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Retro-Inverso CendR Peptide-Mediated Polyethyleneimine for Intracranial Glioblastoma-Targeting Gene Therapy

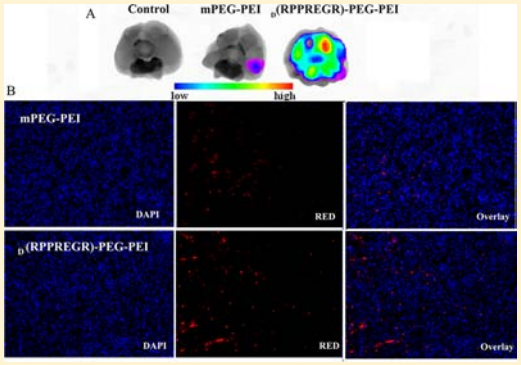
Jing Wang,[†] Yang Lei,[†] Cao Xie,[†] Weiyue Lu,[†] Ernst Wagner,[†] Zuoxu Xie,[†] Jie Gao,[†] Xiaoyu Zhang,[†] Zhiqiang Yan,[‡] and Min Liu*[,†]

[†]Key Laboratory of Smart Drug Delivery(Fudan University), Ministry of Education, Department of Pharmaceutics, School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai 201203, PR China

[‡]National Engineering Research Center for Nanotechnology, Shanghai 200241, PR China

Supporting Information

ABSTRACT: The development of nonviral gene delivery vectors offers the potential to provide effective treatment for glioblastoma in the form of gene therapy. Here, we report the use of retro-inverso C-end rule (CendR) peptide $_{D}$(RPPREGR) as a targeting ligand to prepare a $_{D}$(RPPREGR)-PEG-PEI gene vector. $_{D}$(RPPREGR) peptide specifically recognized the neuropilin-1 receptor that was overexpressed on U87 glioma cells, and showed enhanced tumor spheroid penetration ability. Compared with parental RGERPPR, $_{D}$(RPPREGR) possessed improved biological stability and had a higher affinity for U87 glioma cells; it also showed enhanced penetration of the tumor spheroid. mPEG-PEI/pDNA and $_{D}$(RPPREGR)-PEG-PEI/pDNA complexes were prepared and MTT assay results revealed that the cytotoxicity of $_{D}$(RPPREGR)-PEG-PEI complexes was significantly lower than that of PEI complexes, with cell survival rates above 80%. Qualitative and quantitative *in vitro* transfection results revealed that $_{D}$(RPPREGR)-PEG-PEI complex transfection efficiencies were 1.9 times higher than those of mPEG-PEI. Fluorescent imaging and frozen sections of brain tissue demonstrated that the $_{D}$(RPPREGR) modification improved the *in vivo* transfection efficiency of mPEG-PEI in nude mice bearing U87 gliomas. An antiglioblastoma assay revealed that $_{D}$(RPPREGR)-PEG-PEI carrying the therapeutic gene pORF-hTRAIL significantly prolonged the survival time of intracranial U87 glioma-bearing mice from 25 to 30 days. Therefore, $_{D}$(RPPREGR)-PEG-PEI appears to be suitable for use as a safe and efficient gene delivery vehicle with potential applications in glioblastoma gene therapy.



INTRODUCTION

Ligands are an important part of the targeted drug delivery system, so choosing a ligand with high affinity and stability has become a subject of considerable interest. Since its first report in 1985, phage display technology has been used to screen and obtain a plurality of tumor-homing peptide ligands such as LyP-1, RGD, F3, NGR, SP5−52, CendR peptide, CDTRL, and CKTKRKC.[1−8] However, these peptides consist of L-amino acids that are susceptible to protease degradation in plasma and so readily lose their tumor-homing capability.

The methods often used to improve the stability of peptides include structural transformation, such as cyclization, terminal amino acid modification, and changes to peptide bonds, as well as the introduction of non-natural amino acids such as L-amino acids or D-amino acids. D-Peptides are resistant to protease degradation *in vivo*, so have a high biological stability. However, D-peptides and L-peptides with the same amino acid sequence possess different chiral structures resulting in loss of their biological activity. In 1979, Chorev and Goodman devised a retro-inverso method which involves assembling amino acid residues in the reverse order of their parent peptide sequence and replacing L- with D-amino acids. Compared with parent L-peptides, retro-inverso peptides present an orientation of their side chains very similar to that of the original structure. They not only possess improved biological stability, but may also have similar bioactivities compared with L-peptides.[9−15]

C-end rule (CendR) peptides possessing a consensus R/KXXR/K motif with L-amino acids were identified by Erkki Ruoslahti from phage display technology.[1] They specifically bind neuropilin-1 (NRP-1), which is a transmembrane glycoprotein and a coreceptor of semaphorin3A and VEGF165. NRP-1 plays an important role in tumor angiogenesis, tumor growth, and metastasis,[16−18] and was found not only to be expressed on endothelial tumor cell membranes, but also to be highly expressed on many other tumor cell membranes including glioma, lung cancer, pancreatic cancer, prostate cancer, breast cancer, and melanoma.[19,20] By binding NRP-1, CendR peptides possess both tumor homing and CendR-dependent tissue-penetrating properties, which could be used to deliver drugs into tumor parenchyma, beyond the vascular barrier. However, these peptides have a low biological

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stability, resulting in the loss of binding capability and tissue penetration ability.

In this study, we chose one of the CendR peptides (sequence RGERPPR) as a parent peptide, and synthesized its retro-inverso $_D$(RPPREGR) to study its biological stability and targeting properties. We then conjugated $_D$(RPPREGR) with polyethylene glycol(PEG)-modified polyethylenimine (PEI) to obtain a nonviral gene delivery vector, with the aim of achieving effective treatment of brain gliomas. To our knowledge, no research has previously focused on the retro-inverso $_D$(RPPREGR) peptide or its application in nonviral gene delivery systems.

EXPERIMENTAL PROCEDURES

Materials. CRGERPPR, $_D$(CRPPREGR), RGERPPR, and $_D$(RPPREGR) were purchased from ChinaPeptides (Shanghai, China). High molecular weight PEI (branched, M_W 25 000) was obtained from Sigma-Aldrich (Chemie GmbH, Munich, Germany). CH_3O-PEG-Mal (mPEG-Mal, M_W 2000) and Mal-PEG-NHS (M_W 2000) were from Jenkem Technology (Beijing, China). Rat serum was from Jianlun Biology Technology Co. (Guangzhou, China). Aminopeptidase M (leucineaminopeptidase, microsomal from porcine kidney Type VI−S, lyophilized powder, EC 3.4.11.2) was also obtained from Sigma-Aldrich. Fluorescein-5-maleimide (Mal-FITC) was purchased from Fanbo Biochemicals (Beijing, China), 4′,6-diamidino-2-phenylindole (DAPI) was supplied by Roche (Basel, Switzerland), and the Luciferase Assay Kit was from Promega (Beijing, China). The BCA Protein Assay Kit was purchased from Beyotime (Shanghai, China). The plasmids encoding EGFP (pEGFP-N2) and RFP(pDsRED-N1) were products of Genechem Co. (Shanghai, China), and the luciferase reporter gene plasmid ($pGL_{4.2}$) was from Promega.

The pORF-hTRAIL plasmid was a kind gift from Professor Chen Jiang (School of Pharmacy, Fudan University, China). A Plasmid Mega Kit was used to purify the plasmid DNA before application (Qiagen GmbH, Hilden, Germany). The U87 glioma cell line, U251 glioma cells, and the HEK 293 human embryonic kidney cell line were purchased from the Shanghai Institute of Cell Biology, and cultured in Dulbecco's modified eagle's medium (DMEM) (Invitrogen, Beijing, China) containing 10% fetal bovine serum (FBS) (Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco).

***Ex Vivo* Stability Studies of Peptides.** $_D$(RPPREGR) and RGERPPR peptides (both 0.5 mg, and characterized by high performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS); see Supporting Information, Figure S1) were dissolved in 1 mL of 50% rat serum diluted with water and incubated at 37 °C. After different incubation times, 100 μL serum medium was removed and mixed with 500 μL 15% trichloroacetic acid (TFA), placed at 4 °C for 30 min, then centrifuged at 13 000 rpm for 10 min. The supernatant was analyzed using reverse-phase HPLC (Agilent1100 series, Agilent, Palo Alto, CA, USA) on a YMC C-18 column (4.6 mm × 150 mm, 5 μm) using a gradient method. The mobile phase A was purified water with 0.1% (v/v) TFA. The mobile phase B was acetonitrile with 0.1% TFA. The 30 min linear elution gradient ran from 0% B to 20% B at a flow rate of 0.7 mL/min with a wavelength of 214 nm. We used aminopeptidase to further investigate the stability of L-peptides or D-peptides in proteolytic enzymes. $_D$(RPPREGR) or RGERPPR (both 0.25 mg) was dissolved in 1 mL of PBS, pH 7.4, with aminopeptidase to a final concentration of 1 μg/mL. The peptides were then analyzed as described above. All experiments were carried out three times.

Synthesis of FITC-Labeled Peptides. To prepare fluorescein-labeled peptides, a 1.2× molar excess of Mal-FITC was dissolved in 1 mL of PBS (0.1 M, pH 7.0) with 5 mg $_D$(CRPPREGR) and stirred for 1 h in the dark. The mixture was purified via HPLC (Waters 600E, Milford, MA, USA) on a Symmetry 300 Å C-18 reversed column (300 mm × 19 mm) using a gradient method. The mobile phase A was 10% acetonitrile with 0.1% TFA. The mobile phase B was 35% acetonitrile with 0.1% TFA. The 60 min linear elution ran from 100% A to 100% B at a flow rate of 10 mL/min. FITC-$_D$(CRPPREGR) was collected and measured with a mass spectrometer (Agilent 1100 series). The synthesis of FITC-CRGERPPR was the same as described for FITC-$_D$(CRPPREGR).

Cellular Uptake. U87 glioma cells were seeded in 24-well plates at a density of 4×10^4 cells/well in 0.5 mL of DMEM with 10% FBS and incubated overnight at 37 °C. FITC-$_D$(CRPPREGR) and FITC-CRGERPPR at a final concentration of 5×10^{-6} mol/L were added to the plate and incubated for 4 h. U87 glioma cells were then lysed and suspended in PBS before being measured by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA). The U251 glioma cell line and HEK293 cells were used as controls to examine the cellular uptake of labeled peptides.

Tumor Spheroid Penetration. To further evaluate the tumor-penetrating ability of labeled peptides, a tumor spheroid model was used and prepared as previously described.[21,22] Briefly, U87 cells were seeded in 48-well plates (2×10^3 cells/400 μL per well) precoated with 150 μL of 2% low-melting-temperature agarose to prepare tumor spheroids. After seven days, FITC-$_D$(CRPPREGR) and FITC-CRGERPPR at a final concentration of 5×10^{-6} mol/L were added to the plate and incubated for 4 h. The spheroids were rinsed with ice-cold PBS and fixed with 4% paraformaldehyde for 30 min and then observed with a laser scanning confocal microscope (ZEISS, LSM 710, Oberkochen, Germany).

Synthesis of $_D$(RPPREGR)-PEG-PEI. To attach $_D$(RPPREGR) to Mal-PEG-NHS, 20 μmol of Mal-PEG-NHS and 26 μmol of RGERPPR were dissolved in 1 mL of dimethyl formamide (DMF). Then, 3 μL of triethylamine (TEA) was added to this mixture in a dropwise manner and stirred for 1 h. The resulting $_D$(RPPREGR)-PEG-Mal was purified via an ÄKTA explorer 100 system (GE, Fairfield, CT, USA) equipped with a Sephadex G-15 column (GE). The sample was collected and lyophilized.

To synthesize $_D$(RPPREGR)-PEG-PEI, 3 μmol of $_D$(RPPREGR)-PEG-Mal was added to 2 mL of 0.6 μmol PEI in PBS (0.2 M, pH 7.4), and the reaction mixture was stirred overnight. The resulting $_D$(RPPREGR)-PEG-PEI was then placed in an Amicon Ultra filter tube and washed five times with dH_2O. mPEG-PEI was obtained by conjugating mPEG-Mal with PEI and purified as described above. The mPEG-PEI and $_D$(RPPREGR)-PEG-PEI polymers were characterized by ^{1}H NMR analysis using D_2O as the solvent on a 500 MHz NMR spectrometer (Varian, Palo Alto, CA, USA).

Preparation of Polymer/pDNA Complexes. Equal volumes of pDNA solution were added to polymer solutions dissolved in PBS (pH 7.4). The concentration of polymer was determined according to required polycation−nitrogen/poly-

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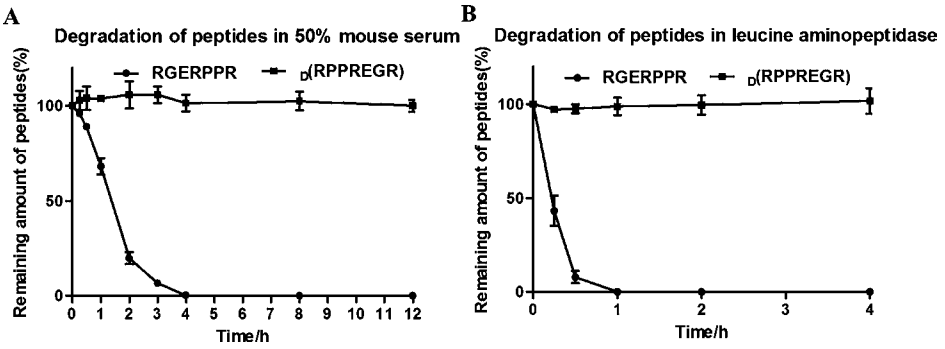

Figure 1. Stability of peptides in rat serum (A) and aminopeptidase M (B).

anion−phosphorus ratios (N/P ratios) of 4, 8, 12, and 16. The final pDNA concentration was 40 μg/mL. The complexes were vortexed for 30 s and then incubated for 30 min at room temperature. The size and zeta potential of the freshly prepared PEI derivatives/$pGL_{4.2}$ complexes with various N/P ratios were measured in triplicate using dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK).

Cytotoxicity Assay. U87 cells were seeded in 96-well plates (5×10^3 cells/well) and incubated for 24 h at 37 °C. Freshly prepared PEI/$pGL_{4.2}$, mPEG-PEI/$pGL_{4.2}$, and $_D$(RPPREGR)-PEG-PEI/$pGL_{4.2}$ complexes (20 μL) with different N/P ratios (containing 0.8 μg of pDNA) were added and incubated with the cells for 12 h in 200 μL of DMEM containing 10% FBS. The medium was then replaced with fresh DMEM and incubated for another 36 h. Twenty microliters of 5 mg/mL MTT solution was added to each well and incubated for 4 h. The medium was then removed, and 150 μL of DMSO was added to dissolve the formazan crystals. After 15 min incubation with DMSO, measurements were performed using a microplate reader (wavelength, 490 nm) (PowerWave XS, Bio-TEK, Winooski, VT). The measurements for each sample were conducted in triplicate.

Gene Transfection *In Vitro*. *Enhanced Green Fluorescence Protein Assay.* U87 cells were seeded in 48-well plates at a density of 2×10^4 cells per well in 0.5 mL of DMEM containing 10% FBS and incubated overnight at 37 °C. The medium was replaced with fresh DMEM, and 50 μL of polymer/pEGFP-N2 complexes containing 2 μg pEGFP-N2 (N/P = 12) were added to each well. After incubating at 37 °C for 12 h, the medium was changed and cells were incubated for a further 48 h. Fluorescence images were visualized with a fluorescence microscope (Leica, DMI4000B, Wetzlar, Germany).

Luciferase Assay. Freshly prepared polymer/$pGL_{4.2}$ complexes were added to U87 cells according to the above procedure and were quantified using a luciferase activity assay. After 48 h of incubation, cells were washed twice with PBS, and then lysed in 100 μL of cell lysis solution for 2 min (Promega, Beijing, China). The relative light units (RLUs) of luciferase activity were determined with an Ultra-Weak Luminescence Analyzer (Chuanghe, Beijing, China). Protein concentration was measured using a BCA protein assay kit. Luciferase activity was expressed as RLU/mg protein. All assays were conducted in triplicate and data were expressed in RLU/mg protein (±SD).

Tumor Spheroid Transfection. U87 tumor spheroids were used to mimic the tumor parenchyma to examine the transfection efficiency. Freshly prepared polymer/pEGFP-N2 complexes (N/P = 12, containing 2 μg pDNA) were added to tumor spheroids and incubated for 12 h at 37 °C, then the medium was replaced with fresh DMEM and incubated for a further 48 h. They were rinsed with ice-cold PBS and fixed with 4% paraformaldehyde for 30 min, then tumor spheroids were observed with a laser scanning confocal microscope (ZEISS, LSM 710).

***In Vivo* Transfection Efficiency and Anti-Glioblastoma Effect.** Male BALB/c nude mice (18 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and kept under specific pathogen-free (SPF) conditions. All animal experiments were evaluated and approved by the Ethics Committee of Fudan University. The intracranial U87 glioma-bearing nude mice model was established as described previously[23] and characterized with H&E staining (see Figure S2).

To investigate the *in vivo* transfection efficiency, freshly prepared mPEG-PEI/pDsRED-N1 and $_D$(RPPREGR)-PEG-PEI/pDsRED-N1 (containing 40 μg pDNA in 200 μL PBS, N/P12) or physiological saline were intravenously injected three times at 15, 17, and 19 days after implantation (n = 3). Two days after the injection, mice were sacrificed, their brains were excised, and red fluorescence intensity was measured with an *in vivo* fluorescent imaging system (FX Pro, Kodak, Rochester, New York, USA). For further examination, the frozen brain sections were prepared and stained with DAPI for 10 min, then examined using fluorescence microscopy.

The model nude mice were randomly divided into three groups (n = 13) and intravenously injected separately with freshly prepared mPEG-PEI/pORF-hTRAIL and $_D$(RPPREGR)-PEG-PEI/pORF-hTRAIL (containing 40 μg pDNA in 200 μL PBS, N/P12) or physiological saline via the tail vein. This was repeated five times on days 11, 13, 15, 17, and 19 after implantation. The survival times were recorded (n = 13) and survival data visualized using Kaplan−Meier plots and analyzed using a log-rank test.

RESULTS

***Ex Vivo* Stability of Peptides.** L-Peptides, especially linear structures, are easily degraded by plasma proteases, thus losing their targeting capacities. We examined the stability of RGERPPR and $_D$(RPPREGR) in rat serum, and, as shown in Figure 1, RGERPPR was found to have a higher degradation rate so was completely degraded in 4 h, while retro-inverso $_D$(RPPREGR) was much more stable, remaining at almost 100% even after 12 h incubation. Aminopeptidase is a common proteolytic enzyme with a hydrolysis site at the amino terminus of the peptide. The stability of the peptides in aminopeptidase

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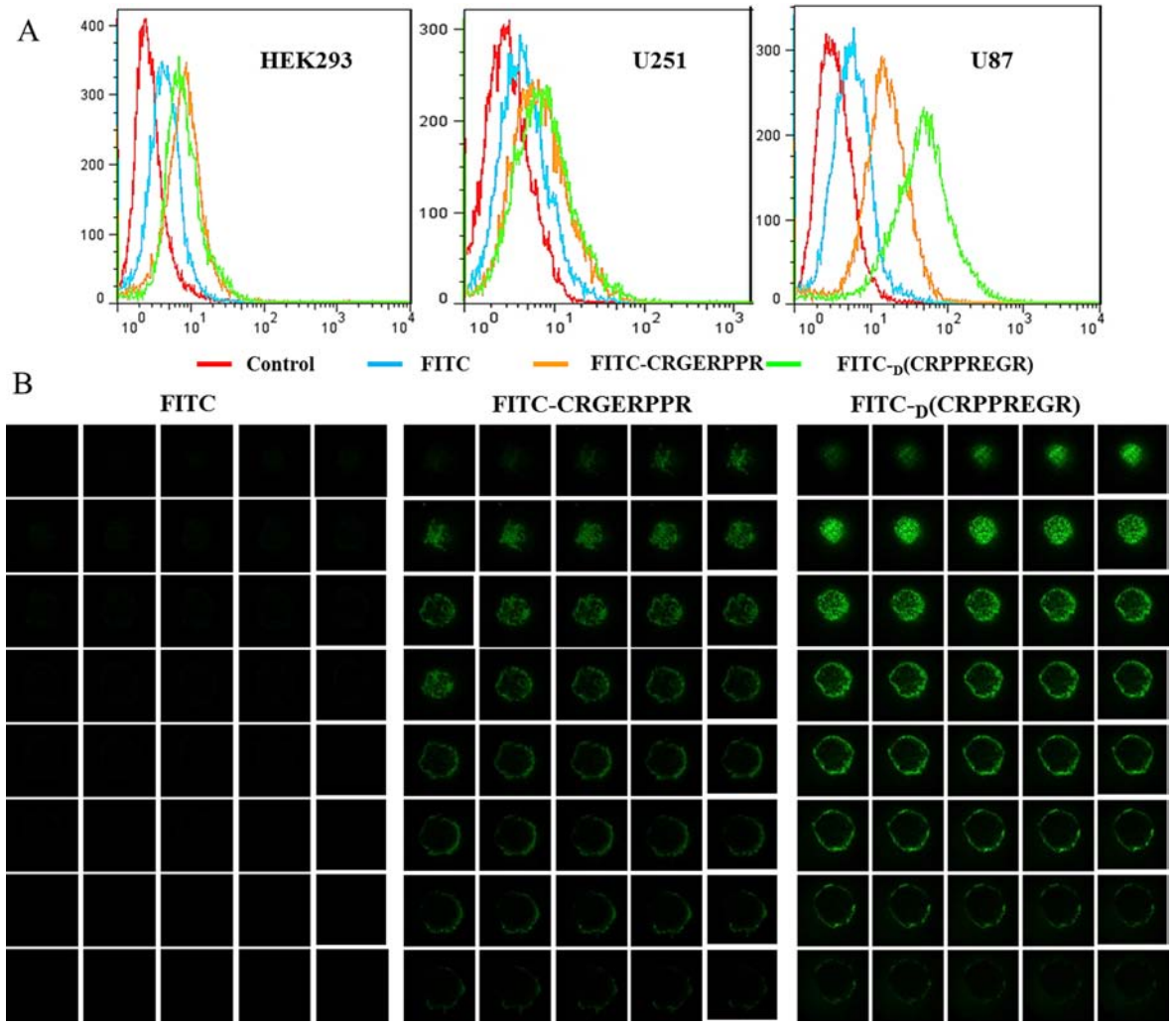

Figure 2. Cellular uptake and tumor spheroid penetration of FITC-labeled peptides. (A) Cellular uptake of FITC-$_D$(CRPPREGR) and FITC-CRGERPPR by U87 glioma cells was examined by flow cytometry. (B) Tumor spheroid penetration of FITC-$_D$(CRPPREGR) and FITC-CRGERPPR was examined by confocal microscopy, with a 5 μm interval between consecutive slides. Green represents the penetration of FITC. Original magnification, 200×.

was consistent with that in serum; thus, RGERPPR was completely degraded in 1 h, while $_D$(RPPREGR) was stable in the presence of aminopeptidase.

Targeting Efficacy of FITC-Labeled Peptides. The ESI-MS data of FITC-$_D$(CRPPREGR) and FITC-CRGERPPR show that their molecular ion peak was m/z 1398.4 ($M+H^+$), which is consistent with their theoretical molecular weight of 1397.0 (see Figure S3). FITC-$_D$(CRPPREGR) and FITC-CRGERPPR were incubated with U87, U251, and HEK293 cells at 37 °C for 4 h, and cellular uptake results are shown in Figure 2A. FITC-$_D$(CRPPREGR) uptake by U87 cells was significantly higher than that of FITC-CRGERPPR, with fluorescent FITC-$_D$(CRPPREGR)-containing cells accounting for 95.3%, compared with only 68.9% of FITC-CRGERPPR-containing cells. For both HEK293 and U251 cells, the uptake of FITC-$_D$(CRPPREGR) and FITC-CRGERPPR was lower, and fluorescent cells accounted for only about 15%. These results indicated that $_D$(RPPREGR) and RGERPPR can specifically recognize U87 cells rather than normal or U251 cells, and retro-inverso $_D$(RPPREGR) has a better affinity for U87 cells than parental RGERPPR.

Evaluation of Tumor Spheroid Penetration. Tumor spheroids were used to imitate the *in vivo* status of solid tumors and evaluate the penetrating ability of peptides by confocal microscopy. As shown in Figure 2B, the penetration of FITC-$_D$(CRPPREGR) was much deeper than that of FITC-CRGERPPR in U87 tumor spheroids as shown by distribution and higher fluorescence intensity properties. These results indicate that the $_D$(CRPPREGR) peptide not only increases uptake by U87 cells, but also effectively increases the tumor penetration efficacy.

Synthesis and Characterization of Polymers. mPEG-PEI and $_D$(RPPREGR)-PEG-PEI were synthesized as indicated in Scheme 1, and ^{1}H NMR results are shown in Figure 3A. The details were as follows: 3.6−3.8 ppm (m, -OCH_2CH_2) for PEG, 2.6−3.4 ppm (m, -CH_2CH_2NH-) for PEI, 3.95 (d, 2H, CH) and 4.15 (s, H, CH) for $_D$(RPPREGR). $_D$(RPPREGR) was successfully introduced into the polymer, and the substituted degrees and molecular weights of the resulting polymers are given in Figure 3B. The substituted degree of PEG was 4.3 for mPEG-PEI and 4.2 for $_D$(RPPREGR)-PEG-PEI.

Characterization of Polymer/pDNA Complexes. Particle sizes and zeta potentials of polymers/$pGL_{4.2}$ complexes

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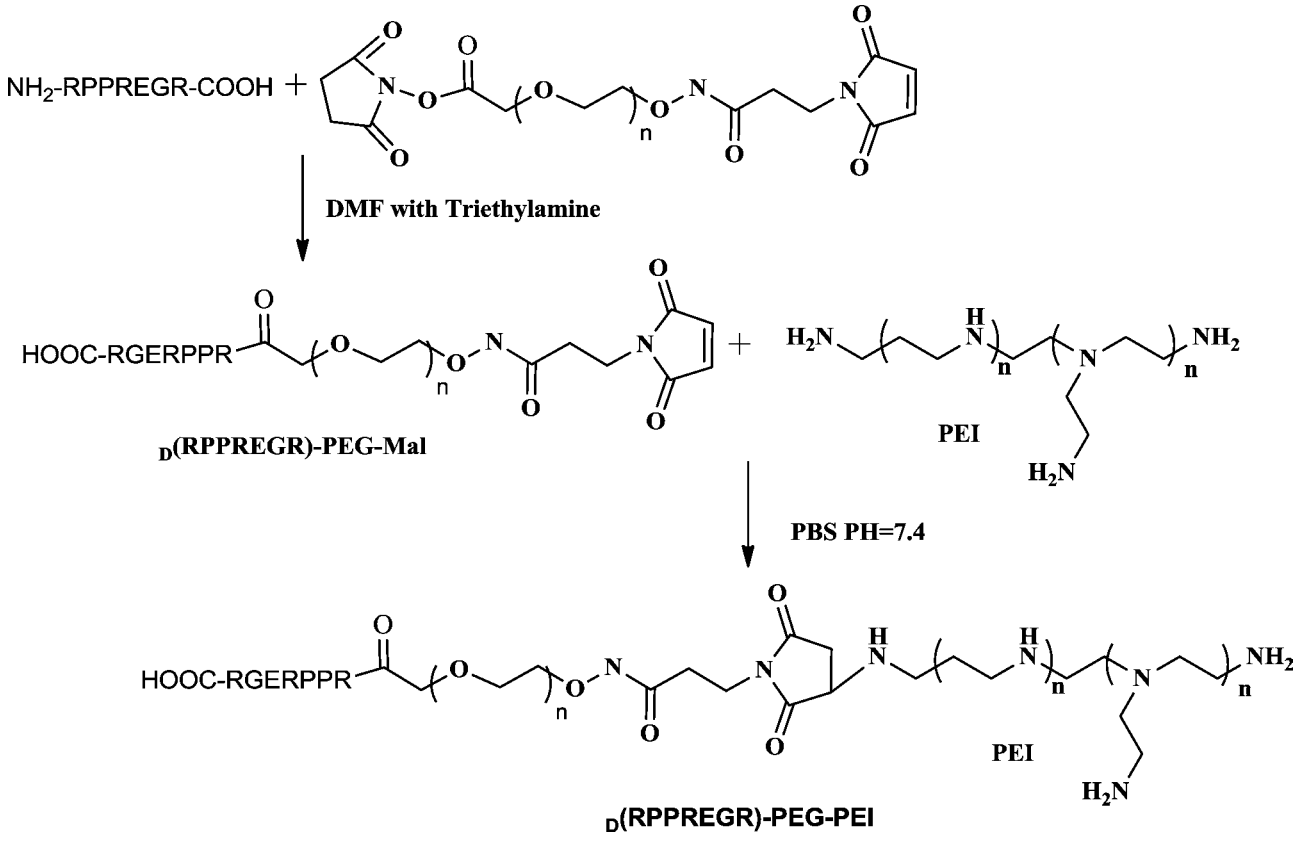
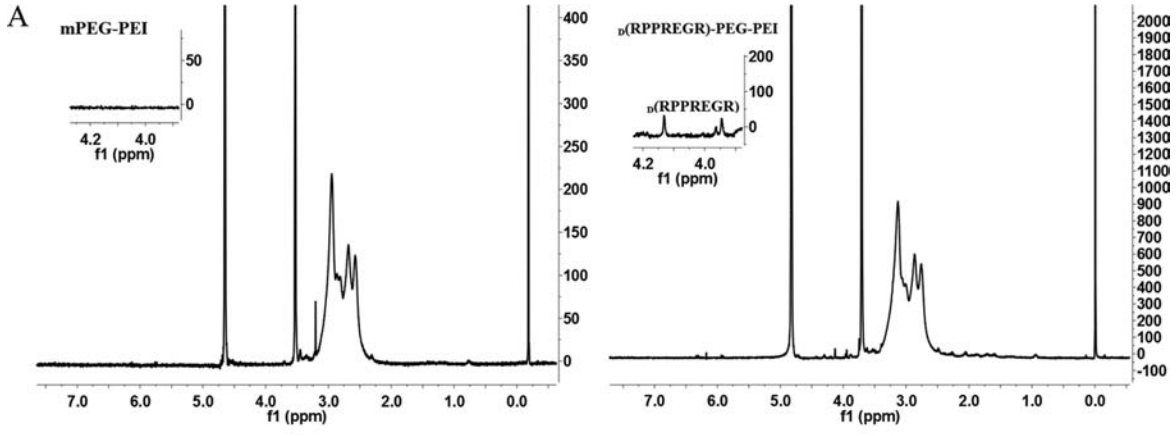
Scheme 1. Synthesis of $_D$(RPPREGR)-PEG-PEI





B Characteristics of mPEG-PEI and $_D$(RPPREGR)-PEG-PEI

Polymer	SD[a] of PEG		Mw (kDa)	
	Theoretical	Calculated	Theoretical	Calculated
mPEG-PEI	5.0	4.3	35.0	33.6
$_D$(RPPREGR)-PEG-PEI	5.0	4.2	38.8	36.6

Figure 3. (A) ^{1}H NMR spectra of mPEG-PEI and $_D$(RPPREGR)-PEG-PEI in D_2O. (B) Characteristics of mPEG-PEI and PEI-PEG-D(RPPREGR).

were measured to investigate the ability of polymers to condense pDNA (Table 1). As the N/P ratios increased from 4 to 16, the sizes of all complexes decreased, while zeta potentials increased. The positive zeta potentials indicated that all polymers were capable of condensing pDNA effectively, which was also confirmed by agarose gel electrophoresis assays

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Table 1. Sizes and Zeta Potentials of Polymer/pGL4.2 Complexes with Different N/P Ratios[a]

	PEI		mPEG-PEI		$_D$(RPPREGR)-PEG-PEI	
N/P	size (nm)	zeta potential (mV)	size (nm)	zeta potential (mV)	size (nm)	zeta potential (mV)
4	1388.3 ± 79.0	16.4 ± 0.6	1998.3 ± 140.3	3.4 ± 0.4	2546.7 ± 502.2	3.0 ± 0.4
8	901.4 ± 89.2	25.8 ± 0.9	357.0 ± 2.7	7.0 ± 0.1	316.9 ± 3.4	5.7 ± 0.2
12	532.1 ± 45.5	30.5 ± 0.5	228.6 ± 1.0	8.7 ± 0.8	225.9 ± 3.8	6.3 ± 0.5
16	300.9 ± 23.2	33.1 ± 0.7	141.1 ± 2.5	10.3 ± 0.2	150.9 ± 2.8	7.7 ± 0.2

[a]Data points represent the mean ± SD (n = 3).

(see Figure S4). At the same N/P ratio, PEI/pDNA complexes showed larger sizes and higher zeta potentials compared with PEGylated polymer/pDNA complexes. Research has shown that higher zeta potentials of PEI/pDNA complexes are caused by strong positive surface charges of PEI. In strong ionic solutions including PBS, PEI derivatives are prone to aggregate, thereby forming large particles. PEGylation can shield part of the positive charge of PEI, enabling complexes with lower zeta potentials to form. Meanwhile, PEGylation can reduce aggregation and decrease particle sizes.[24,25]

Cytotoxicity Assay. We next used an MTT assay to evaluate the cytotoxicity of PEI derivatives/pGL$_{4.2}$ complexes. Figure 4 shows that PEI appears to be the most cytotoxic group

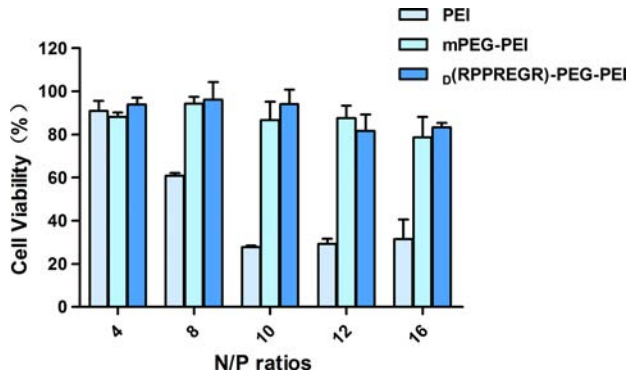

Figure 4. Cell viabilities of mPEG-PEI/pGL4.2 and $_D$(RPPREGR)-PEG-PEI/pGL4.2 compared with PEI/pGL4.2 in U87 glioma cells.

because of its high density positive charge at all N/P ratios. At N/P ≥ 10, its cell viability was lower than 30%. By contrast, the cytotoxicity of mPEG-PEI/pGL$_{4.2}$ and $_D$(RPPREGR)-PEG-PEI/pGL$_{4.2}$ was significantly lower, with a cell viability greater than 80%. It could be inferred that PEGylation of PEI reduces the positive charge of complexes, therefore reducing cytotoxicity.[25,26] Based on cytotoxicity levels, we selected an N/P of 8 for PEI and an N/P of 12 for PEGylation polymers for our remaining experiments.

***In Vitro* Transfection.** We next conducted *in vitro* transfection experiments to examine the targeting specificity of $_D$(RPPREGR)-PEG-PEI/pDNA for U87 glioma cells. An enhanced green fluorescent protein (EGFP) expression assay showed that EGFP expression of $_D$(RPPREGR)-PEG-PEI was higher than that of mPEG-PEI/pEGFP-N2 (N/P = 12) (Figure 5A). Furthermore, the transfection efficiency of PEI derivatives/pGL$_{4.2}$ complexes, as quantified by a luciferase assay (Figure 5C), found that the $_D$(RPPREGR)-PEG-PEI/pGL$_{4.2}$ complex was 1.9 times more efficient relative to mPEG-PEI/pGL$_{4.2}$ at N/P = 12 (P < 0.05). It is reasonable to assume that the modification of $_D$(RPPREGR) facilitated the increase in mPEG-PEI transfection efficiency.

Tumor Spheroid Transfection. We further evaluated the transfection efficiency of $_D$(RPPREGR)-PEG-PEI/pEGFP-N2 using U87 tumor spheroids. As shown in Figure 5B, the EGFP expression of $_D$(RPPREGR)-PEG-PEI was more intense and occurred in a higher percentage of cells than that of mPEG-PEI. These results indicated that the $_D$(CRPPREGR) peptide not only increased the transfection efficiency of U87 cells, but also effectively increased that of tumor spheroids.

Gene Expression *In Vivo*. Figure S2 depicts the successful establishment of an intracranial U87 glioma-bearing nude mouse model. Gene expression of polymer/pDsRED-N1 complexes at an N/P ratio of 12 was investigated in the brain of model mice using a fluorescent imaging system. Compared with mPEG-PEI/pDsRED-N1, $_D$(RPPREGR)-PEG-PEI/pDsRED-N1 demonstrated a stronger fluorescent signal indicating a higher expression of red fluorescence protein, as shown in Figure 6A. These results were confirmed by fluorescence microscope images of the brain sections transfected with polymer/pDsRED-N1. This suggests that the $_D$(RPPREGR) modification facilitated the increase of transfection efficiency *in vivo*.

Anti-Glioblastoma Effect. To study the antiglioblastoma effect of $_D$(RPPREGR)-PEG-PEI/pORF-hTRAIL *in vivo*, we investigated the survival time of intracranial U87 glioma-bearing mice (Figure 7). The median survival time was significantly higher in tumor-bearing nude mice treated with $_D$(RPPREGR)-PEG-PEI/pORF-hTRAIL (30 days) compared with mice treated with either mPEG-PEI/pORF-hTRAIL or saline (both 25 days; n = 13, $^{**}p$ < 0.01, log-rank test).

DISCUSSION

Glioma is one of the most common brain tumors, with a high degree of malignancy, high postoperative recurrence, and rapid speed of invasion. Glioma patients have an average median survival time of less than 16 months.[27,28] However, recent studies have shown that gene therapy is becoming a promising glioma treatment strategy.[29,30]

Efficient treatment of glioma requires both the blood-brain barrier (BBB) and blood-brain tumor barrier (BBTB) to be overcome. Recently, a strategy involving the introduction of ligands that specifically bind to brain tumor cell receptors and aiming to surmount the problems associated with BBTB has gained a lot of attention.[31−34] However, because of the high interstitial pressure in tumors, it is difficult for drug delivery systems to penetrate deeply into tumor tissue.[35] Therefore, another key issue for glioma treatment is that the drug delivery should mediate tissue penetration after reaching the brain tumor site.

The targeting ligand is an important part of the targeting delivery system, so selecting ligands with high affinity and biological activity has been a hot spot of research. Some reported ligands, such as CDX, possess receptor affinity and

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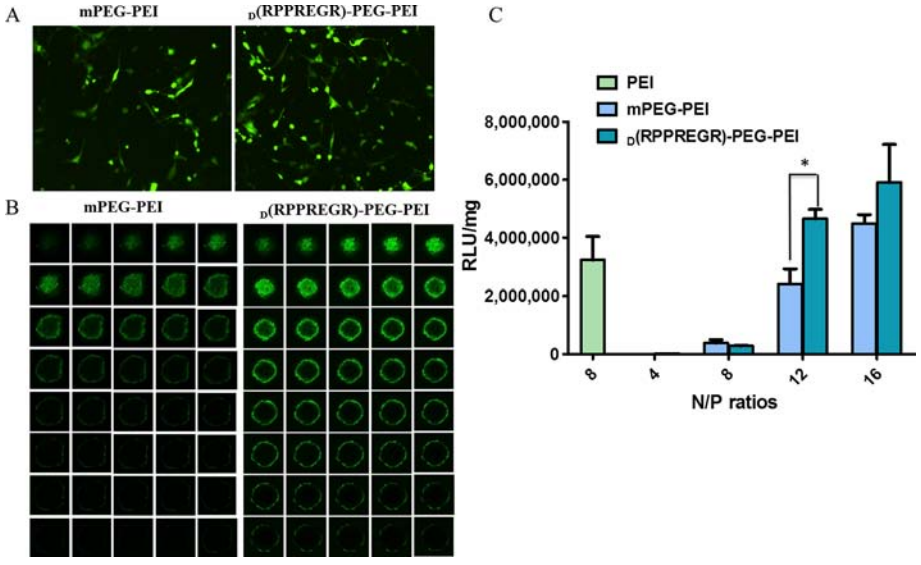

Figure 5. *In vitro* transfection efficiency. (A) Fluorescent images of mPEG-PEI/pEGFP-N2 and $_{D}$(RPPREGR)-PEG-PEI/pEGFP-N2 gene expression in U87 glioma cells at an N/P ratio of 12. (B) U87 tumor spheroid transfection of mPEG-PEI/pEGFP-N2 and $_{D}$(RPPREGR)-PEG-PEI/pEGFP-N2 at an N/P ratio of 12 as assessed by confocal microscopy with a 5 μm interval between consecutive slides. Green represents green fluorescence protein expressed by pEGFP-N2. Original magnification, 200×. (C) Luciferase expression levels of mPEG-PEI/pGL4.2 and $_{D}$(RPPREGR)-PEG-PEI/pGL4.2 in U87 glioma cells at different N/P ratios.

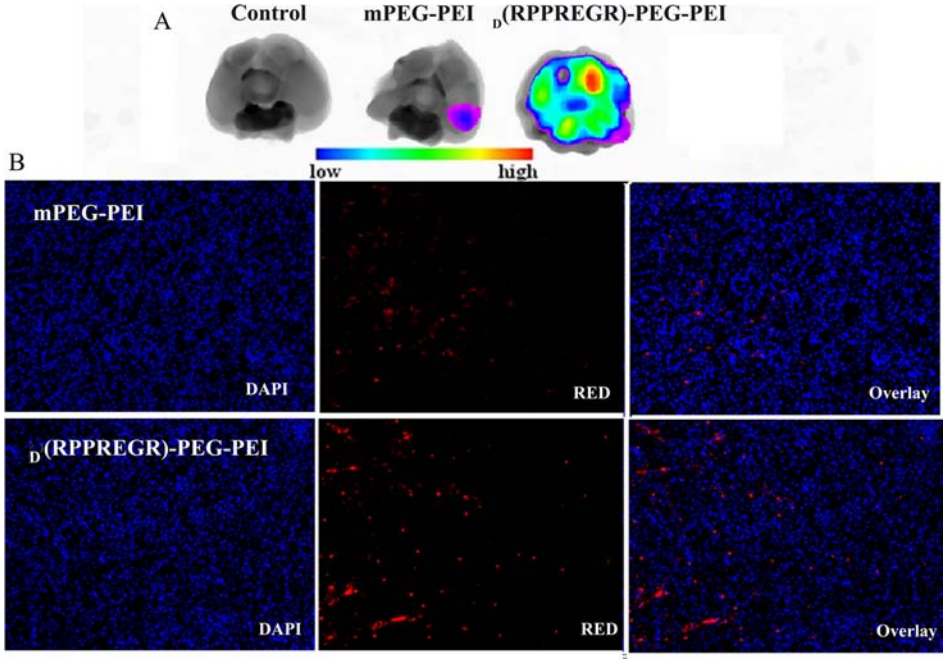

Figure 6. (A) Fluorescent imaging of mPEG-PEI/pDsRED-N1 and $_{D}$(RPPREGR)-PEG-PEI/pDsRED-N1 in the brains of nude mice bearing U87 gliomas at an N/P ratio of 12 as determined by fluorescent imaging. (B) Fluorescence microscopy images of brain sections transfected with polymer/pDsRED-N1 at an N/P ratio of 12: red represents red fluorescence protein expressed by pDsRED-N1; blue represents cell nuclei stained with DAPI. Original magnification, 200×.

selectivity for brain tumor cells. However, these peptides consist of L-amino acids that are susceptible to protease degradation in the plasma, so readily lose their tumor-homing capacity.[36−38] Several researchers have designed retro-inverso peptides which, compared with parent L-peptides, resist protease degradation and thus possess improved biological stability, but also have equal bioactivity because the orientation of their side chains is very similar to the original structure.[39−43] Therefore, retro-inverso peptides have the potential to optimize the properties of L-peptides.

CendR peptides identified from phage display technology both enhance cellular uptake via specific NRP-1 binding and induce extravasation and tissue penetration, which facilitates the delivery of drugs and genes into the tumor parenchyma.[1] In our study, we synthesized $_{D}$(RPPREGR), which is the retro-inverso peptide of the RGERPPR CendR peptide with the aim of using it as a brain tumor binding, tissue-penetrating peptide; it was modified with mPEG-PEI, one of the most successfully and extensively used synthetic carriers for gene delivery, to enable glioblastoma-targeted gene delivery.

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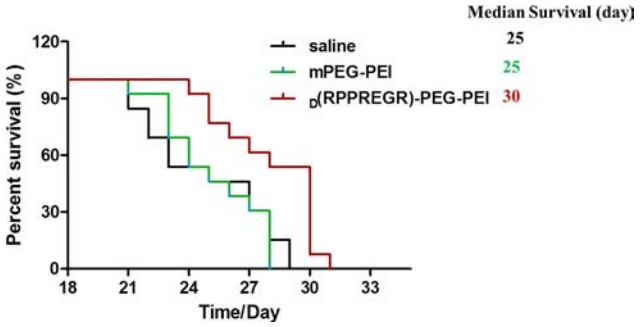

Figure 7. Kaplan–Meier survival curves of mice with intracranial U87 glioblastomas.

First, we showed that $_D$(RPPREGR) exhibited a higher biological stability than RGERPPR in both rat serum and aminopeptidase. Flow cytometry revealed that the uptake of FITC-labeled peptides by U87 cells was significantly greater than that of both HEK293 and U251 cells. This is likely because of the overexpression of NRP-1 in glioblastoma cells,[19,20] which enabled RGERPPR and $_D$(RPPREGR) to specifically bind NRP-1 on the U87 cell surface and enhance cellular uptake.[1] Second, $_D$(RPPREGR) exhibited an increased affinity to U87 cells, compared with RGERPPR, indicating that the D-amino acid replacement and reverse order assembly of parent peptide enhanced the binding ability to the NRP-1 receptor. Confocal microscopy showed that the $_D$(RPPREGR) peptide also effectively increased the tumor penetration efficacy.

$_D$(RPPREGR) was conjugated with branched PEI via heterobifunctional Mal-PEG-NHS to give an $_D$(RPPREGR)-PEG-PEI polymer. An important issue for the systemic delivery of therapeutic genes in clinical applications is the requirement for high levels of efficiency in the targeted vectors, as well as minimal side effects. MTT showed that the cytotoxicity of mPEG-PEI/pGL$_{4.2}$ and $_D$(RPPREGR)-PEG-PEI/pGL$_{4.2}$ was significantly lower than that of PEI/pGL$_{4.2}$, which is consistent with previous reports.[25,26]

In vitro qualitative EGFP expression assays and quantitative luciferase assays revealed a significant increase in the transfection efficiency of $_D$(RPPREGR)-PEG-PEI in U87 cells relative to the nontargeted polymer. This may be explained by the improved affinity of $_D$(RPPREGR) to U87 cells shown earlier (Figure 2A). The U87 tumor spheroid EGFP transfection assay showed that $_D$(RPPREGR)-PEG-PEI effectively increased the EGEP expression of tumor spheroids which was likely to have been caused by both the tumor spheroid penetration efficacy of $_D$(RPPREGR) (Figure 2B, Figure S5) and increased transfection in U87 cells (Figure 5A,C).

We further investigated the *in vivo* transfection efficiency of complexes in intracranial U87 glioma-bearing nude mice. Fluorescent imaging and frozen brain sections showed that $_D$(RPPREGR) transfection resulted in higher expression of red fluorescence protein, presumably because $_D$(RPPREGR) exhibited a better affinity to U87 cells. Necrosis factor-related apoptosis-inducing ligand (TRAIL) can selectively induce apoptosis in glioma cells without affecting normal cells; therefore, TRAIL can be used as a therapeutic gene. We found that the PEI-PEG-$_D$(RPPREGR)/pORF-hTRAIL gene delivery system significantly enhanced the antitumor effect by prolonging the median survival time of tumor-bearing nude mice from 25 to 30 days (** $p < 0.01$).

CONCLUSIONS

We prepared a retro-inverso CendR peptide ($_D$(RPPREGR)) and studied its biological stability, tumor targeting efficacy, and tumor tissue penetration ability. Relative to RGERPPR, $_D$(RPPREGR) not only exhibited improved biological stability, but also possessed the dual ability of specifically targeting U87 cells and tumor spheroid penetration efficacy. Using $_D$(RPPREGR) as a targeting molecule, the $_D$(RPPREGR)-PEG-PEI gene carrier exhibited lower cytotoxicity and increased transfection efficiency both *in vitro* and *in vivo*. Furthermore, the PEI-PEG-$_D$(RPPREGR)/pORF-hTRAIL gene delivery system prolonged the median survival time of tumor-bearing nude mice from 25 to 30 days. In conclusion, $_D$(RPPREGR)-PEG-PEI appears to be a promising gene delivery system for antiglioma treatment.

ASSOCIATED CONTENT

Supporting Information

Additional figures as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel:+86 21 51980092, Fax: +86 21 51980090, E-mail address: liumin@shmu.edu.cn.

Notes

The authors declare no competing financial interest.

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