Enhanced Glioblastoma Targeting Ability of Carfilzomib Enabled by a $^D$A7R-Modified Lipid Nanodisk

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**ABSTRACT:** The robust proliferation of tumors relies on a rich neovasculature for nutrient supplies. Therefore, a basic strategy of tumor targeting therapy should include not only killing regular cancer cells but also blocking tumor neovasculature. D-peptide $^D$A7R, which was previously reported to specifically bind vascular endothelial growth factor receptor 2 (VEGFR2) and neuropilin-1 (NRP-1), could achieve the goal of multitarget recognition. Accordingly, the main purposes of this work were to establish a carfilzomib-loaded lipid nanodisk modified with multifunctional peptide $^D$A7R ($^D$A7R-ND/CFZ) and to evaluate its anti-glioblastoma efficacy in vitro and in vivo. It is testified that the $^D$A7R peptide-conjugated lipid nanodisk can be specifically taken up by U87MG cells and HUVECs. Furthermore, $^D$A7R-ND demonstrated a more enhanced penetration than that of the nonmodified formulation on the tumor spheroid model in vitro and more tumor region accumulation in vivo on the subcutaneous and intracranial tumor-bearing nude mice model. $^D$A7R-ND was shown to co-localize with tumor neovasculature in vivo. When loaded with proteasome inhibitor carfilzomib, the $^D$A7R-decorated nanodisk could remarkably suppress tumor proliferation, extend survival time of nude mice bearing an intracranial tumor, and inhibit neovasculature formation with an efficacy higher than that of the nonmodified nanodisk in vitro and in vivo. The present study verified that the heptapeptide $^D$A7R-conjugated nanodisk is a promising nanocarrier for glioblastoma targeting therapy.

**KEYWORDS:** $^D$A7R peptide, nanodisk, carfilzomib, glioma, neovasculature, target therapy

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**INTRODUCTION**

Glioblastoma (GBM) is the most common and aggressive tumor in brain malignancy.$^1$ In recent years, the incidence of glioblastoma shows an upward tendency, from 4.9 per 100 000 in 1989 to 5.9 in 2010.$^2$ Though tumors of the central nervous system are relatively rare, they are hard to cure and put a heavy burden on patients and their families. Conventional clinical therapy usually relies on surgery, chemo-, and radiotherapy, but glioblastoma grows diffusively and is lacking ofa vivid boundary, which make surgery difficult to resect the tumor completely.$^3$ Besides, the high toxicity and nonselectivity of chemotherapy lead to a poor prognosis.$^4$ A drug delivery system loaded with a variety of chemotherapeutics could avoid the aforementioned disadvantages and improve the antitumor efficacy to some extent,$^5−8$ but the existence of a blood tumor barrier (BTB) sets a natural physiological barrier between the tumor and blood, making a nanocarrier difficult to accumulate in the tumor region.

Meanwhile, the robust angiogenesis provides abundant nutrients for tumor proliferation and metastasis. A regular tumor-targeted drug delivery system cannot meet the demands. In order to cross the BTB, kill tumor cells, and target neovascularization with a single drug delivery system, a multifunctional targeting moiety is essential.

Heptapeptide $^D$A7R is resistant to enzyme degradation optimized from $^A$7R by a retro-inverse strategy.$^9$ The newly designed $^D$-peptide is shown to specifically bind VEGFR2 and NRP-1, which are overexpressed on tumor cells and tumor-related vascular endothelia cells.$^{10,11}$ Numerous researches reveal that VEGFR2 and NRP-1 have a tight connection with tumorigenesis, angiogenesis,
proliferation, and metastasis. Moreover, the A7R peptide is first reported as a therapeutic peptide which can competitively inhibit VEGF binding with NRP-1 to yield an anti-proliferation and anti-angiogenesis effect, latterly the peptide is optimized and reported as an effective tumor and neovascularization targeting ligand.

A nanodisk composed of lipids and polyethylene glycol-lipid (PEG-lipid) is a flat rounded lipid bilayer, the rim of which is surrounded by a PEG-lipid. Research has verified that the disk-shape membrane bilayer is sterically stable. The structure and size would not be affected by temperature changing from 25 to 37 °C. Meanwhile, plenty of work has affirmed that the disk-shape lipid bilayer is a suitable carrier for amphiphilic peptides and hydrophobic drugs. Moreover, comparing with the micelle and liposome, the nanodisk has a larger hydrophobic core to encapsulate insoluble drugs. Furthermore, it has been reported that the disk-shaped lipid membrane could more effectively target the diseased microvascular than the nanoparticles.

Carfilzomib is a second generation proteasome inhibitor for multiple myeloma therapy approved by the FDA in 2012. It is a tetrapeptide with an epoxyketone group, irreversibly binding to the 20S subunit of proteasome. Contrary to its outstanding anti-myeloma efficacy, the effect on the solid tumor is frustrated, which is partially due to the rapid degradation in the body. Therefore, carfilzomib has been encapsulated into nanoparticle systems to avoid degradation, and the antitumor efficacy has been verified on solid tumors.

To this end, a 5A7R-modified lipid nanodisk loaded with carfilzomib was prepared. It was proven on both cell and animal levels that the modified nanodisk showed better anti-glioma efficacy than the unmodified nanodisk.

### EXPERIMENTAL SECTION

**Materials.** Boc-protected α-amino acids were purchased from GL Biochem, Ltd. (Shanghai, China). Rink amide MBHA resin was from Xi’an Innovision Bioscience Co., Ltd. O-Benzotriazole-N,N,N′,N′-tetramethyl-uronium hexafluorophosphatethe (HBTU) was purchased from the American Bioanalytical Co., Ltd. (Natick, MA). Diisopropylethylamine (DIEA) was supplied by Sigma-Aldrich. POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) and mPEG2000-DSPE were purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Mal-PEG3400-DSPE and NH2-PEG3400-DSPE were purchased from Dalian Bio (Arab, AL, USA). FITC was purchased from Sigma-Aldrich (St. Louis, MO, USA). FITC-PEG-DSPE was synthesized via a standard Boc-deprotection liquid chromatography. Carfilzomib(CFZ) was purchased from DALIAN MEILUN Biology Technology Co., Ltd. DiR (1,1’-dioctadecyl-3,3,3’,3’-tetramethyl indocarbocyanine iodide) and DiD (1,1’-dioctadecyl-3,3,3’,3’-tetramethylrhodamine-dicarboxyanine perchlorate) were from Invitrogen (Waltham, MA). All chemicals were of analytical reagent grade.

U87MG cells were purchased from ATCC. HUVECs were obtained from ATCC. All cells were cultured in special Dulbecco’s modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (FBS, HyClone) at 37 °C in a 5% CO2 humidified atmosphere.

Male BALB/c nude mice and SD rats were obtained from Shanghai BK Laboratory Animal Co., Ltd. (Shanghai, China) and kept under SPF conditions. All animal experiments were conducted under the direction of the guidelines approved by the ethics committee of Fudan University.

**Preparation and Characterization.** Synthesis of 5A7R and 5A7R-PEG-DSPE. 5A7R (NH2-R-D-R-D-DR-D-R-D) was synthesized via a standard Boc-protected solid phase peptide synthesis. The 5A7R-PEG-DSPE polymer was synthesized by conjugation of 5A7R-SH (NH2-R-D-R-D-DR-D-R-D) and Mal-PEG3400-DSPE. In brief, 10 mg of 5A7R-SH peptide and 37.5 mg of Mal-PEG3400-DSPE were dissolved in 3 mL of DMF, and 30 μL of DIEA was added. After a 2 h reaction, the solvent was removed by dialysis (MWCO = 14 kDa) against distilled water.

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Preparation of Nanodisk. Nanodisks were prepared via a thin film-hydration method. All lipids and carfilzomib (CFZ) were co-dissolved in 4 mL of chloroform. The organic solvent was removed at a reduced pressure to form a thin film at 37 °C and then hydrated with 1 mL of isotonic PBS. After 1 h of hydration, the mixture was sonicated for 45 min in an ice-bath with a JY92-II sonicator (Scientz, Ningbo, China). Unencapsulated CFZ was removed by filtering against a 0.22 μm filter membrane (Millipore).

The lipid compositions were as follows: DA7R-modified lipid disks (DA7R-ND) were composed of POPC/cholesterol/mPEG2000-DSPE/DA7R-PEG3400-DSPE (35:40:23:2, mol %), and the unmodified nanodisk (mPEG-ND) consisted of POPC/cholesterol/mPEG2000-DSPE (35:40:25, mol %). DA7R-modified FITC-loaded lipid disks comprise POPC/cholesterol/mPEG2000-DSPE/DA7R-PEG3400-DSPE/FITC-PEG3400-DSPE (35:40:21:2:2, mol %) and unmodified FITC-loaded lipid disks. The components and ratios were POPC/cholesterol/mPEG 2000-DSPE/FITC-PEG3400-DSPE (35:40:23:2, mol %).

Characterization of Nanodisk. The size of nanodisks were measured by a Malvern laser particle size analyzer and cryogenic transmission electron microscopy (Cryo-TEM).

To determine the encapsulation efficiency (EE) and loading capacity (LC) of CFZ, drug-loaded nanodisks were demulsified by acetonitrile, and the EE and LC of carfilzomib were measured by HPLC.

The EE% and LC% were calculated based on the following equations:

\[
EE(\%) = \frac{\text{Amount of CFZ in the nanoparticles}}{\text{Total amount of CRZ added}} \times 100% \\
LC(\%) = \frac{\text{Amount of CRZ in the nanoparticles}}{\text{Weight of nanoparticles}} \times 100%
\]

Figure 2. Cellular uptake of 5 μM FITC-labeled different formulations for 1 h. Microscopic observation of (A) U87MG cells uptake and (B) HUVECs uptake by laser scanning confocal microscopy. The bar indicates 10 μm. Quantification of (C) U87MG cells uptake and (D) HUVECs uptake via flow cytometry (* p < 0.05, n = 3, and data are means ± SD).

Figure 3. Trans-BTB ability of different formulations. (A) Transport ratios (%) of FITC, FITC-labeled mPEG-ND, and DA7R-ND on the in vitro BTB model. (B) FITC-labeled DA7R-ND and mPEG-ND uptake on the trans-BTB tumor spheroid model. Pictures were taken by the Z-stack mode of LSCM with an interval of 5 μm. The bar indicates 100 μm (** p < 0.01, n = 3, and data are means ± SD).

The release profiles of CFZ from mPEG-ND/CFZ and DA7R-ND/CFZ nanodisks in vitro were studied in a 0.1 mM phosphate buffered saline (PBS) (pH 7.4) supplemented with 1.5% surfactant Tween 80 or a 0.1 mM PBS (pH 5.5) supplemented with...
1.5% surfactant Tween 80. A 100 μL release media was taken out at each time point. The concentration of carfilzomib was analyzed by HPLC.

The serum stability of CFZ-loaded disks were conducted under a 50% rat serum condition. Drug-loaded nanodisks were put in a dialysis bag (MWCO = 14 kDa) with a serum and PBS mixture, and a 0.1 mM phosphate buffered saline (pH 7.4) supplemented with 1.5% surfactant Tween 80 was used as a dialysis medium to let free carfilzomib release to the outside of the dialysis bag. Samples were taken out at each time point from the inner dialysis bag, multiple additions of acetonitrile were used to remove serum protein, and the residual part of carfilzomib, which was still loaded in nanodisks, was measured by HPLC.

**Tumor Targeting Ability Evaluation. Cellular Uptake Assay.** In order to measure the cellular uptake of 9A7R-ND and mPEG-ND, U87MG cells and HUVECs were seeded in confocal dishes at 5000 cells per well. Twelve hours after seeding, the cells were incubated with FITC and FITC-labeled nanodisks for 1 h at a concentration of 5 μM at 37 °C. The cells were rinsed with PBS and immobilized with 4% paraformaldehyde for 15 min. Intra-cellular fluorescence was observed by a laser scanning confocal microscope (LSCM).

For quantitative measurement, U87MG cells and HUVECs were seeded in 12-well plates at a density of 10^5 cells per well. Twelve hours after seeding, the cells were incubated with FITC and FITC-labeled nanodisks for 1 h at a concentration of 5 μM at 37 °C. Fluorescence-positive cells were captured by flow cytometry.

**Crossing BTB and the Tumor Spheroid Uptake Assay in Vitro.** To assess the BTB transferring ability, the HUVECs/U87 co-culture model was established as described previously. HUVECs were seeded in the apical chamber of the transwell,
and U87 cells were seeded into the basolateral chamber at a ratio of 1:5. Three days after seeding, 5 μM FITC and FITC-labeled D\textsuperscript{A7R}-ND and mPEG-ND solutions were added in the apical compartment; samples were taken out after 0.5, 1, 1.5, 2, 3, and 4 h of incubation at 37 °C; and the transport ratio (%) was measured by a microplate reader (Power Wave XS, Bio-TEK, Winooski, VT, USA). In order to better simulate the tumor environment, tumor spheroids were added into the basolateral chamber, and 100 μL of FITC and FITC-labeled D\textsuperscript{A7R}-ND and mPEG-ND solutions were added into the apical compartment at a concentration of 20 μM in DMEM with 10% FBS. After 1 h of incubation, the tumor spheroids were observed via laser scanning confocal microscope.

**Tumor Targeting Ability Evaluation in Vivo.** To investigate the glioblastoma targeting ability of D\textsuperscript{A7R}-ND and mPEG-ND in vivo, the subcutaneous tumor model and intracranial tumor model were established.

For the subcutaneous tumor model, U87MG cells was inoculated (5 \times 10^6 cells suspended in 100 μL of PBS) into the subcutaneous tissue of the nude mice. For the intracranial tumor model, U87MG cells (5 \times 10^5 cells suspended in 5 μL PBS) were implanted into the right striatum (1.8 mm lateral, 0.6 mm anterior to the bregma, and 3 mm of depth) of male BALB/c nude mice by using a stereotactic mouse adaptor.

When the subcutaneous tumor size reached 0.5–1.0 cm\textsuperscript{3}, six nude mice were randomly assigned into two groups, and DiR-loaded D\textsuperscript{A7R}-ND and mPEG-ND were suspended in 200 μL of PBS and intravenously injected to the tumor-bearing nude mice. The fluorescent images were detected by an in vivo image system (IVIS Spectrum, Caliper PerkinElmer, Waltham, MA, USA) at 2, 4, 6, 8, 12, and 24 h after administration. After 24 h, nude mice were sacrificed for organs, and the distribution of nanodisks in organs ex vivo were measured and semiquantified. To further investigate quantitation biodistribution of two formulations, D\textsuperscript{A7R}-ND and mPEG-ND loaded with 100 μg of near-infrared dye DiD were administrated via tail vein (18 nude mice were randomly assigned into two groups). At 2, 12, and 24 h post administration, three nude mice out of nine in each group were sacrificed, blood samples were collected, and tumors were harvested after heart-perfusion to completely remove residual blood. The tumors were weighed and homogenated and then measured by a microplate reader. The excitation and emission wavelength of DiD were 644 and 665 nm, respectively.

Twenty days after the intracranial glioma bearing nude mice model was established, 12 mice were randomly assigned into two groups (n = 6), and D\textsuperscript{A7R}-ND and mPEG-ND loaded with 100 μg of near-infrared dye DiD were administrated via tail vein. After 4 and 12 h, three nude mice out of six in each group were sacrificed. The tumor-bearing brains were harvested and disposed of, as described above, and measured by a microplate reader.

**Co-Localization Assay.** The FITC-labeled D\textsuperscript{A7R}-ND and mPEG-ND solutions were intravenously injected into the U87MG tumor-bearing nude mice. Eight hours after injection, the mice were sacrificed. The tumors were dissected to make frozen section slices, and they were stained with anti-CD31 antibody to mark neovasculature and DAPI for the nuclei of the tumor cells. Fluorescence was visualized by a laser scanning confocal microscope.

**Anti-Glioblastoma Effect Evaluation. Cytotoxicity Assay.** U87MG cells and HUVECs were seeded in 96-well plates at a density of 3 × 10^3 –5 × 10^5 cells per well and incubated for 12 h.

The medium in each well was replaced by 200 μL of DMEM containing free CFZ, D\textsuperscript{A7R}-ND/CFZ, and mPEG-ND/CFZ. After 72 h of incubation, the cell viability was measured by the standard MTT assay.

**Apoptosis Assay.** U87MG cells and HUVECs were seeded in a 12-well plate at the density of 10^5 cells per well, and free CFZ, D\textsuperscript{A7R}-ND/CFZ, and mPEG-ND/CFZ at a concentration of 10^{-7} mol/L were added into each well. After 48 h of incubation, the standard PI/Annexin V-FITC labeling method was used to measure the apoptosis rate. The extent of apoptosis was detected via a flow cytometer (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA).

**Neovasculature Formation Inhibition Assay.** HUVECs suspended in 300 μL of DMEM were plated on 24-well plates precoated with Matrigel for 12 h. Free CFZ, D\textsuperscript{A7R}-ND/CFZ, and mPEG-ND/CFZ containing 10^{-8} mol/L carfilzomib were given at the same time. After 12 h, samples were observed under fluorescence microscope (DMi4000D, Leica Biosystems, Nussloch, Germany).

**Antitumor Assay in Vivo.** When the subcutaneous tumor volume reached 50–100 mm\textsuperscript{3} (counted by length \times (width)^{2/3}), the dosing schedule started, and the first day of therapy was set as Day 1. Twenty-eight nude mice with relatively uniform tumor sizes were randomly assigned into four groups to receive 100 μL...
of normal saline, free CFZ, 3A7R-ND/CFZ, and mPEG-ND/CFZ injection via tail vein on the first, fourth, seventh, 10th, and 13th day with a total dosage of 5 mg/kg. After the last administration, four nude mice picked up from different groups were sacrificed, and tumors were harvested for TUNEL and CD31 immunohistochemical examination. Organs were harvested for hematoxylin-eosin staining to check the toxicity of each formulation. Three fields of TUNEL and CD31 staining slices under microscope were randomly chosen to do semiquantification. The tumor size and body weight were measured every 2 days. The measurements were done until the 18th day, on which the animals were sacrificed.

The intracranial tumor model mice model was established, and the day of establishing was set as Day 0. The model mice were randomly assigned into four groups (n = 8) and treated with 100 μL of saline, free CFZ, 3A7R-ND/CFZ, and mPEG-ND/CFZ. The nanodisks were administrated via tail vein injection on the 10th, 13th, 16th, 19th, and 22nd day with a total dosage of 10 mg/kg for CFZ after implantation. After the last administration, four nude mice picked up from different groups were sacrificed, and tumors were harvested for TUNEL and CD31 immunohistochemical examination. The survival time was recorded.

**Pharmacokinetics Assay.** To assess the pharmacokinetic properties of 3A7R-ND/CFZ, 3A7R-ND/CFZ, mPEG-ND/CFZ, and free CFZ were analyzed on SD rats with a single dose of 4 mg/kg via tail vein (n = 5). Epoxomocin, an analog of carfilzomib, was used as an interior label. A 0.5 mL of blood sample was collected at each time point, and 500 μL of distilled water and 10 μL of epoxomocin were added and vortexed for 15 min until complete hemolysis. Then, 4 mL of methyl-tert-butyl ether was added to extract compounds for 20 min and centrifuged at 5000 rpm for 5 min. The upper layer was collected and evaporated by nitrogen gas, and the residues were reconstituted in 100 μL of acetonitrile and centrifuged at 10000 rpm for 10 min. The concentration of CFZ was determined by HPLC. The pharmacokinetic parameters were calculated by the software DAS2.0.

**Statistic Analysis.** The IC50 values were calculated by non-linear regression analysis with the GraphPad Prism7.0 version program. All data were presented as mean ± SD, and statistical significance was analyzed using a one-way ANOVA, except for a special declaration. *p < 0.05, **p < 0.01, ***p < 0.001.

## RESULTS AND DISCUSSION

**Characterization of 3A7R-ND/CFZ.** The sizes of 3A7R-ND/CFZ and mPEG-ND/CFZ were respectively 86.3 ± 4.8 and 84.5 ± 5.5 nm. The morphologies of mPEG-ND/CFZ and 3A7R-ND/CFZ were observed by Cryo-TEM (Figure 1A,B). The disk-shaped structure was clearly shown. The rounded shape in the field was the front side of the nanodisk, and the rod was the lateral side. When the mass ratio of carfilzomib and the carrier materials was 1:10, the encapsulation efficiency of CFZ in 3A7R-ND/CFZ and mPEG-ND/CFZ was respectively 88.2% ± 2.2% and 93.6% ± 3.7%, and the drug loading capacity was 88.2% ± 1.5% and 93.6% ± 3.7%.
8.11% ± 0.22% and 8.56% ± 0.37%. CFZ exhibited a sustained release profile from the nanodisks within 72 h, regardless of the tested pH values (7.4 or 5.5). The release behavior of CFZ from DA7R-ND/CFZ and mPEG-ND/CFZ was similar, and no burst release was observed (Figure 1C). This result suggested that the drug loading capacity and morphological features of nanodisks were not affected by DA7R modification. Besides, a more complete drug release in an acidic environment might be favorable for the CFZ release in the tumor region. The CFZ release profile from the nanodisks in the serum was also evaluated (Figure 1D), and no burst release was observed, indicating that the nanodisk was a suitable vector for carfilzomib delivery.

**Tumor Targeting Ability of DA7R-ND.** Tumor Cell Targeting Ability. The tumor cell targeting ability of DA7R-ND was assessed on both U87MG cells and HUVECs with a FITC concentration of 5 μM. As shown in Figure 2, the DA7R modification could effectively enhance the cellular uptake of the nanodisks, which might be attributed to the overexpression of VEGFR2 and NRP-1 on both cell lines.

**Trans-BTB and Tumor Spheroid Penetration Ability in Vitro.** Figure 3A demonstrates that DA7R-ND was much more effectively transferred through the BTB model than mPEG-ND, which may be attributed to the dual-receptor recognition ability of the DA7R peptide. In order to further validate the advantages of the targeting moiety modification in the simulated tumor environment, tumor spheroids were laid into the basolateral chamber with the BTB layer on the top, and the nanodisks accumulating in the tumor spheroid were observed by the Z-stack mode of LSCM (Figure 3B). DA7R-ND exhibited a quite high fluorescence intensity, but mPEG-ND showed no fluorescence, which further demonstrated the advantage of DA7R-ND.

**Subcutaneous Tumor Targeting Ability in Vivo.** The near-infrared dye DiD-loaded nanodisks were administered to the tumor-bearing nude mice via tail vein injection, and fluorescence was captured at 1, 2, 4, 8, 12, and 24 h after injection. DA7R-ND/DiD manifested more accumulation in the tumor region, no matter if in vivo or if the tumors were detached from body (Figure 4A,B). The semiquantification result showed the comparison of DA7R-ND/DiD and mPEG-ND/DiD accumulation at each time point (Figure 4C) and the distribution in organs 24 h post injection (Figure 4D). These results indicated a better tumor retention ability of the DA7R-modified nanodisk. In addition, the neovascularization targeting efficiency of both populations of nanodisks was evaluated in vivo. FITC-labeled nanodisks were used to track whether DA7R-ND could target neovascularization in the tumor. As shown in Figure 4E, the DA7R-modified nanodisk had an obvious co-localization ability with the tumor neovascularization labeled by CD31 staining. This might be due to the overexpression of VEGFR2 and NRP-1 on the endothelial cells.

DA7R-ND and mPEG-ND loaded with DiD were administrated intravenously, and animals were sacrificed at 2, 12, and 24 h post injection to quantify the distribution of disks in the subcutaneous tumor and blood. As shown in Figure 5A, DA7R-ND exhibited a higher tumor accumulation than mPEG-ND at each time point and plateaued at 12 h with 3.26 ± 0.40 ID%/g, while mPEG-ND was only 0.90 ± 0.52 ID%/g. The distribution of the two formulations in the blood was gradually reduced with the passage of time, and there was no significance between the modified and unmodified nanodisks (Figure 5B). The ratio of the distribution in the subcutaneous tumor to blood showed an ascending trend, indicating that nanodisks were continuously transferred from the blood to tumor.

**Intracranial Tumor Targeting Ability in Vivo.** Near-infrared dye DiR-loaded DA7R-ND and mPEG-ND were administrated via tail vein to measure the distribution of the two nanodisks in the brains of the intracranial tumor-bearing nude mice. Samples were taken at 4 and 12 h post injection, and the data are presented in Figure 6. The amount of nanodisks in the brain was in a downward trend from 4 to 12 h, but the DA7R peptide-decorated nanodisk showed more distribution and retention in the brain than the unmodified nanodisk at each time point. The results indicated that the DA7R modification could facilitate more nanodisks across the BTB to accumulate in the brain.

**Antitumor Efficacy of DA7R-ND.** Tumor Associated Cell Cytotoxicity. The cytotoxicity of the nanodisks was evaluated on U87MG cells and HUVECs by the MTT assay and apoptosis assay. Figure 7A,B demonstrates the stronger cytotoxicity of DA7R-ND/CFZ than the unmodified nanodisks on both cell lines. The specific value of IC_{50} was calculated by GraphPad Prism 7. The IC_{50} value on the U87MG cells of free CFZ, mPEG-ND/CFZ, and DA7R-ND/CFZ were 4.036, 16.69, 10.84 nM respectively. The IC_{50} value on HUVECs of CFZ, mPEG-ND/CFZ, and DA7R-ND/CFZ were 4.535, 23.47, 13.74 nM.

**Figure 8.** Anti-angiogenesis ability of different formulations. (A) Images of angiogenesis ability comparison observed by the bright field of fluorescence microscope. (B) Semiquantification of the tube formation inhibition rate calculated by software ImageJ. **p < 0.01, n = 3,** and data are presented as mean ± SD.
respectively. The results of the cell apoptosis assay are shown in Figure 7C,D. There were significantly less viable cells and more apoptosis cells treated with DA7R-ND/CFZ than unmodified disks. However, free carfilzomib exhibited the strongest cytotoxicity, which was testified by either the MTT assay or cell apoptosis assay. This might relate to the incomplete release of carfilzomib from the nanodisks.

**Tumor Neovasculature Formation Inhibition in Vitro.** In order to evaluate the anti-angiogenesis ability, the simulated neovasculature model in vitro was constructed. Free CFZ, mPEG-ND/CFZ, and DA7R-ND/CFZ were incubated with the neovasculature model for 12 h. As shown in Figure 8, the cell model treated with DA7R-ND/CFZ showed less net-like structure, which indicated less angiogenesis than the

![Figure 9](image-url)
mPEG-ND/CFZ-treated group. The semiquantification result calculated by the software ImageJ showed the same outcome. Therefore, the modified nanodisk had a more improved anti-angiogenesis efficacy than that of the nonmodified one, but it was not better than free CFZ.

Antitumor Effect in Vivo. When the subcutaneous tumor volume reached 50−100 mm³, the dosing schedule started, and the first day of therapy was set as Day 1. Saline, CFZ, mPEG-ND/CFZ, and D7R-ND/CFZ were administered via tail vein on the first, fourth, seventh, 10th, and 13th day with a total dose of 5 mg/kg carfilzomib. The curve of the relative subcutaneous tumor volume change and bodyweight change are shown in Figure 9A,B. The D7R-ND/CFZ-treated group exhibited the slowest tumor growth, and all of the treatment groups appeared to have almost the same bodyweight gaining rate, except for the free CFZ-treated group. Eighteen days after the first injection, the animals were sacrificed, and the tumors were dissected for weighing and imaging (Figure 9C,D). The data implied that D7R-ND/CFZ could significantly improve the tumor inhibition efficacy in comparison to mPEG-ND/CFZ and free CFZ. After five injections, four nude mice randomly picked up from four groups were sacrificed to detect the angiogenesis inhibition and tumor cell apoptosis. Figure 9E,F shows that D7R-ND/CFZ could significantly inhibit tumor neovasculature formation. Figure 9G,H shows that D7R-ND/CFZ could induce cell apoptosis in tumor tissue. These results suggested that D7R-ND/CFZ was a promising therapeutic agent.

In order to further evaluate anti-glioblastoma efficacy of D7R-ND/CFZ, saline and different formulations were given via tail vein on the 10th, 13th, 16th, 19th, and 22nd day with a total dose of 10 mg/kg for CFZ after implantation to treat intracranial glioblastoma bearing nude mice. The data shown in Figure 10 indicated that D7R-modified nanodisks loaded with carfilzomib could significantly extend the survival time to 37.5 days, which was 8 days longer than the saline group, 3.2 times longer than the CFZ group, and 1.6 times longer than the mPEG-ND/CFZ group. The strong curative effect was resulted from its glioblastoma angiogenesis inhibition and enhanced glioma cell apoptosis. This work made further efforts to prove that D7R-ND/CFZ could cross the brain-blood tumor barrier (BBTB) to inhibit the proliferation of the intracranial tumor and anti-angiogenesis in glioma.
Besides the strong tumor-homing ability of the targeting molecule, the blood circulation time of the peptide-modified drug delivery system made a great impact on its antitumor efficacy. The pharmacokinetic parameters of CFZ, mPEG-N/P-CFZ, and D4AR-N/P-CFZ were calculated by noncompartmental analysis of the plasma concentrations at preset time points in SD rats (Figure 11B). Free carfilzomib was rapidly eliminated from circulation and already undetectable at 4 h post injection (Figure 11A). On the contrary, carfilzomib loaded in nanodisks showed a prolonged circulation time in vivo. There was no evident difference between the modified and unmodified nanodisks, which demonstrated that the D4AR modification had no obvious effect on the circulation properties of the nanodisks.

CONCLUSION

Our research provided a new insight into a dual-target recognition lipid nanodisk loaded with a hydrophobic drug for anticancer therapy. The easily degraded proteasome inhibitor carfilzomib was loaded within the D4AR-decorated lipid nanodisk to significantly extend its retention time in vivo, yielding better anti-subcutaneous tumor efficacy and prolonged survival time of intracranial tumor bearing nude mice for glioblastoma. Hence, this novel carfilzomib-loaded active targeting nanodrug delivery system holds great potential for anti-glioblastoma therapy.

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Notes
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ACKNOWLEDGMENTS

This work was supported by the National Basic Research Program of China (973 Program, 2013CB932500), National Natural Science Foundation of China (81773657, 81690263, and 81473149), Shanghai Education Commission Major Project (2017-01-07-00-07-E00052), and Shanghai International Science and Technology Cooperation project (16430723800).

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