymes or luminal microorganisms, the acidic environment of the gastrointestinal tract, the epithelial barrier of the small intestine, the unstirred water layer near the epithelial surface, and the various efflux systems [4][5][6]. Several strategies that increase the delivery and bioavailability of peptides are shown in Figure 1. Overcoming these difficulties would pave the way to developing more effective peptide therapeutics.

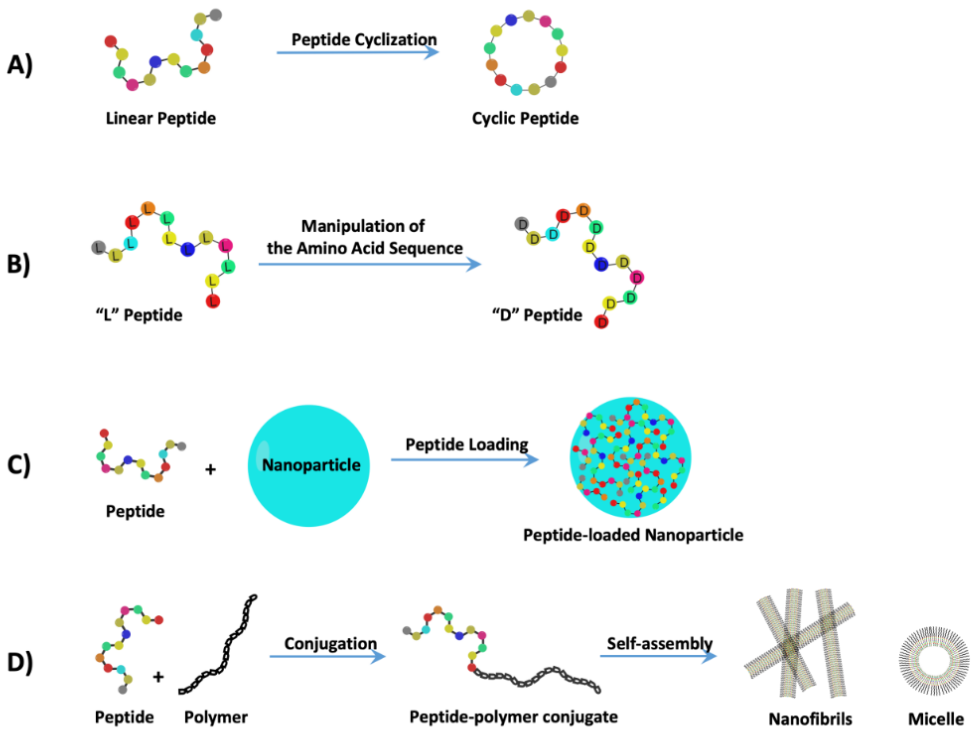

Figure 1. Current methods to enhance the half-life of peptide drugs and improve peptide drug delivery include (**A**) peptide cyclization, (**B**) manipulation of the amino acid sequence, (**C**) peptide-loaded nanoparticles, and (**D**) the conjugation of peptide drugs to natural or synthetic polymers.

2. Treating Cancer with Cell-Targeting Peptide (CTP) and Cell-Permeable Peptide (CPP)

Peptides can be used to cause a therapeutic effect through direct binding with their target or through conjugation to therapeutics and use of the peptide for targeted delivery of the cargo [7][8]. Therapeutic peptides can be divided into two classifications, cell-targeting peptides (CTP) and cell-permeable peptides (CPP). CTPs bind to a molecular marker present on the targeted cell allowing delivery of conjugates to a particular cell type while sparing other cells from the often toxic effects of the therapeutic cargo. The CTP can exert its effect at the cell membrane, or from

binding to its molecular target resulting in internalization of the peptide–therapeutic complex. Instead of binding to molecular markers on the cell surface, CPPs interact with charged components on the cell membrane, which then are internalized through various mechanisms. CPPs intended for anticancer therapy take advantage of the phenomenon that the outer cell membrane on cancer cells is negatively charged relative to normal cells thus enabling a positively charged peptide to preferentially target cancer cells [9][10][11].

2.1. Cell-Targeting Peptides

CTPs are designed to specifically bind to cell membrane proteins that are present in relative abundance on targeted cells compared to the rest of the cell population. The search for cell surface tumor markers has identified many proteins that are enriched in cancer cells versus normal cells, including integrins, epidermal growth factor receptor, G protein-coupled receptors, and other various receptors such as gonadotropin-releasing hormone receptor, vasoactive intestinal peptide receptors 1 and 2, neurotensin receptor 1, aminopeptidase N, and keratin 1, which are overexpressed in breast cancer [7][12][13][14][15][16][17]. Binding of CTPs to these receptors can result in activation or inhibition of signaling from the receptor and/or internalization of the peptide–receptor complex. To achieve a sufficient therapeutic window, a general rule is that at least a 3-fold increase in expression of the targeted protein should be present on cancer cells compared to normal cells. In addition, for peptides used to deliver anticancer agents, the expression level of the cell membrane target must be high enough to deliver a therapeutic dose [18][19].

One cell membrane protein target that has been identified is the epidermal growth factor receptor (EGFR), which is overexpressed in several tumors of epithelial origin including breast cancers of ductal or lobular origin. Antibody–drug conjugates (ADCs) have been effectively used to target EGFR in triple-negative breast cancer and have entered clinical trials [20]. Peptide–drug conjugates (PDCs) have many advantages over ADCs [7]. First, ADCs are large making it difficult to permeate far into tumors. Additionally, ADCs are often immunogenic despite efforts to humanize the antibody. Another problem is that ADCs often accumulate in excretory organs such as the kidneys and liver. In addition, antibody creation is expensive and time consuming. PDCs on the other hand are small and able to penetrate tumors with relative ease. They have low immunogenicity, are less likely to accumulate in excretory organs, are easy to synthesize, and are affordable to produce.

To take advantage of the benefits that peptide therapeutics offer, CTPs are also being developed to target EGFR. GE11 (YHWYGYTPQNVI) is a peptide that was discovered by screening a phage display library for peptides with enriched binding to EGFR. GE11 binds to EGFR with a dissociation constant of 22 nM. This compares with a dissociation constant of 1–2 nM for the interaction of EGFR with EGF, one of the natural ligands for EGFR [21]. Importantly for a potential anticancer therapeutic, the mitogenic activation as a result of ligand binding to EGFR was much lower for GE11 compared to EGF. When proliferation of the human hepatoma cell line SMMC-7721 was measured in the presence of 1 μg/mL of GE11 or EGF over 48 h, treatment with GE11 resulted in an increase in proliferation of approximately 10% while treatment with EGF stimulated a 50% increase in growth. The EGFR/EGF complex is internalized upon receptor/ligand binding and likewise, GE11 results in internalization of the EGFR/GE11 complex. FITC-labeled GE11 was internalized into the high EGFR expressing SMMC-7721, but not

internalized when the EGFR-negative cell line, K562, was treated with the labeled GE11. Furthermore, adding excess EGF or unlabeled GE11 to the cells resulted in decreased uptake of the FITC-labeled GE11. In vivo, intravenous delivery of I125-labeled GE11 resulted in accumulation of GE11 in the tumors of a mouse xenograft of the SMMC-7721 cell line. Four hours after tail vein injection of the radiolabeled GE11, the amount of radioactivity found in the tumor was greater than in any of the other tissues tested (blood, heart, liver, spleen, lung, kidney, and brain). These studies illustrate the ability of GE11 to effectively target EGFR expressing tumor cells.

GE11 on its own does not cause appreciable cell death in the cells that it targets; however, many researchers have conjugated GE11 to polymers to form GE11-coated nanoparticles, micelles, and liposomes, which in turn are loaded with anticancer agents [22]. In this way, a cargo of therapeutic drugs can be delivered to EGFR expressing cells, avoiding much of the off-target toxicity associated with many drugs used to treat cancer. In addition, while uptake of nanoparticles is generally a result of passive diffusion, the presence of GE11 on the surface of nanoparticles results in increased active uptake of the coated nanoparticles via internalization of the EGFR–GE11–nanoparticle complex. GE11 has been used to target delivery of a wide range of agents including PC4, a photosensitive drug used in photodynamic therapy [23]; salinomycin, an inhibitor of breast cancer stem cells [24]; paclitaxel [25]; doxorubicin [26]; curcumin [27] and many other agents. One interesting approach sought to treat laryngeal cancer by conjugating GE11 to liposomes loaded with docetaxel. Treatment with docetaxel often results in upregulated expression of multidrug resistance genes, so the investigators also attached a small interfering RNA (siRNA) to the multidrug resistance gene, ABCG2, to the liposomes using electrostatic attraction. They found that by combining docetaxel with ABCG2-siRNA and using the GE11 to target the delivery, they were able to improve the antitumor efficacies and specificities in laryngeal cancer cells [28].

2.2. Cell-Penetrating Peptides

Unlike CTPs which target a specific molecular marker on the cell membrane, CPPs interact with the outer leaf of the cell membrane using primarily electrostatic forces. Once CPPs adhere to the membrane they translocate through the membrane to the interior of the cell by mechanisms that are not well understood. Preferential targeting of cancer cells with CPPs is achieved by taking advantage of the finding that the outer leaf of the cell membrane in cancer cells is more negatively charged than the membrane of healthy normal cells [9][10][11]. Consequentially, the composition of a CPP generally consists of a high percentage of basic amino acids (arginine, lysine, and histidine), which at physiological pH carry a positive charge on their side chain. The positively charged CPP is then able to electrostatically adhere to the negatively charged membrane. The source of negative charges on a cell membrane originate mainly from the phospholipid heads of the lipid bilayer but can also include other charged membrane components such as proteins. In addition to the charge of a CPP, the hydrophobicity of the peptide is also important. Many CPPs are amphipathic where the hydrophilic and hydrophobic residues influence the conformation of the peptide and cause the CPP to form α-helices or β-sheets upon binding to membrane phospholipids. CPPs forming α-helices have a very hydrophobic area on one face while the other face aggregates charged amino acid sidechains to foster electrostatic interactions. CPPs conformed to β-sheets have a stretch of hydrophilic amino acids and a stretch of hydrophobic amino acids, which facilitate adherence to the membrane and subsequent internalization. Like CTPs, CPPs can be used to deliver therapeutic cargo to target cells. CPPs have been used to

deliver proteins, peptides, siRNAs, plasmid DNA and anticancer drugs. The cargo can be covalently conjugated to the CPP through chemical cross-linking or through cloning to create a CPP fusion product. Cargo with compatible charge to the CPP can also be non-covalently loaded onto the CPP using electrostatic forces. Small proteins, peptides and siRNA have been successfully delivered to targeted cells through non-covalent binding with CPPs [29][30]. The internalization mechanisms employed by CPPs are not well understood but broadly include endocytosis and direct translocation. However, predicting the mode of internalization of a CPP is difficult as it can change based on the concentration of the peptide, the cargo being carried, and the cells being targeted. In general, when CPPs are present on the membrane in high concentration they are internalized using energy-independent direct membrane translocation while at low concentration or when conjugated to cargo they use energy-dependent endocytosis to cross the membrane. There are, however, many examples in the literature where this general rule is not followed [31][32][33].

The promise of CPPs to deliver cargo to specific cell types while avoiding off-target effects has resulted in a skyrocketing of modified CPPs and the applications for their use. CPPsite 2.0 (https://webs.iiitd.edu.in/raghava/cppsite/index.html, accessed on 26 October 2021) is a database dedicated to compiling data on experimentally validated CPPs. At the time of writing there were 1855 entries in the CPPsite 2.0 database.

3. Possible Mechanisms of Therapeutic Peptides

The antitumor mechanism of therapeutic peptides is effectuated through many mechanisms including membrane disruption, apoptosis, tumor angiogenesis inhibition, immune regulation, or through inhibition of discrete internal targets [34][35]. The mechanism of action of many peptides involves the formation of pores or channels in the cell membrane. The pores can result in internalization of the peptide, but they can also be a means of cell death as a result of membrane disruption. The effect of a given peptide in this regard must be experimentally determined as the phenomenon is not well understood. However, several models have been proposed to explain the mechanics involved including the barrel-stave model, carpet model, and toroidal pore model which have been reviewed in several manuscripts [36][37]. In general, these models describe aggregation and arrangement of peptides to form channels into the cell membrane mediated by the amphipathic nature of the peptide and the phospholipid bilayer. The resulting conformational changes enable the peptide to enter the hydrophobic core of the membrane, where disruption of the membrane results in internalization of the peptide or cell breakage and necrosis as a result of dysregulated osmotic pressure. Significantly, cell death via membrane disruption can result regardless of growth rate or multidrug resistance mechanisms, conditions that often foil conventional chemotherapy approaches, while cationic residues in the peptide can enable preferential targeting of the peptide to the relatively anionic cell membrane of cancer cells. In addition to disrupting the cell membrane, peptides can also disrupt mitochondrial membrane potential resulting in the release of cytochrome c, activation of caspases, and induction of apoptosis [37][38][39].

Some peptides do not directly cause cancer cell death, but instead perturb the vascularization of the tumor to inhibit growth. These peptides inhibit vascular endothelial growth factor (VEGF) signaling, which normally signals

for the neovascularization of tumors. By inhibiting VEGF signaling, the peptides prevent tumor growth and metastasis while having minimal effect on normal cells that have low requirements for neovascularization.

Another way that peptides can be used as an anticancer therapeutic is to elicit a tumor-specific immune response. In a recent example of this approach, a cell-penetrating peptide named cytosol localizing internalization peptide 6 (CLIP6) was conjugated to a model antigen, ovalbumin (OVA) [40]. CLIP6 is a CPP which passes through cell membranes exclusively by direct translocation and not by endocytosis, an important characteristic in that endocytosis often leads to endosome entrapment. The investigators found that the CLIP6–OVA complex entered cells effectively and resulted in an enhanced antigen uptake by antigen-presenting cells such as dendritic cells. In vivo, they found that the CLIP6–OVA complex when combined with CpG, an immune adjuvant, was able to trigger a strong antigenic-specific immune response in mice. Using the B16/OVA mouse model, which is a melanoma cancer model with cell surface expression of OVA, the researchers found that two out of six mice immunized with the CLIP6–OVA/CpG became tumor free while mice immunized with OVA or CLIP6–OVA all died within 31–39 days after inoculation with tumors. The results of this study illustrate a role for CPPs in the development of preventative or therapeutic cancer vaccines.

4. Rational of Targeting PCNA-Binding Proteins with a Peptide Derived from Cancer-Associated PCNA

The development of new peptide stability and delivery strategies is opening novel avenues for designing peptide therapeutics that can target key proteins in tumorigenesis. Proliferating cell nuclear antigen (PCNA) is one such target, and it is an essential protein involved in many processes including DNA replication, DNA repair, chromatin organization, transcription, sister chromatin cohesion and cell cycle control [41][42]. PCNA is highly expressed in cancer cells and is critical for cellular proliferation.

A common strategy of designing peptide-based therapy is to disrupt protein–protein interactions (PPIs) with known protein binding sites. PCNA is a homotrimer that encircles DNA and acts as a platform that binds and coordinate proteins at the replication fork [43][44]. It is thought to interact and regulate over a hundred proteins [45] with various functions in the cell. Many, but not all, interacting proteins have conserved motifs that bind to PCNA, such as PIP-box (PCNA-interacting protein box), APIM (AlkB homologue 2 PCNA-interacting motif), and KA box (consisting of residues K-A-(A/L/I)-(A/L/Q)-x-x-(L/V)) [46][47]. The interdomain connector loop (IDCL) and a proximal hydrophobic patch on PCNA is one region where proteins have been indicated to interact [48][49], such as the PIP-box of p21 [50], peptide from the p66 subunit of DNA polymerase δ [48], and FEN1 [51].

To design a therapeutic peptide from PCNA that is specifically cytotoxic toward malignant cells, early studies focused on how PCNA in breast cancer cells was different. An isoform of PCNA was found in malignant breast epithelial cells and tissues but not non-malignant cells, using 2-dimensional PAGE experiments [52]. The cancer-associated isoform of PCNA (caPCNA) was likely from post-translational modifications [53] and not from genetic mutations or alternate splicing [54]. Cells from prostate cancer, hepatic carcinoma, high-grade prostatic intraepithelial neoplasia, and neuroblastoma [55][56][57] also had the unique PCNA isoform associated with cancer.

Antibodies were developed using peptides derived from PCNA and the antibodies were screened for their ability to recognize the caPCNA isoform. Epitope screening studies led to the discovery of an 8-amino acid peptide, dubbed caPeptide, within the IDCL. Conjugating nine D-arginines linked by two cysteines to the *N*-terminus of the peptide led to the development of a cell-permeable peptide, R9-caPeptide, that was found to selectively inhibit malignant cancer growth instead of non-malignant and normal cells [58][59]. R9-caPeptide is an example of a cationic CPP for delivery to cancer cells.

R9-caPeptide was found to disrupt interaction of PCNA with binding partners and growth inhibition experiments showed that R9-caPeptide was cytotoxic in a dose-dependent manner to cancer cell lines derived from breast, lymphoma, neuroblastoma, and pancreas [60][58][59][61]. Treating malignant cancer cells with R9-caPeptide also caused stalled DNA replication forks, DNA damage, cell cycle arrest, and apoptosis. Validating the therapeutic potential, R9-caPeptide inhibited, in mice, xenograft tumor growth from triple negative breast cancer and from neuroblastoma cell lines [58][59].

5. Use of Covalent Warheads in Peptide-Based Therapeutics

Peptide-based therapeutics may provide a viable means to target PPIs, such as for PCNA, as perturbing interactions between partners that involve large surface areas can be a significant challenge for organic small molecule-based therapies. Peptides inhibitors to PPIs can be generated from peptide library screening strategies, or from structural studies that have characterized the PPI interface. Peptides identified by these approaches typically require further optimization of their drug-likeness through improving: affinity, selectivity, stability and cell permeability. An interesting direction to improve affinity and selectivity has been to add functional groups that can form covalent interactions with the protein target side chains in the binding site; such covalent coupling to the protein target provides a greatly enhanced potency of the inhibitor. Acrylamides and chloroacetamides have been extensively studied as moieties for targeting the cysteine side chain, and are being used in covalent targeting strategies for organic small molecules and peptide-based therapies. However, the ‘cysteinome’, proteins containing targetable cysteine residues, is somewhat limited due to the relatively rare occurrence of cysteine in a protein sequence. Thus, recent studies have been exploring potential chemistries to target other side chains and thereby extend the number of proteins that can be targeted through covalent-based approaches.

The discovery that aryl-sulfonyl fluorides and aryl-fluoro sulfates can act as covalent-warheads within peptide inhibitors notably expands the list of covalently targetable residues to now include lysine, tyrosine or histidine side chains. Aryl-fluoro sulfates may be of the strongest interest for therapeutic development, as they were confirmed to be cell permeable and stable in both aqueous buffer and plasma [62]. However, a concern from an initial characterization of this covalent warhead was an observed slow reaction rate, questioning the overall effectiveness of this warhead in forming covalent adducts within the cell. The initial study targeted a lysine residue that was relatively distant from the protein binding site, and encouragingly, a follow up study instead targeted a lysine within the peptide binding site, and rapid covalent adduct formation was readily observed [63]. Similar results were also observed in a separate study targeting human Mcl-1, using a BH3 substrate peptide for generating pro-apoptotic agent [64]. Thus, the rapid bond formation together with the cellular permeability, and stability being akin to that

previously observed with acrylamides targeting cysteine, further suggests that aryl-fluoro sulfates may well expand the targetable residues beyond the cysteinome for novel therapeutic development. Other forms of potential covalent modifications strategies may be focused on the peptide substrate itself, adding stability and structure, to improve its binding characteristics. Cyclization of the peptide has been a common approach in this regard, and more recent studies have suggested other methods, including *N-locking*. A lactam bond is formed between the amino terminus and a glutamic residue at position 4, and this *N-lock* can nucleate helix formation within the peptide. *N-locking* can also be coupled with the covalent warhead strategy, as observed in BH3 peptides that were developed to target the antiapoptotic protein Bfl-1 to produce a peptide that was soluble in aqueous buffer and had low nanomolar affinity to its target [65]. Thus, these covalent-based approaches may provide novel avenues to develop peptide compounds with potentially more suitable ADME characteristics, and higher affinities and activities against their cellular targets.

6. Conclusion

A major consideration in developing peptide therapeutics is addressing delivery problems that prevent adequate quantities of peptide to reach cancer cells. To increase selectivity to malignant tissues and decrease toxicity, peptides can carry a load, such as a chemotherapy agent or another peptide that binds to proteins involved in tumorigenesis. Not only can peptide-based drugs disrupt essential PPIs utilized during the progression of cancer growth, but they can also disrupt membranes, affect the vascularization of the tumor, or induce an immune response that leads to cell death. The strategies and development of peptide therapeutics described here show promise in the laboratory with potential application to future cancer treatments.

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