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Focused Library Approach to Discover Discrete Dipeptide Bolaamphiphiles for siRNA Delivery

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Abstract

In this study, we report a new dipeptide functionalization strategy for developing new dendritic bolaamphiphile vectors for efficient siRNA transfection. A focused library of dipeptides was constructed using four amino acids: L-arginine, L-histidine, L-lysine, and L-tryptophan. The dipeptides were coupled to two dendritic bolaamphiphile scaffolds that we developed previously, allowing us to quickly access a focused library of discrete vectors with multivalent dendritic dipeptide functionalities. The resulting discrete bolaamphiphiles were screened for siRNA delivery in vitro in HEK-293 and HeLa cells. Bolaamphiphiles functionalized with dipeptides containing Lys or Arg and either His or Trp were the most effective for in vitro siRNA delivery. Necessary cationic charge to ensure efficient siRNA binding are provided by Arg and Lys residues, whereas endosomal escape is provided through pH responsive buffering of His or membrane interactions of Trp. The most effective vectors (F10 HR/RH) exhibited greater than 75% gene silencing in multiple cell lines and exhibited serum stability.

Graphical abstract

Introduction

Development of safe and effective delivery vectors is a critical challenge for the application of small interference RNA (siRNA) technology. A variety of synthetic delivery materials have been reported, including polymers,^{1–3} lipids,^{4–6} den-drimers,^{7–9} peptides,^{10–12} and inorganic nanoparticles.^{13,14} Various strategies, including combinatorial screening approaches, have been employed in the discovery of new vectors for siRNA delivery.^{15–17}

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Despite major advances, the low efficiency of endosomal escape and general lack of vectors for targeted delivery to specific tissues warrant further exploration of new strategies and new vectors for siRNA delivery.

Given their biodegradability and biocompatibility, peptides are extensively investigated as vectors for gene delivery.^{10–12,18} Furthermore, functionalization of either small molecule amphiphiles or polymeric constructs with single amino acids or short peptides has proven to be a fruitful approach for developing effective carriers for siRNA delivery.^{19–21} Previously, our group has reported that functionalization of polymers and dendritic amphiphiles with a combination of different amino acids could produce highly effective vectors for siRNA delivery.^{22,23} Our previous functionalization strategy, however, yields a statistical mixture of compounds. The nondiscrete nature of these materials complicates the study of structure/property relationships and limits the potential therapeutic applications. Herein, we devised a new strategy in which dendritic bolaamphiphile (bola) scaffolds are functionalized with a focused small library of dipeptides for the discovery of well-defined, discrete vectors for efficient siRNA delivery.

Our choice of dendritic bola scaffolds to construct the library is based on our recent demonstration that these compounds are highly promising vectors for safe and efficient siRNA delivery. Compared to normal amphiphiles, bolas form more stable nanocomplexes with siRNA and exhibit lower cytotoxicity and membrane lytic properties.²³ Monoamphiphiles have high cytotoxicity and membrane lytic activity due to their structural similarity to phospholipids, whereas the unique architecture of the bolas prevent insertion. The two most effective bola cores from our previous study, a hydrocarbon (C18) core and a fluorocarbon (F10) core, were chosen for current investigation.

For our initial study, a small, focused library of dipeptides was constructed from combinations of L-arginine (Arg, R), L-lysine (Lys, K), L-histidine (His, H), and L-tryptophan (Trp, W). Our choices of the four amino acids are based on the following considerations: *First*, Arg and Lys introduce cationic charges to the vector, necessary for binding to anionic phosphates of siRNA. Several gene delivery carriers and cell penetrating peptides (CPPs) contain Arg and Lys residues.^{24,25} *Second*, an aromatic amino acid, Trp, was included for two reasons: (1) Both natural and synthetic CPPs are rich in Trp,^{26,27} which presumably promotes membrane insertion and cell uptake; (2) furthermore, structural studies of the RNA-induced silencing complex (RISC) have shown that aromatic amino acid residues are abundant and highly conserved in the siRNA binding pocket, playing a critical role for siRNA binding.²⁸ The enhanced binding to siRNA is presumably through intercalation of Trp indole ring to the siRNA base pairs.²⁹ Our previous studies have shown that incorporation of Trp to bola or polymer scaffolds significantly enhanced both the siRNA binding affinity and cell uptake efficiency of the complexes formed.^{22,23} *Lastly*, the pH responsive His was chosen for its ability to enhance delivery via increased endosomal escape.^{19,22,23,30}

A focused library of 12 dipeptides were synthesized using solution-phase coupling chemistry and then functionalized to the two bola cores (Figure 1). The dipeptide library was comprised of binary combinations of the four chosen amino acids (Lys, Arg, Trp, and His)

excluding homodimers. The dipeptides were coupled to generation 1 (G1) C18 and F10 bola cores to afford discrete dipeptide bolas. G1 dendritic bolas were chosen so that most of compounds contain the same net cationic charge (+8) as the most effective bola vectors from our previous study²³ (except HW/WH and RK/KR). In addition to the dipeptide bola vectors, for comparison, we also synthesized a control bola compound having the same net cationic charge as the other bolas but functionalized with only a single amino acid, Arg. The bolas were characterized by HPLC, MALDI-MS, and ¹H NMR (see Supporting Information). The vectors are referred to as X-R₁R₂, where X denotes the G1 bolaamphiphile core (C18 or F10) and R₁ and R₂ indicate the single letter code for the interior and terminal amino acid, respectively. For example, F10-HR refers to a bola containing the F10 core with His in the internal position and Arg in the terminal position.

Experimental Section

Materials

Unless otherwise noted, all reagents were used as received from commercial suppliers without further purification. Protected amino acids were purchased from Advanced Chemtech (Louisville, KY) and Aroz Technologies, LLC (Cincinnati, OH). 1H,1H,12H-perfluoro-1,12-dodecanedioil was purchased from Exfluor Research Corporation (Round Rock, TX). Coupling reagents were purchased from GL Biochem Ltd. (Shanghai, China). Sodium dextran sulfate (25 kDa) was purchased from TCI America (Portland, OR) and was used as received. GelRed siRNA stain was purchased from VWR (Radnor, PA). All siRNA used in this study was purchased from Ambion (Carlsbad, CA) with *Silencer*Select negative control siRNA and *Silencer*Cy-3 labeled negative control siRNA used for control and cellular uptake studies, respectively. The sequences for anti-lucII siRNA are as follows: 5'-AGA CUA UAA GAU UCA AUC Utt-3' (sense) and 5'-AGA UUG AAU CUU AUA GUC Utg-3' (antisense) where capital and lowercase letters represent RNA and 2'-O-methylated RNA, respectively. Lipofectamine RNAiMAX was purchased from Invitrogen (Carlsbad, CA) and used as a positive control following the manufacturer's protocol. All reactions were performed using HPLC grade solvents unless otherwise noted. All water used in biological experiments was nanopure water obtained from Barnstead Nanopure Diamond (Waltham, MA).

Typical Procedure for Synthesis of Dipeptides

In a 25 mL RBF, H-His(Trt)-Ome-HCl (809.0 mg, 1 equiv) and Boc-Trp(Boc)-OH (895.9 mg, 1 equiv) were dissolved in 5 mL of NMP-PyBOP (1248.9 mg, 1.2 equiv) and DIPEA (1.254 mL, 3.6 equiv) were added and stirred under N₂ overnight. After the reaction, the mixture was diluted in 20 mL of EtOAc and washed with brine (3 × 10 mL). The organic layer was dried over Na₂SO₄ and the solvent was removed in vacuo. The crude product was purified by column chromatography (5% MeOH in DCM). In a 25 mL RBF, the purified product was dissolved in 8 mL of 4:1 THF and 2 mL of H₂O. LiOH (720 mg, 10 equiv) was added to the mixture and stirred vigorously for 2 h. After the reaction, the THF was removed in vacuo, then the mixture was diluted in 50 mL of water. A 1 M HCl solution was added to acidify to a pH of 1. The acidified mixture was extracted with EtOAc (3 × 10 mL). The

organic layers were combined and dried over Na_2SO_4 and the solvent was removed in vacuo. All dipeptides were characterized by TLC and MS.

Synthesis of Bola-C18-G1 Dipeptide Vectors (3)

In a 1 dram glass vial were added 10 mg of the TFA salt of the unfunctionalized bola 2 (1 equiv) and Boc protected dipeptide (4.5 equiv). A total of 1.5 mL of NMP was added to dissolve the solids, followed by PyBOP (6 equiv) and DIPEA (15 equiv). The reaction was left to stir for 24 h at rt. The protected bola was precipitated in an excess amount of deionized water. After removing water completely, the solid was dissolved in 1 mL TFA, 2 mL DCM, 2 mL anisole, and 0.25 mL TIPS. After stirring overnight, the solvent was removed in vacuo, the resulting solid was redissolved in MeOH and precipitated in Et_2O . The white precipitate was dissolved in MeOH and purified by dialysis (MWCO = 1000) against MeOH. All bolas were characterized by ^1H NMR and MALDI.

Synthesis of Bola-F10-G1 Dipeptide Vectors

The TFA-salt of the unfunctionalized G1-bola with the fluorocarbon core was subjected to subsequent coupling reactions of boc-protected lysine as described in C18 bola synthesis to give a series of F10-XX compounds. All bolas were characterized by ^1H NMR and MALDI MS.

Gel Shift Assays

The binding of siRNA to the bolaamphiphile vector was studied through agarose gel shift assays. The siRNA was diluted in 10 mM phosphate buffer pH 7.4 and different amounts of vector (5 mg/mL) were added to 5 μL of 4 μM siRNA solution to achieve different N/P ratio (the molar ratio of amino groups from the vector to the phosphate groups from siRNA). A 10 mM phosphate buffer pH 7.4 was then added to achieve a final volume of 10 μL , followed by a 15 min incubation period at room temperature. A total of 2.5 μL of 6 $\times$ loading dye was added to each sample and 10 μL of the mixture was loaded to each well in 1% agarose gel with 1 $\times$ GelRed dye. The electrophoresis was run in TAE buffer at 60 V for 45 min and the gel was visualized under a UV transilluminator.

Dextran Sulfate Competitive Binding Assay

The binding strength of siRNA to bola was studied by competitive binding assay with dextran sulfate (DS). The bolaamphiphile vectors were added to 5 μL of 4 μM siRNA solution to achieve an N/P of 40 and the mixtures incubated for 30 min at rt. A total of 1 μL of DS solution at different concentrations was added to the complex to achieve different S/P ratio (the molar ratio of sulfate groups from DS and phosphate groups from siRNA) and the mixture incubated for another 30 min. The samples were then subjected to agarose gel electrophoresis under the aforementioned condition.

TEM Characterization

siRNA-amphiphile complexes for negative-stain TEM studies were prepared at 2–10 μM siRNA concentration and a N/P ratio of 20 in ddH $_2\text{O}$. In a typical procedure, 10 μL solution containing dendron amphiphiles were added to 10 μL of siRNA solution containing 160

pmol negative control siRNA. The solution was briefly vortexed and incubated at rt for 30 min before imaging. TEM grids (Ultrathin Carbon Type-A, 400 mesh) were glow discharged before use. Sample (8 μL) solution was placed on the grid and allowed to stand for 1 min. The solution was blotted away with a filter paper, while 15 μL of 2% uranyl acetate was pipetted on to the grid from the other side. After 1 min, the staining process was repeated with another 15 μL of 2% uranyl acetate solution. All the solution was removed by a filter paper and the grid was left to air-dry for 10 min before loading into the instrument. Images were obtained on a FEI Tecnai G2 TF20 high resolution TEM operated at an accelerating voltage of 200 kV.

DLS Measurements

The size and zeta potential of bola/siRNA polyplexes were measured at 633 nm using Zetasizer (NanoZS) dynamic light scattering instrument (Malvern Instruments, Malvern, U.K.) at 25 °C with detection angle of 173°. The stock vector solutions (5 mg/mL) were diluted to with ddH₂O and complexed with 6.5 μL of 40 μM siRNA diluted in with ddH₂O PBS (10 mM phosphate, 10 mM NaCl, pH = 7.4) to give a final [siRNA] of 2.5 μM with an N/P ratio of 20 and a final volume of 100 μL . After 5 min' incubation, the samples were analyzed for particle size. The complexes were then diluted to a final volume of 600 μL with PBS (10 mM phosphate, 10 mM NaCl, pH = 7.4) and then transferred into a disposable capillary cell for zeta potential analysis.

General Vector/siRNA Complex Preparation (OptiMEM)

Prior to complexation all vectors and buffers were allowed to equilibrate to room temperature and vortexed. A 1.5 μM solution of siRNA was prepared using OptiMEM as the dilution buffer and the appropriate amount of vector solution (5 mg/mL) required to give the desired N/P ratio diluted with OptiMEM. The vector solution was added to the siRNA solution and gently mixed via pipet to give a complex solution with [siRNA] = 100 nM. After 10 min incubation without agitation, this concentrated solution was gently mixed via pipet, further diluted to the desired concentrations with OptiMEM, and immediately added to the cell culture media.

Flow Cytometry Assay

HEK-293 cells were seeded at a density of 20000 cells/well in 48-well plates 24 h in advance. Prior to transfection, the media was replaced with 200 μL of OptiMEM. Different complex solutions were prepared as described in the general complex procedure (OptiMEM) previously and 50 μL added to each well to make the final siRNA concentration 100 nM. After 4 h incubation, cells were released from each well by trypsin and harvested by centrifugation (5 min, 400G). Fluorescence of transfected cells was measured on a Becton-Dickinson Accuri C6 flow cytometer with argon ion excitation laser. For each sample, data representing 10,000 objects were collected as a list-mode file and analyzed using Becton-Dickinson Accuri C6 software (Becton Dickinson, version 6.1.3). For cell uptake assay, fluorescently labeled negative control siRNA (siRNA-Cy3) was used and the uptake was quantified by the mean Cy3 fluorescence of each cell.

Transfection of Luciferase Expressing HEK 293 and HeLa Cells

Following standard protocols for the handling for HEK-293 and HeLa cells, the knockdown effects of the various vectors were assayed. After passaging, the cells were plated in 96-well plates at 5000 cells/well 24 h in advance. Immediately prior to addition of the complexes the culture media was switched to 80 or 90 μL OptiMEM per well. For initial screening, the vector/siRNA complexes were prepared using the general complex preparation protocol in OptiMEM. The 5 $\times$ vector/siRNA complexes were prepared as described previously and 10 or 20 μL added to each well to achieve the desired concentration. After 48 h of incubation post-transfection, the culture media was removed and replaced with 100 μL of a 150 $\mu\text{g/mL}$ solution of firefly D-luciferin in OptiMEM buffer. Without any further treatment, the cells were incubated at 37 °C for 5 min after which they were imaged using an IVIS lumina II camera. The normalized luciferase knockdown was determined by comparing the overall luminescence of the samples treated with complexes containing antiluc siRNA to those treated with complexes containing negative control siRNA.

LDH Assay in HEK 293 Cells

An LDH assay was performed following manufacturer's protocol to determine cellular toxicity. After 48 h of incubation post-transfection, 50 μL of culture media was removed and placed in a new clear bottom 96-well plate. A total of 50 μL of reaction mixture (prepared using manufactures protocol) and incubated at room temperature for 30 min protected from light. After 30 min, 50 μL of stop buffer was added to the wells. Absorbance was measured on a plate reader at 490 and 680 nm. To determine LDH activity, absorbance at 680 nm was subtracted from absorbance at 490 nm.

Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy was used to observe the trafficking of labeled siRNA in the transfected cells. Unmodified NIH 3T3 fibroblast cells were seeded at a density of 100000 cells/well on an 8-well chamber slide (Lab-Tek, Rochester, NY) 24 h before transfection. Cy3-labeled siRNA was complexed with bolaamphiphile vectors at N/P 60 and transfected to the cells under aforementioned conditions. After transfection, the media was changed back to DMEM supplemented with 10% fetal bovine serum. Confocal fluorescence spectroscopy was performed at different time points after the transfection. The endosome was stained with LysoTracker deep red (50 nM) for 30 min and nucleus was counter-stained 0.5 $\mu\text{g/mL}$ of Hoescht 33342 for 10 min prior to imaging. All confocal images were acquired using a Zeiss LSM 700 inverted laser-scanning confocal microscope. A 63 $\times$ numerical aperture of 1.4 oil immersion planapochromat objective was used for all experiments. A 559 nm helium–neon laser, a SMD640 dichroic mirror, and a 575–620 nm bandpass barrier filter were used to obtain the images of Cy3-labeled siRNA. Images of DAPI-stained nuclei were acquired using a 780 S39 nm two-photon excitation light, a 635 nm dichroic mirror, and a 655–755 nm band-pass barrier filter. The three fluorescent images were scanned separately and overlaid together with the differential interference contrast image (DIC). The cells were scanned as a z-stack of two-dimensional images (1024 $\times$ 1024 pixels) and an image cutting approximately through the middle of the cellular height was selected to present the intracellular siRNA localization.

Statistical Analysis

All quantitative assays were done in triplicates and the data was as mean $\pm$ SEM.

Results and Discussion

siRNA Binding Stability for the Vectors

The siRNA complexation properties of the dipeptide vectors were initially studied via gel shift assay. Increasing amounts of the vectors were mixed with siRNA to give complexes of the desired N/P ratio (molar ratio of the cationic protonated amines on a vector to the anionic phosphates on siRNA) to find the lowest ratio at which the siRNA is fully complexed. As shown in Figure S1, most of the bola vectors fully bound the siRNA at an N/P ratio of 2–8. Bola vectors containing the F10 core generally bound siRNA more strongly (i.e., at lower N/P ratios) than the C18 analogs (Figure S1), with the exception for HW and WH vectors. For the HW and WH vectors, the C18 bola bound much more strongly than the F10 analog, fully complexing at an N/P of 8 compared to N/P 30 respectively (Figure S1). C18-HW or WH, containing net cationic charge +4, formed fibrils (Figure 4E), which significantly enhanced the binding affinity to siRNA, as shown in our previous study.²³ For vectors with a net cationic charge of +8, Trp-containing vectors bound siRNA stronger than other vectors, complexing siRNA fully by N/P 2 for F10-RW and F10-KW, whereas F10-HR and F10-HK fully complex at an N/P of 4 (Figure S1). This is presumably due to intercalation of Trp indole ring into siRNA base pairs.²⁹ In gel shift assays, there is no discernible difference between Arg and Lys variants. siRNA binding strength showed no dependence on the sequence of amino acid in the dipeptide as both dipeptide sequence variants of all combinations bound at similar N/P ratios (Figure S1).

Competitive binding assay with dextran sulfate ($M_n \sim 25$ kDa) further confirmed the trend of binding strength for the library of bolas (Figure S2). Bolas containing the C18 core released siRNA at lower S/P ratios (the molar ratio of sulfate groups from DS and phosphate groups from siRNA) compared to the F10 analogs. Also, Trp-containing vectors released siRNA at higher S/P ratios than the non-Trp counterparts. For example, F10-RW did not release siRNA up to an S/P ratio of 45 whereas F10-RH started to release siRNA at an S/P of 20 (Figure S2). Again, no amino acid sequence dependence was observed in competitive binding assays.

Cell Transfection Study

The transfection efficacy of the bolas was screened in firefly luciferase (Luc)-expressing HEK-293 cells. For initial screening of gene knockdown, complexes were prepared at an N/P of 60 (Figure S3) and the luciferase activity assayed after 48 h. Multiple vectors induced potent gene silencing, with 14 of the 24 dipeptide functionalized bola vectors knocking down luciferase expression by over 75%, similar to commercially available Lipofectamine RNAiMAX (Figure 2). All of the vectors screened showed minimal cytotoxicity, with >90% cell viability for most vectors (Figure S4). The bolas with a net cationic charge of +8 were found to be more effective (HR, RH, HK, KH, KW, WK, RW, and WR), exhibiting low cytotoxicity and excellent gene silencing. Vectors with lower net cationic charge (HW/WH) were much less effective for gene silencing. Presumably, the weak siRNA binding by F10-

HW/WH vectors cause the low transfection efficacy. For C18-HW/WH, the formation of nanofibrils with siRNA prohibited cell uptake, leading to the ineffective gene silencing (vide infra). Control vectors functionalized with only Arg, with net cationic charge +8, were not as effective as the Arg dipeptide variants also containing Trp or His. Presumably, the pH responsiveness of His or the favorable membrane interactions of Trp promote endosomal escape and increase the gene silencing efficiency. Thus, the most effective vectors contain either a pH responsive residue His (H) or an aromatic residue Trp (W), in combination with a cationic residue, Lys or Arg. Such a combination of dual functionality in dipeptides is apparently important for the high transfection efficiency. Arg or Lys provides the necessary cationic charges to ensure efficient siRNA binding, and His or Trp facilitates endosomal escape. On the contrary, purely cationic vectors (RK/KR) or hydrophobic (HW/WH) vectors did not result in effective gene silencing.

Physicochemical Characterizations Using DLS and TEM

To characterize the physicochemical properties of the bola/siRNA complexes, the particle size and zeta potential were measured using dynamic light scattering (DLS). For efficient cellular uptake, siRNA nanoparticles must be smaller than several hundred nm in diameter and have slightly positive zeta potentials.³¹ All of the discrete dipeptide bolas having net cationic charge of 8 formed stable complexes with siRNA with diameters between 180 and 330 nm and zeta potentials ranging from +7 to +23 mV (Figure 3A,B). Bolas containing the F10 core in general formed slightly smaller complexes with higher zeta potentials than the analogous C18 vectors. Lys containing bolas formed larger complexes with higher zeta potentials than their Arg counterparts (e.g., C18-HK vs C18-HR). The morphology of the vector/siRNA complexes was further studied by transmission electron microscopy (TEM) imaging. All bolas imaged with net cationic charge +8 form spherical nanoparticles with sizes consistent with the values obtained by DLS (Figure 3C,D). For vectors containing net cationic charge +4 (HW/WH), the C18 bolas formed nanofibrils (Figure 3E), whereas the F10 bolas still formed spherical nanoparticles (Figure 3F). For C18-HW/WH, the decreased net cationic charge of the dendritic head groups leads to lower charge repulsion, thus, favoring higher packing density to form nanofibrils, in agreement with our previous observations.²³ For F10-HW/WH, the relatively short F10 core presumably does not allow close packing for nanofibril formation.

Cellular Uptake and Intracellular Trafficking

The cellular uptake of the vectors was studied by exposing NIH-3T3 cells to complexes prepared with Cy3-labeled siRNA for 4 h and then measuring fluorescence via flow cytometry. The bolas containing the F10 core generally exhibited greater cell uptake than the analogous C18 vectors (Figure 4A), consistent with our previous observation.²³ In general, vectors containing Arg displayed higher cell uptake than the Lys analogues, likely due to the higher affinity of the Arg side chains for anionic ligands on the cell membrane.³²

Once the vector is endocytosed, efficient endosomal escape is essential for an effective siRNA delivery vector. To directly visualize cellular uptake and monitor intracellular trafficking of the complexes, confocal microscopy was performed 24 h post transfection on cells transfected with Cy3-siRNA and stained with LysoTracker Deep Red (Figure S5).

Endosomal escape was inferred from the presence of Cy3-siRNA, indicated in green, in the cytosol and absence of colocalization with LysoTracker, indicated in red, after cellular internalization.^{33,34} The HR functionalized F10- and C18-bolas showed substantial uptake after 4 h via flow cytometry, and endosomal escape was evident from the Cy3 fluorescence in the cytosol after 24 h (Figure 4B,C). In contrast, control vectors functionalized only with Arg displayed significant cellular uptake after 4 h via flow cytometry, but exhibited minimal endosomal escape as a majority of the internalized Cy3-siRNA was localized in endosome (Figure 4D,E). The increased endosomal escape for F10- and C18-HR vectors was attributed to the pH responsive His residue, which has been shown previously to enhance endosomal escape via the so-called “proton sponge” effect.^{19,22,23,30}

Serum Tolerance and Transfection in Other Cells

We further investigated the serum tolerance of the vectors by performing transfections in media containing 10% fetal bovine serum (FBS). The C18 dipeptide bolas showed minimal gene silencing in the presence of serum (Figure S2). In contrast, the F10 dipeptide bolas afforded significant gene silencing in the presence of serum. A total of 6 of the F10 dipeptide bolas (HR, RH, KW, KW, RW, WR) had greater than 70% gene silencing in the presence of serum (Figure 5). The data is consistent with our previous observation that F10 bolas are significantly more tolerant to serum for transfection.²³

Finally, to demonstrate general efficacy of the dipeptide bola vectors, transfections were also performed in another cell line, Luc expressing HeLa cells (Figure 6). Increased cytotoxicity was observed in HeLa cells, which was directly correlated to the hydrophobicity of the vector. C18 bolas were more cytotoxic exhibiting <70% viability, whereas most F10 bolas were >80% viable (Figure S8). Trp-containing vectors also showed increased toxicity, again presumably because of the increased hydrophobicity. Arg-containing vectors (e.g., HR, RW) exhibited greater gene silencing in HeLa cells, compared to Lys vectors (e.g., HK, KW). Similar to the trend observed for HEK-293 cells, His and Arg functionalized variants (HR and RH) were the most effective vector exhibiting over 75% gene silencing in the F10 bolas, similar to Lipofectamine RNAiMAX (Figure 6). Increased cellular uptake and positive charge provided by Arg coupled with the pH responsive buffering capacity of His creates a highly effective dipeptide moiety for siRNA delivery.

Conclusion

In summary, we created a small focused library of dendritic bola vectors for safe and effective siRNA delivery. The head groups of the dendritic bolas were functionalized with dipeptides consisting of cationic amino acids (Arg, Lys), ionizable (His), and aromatic (Trp) amino acids. Out of the 24 discrete bolas created in this study, 14 exhibited greater than 75% gene silencing in HEK-293 cells. The F10 dipeptide bolas displayed good serum stability exhibiting over 75% knockdown in media containing 10% serum. The most effective bola vectors were functionalized with dipeptides consisting a combination of His and Arg (HR/ RH), with Arg providing additional positive charge to increase siRNA binding and increased cellular uptake and His facilitating in endosomal escape. The dipeptide system allows us to functionalize an amphiphilic scaffold with a single compound to afford a discrete vector

with dual functionality. This provides a simple peptide functionalization strategy to generate bolaamphiphile vectors with discrete structures for safe and efficient siRNA delivery. The methodology is applicable to the functionalization of bola scaffolds with many other peptides for further discovery and optimization of gene delivery vectors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Structure of bolaamphiphiles and the dipeptide library design.

Figure 2. Initial transfection screening in HEK-293 cells. siRNA transfection efficacy was screened by measuring Luc activity in HEK 293 cells after 48 h after treatment with the siRNA/bola complexes, which were prepared at N/P 60 and final [siRNA] = 20 nM.

A	Diameter (nm)	ζ potential (mV)	B	Diameter (nm)	ζ potential (mV)
C18-R	242	+8	F10-R	263	+17
C18-HR	220	+11	F10-HR	185	+14
C18-RH	188	+15	F10-RH	185	+15
C18-HK	322	+17	F10-HK	195	+18
C18-KW	314	+17	F10-KW	222	+21
C18-HW	-	+14	F10-HW	200	+11
C18-RK	265	+14	F10-RK	163	+23

Figure 3.

Physiochemical properties and morphology of siRNA/bola complexes. (A, B) Dynamic light scattering (DLS) measurements. The sizes of the complexes were measured at N/P 60 and [siRNA] = 2.5 μ M. Zeta potentials were measured in low salt PBS at [siRNA] = 0.5 μ M. (C–F) Negative-stain TEM (uranyl acetate) images of bola/siRNA complexes: (C) C18 HR, (D) F10 HR, (E) C18 HW, (F) F10 HW; Scale bars = 500 nm.

Figure 4. Cellular uptake by flow cytometry and intracellular trafficking for siRNA-bola complexes. (A) Cellular uptake of Cy3-siRNA-bola complexes in HEK-293 cells. Fluorescence was measured 4 h post transfection using a flow cytometer (unit: hundreds of RFU). Error bars represents standard deviation from the mean. (B–E) Confocal fluorescence images of NIH 3T3 cells 24 h post-transfection (nuclei were stained blue with Hoechst 33342 indicated in blue, lysosome was stained with LysoTracker Deep Red indicated in red, Cy-3 siRNA is indicated in green, scale bars 10 μ M): (B) F10-HR, (C) C18-HR, (D) F10-R, (E) C18-R.

Figure 5. siRNA transfection efficacy in the presence of serum was screened by measuring Luc activity in HEK 293 cells after 48 h after treatment with the siRNA-bola complex in the presence of 10% FBS at an N/P 90 and [siRNA] = 40 nM. Error bars represent standard deviation from the mean.

Figure 6. siRNA transfection efficacy was screened by measuring Luc activity in HeLa cells after 48 h after treatment with the siRNA-bola complex at an N/P 60 and [siRNA] = 20 nM. Error bars represents standard deviation from the mean.