Gene and doxorubicin co-delivery system for targeting therapy of glioma

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ABSTRACT

The combination of gene therapy and chemotherapy is a promising treatment strategy for brain gliomas. In this paper, we designed a co-delivery system (DGDPT/pORF-hTRAIL) loading chemotherapeutic drug doxorubicin and gene agent pORF-hTRAIL, and with functions of pH-trigger and cancer targeting. Peptide HAiYPRH (T7), a transferrin receptor-specific peptide, was chosen as the ligand to target the co-delivery system to the tumor cells expressing transferrin receptors. T7-modified co-delivery system showed higher efficiency in cellular uptake and gene expression than unmodified co-delivery system in U87 MG cells, and accumulated in tumor more efficiently in vivo. DOX was covalently conjugated to carrier though pH-triggered hydrazone bond. In vitro incubation of the conjugates in buffers led to a fast DOX release at pH 5.0 (intracellular environment) while at pH 7.4 (blood) the conjugates are relatively stable. The combination treatment resulted in a synergistic growth inhibition (combination index, CI < 1) in U87 MG cells. The synergism effect of DGDPT/pORF-hTRAIL was verified in vitro and in vivo. In vivo anti-glioma efficacy study confirmed that DGDPT/pORF-hTRAIL displayed anti-glioma activity but was less toxic.

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1. Introduction

Glioma has been considered as one of the most devastating malignant primary brain tumors because it’s very difficult to treat and cure [1]. Despite surgical debulking, radiation and chemotherapy, median survival has been changed relatively little [2,3]. Whether the operation is available or not, chemotherapy is the most common method for the treatment of gliomas. However, the side effects of chemotherapy could not be disregarded. Recently, gene therapy has been followed with interest because of its non-toxicity and high therapy efficiency [4–6]. As an emerging strategy, the combination of gene therapy and chemotherapy provides hope for the treatment of gliomas. The combination therapy is expected to achieve anticancer effect at lower drug doses, and result in lower undesirable toxicities [7–9].

Currently, combination therapy mainly includes combination of different small molecular chemotherapeutic drugs, and that of small molecular chemotherapeutic drug with gene medicine. In this paper, chemotherapeutic drug doxorubicin (DOX) and gene agent pORF-hTRAIL were combined for the therapy of glioma. It is reported that DOX could enhance the antitumor effect of tumor necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL), which expressed by pORF-hTRAIL, via regulating the expression of death receptors and stimulating mitochondrial apoptotic pathway [10,11]. Therefore, it is expected that synergistic effect will be achieved by the combination of DOX and pORF-hTRAIL.

Two major types of combination therapy are listed as follows: (1) therapeutic drugs were administered respectively [12,13]; (2) different drug carriers, each loading a single therapeutic agent, are administered in combination [14,15]. However, the application of therapies above is limited by their uncontrollable drug ratios in vivo. Recently, single delivery system carrying two drugs has been reported [16]. Co-delivery system loading both drugs ensures that they undergo the same body distribution, thus maximising the benefits of this combination. Although the simultaneous delivery of both drugs can be accomplished, it is powerless to home them specifically to tumor tissue, and control the release of drugs, thus there are a range of toxic effects on normal tissues. Herein, a tumor-homing strategy was developed to enhance the efficacy and specificity of treatment for gliomas. HAiYPRH (T7) peptide, a novel targeting ligand, can specially bind to transferrin receptor (TfR) specifically [17,18]. As known, TfR is over-expressed on the brain capillaries endothelial cells and many malignant tumor cells [19,20]. Thus, T7 peptide targeted to TfR can not only mediate the transport of nanocarriers across the blood–brain barrier (BBB) but also can increase the accumulation of anticancer drugs in brain...
tumors. Therefore, after the delivery system modified with T7, the co-delivery system could accumulate in the tumor tissue specifically.

In addition, it has been reported that the tumor microenvironments are acidic, including the slightly acidic extracellular fluids and the acidic endosomes or lysosomes within cancer cells [21,22]. In order to enhance the release of anticancer drugs in tumor tissues, acid-sensitive spacer was incorporated between the drug and carrier. Glutamic acid (Glu), with two carboxyl groups, has the potential to be used as an acid-sensitive linkage. One carboxyl group can bind to the carrier, and the other can combine with antitumor drug such as DOX using a pH-sensitive hydrazone bond, which was proved to be cleavable in the acidic tumor environment, conversely be stable in normal physiological environment [23–25]. Therefore, after modified with T7, the co-delivery system accumulates in the acidic tumor environment, DOX release selectively and combine pORF-hTRAIL to induce the apoptosis of glioma tumor cells via corresponding targets respectively.

In the present study, tumor-targeted, pH-triggered and co-delivery strategies were applied simultaneously for the therapy of glioma. We designed the multifunctional nanoscale delivery system loaded both DOX and pORF-hTRAIL. DOX was loaded on the surface of T7-modified Dendrigraft poly-L-lysine (DGL) using Glu as an acid-sensitive linkage, then therapeutic gene pORF-hTRAIL was compacted and encapsulated by the DOX conjugated dendrimers. In this paper, the multifunctional delivery system combined DOX and pORF-hTRAIL was synthesized and characterized. In addition, the targeting and antitumor effect was be evaluated, both in vivo and in vitro.

2. Materials and methods

2.1. Materials

2.1.1. DGL generation 3 with 123 lysine groups were purchased from Colcom, France. α-Maleimide-ω-N-hydroxy-succinimidyl polyethyleneglycol (NHS-PEGMAL, MW 3400) was obtained from Nektar Therapeutics (Huntsville, AL, USA), tert-Butyl-oxy carbonyl glutamic acid γ-benzyloxysuccinimidyldiethylamine (TEA) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). tert-Butyl-oxy carbonyl glutamic acid (TFA) and triethylamine (TEA) were purchased from Medpep Co., Ltd. (Shanghai, China). N-hydroxysuccinimide (NHS) was purchased from Shanghai Medpep Co., Ltd. (Shanghai, China). Doxorubicin HCl was from Fudan University. Other reagents, if not specified, were purchased from Sigma-Aldrich.

2.2. Synthesis of DGL–Glu

Boc-Glu(Obzl) (4 mmol) was reacted with NHS (4.8 mmol), EDC (6 mmol) and TEA (6 mmol) in anhydrous DMF (2 ml) for 4 h on ice. DGL (2 mmol) dissolved in DMF (2 ml) was added, and the reaction mixture was stirred for 4 days at room temperature. These compounds were purified using a Sephadex LH-20 column with methanol as the eluent. The yield of DGL–Glu was 68%. 1H NMR (400 MHz, DMSO-d6): δ 1.10–1.86 (br, CH3 of Boc and β, γ-CH2 of lysine), 2.31 (br, γ-CH2 of Glu), 2.95 (m, NH2CH2), 3.90 (br, NHCHCO), 5.02 (s, benzyl), 6.77 and 7.76 (br, NHCO), 7.28 (s, phenyl). 2.3. Synthesis of DGL–Glu–NHNH2

DGL-Glu (1.2 mmol) was dissolved in 4 ml of DMF and was reacted with hydrazine (0.12 mol) for 4 days at 35 °C under nitrogen [26]. These compounds were purified using a Sephadex LH-20 column with methanol as the eluent. The yield of DGL-Glu–NHNH2 was 71%. 1H NMR (400 MHz, DMSO-d6): δ 1.10–1.80 (br, CH3 of Boc, β, γ-CH2 of lysine, and β-CH2 of Glu), 2.02 (br, γ-CH2 of Glu), 2.95 (m, NHCH2CO), 3.84 (br, α-CH2 of Glu), 4.16 (br, NHCHCO), 6.79 and 7.79 (br, NHCO), 8.96 (br, CONHCH2).

2.4. Synthesis of DGL–Glu-NHNH2–DOX (DGD)

Doxorubicin HCl (16.0 mmol) and acetic acid (20 ml) were added to DGL–Glu–NHNH2 (0.8 mmol) dissolved in methanol and the reaction occurred under nitrogen condition for 3 days. The mixtures were purified using a Sephadex LH-20 column, and then were dialyzed for 6 h and lyophilized. UV–Vis spectrophotometry (λ = 480 nm) was used to confirm DOX conjugation.

2.5. Synthesis of T7–PEG–DGL–Glu–NHNH2–DOX (DGDPT)

DGL-Glu–NHNH2–DOX was reacted with NHS-PEG3400–MAL at the molar ratio of 1:2 in PBS 8.0 for 2 h at room temperature. The primary amino groups on the surface of DGL were specifically reacted with the NHS groups of the bifunctional PEG derivative. The resulting conjugate, PEG–DGL–Glu–NHNH2–DOX (DGDPT), was purified by ultrafiltration through a membrane (cutoff = 5 kDa) and the buffer was changed to PBS (pH 7.0). Then DGDPT was reacted with T7, 1:1 (mol/mol) in PBS (pH 7.0) for 24 h at room temperature. The MAL groups of DGDPT were specifically reacted with the thiol groups of T7.

2.6. Preparation of DGDPT/pDNA NPs

DGDPT were freshly prepared and diluted to appropriate concentrations in PBS (pH 7.4). pORF-hTRAIL solution (100 μg pDNA/ml in 50 mM sodium sulfate solution) was added to obtain specified weight ratios and immediately vortexed for 30 s at room temperature. Freshly prepared complexes were used in the following experiments.

2.7. Gel retardation assay

agarose gel retardation assay was carried out to determine the pDNA binding ability of vector. DGDPT/pDNA complexes were prepared at various weight ratios (DGL to pDNA, 0.05:1, 0.1:1, 0.5:1, 1:1, 1:3, 1:6, 1:10, and 15:1). The corresponding N/P ratios were 0.09:1, 0.91:1, 1.82:1, 5.45:1, 10.9:1, 18.2:1 and 27.3:1. The complexes were mixed with appropriate amounts of 6× loading buffer and then electrophoresed on a 0.9% (w/v) agarose gel containing ethidium bromide (0.25 μg/ml of the gel). The location of pDNA in the gel was analyzed on a UV illuminator and photographed using a Canon IXUS 950IS camera.

2.8. Characterization of DGDPT/pDNA

The mean diameter and zeta potential of DGDPT/pDNA, with a DGL to pDNA weight ratio at 3:1, were determined by dynamic light scattering using a Zeta Potential/Particle Sizer Niconps®380 ZLS (PSS Niconp Particle Size System, USA) and transmission electron microscopy (JEM-2100, JCM, Japan).

2.9. In vitro release

DGDPT or DGDPT/pDNA (40 μg DOX-equiv.) in 1 ml of 0.1 M PBS (pH 5.0, 6.0, and 7.4) was sealed in a dialysis bag (MWCO 3500). The dialysis bag was immersed in 40 ml of the same buffer solution and incubated at 37 °C. The absorbance of DOX released at various times was measured on a microplate fluorometer (PerkinElmer) at Ex/Em 537/584 nm. For DOX release from the DGDPT and DGDPT/pDNA, the absorbance of DOX released at various times was directly measured.

2.10. Cell culture

U87 glioblastoma cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured at 37 °C in a humidified 5% CO2 atmosphere. Growth medium was supplemented with fetal bovine serum (FBS) (10%), streptomycin (100 mg/ml) and penicillin (100 units/ml).

2.11. Cellular uptake

U87 glioblastoma cells were seeded at a density of 5 × 104 cells/well in 24-well plates (Corning-Coaster, Tokyo, Japan). The cells were incubated for 48 h and checked under the microscope for confluency and morphology. Then cells were incubated with DGDPT/pORF-hTRAIL, DGDPT/pORF-hTRAIL at the concentration of 15 μg/well measured by DGL in the presence of serum free medium for 30 min at 37 °C, respectively. To evaluate the promotion of transferrin (Tf) for the uptake of DGDPT/pORF-hTRAIL, groups with Tf (25 μM) were added. After 30 min incubation, cells were washed with Hanks then visualized and photographed under an IX2-RFACA fluorescent microscope (Olympus, Osaka, Japan). Plasmid pORF-hTRAIL used here was pre-labeled by TOYO-1 which could intercalate into DNA and emit green fluorescence.

2.12. In vitro gene expression

U87 cells were seeded at a density of 4 × 104 cells/well in 24-well plates. The cells were incubated for 48 h. Here, GFP expression of drug delivery systems were
examined, DGDPT/pEGFP-N2, DGPT/pEGFP-N2 and lipofactmine 2000/pEGFP-N2 with or without Tf (25 μM) at the concentration of 15 μg/well measured by DGL were added to the cells. The mixture was incubated at 37 °C for 2 h, respectively. Then cells were rinsed twice with Hank's solution and further cultured in fresh medium for 48 h. The fluorescence images were acquired and photographed using a fluorescence microscope.

2.13. Cell cytotoxicity assay on U87 MG cells

Cell cytotoxicity assay was conducted using MTT assay kit and followed the manufacturer's protocol. Briefly, 5000 cells/well of the U87 MG cells were plated in 96-well plate 48 h before treatment. Cells were then washed twice with Hank's solution and exposed to different doses of DGDPT/pORF-hTRAIL, DGPT/pORF-hTRAIL and DGDP/pGL-3 with Tf (25 μM) at 37 °C for 2 h in DMEM medium containing 10% FBS. After 2 h of incubation, the cells were washed again twice with Hank's solution and re-fed with fresh media and further incubated at 37 °C for 48 h before the MTT assay was performed. To assess cell viability, 10 μL of MTT (5 mg/ml) solution was added into each well and incubated at 37 °C for 2 h. The medium was removed and 100 μl of DMSO was added to each well to dissolve the formazan crystals formed by the living cells. Cells without treatment were used as control. Absorbance was read at 570 nm and corrected at 630 nm by dual wavelength detection using a Multiskan plate reader. The percentage of control was calculated as the percentage of control.

2.14. Determination of combination index (CI)

The combination of DOX and pORF-hTRAIL towards U87 MG cells was evaluated by the combination index (CI) analysis [24]. The CI was calculated as follows: CI = D2/D2f + D1/D1f = D2f/D1f · D1/D2, where Df is the dose of Drug-1 required to produce x percent effect alone and D2 is the dose of Drug-2 required to produce the same x percent effect in combination with Drug-2; similarly, D1f is the dose of Drug-2 required to produce x percent effect alone and D2 is the dose of Drug-2 required to produce the same x percent effect in combination with Drug-1. Theoretically, CI is the ratio of the combination dose to the sum of the single-drug doses at an isoeffective level. Consequently, CI values of <1 indicate synergism, values of >1 show antagonism, and values of = 1 indicate additive effects.

2.15. In vitro apoptosis detection

Detection of apoptotic cells was performed by TUNEL method using an One Step TUNEL Apoptosis Assay Kit. DGDPT/pORF-hTRAIL, DGDP/pGL-3 and DGDP/pORF-hTRAIL with or without Tf (25 μM) were added to cells and cultured for 48 h. Concentrations of drug delivery systems composed of DOX and pORF-hTRAIL in the medium were DOX = 4 μg/ml and pORF-hTRAIL = 25 μg/ml. Then the cells were fixed with 4% paraformaldehyde solution, and processed for TUNEL according to the manufacturer's instructions. Apoptotic cells marked by FITC were observed using fluorescence microscope.

2.16. Mice

About 4-week old male Balb/c nude mice of 18–22 g body weight were purchased from the Department of Experimental Animals, Fudan University and maintained under standard housing conditions. All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University.

2.17. In vivo imaging analysis

Orthotopic human glioma model, using the human U87 glioma cell line, was created in nude mice. Briefly, 50 × 10^4 U87 cells in 5 μl Hank's solution were slowly (over 2 min) implanted 3 mm deep into the right caudatoputamen. After implantation of the cells, the needle was left in place for 5 min, and then slowly withdrawn from the brain, and the skin incision closed. 20 days after surgery, animals were randomly divided into two groups. One group received DGDPT/pORF-hTRAIL intravenously, the other group DGDPT/pORF-hTRAIL in vivo. To evaluate the biodistribution of nanoparticles (DGDPT/pORF-hTRAIL and DGDP/pORF-hTRAIL) in tumor-bearing mice, DGL were labeled with NIR783, a near-infrared fluorophore.- Fluorescent imaging was performed 1 h, 2 h, 3 h and 4 h post-injection. Then brains were removed carefully, and visualized using a CRi in vivo imaging system.

2.18. In vivo anti-glioma effect

The co-delivery system combined apoptotic pathways of DOX and pORF-hTRAIL. While pORF-hTRAIL-induced apoptosis appeared after the expression of TRAIL, doxorubicin-induced apoptosis had a tendency to appear earlier. Therefore, the appearance time of apoptosis is different among groups, and it is difficult to select the time of caspase-3 analysis. DNA fragmentation is downstream to activation of caspase-3 and a terminal event in the apoptotic pathways. As one of the hallmarks of apoptosis, DNA fragmentation is commonly used to identify apoptotic cells. Therefore, DNA fragmentation was used as an indicator of apoptosis and detected by TUNEL assay in this paper. At the day 12th, 15th and 18th after implantation, the survival percentage of control and experimental groups were measured and compared using the GraphPad InStat (version 3.06). MTT and survival data were analyzed by the GraphPad Prism software.

2.19. Statistical analysis

All data are presented as the mean ± S.D. Statistical analysis was performed by One-way Analysis of Variance (ANOVA) followed by Tukeye Kramer Multiple Comparisons Test using GraphPad InStat (version 3.06). MTT and survival data were analyzed by the GraphPad Prism software.
3. Results

3.1. Synthesis of T7-PEG-DGL-Glu-NHN-DOX (DGDPT)

The dendrimer used in this study was Dendrigraft poly-ε-lysine (DGL) generation 3 with 123 amino groups per molecular. Owning to its rich surface amino groups, DGL can be easily modified by drugs or other molecules. The synthesis of T7-PEG-DGL-Glu-NHN-DOX (DGDPT) was shown in Scheme 1. Fig. 1 shows the $^1$H NMR spectrum of DGL-Glu and DGL-Glu-NHNH$_2$, which were successfully synthesized suggested by the characteristic peaks of $^1$H NMR spectrum. The number of Glu residues incorporated to DGL were evaluated from the integral ratios of signals of DGL dendrimer and Boc-Glu(Obzl) (Fig. 1A). Fig. 1B shows the $^1$H NMR spectrum of DGL-Glu-NHNH$_2$, which was obtained by reaction of DGL-Glu with hydrazine. After the reaction, signals derived from benzyl groups disappeared, but the signal of hydrazide appeared at 8.96 ppm, indicating that benzyl esters of Glu residues were replaced completely by hydrazide groups. In this paper, DGL-Glu (1:2, DGL to Glu, mole ratio) was used. The structure characterization of DGL-Glu-NHN-DOX was not shown, because of the large molecular weight of DGL (22000) and low molar ratio (DOX: DGL, 2:1), characteristic peaks of DOX is not obvious in the $^1$H NMR spectrum of DGL-Glu-NHN-DOX. However, after the isolation, purification and freeze-drying, DGL-Glu-NHN-DOX exhibited a red powder, indicating that DOX were successfully conjugated to DGL. The amount of DOX per DGL was determined by UV–Vis spectrophotometry, the number of DOX molecules per dendrimer was 2. Finally, T7 conjugated to DGD via bifunctional PEG, the synthesis and characterization of DGDPT (DGL: Glu: DOX: PEG: T7 = 1: 2: 2: 1, molar ratio) refers to our previous work [27–29].

3.2. Gel retardation assay

As shown in Fig. 2A, the gel retardation assay demonstrated that pDNA could be effectively packed by vectors at a DGL to pDNA weight ratio greater than 0.5. The specified ratio of 3:1 was used in the following experiments.

3.3. Size and zeta potential of DGDPT/pORF-hTRAIL

The mean diameter of DGDPT/pORF-hTRAIL was 173 ± 5.6 nm, the size distribution data was shown in Fig. 2B. TEM showed that the DGDPT/pORF-hTRAIL NPs was a kind of analogous spherical shape and compacted structure with a size approximately < 200 nm (Fig. 2C). The zeta potential value was 3.2 ± 0.45 mV. DGL have been intensively investigated as a gene delivery module in our laboratory, the zeta potential value of DGL/pDNA (w/w, 3:1) complexes was about 6 mV according to our previous study [30]. In this paper, after the conjugation of DOX, some amino groups on the

Fig. 1. $^1$H NMR spectrum of DGL-Glu (A) and DGL-Glu-NHNH$_2$ (B).
surface of DGL were reacted with DOX molecules, which means that the charge of DGL-Glu-NHN-DOX was lower than that of DGL. Therefore, it is reasonable that the zeta potential value of DGDPT/pORF-hTRAIL was smaller than 6 mv.

3.4. pH-triggered release of DOX

Hydrazone bond is known to be cleaved under mildly acidic conditions. In vitro drug release studies of DOX from the resultant DGDPT and DGDPT/pORF-hTRAIL were performed respectively under physiological conditions (pH 7.4), a slightly acidic environment (pH 6.0) and an acidic environment (pH 5.0) to simulate the pH of the endosomal and lysosomal microenvironments. The release profiles of DOX were shown in Fig. 2D and E. The data revealed that DGDPT and DGDPT/pORF-hTRAIL exhibited a pH-dependent characteristic. The drug released from DGDPT at pH 7.4 was considerably lower, with an initial burst of about 10%. In contrast, the drug release was much faster at pH 5.0, with approximately 80% after 60 h. In addition, the percentage of released drug was about 30% at pH 6.0, less than that at pH 5.0. The drug release of DGDPT and DGDPT/pORF-hTRAIL had no significant difference.
3.5. Cellular uptake

We then characterized the cellular uptake of DGDP/pORF-hTRAIL and DGDPT/pORF-hTRAIL with and without Tf. Plasmid pORF-hTRAIL used here was pre-labeled by YOYO-1, which could intercalate into DNA and emit green fluorescence. A significant increase both in DOX and DNA accumulation by T7-modified co-delivery nanoparticles (DGDPT/pORF-hTRAIL) compared to DGDP/pORF-hTRAIL with and without Tf in U87 MG cells was observed (Fig. 3A). The cellular fluorescence intensity of DGDPT/pORF-hTRAIL co-treated with Tf was superior to that of untreated one. In addition, the energy dependency of co-delivery systems was observed. As shown in Fig. 3B, the cell uptake was inhibited at 4 °C.

3.6. In vitro gene expression

The transfection efficiency of GFP mediated by DGP/pEGFP-N2 and DGPT/pEGFP-N2 NPs with and without Tf was assessed in U87 MG cells. Herein, Lipofectamine 2000/pEGFP-N2 is a positive control group. As shown in Fig. 4, without Tf, DGPT/pEGFP-N2 induced higher formation of GFP than DGP/pEGFP-N2. As expected, the presence of Tf increased the GFP expression in cells treated with DGPT/pEGFP-N2, in comparison with systems without Tf.

3.7. Analysis of combination effects

Cytotoxicity of co-delivery system (DGDPT/pORF-hTRAIL) and mono-delivery systems (DGDPT/pGL-3, DGPT/pORF-hTRAIL) against U87 MG astrocytoma cells was tested. As shown in Fig. 5, co-delivery system exhibited higher cytotoxicity than mono-delivery systems. CI values at 30%, 50% and 70% of growth inhibition effects were 0.11, 0.13 and 0.20, respectively (Table 1). CI < 1 indicate synergy [31].

3.8. In vitro apoptosis detection

One Step TUNEL Apoptosis Assay kits is a novel approach for the detection of apoptosis. This assay is based on incorporation of...
fluorescein-dUTP conjugated to bromodeoxyuridine (BrdU) at the 3’ OH ends of the DNA fragments that are formed during apoptosis. This detection system utilizes a fluorescein conjugated anti-BrdU antibody to be visible by fluorescence microscope. As shown in Fig. 6, apoptosis induced by co-delivery system (DGDPT/pORF-hTRAIL) was more serious than that by mono-delivery system (DGPT/pORF-hTRAIL or DGDPT/pGL-3). And the apoptosis signals caused by systems with Tf were stronger than that by systems without Tf.

3.9. In vivo imaging of tumor homing

The in vivo potential targeting behavior of DGDPT/pORF-hTRAIL was investigated in Orthotopic glioma xenograft nude mice, based on the fluorescence of NIR-labeled nanoparticles. All the animals were tested positively for tumor presence 20 days after glioma cell injection. After intravenously administration of DGPT/pORF-hTRAIL and DGDPT/pORF-hTRAIL, in vivo images were taken at 1 h, 2 h, 3 h and 4 h, respectively. Compared with NIR-loaded DGPT/pORF-hTRAIL group, an obvious accumulation of NIR signal was detected in the brain of nude mice administered with NIR-loaded DGDPT/pORF-hTRAIL. The T7-modified delivery system exhibited a rapid U87 MG tumor targeting as early as 1 h p.i. An increasing tendency in signal intensity was seen in tumors from 1 h to 4 h after the injection of DGDPT/pORF-hTRAIL. Unfortunately, the unmodified delivery system was accumulated mostly in the normal tissues, and cleared up quickly (Fig. 7).

3.10. In vivo apoptosis detection

We examined the induction of apoptosis by co-delivery systems (DGDPT/pORF-hTRAIL and DGDPT/pORF-hTRAIL) and mono-delivery systems (DGPT/pORF-hTRAIL and DGDPT/pGL-3) for brain glioma in orthotopic xenograft nude mice, using the TUNEL method. The results are shown in Fig. 8. Apoptotic cells appeared brown in color. Brown color was observed in tumor cells of xenograft mice after the treatment with drug delivery systems. As expected, the largest number of apoptotic cells was observed in the tumor tissue which was treated targeting co-delivery system. Interestingly, there was no apoptotic cell in normal brain tissue.

3.11. In vivo anti-glioma efficacy

The effect of combination delivery system on mice survival was examined in this study and the results of these experiments are shown in Fig. 9. The median survival time of DGDPT/pORF-hTRAIL group, DGDP/pORF-hTRAIL group, DGDPT/pORF-hTRAIL group, DGDPT/pGL-3 group, DOX group, and saline group were 57, 27, 33.5, 31.5, 34, 23 days, respectively. The change in body weight after treatment was evaluated. After treated with targeting co-delivery system DGDPT/pORF-hTRAIL, the body weight of mice had no obvious change until fifteenth day, while other groups showed a huge decrease in weight after about 20 days (Fig. 9A).

4. Discussion

In this study, DGDPT/pORF-hTRAIL, a targeted co-delivery system, was developed to improve the efficacy of systemic chemotherapy and gene therapy of brain gliomas.

The size of NPs is important physiochemical parameter in designing drug delivery systems. Although each nanoparticle loaded with both DOX and gene medicine, the size was controlled at approximately 173 nm, which is a suitable size for injectable systems and possibility to target cancer tissues via EPR effect [32,33]. This study is also expected to improve cancer cell uptake of drug delivery systems through transferrin receptor-mediated endocytosis. Fortunately, the optimal particle size for endocytosis is less than 200 nm [34]. Therefore, the DGDPT/pORF-hTRAIL NPs can be accumulated in tumor tissue via the EPR effect, and further successfully internalized by tumor cells.

The modification of T7 enhanced the cell uptake of the drug delivery systems, and Tf facilitated the internalization (Fig. 3A). It has been reported that the binding sites of T7 to TIR are different from that of Tf to TIR, which could efficiently avoid competitive inhibition of endogenous Tf [17,18]. And the cellular uptake of T7 was facilitated by endogenous Tf [27]. Moreover, the biodistribution of nanoparticles showed that the accumulation of DGDPT/pORF-hTRAIL NPs in tumor was significantly increased.

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Table 1: Combination index (CI) values at different effect levels for combination treatments of U87 MG cells.
while DGDP/pORF-hTRAIL NPs which was not modified with T7 mostly accumulated in normal tissues, such as liver and kidneys (Fig. 7). These results further demonstrate that T7-modified co-delivery nanoparticles (DGDPT/pORF-hTRAIL) could effectively home to the brain tumors, and accumulate in the tumor cells.

The pH levels replicated physiological conditions (pH 7.4), as well as the acidity characteristic of the tumor extracellular environment (~pH 6.0) [35,36], and of endosomes/lysosomes which are more acidic (~pH 5.0) [22]. Owing to the EPR effect and T7-mediated endocytosis, the delivery systems were accumulated more effectively in the tumor than normal tissues. Furthermore, the pH-triggered hydrazone bond was designed between DOX and carrier, so that DOX could be released from the carrier after accumulation. DOX was released quickly at pH 5.0, but keep stability at pH 7.4 (Fig. 2D and E). This result indicates that DOX can be released preferentially in the endosomal/lysosomal compartment of the cell where it is protected from drug efflux. About 30% of bound drug is released at pH 6.0 in 24 h suggesting that a few DOX would accumulate in the extracellular of tumor, maybe this part of DOX could get into tumor cells by other pathway, such as passive diffusion, and subsequently induce the apoptosis of tumor cells. In addition, the drug release of DGDPT and DGDPT/pORF-hTRAIL had no significant difference, indicating that the release of DOX was not affected by plasmid DNA.

The potential synergy of the combination of DOX and pORF-hTRAIL towards U87 MG cells was evaluated by the CI analysis. In the CI analysis, values of CI < 1, =1 and >1 indicate synergy, additivity, and antagonism, respectively [31]. This combination treatment resulted in a synergistic growth inhibition (CI < 1) in U87 MG cells. This result indicates the effect of this combination treatment was superior to the total effect of treating two agents respectively. Furthermore, the synergistic effect of targeted delivery system combined DOX and pORF-hTRAIL was verified by the apoptosis effect of the co-delivery system (DGDPT/pORF-hTRAIL) and mono-delivery systems (DGDPT/pEGFP and DGPT/pORF-hTRAIL), both in vitro and in vivo (Figs. 6 and 8). 

![Fig. 6.](image1)

**Fig. 6.** Cellular apoptosis of U87 MG cells induced by DGPT/pORF-hTRAIL (A and D), DGDPT/pGL3 (B and E) and DGDPT/pORF-hTRAIL (C and G) was examined by fluorescence microscope after 2 h treatment and 48 h incubation. Cells were incubated without Tf (A, B and C) and with Tf (D, E and F). Green: FITC-labeled apoptotic cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

![Fig. 7.](image2)

**Fig. 7.** In vivo imaging of mice administrated with DGDP/pORF-hTRAIL (left in every image) or DGDPT/pORF-hTRAIL (right in every image). Images were taken at 1, 2, 3 and 4 h after NPs administrated. NPs were labeled with near-infrared probe NIR783. A: 1 h, B: 2 h, C: 3 h, D: 4 h, E: brain, F: tumor was exposed.
paper, the synergistic effect of combination may depend on the apoptosis mechanism of each agent. TRAIL-induced ligation of its cognate death receptors (DR4/DR5), results in formation of the death-inducing signalling complex (DISC) which in turn initiates two pathways: (a) activation of caspase-8 causes activation of effector caspases such as caspase-3, and (b) activation of Bcl-2 inhibitory BH3-domain-containing protein (Bid) to truncated Bid (tBid) by caspase-8, and then tBid activates proapoptotic members Bax and Bak to disrupt mitochondria and induce cell apoptosis [37–39]. DOX increased the expressions of DR4 and DR5 [10,40], as well as augmented TRAIL-induced processing of caspase-8 and Bid [11,41]. Thus, DOX can sensitize tumor cells to TRAIL-induced apoptosis. On the other hand, Multidrug resistance is the most widely studied manifestations of tumor cell resistance to DOX [42,43], Bax and Bak deficiency is a key mechanism for DOX resistance in tumor cells [44,45]. Nevertheless, proapoptotic members Bax and Bak could be activated in TRAIL-induced apoptosis pathway. Consequently, TRAIL could enhance DOX sensitivity of tumor cells by the activating of Bax and Bak.

In the pharmacodynamic evaluation of brain tumor-bearing mice (Fig. 9), DGDPT/pORF-hTRAIL NPs exhibited a best therapeutic efficacy for gliomas. It might be attributed to the targeting ligand T7 peptide modification, pH-triggered DOX release and co-treatment of DOX and pORF-hTRAIL. The anti-glioma effect of targeting mono-delivery systems (DGPT/pORF-hTRAIL or DGDPT/pGL-3) was better than that of co-delivery system (DGDP/pORF-hTRAIL), for T7 could enhance the accumulation of DOX or pORF-hTRAIL in tumor tissue. Although the anti-glioma efficacy of free DOX was comparable to that of mono-delivery systems, the dose of free DOX group was about 14.4-fold than other groups. In anti-tumor studies, the dose of DOX for mice is 5 mg/kg [28,29], which will be accompanied by dose-limiting toxicities including cardiotoxicity and myelosuppression, as shown in Fig. 9A, the weight of mice decreased rapidly after the treatment of free DOX, indicating the dose of DOX possesses side effects for the cancer treatment. However, in the co-delivery system and mono-delivery system, the dose of DOX was less than 0.35 mg/kg, under which the cardiotoxicity of DOX is not significant [46], but the anti-tumor

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**Fig. 8.** TUNEL-stained frozen Coronal sections of brain tumors. dashed line: border of the glial tumor. A: Saline, B: DGDPT/pGL-3, C: DGDP/pORF-hTRAIL, D: DOX, E: DGPT/pORF-hTRAIL. Original magnification: ×400.

**Fig. 9.** The average change in body weight after treatment (A). Kaplan–Meier survival curves of mice bearing orthotopic U87 tumors (B). 12 days after tumor implantation within the brain (a, mice were treated with DGDPT/pORF-hTRAIL, DGDP/pORF-hTRAIL, DGDP/pGL-3, DGPT/pORF-hTRAIL with 50 μg DNA and/or 8 μg DOX/mouse, DOX (5 mg/kg) and saline. 12 animals per group. Median survival for each group was 57 days (DGDP/pORF-hTRAIL), 27 days (DGDP/pORF-hTRAIL), 33.5 days (DGPT/pORF-hTRAIL), 31.5 days (DGDPT/pGL-3), 34 days (DOX) and 23 days (saline). *P < 0.05, **P < 0.01 and ***P < 0.001, blue asterisks indicate DGDPT/pORF-hTRAIL versus DOX. Purple asterisks indicate DGDPT/pORF-hTRAIL versus DGDPT/pGL-3. Green asterisks indicate DGDPT/pORF-hTRAIL versus DGPT/pORF-hTRAIL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
efficacy is still notable. Therefore, the co-delivery system modified T7 can achieve predominant anticancer effect at lower drug dose, and lead to lower undesirable toxicities.

5. Conclusions
Owing to the multifunctional nano-delivery system we designed, DOX synergize with gene agent pORF-hTRAIL in accumulating in tumor site, inducing tumor cell apoptosis, reducing undesirable toxicities and enhancing survival of tumor-bearing mice. In the light of these results, this targeting co-delivery system should contribute to the design and development of novel drug delivery systems for gliomas and other cancer diseases.

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References