Retro-Inverso Bradykinin Opens the Door of Blood–Brain Tumor Barrier for Nanocarriers in Glioma Treatment

Authors: Zuoxu Xie¹, Qing Shen¹, Cao Xie¹, Weiyue Lu¹, Chunmei Peng¹, Xiaoli Wei¹, Xue Li¹, Chunli Gao², Min Liu^{1*}

Affiliations:

¹Key Laboratory of Smart Drug Delivery (Fudan University), Ministry of Education, Department of Pharmaceutics, School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai 201203, PR China

²Department of Otolaryngology-Head and Neck Surgery, Eye and ENT Hospital, Fudan University, 83 Fenyang road, Shanghai 200030, PR China a period.

* Correspondence to: Key Laboratory of Smart Drug Delivery (Fudan University), Ministry of Education, Department of Pharmaceutics, School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai 201203, P.R. of China. Tel: +86 021 51980092, Fax: +86 021 51980090, E-mail address: liumin@shmu.edu.cn

Abstract: The blood–brain barrier and the blood–brain tumor barrier (BBTB) prevent most drugs entering brain tumors. Complicated preparation procedures of drug delivery systems and damage to normal brain tissue have limited the application of many strategies for the treatment of brain tumor in clinical trials. We have designed a bradykinin analog, retro-inverso bradykinin (RI-BK), which is characterized by resistance to proteolysis and high binding activity with the bradykinin type 2 (B₂) receptor. After systemic administration, RI-BK binds to B₂ receptors and induces a change in zonula occluden-1 and depolymerization of F-actin to selectively open the BBTB. RI-BK increased the accumulation of drug-loaded nanocarriers in the glioma but not in normal brain. Co-administration with RI-BK enhanced the therapeutic efficiency of drug-loaded nanocarriers for glioma. These findings suggest that RI-BK could be translated into the clinic as an adjunctive treatment for malignant brain tumors.

Keywords

retro-inverso bradykinin; glioma; blood-brain tumor barrier; polymeric micelles

Introduction

Glioma is the most common type of brain cancer, with high morbidity and mortality [1]. The growth of brain tumors results in the destruction of normal brain tissue, leading to neurological complications [2]. Lesion location, incomplete surgical excision, side effects of radiotherapy, and poor chemotherapy outcome are some of the reasons for the difficulty in treatment of brain tumors [3]. In addition, because of the blood–brain barrier (BBB) and blood–brain tumor barrier (BBTB), antitumor agents are prevented from reaching brain tumor tissues, thus blocking their therapeutic actions [4, 5]. Therefore, it is crucial to find methods that result in the delivery of antitumor drugs to the lesion.

In systemic chemotherapy, the limitation of most antitumor drugs, such as poor solubility, rapid clearance rate, systemic toxicity, or development of multidrug resistance, restricts their clinical use [6, 7]. In order to overcome these drawbacks, various nanoscale drug delivery systems have been developed, such as polymeric micelles and liposomes [8, 9]. Polymeric micelle drug delivery systems have recently attracted much attention in glioma treatment because of their effectiveness in drug delivery, such as the ability to deeply penetrate and accumulate within the target tissue [10].

To facilitate the accumulation of drugs in brain tumors, several delivery strategies have been used to cross the BBB, such as physical stimuli, chemical stimuli, receptor-mediated transcytosis, and transporter-mediated transcytosis [11, 12]. However, these strategies damage the normal brain tissue when used to treat brain tumors. Therefore, researchers have designed drug delivery systems that target the BBTB to treat brain tumors. Most of the current drug delivery systems are too complicated to apply to the clinic. Therefore, our goal was to develop a simple strategy to deliver nanocarriers to brain tumors by selectively opening the BBTB without affecting normal brain tissue.

It has been reported that low-dose bradykinin (BK) may be able to selectively open the BBTB in experimental brain tumors [13]. Subsequently, it was shown that one method of increasing BBTB permeability is through BK type 2 (B₂) receptors [14]. Unfortunately, the rapid degradation of BK in plasma limits its application for clinical use [15]. Because the original spatial orientation of all side chains is retained, retro-inverso peptides, which have high proteolytic resistance, may have the same binding activity as the parent peptide [16-18]. We hypothesized that retro-inverso bradykinin (RI-BK) might selectively open the BBTB and deliver drug-loaded nanocarriers into the brain tumor parenchyma.

For the present study we tested our hypothesis that RI-BK could selectively open the BBTB and deliver drug-loaded nanocarriers into the brain tumor parenchyma. We also evaluated this treatment strategy for clinical applications by performing studies on glioma tumor.

2. Materials and Methods

2.1 Materials

Protected Fmoc-amino acid derivatives and 2-chlorotriyl chloride resin were purchased from GL Biochem (Shanghai, China). Fluorescein-5-maleimide (Mal-FITC) was purchased from Fanbo Biochemicals (Beijing, China). DAPI was purchased from Roche (Basel, Switzerland). Rabbit anti-ZO-1 antibodies and FITC labeled goat anti-rabbit secondary antibody were purchased from Life Technology (USA). Phalloidin-FITC and coumarin-6 was purchased from Sigma-Aldrich (St Louis, MO, USA). Polyester (PET) membrane Transwell (12mm membrane diameter, 0.4 μ m pore size) was purchased from Corning Incorporated Life Sciences (USA). Poly(ethyleneglycol)-distearoylphosphatidylethanolamine (PEG-DSPE) was purchased from Lipoid (Ludwigshafen, Germany). Poly(ethylene glycol)-poly(lactic

acid) (PEG-PLA) block copolymer was purchased from Advanced Polymer Materials (Montreal, Canada). PTX was a kind gift from Prof. Hao Wang (Shanghai Institute of Pharmaceutical Industry).

2.2 Cell Culture

HUVECs and C6 cells were obtained from Shanghai Institute of Cell Biology. The bEnd.3 cells were purchased from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle medium, supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL), which were purchased from Gibco (Grand Island, NY, USA). The *in vitro* BBB model was established by using bEnd.3 cells. The bEnd.3 cells were trypsinized, then the cells were plated onto the insert of Transwell system and cultured for 10 days before the experiments. The *in vitro* BBTB model was established by using bEnd.3 cells and C6 cells. The C6 cells were trypsinized and then added to wells of a 12-well plate, given time to attach (2–3 h) and bEnd.3 cells containing inserts placed in the wells. The co-cultured cells were incubated 10 days before experiments.

2.3 Animal Model

Male BALB/c nude mice weighing 20–22 g were obtained from Shanghai SLAC Laboratory Animal (Shanghai, China) and kept under specific pathogen-free conditions. All animal experiments were evaluated and approved by the Ethics Committee of Fudan University.

The glioma-bearing mouse model was established by using a stereotactic fixation device and microliter syringes to implant 5×10^5 C6 cells suspended in 5 μ L PBS into the right striatum of nude mice (1.8 mm right lateral, 0.6 mm anterior to bregma and 3 mm of depth). Samples of glioma-bearing mice, chosen randomly, were sacrificed 6 days after tumor implantation to confirm by pathological analysis that the tumor was established successfully.

2.4 Synthesis of BK and Derivatives

BK, RI-BK and KD were synthesized by an Fmoc-protected solid phase peptide synthesis strategy on 2-chlorotrityl chloride resin. Resin was swelled with dichloromethane and washed three times with N,N-dimethylformamide (DMF). Fmoc-protected amino acid was dissolved in DMF and O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole solution and diisopropylethylamine was added to the amino acid solution. The activated amino acid solution was added to the resin and mixed by stirring for 45 min. The amino group was deprotected by 25% piperidine in DMF and reacted with the carbonyl group of the next Fmoc-protected amino acid. This cycle was repeated to form the desired peptide chain. The sequence of peptides displayed in the Table S1. After purification to homogeneity by RP-HPLC, all synthetic peptides were analyzed by ESI-MS. To prepare fluorescein-labeled peptides and decrease the interference of fluorescein, the N-terminal of BK was extended with cysteine and ϵ -aminocaproic acid. After

purification to homogeneity by RP-HPLC, all synthetic peptides were analyzed by ESI-MS.

2.5 Surface Plasmon Resonance Studies

Surface Plasmon Resonance (SPR) interaction analyses were performed using a Biacore T200 optical biosensor (GE Healthcare Life Sciences, Uppsala, Sweden). SPR measurements were carried out in HSB-N buffer, and stock solutions were diluted in the same buffer. Data were collected with Biacore control software version 2.0 and analyzed by Biacore T200 evaluation software. To couple the bradykinin B₂ receptor protein to the surface of CM5 sensor chip, the sensor chip was firstly activated with an injection of the mixture of EDC/NHS reagent. Then the B₂ receptor protein dissolved in 10 mM NaOAc (pH 4.5) at 50 µg/mL was injected and followed by covalently coupling to the surface of CM5 sensor chip. A total of 15132.4 RU of B₂ receptor proteins were immobilized. Then BK and RI-BK solution were injected from low to high concentration. At the end of each injection, there was a regeneration of B₂ receptor protein with HSB-N buffer solution. The contacting time of every sample to B₂ receptor protein was 45 s with a buffer flow of 20 µL/min. The whole process of analysis was carried out at 25 °C.

2.6 Homology model for B₂ receptor

The amino acid sequence of B₂ receptor was downloaded from the UniProtKB database (Entry code: P30411), and sequence similarity search was performed using the NCBI BLAST server[19]. Sequence alignment of B₂ receptor with CXCR4 (pdb code: 3odu) and δ opioid receptor (pdb code: 4n6h) was carried out using the ClustalX 2.0 program[20]. Homology modeling was performed with Discovery Studio 3.5. Fifty models were generated after loop refinement, and the one with the lowest Discrete Optimized Protein Energy (DOPE) score was submitted to energy minimization (1000 steps steepest descent with backbone constrained). The PROCHECK[21] statistics showed that over 90% of the residues in B₂ receptor model were in either the most favored or in the additionally allowed regions of the Ramachandran map, suggesting that the overall main chain and side chain structures are reasonable. The 3D structural model of B₂ receptor is shown in Fig. S1.

The structure of BK was predicted by PEP-FOLD server[22], while the residues were mutated to D-amino acid residues with reverse sequence, followed with optimization in SYBYL6.9 to get the structure of RI-BK. The docking program Hex v6.1[23] was used for the preliminary protein-protein docking, and the flexible peptide docking package in RosettaDock program[24] was further used to probe the possible minimum-energy conformations in the binding sites of B₂ receptor. This program has full support for D-amino acid and the parameters of D-amino acid were activated in the docking studies[25]. The solutions given by flexible peptide docking package were rescore by binding affinity with X-Score 1.2 program[26]. The predicted affinities and all of available experimental data were used for the selection of the final complex.

2.7 *Ex vivo* Stability Studies of BK and RI-BK

The stabilities of BK and RI-BK were determined by using a solution containing 50% mouse serum, which was commercially available. Peptides (1 mg) were dissolved in 2 mL serum and incubated at 37 °C. After incubation periods of 0, 1, 2, 5, 10, 20, 30, 60 and 120 min, 100 µL sample of serum medium was mixed with 20 µL 15% trichloroacetic acid solution to precipitate the serum proteins. Then, the mixture was allowed to stand for 30 min, and centrifuged at 10 000 rpm for 15 min. The remaining amounts of peptides were quantified by using RP-HPLC.

2.8 *In vitro* Cell Uptake and Inhibition Studies

The *in vitro* cell uptake of FITC-labeled peptides was performed as follows. Briefly, different cell types were separately seeded in 12-well plates and were cultured for 24 h. Then the cells were incubated with FITC-BK or RI-BK-FITC in cell-culture medium supplemented with 10% FBS for 2 h, washed with PBS, fixed with 4% paraformaldehyde (PFA), stained with DAPI, and examined with a confocal laser scanning microscope (Zeiss LSM 710, Germany). Flow cytometry was used to compare the intracellular uptake of FITC-BK and RI-BK-FITC by C6 cells.

For the *in vitro* cell inhibition study, C6 cells were seeded in 12-well plates and cultured for 24 h. The cells were pretreated with PBS, BK and KD (5 mmol/L), respectively, for 0.5 h, followed by addition of RI-BK-FITC with a final concentration of 5 µmol/L for 2 h incubation. The cells were treated and observed as described above. Quantitative analysis was performed by flow cytometry.

2.9 Immunofluorescence of ZO-1 and F-actin

Both before and after treatment with 1 µM BK or RI-BK, indirect immunofluorescence was used to detect localization and expression of TJ-associated proteins ZO-1 in BBB model and BBTB model. After treatment, the bEnd.3 cells were washed by PBS, fixed with 4% PFA, and then permeabilized with 0.2% Triton X-100. Then the cells were blocked with 5% BSA for 30 min at room temperature and then incubated with rabbit anti-ZO-1 antibody at 4°C overnight. After rinsing with PBS, the cells were incubated with FITC labeled goat anti-rabbit secondary antibody for 1.5h at 37°C followed by DAPI stained. Images were collected on a confocal laser scanning microscope. To labeled F-actin, direct immunofluorescence was conducted: the cells were processed as described above and then incubated with phalloidin-FITC for 1.5h at 37°C. Finally, the cells were observed under the confocal laser scanning microscope.

2.10 Western Blot Studies

The bEnd.3 cells in BBB model and BBTB model were homogenized in lysis buffer containing protease inhibitors. The protein was loaded on an 8% SDS-PAGE. After the electrophoresis, the gel was transferred to a nitrocellulose membrane, which was blocked, cut, and incubated with rabbit anti-ZO-1 antibody overnight. After washing thoroughly, the membranes were incubated with HRP-labeled goat anti-rabbit secondary antibody. Immune complexes were visualized by enhanced chemiluminescence.

2.11 *In vivo* Optical Imaging Studies

Images were acquired using an IVIS Spectrum *in vivo* imaging system. At 11 days past tumor implantation, the glioma-bearing nude mice were randomly divided into three groups, namely PEG-PLA, BK+PEG-PLA, and RI-BK+PEG-PLA. Five minutes after BK or RI-BK injection, DiR-loaded PEG-PLA micelles were intravenously injected into the nude mice of the BK+PEG-PLA and RI-BK+PEG-PLA groups. At 15 and 30 min, and at 1, 2, 8, and 24 h after injection of DiR-loaded PEG-PLA micelles, the mice were transferred to the imaging system for observation. After *in vivo* imaging, the mice were perfused with saline followed by 4% PFA. Brains were carefully excised, and fluorescence images of whole brains were captured.

2.12 Preparation and Characterization of Coumarin-6-Loaded Micelles

Two types of micelles were prepared by using two different amphiphilic copolymers (PEG-PLA and PEG-DSPE). PEG-PLA/coumarin-6 micelles were prepared as follows: 20 mg PEG-PLA and 40 µg coumarin-6 were dissolved together in 2 mL acetonitrile and dried by rotary evaporation to form a thin film at 37 °C. PEG-PLA/coumarin-6 micelles were obtained by hydrating the thin film with physiological saline and filtered three times with 0.1 µm filter membrane (Millipore Billerica, MA, USA) to remove free coumarin-6. PEG-DSPE/coumarin-6 micelles were prepared as follows: 20 mg PEG-DSPE and 20 µg coumarin-6 were co-dissolved in 2 mL acetonitrile and dried by rotary evaporation to form a thin film at 37 °C, and then re-suspended in 10 mM HEPES-buffered saline (pH 7.4) at room temperature. To remove un-incorporated coumarin-6, PEG-DSPE/coumarin-6 micelles were filtered three times with 0.1 µm filter membrane. Micelle size was measured by dynamic light scattering using Zetasizer Nano (Malvern Zetasizer Nano Series, Malvern, UK).

2.13 Biodistribution of Coumarin-6-Loaded Micelles

Nine normal nude mice were randomly divided into three groups, namely PEG-PLA, BK+PEG-PLA, and RI-BK+PEG-PLA. In the PEG-PLA group, the nude mice were injected with 100 µL PEG-PLA/coumarin-6 in the tail vein with a dose of 50 µg/kg coumarin-6. The nude mice in the BK+PEG-PLA and RI-BK+PEG-PLA groups were injected respectively via tail vein with 30 µg/kg BK and RI-BK 5 min before the injection of PEG-PLA/coumarin-6. At 15 min following i.v. injection of PEG-PLA/coumarin-6, the nude mice were sacrificed. The brain tissues were harvested and quickly washed with cold saline and then subjected to n-hexane extraction for HPLC analysis with coumarin-7 as the internal standard.

For the glioma-bearing nude mouse model, the experiments were similar to that described for the normal nude mice, except that the animals used in the study were replaced by the glioma-bearing nude mice at 11 days after tumor implantation.

2.14 Cytotoxicity and Cell Apoptosis Assay

C6 cells in logarithmic growth phase were removed from the culture flask by trypsinization and added to 96-well tissue culture plates in 200 µL of medium to

obtain a concentration of 1×10^3 cells per well, and then the plates were incubated for 24 h at 37 °C in 5% CO₂ atmosphere. Paclitaxel (PTX) -loaded micelles with PEG-PLA (PEG-PLA/PTX), and free PEG-PLA micelles in medium were added to a 96-well plate for 48 h at 37 °C in a 5% CO₂ atmosphere. The viability of the cells was measured using the tetrazolium dye method (MTT assay).

The cell apoptosis assay was analyzed by propidium iodide and annexin V-FITC staining. Briefly, the C6 cells seeded in 12-well plates were incubated with PEG-PLA/PTX micelles at a dose of 0.1 μM PTX. After another 48 h incubation, the cells were rinsed twice with cold PBS and stained with a cell apoptosis kit (250 μL binding buffer, 2.5 μL annexin V-FITC, and 2.5 μL propidium iodide/well), according to the kit directions. The cells were resuspended in 0.5 mL of PBS, and immediately analyzed by flow cytometry.

2.15 Anti-Glioma Effect

The glioma-bearing nude mice were randomly divided into four groups (n = 10). Three groups were intravenously injected separately with PEG-PLA/PTX at a dose of 6 mg PTX/kg on days 6, 9, 12, 15, and 18 after tumor implantation. The mice treated with saline served as control. The BK+PEG-PLA/PTX and RI-BK+PEG-PLA/PTX groups were injected respectively by tail vein with 30 μg/kg BK and RI-BK 5 min before the injection of PEG-PLA/PTX.

2.16 Statistical Analysis

Statistical analysis was performed with the GraphPad Prism software. Results are presented as means ± SD. Differences in variables between groups were assessed using two-tailed Student's t-test (*p < 0.05, **p < 0.01, ***p < 0.005). The survival data are plotted on Kaplan–Meier plots and analyzed using a log-rank test.

3 Results

3.1 Evaluation of Peptides and FITC-Labeled Peptides

We successfully obtained large quantities of highly pure BK, RI-BK, kallidin (KD) (a natural ligand for B₂ receptor), fluorescein isothiocyanate (FITC)-ε-aminocaproyl-BK (FITC-BK), and RI-BK-ε-aminocaproyl-FITC (RI-BK-FITC) characterized by analytical HPLC and electrospray ionization mass spectrometry (ESI-MS) (Fig. S2). Firstly, we verified that RI-BK had high proteolytic resistance after 120 min incubation in 50% mouse serum in water. The RI-BK level in the plasma was close to 100% compared with BK, which was almost totally hydrolyzed by 20 min in 50% mouse serum (Fig. S3). To investigate whether RI-BK is capable of interacting with B₂ receptor, we conducted surface plasmon resonance interaction analyses using a Biacore T200 optical biosensor. RI-BK registered a K_d value of 0.084 nM, which was approximately 40 times lower than that of BK (Table 1; Fig. S4).

3.2 Computational Analyses

The three-dimensional (3D) structure model of the B₂ receptor was built by a homology modeling method using the crystal structures of CXCR4 (pdb code: 3odu) and δ opioid receptor (pdb code: 4n6h) as templates. The structure of BK was predicted by the PEP-FOLD server. To obtain the structure of RI-BK, the residues of BK were mutated to D-amino acid residues with the reverse sequence, followed with optimization (Fig. 1). The peptides were docked into the B₂ receptor using the Flexpep_dock package in the program Rosetta. Examination of the binding modes of BK and RI-BK revealed that the positively charged peptide was attracted to the negative pocket of the receptor, which is conserved in the class A GPCR family. The Arg and ^DArg residues in the terminal regions of BK and RI-BK, respectively, formed electronic interactions with the B₂ receptor. The Arg9 residue in BK formed a salt bridge with Asp311, whereas the Arg1 in BK generated a hydrogen-bond network with The197 and Ser214 (Fig. 2A). Compared with the binding mode of BK, RI-BK adopted a different orientation. The ^DArg9 residue in RI-BK formed hydrogen bonds with the backbone of Phe121 and cation- π interaction with Tyr201, whereas ^DArg1 formed a hydrogen-bond network with Glu221, Tyr215, and Asn225 (Fig. 2B). The benzene fragment in Phe8 of BK and ^DPhe5 of RI-BK was located in the same position and formed aromatic interactions with Trp113 and Phe319 (Fig. 2), whereas the benzene fragment in ^DPhe8 of RI-BK stretched into the hydrophobic pocket formed by Trp52, Val312, and Leu56 (Fig. 2B).

3.3 *In vitro* Cell Uptake and Inhibition Studies

In the cell uptake experiment, we found that RI-BK-FITC exhibited enhanced cellular uptake by C6 cells compared with FITC-BK (Fig. 3A-C). To confirm that RI-BK-FITC is internalized into C6 cells via the B₂ receptor, we used BK and kallidin (KD) (a natural ligand for B₂ receptor) to inhibit the uptake of RI-BK-FITC. We found that KD inhibited the internalization of RI-BK-FITC, but could not obtain data for BK because of the high cytotoxicity (Fig. 3D). The result was confirmed by the quantitative study (Fig. 3G and H). In addition, as shown in Fig. 3, a high fluorescent signal was observed in HUVECs, but not in bEnd.3 cells.

3.4 BK and RI-BK Changes the Distribution of zonula occluden-1 (ZO-1) and F-actin

After administration of BK or RI-BK, we found that ZO-1 was localized to the cellular boundaries with a continuous distribution in the BBB model, but did not show continuous distribution in the BBTB model (Fig. 4). We also observed a difference in F-actin between the models. After administration of BK or RI-BK, F-actin was distinct in the BBB model. However, in the BBTB model RI-BK led to disordered F-actin and the formation of stress fibers in bEnd.3 cells (Fig. 4). In addition, as shown in Fig. 4, western blot analysis revealed that BK or RI-BK suppresses the protein level of ZO-1 in the BBTB model, but not in the BBB model. The results of immunofluorescent staining and western blot analysis indicate that RI-BK may selectively open the BBTB by decreasing the expression level and altering the distribution of ZO-1 and by promoting the formation of stress fibers.

3.5 RI-BK Changes the Biodistribution of Nanocarriers in the glioma-bearing nude mice

In order to test whether RI-BK could facilitate polymeric micelle transport across the BBTB, two types of micelles with different particle size (Fig. S5) were prepared and assembled with either PEG-PLA (20.88 ± 0.33 nm) or PEG-DSPE (12.95 ± 0.65 nm). Using these two types of polymeric micelles, we studied how RI-BK influenced the distribution of polymeric micelles by *in vivo* optical imaging studies. In normal nude mice, administration of RI-BK did not enhance the intensity of fluorescence at any of the time points tested (Fig. S6A). This result was validated by imaging of excised brains 24 h after injection of DiR-loaded micelles (Fig. 5A). In contrast, in glioma-bearing nude mice injected with RI-BK, higher fluorescence intensity was evident in brain tumors at all time points (Fig. S6B). In excised brains of glioma-bearing nude mice it was clearly evident that most of the fluorescence accumulated in the brain tumor rather than being distributed throughout the entire brain (Fig. 5B). These results were confirmed by quantitative analysis, which showed that RI-BK increased access of PEG-PLA/coumarin-6 and PEG-DSPE/coumarin-6 to the brain by 73.4% and 53.2%, respectively. However, in normal nude mice there was no increase in the level of coumarin-6 in the brain after RI-BK injection (Fig. 5C and D; Fig. S7). These results are in agreement with the concept that RI-BK selectively opens the BBTB, allowing the nanocarriers to target the brain tumor.

3.6 *In vitro* and *In vivo* Evaluation of Anti-Glioma

In cytotoxicity and cell apoptosis studies, a cell viability assay (Fig. S8A) showed that the IC₅₀ value of PEG-PLA/PTX on C6 cells after 48 h incubation was 0.148 μ M. C6 cells incubated with PEG-PLA/PTX micelles at a dose of 0.1 μ M resulted in 25% of cells undergoing apoptosis (Fig. S8C). We find the BK and RI-BK did not display obvious cytotoxicity for the C6 cells (Fig. S8B). In addition, we have studied the toxicity of RI-BK *in vivo*. The H&E staining assay showed that RI-BK don't have obviously toxicity for major organs (Fig. S9).

The therapeutic effects were evaluated by survival days in the glioma-bearing nude mice. We intravenously injected four randomly divided groups of glioma-bearing nude mice with saline, PEG-PLA/PTX, PEG-PLA/PTX combined with BK, and PEG-PLA/PTX combined with RI-BK. As shown in Fig.5, single treatment of PEG-PLA/PTX did not improve mouse survival compared with saline treatment, showing a median survival of 22 days versus 21.5 days for the saline group. Similarly, mice treated with PEG-PLA/PTX and BK did not live longer than untreated mice. However, treatment with RI-BK and PEG-PLA/PTX significantly prolonged the average survival time to 31 days ($p < 0.005$, log-rank analysis) compared with saline control.

4 Discussion

The blood–brain barrier and the blood–brain tumor barrier (BBTB) prevent most drugs entering brain tumors. Complicated preparation procedures of drug delivery systems and damage to normal brain tissue have limited the application of many

strategies for the treatment of brain tumor in clinical trials. Accordingly, we devote our energies to find a simple and effective way to across the hindrance. In this work, we utilized the principle of retro-inverso peptides to transform BK to test if RI-BK can temporarily and selectively open the BBTB. Base on a similarity in spatial orientation of a side chain, RI-BK can retain the same activity of BK. Though the computational analyses, we another benzene fragment in Phe5 of BK lost this interaction, which may contribute to the lower binding affinity of BK compared with RI-BK (Fig. 2).

To investigate how RI-BK selectively opens the BBTB without affecting the BBB, We conducted cell uptake studies, immunofluorescence assay and western blot studies. The result indicated that the mechanism of RI-BK-mediated BBTB opening may be related to the level of B₂ receptors expressed on different cells and the change of ZO-1 and F-actin in BBTB. Because of the low level of B₂ receptor expression, low dose RI-BK did not deleteriously affect the BBB. However, RI-BK did selectively open the BBTB, which was caused by the binding of RI-BK to B₂ receptors overexpressed on the BBTB. After RI-BK bind to the B₂ receptors, it will induce a change in ZO-1 and depolymerization of F-actin to selectively open the BBTB.

Because many agents are unable to cross the BBB and BBTB and the toxicity of antitumor drugs, few drugs are suitable for the treatment of brain tumors. For this reason, a variety of drug carriers have been developed that minimize drug degradation, reduce adverse reactions, and allow solubilization of water insoluble drugs, therefore enhancing the amount of active drug reaching the brain lesion[27, 28]. Among these drug carriers, polymeric micelles have shown remarkable potential owing to their properties of water solubility, high stability in blood, deep penetration and accumulation abilities[10, 28]. However, polymeric micelles are unable to reach the brain tumor when the BBB and BBTB are intact; therefore we hypothesized that RI-BK could facilitate polymeric micelle transport across the BBB and BBTB.

Herein, we prepared two widely used polymeric micelles, which are self-assembled by the PEG-PLA or PEG-DSPE [29-31]. We administered these two types of polymeric micelles with high stability and low biological toxicity to glioma-bearing nude mice to evaluate the accumulation of nanocarriers in glioma. Fortunately, our results show that RI-BK can increase the accumulation of nanocarriers in the brain of glioma-bearing nude mice, but not in the brain of normal nude mice. An important consideration to note is that RI-BK could assist the micelles consisting of different materials and of different sizes into the brain of glioma-bearing nude mice. Since B₂ receptors are highly expressed on human brain tumors, we decided to evaluate RI-BK as an adjunctive treatment for glioma[32]. The study of anti-glioma *in vivo* showed that RI-BK was superior to BK by extending additional 6.5 days of median survival time of glioma-bearing nude mice treated with PEG-PLA/PTX. Collectively, these survival data suggest that RI-BK can enhance the therapeutic efficacy of traditional chemotherapy drugs for glioma. These results are consistent with the high accumulation of nanocarriers in the brain tumor.

According to what we've know, many drugs based on the technology of nanocarrier were applied in clinic, such as liposomal doxorubicin (Doxil), Albumin-bound

nanoparticles of paclitaxel (Abraxane) and polymeric micelle formulation of paclitaxel (Genexol-PM) [33, 34]. In addition, it is reported that a B₂ receptor peptide agonist was discovered called Cereport (known commercially as labradimil and also formerly referred to as RMP-7). Previous studies have revealed that Cereport is capable of enhancing the BBTB transport of a number of drugs in pre-clinical and clinical trials [35, 36]. Cereport contains unnatural amino acids and the reduced peptide bond, so its preparation is complicated [35]. It is reported that half-life of Cereport in 20% rat plasma less than 90 minutes [37]. In addition, Cereport has lower affinity than BK to B₂ receptor [38]. In a summary, compare to the Cereport, RI-BK have higher affinity, better stability and simpler preparation. We envision that our findings may be promising translated into the clinic as an adjunctive treatment for malignant brain tumors.

In the present study, we described the novel B₂ receptor agonist RI-BK, which is characterized by proteolytic resistance. Because of the low level of B₂ receptor expression, low-dose RI-BK did not deleteriously affect the BBB. However, RI-BK did selectively open the BBTB by altering the expression level and distribution of ZO-1 and promoting the formation of stress fibers. Therefore, the combination of RI-BK with nanocarriers can facilitate delivery of antitumor drug to brain tumor tissue where it can exert its effect (Fig. 6).

Conflict of Interest

The authors declare no competing financial interest.

Acknowledgment

This work was supported by the National Science Foundation of China (Grant no. 81473148), the National Key Basic Research Program of China (Grant no. 2013CB932502), and the National Science Foundation of China (Grant no. 81473149).

Reference

- [1] L. Perryman, J.T. Erler, Brain Cancer Spreads, *Sci Transl Med*, 6 (2014).
- [2] D. Ricard, A. Idbaih, F. Ducray, M. Lahutte, K. Hoang-Xuan, J.Y. Delattre, Primary brain tumours in adults, *Lancet*, 379 (2012) 1984-1996.
- [3] A. Omuro, L.M. DeAngelis, Glioblastoma and Other Malignant Gliomas A Clinical Review, *JAMA-J. Am. Med. Assoc.*, 310 (2013) 1842-1850.
- [4] N.S. Ningaraj, M. Rao, K. Hashizume, K. Asotra, K.L. Black, Regulation of blood-brain tumor barrier permeability by calcium-activated potassium channels, *J. Pharmacol. Exp. Ther.*, 301 (2002) 838-851.
- [5] R. Daneman, The blood-brain barrier in health and disease, *Ann Neurol*, 72 (2012) 648-672.

- [6] C.M.L. vanHerpen, Q. vanHoesel, C.J.A. Punt, Paclitaxel-induced severe hypersensitivity reaction occurring as a late toxicity, *Ann. Oncol.*, 6 (1995) 852-852.
- [7] F.C. Campos, V.J. Victorino, M.C. Martins-Pinge, A.L. Cecchini, C. Panis, R. Cecchini, Systemic toxicity induced by paclitaxel in vivo is associated with the solvent cremophor EL through oxidative stress-driven mechanisms, *Food Chem. Toxicol.*, 68 (2014) 78-86.
- [8] A. Sharma, E. Mayhew, L. Bolcsak, C. Cavanaugh, P. Harmon, A. Janoff, R.J. Bernacki, Activity of paclitaxel liposome formulations against human ovarian tumor xenografts, *Int. J. Cancer*, 71 (1997) 103-107.
- [9] V.P. Torchilin, Targeted polymeric micelles for delivery of poorly soluble drugs, *Cell. Mol. Life Sci.*, 61 (2004) 2549-2559.
- [10] K. Bourzac, NANOTECHNOLOGY Carrying drugs, *Nature*, 491 (2012) S58-S60.
- [11] M. Aryal, N. Vykhodtseva, Y.Z. Zhang, J. Park, N. McDannold, Multiple treatments with liposomal doxorubicin and ultrasound-induced disruption of blood-tumor and blood-brain barriers improve outcomes in a rat glioma model, *J. Control. Release*, 169 (2013) 103-111.
- [12] Y. Chen, L. Liu, Modern methods for delivery of drugs across the blood-brain barrier, *Advanced Drug Delivery Reviews*, 64 (2012) 640-665.
- [13] T. Inamura, K.L. Black, BRADYKININ SELECTIVELY OPENS BLOOD-TUMOR BARRIER IN EXPERIMENTAL BRAIN-TUMORS, *J. Cereb. Blood Flow Metab.*, 14 (1994) 862-870.
- [14] P.J. Elliott, N.J. Hayward, M.R. Huff, T.L. Nagle, K.L. Black, R.T. Bartus, Unlocking the blood-brain barrier: A role for RMP-7 in brain tumor therapy, *Exp. Neurol.*, 141 (1996) 214-224.
- [15] F. Marceau, M. Gendreau, J. Barabe, S. Stpierre, D. Regoli, THE DEGRADATION OF BRADYKININ (BK) AND OF DES-ARG-9-BK IN PLASMA, *Can. J. Physiol. Pharmacol.*, 59 (1981) 131-138.
- [16] G. Guichard, N. Benkirane, G. Zederlutz, M.H.V. Vanregenmortel, J.P. Briand, S. Muller, ANTIGENIC MIMICRY OF NATURAL L-PEPTIDES WITH RETRO-INVERSO-PEPTIDOMIMETICS, *Proc. Natl. Acad. Sci. U. S. A.*, 91 (1994) 9765-9769.
- [17] J. Wang, Y. Lei, C. Xie, W. Lu, E. Wagner, Z. Xie, J. Gao, X. Zhang, Z. Yan, M. Liu, Retro-inverso CendR peptide-mediated polyethyleneimine for intracranial glioblastoma-targeting gene therapy, *Bioconjug Chem*, 25 (2014) 414-423.
- [18] X. Wei, C. Zhan, Q. Shen, W. Fu, C. Xie, J. Gao, C. Peng, P. Zheng, W. Lu, A d-Peptide ligand of nicotine acetylcholine receptors for brain-targeted drug delivery, *Angew Chem Int Ed Engl*, 54 (2015) 3023-3027.

- [19] S.F. Altschul, T.L. Madden, A.A. Schaffer, J.H. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nucleic Acids Res*, 25 (1997) 3389-3402.
- [20] M.A. Larkin, G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J. Gibson, D.G. Higgins, Clustal W and clustal X version 2.0, *Bioinformatics*, 23 (2007) 2947-2948.
- [21] R.A. Laskowski, J.A.C. Rullmann, M.W. MacArthur, R. Kaptein, J.M. Thornton, AQUA and PROCHECK-NMR: Programs for checking the quality of protein structures solved by NMR, *J Biomol Nmr*, 8 (1996) 477-486.
- [22] H. Kaur, A. Garg, G.P.S. Raghava, PEPstr: A de novo method for tertiary structure prediction of small bioactive peptides, *Protein Peptide Lett*, 14 (2007) 626-631.
- [23] D.W. Ritchie, G.J. Kemp, Protein docking using spherical polar Fourier correlations, *Proteins*, 39 (2000) 178-194.
- [24] B. Raveh, N. London, O. Schueler-Furman, Sub-angstrom modeling of complexes between flexible peptides and globular proteins, *Proteins*, 78 (2010) 2029-2040.
- [25] V.K. Mulligan, *Working with D-Amino Acids in Rosetta*, 2014.
- [26] R. Wang, L. Lai, S. Wang, Further development and validation of empirical scoring functions for structure-based binding affinity prediction, *Journal of computer-aided molecular design*, 16 (2002) 11-26.
- [27] C.Y. Wang, J. Mallela, S. Mohapatra, Pharmacokinetics of Polymeric Micelles for Cancer Treatment, *Curr. Drug Metab.*, 14 (2013) 900-909.
- [28] Z. Ahmad, A. Shah, M. Siddiq, H.B. Kraatz, Polymeric micelles as drug delivery vehicles, *RSC Adv.*, 4 (2014) 17028-17038.
- [29] H. Wu, L. Zhu, V.P. Torchilin, pH-sensitive poly(histidine)-PEG/DSPE-PEG co-polymer micelles for cytosolic drug delivery, *Biomaterials*, 34 (2013) 1213-1222.
- [30] W. Xu, I.A. Siddiqui, M. Nihal, S. Pilla, K. Rosenthal, H. Mukhtar, S. Gong, Aptamer-conjugated and doxorubicin-loaded unimolecular micelles for targeted therapy of prostate cancer, *Biomaterials*, 34 (2013) 5244-5253.
- [31] L. Yao, Q. Song, W. Bai, J. Zhang, D. Miao, M. Jiang, Y. Wang, Z. Shen, Q. Hu, X. Gu, M. Huang, G. Zheng, X. Gao, B. Hu, J. Chen, H. Chen, Facilitated brain delivery of poly (ethylene glycol)-poly (lactic acid) nanoparticles by microbubble-enhanced unfocused ultrasound, *Biomaterials*, 35 (2014) 3384-3395.
- [32] D.M. Raidoo, S. Sawant, R. Mahabeer, K.D. Bhoola, Kinin receptors are expressed in human astrocytic tumour cells, *Immunopharmacology*, 43 (1999) 255-263.

- [33] C. Riggio, E. Pagni, V. Raffa, A. Cuschieri, Nano-Oncology: Clinical Application for Cancer Therapy and Future Perspectives, *Journal of Nanomaterials*, 2011 (2011) 1-10.
- [34] E.K. Chow, D. Ho, Cancer nanomedicine: from drug delivery to imaging, *Sci Transl Med*, 5 (2013) 216rv214.
- [35] D.F. Emerich, R.L. Dean, C. Osborn, R.T. Bartus, The development of the bradykinin agonist labradimil as a means to increase the permeability of the blood-brain barrier - From concept to clinical evaluation, *Clin. Pharmacokinet.*, 40 (2001) 105-123.
- [36] C.V. Borlongan, D.F. Emerich, Facilitation of drug entry into the CNS via transient permeation of blood brain barrier: laboratory and preliminary clinical evidence from bradykinin receptor agonist, *Cereport, Brain Res Bull*, 60 (2003) 297-306.
- [37] J.A. Straub, A. Akiyama, P. Parmar, In vitro plasma metabolism of RMP-7, *Pharm Res*, 11 (1994) 1673-1676.
- [38] S. Belanger, V. Bovenzi, J. Cote, W. Neugebauer, M. Amblard, J. Martinez, B. Lammek, M. Savard, F. Gobeil, Jr., Structure-activity relationships of novel peptide agonists of the human bradykinin B₂ receptor, *Peptides*, 30 (2009) 777-787.

Table 1. Experimental determined and predicted free energies of binding of the peptides with B₂ receptor ($\Delta G=1.3636\log K_d$)

Peptide	$K_d(\text{mol}\cdot\text{L}^{-1})$	Experimental ΔG ($\text{kcal}\cdot\text{mol}^{-1}$)	Predicted ΔG ($\text{kcal}\cdot\text{mol}^{-1}$)
BK	3.488×10^{-9}	-11.53	-12.07
RI-BK	8.402×10^{-11}	-13.70	-12.78

Fig. 1. The 3D structure of BK (yellow) and RI-BK (pink)

Fig. 2. Binding mode of BK (yellow) and RI-BK (pink) in B₂ receptor. The B₂ receptor is shown in white, residues involved in binding are represented with sticks, and hydrogen bonds are denoted by a black dashed line.

Fig. 3. Uptake efficiency of FITC-labeled peptides in different types of cells and inhibitory effects of KD, RI-KD, RI-BK on the uptake of RI-BK-FITC in C6 cells analyzed by confocal laser scanning microscopy (CLSM) or flow cytometry. (A) Control; (B) FITC-BK with C6 cells; (C) RI-BK-FITC with C6 cells; (D) inhibition by KD; (E) RI-BK-FITC with bEnd.3; (F) RI-BK-FITC with HUVECs (blue, DAPI-stained cell nuclei; green, FITC-labeled peptides; scale bars, 10 μ m). (G) Quantitative analysis of the amount of internalized FITC-labeled peptide based on the CLSM results. (H) Inhibitory effects of BK analogs were quantified by flow cytometry.

Fig. 4. Immunofluorescence localization of ZO-1 (A–F) and F-actin (G–L) in BBB model (A–C, G–I) and BBTB model (D–F, J–L) after infusion with BK or RI-BK for 15 min analyzed by confocal laser scanning microscopy. Left, control; middle, BK treated; right, RI-BK treated (blue, DAPI-stained cell nuclei; green in A–F, FITC-labeled ZO-1; green in G–L, FITC-labeled F-actin; scale bars in A–F, 50 μ m; scale bars in G–L, 20 μ m; yellow arrow, absence of ZO-1; red arrow, stress fiber). (M) Western blot analysis of protein level of ZO-1 revealed that BK and RI-BK induced different results in the BBB model and BBTB model

Fig. 5. Optical imaging results of excised normal (A) and glioma-bearing (B) brains at 24 h after administration (top brain = saline; middle brain = BK; bottom brain = RI-BK). Biodistribution of coumarin-6-loaded nanocarriers in the brain of normal nude mice (C) and glioma-bearing nude mice (D) was quantitatively analyzed 15 min after i.v. injection of nanocarriers. (E) Kaplan–Meier survival curves of glioma-bearing nude mice (n = 10).

Fig. 6. Overview of how RI-BK selectively opens the BBTB to nanocarriers, resulting in antitumor drug delivery to the glioma.

Figure 1

BK

RI-BK

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6