Functional Delivery of siRNA by Disulfide-Constrained Cyclic Amphipathic Peptides

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S Supporting Information

ABSTRACT: The promise of oligonucleotide therapeutic agents to perturb expression of disease-related genes remains unrealized, in part due to challenges with functional cellular delivery of these agents. Herein, we describe disulfide-constrained cyclic amphipathic peptides that complex with short-interfering RNA (siRNA) and affect functional cytosolic delivery and knockdown of target gene products in cell culture and *in vivo* to mouse lung. Reduction of the constraining disulfide bond and subsequent proteolytic clearance of the peptide are key design features that allow unmasking of the siRNA cargo and presentation to the RNA interference machinery.

KEYWORDS: siRNA delivery, cell-penetrating peptides, cyclic peptides

The use of oligonucleotides as gene-based therapeutic agents has been pursued for decades.¹ The discovery of RNA interference (RNAi) mechanisms for gene silencing has inspired renewed efforts to develop oligonucleotides for therapeutic applications in the form of exogenous short-interfering RNA (siRNA).^{2–4} Despite significant effort, the clinical application of siRNA therapeutics remains extremely limited.⁵ A major challenge for the development of siRNA and other oligonucleotide-based therapeutics is the transport of these negatively charged macromolecules into the cell.⁶ Translocation of siRNA into cells requires vectors that facilitate cell binding, internalization, unpackaging, and presentation of the siRNA duplex to the RNAi machinery. Numerous siRNA delivery systems have been explored, including lipid nanoparticles, cationic polymers, antibody constructs, and RNA aptamers.^{5,6} While significant progress in siRNA delivery has been made with these various transfection agents, none are ideal and significant formulation/characterization barriers preclude their immediate clinical application.

Cell-penetrating peptides (CPPs) are promising siRNA transfection agents.^{7–9} CPPs have been validated for the translocation of large macromolecular cargo, including proteins, into cells without appreciable toxicity.^{10,11} CPPs, including

TAT,^{12,13} penetratin,^{14,15} transportan,¹⁶ and oligoarginines,¹⁷ have been covalently appended to siRNA to promote cell internalization.¹⁸ These strategies have been only moderately successful, as the chemical modification to siRNA that is required can perturb the biological activity of the internalized oligonucleotide.^{19,20} Accordingly, noncovalent CPP-siRNA complexes have been pursued for delivery of siRNA.^{18,21–23} Noncovalent complexation of siRNA with oligoarginine peptides,^{24,25} TAT-double-stranded RNA binding protein fusions,²⁶ or multidomain peptides containing polycationic and hydrophobic segments^{25,27–30} has provided CPP-siRNA nanoparticle-like aggregates that facilitate cellular translocation of the siRNA. These approaches have yielded encouraging results; yet efficient functional delivery of siRNA remains challenging, most likely due to localization of CPP-siRNA complexes in the endosome after translocation into the cell.^{20,28,31}

Herein we describe the use of redox-sensitive cyclic peptides as an effective method for the functional delivery of siRNA to

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cells in culture and to lung cells in an *in vivo* animal model. Amphipathic cyclic peptides (cyclized *N*-terminus to *C*-terminus) have recently been shown to carry complexed small molecule cargo into the nucleus of cells via a nonendocytic process.^{32,33} We hypothesized that similar disulfide-constrained cyclic Ac-C(FKFE)₂CG-NH₂ peptides³⁴ could form noncovalent complexes with siRNA that would have advantages over existing CPP-siRNA noncovalent delivery complexes. First, cyclic peptide-siRNA complexes should be stabilized to proteolytic and nucleolytic degradation of the cyclic peptide and its siRNA cargo, respectively. Second, Ac-C(FKFE)₂CG-NH₂ and similar sequences are relatively simple compared to existing CPPs that have been explored for siRNA delivery. For example, the MPG peptide is 27 amino acids in length²⁷ and PepFect14 is 21 amino acids and is side chain conjugated to other agents.^{31,35} In contrast, our disulfide-constrained cyclic peptides are merely ten amino acids; synthesis is straightforward.³⁴ Finally, we predicted that disulfide-constrained cyclic peptides would undergo reduction by intracellular glutathione, facilitating proteolytic degradation and presentation of the siRNA cargo to the RNAi machinery.

We chose amphipathic peptides with alternating hydrophobic and hydrophilic residues to evaluate disulfide-constrained cyclic peptides as siRNA delivery vectors. We initially used the Ac-C(FKFE)₂CG-NH₂ peptide;³⁴ we also used the cationic Ac-C(WR)₄CG-NH₂ peptide. Trp/Arg-rich peptides have been previously exploited as CPPs.³² We further reasoned that Trp/Arg-containing peptides would exhibit improved binding to siRNA due to attractive charge interactions and based on the demonstrated ability of Trp to bind to oligonucleotides via π - π interactions.³⁶ In a linear form, these peptides have a strong tendency to form β -sheet secondary structures that readily self-assemble into fibrils; however, cyclization via a disulfide bond between the flanking Cys residues favors the formation of transient, amorphous aggregates that lack the stabilizing hydrogen bond network found in fibrillar assemblies.³⁴ The proposed cyclic peptides present hydrophobic and hydrophilic side chains on opposite faces of the cyclic peptides (Figure 1), providing an ideal sequence pattern for binding to siRNA and facilitating its delivery into the cell. Enantiomeric *L* and *D* variants of each peptide, as well as a variant with a redox-insensitive thioether constraint (which cannot be reduced), were prepared to determine if the proteolytic degradation of

Figure 1. Structural models of the cyclic, disulfide-constrained Ac-C(FKFE)₂CG-NH₂ peptide shown the hydrophobic face (A) and the hydrophilic face (B). Hydrophobic Phe side chains are shown in green, hydrophilic Lys and Glu side chains are shown in blue, the cystine disulfide sulfurs are shown in yellow, and backbone and other functionality is shown in gray. This class of cyclic amphipathic peptide presents a hydrophobic and a hydrophilic face, as is apparent in these structural representations.

the reduced peptide in the cell is necessary for functional knockdown by internalized siRNA.

Peptides were prepared by solid phase peptide synthesis, and cyclization was performed as previously described (see [Supporting Information](#) for experimental details and structures and characterization data for all peptides, [Figures S1–S11](#)).³⁴ The cyclic Ac-C(FKFE)₂CG-NH₂ and Ac-C(WR)₄CG-NH₂ peptides were tested for stability in water, cell culture media, and fetal bovine serum (FBS) supplemented media ([Figure S12](#)). Neither peptide showed evidence of degradation in water or in cell culture media, but both were slowly degraded over 24 h in FBS-supplemented media, most likely due to the presence of glutathione in this media.³⁷ The rate of degradation was slow, however, related to the length of time peptide/siRNA complexes were incubated with cells in subsequent siRNA delivery analyses.

Transport of siRNA into cells using the proposed cyclic amphipathic peptides was assessed *in vitro* by fluorescence imaging of A549 lung cancer cells incubated with rhodamine (Rh)-labeled siRNA in the presence or absence of the cyclic peptides ([Figure 2](#)). The Rh-labeled siRNA was incubated with cyclic peptide (100 μ M peptide, 100 nM siRNA in phosphate-buffered saline) at room temperature for 30 min after which these mixtures were applied to A549 lung cancer cells in culture for 2 h. Upon rinsing, the cells were incubated for 48 h, after

Figure 2. Transport of rhodamine-labeled (Rh) siRNA into A549 lung cancer cells by disulfide-constrained cyclic amphipathic peptides. Nuclei are stained with DAPI. Scale bar is 20 μ m and corresponds to all images. (A) Rh-siRNA alone, (B) cyclic L-Ac-C(FKFE)₂CG-NH₂ + Rh-siRNA, (C) cyclic D-Ac-C(FKFE)₂CG-NH₂ + Rh-siRNA, (D) cyclic L-Ac-C(WR)₄CG-NH₂ + Rh-siRNA. Peptide concentrations were 100 μ M; Rh-siRNA concentrations were 100 nM.

which the cells were washed and analyzed by fluorescence microscopy to determine siRNA transport efficiency. It was found that Rh-siRNA without peptide did not enter the cells to any appreciable degree (Figure 2A). The cyclic L- and D-Ac-C(FKFE)₂CG-NH₂ (Figure 2B and C respectively) peptides were found to aid siRNA translocation into the cytosol to a modest degree. In contrast, cyclic L- and D-Ac-C(WR)₄CG-NH₂ (L-Ac-C(WR)₄CG-NH₂ is shown in Figure 2D) were found to affect significant cytosolic uptake of Rh-siRNA. The chirality of the cyclic peptides did not significantly influence siRNA uptake. The higher efficiency of cytosolic delivery of siRNA with cyclic Ac-C(WR)₄CG-NH₂ peptides is most likely due to enhanced complexation affinity of these cationic peptides with siRNA and to enhanced translocation ability for Arg/Trp-containing peptides, as has been previously demonstrated.^{17,24} Addition of up to 1 mM peptide with siRNA to A549, primary rat alveolar epithelial type II cells, human smooth muscle cells, human fibroblasts, and mouse lung epithelial MLE15 cells, did not appear to distress the cells based on microscopic analysis.

Functional gene knockdown analyses were conducted in order to determine whether this siRNA has been delivered effectively to RNAi machinery (Figure 3). Cyclic L-Ac-

Figure 3. Knockdown efficiency of cyclic peptide-siRNA complexes against TTF-1 expression in A549 lung cancer cells. L-Peptide is cyclic L-Ac-C(FKFE)₂CG-NH₂, D-peptide is cyclic D-Ac-C(FKFE)₂CG-NH₂, and WR₄ is cyclic L-Ac-C(WR)₄CG-NH₂. (A) Western blot densitometry of TTF-1 expression relative to actin expression. (B) Relative TTF-1 levels expressed as percent knockdown normalized against actin expression ($n = 3$).

C(FKFE)₂CG-NH₂, D-Ac-C(FKFE)₂CG-NH₂, and L-Ac-C(WR)₄CG-NH₂ (100 μ M) were premixed for 30 min with 600 nM siRNA targeted to the TTF-1 transcription factor, which is a master regulator of cells in the thymus and lung. Human pulmonary epithelial A549 and H441 cells were treated with the resulting cyclic peptide-siRNA complexes for 48 h, at which time TTF-1 expression was quantified by Western blot and densitometry analysis normalized to actin expression (Figure 3). This time (48 h) was determined previously to show optimal TTF-1 knockdown using standard liposomal reagents to deliver TTF-1 siRNA.³⁸ While naked siRNA failed to knockdown TTF-1 expression, each of the peptide-siRNA complexes displayed knockdown efficiencies of varying levels in both cell lines. In A549 cells, cyclic D-Ac-C(FKFE)₂CG-NH₂ and L-Ac-C(FKFE)₂CG-NH₂ provide TTF-1 knockdown of ~20% and ~85%, respectively. These results are interesting

since both enantiomers appeared to transport siRNA into the cytosol at similar levels. We hypothesize that the higher proteolytic stability of the D-peptide results in less efficient unpackaging of the siRNA cargo and thus less efficient knockdown due to lack of siRNA access to the RNAi machinery. We were gratified to find that the cyclic L-Ac-C(WR)₄CG-NH₂ peptide promoted >90% siRNA knockdown of TTF-1, consistent with the high transport capability of this peptide. Interestingly, knockdown of TTF-1 with cyclic L-Ac-C(FKFE)₂CG-NH₂ delivery is still surprisingly effective considering the difference in siRNA delivery efficiency of this peptide relative to cyclic Ac-C(WR)₄CG-NH₂.

Knockdown analyses utilizing human H441 cells showed a similar trend (Figure S13, Supporting Information). It was found that cyclic D-Ac-C(FKFE)₂CG-NH₂ and L-Ac-C(FKFE)₂CG-NH₂ provide TTF-1 knockdown of ~30% and 40%, respectively. Commercial transfection agents, Pepfect 14 and Lipofectamine, were assessed as comparative controls. Pepfect 14 (~50% TTF-1 knockdown) and Lipofectamine 2000 (~40% TTF-1 knockdown) provided knockdown levels that were comparable to the Ac-C(WR)₄CG-NH₂ (Figure S13). Significantly, these preliminary studies indicate favorable RNAi knockdown of a gene target in cells compared to existing CPP-siRNA transport vectors that range in effectiveness from 20–90% knockdown.^{22,28,31,39} The simplicity of the cyclic peptides described herein is a distinct advantage of existing CPPs for siRNA delivery.

A thioether cyclized peptide that is insensitive to reductive ring-opening, thioether-(WR)₄CG-NH₂ (Figure S1.F, Supporting Information), was synthesized in order to test the hypothesis that reductive ring-opening and proteolytic degradation of the cyclic CPPs is necessary for efficient gene knockdown. Gene knockdown with thioether-(WR)₄CG-NH₂ was tested in human H441 cells. It was found that thioether-(WR)₄CG-NH₂ displayed negligible knockdown in comparison to cyclic Ac-C(WR)₄CG-NH₂, with ~0% and ~45% TTF-1 knockdown with these peptides, respectively (Figure S13). These findings, coupled with the poor knockdown observed with cyclic D-Ac-C(FKFE)₂CG-NH₂, further indicate that reductive cleavage of the constraining disulfide bond and proteolytic degradation of the delivery peptide are essential for the unpackaging of siRNA leading to functional gene knockdown in the cell.

In order to further validate the functional ability of the disulfide-constrained cyclic peptides Ac-C(FKFE)₂CG-NH₂ and Ac-C(WR)₄CG-NH₂ as effective siRNA transfection agents, we delivered TTF-1 siRNA-peptide complexes *in vivo* to the lungs of C57B6 mice and assessed whole lung expression of TTF-1 via Western blot (Figure 4 and Figure S14). As identified earlier either the D- or L-isoform of both C-(FKFE)₂CG-NH₂ and Ac-C(WR)₄CG-NH₂ (100 μ M) were complexed with TTF-1 siRNA (100 nM) and then suspended in a total volume of 50 μ L of saline solution. Mice were anesthetized with isoflurane and placed in the supine position. The tongue of each animal was pulled out, gently, with blunt-ended forceps, and the 50 μ L of siRNA solution was delivered via pipet to the back of the tongue. Using a second pair of forceps the animals' noses were gently pinched shut, and the tongues remained held until the animal aspirated the solution into their lungs. After aspiration animals were allowed to recover for 48 h, at which time they were euthanized and lungs were harvested and lysed for Western blot detection of TTF-1. Similarly to the *in vitro* studies, animal lungs treated

Figure 4. *In vivo* knockdown of TTF-1 expression in mouse lung by TTF-1-targeted siRNA. TTF-1 expression was quantified by Western blot analysis relative to actin expression in mouse lung (see [Supporting Information and Figure S14](#) for details). T indicates TTF-1 specific siRNA, while Sc indicates a scrambled control ($n = 4$, $*p < 0.05$).

with either the D- or L-enantiomer of both Ac-C(FKFE)₂CG-NH₂ and Ac-C(WR)₄CG-NH₂ showed reduced TTF-1 protein expression compared to control groups, with the L-enantiomer of both groups showing the least expression of TTF-1, suggesting functional delivery of siRNA into cells and targeted degradation of TTF-1 mRNA. These results also identify the lung as an amenable target for RNAi based therapies.^{40,41}

Studies were conducted to characterize the formation of cyclic peptide-siRNA noncovalent complexes. A gel shift assay was utilized in which varying concentrations of cyclic L-Ac-C(FKFE)₂CG-NH₂ and L-Ac-C(WR)₄CG-NH₂ were incubated with siRNA ([Figure S15](#)). It was found that cyclic L-Ac-C(FKFE)₂CG-NH₂ complexed with siRNA only weakly, requiring much greater than 100-fold excess of peptide to siRNA before shifting of the siRNA band was observed on the agarose gel; even at large excesses of peptide, the shifted siRNA band was observed to streak on the gel, suggesting that the complex is weak. In contrast, at 100-fold excess of cyclic L-Ac-C(WR)₄CG-NH₂, the siRNA band was cleanly shifted indicating higher binding affinity for the Trp/Arg peptide to siRNA; this may account, in part, for its higher translocation efficiency for siRNA into the cytosol. These results support noncovalent complexation between the peptide and siRNA as the mode of siRNA transport, as opposed to independent poration of the membrane by the peptides independent of siRNA complex formation.

Dynamic light scattering (DLS) and transmission electron microscopy (TEM) were employed to determine the physical characteristics of the siRNA complexes formed with 10-fold and 1000-fold cyclic L-Ac-C(WR)₄CG-NH₂. DLS indicated the presence of polydisperse nanoparticles with an average hydrodynamic radius of 84 ± 12 nm ([Figure S16](#)). TEM images showed spherical nanoparticles with an average diameter of 18 ± 2 nm ([Figure S15](#)). These nanoparticles formed higher order aggregates consistent with the particle sizes observed by DLS experiments. These cyclic amphipathic peptides presumably encapsulate the siRNA duplex via hydrophobic/aromatic and/or charge interactions between the peptide and the siRNA. The opposite facial orientation of hydrophobic and hydrophilic side chain functionality in these peptides also enables presentation of functionality (guanidinium or ammonium) to the cell surface that promotes siRNA

translocation into the cell. In contrast, TEM showed no evidence of nanoparticle formation with between cyclic L-Ac-C(FKFE)₂CG-NH₂ and siRNA (in 1000-fold peptide excess); DLS shows polydisperse aggregates with no consistent radius. These results are consistent with a weaker binding affinity of cyclic Ac-C(FKFE)₂CG-NH₂ compared to cyclic Ac-C(WR)₄CG-NH₂ to siRNA. The correlation of cyclic peptide/siRNA binding affinity to translocation efficiency and a more detailed characterization of the resulting complexes are topics of ongoing research in our group.

Our data provides early insight into the basis for functional delivery of siRNA by these cyclic amphipathic peptides. While fluorescence microscopy indicates that cyclic Ac-C(WR)₄CG-NH₂ enables siRNA translocation much more efficiently than cyclic Ac-C(FKFE)₂CG-NH₂, TTF-1 knockdown *in vitro* and *in vivo* indicates that gene knockdown with cyclic L-Ac-C(WR)₄CG-NH₂ peptide was only slightly more efficient than was observed with cyclic L-Ac-C(FKFE)₂CG-NH₂. This suggests that high-density siRNA delivery is not absolutely essential for functional downregulation of gene expression. Additionally, D- and L-enantiomers of these cyclic peptides affected similar levels of siRNA translocation into the cytosol, but functional gene knockdown by siRNA was significantly more effective with the L-enantiomers. This data, combined with the inability of the permanently cyclized peptide to knockdown gene expression, is consistent with our hypothesis that disulfide bond reduction and proteolytic degradation of the delivery peptides contributes to unpackaging and presentation of siRNA to the RNAi apparatus. The mechanism by which disulfide-constrained cyclic peptides assist siRNA delivery to the cytosol is as yet unknown. Previous work by Parang and co-workers with delivery of small molecule drugs using N-to-C terminal cyclic Trp/Arg peptides suggests that this class of material does not utilize endocytic pathways, thus avoiding problems with endosomal escape.³² However, the possibility that our disulfide-constrained cyclic peptides exploit endocytic pathways for cargo translocation cannot, at present, be ruled out. Studies to elucidate the mechanism(s) by which our cyclic peptides enable siRNA transport into the cytosol are currently underway.

In conclusion, we have demonstrated that disulfide-constrained cyclic amphipathic peptides affect functional delivery of siRNA to cells both *in vitro* and *in vivo*. Our data supports the formation of noncovalent peptide-siRNA complexes that are able to penetrate into the cytosol. Our data also suggests that disulfide bond reduction in by chemical reductants in the cytosol likely plays a role in peptide linearization and proteolytic clearance, enabling unpackaging of the siRNA cargo. Functional knockdown of gene targets is highly efficient and is comparable to the best available siRNA transfection agents. Our simple peptides appear to be ideal for *in vitro* siRNA delivery in cell culture and for localized *in vivo* delivery to lung tissues. However, the peptide-siRNA complexes appear to be weak, suggesting that these peptides may not be ideal for systemic *in vivo* delivery siRNA. Evidence shown herein that the cyclic peptides are slowly degraded in fetal bovine serum (most likely due to the presence of glutathione) suggests that applications that require prolonged exposure of siRNA/cyclic peptide complexes to blood prior to reaching target tissues may be problematic. Additional studies are currently underway to characterize the mechanisms of siRNA delivery by these peptides as well as to correlate the strength of the noncovalent complexes to functional gene knockdown efficiency. These

studies will enable the design of next-generation siRNA delivery agents based on the disulfide-constrained cyclic peptides described herein.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsmedchemlett.6b00031](https://doi.org/10.1021/acsmedchemlett.6b00031).

Additional figures, experimental procedures, and characterization data (PDF)

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Notes

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