Liposome-based glioma targeted drug delivery enabled by stable peptide ligands

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ABSTRACT

The treatment of glioma is one of the most challenging tasks in clinic. As an intracranial tumor, glioma exhibits many distinctive characteristics from other tumors. In particular, various barriers including enzymatic barriers in the blood and brain capillary endothelial cells, blood–brain barrier (BBB) and blood–brain tumor barrier (BBTB) rigorously prevent drug and drug delivery systems from reaching the tumor site. To tackle this dilemma, we developed a liposomal formulation to circumvent multiple-barriers by modifying the liposome surface with proteolytically stable peptides, DCDX and c(RGDyK). DCDX is a D-peptide ligand of nicotine acetylcholine receptors (nAChRs) on the BBB, and c(RGDyK) is a ligand of integrin highly expressed on the BBTB and glioma cells. Lysosomal compartments of brain capillary endothelial cells are implicated in the transcytosis of those liposomes. However, both peptide ligands displayed exceptional stability in lysosomal homogenate, ensuring that intact ligands could exert subsequent exocytosis from brain capillary endothelial cells and glioma targeting. In the cellular uptake studies, dually labeled liposomes could target both brain capillary endothelial cells and tumor cells, effectively traversing the BBB and BBTB monolayers, overcoming enzymatic barrier and targeting three-dimensional tumor spheroids. Its targeting ability to intracranial glioma was further verified in vivo by ex vivo imaging and histological studies. As a result, doxorubicin liposomes modified with both DCDX and c(RGDyK) presented better anti-glioma effect with prolonged median survival of nude mice bearing glioma than did unmodified liposomes and liposomes modified with individual peptide ligand. In conclusion, the liposome suggested in the present study could effectively overcome multi-barriers and accomplish glioma targeted drug delivery, validating its potential value in improving the therapeutic efficacy of doxorubicin for glioma.

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1. Introduction

Gliomas, accounting for 29% of all primary brain and CNS tumors and 80% of malignant tumors, severely threaten human health for fast development and poor prognosis [1]. The survival rate of patients remains very low after current multimodal treatment-aggressive surgical resection followed by concurrent or sequential radiation and temozolomide chemotherapy [2]. The infiltrative growth of gliomas makes it impossible for surgeons to completely remove tumor tissues without affecting normal brain functions, leading to rapid recurrence. Furthermore, the poor prognosis is also ascribed to the side effects of radiotherapy and poor outcome of chemotherapy. In recent decades, actively targeted drug delivery system has attracted extensive attention for effective delivery of chemotherapeutics to the tumor region, but there is still a long way to go. As an intracranial tumor, glioma possesses many distinctive characteristics from peripheral tumors. Its oncogenesis and development are complicated with various barriers such as enzymatic barrier, blood–brain barrier (BBB), and blood–brain tumor barrier (BBTB), preventing drug or drug delivery system from reaching the tumor sites [3–5]. In this regard, we hypothesized that nanocarriers modified with both stable peptide DCDX and c(RGDyK), which were respective ligand of nicotine acetylcholine receptors (nAChRs) on the BBB and integrin on the BBTB and glioma cells, could overcome enzymatic barrier and traverse BBB and BBTB, thus effectively accomplish glioma targeting.

The blood–brain barrier, which is mainly formed by capillary endothelial cells, remains intact at the early stage of glioma and around the
infiltrative tumor edge [3]. It behaves as the main obstacle to chemotherapy and nanocarriers, preventing approximately 98% of small molecules and nearly 100% of large molecules from transport into the brain [6]. It functions as not just physical (tight junctions) barrier but also enzymatic barrier [7]. According to previous reports, receptor-mediated transcytosis (RMT) is exploited as a successful pathway to circumvent BBB. The receptors highly expressed on the capillary endothelium of the brain have been exploited to facilitate receptor-mediated brain transport of drug delivery systems, including nicotine acetylcholine receptors [8,9], low density lipoprotein receptor-related protein-1 [10,11], and transferrin receptors [12]. They can recognize the corresponding ligands or monoclonal antibodies in the circulating blood and facilitate transport to the brain. The peptide-based ligands being rationally identified by phage display or structure-guided design have attracted increasing attention. When conjugated with delivery systems, these ligands can target the corresponding receptors and initiate the RMT process, enabling the transport of conjugates to the brain. However, the enzymatically protective barriers in brain capillary endothelial cells, rigorously threaten the stability of these peptide-based ligands, thus attenuating their efficacies to complete the unidirectional transport and targeting ability. Previously, a stable 16 residue peptide, glutamate-aspartic acid peptide-c(RGDyK) was synthesized and adopted as BBTB to improve peptide stability. Hence, stable cyclic arginine-glycine-aspartic acid peptide-c(RGDyK) was synthesized and adopted as BBTB targeting moiety. Liposomes are a class of versatile drug delivery carriers, as which are known to be nontoxic and nonimmunogenic. Furthermore, coating the surface of liposomes with PEG provides ‘stealth’ properties and greatly prolongs circulation [19]. Liposomes are also easily to be functionalized with various targeting ligands to trigger RMT and to accomplish targeted drug delivery. During the long blood circulation process, stability of targeting moiety encounters tremendous challenge in enzymatic microenvironments in vivo. Therefore, stable targeting ligands may greatly improve the targeting ability of liposomes.

In this study we designed stable peptides modified liposomes to inspire glioma targeting drug delivery. Doxorubicin liposomes were decorated with both \( ^{2} \text{CDX} \) and \( c(\text{RGDyK}) \) \( ^{2} \text{CDX}/c(\text{RGDyK}) \)-LS. The objective was to achieve glioma targeting with reduced side effects by overcoming multiple barriers. The targeting ability of \( ^{2} \text{CDX}/c(\text{RGDyK}) \)-LS was investigated in vitro and in vivo. The potential of \( ^{2} \text{CDX}/c(\text{RGDyK}) \)-LS containing doxorubicin in the treatment of intracranial glioma was evaluated.

2. Materials and methods

2.1. Materials

HSPC (hydrogenated soy phosphatidylcholine) and mPEG\(_{2000}\)-DSPE were purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was from Sinopharm Chemical Reagent Co. LTD. (Shanghai, China). Protected Fmoc-amino acid derivatives and Benzotriazole-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) were acquired from GL Biochem Ltd. (Shanghai, China). Protected Boc-amino acid derivatives were from Peptide Institute (Osaka, Japan). \( O \)-Benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluorophosphate (HBTU) was purchased from American Bioanalytical CO. (Natick, MA). Diisopropylethylamine (DIEA) and Boc-Gly-PAM resin were supplied by Fluka (USA). Mal-PEG\(_{5000}\)–DSPE was obtained from Laysan Bio Co. (Arab, AL). Sephadex G50 and 5-carboxyfluorescein (FAM) were purchased from Sigma (St. Louis, MO). Fluorescein-5-maleimide was purchased from FanboBiochemicals (Beijing, China). Near infrared dye DiR and LysoTracker®Red DND-99 were from Invitrogen (Grand Island, NY). Rat tail collagen Type I was obtained from Shengyou Biological Technology Co. (Hangzhou, China). DNase I and collagenase were from Dingguo Biological Technology Co. (Shanghai, China). DAPI was supplied by Roche (Basel, Switzerland). EBM-2 was from Lonza (Visp, Switzerland). DOX hydrochloride was purchased from Zhejiang Haizheng Co. (Zhejiang, China).

U87 cells and human umbilical vascular endothelial cells (HUVECs) were obtained from Shanghai Institute of Cell Biology and were maintained in Dulbecco’s Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum.

![Fig. 1. Stability of peptides in 50% rat plasma (A) and lysosomal homogenate (B) at 37 °C. Fresh rat serum was diluted with phosphate buffered saline and incubated with \( ^{2} \text{CDX} \) or \( ^{1} \text{CDX} \) or \( c(\text{RGDyK}) \) at a concentration of 0.1 mg/mL at 37 °C for different time periods, and the remaining peptide amounts were determined by HPLC to monitor and quantify peptide hydrolysis.](image-url)

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with 10% FBS (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C under a humidified atmosphere containing 5% CO₂.

Sprague-Dawley (SD) rats, ICR mice and male BALB/c nude mice of 4–6 weeks age were purchased from Shanghai SLAC laboratory animal Co. LTD (Shanghai, China) and kept under SPF conditions. All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University.

2.2. Study of peptide stability in vitro

The stability of ³⁵CDX and c(RGDyK) in rat plasma and in rat liver lysosomal homogenate was examined as previously reported [20,21].

2.3. Synthesis and characterization of materials

³⁵CDX and c(RGDyK) were synthesized via solid phase peptide synthesis using active ester chemistry to couple Boc/Fmoc-protected amino acid to the deprotected resin.

³⁵CDX-PEG₃₄₀₀-DSPE or c(RGDyK)-PEG₃₄₀₀-DSPE was synthesized through the covalently conjugation of ³⁵CDX-SH or c(RGDyK)-SH with Mal-PEG₃₄₀₀-DSPE by a sulfhydryl-maleimide coupling method [18]. The successful synthesis was confirmed by the disappearance of the peak in HPLC spectrum and the ¹H NMR.

2.4. Preparation and characterization of liposomes

2.4.1. Preparation of liposomes

All the liposomes loading with FAM, DiR or DOX, including liposomes without any targeting moiety (LS), liposomes modified with ³⁵CDX (³⁵CDX-LS), liposomes modified with c(RGDyK) (c(RGDyK)-LS) and those modified with both ³⁵CDX and c(RGDyK) (³⁵CDX-(c(RGDyK))-LS), were prepared respectively for different use by the thin-film hydration and extrusion method [22]. For blank liposomes, a mixture of HSPC/cholesterol/mPEG₂₀₀₀-DSPE(52/43/5, by mole) or HSPC/cholesterol/mPEG₂₀₀₀-DSPE/³⁵CDX-PEG₃₄₀₀-DSPE(52/43/4/1, by mole) or HSPC/cholesterol/mPEG₂₀₀₀-DSPE/c(RGDyK)-PEG₃₄₀₀-DSPE(52/43/2/2/1, by mole) in CHCl₃ solution was rotary evaporated to form a thin film. The dried lipid film was subsequently hydrated in saline at 60 °C for 1 h. Then the lipid dispersion was extruded through a series of polycarbonate membranes with the pore size ranging from 200 nm to 50 nm using an Avanti Mini Extruder (Avanti Polar Lipids). DOX-loaded liposomes were prepared using a traditional ammonium sulfate gradient loading method according to the previously reported procedure [23].

2.4.2. Particle size and encapsulation efficiency

The particle size distributions of different liposomes were measured by dynamic light scattering method (Nicomp™ 380ZLS, USA). The encapsulation efficiency of liposomes was determined as previously reported [24].

2.5. Study of cellular uptake in vitro

Both brain capillary endothelial cells isolated according to previous report [25] and U87 cells were seeded into confocal disk chambered cover glasses or 12-well plates. 24 h later, the culture medium was changed with 5 μM FAM loaded liposomes (lipid ~0.6 mM) of different formulations in DMEM with 10% FBS for 4 h, respectively. The fluorescent intensity was captured by confocal laser microscopy and flow cytometer after three times rinse by PBS.

2.6. Evaluation of brain targeting ability on BBB model

The BBB model was established as previously reported [25,26]. Rat primary brain capillary endothelial cells were isolated and seeded onto rat tail collagen coated Transwell chamber. Transendothelial electrical resistance (TEER) was detected by an epithelial volt-Ωm (Millicell-RES, Millipore, USA) to evaluate cell monolayer integrity. Monolayers with TEER over 200 Ω · cm² were used for further experiments. Sucrose
permeability coefficient was detected to evaluate the permeability of the BBB [27].

The culture medium in each apical chamber was replaced by 50 μM FAM-loaded liposomes of different formulations in DMEM with 10% FBS, respectively. After 0.5, 1, 2, and 4 h incubation at 37 °C, fluorescence intensity of the solutions collected from lower compartment was detected by a fluorescence spectrophotometer. At the end, the TEER was measured again to verify the integrity of the BBB model.

2.7. Evaluation of BBTB targeting ability on HUVECs/U87 co-culture model

HUVECs/U87 co-culture model was established according to the previous report [28]. In brief, HUVECs were plated in the inserts of Transwell and U87 cells were seeded into the chamber at a 1:5 HUVECs: U87 ratio. Three days later, the culture medium in each apical chamber was replaced by 50 μM FAM-loaded liposomes of different formulations in DMEM with 10% FBS. Fluorescence intensity of the solutions collected from lower compartment after different incubation periods was detected by a fluorescence spectrophotometer.

2.8. Evaluation of targeting ability on BBB/U87 tumor spheroids co-culture model and lysosome colocalization

U87 cells were seeded onto 48-well plates coated by agarose at a density of $2 \times 10^3$–400 μL per well to culture tumor spheroids. Ten days later, the tumor spheroids were transferred to the Transwell basolateral chamber of BBB model. In each apical chamber, the culture medium was replaced by 50 μM FAM-loaded liposomes of different formulations in DMEM with 10% FBS or those which were pre-incubated with 50% rat plasma. The inserts were removed after 4 h incubation. Transwell membranes were treated with LysoTracker® Red DND-99, and then carefully detached and imaged by a confocal laser microscope. After another 4 h incubation, the tumor spheroids were gently rinsed with pre-warmed PBS for three times and fixed by 4% paraformaldehyde for 30 min. Fixed tumor spheroids were observed by confocal laser microscopy.

2.9. Cytotoxicity assay

The in vitro cytotoxicity of doxorubicin liposomes was determined using MTT assay [29]. U87 cells were seeded into 96-well culture plates at a density of 3 × 10^3 cells/200 μL per well. After 24 h cultivation at 37 °C, the cells were treated with different concentrations of doxorubicin loaded liposomes of different formulations. 4 h later, cells were washed by warmed PBS and the medium was replaced by fresh DMEM containing 10% FBS. The cytotoxicity was determined at 72 h by MTT assay.

2.10. In vivo glioma distribution

To investigate brain tumor targeting efficiency in vivo, different formulations of DiR loaded liposomes were prepared. The intracranial U87 tumor xenograft orthotrophic glioma model was established as described previously [9]. At various stage of the glioma (8 days, 15 days post implantation), 100 μL DiR (0.25 mg/kg) loaded liposomes (lipids ~6 mM) of different formulations were administrated to the intracranial glioma bearing mice systematically. 12 h later, the mice were sacrificed and brains were collected to be imaged using Maestro version 2.10.1.

And also, the intracranial glioma bearing mice were injected with 5-FAM (0.4 mg/kg) loaded liposomes (lipids ~6 mM) after 12 days post-implantation. Four hours later, the mice were anesthetized and brains were collected and frozen in Tissue-Tek® O.C.T. compound after heart perfusion with saline and 4% paraformaldehyde. Frozen sections of 10 μm thickness were prepared and stained with 5 ng/mL DAPI for 10 min at room temperature. The sections were examined under the fluorescence microscope.

2.11. Study of in vivo antitumor effect

BALB/c nude mice bearing orthotopic glioma model was established as above. The mice were randomly divided into six groups (n = 8) and were administered with saline, free DOX, LS/DOX, DCDX-LS/DOX, c(RGDyK)-LS/DOX and DCDX/c(RGDyK)-LS/DOX via tail vein at a dose of 2 mg/kg doxorubicin at 6, 9, 12, and 15 days after implantation, respectively. Survival time was recorded every day and Kaplan–Meier survival curves were plotted for each group.

3. Results

3.1. Characterization of liposomes and targeting moieties

3.1.1. Characterization of liposomes

Liposomes loaded with DOX with or without modification were of similar vesicle size and polydispersity index, as well as encapsulation efficiency. The average size of LS/DOX, LS/DOX/c(RGDyK)-LS/DOX and DCDX/c(RGDyK)-LS/DOX were 90.6 ± 3.4, 92.4 ± 5.5, 91.9 ± 7.1, and 93.9 ± 6.7 nm, respectively. The encapsulation efficiency was 95.5 ± 2.0%, 94.8 ± 1.6%, 94.4 ± 1.8%, and 95.1 ± 1.7%, indicating that incorporation of DCDX-PEG3400-DSPE and c(RGDyK)-PEG400-DSPE into liposomes did not affect the physical properties of liposomes significantly.

![Fig. 3. Transcytosis efficiency of FAM-loaded LS, DCDX-LS, c(RGDyK)-LS and DCDX/c(RGDyK)-LS in vitro BBB model (A) and BBTB model (B). 50 μM FAM-loaded liposomes of different formulations in DMEM with 10% FBS were added into the inserts. Fluorescence intensity of the solutions collected from lower compartment after different incubation periods was detected by fluorescence spectrophotometer. Mean ± SD, n = 3, ***p < 0.001.](image-url)
3.1.2. Peptide stability

To assess peptide stability, we studied the proteolysis of peptides in 50% rat plasma and liver lysosomal homogenate. Both DCDX and c(RGDyK) exhibited no perceptible degradation. The stability of L-peptide was investigated as control. The retro-inverso isomer of DCDX, termed LCDX, displayed very fast degradation under both conditions (Fig. 1). These results demonstrated the superiority of D-peptide and cyclic peptide with respect to proteolytic stability.

3.2. Targeting ability in vitro and in vivo

3.2.1. In vitro cellular uptake by brain capillary endothelial cells and U87 cells

Cellular uptake of liposomes by primary brain capillary endothelial cells was studied with confocal microscope and flow cytometer. As shown in Fig. 2, the uptake of DCDX decorated liposomes and the liposomes modified with both targeting moieties was significantly greater than that of unmodified liposomes and liposomes modified with c(RGDyK), suggesting that the presence of DCDX effectively increased liposomes uptake by primary brain capillary endothelial cells. The percentage of fluorescein positive cells after treatments with DCDX-LS/FAM and DCDX/c(RGDyK)-LS/FAM was respective 76.8% and 75.7%, in marked comparison to 0.5% of LS/FAM and 2.9% of c(RGDyK)-LS/FAM. These results indicated the presence of DCDX on the surface of liposomes, and the cyclic peptide c(RGDyK) executed no obvious influence on the brain targeting ability of liposomes.

Cellular uptake of liposomes by U87 cells was performed in the similar way to verify the existence of c(RGDyK) on liposomal surface and its glioma targeting ability. From the fluorescent images, significant uptake was found of liposomes modified with c(RGDyK) and those modified...
with both ligands. Nearly no fluorescence was observed from the cells treated with unmodified liposomes or liposomes modified with \(^{10}\)CDX. The percentage of fluorescent positive cells after treated with c(RGDyK)-LS/FAM and \(^{10}\)CDX/c(RGDyK)-LS/FAM was 99.5% and 99.6%, whereas that of LS/FAM and \(^{10}\)CDX-LS/FAM was only 17.3% and 15.2%, indicating that the presence of \(^{10}\)CDX did not exert obvious impact on the glioma targeting ability of c(RGDyK).

3.2.2. Evaluation of brain targeting ability on BBB model

The BBB model was utilized here to evaluate the brain targeting ability of the liposomes in vitro. The cell monolayer with qualified transendothelial electrical resistance (TEER over 200 $\Omega \cdot cm^2$) was characterized for further experiments. The permeability of \(^{14}\)C radiolabeled sucrose was studied to monitor the integrity of the in vitro BBB model and permeability coefficient ($P_{e}$) was calculated according to the previous report [27]. It turned out that, the $P_{e}$ value of sucrose was determined as $0.16 \times 10^{-3}$ cm/min, validating well performance of the established in vitro BBB model. As shown in Fig. 3A, \(^{10}\)CDX modification significantly boosted the transcytosis of liposomes across the BBB. After 4 h, 2.38 $\pm$ 0.07% of \(^{10}\)CDX modified liposomes and 2.28 $\pm$ 0.14% of the liposomes modified with both targeting moiety traversed the BBB, which was significantly greater than that of unmodified liposomes (0.23 $\pm$ 0.02%) and liposomes modified with c(RGDyK) (0.29 $\pm$ 0.04%).

3.2.3. Evaluation of BBTB targeting ability on HUVECs/U87 co-culture model

When co-cultured with U87 cells, HUVECs exhibited higher proliferative rate ($p = 0.045$) in three days. It was reported that stimulation by tumor cells endowed endothelial cells with some characteristics of angiogenesis [28]. Here HUVECs/U87 co-culture model was established as a BBB model to assess the BBTB transcytosis efficiency of the liposomes. As shown in Fig. 3B, c(RGDyK) decoration significantly boosted liposomal transcytosis across the BBTB. After 4 h, 5.44 $\pm$ 0.01% of c(RGDyK) modified liposomes and 5.37 $\pm$ 0.04% of the liposomes modified with both targeting moiety traversed the BBTB, which was significantly greater than that of unmodified liposomes (2.71 $\pm$ 0.08%) and liposomes modified with \(^{10}\)CDX (2.89 $\pm$ 0.05%).

3.2.4. Evaluation of targeting ability on BBB/U87 tumor spheroids co-culture model and lysosomal colocalization

In order to better mimic the situation of brain tumor at the early stage and around the infiltrative tumor edge during all stages, a BBB/U87 tumor spheroids co-culture model was established to assess the targeting ability of liposomes modified with \(^{10}\)CDX and c(RGDyK) in vitro. The model was treated with FAM loaded LS, \(^{10}\)CDX-LS, c(RGDyK)-LS, \(^{10}\)CDX/c(RGDyK)-LS and \(^{10}\)CDX/c(RGDyK)-LS in the apical chamber, respectively. Only the tumor spheroid in the Transwell treated with liposomes modified with both peptide ligands exhibited obvious uptake (Fig. 4). As shown in Fig. 5, the intracellular distribution of the endocytosed \(^{10}\)CDX/c(RGDyK)-LS and \(^{10}\)CDX/c(RGDyK)-LS in the in vitro BBB monolayer demonstrated the similar pathway to that of \(^{10}\)CDX-LS as our previous report [14]. Since most of the liposomes colocalized with lysosome, the stability of glioma targeting moiety during the BBB transcytosis process became particularly important. The fluorescent intensity of tumor spheroid treated with \(^{10}\)CDX/c(RGDyK)-LS was higher than that of \(^{10}\)CDX/c(RGDyK)-LS due to the higher binding affinity of \(^{10}\)CDX to nAChRs and higher stability to the BBB enzymatic barrier. Laser scanning confocal images of tumor spheroids showed that the liposomes penetrated into the tumor spheroid to a certain extent (Fig. 4B). After incubation with 50% rat plasma, tumor spheroid cellular uptake of pre-incubated \(^{10}\)CDX/c(RGDyK)-LS exhibited no perceptible difference with that of non-treated group, whereas pre-incubation with rat plasma significantly impaired cellular uptake of \(^{10}\)CDX/c(RGDyK)-LS due to its proteolytic degradation (Fig. 4A), emphasizing the importance of the stability of targeting ligand.

3.2.5. In vivo glioma distribution

During the development of gliomas, various barriers emerge as obstacles for nanocarriers to reach the tumor site at different tumor stages. At the early stage, blood–brain barrier almost remains intact and prevents the entry of nanocarriers into the tumor. During the progression of glioma, the BBTB emerges as the main obstacle for nanocarriers, while the BBB also exists around the infiltrative tumor edge. Here we studied the biodistribution of various liposomes containing near infrared dye DiR in nude mice bearing intracranial glioma 8 and 15 days

*Fig. 5. Colocalization of endocytosed liposome and lysosome in the in vitro BBB monolayer. FAM loaded liposomes was incubated with in vitro BBB monolayer at 37 °C for 4 h, followed by lysosome staining. After fixation with formaldehyde, cells were subject to DAPI staining. Transwell membrane was detached and imaged by a confocal laser scanning microscope. Scale bar = 10 μm.*
after tumor implantation. All mice were sacrificed and the organs were dissected for imaging 12 h post-injection. As shown in Fig. 6, at various stages unmodified liposomes rarely distributed in the brain, while \(^{125}\text{CDX-LS}\) distributed in the whole brain without selectivity due to its BBB targeting ability. In contrast, \(c\text{(RGDyK)}\text{-LS}\) slightly accumulated in the brain tumor. It was apparent that significant fluorescence accumulated in the tumor region after the treatment of liposomes modified with both \(^{125}\text{CDX}\) and \(c\text{(RGDyK)}\), suggesting effective and precise targeting for glioma. \(^{125}\text{CDX}/c\text{(RGDyK)}\text{-LS}\) could traverse the BBB and BBTB by receptor-mediated transcytosis and target tumor cells through the interaction between \(c\text{(RGDyK)}\) and integrin. Multiple barriers targeting ability endowed the liposome with glioma targeting efficacy at all glioma stages.

In order to investigate more precise distribution of the liposomes in tumor region, the brain glioma slides were examined with a fluorescent microscope (Fig. 6B). Nearly no fluorescence was observed in the normal brain or the glioma of the mice treated with LS. In the \(^{125}\text{CDX-LS}\) and \(c\text{(RGDyK)}\text{-LS}\) groups, weak fluorescence appeared on the edge of the tumor. However, significantly greater distribution of liposomes in the glioma was observed when treated with liposomes modified with both \(^{125}\text{CDX}\) and \(c\text{(RGDyK)}\) and most of fluorescence was near the nuclei, indicating intracellular delivery of liposomes. The results of microscopic observation were consistent with that of ex vivo imaging, underlying the glioma targeting ability of liposomes modified with both proteolytically stable ligands.

3.3. Anti-tumor effect in vitro and in vivo

3.3.1. Cytotoxicity assay

In vitro cytotoxicity of free DOX and DOX loaded liposomes of different formulations was evaluated with U87 cells by the MTT assay (Fig. 7). After treatment with liposomes for 4 h, the culture medium was exchanged with fresh DMEM containing 10% FBS. Inhibition of cell growth was studied after 72 h. Free doxorubicin exhibited the strongest anti-proliferative effect due to quick cellular uptake and accumulation of small molecule chemotherapeutics during 4 h treatments, registering an IC\textsubscript{50} value of 0.5 \(\mu\text{M}\). Less cellular uptake of PEGylated liposomes led to higher IC\textsubscript{50} value than that of free DOX. The IC\textsubscript{50} values for unmodified liposomes and \(^{125}\text{CDX}\) modified liposomes towards U87 cells were 83.1 \(\mu\text{M}\) and 75.8 \(\mu\text{M}\), respectively, while the IC\textsubscript{50} values for \(c\text{(RGDyK)}\text{-LS}\) and \(^{125}\text{CDX}/c\text{(RGDyK)}\text{-LS}\) were 13.2 \(\mu\text{M}\) and 12.0 \(\mu\text{M}\). Higher cytotoxicity from \(c\text{(RGDyK)}\text{-LS}\) and \(^{125}\text{CDX}/c\text{(RGDyK)}\text{-LS}\) may be related to increasing cellular uptake. The similar IC\textsubscript{50} values of \(c\text{(RGDyK)}\text{-LS}\) and \(^{125}\text{CDX}/c\text{(RGDyK)}\text{-LS}\) further verified that the presence

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**Fig. 6.** (A) The ex vivo imaging of liposomes encapsulating DiR in nude mice bearing intracranial glioma 8 days and 15 days post-implantation. (B) The distribution of FAM-loaded liposomes in the brain with intracranial glioma 12 days post-implantation. Frozen sections were examined under fluorescence microscope. Nuclei were stained with DAPI (blue), while green represented the liposomes. The yellow lines were the margins of the glioma and the arrows were pointing to the glioma (scale bar = 100 \(\mu\text{m}\)).
of DCDX on the multi-targeting liposomes did not exert any impact on the glioma targeting ability of c(RGDyK).

3.3.2. In Vivo antitumor effect

Fig. 8 showed the survival of nude mice bearing intracranial U87 glioma treated with various liposomal formulations. In the absence of any targeting moiety, treatments with free or liposome-formulated doxorubicin at a dose of 2 mg per kg body weight (at 6, 9, 12, and 15 days post-tumor implantation) did little in improving mouse survival, registering a median survival of 28 days (p = 0.015) and 29 days (p = 0.006) versus 26 days for the saline-treated group. By contrast, the median survival of the groups treated with 0CDX-LS (32.5 days, p < 0.005) and c(RGDyK)-LS (30.5 days, p < 0.005) containing DOX was much longer than that of the saline group. In particular, the median survival of the mice administrated 0CDX/c(RGDyK)-LS containing DOX was much longer than that of c(RGDyK)-LS/DOX (p < 0.005) and 0CDX-LS/DOX(p < 0.05), registering a survival of 36.5 days. These results indicated that liposomes modified with both 0CDX and c(RGDyK) exhibited a significant improvement in anti-tumor activities than liposomes functionalized with a single ligand.

4. Discussion and conclusion

Although plenty of actively targeted drug delivery systems for glioma have been reported, an important issue with regard to the stability of targeting moiety was always neglected. Surface-modification of nanoparticles with PEG has been developed as a powerful strategy to prolong blood circulation time to reach their therapeutic targets [19]. During the long circulation process, the stability of targeting moiety encounters enormous challenges. It was found that peptides consisting of L-amino acids were susceptible to proteolytic degradation in plasma and the BBB enzymatic barrier, readily losing their brain targeting and tumor-homing capability [30,31]. The methods often adopted to increase peptide stability include cyclization, partial D-amino acid substitution and retro-inverso isomerization [32]. DCDX was designed by retro-inverso isomerization and exerted high binding affinity to nAChRs expressed on the BBB. High stability of c(RGDyK) was accomplished due to its short peptide sequence, cyclization and partial D-amino acid substitution. After incubation with 50% rat plasma, the targeting ability of pre-incubated liposomes modified with DCDX and c(RGDyK) was not affected by its short peptide sequence, cyclization and partial D-amino acid substitution. With the deterioration of brain glioma, angiogenesis and gradual impairment of BBB, BBTB becomes the main obstacle for nanocarriers. c(RGDyK) on the surface of liposomes plays a critical role due to its ability of BBTB transcytosis and tumor cells targeting [33-35]. Even though the BBB is compromised under the situation of malignant gliomas, glioma cells around the tumor edge utilize the available brain vasculature for nutrition and the BBB presents the main obstacle to glioma targeted transport of chemotherapeutic agents in this area. Liposomes modified with both DCDX and c(RGDyK) possess the similar brain targeting ability as DCDX-LS and glioma targeting ability as c(RGDyK)-LS. Two targeting moieties functioned individually and no reciprocal interference was observed. Liposomes modified with DCDX and c(RGDyK) could traverse the in vitro BBB monolayer and penetration into tumor spheroids, demonstrating significantly higher efficiency than that of liposomes modified with a single ligand. In vivo biodistribution further demonstrated that DCDX/c(RGDyK)-LS resulted in a precise and high glioma retention. However, most of the liposomes were only delivered into the periphery of tumor. It is hard to penetrate deeply into tumor tissue due to the high interstitial pressure in tumors and dense tumor extracellular matrix [36], thus offering modest survival benefits. It is possible that other strategies would further enhance anti-glioma effects by incorporating tumor penetrating peptide and reducing particle size to improve penetration [37].

In actual research practice, two or several targeting moieties modified delivery systems have been employed to achieve better therapeutic effects. However, the enzymatic barriers have been rarely taken into consideration. The liposomal formulation suggested in the present study could overcome multi-barriers and achieve glioma targeting effectively at different developing stages of glioma, validating its potential value in improving therapeutic efficacy of doxorubicin for glioma.

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