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PII: S0168-3659(16)30011-6
DOI: doi: 10.1016/j.jconrel.2016.01.015
Reference: COREL 8071

To appear in: Journal of Controlled Release

Received date: 26 June 2015
Revised date: 10 December 2015
Accepted date: 8 January 2016

Please cite this article as: Ying-Zheng Zhao, Qian Lin, Ho Lun Wong, Xiao-Tong Shen, Wei Yang, He-Lin Xu, Kai-Li Mao, Fu-Rong Tian, Jing-Jing Yang, Jie Xu, Jian Xiao, Cui-Tao Lu, Glioma-targeted therapy using Cilengitide nanoparticles combined with UTMD enhanced delivery, Journal of Controlled Release (2016), doi: 10.1016/j.jconrel.2016.01.015

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Glioma-targeted therapy using Cilengitide nanoparticles combined with UTMD enhanced delivery

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Running Title: Cilengitide multi-stage targeting therapy for gliomas

Acknowledgments
This research was supported by National Natural Science Foundation of China (Grant No. 81360195, 81301982, 81571392, 81272160 and 81302726), Key support of high level talent innovation and technology project of Wenzhou (Zhao Ying-Zheng, 2015), Zhejiang Provincial Foundation for Health Department (Grant No. 2015ZDA023 and 2016139678), Medicine Grant from Wenzhou Bureau of Science and Technology (Grant No. Y2014730).

Declaration of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.
ABSTRACT

Malignant gliomas especially glioblastoma (GBM) are poorly responsive to the current treatments. Cilengitide (CGT) is a cyclic pentapeptide that demonstrated efficacy for GBM treatment by targeting the integrins αvβ3 and αvβ5 over-expressed on GBM cells. However, clinical translation of this therapy has been limited by issues including fast blood clearance, high kidney and liver uptake, poor blood-brain barrier (BBB) penetration, low tumor specificity and rapid washout from tumors. In this study, these issues were tackled in an integrated manner using a multi-stage strategy combining ultrasound-targeted microbubble destruction (UTMD) with CGT nanotherapy. CGT nanoparticles (CGT-NP) prepared using gelatin and Poloxamer 188-grafted heparin copolymer demonstrated significant apoptotic and cytotoxic effects in C6 GBM cells. Biodistribution study in a rat GBM model demonstrated buildup of high CGT level in tumors subjected to CGT-NP+UTMD combined therapy. The tumor CGT level in these animals was increased over 3-fold, tumor retention of CGT prolonged and renal clearance significantly reduced when compared with free CGT with or without UTMD. CGT-NP+UTMD treatment was further shown to extend the median survival period from less than 20 days in the control and about 30 days in free CGT group to about 80 days. This was achieved with low CGT dosing level (2 mg/kg twice weekly). In situ monitoring of GFAP, Ki67, caspase-3, Beclin-1, and LC-3 in the tumor samples together with TUNEL assay, transmission electron microscope imaging and Western blot assay all demonstrated high apoptotic and autophagy activities induced by the combined therapy. In conclusion, this study has provided extensive preclinical data supporting the use of this combined therapy to overcome the limitations of standard CGT treatment of gliomas.

Key words: Glioma; Cilengitide; nanoparticles; ultrasound-targeted microbubble destruction; BBB opening technique

Abbreviations list:
CNS – central nervous system
BBB – blood-brain barrier
EPR – enhanced permeability and retention
CGT – Cilengitide
UTMD – ultrasound-targeted microbubble destruction
TEM – transmission electron microscope
GBM – glioblastoma
RGD – arginine-glycine-aspartic acid
US – ultrasound
MBs – microbubbles
NPs – nanoparticles
PMB – phospholipid-based microbubbles
1. INTRODUCTION

Gliomas are tumors that originate from the glial cells in the central nervous system (CNS). A malignant glioma is considered as the most pernicious form of cancers [1]. This malignancy can be classified by cell type into astrocytoma, glioblastoma, oligodendroglioma, ependymoma, and mixed glioma, or into four histopathologic grades according to the malignant level [2], in which glioblastoma (GBM) is classified as the most tough-to-treat grade IV glioma [3]. The most unique feature of GBM is its aggressiveness. Although there is a low tendency for malignant glioma to spread away from the CNS, glioma cells often aggressively invade and penetrate into the surrounding brain parenchyma and may even cross the midline to the heterolateral brain [4]. Another distinct nature of these tumors is their angiogenesis and vasculogenesis [5]. Therefore, it is extremely difficult for surgical resection to completely eliminate malignant glioma even when combined with radiotherapy and chemotherapy [6].

Cyclo(L-arginylglycyl-L-aspartyl-D-phenylalanyl-N-methyl-L-valyl) (Cilengitide or CGT) is a cyclic arginine-glycine-aspartic acid (RGD) pentapeptide antagonist of the integrins αvβ3 and αvβ5, two transmembrane receptors over-expressed on GBM and tumor-invaded endothelial cells [7]. CGT has been widely researched in preclinical and clinical studies including a randomized Phase III study. It has the ability to actively target the cells in which αvβ3/αvβ5 are over-expressing, to deprive their signals for survival and proliferation, and consequently inhibit angiogenesis and tumor growth [8,9,10].

Given its promising anticancer activities, clinical translation of CGT therapy has been limited by several delivery-related issues. First of all, the fast clearance from blood, high kidney and liver uptake, and rapid elimination from tumors make this receptor-targeting therapeutic agent hard to achieve therapeutic level in the glioma [11]. Moreover, although the αvβ3 integrin is present and over-expressed in glioblastoma, targeting of this molecule is complex because of the dose dependent opposing effects of CGT [12]. This lack of cancer specificity may lead to unwanted systemic side-effects. Most importantly, the blood-brain barrier (BBB) provides a critical barrier to drug therapy by preventing all large molecules and more than 98% of small molecule drugs from entering the brain parenchyma [13]. As a general rule, only lipid soluble (lipophilic) molecules with a molecular mass of less than 400 Da can cross from blood to brain [14]. The CNS penetration of CGT, a molecule with MW over 500 Da, is thus severely limited. It is noteworthy that the BBB might be leaky in the malignant brain tumor center, the well-vascularized actively proliferating tumor edge and the normal brain tissue adjacent to the tumor still have complex barrier integrity [15]. Currently, twice weekly intravenous administration without interruption for long term is needed for CGT to have some anticancer effects [16]. A more efficient way to deliver this drug is clearly warranted.

Nanotechnology may help overcome the aforementioned limitations. It may enhance CGT delivery to the cancer sites by the enhanced permeability and retention (EPR) effect [17]. In addition, intravenously injected nanoparticles have been shown to improve drug penetration into the CNS by increasing the BBB permeability [18-20]. Encapsulation of CGT in a well-designed nanocarrier can also avoid exposure of the off-target tissues to the drug, delay the drug clearance, and allow sustained or triggered release. This is particularly significant for CGT therapy as this can reduce its non-specific effects on the healthy cells, extend its circulation time and achieve more favorable pharmacokinetics [21], so safer and more effective cancer treatment becomes feasible.

Although nanocarriers alone are likely to enhance CGT delivery across the BBB, the enhancement is expected to be limited considering the high resistance of this barrier. The technique that combines low-intensity ultrasound (US) with microbubbles (MBs), often referred as
ultrasound-targeted microbubble destruction (UTMD), has been shown capable of improving the efficiency of nanoparticle-based drug delivery across biological barriers without causing cellular damages [22-24]. Studies have confirmed that this technique can lead to non-invasive and reversible BBB disruption [25]. In addition, with the recent advance of US technology, it becomes feasible to focus the US beam on the tumor, so the US only triggers cavitation of MBs (oscillation and collapse) to facilitate drug delivery specifically to the brain tumor site [26].

This study objectives at developing a multi-stage targeting strategy (see Fig. 1), in which the UTMD technology helps focus and enhance the delivery of CGT loaded nanoparticles (CGT-NP) across the BBB near the tumor site, the nanoparticles facilitate the localization and accumulation of CGT in the brain tumor by EPR effect, and a high and persistent local level of CGT in the tumor will be established to effectively target the overexpressing integrins on the GBM cell surface with minimal interference of the non-cancer tissues. CGT-NP was prepared with Poloxamer 188-grafted heparin copolymer which has recently been tested for brain delivery of bFGF with no detectable toxicity [27]. In our previous study, only MBs or only ultrasound had little effects on cavitation that plays a key role in cellular penetration for exogenous drugs [28, 29]. Therefore, this study further investigated the glioma-targeted therapy using CGT nanoparticles combined with UTMB treatment. To be specific, three objectives were studied: (i) to evaluate the various physicochemical and in vitro biological characteristics of CGT-NP, (ii) to study the impact of the proposed multi-stage targeting strategy integrating UTMD and CGT-NP on the in vivo biodistribution of CGT in a rat model with glioma, and (iii) to extensively study the in vivo therapeutic effects of this strategy on this model. The findings have provided valuable preclinical data to validate a noninvasive, efficient targeted peptide-nanotherapy for treatment of GMB, one of the most untreatable and deadly malignant diseases.

Fig. 1 Schematic diagram of dual target under combination of CGT-NP and UTMD.
2. MATERIALS AND METHODS

2.1 Chemicals
Related details can be found in supplement.

2.2 Preparation of phospholipid-based microbubbles (PMB)
Phospholipid-based microbubbles (PMB) were prepared by sonication–lyophilization method. PMB concentration was about $2 \times 10^9$ bubbles/mL with an average diameter of 3.4 μm. Related details can be found in supplement.

2.3 Preparation and characterization of CGT-loaded NP
Preparation and characteristics of CGT-NP, CGT-modified-NP and blank NP can be found in supplement.

2.4 Experiment in vitro

2.4.1 Cell culture
C6 rat glioma cells were used as the standard cell model of glioblastoma (GBM). Related details can be found in supplement.

2.4.2 UTMD system in vitro
To verify the anti-tumor efficacy of the CGT-NP and UTMD in vitro, the C6 glioma cells received different treatments. PMB (Mean diameter =3.4 μm) and CGT-containing solution (CGT-NP, CGT-modified-NP or CGT solution) were mixed for 20min in a sealed container and then added into cell plates. Fresh growth media containing 10% FBS was then added to a final volume of 400 μL /well. In each cell, the PMB concentration was about $1 \times 10^6$ microbubbles /mL and the CGT (CGT-NP, CGT-modified-NP or CGT solution) concentration was 10μg CGT/mL. Cultured C6 glioma cells on the bottom of the cell plates were exposed to ultrasound radiation in a device, as reported in our previous study [30]. The ultrasound transducer was inserted in a 37°C water tank and directly faced the bottom of the cell plate. A spongy rubber ultrasound shield was used to focus ultrasound on experimented cells. After the mixture containing PMB and CGT / CGT-NP were added into the well, the cell plate was rotated at approximately 30 rpm for 60 seconds and then immediately exposed to ultrasound treatment. Each sample received designed ultrasound exposure in the water bath. The cell plate was held 4 cm from the submerged transducer (Acuson Sequoia 512C system, Siemens). The microbubble destruction function (using MBD key) attached to the Acuson Sequoia 512C system was used to burst PMB (ultrasound frequency =14 MHz, exposure time = 10s, repeat three times with off intervals of 1s). After the UTMD treatment, the cell plates were incubated for about 4 hours and then the media were changed to fresh growth media.

2.4.3 Measurements of cell apoptosis
Related details can be found in supplement.

2.4.4 Cancer cytotoxicity test
Related details can be found in supplement.
2.4.5 Enhancement effects of UTMD on CGT-NP cellular uptake
Related details can be found in supplement.

2.5 C6 rat model construction and experiment design

2.5.1 Brain glioma tumor model
All animal experiments were performed under the approval and guidance of the Institutional Animal Care and Use Committee of Wenzhou Medical University. Male Sprague-Dawley rats (250-350 g) were purchased from Shanghai, China. Each experimental rat was anesthetized with intraperitoneal injection of chloral hydrate (350 mg/kg bodyweight) 5 min before the experiment and then immobilized on a stereotactic frame (KOPF900, USA). To implant C-6 glioma cells, a sagittal incision was made through the skin overlying the calvarium, and a small dental drill was used to create a hole (1.0 mm diameter) in the cranium 1.0 mm posterior to the bregma and 3 mm lateral to the sagittal suture (right hemisphere of the brain) without duramatal damage. A total of 1×10^6 cells/10 μL (suspended by DMEM) of C-6 glioma cell suspension was injected. The needle fell 6.0 mm followed with 1.0 mm rise perpendicular to the hole, so the net depth of the injection needle in the hole was 5.0 mm from the brain surface. The injection was performed over a 10-min period. The growth of rat brain tumor was monitored longitudinally by MRI.

2.5.2 Experiment design
Fig. 2 showed the experiment design of the glioma-targeted therapy using CGT-NP combined with UTMD enhanced delivery. To verify the treatment efficacy of the drug-loaded NPs and UTMD, ninety six C-6 tumor-implanted rats (C6 rats) were randomized into eight groups (n=12) as follows:
- Group 1 (Control): normal saline (0.9% NaCl).
- Group 2 (CGT solution alone): CGT solution.
- Group 3 (CGT-modified-NP alone): CGT-modified-NP only.
- Group 4 (CGT-NP alone): CGT–NP only.
- Group 5 (UTMD): PMB only.
- Group 6 (CGT + UTMD): CGT solution/PMB mixture.
- Group 7 (CGT-modified-NP + UTMD): CGT-modified NP/PBM mixture.
- Group 8 (CGT-NP + UTMD): CGT-NP/PMB mixture.
Fig. 2 Experiment design of the glioma-targeted therapy under CGT-NP combined with UTMD enhanced deliver in vivo

Treatment began from day 7 after tumor implantation and all treatments were intravenously administered. They were administered twice weekly (Tuesday and Friday) for a total of eight injections in 28 days. The drug treatments in groups (6) to (8) were administered prior to US treatment. CGT dose in CGT-containing groups (CGT-NP, CGT-modified-NP or CGT solution) was 2 mg/kg weight. PMB dose in PMB containing groups was $1 \times 10^6$ microbubbles/mL in final injection. For each group, the total volume of tail vein injection was 1 mL.

C6 rats were anesthetized with chloral hydrate (350 mg/kg bodyweight) 5 min before the experiment. Ultrasound transmission gel was coupling on the surface of rat brain corresponding to the glioma. Experimental solution was infused via tail vein through a 20-gauge cannula. Immediately after the intravenous injection, a linear array transducer (Acuson Sequoia 512C system, Siemens) was applied to generate the UTMD effect. All the basic parameters of ultrasound in vivo were
consistent to those in vitro. In the groups with UTMD, the linear array transducer was placed over the glioma tumor. The microbubble destruction function (using MBD key) attached to the Acuson Sequoia 512C system was used to burst PMB (ultrasound frequency =14 MHz, exposure time = 10s, repeat three times with off intervals of 1s to allow refill of the glioma with more PMB).

2.6 Evaluation of the effects of CGT treatment on glioblastoma function of C6 rats

2.6.1 Tumor volume monitoring by magnetic resonance imaging (MRI)

All MRI images were acquired on a 3T scanner (Trio with Tim, Siemens, Erlangen, Germany) using the standard wrist coil (Chenguang Medical Technologies Company, Shanghai, China) with an inner diameter of 13 cm. Tumor progression was assessed 6 days after tumor implantation and the tumor volume monitoring was performed once every week. Animals were anesthetized with chloral hydrate (350 mg/kg) 5 min before MRI procedure.

The tumor location was determined by performing a gradient echo FLASH sequence to acquire T1W images with the following imaging parameters: pulse repetition time (TR)/echo time (TE) =560/27 ms; NEX=2; matrix size=256×256; FOV= 56mm×70mm; slice thickness = 2.4 mm; slice spacing=2.9 mm.. Tumor size of animals in experiment group was quantified using turbo-spin-echo based T2-weighted images with the following parameters: pulse repetition time (TR)/echo time (TE) = 2300/110 ms; NEX=2; matrix size=256×256; FOV= 56mm×70mm; slice thickness = 2.4 mm; slice spacing=2.9 mm.

To achieve enhanced T1-weighted and enhanced T2-weighted images, gadopentetate dimeglumine (Gd-DTPA, 0.4 mmol/kg) MRI contrast agent (Magnevist, Berlex Laboratories, Wayne, NJ) was administered as intraperitoneal injection 10 min before scanning.

2.6.2 GFAP, Ki67, caspase-3, Beclin-1, LC-3 immunohistochemical staining

According to previous reports [31-35], the immunohistochemical staining of GFAP, Ki67, caspase-3, Beclin-1 and LC-3 were performed to investigate the inhibition on gliomas after CGT treatment.

The antibodies used were the following: a rabbit polyclonal antibody to GFAP (1:2000, Abcam, Cambridge, UK), a rabbit monoclonal antibody to Ki67 (1:50, Abcam, Cambridge, UK), and a rabbit polyclonal antibody to caspase-3 (1:500, Abcam, Cambridge, UK), a rabbit polyclonal antibody to Beclin 1 (1:200, Abcam, Cambridge, UK), a rabbit polyclonal antibody to LC3A/B (1:50, Abcam, Cambridge, UK).

Paraffin embedded tumors were cut into 4-μm-thick sections, deparaffinized in xylene series and hydrated in distilled water. Antigen retrieval was obtained by citrate buffer and washed with PBS. Samples were blocked with 3% H2O2 for 5 min, followed by treatment with primary antibodies for 2 h at 37 °C and HRP-secondary antibody for 30 min at 37 °C, and stained with DAB and counterstained with hematoxylin.

Morphology of stained cells was examined and recorded with an optical microscope (Nikon ECLIPSE Ti-S, Ruizezhongyi Company, Beijing, China). The number of stained cells was evaluated by manually counting four random microscopic fields (400X original magnification) per subject. About 10,000 cells per group were counted by an experienced technician who was “blinded” to the content of the samples as well as the results of the experiment.

2.6.3 Histology of brain tumors
Brain tumors were fixed in 10% buffered formalin, embedded in paraffin, and sectioned at 4 μm thickness. Sections were stained by H&E. Under an optical microscope (Nikon ECLIPSE Ti-S, Ruikeshongyi company, Beijing, China), the tumor histology was viewed and imaged.

2.6.4 Apoptosis assay (TUNEL)
DNA fragmentation in situ was analyzed using the modified terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Boster biological engineering Co., Wuhan, China) following the manufacturer’s instructions. Cell staining and morphology were recorded by an optical microscope (Nikon ECLIPSE Ti-S, Ruikeshongyi Company, Beijing, China). The number of apoptotic tumor cells was evaluated by counting stained and featured apoptotic cells in four random microscopic fields (400X original magnification) per subject.

2.6.5 Transmission electron microscope (TEM)
After animals’ sacrifice and perfusion, tumors were cut into samples of 1 mm³ volume, immersed in 2.5% glutaraldehyde for 1 min, washed in PBS, immobilized with 1% osmic acid for 2.5 h, washed again with PBS and progressively dehydrated by immersing in 30%, 50%, 70%, 80%, 90%, 100% acetone (30 min each). The dehydrated samples were permeated by embedding agent Epon 812, sliced into semi-thin and then ultra-thin sections, and stained by uranium and plumbum. Stained tumor sections were examined and imaged under transmission electron microscope (Hitachi H-600, Japan).

2.7 Western-Blot assessment
Animals were deeply anesthetized and perfused with saline via the left ventricle until colorless perfusion fluid appeared from the right atrium and the tissues of interest were obtained. A microvessel fraction was isolated from the tumor tissue by centrifugation in 15 mL with 18% (w/v) dextran solution at 10,000g and 4°C for 10 min. Protein homogenates of microvessels were prepared by rapid homogenization in 10 volumes of lysis buffer (2 mM EDTA, 10 mM EGTA, 0.4% NaF, 20 mM Tris-HCl protease inhibitors, pH 7.5). Samples were centrifuged (17,000g, 4°C) for 1 h and the protein concentration of the soluble material was determined by the Coomassie G250 Binding method. Equal amounts of protein (10–20 µg) were fractioned on 12% SDS–polyacrylamide gels, followed by transferring to nitrocellulose membranes (Santa Cruz Biotechnology). The membranes were blocked in blocking buffer (5% non-fat dairy milk dissolved in Tween-Tris-buffered saline) overnight at 4°C. The blots were then incubated with rabbit polyclonal GFAP (1:50000, Abcam, Cambridge, UK), a rabbit monoclonal antibody to Ki67 (1:200, Abcam, Cambridge, UK), and a rabbit polyclonal antibody to caspase-3 (1:500, Abcam, Cambridge, UK), a rabbit polyclonal antibody to Beclin 1 (1:200, Abcam, Cambridge, UK) and a rabbit polyclonal antibody to LC3A/B (1:100, Abcam, Cambridge, UK) respectively, and mouse polyclonal anti-β-actin antibody was applied as an inner control. Protein bands were scanned using ChemiImager 5500 V2.03 software, and the relative integrated density values (IDV) were calculated by computerized image analysis system (Fluor Chen 2.0) and normalized with that of β-actin.

2.8 Statistical analysis
The size of tumor was evaluated with image analysis software using MATLAB (Math-Works, Natick, MA, USA). Tumor volumes (V) were calculated using an ellipsoid approximation \[V \approx \frac{4}{3} \pi \times (0.5)^3 \times (abc)\], where a, b and c are the maximum diameters of the tumor measured in three
orthogonal planes on 2-D T2-weighted MR images. Least-squares nonlinear regression analyses were performed to compare the rate of tumor growth between groups. Population survival curves were also plotted using the Kaplan-Meier method (Kaplan and Meier 1958). Survival curves were compared between groups using the log-rank test. Statistical analyses were performed using either GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA) or Excel 2002 (Microsoft Corporation, Redmond, WA, USA).

All results are expressed as mean ± SD for each group. A Student’s t-test was performed to determine the significant difference between two groups. One-way analysis of variance (ANOVA) was utilized to determine the significant difference between multiple groups. \( P<0.05 \) was considered statistically significant.

3. RESULTS

3.1 Characterization of CGT loaded NP
Related details can be found in supplement.

3.2 Apoptosis of C6 glioma cells
Quantitative analysis of apoptotic cells (Fig. 3 A and B) using flow cytometry indicated that cells of CGT-NP+UTMD group reached the highest apoptosis level of 72.98±6.00%. In comparison, the UTMD group, CGT+UTMD group and CGT-modified-NP+UTMD group resulted in 4.34±3.37%, 10.10±5.83% and 25.75±5.54% cell apoptosis ratios, respectively, all of them several fold lower than CGT-NP+UTMD group. Without UTMD, the apoptosis ratios were generally lower; the cell apoptosis ratios for the Control, CGT solution alone, CGT-modified-NP and CGT-NP alone groups were 4.03±2.11%, 7.90±4.24%, 13.01±3.16% and 35.20±3.03%, respectively. This result showed that among the three forms of CGT treatments (CGT, CGT-modified-NP and CGT-NP), CGT-NP achieved the strongest apoptotic effect, and the combination with UTMD could further enhance this effect especially with CGT-NP treatment (+UTMD vs no UTMD: 72.98% vs 35.20%, ~2-fold improvement).
Fig. 3 Quantitative analysis of apoptotic cells (A & B) and cytotoxicity on C6 cells (C). Cell apoptosis was measured by flow cytometry. Cell viability was measured by MTT assay. (* p<0.05 vs control; **p<0.01 vs control; #p<0.05 vs CGT group; ##p<0.01 vs CGT group)

3.3 Cytotoxicity of C6 glioma cells
As showed in Fig. 3C, CGT-NP+UTMD combined treatment achieved the strongest anticancer cytotoxic effect (i.e. lowest cell viability measured by MTT assay) among all groups, with
CGT-modified-NP+UTMD group being the second most effective. In comparison, the decreases in cell viability in Control+UTMD and CGT (with or without UTMD) groups were not significant compared with the Control. Overall, the data suggest that encapsulating CGT in NP could effectively increase its anticancer cytotoxic effect, which could be further enhanced when combined with UTMD technique, and UTMD alone did not cause significant reduction in cell viability.

### 3.4 Enhancement effects of UTMD on CGT-NP cellular uptake

Green fluorescence was used to evaluate the cellular uptake of CGT by C6 cells. There was no fluorescent signal detected in Control group and UTMD group, ruling out the presence of strong auto fluorescence from the microbubbles and tested cells. As shown in Fig. 4A, only weak CGT signals were noted in the cells treated with CGT-NP group. It confirmed the limited cell permeability of CGT-NP without UTMD. In comparison, under drug concentration of 5μg/mL, 10μg/mL and 25μg/mL, the fluorescent signal of detected in the CGT-NP+UTMD group were stronger than CGT-NP group. In the CGT-NP+UTMD group, most of the fluorescence signals appeared in the cell interior rather than the cell surface, which proved the high efficiency of CGT-NP+UTMD technique for intracellular transportation. Flow cytometry quantitative analysis confirmed the results of fluorescence imaging results. As shown in Fig. 4B, the fluorescent signals in CGT-NP+UTMD group at 10μg/mL CGT concentration was significantly stronger than those in Control group (p < 0.01) and CGT-NP group (p < 0.01). The fluorescent signal of CGT-NP+UTMD group at 25μg/mL CGT concentration was similar to that of CGT-NP+UTMD group at 10μg/mL CGT concentration. C6 cells treated with only CGT-NP produced a little shifts (right-shift indicating increased fluorescence from cells), compared with Control group. Meanwhile, strong shifts were observed in the cells treated with CGT-NP+UTMD. All these results showed that UTMD treatment could further significantly increase the cellular uptake of CGT-NP.
Fig. 4 Green fluorescence (A) and flow cytometry quantitative analysis (B). The fluorescent signal (right-shift) indicates increased fluorescence from cells. (* \( p < 0.05 \) vs control; ** \( p < 0.01 \) vs control; \( # p < 0.05 \) vs CGT-NP group; \( ## p < 0.01 \) vs CGT-NP group)
3.5 Antitumor efficacy and animal survival in vivo

The final goal of this study was to investigate whether CGT-NP combined with UTMD could further improve the efficiency of drug delivery and CGT-mediated brain tumor growth inhibition. Tumor progression was longitudinally monitored by enhanced T2-weighted MRI (Fig. 5A and B) and animal survival was also monitored (Fig. 5C). Tumors in untreated control rats grew rapidly from 10.35± 6.67 mm$^3$ on day 7 to 672.75± 86.33 mm$^3$ on day 28 (relative tumor volume from 1 on day 7 to 63.71±6.44 fold on day 28, $p<0.001$). Meanwhile, tumors in treated rats in groups UTMD, CGT, CGT+UTMD, CGT-modified-NP, CGT-modified-NP+UTMD showed relative tumor volume 60.39±4.43, 50.46±3.96, 53.39±5.41, 45.73±3.64, 43.21±3.86, respectively (day 7 vs day 28: all $p<0.001$). In comparison, the tumor growth in CGT-NP and CGT-NP+UTMD groups were much slower (CGT-NP+UTMD vs. CGT-NP; $p<0.05$). For CGT-NP only, the tumor grew from 11.27±5.38 mm$^3$ on day 7 to 225.40±49.36 mm$^3$ on day 28, translating into a 21.38±2.45-fold tumor increase (day 7 vs day 28: $p<0.05$). The anticancer effect was even more obvious for CGT-NP+UTMD; the tumor grew from 11.35±4.97 mm$^3$ on day 7 to 11.12±3.22 mm$^3$ on day 28, translating into a mere 0.98±0.90-fold tumor change. In other words, CGT-NP+UTMD treatment has led to complete suppression of the brain tumor growth.

Survival (Fig. 5C) in CGT-NP+UTMD group (81.2±1.7 days) was significantly prolonged compared to the control group (16.5±2.1 days, $p<0.001$) and CGT group (30.4±2.9 days, $p<0.01$). The median survival days in CGT-modified-NP group (35.0±1.8 days) and CGT-modified-NP+UTMD group (41.1±2.0 days) were both longer than control group, $p<0.05$. It should be noted that in this study, “survival” is defined as “median days of survival”, there were still a few live rats in control group on Day 28; however, other groups have more.
Fig. 5 Tumor progression monitored by enhanced T2-weighted MRI (A & B) and glioma animal survival were analyzed by the median survival days (C). CGT-NP+UTMD treatment completely suppressed the growth of brain tumor and significantly prolonged the animal survival. (#p<0.01 vs CGT-NP; ***P<0.001 vs other groups)

3.6 GFAP, Ki67, caspase-3, Beclin-1 and LC-3 in situ immunohistochemical staining

Immunohistochemistry with GFAP (Glial Fibrillary Acidic Protein), Ki67, caspase-3, Beclin-1 and LC-3 assay were performed using paraffin-embedded tumor tissues (Fig. 6). GFAP was used to evaluate the malignancy of tumor, Ki-67 to indicate the proliferation of tumor cells, and caspase-3 to evaluate apoptosis of tumor cells. Otherwise, Beclin-1, LC-3 were used to evaluate autophagy of tumor cells.

Evidence confirming the expression of GFAP, Ki67, caspase-3, Beclin-1 and LC-3 proteins in tumor tissue were provided by in situ immunohistochemistry assay. The brown stains indicated GFAP, Ki67, caspase-3, Beclin-1, and LC-3 proteins expressed in tumor tissues, and the cell nuclei were stained blue as a contrast.

The expression of GFAP (Fig. 6A) in the CGT-NP+UTMD group was 610.5±17.8% of control
group ($p<0.01$). CGT-NP+UTMD group was significantly higher than CGT group (121.7±29.6% of control, $p<0.01$). CGT-NP+UTMD group showed the highest expression of GFAP. CGT-modified-NP group, CGT-modified-NP+UTMD group and CGT-NP group demonstrated 296.3±6.9%, 333.3±34.6%, 411.7±38.4% GFAP expression of the control, respectively (Fig. 6A, all $p<0.05$).

Fig. 6 Immunohistochemical assays of GFAP, Ki67, caspase-3, Beclin-1, LC-3 protein in tumor tissue. Brown staining indicated expression of GFAP, Ki67, caspase-3, Beclin-1, LC-3 protein and blue staining showed the nuclei. In histogram, control group was set as 100% for comparison. (*$p<0.05$ vs control; **$p<0.01$ vs control; *** $p<0.001$ vs control; # $p<0.05$ vs CGT group; ## $p<0.01$ vs CGT group)

The Ki-67 proliferation indices of CGT-NP group (25.0±5.8% of control, $p<0.01$) or CGT-NP+UTMD group (11.2±3.3% of control, $p<0.001$) were both significantly lower than that of
the other groups (Fig. 6B).

As shown in Fig. 6C, the proportion of apoptotic tumor cells in the CGT-NP+UTMD group was significantly higher than that in other groups ($p<0.05$), as indicated by the presence of active Caspase-3 (2.5-fold of control and 2.4-fold compared with CGT group, $p<0.05$) as well as in the TUNEL assay.

As demonstrated by Beclin-1 and LC-3 expression, all three indicators showed that CGT-NP+UTMD group achieved the highest level of tumor cell autophagy (Fig. 6D, 888.7±25.6% of control, $p<0.001$; Fig. 6E, 351.1±9.9% of control group, $p<0.01$).

### 3.7 HE staining and TUNEL assay

Immunohistochemistry with HE staining and TUNEL assay were performed using paraffin-embedded tumor tissues (Fig. 7). The histological changes of the tumors after treatments were compared using HE staining. The HE stained sections of tumor tissues from the control group, UDMD group, CGT group, CGT+UTMD group all appeared more hypercellular and showed obvious nuclear polymorphism (Fig. 7A). On the contrary, tumor tissues from CGT-NP+UTMD group were hypocellular and exhibited the highest level of tumor apoptosis and necrosis, indicating the achievement of the strongest anticancer effects using CGT-NP with the help of UTMD.

TUNEL assays were carried out to correlate the cell apoptosis with the up-regulation of cleaved caspase-3 in tumor tissues. Apparently, cell apoptosis was most significant in CGT-NP+UTMD group among all groups (Fig. 7B).
Fig. 7 HE staining (A) and TUNEL assays (B) of C6 glioma sections from rats receiving different therapies on Day 28 after the first treatments. Nuclei were stained blue while extracellular matrix and cytoplasm were stained red in HE staining. In TUNEL analyses, brown staining indicated apoptotic cells and green staining indicated normal C6 glioma cells. About 10,000 cells per group were counted by an experienced technician who was “blinded” to the content of the samples as well as the results of the experiment. (**p<0.01 vs control; *** p<0.001 vs control; ###p<0.001 vs CGT group)

3.8 Transmission electron microscope imaging

In control group and UTMD group, glioma cells demonstrated cell morphology of healthy cells, including large nuclei, dispersed chromatin and distinct nucleolus (Fig. 8A). The surrounding cytoplasm was also rich in free ribosomes and contained rod-shaped mitochondria and solitary cisterns of the granular endoplasmic reticulum. In comparison, some moderate signs of cellular damages were observed in groups including CGT, CGT+UTMD, CGT-modified-NP and CGT-modified-NP+UTMD. These signs included elongated mitochondria with frequent intramembranous dilatious, minimum (CGT) to some (CGT-modified-NP) degree of condensation of chromatin, and some nuclear pores being capped with a dense lamina. An autophagosome was also observed in the image of CGT+UTMD group and CGT-modified-NP+UTMD group. Obvious signs of cellular damages were detected in CGT-NP group and even more so in CGT-NP+UTMD group. For instance, large clumps of densely packed chromatin close to the nuclear envelope were observed in CGT-NP+UTMD treated cells. The perinuclear cistern was clearly swollen and the nuclear pores were still visible. Large blebs of cytoplasm were also seen, which were associated with an advanced stage of cytoplasmic condensation containing recognizable nuclear fragment, mitochondria and accumulations of monoribosomes.
Fig. 8 Results of TEM imaging (A) and Western blot analysis (B). In TEM images, the green arrows indicate the nucleolus, blue arrows indicate the nuclear envelope, red arrows indicate the mitochondria, yellow arrows indicate the autophagosomes, white arrows indicate the densely packed chromatin. The Western blot analysis revealed different protein expression of GFAP, Ki67, caspase-3, Beclin-1 and LC-3 in brain tissue in different groups.
3.9 Results of Western-Blot assay

Protein levels of GFAP, Ki67, caspase-3, Beclin-1 and LC-3 in different groups were indicated by the Western-Blot band intensities (all results were compared to β-actin as standard). As consistent with the immunohistochemistry results, Ki-67 was expressed at the highest level in CGT-NP+UTMD group and the lowest in the Control group (Fig. 8B). However, expression of GFAP, caspase-3, Beclin-1 and LC-3 in CGT-NP+UTMD group were lower than any other groups. These results indicate that the CGT-NP+UTMD combined treatment has the strongest effect in promoting apoptosis and autophagy of tumor and inhibiting its proliferation.

4. DISCUSSIONS

Currently, CGT is almost exclusively used for surface modification of nanocarriers to achieve active targeting of the integrin receptors αvβ3/αvβ5 on endothelial cells. Its own targeted therapy activities, including anticancer effects against gliomas and synergism to anti-tumor drugs, were observed in both preclinical and clinical studies. CGT is in fact the first one entering randomized Phase III study among various cyclic RGD pentapeptides. Studies [36,37] indicated that CGT had anti-tumor activity in glioma animal model of home position transplantation. Preliminary randomized Phase I and II study also demonstrated therapeutic benefits in CGT over the current concomitant radiotherapy when combined with temozolomide chemotherapy. However, these studies eventually failed and revealed the limitations of CGT as a therapeutic agent by itself for glioma treatment. Issues such as quick clearance from circulation, inefficient penetration across the BBB and subsequent low brain accumulation, and again quick clearance from the tumor tissues still need to be resolved. As a non-viral technique, UTMD has evolved as a new, promising tool for site-specific drug and gene delivery in vitro and in vivo, targeting delivery via a process called sonoporation, allowing for direct transfer into the cells. Significant efforts have been made to demonstrate the application of UTMD in facilitating drug delivery specifically to the brain tumor site. The exact mechanisms behind this method remain unclear. It is assumed that mechanosensitive ion channels in the cell membranes of endothelial cells can respond to the mechanical forces (acoustic radiation force, circumferential stress, shear stress, etc) of the UTMD. These effects can increase the BBB opening through paracellular pathway (mainly introduced by tight junctions) [38] and transcellular pathway (mainly introduced by endocytosis) [39]. A unique advantage of UTMD introduced BBB opening technique over other conventional BBB disruption schemes, is the selective and regional permeability increases that result in enhanced local delivery within the brain [40]. Though the total duration of UTMD exposure are conventionally less than 30s [41], the ensuing BBB opening effect can last at most for approximately 4 hours [42]. The safety of FUS disruption of the BBB is well documented and the overall effects are transient and reversible with no overt neuronal injury [43].

In this study, we proposed to tackle the quick clearance issues with nanotechnology and overcome the BBB by combining the nanotherapy with UTMD, so CGT can fully realize its potential as an effective cancer targeting drug for glioma treatment. Overall, the data have extensively supported the effectiveness of this “multi-stage targeting” strategy (as summarized in Fig. 1).

Heparin-poloxamer based nanoparticles were developed for encapsulation and delivery of CGT. As studies have previously reported that by simple modification of the nanoparticle surface with CGT (e.g. by passive adsorption of CGT), issues such as fast blood clearance, high kidney and liver uptake and rapid tumor elimination can be conveniently addressed. We therefore included the CGT-modified-NP (by surface CGT adsorption) in addition to the more standard CGT-NP for comparison. As shown in Table S1, all nanoparticles demonstrated properties suitable for systemic
delivery, including size (≈100 nm with PDI<0.2), moderately negative zeta potential (≈−15 mV) and uniform morphology that are comparable to anticancer nanosystems such as Doxil (108 nm, zeta potential −13.3 mV) [44]. Both CGT-NP and CGT-modified-NP showed good CGT encapsulation efficiency (>60%), with CGT-NP being a more efficient carrier (87.5%). This discrepancy in CGT loading has been adjusted later in the in vivo experiments to ensure the administration of same quantity of CGT.

Two in vitro assays (apoptosis assays and MTT assays, Fig. 3) were performed and their findings have clarified three key issues before we proceeded to extensive in vivo works. First of all, the data confirmed the therapeutic activity of CGT in CGT-NP. As shown in cell apoptosis assay (Fig. 3A,B), cell apoptosis ratios of CGT-NP group and CGT-NP+UTMD group are significantly higher than the Control and CGT group. A similar trend was observed in MTT assay (Fig. 3C). The data confirmed that, at a minimum, the anticancer activities of CGT were well preserved after encapsulation of CGT in the nanoparticles prepared from gelatin and Poloxamer 188-grafted heparin copolymer. Moreover, the superior performance of CGT-NP (with CGT encapsulated within nanoparticles) over CGT-modified-NP (with CGT adsorbed on nanoparticle surface) especially when combined with UTMD indicated that CGT molecules perform better when strongly associated with the nanoparticles. The nanoparticles probably have ensured a high local concentration of CGT molecules near the cell membrane, so when subjected to UTMD, a steep CGT gradient could be achieved [45]. Encapsulation in nanoparticles may also have better protected the CGT molecules from early degradation. Finally, the data clearly showed that UTMD by itself did not cause cell death. This is consistent with the previous findings, which demonstrated that UTMD only results in transient, reversible, and localized BBB disruption [46,47]. It is expected that brain cellular/tissue damages by the US should be negligible and the in vivo cell killing activities of CGT-NP/US combined therapy would be exclusively provided by the CGT-NP component, which is desirable as non-specific cell death should be minimized as much as possible in any brain-related treatments.

Under our second objective, we studied if the proposed multi-stage targeting strategy could achieve in vivo biodistribution of CGT favorable for glioma treatment. The data in Fig. 4 strongly support this strategy. Not only that the peak tumor CGT level was significantly elevated in CGT-NP+UTMD group, this strategy was also able to modestly delay the time to reach the peak level and sustain a high tumor CGT level in the duration of the study. The data suggest a gradual CGT-NP build-up, slow tumor clearance and hence extended tumor retention of CGT. This could be attributed to the ability of nanoparticles to accumulate in the tumor by EPR and sustain CGT release. In short, the data are consistent with the proposed scheme in Fig. 1, in which the UTMD facilitated BBB passage of the nanoparticles and the nanoparticles were able to localize, accumulate and retain CGT in the targeted brain tumor tissue.

In terms of tumor specificity, when the CGT-NP+UTMD biodistribution data are compared with CGT+UTMD and CGT-NP (no UTMD), the CGT levels in non-cancer locations including blood, liver, spleen, lung and most noticeably kidney have all been reduced in CGT-NP+UTMD. This finding indicates that CGT encapsulation with nanoparticles and the use of UTMD both contributed to improving the specificity of the CGT therapy of glioma. The substantial decrease in the kidney CGT level suggests significantly reduced renal clearance in CGT-NP+UTMD group, which probably has contributed to the higher tumor level as less drug was eliminated before it reached the intended brain target.

Highly promising in vivo anticancer efficacy of CGT-NP+UTMD therapy was obtained (Fig. 5), which is a very difficult task to accomplish given that GMB is known for its poor response to drug
therapy. Our third specific objective is thus achieved. Under this objective, immunohistochemical staining of several key cancer markers was performed. In general, loss of GFAP expression is a significant indicator of increased tumor malignancy\[48\].

The result in Fig. 6 shows that both CGT-modified-NP and CGP-NP could significantly increase GFAP expression with or without UTMD, but CGT-NP+UTMD clearly achieved the strongest improvement. The expression of Ki-67, which was used to indicate the proliferation of tumor cells, exhibited a trend opposite to GFAP. The data showed that by delivering CGT with nanoparticles, a decrease in the degree of glioma malignancy is feasible. UTMD further magnified this effect likely by increasing the nanoparticles that could cross the BBB.

An anticancer treatment can lead to apoptosis and autophagy of cancer cells as response(s). Markers of apoptosis (Caspase-3) and autophagy pathway (Beclin-1, LC3) were thus evaluated to learn more about the anticancer mechanism mediated by CGT-NP. Results indicated that both pathways were involved. The role of autophagy in cancer treatment remains to be less understood. It may promote the survival of cancer cells especially during stressful situations, but may also serve as a possible pathway to kill highly resistant cancer cells that have high apoptotic threshold. The finding that CGT-NP+UTMD treatment utilize dual pathways deserves further investigation in future, as simple apoptosis induction may not be fully effective in eradicating a highly malignant disease like GMB.

One noteworthy issue is the CGT dosing rate used in this study. The CGT dose used was 2 mg/kg twice weekly. This was substantially lower than other studies that use CGT as the primary drug for anticancer therapy. For instance, previous studies have used dosing rates such as 25 mg/kg x 5 days/week \[49,50\], 20 mg/kg daily \[51\], 1000 mg/rat three days per week \[52\]. Considering the extremely high cost of RGD peptide drugs in general, the use of nanotechnology and UTMD could drastically spare the dose and injection frequency, which will definitely put RGD peptide therapy one step closer to being an affordable clinical application.

The other noteworthy issue is the effects of the difference between the in vitro and in vivo situation on glioma inhibition. Though the UTMD parameters in vivo are consistent with those in vitro, the internal and external condition between the in vitro and in vivo situation are different. Because the cultured glioma cells in vitro can directly contact with CGT-NP during UTMD treatment, the glioma inhibition can be observed in short time. However, the PMB in the in vivo situation can not directly contact with glioma tissue because of the biological barrier of blood vessel. Only CGT-NP can penetrate into the glioma after UTMD treatment. Therefore, the glioma inhibition in vivo of CGT-NP+UTMD treatment can be observed after twenty days.

The failure of CGT in clinical use does not frustrate the enthusiasm of scientists in glioma-targeted therapy. Some researchers try to use different strategies to solve the limitations of CGT in clinical application \[53\]. Our study focused on using novel drug targeted delivery system to improve the treatment efficiency of CGT. With novel nanocarriers, the half-life and pharmacokinetic behaviour of loaded drug can be improved \[54\]. Reversible BBB opening and high CGT concentration in glioma were realized by NP combined the UTMD technique, which enhance the glioma-targeted therapy of CGT. The data of CGT-NP+UTMD therapy, both in terms of biodistribution and efficacy, are very promising. This study has provided preclinical data supporting that this multi-stage targeting therapy could efficiently deliver a RGD peptide like CGT to cross the BBB, accumulate in the GMB tumor and minimize its distribution to the non-targeted organs. This strategy could also effectively promote apoptosis of GMB cells and suppress the tumor growth. Put everything together, this technology offers hope to bring CGT therapy back to clinical setting and
provide a much needed solution to gliomas.

References


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Graphical abstract