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REVIEW ARTICLE

Tissue-Specific Cell Penetrating Peptides for Targeted Delivery of Small Interfering RNAs

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ABSTRACT

Cell penetrating peptides (CPPs) offer unique and promising solutions to overcome barriers to intracellular delivery of potential therapeutics for a variety of diseases, particularly when used as a delivery method for RNA interference agents such as siRNA. CPP mediated siRNA delivery provides promising therapeutic potential for various pathologies including many different types of cancer. Both cell-specific and non-cell-specific CPPs can play roles in transporting several types of cargo into cells, including but not limited to drugs, viral vectors, peptide nucleic acids, nanoparticles, liposomes, and siRNA. CPPs mediate siRNA delivery through a variety of delivery methods, including covalent conjugation and nanoparticle formation, and can be fashioned to facilitate endosomal escape. While no therapeutics currently utilize CPP-mediated siRNA delivery, two major approved therapeutics use RNA interference as a treatment modality, and many clinical trials are in progress testing the use of CPPs, again with an emphasis on the treatment of cancer. Further research is needed before the clinical use of CPP/siRNA complexes is commonplace, but advances in both CPP and siRNA technology appear promising for this method of treatment.

Introduction

The design of novel gene therapies remains rare despite advances in knowledge surrounding the genetic makeup of various pathologies previously believed to be untreatable. The use of RNA interference (RNAi) to treat disease has potential to change medicine as we know it, with a handful of therapies already in clinical use[1-5]. Perhaps one of the largest barriers to this type of gene therapy is the delivery of genetic material such as synthetic small interfering RNA (siRNA) into target cells, which must overcome obstacles such as immunogenicity, degradation prior to reaching target cells, poor cellular uptake, and lack of targeted delivery. So far, RNA-based therapeutics have been limited to pathologies where the culprit or target organ of interest is the liver, where siRNA/miRNA naturally accumulate, or have been designed to be taken up by hepatocytes by addition of N-acetylgalactosamine to the siRNA[6]. Therefore, the ability to direct RNAi therapies in an organ or site-specific manner would represent a major breakthrough for multiple disease processes.

Cell penetrating peptides (CPP), also known as protein transduction domains, could provide a solution to many of these barriers, with promising clinical implications. The discovery of the ability of Trans-Activator of Transcription (Tat) protein, expressed by human immunodeficiency virus, to transfect cultured cells and induce viral gene expression without a receptor[7,8] has led to the discovery of novel CPPs effective at delivering various diagnostic or therapeutic cargo across the cell membrane. Cross-linking of proteins such as horseradish peroxidase, β-galactosidase, RNase A and domain III of pseudomonas exotoxin A to Tat demonstrated Tat's ability to carry large cargo into cells in a non-cell-specific manor[9]. This ability was later further confirmed to preserve the functionality of such large cargo when used *in vivo*[10]. Similarly, the homeobox Antennapedia (Antp) transcription factor of *Drosophila melanogaster* was demonstrated to enter nerve cells without the need for a receptor, allowing for functional regulation of neural morphogenesis[11]. Further studies of Tat and Antp led to the identification of the domains of each protein responsible for these cell penetrating properties, and upon mapping, the first two CPPs were determined: Tat's cationic, lysine- and arginine-rich domain (YGRKKRRQRRR)[12] and Antp's 16 amino acid domain, termed penetratin (RQIKIWFQNRRMKWKK)[13]. Since then, the discovery of additional non-specific CPPs as well as cell-specific CPPs has increased exponentially[14], especially with the development of techniques such as phage display reported by Smith in 1985[15]. Using phage display led to the discovery of CPPs able to target specific cell types such as synovial fibroblasts[16], pancreatic islet cells[17], vascular endothelium[18], dendritic cells[19], and cardiomyocytes[20]. There is continued significant interest in the identification of cell specific CPPs and their clinical implications, leading to an explosion of developments as well as publications surrounding this area of study, making it impossible to cover all aspects of these novel peptides. Therefore, out of necessity, this review will be limited to the uses and applications of CPPs in the delivery of siRNA. Interested readers are referred to earlier reviews of CPPs by the authors[21,22] as well as several other reviews on the identification and uses of CPPs[23-25].

2. Cell Penetrating Peptide Overview

2.1. *Identification*

Cell-specific CPP's were identified by various screening methods. Plasmid, microorganism surface, ribosome, or phage display are included in these methods, but phage display libraries were the primary method in identifying these peptides[22]. Phage display methods can be traced back to 1985[15]. This method involves using a large phage library to identify which amino acid lengths and sequences[14] are taken up by a target cell. Once internalized, the phage undergoes isolation, expansion, and several (usually 3-5) rounds of screening[22], followed by the isolation and sequencing of a few peptides that are able to enter the target cell of interest. False positives are a concern using the phage display library method, but can be minimized by using *in vitro* relevant cell types as a pre-screen, followed by multiple rounds of screening to enrich for targeting phage[22].

2.2. *Types of Cell Penetrating Peptides*

Although there are multiple classification systems, for the purpose of this review, we will classify CPPs into cell- and non-cell-specific types (Figure 1). Cell-specific CPPs can target specific cells and deliver cargoes selectively thereby diminishing doses needed as well as limiting off-target side effects. Using cell-specific CPPs would also be advantageous in the process of upscaling the experiments to human trials because less peptide is required due to its specificity[22].

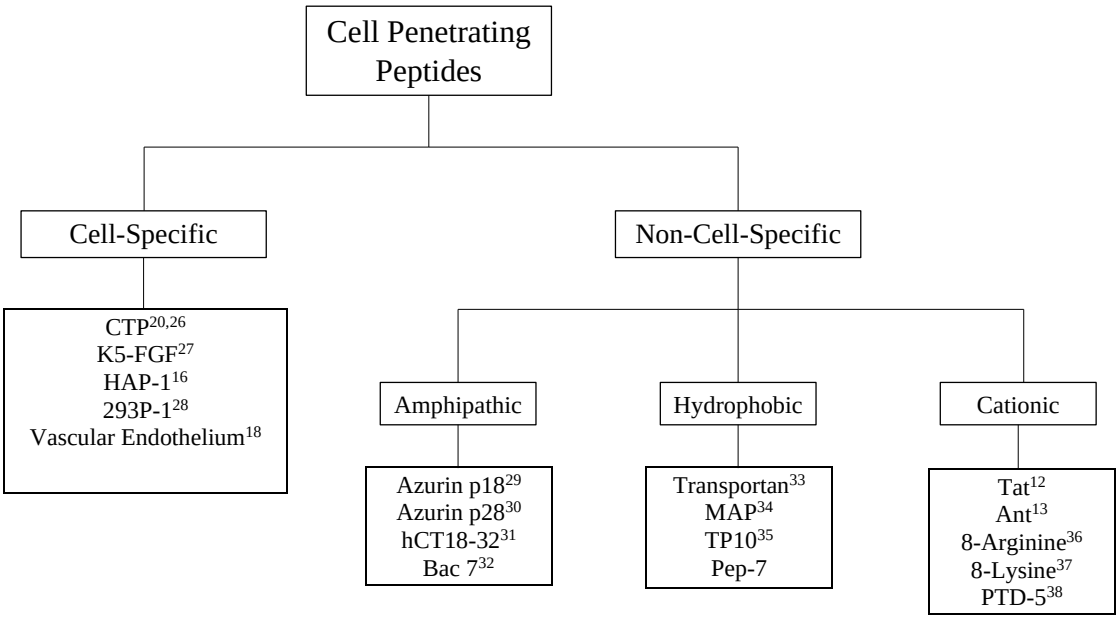

Figure 1. Classification of CPPs with various examples in each category.

Non-cell specific CPPs can be divided into three categories: amphipathic, hydrophobic, and cationic. Amphipathic CPPs are synthesized by covalently bonding a nuclear localization signal to the hydrophobic domain of the CPP. Hydrophobic CPPs, on the other hand, are created by signal sequences on peptides that allow secretion. It stands to reason that peptides that allow materials out of the cell would also allow them to enter[22]. Therefore, most leading sequences of amino acids of various secreted proteins can be used as CPPs, some specific examples of which can be found here[39]. Cationic CPP's primarily involve the presence of positively charged amino acids (arginine, histidine, and lysine). Histidine can function as a CPP below a pH of 6.0 (protonates at a low pH, so positively charged)[40]. Homopolymers of arginine of various lengths have been shown to act as non-specific CPPs with size ranging ideally from 8-10 amino acids, though as few as 6 or as high as 12 arginine residues can still act as non-specific CPPs[41]. Lysine homopolymers can similarly act as non-specific CPPs with no cytotoxicity at small lengths (6-12 amino acids)[37], with larger than 12 amino acids showing reduced efficiency[22].

2.3. *Transduction*

The mechanism of transduction of these CPPs has been difficult to elucidate despite nearly 25 years of research. Firstly, CPPs are only a few amino acids long. Their small nature makes them difficult to identify using standard techniques[22]. Next, because the transduction occurs so quickly (usually within 30 minutes), the mechanisms involving entry into the cell are hard to identify. Third, it is likely that different CPPs will have different methods of entering the cell[22]. These would vary depending on concentrations used, cargoes, as well as the chemical nature of each CPP. Since the entry mechanism for cell-specific CPPs are likely to be unique to the cell type, and distinct from the mechanisms for non-specific CPPs like Tat or Antp, a common transduction pathway is unlikely, and adds to the complexity of identifying specific mechanisms by which transduction occurs.

Current evidence exists for both energy-dependent and energy-independent pathways[42]. Surface binding through electrostatic interactions is predicted to be a shared initial step among non-cell-specific cationic CPPs, followed by cell entry[43,44]. Transduction is also predicted to occur in two steps, with surface binding to negatively charged heparan sulfate being the first step, and either translocation across the lipid membrane (for cationic CPPs with small cargoes), or micropinocytosis (for CPPs with large cargoes) being the second step[45]. Adenosine-triphosphate stores are also depleted in the process of transduction[37], which suggests an energy-dependent process. However, these stores are depleted less than what would be predicted for the process, which suggests there are other non-energy-

dependent pathways. CPPs that are hydrophobic are also seen to function more effectively as transporters within the transduction process[46].

2.4. *Cargoes*

Although CPPs can be used to deliver a variety of cargoes, including but not limited to drugs[47-50], viral vectors[47-50], radioisotopes[26,51-53], plasmid DNA[54], proteins[55-60], peptide nucleic acids[61], nanoparticles[62], liposomes[63], quantum dots[64,65], and photosensitizers[66,67], the most research has been with siRNA[22,68-75]. For this reason, we will limit this review to delivery of siRNA as a cargo by various CPPs.

3. Cell Penetrating Peptide Mediated Small Interfering RNA Delivery

Development of new methods of delivery is paramount to the development of RNAi therapies, with cellular uptake of small nucleic acids being one of the largest barriers to RNAi treatment. Therapeutics involving siRNA have become a topic of interest in recent years because they allow specificity in treating diseases, including cancers[76]. However, several barriers still must be overcome: poor serum stability, serum degradation and loss of function, efficiency of uptake, targeted delivery, endosomal escape, and release of the cargo siRNA from the vector are all concerns that must be addressed before these therapeutics are ready for clinical trials. siRNA is, at its core, a small, negatively charged molecule that has difficulty crossing membranes due to its hydrophilic nature. Therefore, encapsulating the RNA and linking it to delivery vectors (such as CPPs) prove to be an effective solution in addressing many of the aforementioned concerns[77].

Once siRNA is delivered to cells, and released from the vector CPP, it is free to be recognized by the RNA-induced silencing complex (RISC). The RISC complex mediates the cleavage of the siRNA/target mRNA complexes to achieve gene silencing[78] (Figure 2). Therefore, for gene silencing to occur, the chosen siRNA must be able to survive serum degradation, penetrate the cell, and form the RISC complex. CPPs, therefore, can be the vectors for this process to occur successfully.

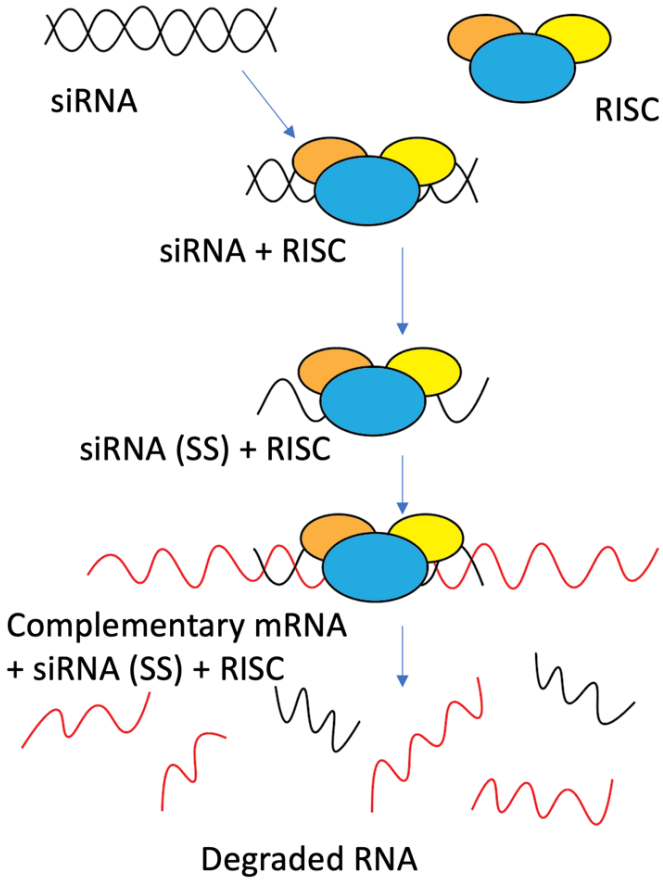

Figure 2. Gene silencing using siRNA. Gene silencing is mediated through the formation of the RISC complex following the delivery of siRNA into cells. Upon delivery into human cells, the double stranded siRNA complexes with the Argonaute2 protein, which assembles with the RNA binding protein TRBP and Dicer, an enzyme responsible for the cleavage of dsRNA and the formation of guide siRNA. After the formation of guide siRNA, the target mRNA is free to bind, and gene silencing occurs with the degradation of the target mRNA[78].

3.1. *Small Interfering RNA Delivery Methods*

Early attempts at the delivery of small nucleic acids attempted to do so through the conjugation of siRNA with CPPs through covalent bonds. In one of the earliest examples of CPP-nucleic acid conjugation, investigators showed that the addition of four lysines linked to the C-terminus of PNA oligomers (PNA-4K) injected into GFP mice allowed for successful upregulation of EGFP-654 compared to PNA sequences with only one lysine (PNA-1K) in a mouse model expressing EGFP interrupted by an aberrantly spliced mutated intron of the human β-globin gene[79]. Most conjugation of CPPs with small nucleic acids is accomplished through disulfide linkages, allowing for cleavage by enzymes such as glutathione reductase in the reducing environment of the cell[69] (Figure 3). Covalently bonded penetratin-siRNA complexes have been shown to successfully downregulate luciferase activity at an equivalent level to delivery using cationic liposomes[70], with similar results demonstrated in various cell types[80]. *In vivo*, however, successful delivery of covalently linked siRNA and CPPs proves to be elusive. Instead, non-covalent siRNA-CPP complexes seem to be more effective; in particular, nanoparticle (NP) formation has promising implications for clinical therapies. Early methods of CPP-nucleic acid NP formation were exceedingly complicated. For instance, researchers developed a multifunctional envelope-type nano device (MEND) in order to deliver nucleic acids such as siRNA, by MEND PEGylation[81]. However, this method inhibited both uptake and endosomal escape. A solution was found to achieve significant gene silencing by siRNA through first creating a PEG-peptide-DOPE (PPD) complex, further modifying it with a pH-sensitive fusogenic GALA peptide, and using the GALA/PPD-MEND complex for siRNA delivery[81]. Other early methods were similarly complicated, requiring the development of large lipidoid libraries to facilitate CPP delivery[82] and complex formation of liposomes containing CPPs and siRNA[83].

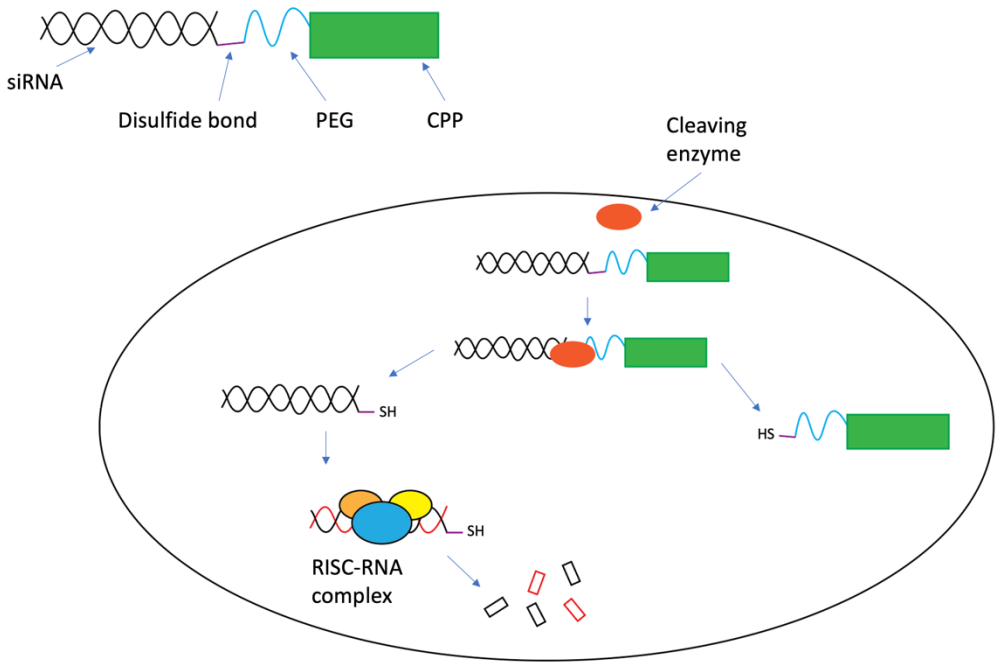

Figure 3. *Covalently conjugated CPP/siRNA complexes generally work by covalently linking the desired siRNA (shown in black) to PEG (shown in blue) through a disulfide bond (shown in purple). Then PEG is attached to the cell-specific CPP (shown in green), and cleavage of the disulfide bond by a cleaving enzyme such as glutathione (shown in orange) once the CPP has mediated cell entry allows for the now-free siRNA to be recognized by the RISC complex. Degradation of the target mRNA then occurs, and the CPP is metabolized and excreted.*

Later methods proved to be much more efficient. Simply mixing a novel peptide, PepFect 6, with siRNA in water created stable NPs with week-long stability, which were reported to be both of suitable size and stability for treatment *in vivo*.[84] . As discoveries concerning the delivery of siRNA by CPPs continue to be made, new methods of controlling the release of CPP cargo are being sought. In one study, NPs were modified with both pH-responsive and photo-responsive CPPs. The pH-

responsive CPP activity was inhibited by the lower pH environment of tumor cells, allowing for NP accumulation at the membrane[85]. The CPP modified NPs, which were filled with siRNA, were then internalized into the tumor cells by near-infrared light illumination, leading to anti-tumor effects[85]. Covalently conjugated CPP-siRNA complexes have been delivered to cancer cells both *in vitro* and *in vivo* by loading such complexes in ultrasound-sensitive microbubbles, which are disrupted by the application of ultrasound. This method was found to significantly augment *c-myc* silencing and delay tumor progression[71]. Finally, Qi et al. created CPP NPs containing Fe_3O_4 to create cell-penetrating magnetic nanoparticles, which reportedly were able to reduce EGFP expression to 12% of original levels *in vitro* when a magnetic field was applied.

3.2 *Endosomal Escape*

Uptake of many CPPs is mediated by endocytosis, which is a problem as the inside of an endocytic vesicle is still outside of the intracellular environment. Additionally, it renders the CPP/cargo complex vulnerable to degradation. Therefore, the importance of endosomal escape in drug development using CPPs cannot be overemphasized. It has been shown that increasing endosomal escape can increase CPP internalization, and significantly increase efficient gene expression[86]. Some peptides, like arginine-rich cationic peptides as well as amphiphilic peptides, have inherent endosomal escape abilities[87]. Some peptides (such as Tat) which have minimal endosomal escape activity can be modified to facilitate escape into the cytoplasm[88]. It is possible that the cationic nature of these peptides facilitates endosomal escape. Brock et al. found that blocking the contact and fusion of endosomal bilayers containing bis(monoacylglycero)phosphate, an anionic lipid present in cell membranes, prevented the endosomal escape of Tat, suggesting that cationic peptides interact with anionic lipids to induce leakage from endosomal membranes[89]. Increasing the endosomal escape activity of CPPs is an ongoing field of research, and novel strategies such as integration of CPPs with arginine–glycine–aspartate motifs[90], coating CPP nanoparticles with metal-phenolic networks[91], photochemical internalization treatment[92], and the use of ultrasound to disrupt endosomal membranes and deliver small nucleic acids[93] are continuously being developed.

Recently, Dastpeyman et al.[94] showed that treatment with peptides synthesized with distinct cell penetrating and endosomal escape motifs linked to antisense oligonucleotides (ASOs) not only were able to cross the blood brain barrier, but also significantly increased gene expression of the survival motor neuron 2 (SMN2) gene *in vivo*. Although enhanced endosomal escape has also the potential for increased cellular toxicity, it has been demonstrated to not be the case for at least dfTAT, a disulfide-bound dimer of Tat, that did not induce recruitment of Gal3 or Chmp1b during endosomal escape, known markers of endocytic degradation and indicators of possible cellular toxicity[95].

3.3 *Small Interfering RNA Potential therapeutics*

3.3a Cancer Therapeutics Application

The vast majority of the body of research surrounding the delivery of RNAi agents such as siRNA to target cells has been performed in the context of cancer. Numerous strategies have been employed to tackle the hurdles of small nucleic acid delivery into cells, with much success. For instance, Qiu et al[96]. used chimeric CPP-functionalized lipopepsomes (a liposome decorated with various CPPs) to enhance the delivery of anti-tumor siRNA to human lung tumor-bearing mice. This method led to significant gene silencing *in vivo*, with enhanced peptide accumulation at the tumor, highly selective internalization, efficient endosomal escape, and prolonged blood circulation[96]. Tumor-bearing mice treated with the CPP-functionalized lipopepsomes containing siRNA showed a median survival time of 45 days, as compared to mice treated with siRNA alone with median survival of 36 days. The CPP/siRNA treated mice also displayed negligible weight loss and well-organized lung structure. It was theorized in this study that the nature of the sturdy membrane of lipopepsomes prevented RNA degradation, leading to high levels of gene silencing[96]. In studying the treatment of melanoma, it was found that a CPP/cholesterol/PEG conjugate was able to form nanoparticles to facilitate delivery of Wee1 siRNA, leading to significant silencing of the *WEE1* gene and melanoma tumor cell apoptosis *in vitro* as well as inhibition of tumor growth *in vivo*[97]. Other studies on the uses of CPPs to target melanomas use different CPP-based delivery methods, such as microneedles[98]. Delivery of Tat-bound siRNA encapsulated in engineered extracellular vesicles has been shown to be successful as well, facilitating androgen receptor downregulation and inducing apoptosis in human prostate cancer cells *in vitro*[99]. A delivery system composed of CPPs and polyethylene oxide were attached on a PEI backbone and dispersed in water as nanoparticles. Association of those nanoparticles with STAT3 targeting siRNA showed strong antitumor effects when delivered both locally onto C26 cancer cells and intravenously in a mouse model of colon cancer *in vivo*, with treated mice

exhibiting 66 metastatic nodules in contrast to 150 in control mice[100].

Antibodies provide another interesting siRNA delivery method. In one study, antibodies were conjugated to siRNA using a peptide composed of both a CPP and a substrate peptide (a peptide extensively expressed in tumor cells)[101]. The substrate peptide was then cleaved by tumor specific enzymes upon antibody attachment to tumor cells, allowing the CPP-siRNA complex to be released from the antibody and facilitate entry into the tumor cells. Efficacy was demonstrated *in vivo*, with 66.7% EGFP downregulation efficiency in tumor model mice xenografted with human colon cancer cells expressing the EGFP protein[101]. This suggests that oncogene-specific siRNA could be delivered using the same delivery system, leading to potential therapeutics. Other studies have also used immunogenic responses in combination with the transduction abilities of CPPs, leading to interesting possibilities for the treatment of various cancers[102]. One study demonstrated the ability of a CPP called tLyp-1conjugated in NPs with siRNA to cross the blood-brain barrier and target glioblastomas; linkage to anti-tumor monoclonal antibodies allowed for not only cytosolic entry of the siRNA and inhibition of LSINCT5-activated signaling pathways, but also activation of anti-tumor immunity[103].

CPP mediated siRNA delivery can also be used to complement other existing therapies; for instance, glycolysis inhibiting siRNA, delivered by a guanidine rich CPP, was able to sensitize tumor cells to photothermal therapy, leading to both increased effectiveness of photothermal therapy and tumor cell starvation[104]. The body of research on the use of CPPs to treat cancer is vast, and continues to grow with many studies on other types of cancer showing promise including glioblastomas[75,105], hepatocellular carcinoma[106], pancreatic cancer[107], and more[102,108-111]. Further research is necessary to test the effectiveness of these therapies *in vivo*, eventually leading to possible clinical trials and new CPP/siRNA-based drugs.

Interestingly, the use of RNAi to treat triple negative breast cancer (TNBC), the current leader in types of breast cancer that threaten women's health[62], has shown promise. Tumor growth in TNBC implicates Rictor, a protein involved in cell metabolism and cell growth regulation[112,113]. One study was able to target Rictor as a mechanism to treat breast cancer with the vector GO-PEI-PEG-CPP[62], a molecule made up of graphene oxide (a molecular carrier with unique mechanical, electrical, and thermal properties[114]), PEI, PEG, and a CPP. Treatment with the GO-PEI-PEG-CPP/siRNA complex led to a decrease in cancer cell viability, the induction of apoptosis, decreased expression of Rictor, and an overall inhibitory effect on breast cancer, as shown in vitro and in vivo studies[114]. This CPP/siRNA delivery vector also showed reduced tumor volume, weight, and size in mice, with even greater reductions than previously observed[62]. These results show a promising future in the use of nanomaterials, specifically CPP and GO-based ones, in treating TNBC.

Other techniques using nanomaterials to target TNBC also exist. Numerous studies focus on the proteins EGFR, which functions in suppression of cell apoptosis and encouraging cell proliferation[115,116], and BRD4, which allows cancer cells to remain undetected by the immune system[117]. Overexpression of both proteins is implicated in TNBC[115,116,118,119], and are therefore ideal candidates to target using siRNA. Readers are referred to other studies highlighting the use of CPPs in TNBC treatment [73,77,120-122].

3.3b Treatment of Other Pathologies

CPP mediated siRNA delivery has shown potential to treat other pathologies as well, the etiologies of which usually involve inflammation. As discussed above, the non-cell-specific properties of Tat have been extensively studied, but Tat has also been utilized in a tissue-specific manner by being administered locally. One study showed Tat's ability to deliver siRNA to osteoarthritic chondrocytes *in vitro* and to induce *in vivo* genetic recombination, showing its promise as a delivery agent for intra-articular therapeutics[123]. Other CPP/siRNA nanocomplexes have shown similar results as well *in vitro*[72]. In another study, Liang et al[124]. reported that delivery of siRNA targeting RAGE (a regulator of the pro-inflammatory cascade) using benzyl-modified polypeptide (P-Ben) in an ischemia-reperfusion model in rats reduced RAGE mRNA by 84%, as well as significantly reduced expression of TNF-α and IL-6. Rats receiving P-BEN/siRAGE complexes had an infarct size of 6.9%, compared to 66.3% in saline treated rats. Similar studies have shown that small extracellular vesicles loaded with anti-RAGE siRNA and labeled with a cardiac-specific CPP, termed cardiac targeting peptide, were able to reduce inflammation both *in vitro* and in a mouse model of myocarditis[125]. CPP mediated siRNA delivery has been established as a possible treatment for ulcerative colitis, as well. Intravenous administration of MPEG-PCH-CH2R4H2C/siRNA nanomicelles (a peptide-based drug delivery system showing enhanced permeability and retention) showed accumulation of siRNA in the inflamed region of the large intestine as well as reduction of colitis symptoms as well as inflammation[126]. The same

peptide/siRNA complex has also shown promising results *in vivo* in inflammation-based pathologies such as contact dermatitis[127] and rheumatoid arthritis[128]. Finally, a CPP called lung targeting peptide was shown to be effective at delivering siRNA against SARS-CoV2 when anti-spike siRNA was attached to a cyclic version of the lung targeting peptide, demonstrating anti-viral efficacy *in vitro*[129]. More work is needed to confirm these findings; however, a simple inhaled CPP/siRNA-based treatment for SARS-CoV2 would be extremely advantageous in today's day and age.

The possibilities for RNAi treatments delivered by CPPs are numerous. Other studies include experimenting with using antisense oligonucleotides as a potential treatment for multi-drug resistant bacteria[130], as vectors for gene delivery in anti-atherosclerotic therapies[131], and for the treatment of peripheral vascular disease[132]. The development of novel CPP/siRNA-based treatments continue, but as mentioned above, additional studies are needed to confirm the efficacy of these treatments *in vivo*.

4. CPP-siRNA complexes

Seeing as the usage of RNAi to treat to disease is a very new field of study, there are few therapeutics using RNAi in existence, none of which use CPPs as targeted siRNA delivery. There are, however, two approved RNAi based therapeutics. One of these is Inclisiran, an siRNA-based therapy shown to significantly reduce levels of LDL in adults with familial hypercholesterolemia[133]. Another is Vutrisiran, a transthyretin-targeting siRNA used to treat transthyretin-mediated amyloidosis[134]. Predictably, both therapeutics act on the liver: Inclisiran targets PCSK9 mRNA, therefore inhibiting the PCSK9 gene and increasing the recycling and expression of LDL-C receptors on the surface of liver cells, leading to increased LDL-C uptake[135]. Vutrisiran works by inhibiting the synthesis of TTR, a protein synthesized by liver cells that when accumulated plays a key role in the development of hereditary TTR-mediated amyloidosis[134]. Neither of these drugs uses CPPs in conjunction with their siRNA therapies, but their success is indicative of the possibilities that RNAi treatments present, especially with the potential that CPPs provide as carriers of siRNA. Early CPP-based trials demonstrated CPPs to be safe for clinical use but were too small to determine whether they provided a benefit over established treatments[22,136]. The future of RNAi treatments rests, then, on finding carriers for small nucleic acids for targeted delivery to organs other than the liver, and CPPs provide promising tissue-specific delivery methods.

Although there are no current therapeutics that are based on CPP/siRNA complexes, there are several clinical trials currently targeting cancer using CPPs. Of the known clinical trials, however, none use CPPs to deliver siRNA or miRNA to cells. *In vitro* and *in vivo* studies are still being done to test the viability of CPPs as RNA delivery vectors. These studies show promising results, as discussed in the previous section, in targeting cancers and inhibiting the growth of tumors[137].

5. Barriers to Treatment

Regardless of the promise CPPs show in delivering siRNA to cells, there are a number of hurdles to surmount prior to undertaking clinical trials. First, CPPs are expensive to develop[14]. If used in clinical trials, the upscaling of production of a particular CPP would require significant funding. It is currently estimated that it takes ~2.6 billion dollars to bring a new therapeutic to market[22]. However, targeted CPP-carried cargoes like siRNA could be long-lasting, therefore reducing the treatment frequency, as well as dose requirement and perhaps making the method more affordable[22].

Another factor that would pose a barrier to CPPs as therapeutics would be the delivery of these peptides[14]. To eventually use CPPs in a long-term clinical setting, oral bioavailability will be critical. However, long-acting formulations to allow for monthly or less frequent subcutaneous injections, as in the field of anti-PCSK9 inhibitor therapies, would be an attractive solution. Toxicity of therapeutics, particularly to organs involved with elimination, would be a concern[14], and would need to be extensively studied prior to clinical trials. As mentioned before, however, cell-specific CPP treatments might reduce potential toxicity by facilitating targeted therapies and allowing for reduced dosage requirements targeted therapies and reduced dosage requirements. Lastly, the possibility of immunogenic response due to CPP and cargo conjugates exposing the immune system to newly generated epitopes must be kept in mind[22].

6. **Conclusions**

CPPs and siRNA both were discovered nearly 25 years ago, and research interest, scientific study, and resulting publications continue to grow exponentially with two siRNA-based therapies in clinical usage already. These are likely to be followed by many more. These initial therapies target the liver where siRNA accumulate, with modifications applied to facilitate further hepatic uptake. With the advent of cell-specific penetrating peptides, siRNA therapeutics are poised for an expansion of their applications.

Although in this review we identified many hurdles to successful therapies, and there is much to be accomplished, the potential of CPP-based, targeted siRNA is huge, and could open up myriad avenues of new therapeutics for a multitude of pathologies.

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