Co-delivery of pEGFP-hTRAIL and paclitaxel to brain glioma mediated by an angiopep-conjugated liposome

Xiyang Sun, Zhiqing Pang, Hongxing Ye, Bo Qiu, Liangran Guo, Jingwei Li, Jinfeng Ren, Yong Qian, Qizhi Zhang, Jun Chen, Liangran Guo, Jingwei Li, Jinfeng Ren

* Department of Pharmacy, School of Pharmacy, Fudan University, 826 Zhangheng Rd., Shanghai 201203, People’s Republic of China

** Key Laboratory of Smart Drug Delivery, Ministry of Education & PLA, 826 Zhangheng Rd., Shanghai 201203, People’s Republic of China

** Department of Neurosurgery, Huashan Hospital, Fudan University, Shanghai 200040, People’s Republic of China

** Department of Neurosurgery, First Hospital of Chinese Medical University, Shenyang 110001, People’s Republic of China

** Corresponding author. Department of Pharmaceutics, School of Pharmacy, Fudan University, 826 Zhangheng Rd., Shanghai 201203, People’s Republic of China

E-mail address: xjjiang@shmu.edu.cn (X. Jiang).

** Crown Copyright © 2011 Published by Elsevier Ltd. All rights reserved.

** Contents lists available at SciVerse ScienceDirect

Biomaterials journal homepage: www.elsevier.com/locate/biomaterials

** Biomaterials 33 (2012) 916–924

1. Introduction

The treatment of brain cancer is one of the most difficult challenges in oncology. A single medication cannot obtain optimal efficacy because cancer cells often exhibit major resistance to one type of chemotherapeutic drug [1]. Combination drug therapy has attempted to overcome this problem in the clinic [2,3].

The tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is the most promising cytokine for anticancer therapy because its cytotoxic activity is relatively selective for cancer cells over normal cells [4]. However, some studies have suggested that many tumours, including glioma, easily gain resistance to TRAIL-based therapies [5,6]. TRAIL may ultimately prove to be an effective co-delivery system. More importantly, the median survival time of brain tumour-bearing mice treated with ANG-CLP/PTX/pEGFP-hTRAIL was 69.5 days, significantly longer than that of other groups, even longer than the commercial temozolomide group (47 days). Therefore, the dual targeting co-delivery system is a promising drug delivery strategy against glioma.

TRAIL and the microtubule-targeting drug paclitaxel (PTX) have been shown to have a remarkable cooperative anti-tumor effect in subcutaneous xenografts derived from GBM cells [8]. However, the therapeutic usefulness of this combination treatment against GBM is limited by the following factors: 1) TRAIL has a short biological half-life and is rapidly cleared from circulation after systemic administration [9]. 2) Because of the low aqueous solubility and low therapeutic index of PTX, its clinical application is extremely limited. 3) Multiple dosing of TRAIL or PTX and respective chemotherapeutic drug will usually cause side effects or toxicity on normal brain endothelial cells [10]. Even if it overcomes the BBB/BBT, a drug will usually cause side effects or toxicity on normal brain endothelial cells [11]. It was reported that the activity of PTX against brain tumours was disappointing in a phasell study because of drug resistance and poor penetration across the BBB. Thus far, the effectiveness of a combination therapy of TRAIL and PTX on intracranial glioblastoma-bearing model systems has not been reported.

First, gene therapy may offer a promising cure for primary brain glioma for its high dose, relatively stable and long-term expression...
Moreover, compared with single gene therapy or chemotherapy, a combination therapy is expected to achieve a breakthrough in glioma treatment because therapeutic genes and chemical drugs could act on different targeting sites with different mechanisms and achieve synergistic therapeutic efficacy [13–15]. In addition, increasing the therapeutic effects might reduce the drug dosage as well as the side effects [16]. Second, cationic liposome (CLP) represents one of the most promising systemic delivery strategies for glioma therapy because of their ready entry into the central nervous system (CNS). Moreover, a CLP-mediated transfection is a useful and promising method for in vitro [17] and in vivo [18] gene transfer due to low immunogenicity and toxicity, ease of preparation, and potential applications for active targeting. In addition, lipids and lipophic drug PTX are easily mixed to prepare liposomes with stability, high efficiency and low toxicity. Third, angiopiep can target the low-density lipoprotein receptor-related protein (LRP), which is over-expressed on the BBB and glioma cells [19,20]. Recently, angiopiep-2 (TFFYGGSRGKRNNFKTEYEY, molecular weight 2.4 kDa) has been used for not only for enhancing delivery across the BBB but also for targeting brain tumours, called the “dual targeting effect” [21]. Therefore, the goal of this study was to develop an angiopiep-modified CLP (ANG-CLP) for the co-delivery of PTX and pEGFP-hTRAIL to glioma. A unique feature of this system of the combination therapy is that it has a dual targeting modification that has not been reported and it is expected to provide a breakthrough in the treatment of brain glioma. The system was carefully characterised, and its targeting efficiency and therapeutic efficacy were systematically evaluated in vitro and in vivo.

2. Materials and methods

2.1. Materials and animals

DC-chol, DOPE, rhodamine-DOPE and COOH–PEG2000–DSPE were provided by Avanti Polar Lipids (Alabaster, AL, USA). Investigated by Bingliang Fang, pEGFP-hTRAIL (Addgene plasmid 109953) was purified using a QIAGEN Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). Angiopiep-2 was synthesised by Shanghai Gene-Pharma Co., Ltd Company (Shanghai, China). The 1,1-dioctadecyl-3,3,3,3-tetramethyl indocarbocyanine iodide (DiR) was obtained from Biotium (Hayward, CA). The 1-dimethylaminopropyl-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma–Aldrich (Saint Louis, MO). The paclitaxel was purchased from Fujian South Bio-Engineering Co. Ltd. (Fujian, China). The temozolomide capsules were purchased from JiangSu Tasy Diy Pharmaceutica Co. LTD (Jiangsu, China). The TUNEI in situ apoptosis detection kit (Fluo-3 AM) and BCA protein assay kit were purchased from R&D Systems (Minneapolis, MN). All other materials were analytical grade and used without further purification. The U87 MG cell line and bovine caprine epithelial cells (BCECs) were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco’s modified eagle medium (DMEM), supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin sulphates. All cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO2. The cells were maintained in their exponential growth phase. The BALB/c mice (BK Lab. Animal Ltd., Co, Shanghai, China) used in this study were treated according to protocols approved by the ethical committee of Fudan University.

2.2. Preparation and characterisation

2.2.1. Preparation of dual targeting co-delivery system

The CLP was prepared as previously described with minor modifications [22]. Briefly, the lipid components, DC-chol, DOPE, and DSPE-PEG2000-COOH, were dissolved in chloroform containing PTX or DiR in a glass flask at a molar ratio of 1:2:0.03. The solution was then evaporated to dryness under vacuum using rotary evaporation (Buchi AG, Flawil, Switzerland) for 2 h at 40 °C. The dried lipid film was subsequently hydrated in HEPES buffer (10 mM, pH 7.4) at the final lipid concentration (20 mg/ml) for 30 min. After ultrasonication for 10 min using a probe-type ultrasonicator, the lipid dispersion was extruded by turns through a 200 mm, 100 mm, and 50 mm polycarbonate filter using an Avanti Polar Lipids Mini-Extruder. On the surface of the liposomal vesicle, angiopiep-2 was coupled with the COOH group of COOH–PEG2000–DSPE using EDC as a coupling agent. Adequate amounts of EDC and NHS were added to the blank liposomes (COOH–PEG2000–DSPE–EDC–NHS = 0.03:1.25:2.1, μmol/μmol) and incubated at room temperature for 10 min. Afterwards, 0.03 μmol of angiopiep-2 was added and incubated at room temperature with slow stirring for 8 h. The dissoctive proteins, unencapucilated Dir or PTX, were removed by passing the liposome suspension through a Sepharose CL–4B gel column. The plasmid pEGFP-hTRAIL was mixed with CLP/PTX or ANG-CLP/PTX and incubated for 30 min at room temperature, the unmodified co-delivery system (CLP/PTX/pEGFP-hTRAIL) or modified co-delivery system (ANG-CLP/PTX/pEGFP-hTRAIL) was formed.

2.2.2. Characterisation

2.2.2.1. DNA retardation assays. The co-delivery system at lipid/DNA ratios of 1:0.1, 1:0.15, 1:0.2, 1:0.3, 1:0.5 and 1:1 (w/w) were mixed with loading buffer containing a tracking dye (xylene cyanol) and loaded into individual wells of a 0.8% agarose gel and electrophoresed at 100 V for 45 min. The gels were stained by ethidium bromide, and the bands corresponding to plasmid DNA were visualised under UV light.

2.2.2.2. Particle size distribution and zeta potential. The particle size distribution and zeta potential of the co-delivery system were measured by a dynamic light scattering detector (Zetasizer, Nano-ZS, Malvern, UK). The change in their size before and after BSA addition (10%) was measured.

2.2.2.3. Atomic force microscopy (AFM). A Nanoscope atomic force microscope (Digital Instruments, Santa Barbara, CA) was used to collect the images of the co-delivery system. The E scanner heads was used with a rectangular beam silicon crystal sharpened tip tapping mode cantilever (Digital Instruments).

2.2.2.4. Determination of angiopiep-2 in the dual targeting co-delivery system. The average amount of angiopiep-2 was quantified by the Micro BCA method, where the absorbance was measured at 562 nm using a spectrophotometer (Molecular Devices, Sunnyvale, USA). The coupling efficiency was obtained through dividing the weight of angiopiep-2 in the co-delivery system by the weight of the feeding protein.

2.2.5. Drug-loading coefficient, encapsulation ratio and in vitro release. The drug loading coefficient (DL) and encapsulation ratio (ER) of the complexes were investigated. In the in vitro release behaviour of PTX or Dir from the co-delivery system was monitored by a dialysis method as described previously [23]. The concentration of PTX in samples was determined by HPLC as described above with correction for the volume replacement. The concentration of Dir in samples was determined by a fluorospectrophotometer (Fl-1000, Hitachi, Tokyo, Japan).

2.3. Cellular uptake

Rhodamine-DOPE was incorporated to label the lipid composition for cell uptake. U87 MG and BCECs cells were each seeded at a density of 5 × 106 cells/well in a 24-well plate. After incubation for 48 h, the cells were checked under the microscope for confuency and morphology. Then, each was incubated with the modified or unmodified co-delivery system (at dose of 10 μg/100 ng PTX per well) in serum-free media for 60 min at 37 °C. The cells were washed with Hank’s, then visualised and photographed under an O20 SFICA fluorescence microscope (Olympus, Osaka, Japan).

2.4. In vitro gene expression

The same cells from above were incubated for 48 h. The media was then aspirated from the wells, and the cells were washed once with serum-free media. The modified or unmodified co-delivery system (in the quantity required to deliver 2 μg pEGFP-hTRAIL and 200 ng PTX per well) was added gently to each well with serum-free media. After 2 h, the cells were rinsed twice with Hank’s solution and further cultured in fresh media containing FCS for 24 h at 37 °C. GFP expression was visualised by an inverted fluorescent microscope.

2.5. For quantitative apoptosis detection in vitro

The BCECs-U87 MG cells co-culture model was established with reference to a previous study [24]. The co-cultured model was incubated for 30 min with serum-free DMEM. After removing the solution, the blank group (CLP, ANG-CLP), the unmodified group (CLP/PTX, CLP/pEGFP-hTRAIL, CLP/PTX/pEGFP-hTRAIL) or the modified group (ANG-CLP/PTX, ANG-CLP/pEGFP-hTRAIL, ANG-CLP/PTX/pEGFP-hTRAIL) was applied to the apical chamber of the transwells at dose of 2 μg pEGFP-hTRAIL and 200 ng PTX. All of the co-cultured cells were incubated for 8 h after the drug treatment. After removal of the transwells, the U87 MG cells were cultured for another 36 h, then analysed using a flow cytometer with the Annexin V-FITC apoptosis detection kit.

2.6. In vivo imaging

U87 MG cells (5.0 × 106 cells suspended in 5 μl PBS) were implanted into the right striatum (1.8 mm lateral to the bregma at 3 mm depth) of male BALB/c nude mice using a stereotactic fixation device with a mouse adaptor. The intracranial U87 MG glioma-bearing mice were injected with the modified or unmodified co-delivery
system at a dose of 50 μg DNA/mouse and 5 μg PTX/mouse via the tail vein 16 days after implantation. The fluorescence scans (from 620 nm to 900 nm) were performed at 2, 6, 12 and 24 h post-i.v. using an in vivo image system (CRI, Woburn, MA, USA).

To compare the tissue and tumour distributions of the modified or unmodified co-delivery system, the mice were sacrificed at 24 h post-injection. Tumour-bearing brains and major organs, including livers, lungs, spleens, kidneys, and hearts, were dissected, washed with saline, and subjected to the in vivo image system to obtain the fluorescence images.

2.7. In vivo anti-tumour effect

2.7.1. Gene expression in tumour-bearing mice

On the 16th day after implantation, the modified or unmodified co-delivery system was injected into the tail vein of mice at a dose of 50 μg pEGFP-hTRAIL and 5 μg PTX per mouse. Approximately 48 h later, the animals were anesthetised with diethyl ether and killed by decapitation. Afterwards, the brains were frozen in OCT embedding medium (Sakura, Torrance, CA, USA) at –80 °C. Frozen sections of 20-μm thickness were prepared and stained with 300 nM DAPI for 10 min at room temperature. After washing twice with PBS (pH 7.4), the sections were immediately examined under the fluorescence microscope.

2.7.2. In situ tumour apoptosis detection and survival monitoring

At 7, 9, 11, and 13 days after implantation, the mice were dosed in the negative control groups (saline, CLP, ANG-CLP), the unmodified group (CLP/PTX, CLP/pEGFP-hTRAIL, ANG-CLP/PTX, ANG-CLP/pEGFP-hTRAIL) or modified group (ANG-CLP/PTX, ANG-CLP/pEGFP-hTRAIL, ANG-CLP/PTX/pEGFP-hTRAIL) via the tail vein in the quantity required to deliver 50 μg pEGFP-hTRAIL and 5 μg PTX per mouse. Temozolomide was administered at a dosage of 50 mg/kg. At day 16 after implantation, the mice were sacrificed, except for 10 mice from each group, which were monitored for survival. Frozen tissue sections (20 μm) were prepared from the sacrificed mice. Apoptotic cell death in tumour tissues were detected with TUNEL assays using an in situ cell death detection kit following the manufacturer’s instructions before observation under the microscope.

2.8. Statistical analyses

All experiments were performed at least three times, and representative results are presented (quantitative data expressed as the mean ± SD). Statistical comparisons were made with Student’s t test. Survival analysis was computed by the Kaplan–Meier method and compared by the log-rank test.

3. Results

3.1. Characterisation

The CLP neutralizes the negative charge of the phosphate groups on the DNA backbone, thus retarding the DNA mobility under the influence of an electric field. As shown in Fig. 1, only when the lipids/DNA weight ratio of CLP/PTX/pEGFP-hTRAIL increased up to 1:0.3, and the lipids/DNA weight ratio of ANG-CLP/PTX/pEGFP-hTRAIL increased up to 1:0.2, did the complexes produce sufficient retardation of DNA.

Quantitative analysis of the particle size and zeta potential are shown in Table 1. CLP/PTX/pEGFP-hTRAIL was positively charged at lipids/DNA ratios of 1:0.1, 1:0.15, and 1:0.2. With an increased amount of DNA and the addition of angiopep-2, the average diameter increased slightly, but the zeta potential led to a decrease in surface charge. The measured diameters increased upon addition of BSA to CLP/PTX/pEGFP-hTRAIL at a final concentration of 10 mg/mL. But when BSA was added to ANG-CLP/PTX/pEGFP-hTRAIL, they did not show apparent increases in size under similar conditions. The polydispersity of all the formulations also showed a narrow size distribution (PDI < 0.18), and the mean diameter of ANG-CLP/PTX/pEGFP-hTRAIL with lipids/DNA ratios of 1:0.2 was less than 150 nm after the addition of BSA. To load more of the gene and to make the complexes more stable after injection, the co-delivery system with a lipids/DNA ratio of 1:0.2, which appeared spherical in shape with a relatively monodispersed size (Fig. 2A), were used in the following experiments.

The PTX-loading coefficient was 2.2 ± 0.3% with a 97.3 ± 2.1% encapsulation efficiency when adding 1 mg. The feeding ratio of PTX was fixed at 1 mg when 50 mg of lipids was used. According to the protein assay, the coupling efficiency was 96.4 ± 3.2%. The amount of modified angiopep-2 was approximately 36.1 ± 1.7 μg/mg lipids (mean ± SD; n = 4).

3.2. Cellular uptake

As shown in Fig. 3, the cellular uptake of ANG-CLP/PTX/pEGFP-hTRAIL in U87 MG cells and BCECs were both enhanced. The results suggested that conjugating angiopep-2 to CLP might facilitate its uptake into the BCECs or glial cells.

3.3. In vitro gene expression

As shown in Fig. 4, U87 MG cells and BCECs treated with ANG-CLP/PTX/pEGFP-hTRAIL showed a significantly higher GFP expression level than those treated with CLP/PTX/pEGFP-hTRAIL after transfection at 48 h. Moreover, the transfected U87 MG cells treated with the modified co-delivery system underwent apparent apoptosis, and the transfected BCECs suggested low cytotoxicity.

![Fig. 1. Agarose gel retarded electrophoresis assay of ANG-CLP/PTX/pEGFP-hTRAIL (M2–F2) or CLP/PTX/pEGFP-hTRAIL (M1–F2). Lane M: marker; Lanes A, B, C, D, E and F: CLP/PTX/pEGFP-hTRAIL or ANG-CLP/PTX/pEGFP-hTRAIL at lipids/DNA ratios of 1:0.3, 1:0.15, 1:0.2, 1:0.3, 1:0.5 and 1:1, w/w.](image-url)
3.4. In vitro apoptosis detection

As shown in Fig. 5, the apoptosis of U87 MG cells induced by the CLP/PTX/pEGFP-hTRAIL (60.51 ± 2.71%) after crossing the BCEC monolayer was more significant than that by CLP/PTX (41.28 ± 1.95%) or CLP/pEGFP-hTRAIL (29.17 ± 1.26%). The apoptosis of U87 MG cells induced by ANG-CLP/PTX/pEGFP-hTRAIL (81.99 ± 3.28%) after crossing the BCECs monolayer was also more significant than that by ANG-CLP/PTX (58.67 ± 2.21%) or ANG-CLP/pEGFP-hTRAIL (40.12 ± 2.12%). The results demonstrated that the apoptosis of tumour cells induced by the co-delivery system was more evident than that by the single delivery system. Furthermore, ANG-CLP/PTX/pEGFP-hTRAIL showed a higher apoptosis percentage than did CLP/PTX/pEGFP-hTRAIL.

3.5. In vivo real-time imaging analysis

As shown in Fig. 6, compared with CLP/PTX/pEGFP-hTRAIL, the fluorescence signal in the tumour-bearing brain of ANG-CLP/PTX/pEGFP-hTRAIL was much stronger at any time post-injection, from

<table>
<thead>
<tr>
<th>Formulation(lipids/DNA, w/w)</th>
<th>Mean diameter in H2O (nm)</th>
<th>PDI</th>
<th>Mean diameter in BSA (nm)</th>
<th>PDI</th>
<th>Zeta potential in H2O (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLP/PTX/pEGFP-hTRAIL(1:0.1)</td>
<td>64.7 ± 3.4</td>
<td>0.103 ± 0.012</td>
<td>257.7 ± 21.3</td>
<td>0.358 ± 0.042</td>
<td>38.7 ± 3.1</td>
</tr>
<tr>
<td>CLP/PTX/pEGFP-hTRAIL(1:0.15)</td>
<td>81.5 ± 5.3</td>
<td>0.125 ± 0.032</td>
<td>191.9 ± 12.1</td>
<td>0.243 ± 0.057</td>
<td>25.9 ± 2.7</td>
</tr>
<tr>
<td>CLP/PTX/pEGFP-hTRAIL(1:0.2)</td>
<td>101.2 ± 8.7</td>
<td>0.166 ± 0.025</td>
<td>156.2 ± 14.5</td>
<td>0.211 ± 0.042</td>
<td>12.9 ± 1.3</td>
</tr>
<tr>
<td>ANG-CLP/PTX/pEGFP-hTRAIL(1:0.1)</td>
<td>74.8 ± 5.6</td>
<td>0.128 ± 0.017</td>
<td>190.6 ± 25.6</td>
<td>0.196 ± 0.035</td>
<td>35.8 ± 3.6</td>
</tr>
<tr>
<td>ANG-CLP/PTX/pEGFP-hTRAIL(1:0.15)</td>
<td>100.5 ± 11.6</td>
<td>0.139 ± 0.034</td>
<td>182.9 ± 19.7</td>
<td>0.167 ± 0.015</td>
<td>22.4 ± 2.3</td>
</tr>
<tr>
<td>ANG-CLP/PTX/pEGFP-hTRAIL(1:0.2)</td>
<td>118.9 ± 4.6</td>
<td>0.171 ± 0.031</td>
<td>131.4 ± 16.4</td>
<td>0.149 ± 0.028</td>
<td>8.2 ± 0.6</td>
</tr>
</tbody>
</table>

Fig. 2. Atomic force microscopy pictures of (A) CLP/PTX/pEGFP-hTRAIL and (B) ANG-CLP/PTX/pEGFP-hTRAIL at lipids/DNA weight ratios of 1:0.2. (C) PTX and Dir release profiles from the unmodified or modified co-delivery system in vitro.
2 h to 24 h (Fig. 6A). Ex vivo evaluation of excised tissues (heart, liver, spleen, lung, and kidney) and the tumour-bearing brain at 24 h post-injection showed an obvious tumour-bearing brain accumulation of ANG-CLP/PTX/pEGFP-hTRAIL (Fig. 6B). The fluorescence was located on the tumour position of brain for CLP/PTX/pEGFP-hTRAIL, indicating that it could accumulate in glioma tissue via the EPR effect. However, the fluorescence was distributed throughout the brain, including the glioma bed and infiltrating margin for ANG-CLP/PTX/pEGFP-hTRAIL, suggesting that the co-delivery system modified with angiopep-2 not only accumulated in the glioma bed via the EPR effect but was also transported across the BBB and then targeted the glioma infiltrating margin.

3.6. In vivo anti-tumour effect

3.6.1. Distribution of gene expression in the mouse brain

As shown in Fig. 7, at 16 days after brain glioma implantation (48 h after i.v. injection of the co-delivery system), for the CLP/PTX/pEGFP-hTRAIL (Fig. 7D–F), the gene expression was exhibited at the border outside of the glioma, but for ANG-CLP/PTX/pEGFP-
hTRAIL (Fig. 7A–C), the gene expression was higher and more widespread both inside and outside of the glioma.

3.6.2. In situ tumour apoptosis detection and survival monitoring

The brain tumour apoptosis was assessed by TUNEL. As shown in Fig. 8, at 16 days after the brain glioma implantation (3 days after the last administration of medicine), no obvious or detectable apoptosis phenomena were detected in the negative controls (Fig. 8A, E, I). In the unmodified group, induced tumour apoptosis usually occurred at the border of the glioma with lower efficiency (Fig. 8D), while a relatively more widely extended apoptosis was observed not only on the edge of the glioma, but also inside the tumour site when induced by the angiopep-modified system (Fig. 8B, C, D). More importantly, a more evident apoptosis of tumour cells was induced by the co-delivery system in comparison to the single delivery system. The group that was treated with temozolomide displayed obvious apoptosis phenomena (positive control, Fig. 8J).

![Fig. 5. Advanced apoptosis (%) of blank CLP, CLP/PTX, CLP/pEGFP-hTRAIL, CLP/PTX/pEGFP-hTRAIL, blank ANG-CLP, ANG-CLP/PTX, ANG-CLP/pEGFP-hTRAIL, and ANG-CLP/PTX/pEGFP-hTRAIL against U87 MG cells in in vitro BBB models (n = 3).](image)

![Fig. 6. In vivo fluorescence imaging of intracranial U87 MG glioma tumour-bearing nude mice and the dissected organs after intravenous injection of (A) CLP/PTX/pEGFP-hTRAIL and (B) ANG-CLP/PTX/pEGFP-hTRAIL.](image)
However, among all of the groups in the experiment, the most obvious and extended apoptosis phenomena were inside the tumour site of the group treated with ANG-CLP/PTX/pEGFP-hTRAIL (Fig. 8H). The anti-tumor effect was also reflected in the median survival time of the mice bearing brain tumour xenografts. As shown in Fig. 9, the mice treated with the co-delivery system achieved a much longer median survival time than those treated with the single medication systems. The median survival time of the mice treated with the dual targeting co-delivery system was up to 69.5 days, significantly longer compared with that of the other groups, including the temozolomide positive control group (47 days).
4. Discussion

The dual targeting co-delivery system constructed in this work was stable under physiological conditions. The results of the DNA retardation assays have revealed that angiopep-2 can loosen the CLP/DNA complex to some extent through electrostatic interactions, but they could not completely cause the complexes to fall apart when the lipids/DNA weight ratio was increased up to 1:0.2. Conjugation with angiopep-2 induces the decrease of positive potential and results in a lower likelihood to integrate with BSA. This result confirms that modification by angiopep-2 can minimise interactions with serum proteins. The cumulative release profiles of PTX or Dir in vitro from the unmodified or modified co-delivery system were similar. This profile confirmed that the dual targeting co-delivery system did not change the PTX or Dir release behaviour. All of these factors are beneficial for the biodistribution of the system.

Additionally, the co-delivery system exhibits a potential dual targeting effect by conjugation with angiopep-2 in vitro and in vivo. This datum is in agreement with the previous results of nanoparticles modified with angiopep-2 [21]. Moreover, from the results of the pharmacodynamics studies, the dual targeting co-delivery system did not influence the respective effect of PTX or pEGFP-hTRAIL on tumour cells. The system increased efficacy, reduced side effects and had low cytotoxicity on BCECs after transfection. Its enhanced therapeutic effects are a result of higher drug uptake by angiopep-2 modification, and the combined treatment of TRAIL and PTX has been demonstrated to lead to a markedly greater caspase activation and abrogation of the mitotic checkpoint than either TRAIL or PTX alone, causing significantly greater levels of cancer cell death [25]. Reducing the toxicity towards BCECs could be attributed to the therapeutic selectivity of TRAIL because TRAIL is able to induce cell apoptosis of glioma selectively, while exerting few toxic effects on normal cells including BCECs [26]. The lower dosage of chemotherapeutics may lead to a reduction of side effects, and the dual targeting co-delivery system could induce relatively more widespread apoptosis not only on the edge of the glioma but also on the interior of the tumour site (Fig. 8). Presumably, the unmodified system could access the glioma tissues through the EPR effect but could not penetrate into the glioma passively due to the densification of the tumour tissue, and thus could not induce apoptosis inside the glioma. However, the angiopep-2 modified system could reach the inside of the glioma and further accumulate in the glioma cells, thus inducing apparent apoptosis at the edge and interior of the tumour by the accumulation of higher concentrations of therapeutic agents. Furthermore, the median survival time of the brain tumour-bearing mice treated with the dual targeting co-delivery system was up to 69.5 days, which was significantly longer compared with the single medication and unmodified system groups, including the one treated with commercial temozolomide. Additionally, the median survival in previous studies with nanoparticles loaded with pORF-TRAIL was no more than 61 days [27]. These results are due to the ability of the dual targeting combined delivery platform to significantly improve the anti-glioma effect as well as reduce side effects. Finally, the low cytotoxicity of the vector (Fig. 5) allowed for repeated i.v. administrations.

5. Conclusions

In summary, the dual targeting co-delivery system exhibited multiple functions: 1) efficiently delivering both genes and chemotherapeutic drugs to target cells, 2) improving the anti-tumour effect in vitro and in vivo and causing low toxicity, and 3) prolonging the survival time of U87 MG glioma bearing-mice. The dual targeting co-delivery system thus showed great potential for glioma therapy in clinical applications.

Acknowledgements

This work was supported in part by grants from the National Basic Research Program of China (973 Program) (2007CB935800).

References


