Choline transporter-targeting and co-delivery system for glioma therapy

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ABSTRACT

Combination of gene therapy and chemotherapy is a promising approach for glioma therapy. In this study, a co-delivery system of plasmid encoding human tumor necrosis factor-related apoptosis-inducing ligand (pORF-hTRAIL, Trail) and doxorubicin (DOX) has been simply constructed in two steps. Firstly, DOX was intercalated into Trail to form a stable complex. Secondly, DOX-Trail complex was condensed by Dendrigraft poly-L-lysine (DGL) to form a nanoscaled co-delivery system. Choline transporters are both expressed on blood—brain barrier (BBB) and glioma, Herein, a choline derivate with high choline transporter affinity was chosen as BBB and glioma dual targeting ligand. Choline-derivate modified co-delivery system showed higher cellular uptake efficiency and cytotoxicity than unmodified co-delivery system in U87 MG cells. In comparison with single medication or unmodified delivery system, Choline-derivate modified co-delivery system induced more apoptosis both in vitro and in vivo. The therapeutic efficacy on U87 MG bearing xenografts further confirmed the predominance of this dual targeting and co-delivery system.

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1. Introduction

Current treatments for glioblastoma multiforme (GBM) are insufficient with a nearly universal recurrence after surgery, radiation therapy, and chemotherapy [1]. Poor chemotherapy outcome after surgery contributes to this high recurrence. The blood—brain barrier (BBB) existing at the early stage or the tumor margin of glioma limits the penetration of most therapeutic agents [2]. Even though some chemotherapeutics could permeate across the BBB, single medication could not obtain optimal efficacy due to the drug resistance. Based on the above consideration, two strategies including glioma specific targeting and combination therapy were employed for better outcomes.

For glioma targeting, two-order targeting strategy was commonly employed in designing drug delivery or diagnosis systems to overcome the BBB and accumulate into tumor. In brief, one ligand with binding affinity to the receptors or transporters expressed on the vasculature endothelial cells could facilitate the transcytosis across the BBB. Another ligand with high affinity to the receptors overexpressed on glioma cells could enhance the accumulation into tumor [3–5]. Dual targeting to BBB and glioma with one ligand was another choice. For example, Angiopep-2, one of the family of Kunitz domain-derived peptides, could target to the low-density lipoprotein receptor-related protein-1 (LRP1) expressed on brain capillary endothelial cells (BCECs) and glial cells [6,7]. In our previous study, a choline derivate with high BBB choline transporter affinity was challenged to mediate the gene delivery into brain [8]. Because of high malignancy, choline transporters are overexpressed on glioma cells. This indicates that choline transporters may facilitate the glioma targeting [9]. In another work, we developed a choline-derivate modified nanoprobe for glioma MRI imaging and gained notable signal contrast between tumor and normal brain region [10]. Herein, choline derivate was selected as a BBB and glioma dual targeting ligand.

Combination therapy in glioma has attracted wide attention due to the inefficiency of single medication. Attempts have been made on the co-delivery of gene drug and chemotherapeutics. The synergistic effect arisen from the co-delivery of human tumor necrosis factor-related apoptosis-inducing ligand (pORF-hTRAIL, Trail) and paclitaxel or doxorubicin (DOX) has been proved to gain enhanced anti-tumor effect [11,12]. In this study, Trail and DOX were chosen as model drugs.

A co-delivery system of DOX and nucleic acid was reported by Bagalkot et al. [13]. DOX is known to intercalate within the double helix of DNA strand due to the presence of flat aromatic rings in this molecule. At present, aptamer and plasmid DNA were developed as
vectors which could form stable complex upon DOX intercalation. Combined chemoimmunotherapy using plasmid-DOX complex achieved comparable therapeutic effect and reduced cardiotoxicities to DOX in two different tumor models. The therapeutic benefits came from the improved pharmacokinetics of DOX by increasing blood circulation. In turn, intercalation (complexation) can also protect the plasmid against nucleases [14]. While, a flaw that mars perfection may be the small size of complex resulting in rather fast renal clearance. To take the advantage of enhanced permeability and retention (EPR) effect, plasmid-DOX complex was further condensed by dendrimer to form nanoparticles (NPs). This nanosized co-delivery system preferentially accumulated in tumor and significantly inhibited its growth in a subcutaneous tumor model [15].

In this study, a co-delivery system for glioma therapy was established. Firstly, DOX was intercalated into Trail to form a stable complex. Trail served both as a DOX carrier and a gene drug. Secondly, DOX-Trail complex was condensed by Dendrigraft poly-L-lysine (DGL) to form a nanoscaled co-delivery system (Scheme 1). Choline derivate was further conjugated to this system to realize the glioma specific drug delivery. Targeting efficiency and anti-tumor effect of the system were evaluated both in vitro and in vivo.

2. Materials and method
2.1. Materials

DGL generation 3 with 123 lysine groups were purchased from Colcom, France. α-Maleimidyl-ω-N-hydroxysuccinimidyl polyethylene glycol (NHS-PEG-MAL, MW 3500) was obtained from Jenkem Technology (Beijing, China). Doxorubicin hydrochloride (DOX $\text{HCl}$) was purchased from Beijing Huafeng United Technology Corp. The plasmid Trail and pGL2-control vector (InvivoGen, San Diego, CA, USA) were purified using QIAGEN Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). Annexin V-FITC TUNEL Apoptosis Assay Kit and TdT In Situ Apoptosis Detection Kit were purchased from KeyGEN (Nanjing, China). Near-infrared probe (NIR783) was kindly

Scheme 1. Construction of dual targeting and co-delivery system. DOX was intercalated into Trail plasmid to form a stable complex which was further condensed by choline-derivate modified DGL. This co-delivery system could accumulate into glioma cells by EPR and dual targeting effect. After cytoplasm releasing, Trail and DOX educe combination therapy on glioma.
provided by Prof. Cong Li (Molecular Imaging Institute, School of Pharmacy, Fudan University). Other reagents, if not specified, were purchased from Sigma–Aldrich.

2.2. Cell lines and animals

U87 MG 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 Dulbecco’s modified Eagle’s Medium (DMEM) which supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Male Balbc nude mice of about 20 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. For U87 MG xenograft model, nude mice were anesthetized by intraperitoneal injection of 10% Chloral hydrate. U87 MG cells (1 × 10⁶ in 5 μL PBS) were implanted into the right striatum (18 mm right lateral to the bregma and 3 mm of depth) of the mice by using a stereotactic fixation device with mouse adator.

2.3. Preparation and characterization of DOX-Trail complex

A physical complex between Trail and DOX was made as described previously [15]. Briefly, DOX was dissolved in methanol at the concentration of 1 mg/ml as a stock solution. Different picomoles of Trail were added in a fixed concentration of DOX (3 μM) in PBS buffer, the mixture were vortexed for 1 min and the fluorescence of DOX was monitored at excitation 480 nm and emission was recorded in the interval of 520–680 nm on a Perkin Elmer LS-55 spectrofluorometer. The fluorescence quenching was visualized by Cambridge Research & Instrumentation (CRI) in vivo imaging system (CR, MA, USA).

2.4. Preparation and characterization of dendrimers/DOX-Trail NPs

DP (DGL-PEG) and DPC (DGL-PEG-10) were synthesized as described previously [8]. Dendrimers (DGL-PEG, and DGL-PEG-10) were diluted to appropriate concentrations in PBS (pH 7.4). DOX-Trail complex or Trail solution (100 μg DNA/ml, 50 μM sodium sulfate solution) was added at weight ratio of 6:1 (DGL to DNA) and immediately vortexed for 30 s. The particle size distribution of DPC/DOX-Trail was measured by a dynamic light scattering detector (Zetasizer, Nano-ZS, Malvern, UK) and transmission electron microscope (Tecnai G2 spirit Biotwin, FEI).

2.5. Cellular uptake and release of DOX from complex and NPs

U87 MG cells were seeded at a density of 5 × 10⁴ cells/well in 6-well plates (Corning-Coaster, Tokyo, Japan). Cells were incubated for 48 h and checked under the microscope for confluency and morphology. Then cells were incubated with free DOX, DOX-Trail complex, DP/DOX-Trail and DPC/DOX-Trail at the concentration of 1 μg/ml measured by DOX in the presence of serum free medium for 30 min at 37 °C, respectively. Then cells were digested, centrifuged and washed twice with Hank’s solution. The fluorescence intensity was analyzed using a flow cytometer (FACScalibur, BD Biosciences, Bedford, MA, USA) equipped with an argon ion laser (488 nm) as the excitation source. To evaluate the DOX release from complex and NPs, cells were cultured in fresh medium containing FBS for another 2 h following the 30 min incubation. For each flow cytometer analysis, 1 × 10⁶ events were collected.

2.6. Cytotoxicity assay on U87 MG cells

Cytotoxicity assay was evaluated by MTT assay. U87 MG cells were seeded at a density of 5 × 10⁴ cells/well in a 96-well plate. After 48 h incubation, cells were then washed twice with Hank’s solution and exposed to different concentrations of DOX, DPC/Trail, DP/DOX-Trail, DPC/DOX-Trail and DPC/DOX-NEG (negative control, DPC/DOX-pGDL) at 37 °C for 2 h in serum free medium. After 2 h of incubation, cells were washed twice with Hank’s solution and re-fed with 60 μl of DMEM medium containing 10% FBS for 48 h. To assess cell viability, 40 μl of MTT (1.25 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 served as control. The absorbance was read at 570 nm and corrected at 630 nm by dual wavelength detection using a Multiskan MK3 microplate reader (Thermo Scientific). Cell viability was calculated as the survival percentage of control.

2.7. Apoptosis detection in vitro

U87 MG cells were seeded at a density of 5 × 10⁴ cells/well in 24-well plates. Cells were incubated for 48 h. Then DOX, DPC/Trail, DP/DOX-Trail, DPC/DOX-Trail and DPC/DOX-NEG at the concentration of 3.16 μM were measured by DGL were added to the cells. The mixture was incubated at 37 °C for 2 h. The cells were washed twice with Hank’s solution and further cultured in DMEM medium containing 10% FBS for 48 h. Then medium was removed. The cells were incubated with Annexin V-FITC Apoptosis Detection Kit for 15 min. The fluorescence labeled apoptotic cells were imaged using a fluorescence microscope.

2.8. In vivo imaging

At 18th day after glioma implantation, DP/DOX-Trail and DPC/DOX-Trail (6:1:0:16, DGL to DNA to DOX, w/w/w) at the dose of 50 μg DNA/mouse were injected into the tail vein of model mice, respectively. Images were taken 2 h after injection by Cambridge Research & Instrumentation (CR, MA, USA). Then mice were anesthetized with diethyl ether and killed by decapitation and main organs were further visualized. DGL was pre-labeled by NIR783 dye.

2.9. In vivo anti-glioblastoma effect in U87 grafted model

At the 12th, 15th and 18th day after implantation, model mice were administrated of DOX (5 mg/kg), DPC/Trail, DP/DOX-Trail and DPC/DOX-Trail (6:1:0:16, DGL to DNA to DOX, w/w/w) at a dose of 50 μg Trail or/and 8 μg DOX/mouse. At the 21st day, animals were anesthetized with diethyl ether and killed by decapitation. Brains were removed, fixed in 4% parafomaldehyde for 48 h, placed in 15% sucrose PBS solution for 24 h until subsidence, then in 30% sucrose for 48 h until subsidence. Afterwards, brains were frozen in OCT embedding medium (Sakura, Torrance, CA, USA) at –80 °C. Frozen sections of 20 μm thickness were prepared with a cryotome Cryostat (Leica, CM 1900, Wetzlar, Germany). Detection of apoptotic cells was performed on the basis of TUNEL method using an in situ apoptosis detection kit. Sections were incubated with proteinase K for 15 min at room temperature and endogenous peroxidase was blocked with a solution PBS and 3% H2O2 for 10 min. The slides were then incubated with a solution of digoxigenin-conjugated nucleotides and terminal deoxynucleotidyl transferase (TdT) at 37 °C for 60 min. Subsequently, the anti-digoxigenin antibody was applied and incubated for 30 min at room temperature. Detection of the antigen antibody link was made through immunoperoxidase followed by diaminobenzidine (DAB) chromogenic. The sections were counterstained with hematoxylin, rinsed in distilled water and observed by microscope. The survival of brain tumor-bearing mice was evaluated using the same course of treatment.

3. Results

3.1. DOX-DNA complex and NPs formation

The DOX-DNA complex was formulated by insertion doxorubicin into plasmid DNA and monitored by fluorescence scanning and imaging. The total fluorescence quenching was observed with a binding ratio of doxorubicin to plasmid of 3000:4 (Fig. 1A). As speculated, one Dox molecule could occupy 5-6 base pairs of plasmid DNA. Fluorescence imaging further demonstrated this intercalation induced fluorescence quenching (Fig. 1B). Dynamic light scattering showed that the hydrodynamic diameter of DPC/DOX-Trail NPs was 91.28 nm (Fig. 1C). Transmission electron microscope conformed that NPs had a narrow distribution (Fig. 1D).

3.2. Cellular uptake and release of DOX from complex and NPs

U87 MG cells were treated with free DOX, DOX-Trail complex, DP/DOX-Trail and DPC/DOX-Trail NPs for 30 min. Then cellular uptake was examined by flow cytometry (Fig. 2). The fluorescence intensity in cells treated with free DOX was the highest. Although the fluorescence intensity in cells treated with DP/DOX-Trail and DPC/DOX-Trail NPs showed no significant difference, both NPs treated cells had superior fluorescence intensity than that in DOX-Trail treated cells. After 2 h additional incubation, the fluorescence of DOX was examined again. Once DOX was released from the DOX-Trail complex, the fluorescence quenching was inverted. The fluorescence intensity both enhanced in DOX-Trail complex and NPs.
treated cells. The fluorescence intensity in DPC/DOX-Trail NPs treated cells was higher than that in DP/DOX-Trail NPs treated cells.

3.3. Cytotoxicity in vitro

The dominance of combination therapy of DOX and Trail in U87 MG cells was investigated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Fig. 3). After 2 h-treatment followed by 48 h-incubation, the IC₅₀ values of DPC/Trail, DP/DOX-Trail, DPC/DOX-Trail, DPC/DOX-NEG and free DOX was 21.98 µM, 2.318 µM, 1.645 µM, 3.458 µM and 3.154 µM, respectively. DPC/DOX-Trail showed the highest cytotoxicity, which has an IC₅₀ value even lower than that of free DOX. This indicated that the transfection of Trail elevated the anti-glioblastoma effect of DOX. This enhancement was demonstrated by the replacement of Trail by a negative plasmid DNA (NEG), which resulting in increased IC₅₀ value.

3.4. Apoptosis detection in vitro

Annexin V-FITC could label the membrane phospholipid phosphatidylserine, which is translocated from the inner to the outer side of cell membranes in early cell apoptosis. PI is a molecular
probe that can label nucleus through destructed membranes. Cells labeled only with green fluorescence are at the early state of apoptosis, and those labeled only with red fluorescence are dead. Cells labeled both with green and red indicating the cells are at the late state of apoptosis. As shown in Fig. 4, DPC/Trail NPs treatment led to obvious early apoptosis with little late apoptosis or cell death. While combination treatment caused significantly more late apoptosis or cell death. Among these, DPC/DOX-Trail NPs treatment showed most significant anti-glioblastoma effect.

3.5. In vivo imaging

NIR imaging was performed to evaluate the in vivo targeting efficiency of choline derivate as glioma homing ligand. At 18th day, nude mice were administrated with NIR783 labeled DP/DOX-Trail and DPC/DOX-Trail NPs via tail vein. In vivo images were taken 2 h after administration (Fig. 5). Compared to DP/DOX-Trail NPs treated mice, an obvious accumulation of NIR signal was detected at the tumor region of mice injected with DPC/DOX-Trail NPs. Ex vivo imaging also exhibited the targeting behavior of choline derivate.

3.6. Apoptosis detection in U87 grafted model

At the 21st day after glioma implantation, TUNEL assay was performed to detect the DNA fragmentation in nuclei of apoptotic tumor cells (Fig. 6). Although with high cytotoxicity in vitro, DOX induced only little apoptosis in vivo. An extended apoptosis was observed after the combination therapy of Trail and DOX, especially in DPC/DOX-Trail treated group.

3.7. In vivo anti-glioblastoma effect in U87 grafted model

The anti-tumor effect of dual targeting and co-delivery system was evaluated as the median survival time of model mice. Median survival time of DPC/DOX-Trail, DP/DOX-Trail, DPC/Trail, DOX and saline treated mice was 47 (p < 0.01, compared to other groups), 37, 34.5, 32, 24.5 days, respectively (Fig. 7B). The dominance of targeted combination therapy (DPC/DOX-Trail) was also reflected on the body weight change. The body weight showed a slow decrease after the 27th day, while other groups had a rapid decrease (Fig. 7A).
4. Discussion

Despite recent advances in brain cancer therapy, the prognosis remains poor for patients diagnosed with malignant gliomas. The median survival time of glioblastoma patients is less than 15 months [16]. Patients have a universal recurrent after the surgery followed by chemotherapy. The effectiveness of single medication arises from the impermeability across the BBB and drug resistance. Thus, combination medication with BBB permeability may be a promising pathway to treat glioma. The synergistic effect of combination of DOX and Trail was reported and a significant therapeutic was achieved[11]. In this study, DOX and Trail were selected as model drugs. The co-delivery system was established in the following two steps.

In the first step, DOX was interacted into the double-stranded Trail to form a stable complex. The native fluorescence spectrum of doxorubicin was totally quenched at the binding ratio of doxorubicin to Trail of 3000:4 (Fig. 1A). The fluorescence images also showed an obvious quenching effect (Fig. 1B). In the second step, DOX-Trail complex was condensed by choline derivate modified DGL. And the cellular uptake of U87 MG cells was evaluated by flow cytometry (Fig. 2). Compared to free DOX, the cellular uptake of DOX-Trail complex was significantly reduced. Although the interaction of DOX into DNA could improve the pharmacokinetics of DOX, the cellular uptake of complex was hindered by the negative charge of plasmid DNA. While, condensation by positively charged DGL led to an increased DOX accumulation. Unfortunately, no difference was found in cellular uptake of DP/DOX-Trail and DPC/DOX-Trail NPs in the first 30 min. It was reported that DNA-DOX complex was very stable with only less than 20% leakage after incubation in PBS 7.4 for 120 h [15]. The fluorescence quenching may hind the targeting efficiency of choline derivate. We then evaluated the cellular DOX fluorescence of treated cells 2 h later. A recovery of fluorescence was observed both in complex treated or NPs treated cells. The fluorescence intensity of DPC/DOX-Trail treated cells was comparable to that of free DOX treated cells and higher than that of DP/DOX-Trail treated cells. DOX could escape from the complex or NPs in a accelerate manner in the intracellular component.

The synergistic effect of the combination of DOX and Trail towards U87 MG cells was evaluated by MTT assay and apoptosis detection in vitro (Figs. 3 and 4). The apoptosis detection showed that single medication of DPC/Trail induced the significant early apoptosis. While less late apoptosis or cell death was detected.
This was agreed with the high IC₅₀ value of DPC/Trail. Combination medication of Trail and DOX had lower IC₅₀ values than free DOX indicating the synergistic effect. This may depend on the apoptosis mechanism of each drug. On one hand, TRAIL induces apoptosis via its cognate receptors (DR4/DR5). DOX increased the expressions of DR4 and DR5 thus sensitizing tumor cells to Trail-induced apoptosis [17]. On the other hand, Bax and Bak deficiency is responsible for DOX resistance in tumor cells. Proapoptotic members Bax and Bak could be activated in Trail-induced apoptosis pathway. Thus, Trail could in turn enhance DOX sensitivity of tumor cells [11].

Blood-tumor barrier (BTB) is another major concern that impairs the therapeutic effects in systematic administration. Brain tumor vascular pore cutoff size was significantly reduced in cranial microenvironment compared with that in peripheral ones [18]. Thus, for glioma therapy, one more principle for drug delivery system other than long circulation time should be the suitable size of NPs. NPs with a diameter around 100 nm might permeate into glioma more efficiently. Herein, DOX-Trail complex was condensed by DGL at an optimal weight ratio to gain the desired particle size (Fig. 1C, D). Then the in vivo targeting efficiency was evaluated (Fig. 5). None targeting co-delivery system showed less accumulation in tumor via EPR effect. Choline derivate modified system revealed more accumulation by both EPR effect and dual targeting. Choline transport via choline transporter is in a conformation dependent manner. Here, the targeting mechanism was hypothesis that choline derivate with high choline transporter affinity could facilitate the NPs anchoring to the choline transporters overexpressed on tumor cells.

In the evaluation of anti-glioblastoma effect in U87 grafted model (Figs. 6 and 7), DPC/DOX-Trail NPs exhibited the highest therapeutic efficacy for gliomas. The median survival time of free DOX treated mice is only 32 days. The dosage of free DOX for mice therapeutic efficacy was evaluated (Fig. 4). None targeting co-delivery system showed less accumulation in tumor via EPR effect. Choline derivate modified system revealed more accumulation by both EPR effect and dual targeting. Choline transport via choline transporter is in a conformation dependent manner. Here, the targeting mechanism was hypothesis that choline derivate with high choline transporter affinity could facilitate the NPs anchoring to the choline transporters overexpressed on tumor cells.

In summary, a co-delivery system for glioma therapy was established in two steps and achieved enhanced tumor accumulation, tumor apoptosis and better therapeutic outcomes. Choline derivate employed here exhibited as a glioma targeting ligand. This easily established co-delivery system holds great potential for cancer therapy in clinical application.

5. Conclusions

In summary, a co-delivery system for glioma therapy was established in two steps and achieved enhanced tumor accumulation, tumor apoptosis and better therapeutic outcomes. Choline derivate employed here exhibited as a glioma targeting ligand. This easily established co-delivery system holds great potential for cancer therapy in clinical application.

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