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Targeted Systemic Delivery of a Therapeutic siRNA with a Multifunctional Carrier Controls Tumor Proliferation in Mice

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Abstract: In this study, novel peptide-targeted delivery systems were developed for systemic and targeted delivery of therapeutic siRNA based on a multifunctional carrier, (1-aminoethyl)iminobis[*N*-(oleicylcysteinylhistinyl-1-aminoethyl)propionamide] (EHCO), which showed pH-sensitive amphiphilic cell membrane disruption. EHCO formed stable nanoparticles with siRNA. Targeted siRNA delivery systems were readily formed by surface modification of the nanoparticles. PEGylation of the siRNA/EHCO nanoparticles significantly reduced nonspecific cell uptake. The incorporation of a bombesin peptide or RGD peptide via a PEG spacer resulted in receptor-mediated cellular uptake and high gene silencing efficiency in U87 cells. Fluorescence confocal microscopic studies demonstrated that EHCO/siRNA nanoparticles and PEG modified EHCO/siRNA nanoparticles were able to facilitate endosomal escape of the siRNA delivery systems. Systemic administration of a therapeutic anti-HIF-1α siRNA with the peptide-targeted delivery systems resulted in significant tumor growth inhibition than a nontargeted delivery system or free siRNA via intravenous injection in nude mice bearing human glioma U87 xenografts. The results indicate a great promise of the multifunctional carrier EHCO for systemic and targeted delivery of therapeutic siRNA to treat human diseases with RNAi.

Keywords: RNAi; systemic siRNA delivery; targeted siRNA delivery; multifunctional carriers; HIF-1α

Introduction

Tumor hypoxia poses a great challenge for effective cancer treatment.[1] Hypoxic cancer cells are resistant to apoptosis and produce factors that elevate glycolysis and angiogenesis and promote cell survival under hypoxic conditions.[2] Hypoxia inducible factor-1α (HIF-1α) is believed to act as an "angiogenic switch" in hypoxic cells to regulate the expression of vascular endothelial growth factor (VEGF) and is associated with tumor malignancy and poor prognosis.[3−5]

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In normoxia, HIF-1α protein is constitutively expressed, but it is rapidly degraded by proteasomal degradation and maintained at an almost undetectable level in normoxic cells. The degradation process is inhibited in hypoxic cells, leading to increased level of HIF-1, a heterodimer of HIF-1α and HIF-1β protein, a stable protein in normoxic cells.[6] The stabilization of HIF-1α protein is observed in malignant and metastatic tumors, but not in normal tissues.[7,8] Effective inhibition of HIF-1α in tumor tissues has a potential to prevent tumor proliferation and metastasis.[9]

RNA interference (RNAi) is an effective biochemical process that can silence specific disease related genes with small interfering RNA (siRNA).[10−14] Inhibition of HIF-1α expression with RNAi may result in suppression of tumor proliferation and alleviation of resistance to anticancer therapies. A recent study has demonstrated that silencing HIF-1α expression with RNAi can inhibit tumor growth in an animal model through local injection of anti-HIF-1α siRNA.[15] However, a practical approach for application of RNAi of HIF-1α in cancer treatment would be the systemic and specific delivery of anti-HIF-1α siRNA. Unfortunately, broad clinical development and application of RNAi are limited by the lack of efficient and specific delivery systems of therapeutic siRNA for systemic administration.[16−19] siRNA has poor in vivo stability, and its plasma half-life is only a few minutes after intravenous administration.[20] In order to achieve effective RNAi activity of clinical significance, suitable delivery systems are required to protect siRNA from degradation and specifically deliver siRNA to targeted cells. Currently available delivery systems are mainly adopted from the available nonviral gene delivery systems, e.g. cationic lipids and polymers.[17] These systems are either toxic or inefficient for systemic delivery of therapeutic siRNA. Novel efficient systemic and targeted siRNA delivery systems are needed for broad clinical applications of RNAi.[16]

Recently, we have designed and developed a novel class of polymerizable surfactants with pH-sensitive amphiphilicity as the multifunctional carriers for efficient siRNA delivery.[21,22] A distinct and advantageous feature of the carriers is the fine-tuned pH-sensitive amphiphilicity and amphiphilic cell membrane disruption at the endosomal−lysosomal pH, which can facilitate specific endosomal−lysosomal escape of the delivery systems.[22] The multifunctional carriers can readily form targeted siRNA delivery systems by modifying the surface of siRNA nanoparticles of the carriers with targeting ligands. In this study, we develop targeted delivery systems based on a multifunctional carrier, (1-aminoethyl)iminobis[*N*-

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(oleicylcysteinylhistinyl-1-aminoethyl)propionamide] (EHCO), for systemic and targeted delivery of an anti-HIF-1α siRNA to treat tumors by silencing HIF-1α expression with RNAi. The targeted delivery systems can facilitate cell-specific siRNA delivery and endosomal−lysosomal escape of siRNA after cell uptake, and result in significant suppression of tumor growth in vivo after intravenous administration.

Experimental Section

Materials. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) was purchased from Pierce Inc. (Rockford, IL). TransFast that is formulated with *N*,*N*-[bis-(2-hydroxyethyl)-*N*-methyl-*N*-[2,3-di(tetradecanoyloxy)propyl] ammonium iodide and dioleoyl phosphatidylethanolamine (DOPE) was purchased from Promega. Hyperbranched PEI (MW = 25 KDa), *N*-(2,3-dioleoyloxy-1-propyl)trimethylammonium methyl sulfate (DOTAP), and 2,5-diphenyl-3-(4,5-dimethyl-2-thiazolyl)tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). ISOLUTE column reservoirs (Charlottesville, VA) were used for the solid phase synthesis. Fluorescein-5-maleimide was purchased from Invitrogen (Carlsbad, California). NHS-PEG3400-maleimide and mPEG5000-Mal were purchased from Nektar (Huntsville, AL). Cyclic peptide c(RGDfK) was purchased from Peptide International (Louisville, KY).

(1-Aminoethyl)iminobis[*N*-(oleicylcysteinylhistinyl-1-aminoethyl)propionamide] (EHCO) was synthesized according to our previously described report.[16] Tumor specific peptide bombesin (sequence 7−14) with two consecutive 6-aminohexanoic acid units was synthesized on resin using standard peptide chemistry and was purified using preparative HPLC. Bombesin PEG conjugate with a thiol reactive maleimido end group (BN−PEG-Mal) was then prepared by reacting the bombesin peptide with NHS-PEG3400-Mal. Cyclic peptide c(RGDfK) PEG conjugate with a thiol reactive maleimido end group (RGD−PEG-Mal) was similarly prepared as a tumor specific targeting peptide for targeted siRNA delivery. The peptide conjugates were characterized by MALDI-TOF mass spectrometry.

Small Interfering RNA. siRNAs were purchased from Dharmacon (Chicago, IL). siGENOME nonspecific siRNA which has no gene targets in human, mouse and rat cells was used as a negative control (mismatch siRNA). An anti-Luc siRNA targeting firefly luciferase used for in vitro RNAi study has an antisense sequence of 5′-UCGAAGUACUCAGCGUAAGdTdT-3′ and a sense sequence of 3′-dTdTAGCUUCAUGAGUCGCAUUC-5′. A siRNA targeting alpha subunit of hypoxia-inducible factor (HIF-1) with the antisense sequence of 5′-UCACCAAAGUUGAAUCAGAdTdT-3′ and the sense sequence of 3′-dTdTAGUGGUUUCAACUUAGUCU-5′ is used for in vivo study. For in vivo study, siRNAs (anti-Luc siRNA and anti-HIF-1α siRNA, with the same respective sequence as mentioned above) were modified with Dharmacon's siSTABLE technology and purchased from Dharmacon.

Cell Lines. The human astrocytoma U87 cell line was obtained from ATCC (American Type Culture Collection, Rockville, MD). U87 cells were maintained in minimal essential medium (MEM, ATCC) at 37 °C in a humidified 5% CO_2 atmosphere. Cell culture medium was supplemented with fetal bovine serum (FBS, 10%), streptomycin (100 μg/mL) and penicillin (100 units/mL). U87 with constitutive firefly luciferase expression (U87-Luc) was generated by infecting U87 MG cells with recombinant retroviruses containing a luciferase gene. U87-Luc cells were maintained in minimal essential medium containing 10% FBS, G418 (300 μg/mL), treptomycin (100 μg/mL) and penicillin (100 units/mL).

Preparation of Peptide Targeted EHCO/siRNA Nanoparticles. The general scheme of the preparation of tumor-specific peptide targeted EHCO/siRNA nanoparticles is illustrated in Figure 1. EHCO/siRNA nanoparticles (N/P = 12) were prepared by mixing siRNA (1 μg) with 3.6 μL of EHCO (2.5 mM) in 5 mM Tris buffer (pH = 7) for 30 min. The peptide targeted nanoparticles were prepared by adding appropriate amount of maleimide-containing PEG peptide conjugates (0.225 nmol, 2.5 mol % BN−PEG-Mal or RGD−PEG-Mal relative to EHCO) 15 min after mixing siRNA (1 μg) with 3.6 μL of EHCO (2.5 mM) in 5 mM Tris buffer (pH = 7). Nontargeted pegylated EHCO/siRNA nanoparticles were prepare similarly by adding PEG-MAL (5,000 Da, 2.5 mol % relative to EHCO) to the EHCO and siRNA mixture. Fluorescein-labeled nanoparticles were obtained by adding fluorescein-5-maleimide (0.5 mol % relative to EHCO) in various siRNA nanoparticles during preparation. After 2 h incubation at room temperature in the dark, free maleimide derivatives were removed by ultrafiltration (Nanosep, MWCO = 100K, 5000 g, 5 min) and the siRNA nanoparticles were collected for further experiments. Fluorescence-tagged siRNA nanoparticles were similarly prepared. The sizes of the siRNA nanoparticles were measured by dynamic light scattering with a Brookhaven Instruments BI-200SM system equipped with a 5 mW helium neon laser with a wavelength output of 633 nm.

Flow Cytometry. Approximately 5×10^5 U87 cells per well were cultured in a 6-well plate for 24 h. Various fluorescein-labeled EHCO/siRNA nanoparticles containing 1 μg of siRNA per well were added to U87 cells and incubated at 37 °C for 4 h. The medium was then removed by aspiration, and the cells were washed twice with cold phosphate buffered saline (PBS) and trypsinized. The cells were collected and fixed with 2% PFA in PBS at 4 °C for 20 min. Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences). Results were analyzed using WinMDI software version 2.9 (Joseph Trotter).

Confocal Microscopy. Approximately 1×10^5 U87 cells were seeded in a 4-well cell culture chamber (Laboratory-Tek II Chamber Slide System, NUNC). Various nanoparticles of fluorescein-labeled siRNA containing 0.5 μg siRNA per well were applied to cells and incubated at 37 °C for 4 h. To label late endosomes and lysosomes, 75 nM LysoTracker Red (Molecular Probes) was added into cells 30 min before cell fixation according to the manufacturer's recommendation. The medium was removed by aspiration, and the wells

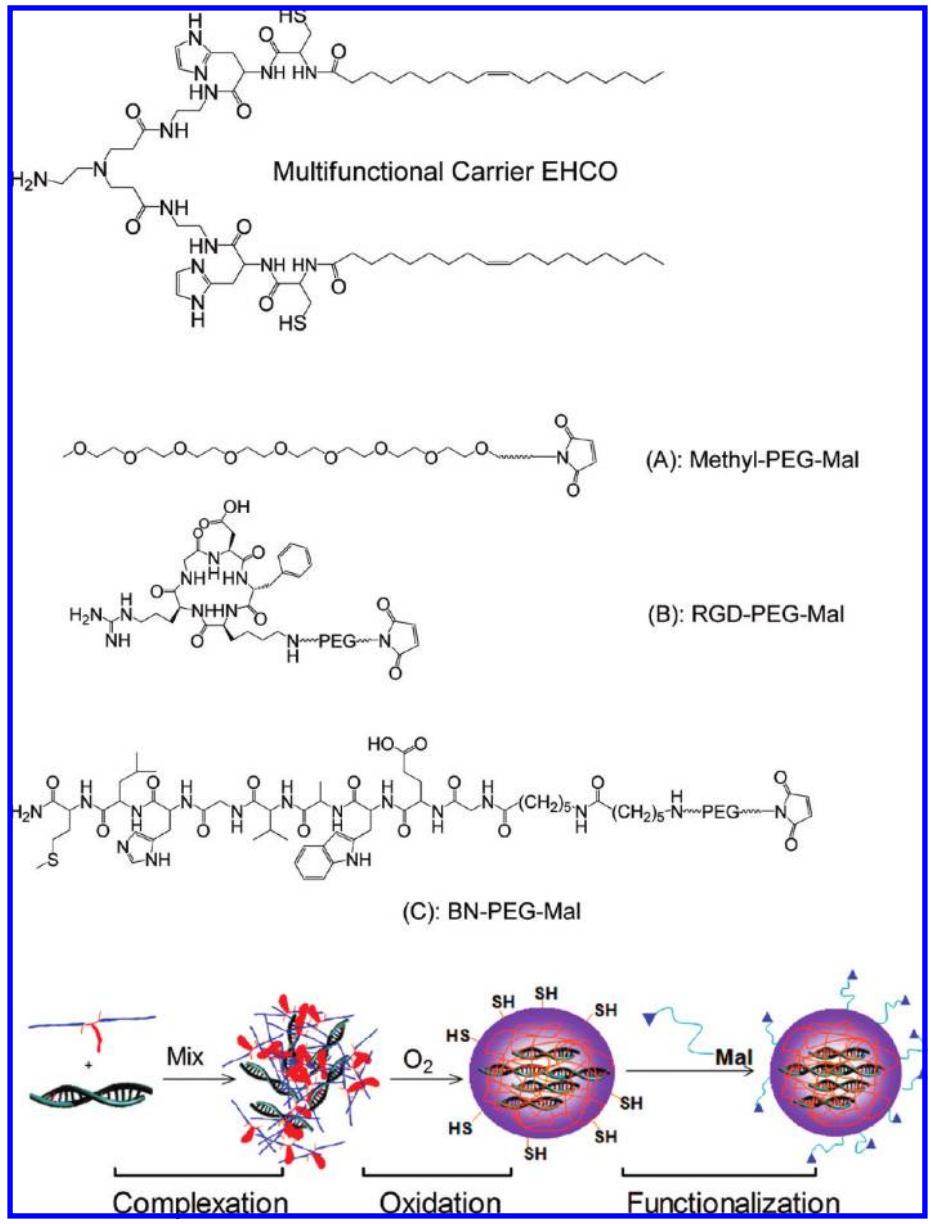

Figure 1. Chemical structures of EHCO, thiol reactive PEG and peptide PEG conjugates, and the scheme of preparation of ligand-directed siRNA delivery system.

were washed with cold phosphate buffered saline (PBS) and fixed with 1 mL/well of 4% paraformaldehyde (PFA) in PBS at 4 °C for 20 min. The fixation agent was aspirated; the cells were washed with PBS twice. The samples were visualized by confocal microscopy based on a Zeiss (Thornwood, NY) LSM 510 confocal imaging system with a Zeiss Axioplan 2 microscope and an argon laser (for fluorescein, excitation 488 nm, emission 505 nm long-pass filter; for LysoTracker-red, excitation 543 nm, emission 560 nm long-pass filter).

Intracellular siRNA Delivery. U87-Luc cells were seeded 24 h prior to transfection into a 96-well plate at a density of 5×10^3 cells/well. Transfast, DOTAP and PEI were used as controls. Transfast was used according to manufacturers' instructions. The siRNA nanoparticles of DOTAP and PEI were prepared at the N/P ratio of 3.6 and 10, respectively. At the time of siRNA transfection, the medium in each well was replaced with fresh serum-free medium. EHCO nanoparticles, pegylated, targeted nanoparticles and control nanoparticles of the antiluciferase siRNA were incubated with the cells at a siRNA concentration of 20 nM for 4 h at 37 °C. The medium was then replaced with 100 μL of fresh complete medium, and cells were incubated for an additional 44 h. All transfection tests were performed in triplicate wells. After the incubation, cells were washed with prewarmed PBS, treated with 200 μL of cell lysis buffer and then subjected to a freezing−thawing cycle. Cellular debris was removed by centrifugation at 14000*g* for 5 min. The luciferase activity in cell lysate (20 μL) was measured using a luciferase assay kit (100 μL luciferase assay buffer) on a luminometer for 10 s (Lumat 9605, EG&G Wallac). The gene silencing efficiency of various siRNA nanoparticles was

calculated as the percentage of the luciferase expression reduction in treated cells as compared to luciferase expression in untreated cells.

In Vivo Gene Silencing of Luciferase Expression. In vivo gene silencing of the EHCO/siRNA nanoparticles was investigated in a U87-Luc tumor model expressing luciferase with an antiluciferase siRNA. The animal model was developed by subcutaneous inoculation of approximately 2 $\times$ 10^6 U87-Luc cells in Matrigel mixture (v/v = 1) in female athymic nude mice. DOTAP/siRNA complexes were used as a control. EHCO/siRNA nanoparticles (300 μL) and the control were intraperitoneally injected into the mice (three in each group) at a siRNA dose of 2.5 mg/kg. Anti-Luc siRNA nanoparticles were injected on the days 15, 16 and 18 after tumor inoculation. For luminescence imaging, mice were intraperitoneally injected with 50 mg/kg firefly D-Luciferin (Xenogen Corp., Alameda, CA), 100 mg/kg ketamine, and 10 mg/kg xylazine hydrochloride (both from Sigma) in PBS. Luminescence images of the mice were then acquired 10 min after the injection with an IVIS 100 imaging system (Xenogen) with an exposure time of 1 s, medium binning. Quantitative analysis of luminescence intensity in the images was performed using LivingImage software (Xenogen). Luminescence imaging was taken on the 14th, 16th and 19th days after tumor inoculation to obtain the bioluminescence signal of mice before the injection and 2 and 5 days after the first injection.

Tumor Treatment with Systemic Delivery of Anti-HIF-1α siRNA. Antitumor efficacy of targeted nanoparticles (2.5% RGD or BN) of EHCO and anti-HIF-1α siRNA was investigated in a mouse model bearing U87 tumor xenografts. The animal model was similarly prepared as described above. Free anti-HIF-1α siRNA, pegylated EHCO/siRNA nanoparticles (2.5% PEG) and PEI/siRNA nanoparticles were used as controls. Anti-HIF-1α siRNA nanoparticles and free siRNA were administered to the mice (five in each group) at a siRNA dose of 2.5 mg/kg via tail vein injection on day 21 after tumor cell inoculation. Tumor growth was monitored every 2 to 4 days by measuring the tumor volume with a digital caliper. Tumor volumes were calculated using the formula $(1/6)\pi D_1^2 D^2$, where D_1 was the smaller diameter measured. The day that mice received siRNA was set as day 0, and the relative tumor growth after the treatment was calculated as the percentage of the tumor volume after the treatment as compared to the tumor volume at day 0. Mice received siRNA complexes on days 0, 2, 4, 8, 12 and 15, respectively. All of the mice treated with PEI/siRNA nanoparticles died instantly after the injection.

Statistical analysis was performed for multiple comparisons in quantitative data analysis using one-way analysis of variance (ANOVA) with post hoc Dunnett C-testing among the experimental data, and probability values of $P < 0.05$ were considered significant.

Results and Discussion

The targeted delivery systems for anti-HIF-1α siRNA were prepared by complexing siRNA with a multifunctional carrier, (1-aminoethyl)iminobis[*N*-(oleicylcysteinylhistinyl-1-aminoethyl)propionamide] (EHCO),[22] followed by the modification of the surface of the siRNA nanoparticles with tumor specific peptides (Figure 1). Peptides RGD and bombesin (BN) were used as the targeting agents, respectively, for the tumor specific delivery system. RGD can specifically bind to $\alpha_v\beta_3$ integrin that is overexpressed in angiogenic blood vessels and some endothelial cancer cells.[23] Bombesin is an analogue of mammalian gastrin-releasing peptide and neuromedin B and can bind specifically to the gastrin-releasing peptide receptor, neuromedin B receptor (NMB-R) and the orphan receptor bombesin receptor subtype 3 with high affinity.[24] These receptors are overexpressed in various cancers, including prostate, breast, lung, colon, ovarian and pancreatic cancers and malignant gliomas.[24] The peptides were conjugated to a PEG (3,400 Da) spacer with

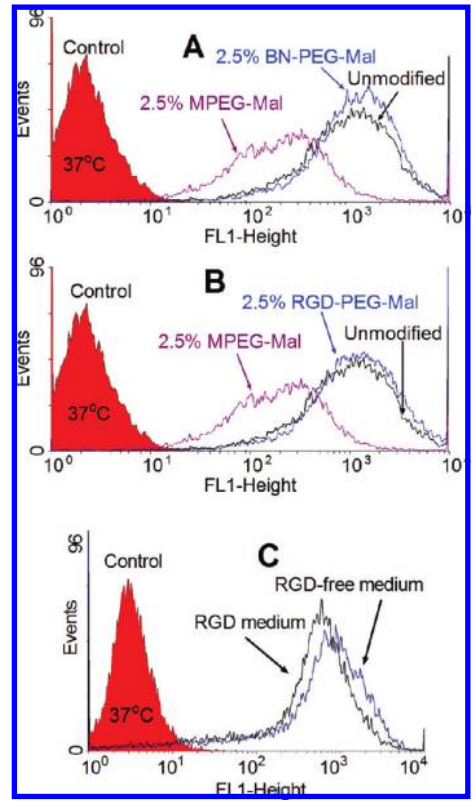

Figure 2. Flow cytometric analyses of cellular uptake of siRNA nanoparticles in U87 cells. Samples were measured on a FACSCalibur flow cytometer (BD Biosciences). (A) 2.5% BN−PEG-Mal modified siRNA nanoparticles. (B) 2.5% RGD−PEG-Mal modified siRNA nanoparticles. (C) Cellular uptake of 2.5% RGD−PEG-Mal modified siRNA nanoparticles in U87 cells with/without presence of RGD-containing medium.

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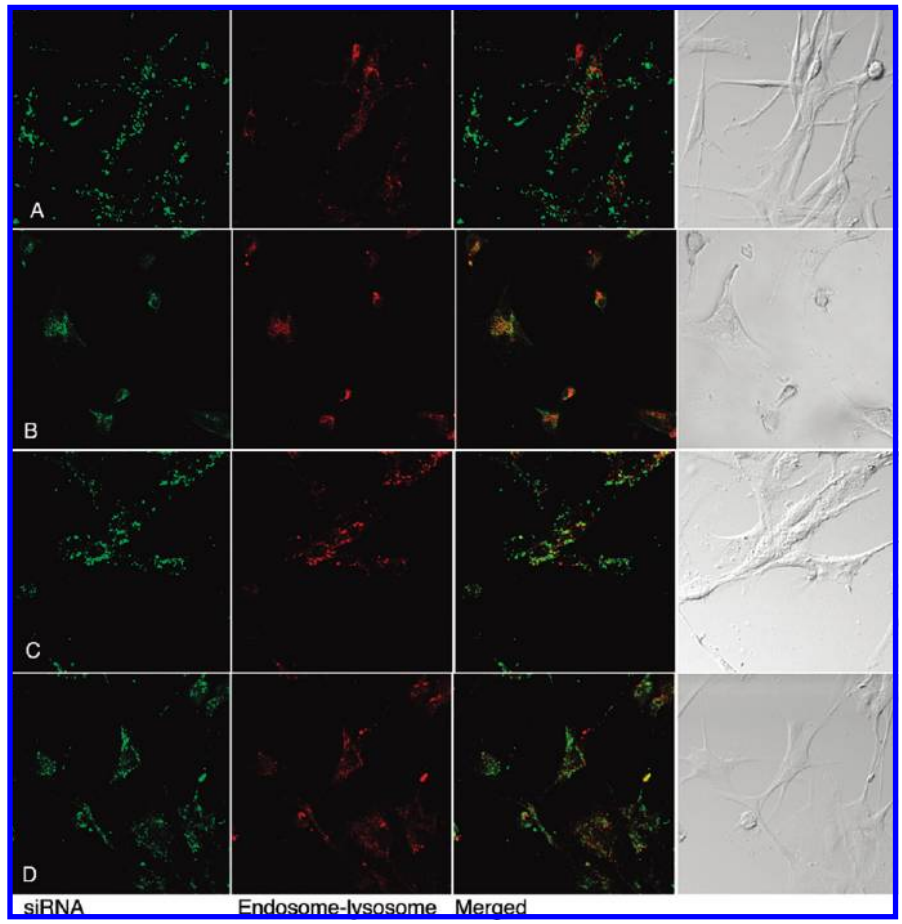

Figure 3. Confocal fluorescence microscopic images of different siRNA formulations treated U87 cells (fluorescein, excitation 488 nm, emission 505 nm long-pass filter; LysoTracker Red, excitation 543 nm, emission 560 nm long-pass filter): (A) siRNA/EHCO nanoparticles; (B) pegylated (PEG, 5,000 Da, 2.5%) nanoparticles; (C) bombesin targeted nanoparticles; (D) RGD targeted nanoparticles.

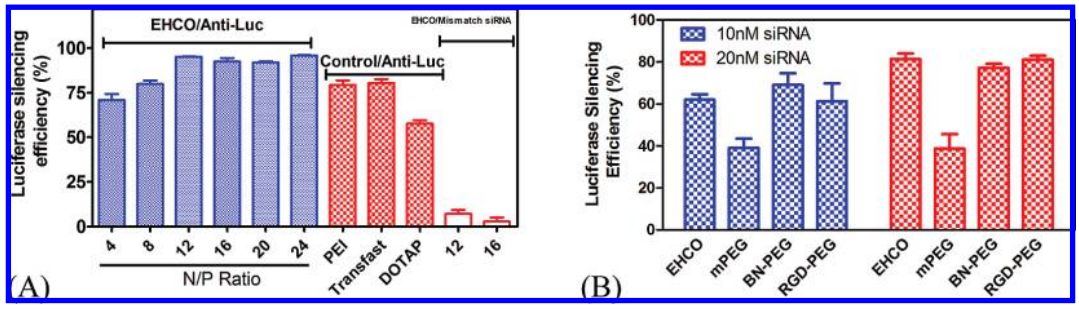

Figure 4. In vitro silencing of luciferase gene expression in U87-Luc cells. (A) Luciferase silencing efficiency by EHCO/anti-Luc siRNA complexes at different N/P ratios. PEI, TransFast and DOTAP were used as control carriers, and mis-match siRNA was used as a siRNA control. (B) Luciferase silencing efficiency by EHCO/anti-Luc complexes or pegylated EHCO/anti-Luc siRNA complexes at N/P ratio of 12.

a maleimido group to give RGD−PEG-MAL or BN−PEG-MAL. The peptides were incorporated to the siRNA nanoparticles by reacting maleimido group with the thiols on the surface of the nanoparticles. The PEG spacer was designed to minimize the nonspecific tumor uptake, prolong the blood circulation and improve tumor targeting efficiency of the targeted delivery systems. Unmodified EHCO/siRNA nanoparticles and pegylated EHCO/siRNA nanoparticles were prepared as the nontargeted controls. The sizes of EHCO/siRNA, RGD−PEG/EHCO/siRNA, BN−PEG/EHCO/siRNA and mPEG/EHCO/siRNA nanoparticles were 179 ± 9, 184 ± 6, 170 ± 10 and 186 ± 9 nm, respectively, as measured by dynamic light scattering (DLS). The nanoparticles were also labeled with fluorescein by reacting fluorescein-5-maleimide (0.5% molar ratio based on EHCO) with the surface thiols to allow us determining cellular uptake of targeted siRNA delivery system.

Cellular uptake of the peptide targeted siRNA nanoparticles was evaluated by flow cytometry in human glioma U87 cells with the pegylated and unmodified nanoparticles as the

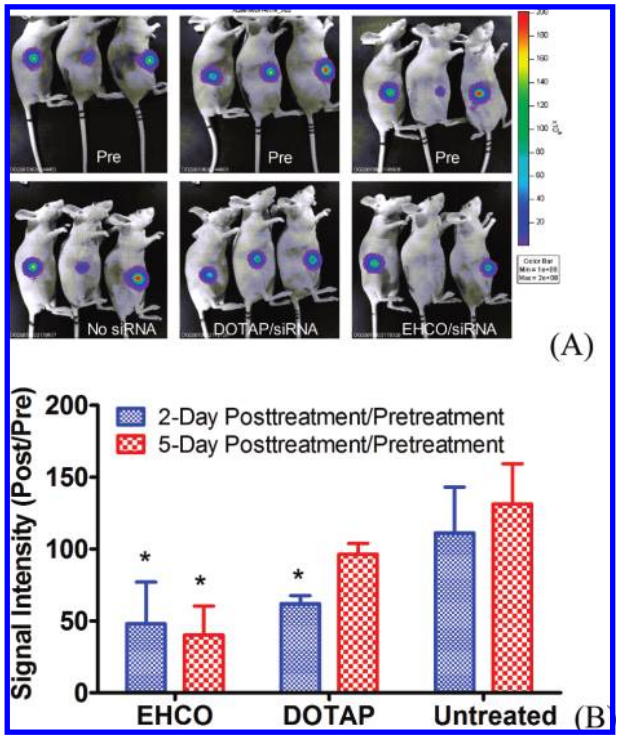

Figure 5. (A) Representative bioluminescence images of mice before and 2 days after the first injection of siRNA nanoparticles. (B) Luciferase expression knockdown efficiency in different mice groups. The bioluminescent signal intensity was quantified using LivingImage software (Xenogen), and gene silencing was calculated by average signal intensity of post/pre in different mouse groups. Data represent the mean ± SD ($n = 3$). *: $P < 0.05$ versus untreated groups.

nontargeted controls, Figure 2. High cell uptake was observed for the unmodified EHCO/siRNA nanoparticles, possibly due to nonspecific internalization through charge interaction of the positively charged nanoparticles with the negatively charged cell surface. The pegylation of the EHCO/siRNA nanoparticles limited the nonspecific charge interaction and resulted in reduced cellular uptake of mPEG/EHCO/siRNA nanoparticles. The incorporation of tumor specific peptides, RGD and bombesin, into the nanoparticles via the PEG spacer facilitated receptor-mediated endocytosis. As a result, the cellular uptake of RGD−PEG/EHCO/siRNA or BN−PEG/EHCO/siRNA nanoparticles was significantly increased compared to mPEG/EHCO/siRNA nanoparticles, Figure 2A,B. A binding competition study showed that the presence of free RGD reduced cell uptake of the RGD targeted siRNA nanoparticles, Figure 2C, because free RGD peptide competed with the targeted nanoparticles for the same target at the cell surface.

One of the key features of EHCO is the pH-sensitive amphiphilicity, which only causes hemolysis at pH 5.4, the endosomal−lysosomal pH, but not at the physiological pH (7.4) at the concentration for transfection. The pH-sensitive amphiphilicity of EHCO may facilitate endosomal−lysosomal escape of siRNA nanoparticles at the reduce pH in the endosomal−lysosomal compartments. The endosomal−lysosomal escape of the siRNA/EHCO nanoparticles, pegylated (PEG, 5,000 Da, 2.5%) nanoparticles, bombesin and RGD targeted nanoparticles with a PEG spacer (3,400 Da, 2.5%) was investigated using fluorescence confocal microscopy. Figure 3 shows the confocal microscopic fluorescence images of the intracellular location of the nanoparticles of labeled siRNA and late endosomes and lysosomes in U87 cells. It clearly shows that most labeled siRNA nanoparticles did not overlap with the late endosomes and lysosomes for all siRNA nanoparticles. This observation suggested that the siRNA nanoparticles, pegylated nanoparticles and targeted nanoparticles with a PEG spacer can readily escape from endosomal−lysosomal compartments to cytoplasm. The results demonstrated that EHCO with pH-sensitive amphiphilic cell membrane disruption at the endosomal−lysosomal pH facilitated the endosomal−lysosomal escape of the delivery systems, validating the hypothesis that pH sensitive amphiphilicity of the multifunctional carriers could promote the escape of siRNA delivery systems from the endosomal−lysosomal compartments.

Cellular gene silencing activity of the targeted siRNA nanoparticles was evaluated with an antiluciferase siRNA in U87-Luc cells with constitutive luciferase expression. At 20 nM siRNA, EHCO showed the superior cellular siRNA delivery efficiency to some of the commonly used transfection agents, including PEI, Transfast and DOTAP in U87-luc cells (Figure 4A), most likely due to its specific pH-sensitive amphiphilicity induced endosomal−lysosomal escape. The nanoparticles of a mismatch siRNA with EHCO did not show any efficacy on silencing luciferase expression. The pegylated EHCO/siRNA nanoparticles showed significant decrease of luciferase gene silencing efficiency as compared to unmodified EHCO/siRNA nanoparticles, Figure 4B. In contrast, both peptide-PEG modified nanoparticles exhibited comparable luciferase gene knockdown efficiency to unmodified ECHO nanoparticles, much higher gene silencing efficiency than that of mPEG modified nanoparticles, Figure 4B. The luciferase knockdown efficiency of the targeted siRNA nanoparticles correlated well to their cellular uptake as determined by flow cytometry. Receptor-mediated endocytosis of the targeted EHCO/siRNA nanoparticles with a PEG spacer resulted in high cellular uptake, which consequently resulted in high gene silencing efficiency as compared to the nontargeted pegylated nanoparticles.

The systemic in vivo delivery efficiency of EHCO/siRNA nanoparticles was first investigated in mice bearing human U87-Luc xenografts using an antiluciferase siRNA. DOTAP was used as the control because it is also an amphiphilic carrier for siRNA.[25] Both EHCO and DOTAP formed turbid solutions after complexation with siRNA at a relatively high siRNA concentration (0.5 mg/mL) for a dose of 2.5 mg/kg at an injectable volume (100 μL/mouse). Apparent precipitate was observed in the DOTAP/siRNA complexes at this

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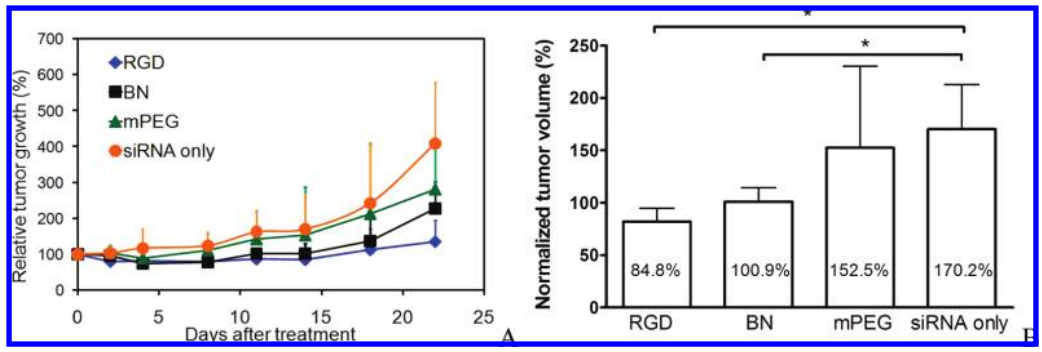

Figure 6. Intravenous administration of 2.5% peptide-modified EHCO/siRNA nanoparticles significantly inhibited tumor growth rate in mice as compared to nontargeted pegylated EHCO/siRNA nanoparticles (2.5% mPEG-Mal modified nanoparticles) or free siRNA. Normalized tumor growth curve in the mice ($n = 5$) treated with different siRNA formulations (A). Normalized tumor volume of mice 14 days after the first injection of different siRNA formulations. Data are represented as the mean ± SD ($n = 5$). *, $P < 0.05$ versus untreated groups (B).

concentration and no precipitation was observed for the EHCO/siRNA complexes. The siRNA complexes were administered by intraperitoneal injection. Dilution of the DOTAP/siRNA complexes to 0.1 mg/mL minimized the precipitation of complexes. Since both formulations were turbid and not suitable for intravenous injection, they were administered by intraperitoneal injection. Anti-Luc siRNA nanoparticles were injected on days 15, 16 and 18 after tumor inoculation, and bioluminescence imaging was taken on days 14, 16 and 19 after tumor inoculation, which corresponded to one day before the injection and 2 days and 5 days after injection, respectively. Figure 5A shows the bioluminescent images of the mice before and 2 days after the injection of saline, DOTAP/siRNA and EHCO/siRNA complexes and normalized bioluminescence signal intensity (post/pre) in U87-Luc tumor after the treatments. The bioluminescence signal intensity in the tumor decreased after the first injection of both DOTAP and EHCO siRNA complexes, while the signal in the control group increased. Quantitative analysis showed that DOTAP/siRNA complexes resulted in approximately 38% inhibition of luciferase expression 2 days after the first injection, while the silencing efficiency significantly decreased to approximately 4% on the fifth day and two more injections. In contrast, EHCO/siRNA complexes resulted in approximately 52% inhibition of luciferase expression 2 days after the first injection and up to 60% on the fifth day and two more injections, Figure 5B. The results have demonstrated that the multifunctional carrier EHCO was more effective than DOTAP for systemic in vivo delivery of siRNA.

Intraperitoneal injection has been considered less efficient than intravenous injection in terms of the bioavailability of therapeutic siRNA. The turbidness of EHCO/siRNA complexes decreased significantly after the modification with PEG and the peptide−PEG conjugates because pegylation of EHCO might stabilize the nanoparticles and prevent aggregation. The efficacy of the systemic and targeted delivery of the therapeutic anti-HIF-1α siRNA of the peptide-targeted delivery systems was investigated via intravenous injection in mice bearing human glioma U87 xenografts. Pegylated EHCO/siRNA nanoparticles, PEI/siRNA complexes and free siRNA were used as controls and administered intravenously at the same siRNA dose. PEI is one of the most commonly investigated carriers for siRNA delivery and generates substantial cellular siRNA delivery.[26] Unfortunately, the mice treated with PEI/siRNA complexes died immediately after the first injection, most likely due to the toxicity of PEI. The mice in the other groups were injected five more times in a period of two weeks. The mice treated with RGD and BN targeted siRNA nanoparticles had significantly slower tumor growth rate than those treated with the pegylated delivery system and free siRNA, Figure 6. At 14 days after the first treatment, the average tumor size of the mice treated with RGD targeted nanoparticles, bombesin targeted nanoparticles, pegylated nanoparticles and free siRNA were approximately 84.8%, 100.9%, 152.5% and 170.2% of corresponding initial tumor size before the treatment. The peptide targeted siRNA/EHCO nanoparticles resulted in more significant suppression of tumor growth than the free siRNA ($p < 0.05$) during the two-week period of treatment. It is interesting to note that the RGD-targeted delivery system was more effective than the bombesin targeted system in tumor inhibition after the last injection, possibly because RGD could deliver siRNA into both angiogenic epithelial cells and cancer cells.[23] The results indicated that tumor specific peptide targeted EHCO/siRNA nanoparticles were effective for systemic targeted delivery of the therapeutic anti-HIF-1α siRNA into tumor tissue, resulting in significant tumor growth inhibition.

The results in this study have shown that systemic and targeted delivery of an anti-HIF-1α siRNA is effective to control tumor growth in mice by silencing the expression of HIF-1α protein. The novel peptide targeted delivery systems is critical to achieve tumor-specific siRNA delivery and effective RNAi activity. The systemic injection of the free anti-HIF-1α was not able to suppress tumor

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growth, although some of recent studies showed that the naked siRNA might stimulate immune response.[27] The targeted delivery systems demonstrated several advantageous features, including easy modification in formulations, pH-sensitive amphiphilic endosomal−lysosomal escape, low toxicity for systemic injection and high in vitro and in vivo siRNA delivery efficiency. Unlike other lipid delivery systems, no helper materials are needed for the delivery systems based on the multifunctional carriers to achieve efficient in vitro and in vivo siRNA delivery. The targeted delivery systems with the multifunctional carrier are promising for highly efficient systemic and targeted delivery of therapeutic siRNA.

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