Peptide-Based Vectors: A Biomolecular Engineering Strategy for Gene Delivery

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Abstract

From the first clinical trial by Dr. W.F. Anderson to the most recent US Food and Drug Administration–approved Luxturna (Spark Therapeutics, 2017) and Zolgensma (Novartis, 2019), gene therapy has revamped thinking and practice around cancer treatment and improved survival rates for adult and pediatric patients with genetic diseases. A major challenge to advancing gene therapies for a broader array of applications lies in safely delivering nucleic acids to their intended sites of action. Peptides offer unique potential to improve nucleic acid delivery based on their versatile and tunable interactions with biomolecules and cells. Cell-penetrating peptides and intracellular targeting peptides have received particular focus due to their promise for improving the delivery of gene therapies into cells. We highlight key examples of peptide-assisted, targeted gene delivery to cancer-specific signatures involved in tumor growth and subcellular organelle–targeting peptides, as well as emerging strategies to enhance peptide stability and bioavailability that will support long-term implementation.

INTRODUCTION

Gene therapy holds tremendous potential for the treatment of a wide variety of genetic diseases, including cancer, and it offers new options to improve upon conventional symptomatic treatments as well as provide real cures (1). Generally, to efficiently deliver therapeutic genes to their target sites, a safe, reliable, and efficient delivery vehicle is necessary for feasible and viable clinical application. Viral-based gene carriers comprise the majority of gene therapies, and

the past year has revealed both successes and challenges for this approach (2, 3). The rapid development and global distribution of multiple adenovirus vector vaccines represent a truly unprecedented advancement that is poised to aid in mitigating the catastrophic effects of the COVID-19 pandemic (4). During the same period, several high-profile gene therapy assets encountered setbacks, with clinical trials being halted due to safety concerns or inability to achieve efficacy targets. These successes and failures highlight the need for a wider range of gene delivery vehicles that can address concerns surrounding high immunogenicity, long-term expression, limited packaging capacity, risk of insertional mutagenesis, potential for carcinogenicity, poor tissue target specificity, complex manufacturing processes, and the challenges of large-scale production that exist with current technologies (5). Rising to meet these challenges, pharmaceutical and biotech firms are testing numerous nonviral-based carriers for gene therapy.

Gene delivery vehicles must overcome a variety of cellular and extracellular barriers, such as nonspecific protein binding, nuclease degradation, target site transportation, cellular internalization, endosomal entrapment, and subsequent intracellular cargo release. In the last few decades, nonviral counterparts such as various naturally derived, synthetic, or hybrid (natural and synthetic) peptides with varying sizes and structural features have become promising alternatives to viral carriers due to their larger genetic payload capacity, biocompatibility, improved pharmacokinetics by chemical modifications, and scalability 6–9). Cell-penetrating peptides (CPPs), a subclass of membrane-active peptides, are particularly intriguing due to their unique interactions with cellular membranes. CPPs can deliver bioactive cargo ranging from small drug molecules to large plasmid DNA (pDNA) into cells. CPPs typically are composed of short sequences of basic amino acids, allowing insertion and/or interaction with cell membranes and efficient translocation into cells. CPP–cell membranous lipid interactions are critical for CPP activity, and the extent of CPP–lipid interactions often correlates with permeation efficiency across the lipid bilayer (10). The reader is referred to informative reviews that were published recently in this field for more information about the background and uptake mechanisms of CPPs (11, 12). Another major focus of gene therapy is delivering nucleic acids to intracellular locations where they are therapeutically active. Intracellular targeting peptides (ITPs) offer promise based on their capacity to more specifically direct conjugated cargo across membrane barriers and into specific organelles. In some cases, when CPPs encounter barriers inhibiting

access to organelles such as mitochondria, the nucleus, or the cytoplasm, CPPs are fused with ITPs (13, 14).

We highlight promising peptide-based gene delivery strategies that have advanced through preclinical and clinical research. This review is structured into four sections. First, we review peptides targeting the tumor microenvironment (TME) and premetastatic niche. Second, we highlight peptides that aid in targeting genes to subcellular organelles (**Figure 1**). Third, we summarize a few strategies to enhance the resistance of peptide-based gene carriers to proteolytic enzymes. Finally, we discuss some of the interesting updates on CPPs that are currently under clinical trial evaluation for the treatment of various genetic diseases and disorders (**Figure 2**).

PEPTIDES TARGETING THE TUMOR MICROENVIRONMENT AND (PRE)METASTATIC NICHE

In this section, we review some of the recent advances in peptide-based delivery systems, focusing on peptides that target cancer specific signatures involved in tumor growth, invasion, vasculature, and premetastatic niche formation.

VEGFR-2- and NRP-1-Targeting Peptide

To maintain the balance between the development of new blood vessels and the maintenance and remodeling of existing ones during development and in adult tissues, vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) are essential (15). VEGFR-2 is overexpressed on endothelial cells, and neuropilin-1 (NRP-1), a cell-surface glycoprotein receptor, is also involved in angiogenesis and tumor growth (16). Therefore, VEGFR-2 and NRP-1 are two ideal targets for gene therapies because of their synergistic effects on angiogenesis (17). AT7 (ATWLPPR), identified by a phage display peptide library, exhibits high affinity for both VEGFR-2 and NRP-1 (18). Drug or gene delivery systems modified with AT7 possess efficient and effective active targeting ability for anticancer treatment. For instance, Lu et al. (19) elucidated the therapeutic potential of PPTA [polyethylenimine-polyethylene glycol (PEI-PEG) conjugated TAT-AT7] nanocarriers loaded with secretory endostatin genes for the treatment of glioma. Results from in vitro surface plasmon resonance (SPR) analyses showed good binding affinities between TAT-AT7 and both VEGFR-2 (K_D : 2.276×10^{-8} M) and NRP-1 (K_D : 2.740×10^{-8} M), with improved binding as compared to either peptide alone. In vitro biomimetic blood–brain barrier (BBB) model transport experiments and fluorescence imaging experiments in brain tissue from

orthotopic U87 glioma-bearing nude mice demonstrated that TAT (YGRKKRRQRRR) and TAT-AT7 increased penetration across the BBB (1.78 ± 0.11% penetration with TAT-AT7; 1.70 ± 0.06% penetration with TAT; 0.95 ± 0.07% penetration without peptide). Following intravenous (i.v.) injection of the PPTA/gene complex in an orthotopic U87-bearing nude mouse model, significant gene transfection efficiency, improved glioma targeting ability, and superior inhibitory effects on angiogenesis and glioma growth were observed. These results show that tandem peptide TAT-AT7 is a potential targeting ligand for the treatment of glioma.

Plectin-1-Targeting Peptide

Pancreatic ductal adenocarcinoma is a potentially lethal cancer that metastasizes in more than 80% of cases. Plectin-1 (PL-1) is a novel biomarker that was identified in 100% of the invasive pancreatic ductal adenocarcinoma lesions based on analysis of patient samples obtained from the pathology department of Massachusetts General Hospital in Boston (20). However, this cytoskeletal component is not expressed in normal human pancreas, liver, or lungs. By using phage display screening, plectin-targeted peptides (PTPs) were found to specifically bind to PL-1 (21). To treat pancreatic cancer, Li et al. (22) developed a redox-sensitive co-delivery system based on branched PEG with G2 dendrimers through disulfide linkages (PSPG), which was responsive to the intracellular glutathione (GSH). PTP (KTLLPTP), a PL-1-targeting peptide, was then coupled with the PSPG vector to form peptide-conjugated nanoparticles (PSPGP NPs) for targeted co-delivery of testicular receptor 3 (TR3), an orphan nuclear receptor silencing RNA (siRNA) (siTR3) and paclitaxel (PTX) in pancreatic cancer cells. The PSPGP NPs exhibited excellent loading capacity for both siTR3 and PTX with a flexible mass ratio of the two cargoes. In vitro results showed that PSPGP NPs effectively facilitated cellular uptake and high transfection efficiency in Panc-1 cell lines. Moreover, siTR3-mediated knockdown of *TR3* gene decreased the expression of antiapoptotic proteins including Bcl-2 and survivin in cancer cells. Interestingly, in vivo results also showed that systemic administration (i.v.) of the co-delivery system into Panc-1 tumor bearing mice resulted in a significant synergistic effect of PTX and siTR3 in treating cancer by inhibiting tumor growth and inducing apoptosis in pancreatic cancer cells.

LRP-1-Targeting Peptide

Lipoprotein receptor related protein 1 (LRP-1) is highly expressed in glioblastoma (GB) cells and GB-associated endothelial cells, and it binds to multiple ligands and controls blood–brain barrier permeability (23, 24). Demeule et al. (25) developed angiopep-2 (ANG), a shortened peptide variant of the active LRP-binding domain (Kunitz-type) of aprotinin. When compared to other aprotinin-derived peptides and the original aprotinin, ANG (TFFYGGSRGKRNNFKTEEY) demonstrated the highest permeability and LRP-mediated transcytosis. ANG has been used successfully in preclinical studies and clinical trials to deliver therapeutic and imaging agents to primary and secondary brain tumors. In another report, Srimanee et al. (26) established a PF14 (stearyl-AGYLLGKLLOOLAAAALOOLL-NH2) modified with ANG via noncovalent complex formation for oligonucleotide delivery into human GB U87 cells. In vitro luciferase assays revealed that the PF14/ANG/siRNA complex outperformed the parent PF14/siRNA complex in terms of glioma-targeted specificity and gene-silencing efficiency, which was enhanced by receptor-mediated gene uptake. These results suggest its great potential for further in vivo study and, in the future, gene therapeutic applications.

Integrin-Targeting Peptide

Integrins are transmembrane proteins that facilitate cell adhesion, signal transduction, cell migration, and differentiation. They exist as obligate heterodimers composed of α and β subunits (27). In addition to binding the extracellular matrix (ECM), integrins also bind to endogenous ligands such as soluble proteins and cell surface proteins. The vitronectin receptor, also known as integrin αvβ3, is a key player in angiogenesis and tumor metastasis and binds to a variety of ECM proteins with the RGD motif (28). In this context, Cen et al. (29) prepared a novel methoxy-modified phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta (PIK3CB) siRNA molecule (siPIK3CB) that was covalently linked to a [cyclo(Arg-Gly-Asp-D-Phe-Lys)-Ahx]2-Glu-PEG-MAL (biRGD) peptide, which selectively binds to integrin αvβ3 receptors (Ahx, 6-aminocaproic acid; MAL, maleimidopropionic acid). The authors selected the αvβ3-positive U87MG cell line as a representative for human GB cells. In vitro results showed that biRGD-siPIK3CB specifically entered and silenced PIK3CB expression in GB cells via receptor-mediated endocytosis, resulting in induction of apoptosis and cell cycle and migration arrest. In vivo, in an orthotopic GB xenograft model based on luciferase-expressing U87MG, i.v.-injected biRGD-siPIK3CB substantially slowed tumor growth and prolonged the survival

time of mice by reducing tumor viability via silencing PIK3CB expression. Furthermore, these findings revealed the great translational potential of the biRGD-siPIK3CB conjugate as a novel integrin αvβ3 targeting ligand for GB therapy.

Fibronectin-Targeting Peptide

The TME plays an essential role in tumor progression and metastasis. A major component of the TME is the ECM, which is composed of distinct components including collagen, proteoglycans, fibronectins, and laminins (30). Fibronectin has been used to develop antibody-targeted vehicles for specific and effective delivery of imaging agents and therapeutic drugs to metastatic sites (31). The tumor homing linear pentapeptide Cys-Arg-Glu-Lys-Ala (CREKA) has specific affinity toward fibrin clots in tumor ECM, with negligible binding in healthy tissues. Based on this, pH- and GSH-sensitive GALA-PEG-SS-PEI-CREKA NPs were developed to treat triple-negative breast cancer (TNBC) in combination with siEGFR and siBR4 (32). The epidermal growth factor receptor/phosphatidylinositol 3-kinase and protein kinase B (EGFR/PI3K/Akt) signal pathways and the bromodomain-containing protein 4 (BRD4)/c-Myc signal pathway are associated with TNBC pathogenesis and progression. In this system, GALA, a 30-residue amphiphilic and pH-sensitive peptide with the repeat sequence Glu-Ala-Leu-Ala, was demonstrated to effectively cross lipid bilayers in cells and aid gene-loaded vesicles in eluding endosomes through endocytosis. In vitro gene silencing study results illustrated that the gene delivery system significantly inhibited MDAMB-231 cell proliferation, invasion, and migration via synergistic inhibition of the EGFR/PI3K/Akt and BRD4/c-Myc pathways. Significant cellular uptake, intracellular localization, and lysosome escape of the siRNA were also observed. Together, the co-delivery of siEGFR and siBRD4 using TME-targeting peptides (GALA and CREKA) may provide a more effective strategy to treat TNBC.

Ganglioside-Specific Binding Peptide

Buforin IIb is an anticancer peptide derived from histone H2A that can specifically deliver therapeutic nucleic acids into cancer cells. However, buforin IIb is cytotoxic to normal cells at high concentrations. The stepwise elimination of the C-terminal RLLR repeats of buforin IIb results in BR2 (RAGLPFQVGRLLRRLLR), a cancer cell–specific peptide that can penetrate cell membranes through interactions with cell surface gangliosides (via lipid raft–mediated macropinocytosis), with reduced collateral cytotoxicity in normal cells (33, 34). In one example,

Lee et al. (35) developed cancer-specific CPP carriers based on BR2 to selectively deliver antivascular endothelial growth factor siRNAs (siVEGF) into HeLa cells. BR2-mediated siRNA delivery was achieved by means of electrostatic interaction between anionic siRNAs and cationic CPPs. Notably, the BR2-VEGF siRNA complex demonstrated significant serum stability as well as high levels of gene silencing in vitro. Higher levels of siVEGF internalization were found in human colon cancer cells and HeLa cells, resulting in a significant reduction in intracellular VEGF levels and overall improved antitumor efficacy in the absence of significant toxicity. These findings suggest that BR2 has significant potential for the safe, efficient, and targeted delivery of siRNA for various applications.

Additional Targets

Transmembrane receptors such as epidermal growth factor receptor (EGFR) could also be a target for cancer therapy. EGFR activates the PI3K/Akt signaling pathway which is overexpressed in various malignancies including TNBC. Recent research and review articles have highlighted the potential use of peptides that specifically targeting the EGFR for cancer treatment (35a). For a deeper understanding of the potential use of EGFR targeting peptides in gene delivery, the reader is referred to these articles (35b).

INTRACELLULAR TARGETING PEPTIDES TO TARGET SPECIFIC CELL ORGANELLES

Several therapeutic gene targets are located inside subcellular organelles. Autoimmune diseases, congenital disorders of glycosylation, cancers, and neurogenerative diseases are all caused by dysfunction at the subcellular level (36–39). For instance, the endoplasmic reticulum's (ER's) primary function is to facilitate the folding of secretory and membrane proteins. Exposure to free radicals, oxidative stress, elevated protein synthesis, and gene mutations causes ER dysfunction and ER stress, which may result in protein misfolding or in the production of mutant proteins. Familial hypoparathyroidism, coagulation factor X deficiency, and Crigler–Najjar syndrome type II are some of the diseases related to ER dysfunction (40). Genes such as pDNAs, siRNAs, and messenger RNAs (mRNAs) can be used to treat these diseases by interfering with the production of disease-causing proteins, inducing the destruction of the mRNAs of targeted genes, or by

targeting other subcellular targets. Typically, siRNA and mRNA cargoes reach their final destination, the cytosol, during the CPP-mediated intracellular delivery of genes by evading the endosome, whereas DNA cargoes need additional translocation to the nucleus or to the mitochondria. Consequently, to successfully deliver nucleic acids, intracellular barriers such as endosomal entrapment, lysosomal degradation, the complex cytoskeleton, the mitochondrial double membrane, or the nucleus envelope must be overcome (41). In this context, ITPs serve as a promising approach to specifically direct their cargo to the respective organelles and ensure membrane interactions that support delivery. Targeting specific organelles would also result in a maximized therapeutic effect and minimized side effects. In the following section, we spotlight various key classes of organelle-specific targeting peptides that are beneficial for enhancing nucleic acid delivery.

Nuclear Delivery

The sequestering of genetic material within the nucleus makes it a key component of the cellular machinery for regulating gene expression and for the selective translocation of proteins between the nucleus and the cytoplasm (42). Moreover, various DNA viruses spread toxicity by delivering their genetic material to the nucleus, where it undergoes further replication and generates viral particles (43). The study of these viral delivery mechanisms has aided the development of nuclear localization signaling (NLS) peptides. For instance, small peptide sequences such as KKKRKV from the SV40 large T antigen exhibit nuclear localization (44). Proteins with such NLS sequences can be recognized in the cytoplasm by importin and translocated into the nucleoplasm via nuclear pore complex structures on the nuclear envelope, which actively control molecular translocation in and out of the nucleus (45). The other most common studied NLS peptides are nucleoplasmin (KRPAATKKAGQAKKKK) from *Xenopus* protein (46) and polyarginine (47). Several peptide gene carriers modified with these NLS peptides have shown improved accumulation within the nucleus (48, 49).

Aberrant gene expression is implicated in a variety of diseases, including cancer and neurodegenerative and cardiovascular disease, at both the transcriptional and/or translational levels (50, 51). For these applications, several interesting peptide-based gene delivery systems have been developed, including a recent approach of using peptide carriers to deliver therapies for the treatment of breast cancer. In this study, anti–B-cell lymphoma 2 (BCL2) antisense oligonucleotides (ASOs) loaded into an amphiphilic peptide carrying an N-terminal KRKR

sequence that functions as a NLS peptides were used to knock down the *BCL2* gene, which encodes an antiapoptotic protein in MCF-7 cancerous cell lines (52). This amphiphilic peptide (KR)2(HR)2(WL)7W was composed of a nuclear targeting moiety together with the integration of a CPP sequence to promote nuclear targeting in addition to enhancing cellular uptake. In vitro self-assembly studies demonstrated that the amphiphilic NLS peptide initially formed micelles that ultimately assembled into multicompartment micelles (MCMs) with the entrapment of DNA in the micelles. A surface plasmon resonance study revealed that the increased cellular uptake of NLS MCMs was due to NLS interactions with importin receptors via karyopherin α proteins. Importantly, treatment of MCF-7 cells with anti-BCL2 ASO-loaded NLS MCMs resulted in up to 86% knockdown of *BCL2*, an apoptosis inhibitor that is overexpressed in more than half of all human cancers. Overall, these findings motivate the further development of these NLS MCMs for systemic gene therapy and vaccination applications.

In another report, Panigrahi et al. (53) designed a multifunctional CPP and NLS cyclopeptide CSP2 (Cyclo [WWWWGGRRRRGC]) to transport VEGF siRNA and ASO into colon cancer cells. The authors reported previously that a cyclic peptide-based nanostructure with arginine and tryptophan residues (Cyclo [WWWWGGRRRGG]) could successfully deliver siRNA (54). Incorporating cysteine residues in the CPPs enabled gene delivery into the nucleus. At mRNA and protein levels, CSP2-mediated nucleic acid carriers showed excellent protein silencing efficiency compared to a well-established TAT (YGRKKRRQRRR) peptide with negligible cytotoxicity. Thus, this new cyclic peptide CPS2 provides a promising tool for nucleic acid delivery for future RNA interference (RNAi) therapeutic development, especially for cancer treatment.

Overall, studies have demonstrated the potential role of NLS peptides and CPPs bearing NLS peptides in effectively transporting genetic cargoes across the nuclear membrane and into the nucleus. NLS sequences also have been widely used to deliver cargoes such as proteins, peptides, and oligonucleotides for the treatment of various genetic diseases and cancer (55, 56).

Mitochondria Delivery

Mitochondria play a key role in generating adenosine triphosphates (ATPs), modulating intracellular calcium concentration, and regulating apoptosis. Mutations in apoptosis -related genes may be involved in the development of chemoresistance in most human cancers. Mitochondria also perform oxidative phosphorylation via the electron transport chain, which is

an important process for removing reactive oxygen species (ROS). As a result, mitochondrial dysfunction is found in a variety of adult-onset diseases, including obesity; diabetes; stroke; and Parkinson's, kidney, and liver diseases (57). To overcome these challenges and advance mitochondrial medicine, peptides that can translocate into cells and localize to mitochondria could be very helpful in developing new treatments for mitochondrial diseases (58, 59).

In a recent report, to regulate levels of ROS in a cell, a combinatorial covalent fusion of a mitochondrial-penetrating peptide, mtCPP1 (D-Arg-Dmt-Orn-Phe-NH2, Dmt: 2,6-dimethyl-L-tyrosine; Orn: Ornithine), and PepFect14 (Stearyl- AGYLLGKLLOOLAAAALOOLL) was used for intracellular localization of ASO to silence mRNA coding uncoupling protein 2 and cytochrome c oxidase subunit II (60). These peptides were noncovalently complexed with oligonucleotides, resulting in nano-complexes that impacted mitochondrial biological processes. The silencing of uncoupling protein 2 and cytochrome c oxidase subunit II, a mitochondrial anion carrier and a mitochondrial protein that make up complex IV of the electron transport chain, yielded significant changes in the levels of ROS and mitochondrial membrane potential, respectively. These findings might have significant implications for the development of therapeutic modality for mitochondrial diseases.

For an efficient mitochondrial transfection, an understanding of the physicochemical mechanism of peptide/gene self-assembly is necessary, which in turn contributes to the understanding and further improvement of the biophysical properties of peptide/gene systems. Chuah et al. (61) assessed the critical physicochemical and biological parameters (size, surface charge, complex stability, peptide and DNA dose) for their capacity to govern the efficiency of mitochondrial-targeted delivery systems. In this study, dual-domain peptides containing lysine-histidine (KH)-rich sequences (for DNA condensation, cell penetration, and endosome disruption) were fused with mitochondria-targeting sequences (MTSs) of cytochrome c oxidase (cytocox) subunit IV and the human hepatic enzyme ornithine transcarbamylase (OTC). Cytocox-KH was produced by the MTS (MLSRQSIRFFK) of cytocox fused to KH sequences, whereas OTC-KH was produced by the MTS (MLFNLRILLNNAAFRNGHNFMVRNFRCGQPLQ) of OTC fused to KH sequences. Cytocox-KH and OTC-KH self-assembled upon mixing with DNA into nanocomplexes. An optimized peptide/pDNA formulation, identified through qualitative and quantitative studies, identified key physicochemical parameters for mitochondria-specific gene delivery and

successfully transfected a high proportion of mitochondria in a human embryonic kidney 293 cell line. Proton nuclear magnetic resonance studies confirmed the efficacy of a dual-domain peptide design with distinct functions: A helical MTS domain was required for intracellular transfection, and an unstructured KH-rich region was required for pDNA interaction. Further investigation revealed that the lysine-specific interaction aided in the self-organization and structural arrangement of the formed peptide/pDNA complex, which is crucial for its biological efficiency. Through these results, the authors concluded that the reported nonviral gene vector represented a novel tool for investigating mitochondrial transfection.

Overall, these findings along with the other recent reports demonstrated effective targeting of mitochondrial genes, offering a framework for the development of peptide-based gene delivery systems that could be used to treat patients with mitochondrial disorders (62, 63).

Lysosomal Delivery

The lysosome plays a critical role in maintaining cellular homeostasis. Dysregulation of lysosome functions may affect several components of the cellular metabolic machinery, such as the transport and biogenesis of proteins, nucleic acids, sugars (glycolysis), and lipids, resulting in alteration of cellular metabolic pathways. Genetic deficiencies of lysosomal enzymes lead to lysosomal dysfunctions known as lysosomal storage disorders (LSDs). Enzyme replacement therapy is one of the clinically proven strategies for treating LSDs by delivering lysosomal enzymes specific to the condition (64). For instance, Fabry disease (FD) is an inherited lipid metabolism disorder caused by deficiency of a lysosomal enzyme known as alpha-galactosidase A (GLA) (65). Mutations in the GLA gene cause this enzymatic deficiency. In light of this, a simple enzyme replacement therapy was developed in a report using poly-lysine and poly-histidine peptide (K10H16) as a carrier to facilitate GLA delivery into lysosomes (66). Initially, the lysosome-specificity of K10H16 was demonstrated by K10H16-mediated delivery of maltose-binding protein-fused green fluorescent protein (MBP-GFP), an acidic model protein that was electrostatically complexed with K10H16. Subsequently, GLA was coloaded with MBP-GFP resulting in delivery into the lysosomes of human fibroblast cells via macropinocytosis. Additional in vitro studies showed that the combination of GLA and the K10H16 peptide restored cell proliferation by 32% in fibroblasts from FD patients, demonstrating the potential efficacy of the K10H16 delivery system for enzyme replacement therapy. In another approach, K10H16-modified liposomes were designed to deliver GLA to

intracellular lysosomes and improved the proliferation of GLA-knockdown HT1080 cells (67). These findings indicate that this K10H16 and liposome combination is an effective gene carrier for lysosomal enzymes in a lysosome-targeting nucleic acid delivery for the treatment of LSDs.

Appropriate organelle targeting improves therapeutic outcomes and minimizes adverse side effects. The above strategies ensure the delivery of therapeutic genes to lysosomes and aid in restoring normal lysosome function.

Golgi and Endoplasmic Reticulum Apparatus Delivery

The Golgi and ER apparatus are involved in the secretory pathway for the transport of newly synthesized proteins, and they also support important posttranslational modifications such as phosphorylation, acylation, glycosylation, methylation, and sulfation (68). Mutations in genes that encode glycosylation enzymes or glycosylation-linked transport proteins can cause congenital disorders of glycosylation and muscular dystrophies. Furthermore, both the Golgi apparatus and the ER also have been proposed as future anticancer targets. In this context, a recent approach established by Acharya et al. (69) has shown promise for enabling the long-term development of therapeutic strategies to counteract muscular dystrophy with the help of an ER-targeting peptide (KDEL) that directs disease-targeting genes into the muscle cells. In this report, gold NPs modified with KDEL (Lys-Asp-Glu-Leu) peptides were developed to deliver siRNA directed against NADPH oxidase 4 into C2C12 myoblasts and myotubes. KDEL receptors are expressed ubiquitously across various mammalian cell types (70). The KDEL amino acid sequence is recognized by the KDEL receptor and is a highly conserved motif in the directed retrograde transport of proteins to the ER. It serves as a retention signal for both soluble and transmembrane proteins that are transported in vesicles through a coat protein-I–mediated mechanism. Fluorescence microscopy analysis revealed the intracellular colocalization of NPs and retrograde cellular transport pathways in both undifferentiated C2C12 myoblasts and differentiated C2C12 myotubes. In vitro results also showed that the gold-KDEL nano system was less cytotoxic but equally as effective as lipofectamine at delivering siRNA and silencing targeted mRNA in both C2C12 myoblasts and myotube cells. This study demonstrates the potential use of gold-KDEL nanoconjugates as an ER-targeting gene delivery system.

Another intriguing study used pardaxin, a Golgi- and ER-targeting peptide, to investigate the mechanism by which dendritic cells (DCs) promote $CD8^+$ T cell responsiveness to exogenous antigens, resulting in increased overall host defense against infections or cancer (71). Pardaxin

(also known as FAL) is a cationic polypeptide chain (H-GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE-OH) derived from the Red Sea Moses sole (*Pardachirus marmoratus*) (72). ER-targetable pardaxin-based liposomes were designed, loaded with ovalbumin (OVA) as a model antigen, and characterized to demonstrate a booster effect on DC activation and maturation. Furthermore, both in vivo and in vitro, OVA liposome–pulsed DCs demonstrate a significantly superior ability to induce a cytotoxic T lymphocyte response. Data showed that OVA liposomes modulate the antigen's intracellular trafficking and presentation pathway, promoting cross-presentation and bearing a close relationship to ER-associated degradation. These findings may shed light on the relationship between cross-presentation and ER-targeted antigen delivery, adding to our understanding of ER-associated degradation–mediated cross-priming.

Overall, these reports have highlighted the potential functions of Golgi- and ER-targeting peptides. An exciting opportunity exists to take advantage of these peptides to promote intracellular transport of nucleic acids and to ultimately increase its transfection efficiencies. **Table 1** lists the various subcellular organelle-specific targeting peptides and their sequences.

APPROACHES FOR ENHANCING PEPTIDE STABILITY AND DELIVERY

Proteolysis is a potential problem associated with peptides, which limits the widespread use of peptides in gene or drug delivery applications. Peptides are rapidly degraded by endogenous proteases such as exoproteases and endoproteases. As a consequence, most linear peptides with all natural L stereo forms of amino acids have a half-life of less than 30 min in serum, which is insufficient to enable efficient targeting, especially of large therapeutics. Different strategies are being used to enhance peptide resistance to proteases by incorporating alpha amino acids with noncanonical side chains, via cyclization and conjugation of peptides with another molecule or NP (73–75). In this section, we discuss some of the strategies commonly used to enhance the peptide carrier's stability and bioavailability.

L/D-Amino Acid Stereochemistry

Incorporation of D-amino acid residues with noncanonical side chains is an alternative strategy for decreasing protease enzyme recognition. Replacement of all the L-amino acids with D-amino acids in the peptide sequence forms a retro-inverso peptide. The retro-inverso peptides often

have similar biological activity to the parent peptide sequence while presenting full resistance to proteolytic degradation. For example, the CADY-K (GLWRALWRLLRSLWRLLWK) peptide, a short version of CADY (Acetylated-GLWRALWRLLRSLWRLLWRA-cysteamide), has higher biological activity than the parental peptide. However, proteolysis compromised its in vivo application (76). Therefore, a peptide–gene complex was formulated with a retro-inverso analog of CADY-K, called RICK (77). This complex showed that RICK [$_{D}$(KWLLRWLSRLLRWLARWLG)] retained the main unique properties of CADY-K, such as conformational versatility, formation of RICK:siRNA NPs, and remarkable efficacy in siRNA cellular delivery in U87 cell lines, and also demonstrated proteolytic stability over preincubation with trypsin and serum.

In another approach, Li et al. (78) investigated the retro-inverso peptide D(PRPSPKMGVSVS) (D-SP5) as a targeting peptide to develop gene therapies for gastric adenocarcinoma. D-SP5 has a higher affinity for human gastric adenocarcinoma (SGC7901) cells because of its unique interface geometries, which are not available to L-SP5. In vitro results also showed that the expression of tumor necrosis factor–related apoptosis inducing ligand (TRAIL) protein in SGC7901 cells treated with D-SP5-PEG-PEI/p*TRAIL* showed higher cellular viability compared with those treated with mPEG-PEI/p*TRAIL* (control). In vivo pharmacodynamics studies and tunnel assays with i.v.-administered D-SP5-PEG-PEI/p*TRAIL* in BALB/c nude mice with SGC7901 xenografts revealed a better treatment effect and a higher apoptotic level in the treatment group, respectively.

Follicle stimulating hormone (FSH) is a glycoprotein hormone consisting of α and β chains, and some FSH receptor–binding domains have been identified, including amino acids 1–15, 33–53, 51–65, and 81–95 of the FSH β chain (79). Because FSH β 33–53 amino acids have the strongest binding affinity, a 21-amino-acid peptide, YTRDLVYGDPARPGIQGTGTF (FP21), corresponding to this site was designed as a target-specific ligand to distribute nucleic acids to ovarian tumor tissue. In a study aimed at delivering growth-regulated oncogene alpha (p*GRO-α*) short hairpin RNA to ovarian cancer cells, Agris et al. (80) designed a PEG-modified PEI nanovehicle conjugated with D-FP21 peptides (D-FP21-PEG-PEI/p*GRO-α*). In vivo results showed that when the (D-FP21-PEG-PEI/p*GRO-α*) complex was i.v. injected into the bloodstream, it demonstrated superior antitumor activity, reducing tumor spheroid volumes in HO8910 tumor-bearing BALB/c nude mice from 33.3% to 58.5%. This is most likely due to the

ligand's resistance to hydrolysis by systemic endogenous peptidases, which results in improved biostability and tumor targeting (81). Together, these results demonstrate the utility of peptide stereochemistry, as well as the importance of a key D-amino acid modification, in advancing the peptide-based carrier design for the enhancement of gene silencing in cancer cells.

Peptide Cyclization

Linear peptides are more prone to proteolytic degradation when compared to cyclic peptides. Cyclization can improve the hydrolytic stability and targeting efficacy of a linear peptide based upon the constrained geometry of the peptide incurred by cyclization, as well as the inaccessibility of terminal residues to exoproteases. When compared to their linear counterparts, cyclic peptides generally exhibit increased biological activity.

Mandal et al. (82) designed redox-responsive disulfide cyclic and hybrid peptides using tryptophan (W) and arginine (R) residues to deliver STAT3-targeting siRNA into TNBC MDA-MB-231 cells. Among the different combinations of R and W amino acids, C4 (CR5W5C), a cyclic peptide containing R and W residues and two cysteines (C) cyclized through a disulfide bridge, and H4 (W4CR5CW4), a hybrid cyclic-linear peptide containing R and C residues on the ring attached to W chains, showed superior efficiency in delivering siRNA into the TNBC cells. For a comparative study, the linear counterparts of cyclic and hybrid peptides were also synthesized. However, the strongest binding affinity of siRNA was observed for C4 and H4 peptides compared to linear and other combination peptides. In vitro results showed effective cellular internalization of siRNA (~70% of the cell population), siRNA protection against early degradation by nucleases, and efficient protein silencing (70–75%), resulting in reduced STAT3 expression in human TNBC MDA-MB-231 cells via use of C4 and H4 peptides. By balancing the number, location, and orientation of hydrophobic and positively charged amino acid residues, it was shown that the overall structure of the peptide played a crucial role in appropriate siRNA interactions and delivery.

Suicide gene therapy is a potential treatment for uterine fibroids (UFs). UFs are the most common benign tumors in reproductive-age women. DNA therapeutics used for this treatment should be delivered specifically to UF cells for successful suicide gene therapy. Recently, polycondensed peptide carriers modified with a cyclic RGD moiety (R6p-cRGD/pPTK) were established to deliver genes to UF cells (83). CPPs with R residues aided in the formation of stable complexes with nucleic acids and actively promoted their intracellular uptake via

hydrogen bond formation with cell surface glycosaminoglycans. Threefold increases in the transfection efficiency were observed in αvβ3-positive PANC-1 cells with the conjugation of cRGD to R6p/pPTX plasmid polyplexes. In vitro results also demonstrated that the treatment with R6p-cRGD/pPTK complexes induced suicide-specific cell death, with the majority of the cells being detected at an early stage of apoptosis. Furthermore, the polyplex treatment significantly reduced the proliferation activity and the number of viable UF cells.

Although cyclic CPPs typically are more effective than linear CPPs for gene delivery, their cytosolic entry efficiency is often significantly diminished at low concentrations or in the presence of serum proteins. This is because CPP binding to serum proteins reduces the concentration of free CPPs; moreover, the endocytic uptake or the endosomal escape is CPP concentration dependent. Cyclic CPP12 is one such example; CPP12, cyclo(F_DF-Nal-R_DRR_DRQ) (Nal, l-2-naphthylalanine) is very effective at delivering a variety of cargo molecules into the cytosol of mammalian cells. However, at lower concentrations, its cytosolic entry efficiency is reduced significantly. The ability of CPP12 analogs to enter cells was tested by swapping out the hydrophobic residues with amino acids with varying degrees of hydrophobicity, resulting in CPP12-2, which demonstrated up to 3.8-fold-higher cytosolic entry efficiency even at low concentrations than its parent form (CPP12). CPP12-2 was also tested for cytotoxicity against HeLa cells. CPP12-2 had no effect on the viability of HeLa cells at concentrations up to 25 μM. Thus, CPP12-2 is an excellent choice for cytosolic delivery of highly active cargoes to achieve biological activity at low concentrations (84). Overall, the above results prompted researchers to develop structurally constrained and cyclic peptides as second-generation CPPs for effective and efficient nucleic acid delivery.

CLINICAL DEVELOPMENTS IN PEPTIDE-CARRIER TECHNOLOGY

More and more CPPs are being investigated in clinical trials to treat cancer and other diseases. In this section, we summarize a few clinical trials based on CPPs used in drug and gene delivery systems aimed at lowering postoperative pain, treating intraocular inflammation, and reducing chemotherapy-induced side effects, including CPPs under exploration within delivery vehicles intended for treatment of brain tumors, breast cancer, colorectal cancer, and other diseases.

Capstone Therapeutics Corp. developed a CPP-conjugated peptide called AZX100 to disrupt the expression of connective tissue growth and reduce fibrosis and scarring. The AZX100 protein

is a phosphorylated peptide analog of HSP20 (WLRRAS[P]APLPGLK) and PTD4 (YARAAARQARA). A phase II clinical trial was conducted and completed in 2012 to determine its potential to alleviate keloid scarring (85, 86).

PEP-Therapy and Institut Curie are conducting a first-in-human clinical trial of PEP-010 for the treatment of advanced solid tumors. Both CPP and interfering peptides (to block caspase-9/PP2A interaction) were included in PEP-010's single peptide technology. PEP-010 has been demonstrated to facilitate an 85% reduction in tumor growth in patient-derived xenograft tumor models of breast and ovarian cancer (87).

AMAL Therapeutics, a Boehringer Ingelheim subsidiary, developed the protein vaccine known as ATP128 to treat stage IV colorectal cancer. The ATP128 chimeric recombinant protein vaccine contains a TLR peptide agonist, multiple antigenic domains, and a CPP for antigen delivery. AMAL conducted KISIMA-01, an international phase Ib clinical study, to evaluate the combination of ATP128 with the anti-PD1 antibody ezabenlimab in microsatellite-stable patients with stage IV colon cancer. Studies show that enhanced T cell quality correlates with an ATP128-specific immune response in the peripheral circulation and increased infiltration of tumor-infiltrating lymphocytes (TILs) into liver metastases (88).

ATX-101, a peptide with nuclear localization domains and cell-penetrating properties developed by APIM Therapeutics, targets the proliferating nuclear antigen, a critical scaffold protein involved in numerous cellular processes. Currently, two clinical trials are underway: a phase Ib/IIa study in platinum-sensitive ovarian cancer, in which ATX-101 is being tested in combination with platinum-based chemotherapy, and a phase II study in sarcoma, in which ATX-101 is being tested as monotherapy (89).

Aileron Therapeutics is currently in phase Ib clinical trials with ALRN6924, a biomarker-driven selective cell-permeable stapled peptide that targets p53, which temporarily pauses the cell cycle in normal cells. It is designed to selectively protect healthy cells in patients with cancers that harbor p53 mutations to reduce or eliminate chemotherapy-induced side effects without interrupting chemotherapy's destruction of cancer cells. In recently generated data, higher ALRN-6924 (1.2 mg/kg) dose levels yielded longer-lasting pharmacodynamic effects (90). Additional clinical trials are listed and summarized in **Table 2**.

CONCLUSIONS

In recent years, gene therapy has made significant advances in several therapeutic areas, targeting various specific cell groups. However, most of the enrolled clinical studies used traditional viral vector systems, which are challenging to scale cost-effectively. Peptides have been a fast-paced research topic in gene delivery, such as those from natural or synthetic sources and/or combinations of different hybrid types. Although peptide carriers offer targetability, nucleic acid condensation, subcellular localization, and protease and endosomal escape, attracting more and more researchers to explore the promising delivery system, they do not carry ideal characteristics and have faced critical challenges, such as short circulation half-lives and inadequate biodistribution, gene transfer efficiency, and use of multiple peptides may also increase complexity, cost of production, stability and safety issues (90a). Developing an effective and safe nucleic acid vector necessitates a thorough understanding of the biological properties of nucleic acid materials, physicochemical properties of carriers, and the physiology of the specific cells to be targeted, as well as a molecular-level understanding of peptide carrier-induced transfection.

Despite many challenges, advances in the development of novel peptide-based gene therapeutic modalities can be seen in the increasing number of these treatments that are currently being tested in clinical trials. The US Food and Drug Administration (FDA) recently lifted the clinical hold on Sarepta Therapeutics's SRP-505, a CPP-based RNA technology for the treatment of Duchenne muscular dystrophy, on September 6, 2022 (91). The FDA approved Revance's DAXXIFY® (daxibotulinumtoxinA-lanm) as the first and only CPP-based neuromodulator formulation (92). Melflufen (melphalan flufenamide, PEPAXTO®), the first peptide-coupled drug, was also approved by the FDA on February 27, 2021, for the treatment of multiple myeloma (93). These recent successes have fueled excitement and hope that clinically relevant gene delivery technologies will emerge and provide a new approach to disease treatment in the coming decade.

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The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Figure 1 Schematic illustration of (*a*) construction of an ST21-H_3R_5-PEG–based nano system for the simultaneous co-delivery of fasudil and miR195 to SK-Hep-1 human hepatocellular carcinoma cells (adapted with permission from Reference 17; copyright 2016 Dove Medical Press Ltd.). (*b*) Self-assembled DNA-loaded NLS-functionalized MCMs loaded with anti-BCL2 ASO to knock down *BCL2*, an inhibitor of apoptosis. (adapted with permission from Reference 52). (*c*) LAH4-L1, an amphipathic cationic polypeptide designed to deliver siMDR1 for overcoming ovarian cancer cell MDR (adapted with permission from Reference 9). (*d*) Self-assembled peptide nanospheres, CPS2 (cyclo [WWWWGGRRRRGC]) loaded with VEGF ASOs and siRNA for targeting nucleus and cytoplasm (adapted with permission from Reference 53). Abbreviations: ASO, antisense oligonucleotide; H3R5, disulfide cross-linked stearylated polyarginine peptide modified with histidine; LSC, LAH4-L1-siRNA complexes; MCM, multicompartment micelle; MDR, multidrug resistance; miR195, miRNA-195; NLS, nuclear localization signal; NPC, nuclear pore complex; PEG, polyethylene glycol; RISC, RNA-induced

silencing complex; siRNA, silencing RNA; ST21, cell-penetrating peptide–modified aptamer; VEGF, vascular endothelial growth factor.

Figure 2 Schematic illustration of three therapy mechanisms (*a*) PEP-010 (PEP Therapy). PEP-010 breaks down caspase-9 and PP2A's interaction (key proteins in apoptotic pathway). Free caspase-9 will allow cancer cells to undergo normal apoptosis. (*b*) The KISIMA™ cancer vaccine (ATP-128). TLR activates dendritic cells, whereas CPP facilitates vaccine delivery into them. The presentation of the CRC-specific MAD activates antigen-specific $CD8^+$ and $CD4^+$ T cells, increasing their activity and ability to recognize and target tumor cells. (*c*) ATX-101 (APIM Therapeutics). PCNA is the scaffold responsible for cell division; however, when DNA is damaged or cellular stress occurs, PCNA is post-translationally modified increasing its affinity for cellular defense proteins. ATX-101 inhibits PCNA scaffold functions and affects several cellular mechanisms. This reduces cell survival time and improves the efficacy of multiple anticancer drugs. Figure adapted from images created with BioRender.com. Abbreviations: AlkB, alpha-ketoglutarate dependent dioxygenase; APIM, AlkB homolog 2 PCNA interacting motif; CPP, cell-penetrating peptide; CRC, colorectal cancer; MAD, multiple-antigenic domain; PCNA, proliferating cell nuclear antigen; PTM, post-translational modification; TLR, toll-like receptor.

Table 1 List of different organelle-specific targeting peptides and their sequences

Organelle	Peptide	Sequence	Reference
Nucleus	Octaarginine	RRRRRRRR	47
	Nucleoplasmin	KRPAATKKAGQAKKKK	46
	SV40 large T-antigen	PKKKRKV	44
	M918	MVTVLFRRLRIRRACGPPRVRV	94
	HNLS-3	PFVYLIPKKKRKVHHHHHHGC	95
	hPP10	KIPLPRFKLKCIFCKKRRKR	96
	Transportan	GWTLNSAGYLLGKINLKALAALAKKI L	97
	c-Myc	PAAKRVKLD	98
	CHIKV	KPRRNRKNKKQKQKQQA	99
	N50-sC18	VQRKRQKLMPGLRKRLRKFRNK	100
	NrTP-sC18	YKQCHKKGGKKGSGGLRKRLRKFRN K	100
	M9	NQSSNFGPMKGGNFGGRSSGPYGGGG QYFAKPRNQGGY	101
	Vpr	DTWTGVEALIRILQQLLFIHFRIGCRHS RIGIIQQRRTRNGA	102
Mitochondria	MCoxIV	MKSFITRDKTAIGSGIM	103
	Cytcox-KH9	MKSFITRDKTAIGSGIM-(KH9)	104
	BP100	KKLFKKILKYL	105
	PepFect14	Stearyl- AGYLLGKLLOOLAAAALOOLL	60
	MitP	INLKKLAKL(Aib)KKIL	106
	mtCPP1	(D)Arg-Dmt-Orn-Phe- NH_2	107
	MTP-KH9	MLSLRQSIRFFK-KH9	108
	MTS	MLRAALSTARRGPRLSRLL	109
	m(KLA)-iRGD	D(KLAKLAKKLAKLA)K-GGiRGD	110
	CpMTP	ARLLWLLRGLTLGTAPRRA-NH2	111

	pHK	MIASHLLAYFFTELN	112
	linTT1	AKRGARSTA	113
Lysosome	K10H16	KKKKKKKKKKGHHHHHHHHHHHHHH HHH	66
	H16	HHHHHHHHHHHHHHHH	67
	YXXϕ	Y*XXϕ*	114
	DXXLL	D*XX*LL	114
Golgi and endoplasmic reticulum	altORF	*X*L*X**C*(R/K)*X*	115
	KKXX	KAAAAK	116
	KDEL	KDEL	117
	Pardaxin	H-GFFALIPKIISSPLFKTLLSAVGSALSSS GGQE-OH	118
	R4	DHA-RRRR-K-NBD-NH_2	119

Abbreviations: *ϕ*, any hydrophobic amino acid; Aib, 2-aminoisobutyric acid; (D)Arg, D-arginine; DHA, docosahexaneoic acid; Dmt, 2,6-dimethyl-L-tyrosine; NBD, 4-nitrobenzo-2-oxa1,3-diazole; Orn, ornithine; Phe, phenylalanine; *X*, any amino acid.

Table 2 List of selected peptides developed by pharmaceutical companies, along with their clinical trial status

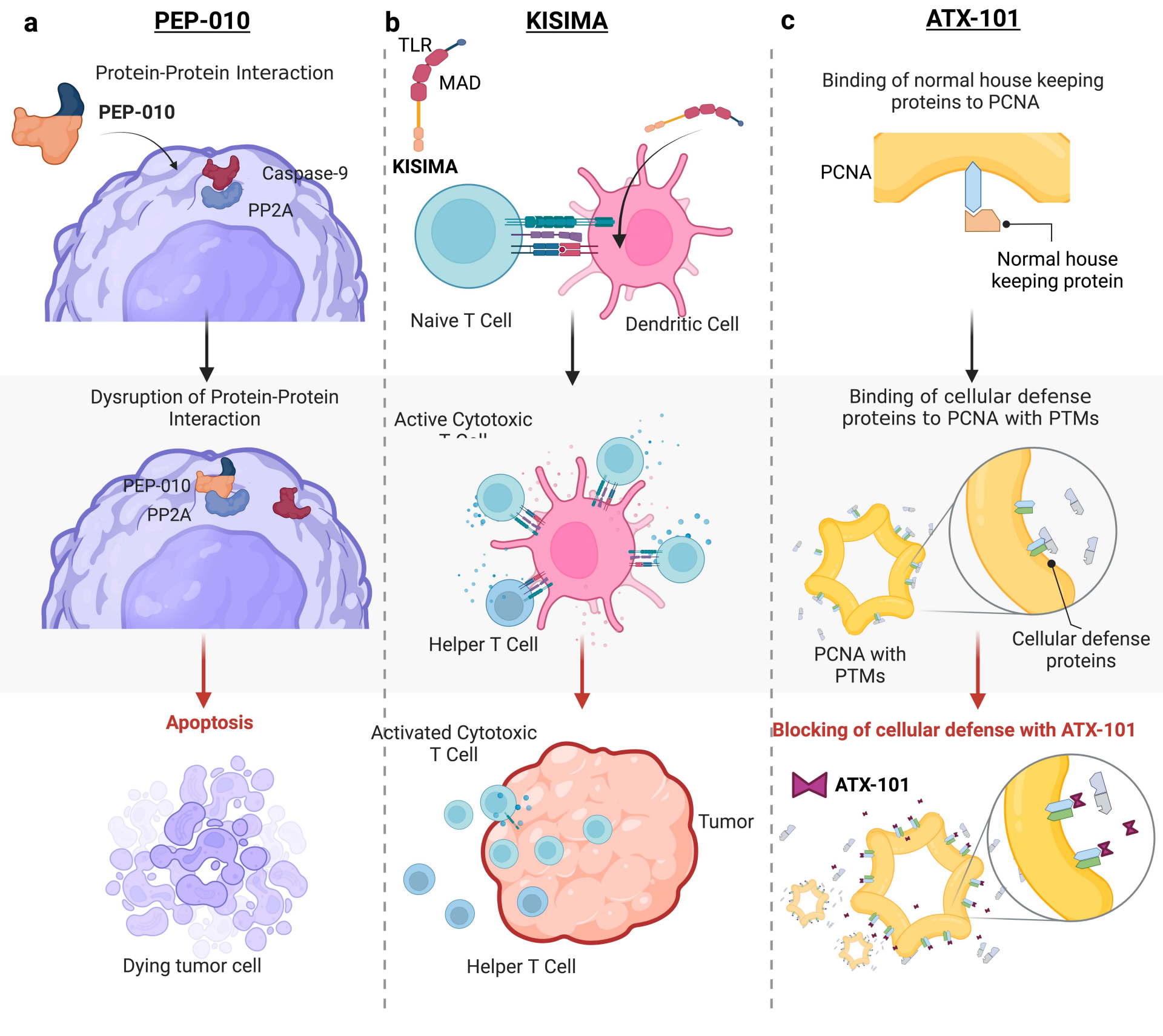
Name/peptide	Company	Treatment	Function	Status	Reference
KAI-1678/11-amino-acid (YGRKKRRQRRR) peptide derived from the TAT protein	KAI Pharmaceuticals	Postoperative pain	Inhibits epsilon protein kinase C	Phase IIa (discontinued because of postherpetic neuralgia occurrence in ~10–20% of patients)	120
ATX-101	APIM Therapeutics	Several cancers	Impairs interactions between PCNA- and APIM-containing proteins	Phase Ib/IIa (NCT04814875, NCT04814875)	89
DaxibotulinumtoxinA/poly-lysine backbone peptide (RTP004)	Revance Therapeutics	Neuromodulator category for the treatment of glabellar (frown) lines	Novel botulinum	Phase III completed (NCT03014635), now under regulatory review	121
XG-102 (D-JNKI1)/TAT-coupled dextrogyre peptide	Xigen	Intraocular inflammation	Inhibits the c-Jun N-terminal kinase	Phase III completed (NCT02508337)	122
PEP-010/14-amino-acid (RGERTAFIKDQSAL) peptide sequence derived from mouse furin convertase protein	PEP-Therapy	Triple-negative breast cancer and ovarian cancer	Dissociates the interaction between caspase-9 and PP2A Released caspase-9 restores normal apoptosis in cancer cells	Phase Ia (NCT04733027)	87

P28/28-amino-acid (Leu50-Asp77 LSTAADMQ GVVTDGMA SGLDKDYL KPDD) peptide sequence derived from a protein azurin	CDG Therapeutics	Brain tumor	Non-HDM2-mediated peptide inhibitor of p53 ubiquitination	Phase Ib (NSC7451040)	123
ATP-128/monotherapy and in combination with the PD-1 inhibitor BI 754091	AMAL Therapeutics	Colorectal cancer	CPP with a multi-antigenic cargo tailored to raise an immune response against colorectal tumors and a toll-like receptor peptide agonist	Phase Ib (NCT04046445, KISIMA-01)	88
ALRN-6924/ structure based on the α-helical domain of p53	Aileron Therapeutics	To eliminate or reduce chemotherapy-induced side effects in cancer patients	Targets P53 protein	Phase IIa (NCT02909972)	90
AZX100/ phosphorylated peptide analog of HSP20 (WLRRAS[P] APLPGLK) and PTD4 (YARAAARQ ARA)	Capstone Therapeutics Corp.	To reduce fibrosis and keloid scarring	Disrupts expression of connective tissue growth	Phase II (NCT00451256, NCT00892723, NCT00811577)	85, 86

Abbreviations: APIM, AlkB homolog 2 PCNA interacting motif; CPP, cell-penetrating peptides;

PCNA, proliferating cell nuclear antigen.

Peptide Based Gene Delivery: Clinical Research Outcomes



Peptide Based Gene Delivery: Pre-clinical Research Outcomes