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β-elemene combined with temozolomide in treatment of brain glioma

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ABSTRACT

Temozolomide (TMZ) is a widely used chemotherapeutic agent for malignant glioma. β-Elemene has been reported to have the ability of passing through the blood-brain barrier and reverse multidrug resistance. In the present study, transport of drugs through the in vitro blood-brain barrier (BBB) model also suggested that β-elemene can assist in TMZ transport to the brain. Plasma and brain pharmacokinetics demonstrated that when β-elemene is used in combination with TMZ, the metabolic rate of TMZ in plasma is slowed, and mean residence time (MRT) in brain is prolonged. The brain tissue distribution at 1 h indicated that the combination of TMZ and β-elemene promotes the distribution of β-elemene in the brain but slightly reduces the distribution of TMZ in the brain. Furthermore the antitumor effect and toxicity in vivo were also investigated. The combination of β-elemene and TMZ was well tolerated and significantly inhibited tumor growth in glioma xenografts. In summary, the present study indicates a synergistic antitumor effect of β-elemene and TMZ in glioma.

1. Introduction

Glioma is the most common central nervous system tumor, accounting for approximately 45% of intracranial tumors [1]. For the treatment of malignant gliomas, the most fundamental solution is surgical resection [2,3]. However, due to the extensive invasion of malignant gliomas, the surrounding normal brain tissues cannot be distinguished from malignant tissues by imaging examination or intraoperative microscopy. At the boundary of the brain, it is difficult to completely remove gliomas during surgery. The postoperative recurrence rate is extremely high. The 1-year survival rate of patients who only undergo surgery is approximately 5% [4]. The current standard treatment for malignant glioma is surgical resection followed by radiotherapy combined with concurrent and/or adjuvant TMZ chemotherapy [5,6].

TMZ is a member of a class of DNA-alkylating antitumor drugs shown to have good efficacy over the past 10 years [7]. Compared with traditional chemotherapy drugs, TMZ can better improve the recovery of

patients with glioma. One study showed that patients who underwent accepted surgical operations and were supplemented with radiotherapy and TMZ had significantly longer survival rates [8].

TMZ could alkylate the DNA at the N7 or O6 position of guanine residues to achieve therapeutic effect [9], and it was demonstrated that high expression levels of the cellular repair enzyme O6-methylguanin-DNA-methltransferase (MGMT) could protect tumor cells from the cytotoxic impact of TMZ, which resulted in treatment resistance [10,11]. However, in some newly treated patients with glioma and a large number of patients with recurrent malignant glioma, the promoter of MGMT is unmethylated, and the expression is positive. It is difficult for these patients to benefit from TMZ chemotherapy, and high-dose TMZ chemotherapy can also increase toxicity and side effects. In recent years, further improving the efficacy of and reversing resistance to TMZ by combining it with other chemotherapy drugs has been an area of much glioma research [12].

β-Elemene (ELE) is a compound extracted from the medicinal herb Curcuma wenyujin [13]. It has a broad spectrum of antitumor activity

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[14–16], high efficiency, and low toxicity and can pass through the blood-brain barrier [17,18]. Compared with traditional chemotherapeutic drugs, in addition to its tumor killing effect, it can also have an immune-protective effect. At the same time, it has a particularly marked killing effect on tumor cells with high expression of multidrug resistance genes [19]. The mechanism of action may be related to the reversal of multidrug resistance gene expression [13,20,21]. There have been few reports on the use of TMZ and elemene in combination in anti-glioma studies in vitro or in vivo [22,23]. We carried out this study to systematically study the effect of the combination of β-elemene and TMZ. The ability of drugs to penetrate the BBB in vitro was investigated. Plasma and brain pharmacokinetics were investigated in detail, and the distribution in brain tissue at 1 h was also examined. We also investigated the antitumor effect and toxicity in vivo.

2. Materials and methods

2.1. Materials

The elemene injection (β-E) was purchased from Dalian Jingang Medicine Company (Dalian, China). Penicillin-streptomycin, RPMI1640 media, fetal bovine serum (FBS), 0.25% (w/v) trypsin, and 0.03% (w/v) EDTA solution were purchased from Gibco (Australia). Methanol (HPLC grade) was obtained from Hangzhou Hede Chemical Co. Ltd (Hangzhou, China). Acetonitrile (HPLC grade) was obtained from Nanjing Xinhuayuan Chemical Agents Co. (Nanjing, China). Temozolomide was purchased from Guangzhou Aichun Pharmaceutical Technology Co., Ltd. Diazepam was obtained from Zhejiang Medicon Trading Co., Ltd. Sodium salicylate was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd.

2.2. In vitro BBB model setup

Both mouse brain endothelial cells (bEnd3) and rat glioma (C6) were first cultured in complete media (DMEM with 10% FBS and 1% P/S) in a flask at 37 °C in a humidified incubator with 5% CO_2 to reach confluence before being moved to inserts. Next, C6 cells were seeded on the bottom of 24-well plates at a density of 1×10^5 cells/cm^2. After 48 h of adhesion, endothelial cells were seeded onto the upper side of 6.5 mm Transwell® collagen-coated 0.4-μm-pore polytetrafluoroethylene membrane inserts at a density of 5×10^4 cells per cm^2, and the inserts were placed in 24-well plates containing C6. The well plates with inserts were incubated, the fluid was changed every day, and the cell growth status was observed under an inverted microscope. After approximately 2–3 days, a transepithelial electrical resistance (TEER) experiment was performed on the model after the cells were confluent to prove that the in vitro BBB model was successfully established and reached the standard for the next experiment.

TEER values were obtained by applying a transendothelial current to the membrane testing the membrane potential generated, and finally translating the value into resistance (current, Ohm) multiplied by the area (cm^2) of the endothelial monolayer (Ohm cm^2) [24]. We used Millicell® ERS-2 to measure and plot the transendothelial resistance of the model. The electrode was inserted vertically into the Transwell cell, with the short electrode in the upper chamber, the long electrode in the lower chamber, and the electrode pad below the liquid level. The resistance value of the display (Ω) was recorded, and the effective resistance value was calculated.

2.3. Drug transport through the in vitro BBB model

The co-cultured BBB model described above was then used to test the permeability of the different drugs. A total of 100 μL of drug-containing culture medium, β-E (500 μg/mL), TMZ (500 μg/mL) and T + β-E (the mass ratios of TMZ and β-elemene were 1:1, 1:2 and 1:3) were added to the Transwell chamber. At 0.5, 1, 2 and 3 h, 10 μL of the culture solution in the lower chamber was collected, 190 μL of acetonitrile was added for extraction, and the supernatant was collected after centrifugation at 13,000 rpm for 10 min. The supernatant was taken for HPLC analysis of the β-elemene and TMZ that had passed through the BBB.

2.4. In vivo pharmacokinetic studies and the distribution of drugs in the brain

ICR mice (20 ± 2 g) were used to investigate the pharmacokinetics and brain distribution of different drugs. The experiment was divided into four groups: tail vein injection of β-E (40 mg/kg, iv), intragastric administration of TMZ (30 mg/kg, po) and TMZ + β-E (30 + 40, po + iv). Blood and brain samples were collected at different time points (5, 15, 30, 60, 120 and 240 min). EDTA-Na$_2$ was used as an anticoagulant, and samples were centrifuged at 4000 rpm for 10 min to obtain the plasma. For β-elemene, diazepam was added to the collected plasma as the internal standard and acetonitrile was used to extract β-elemene and diazepam from the plasma. Similar to the β-elemene assay in plasma, sodium salicylate was used as internal standard for TMZ.

The brains of the sacrificed mice in the pharmacokinetic studies were collected immediately and homogenized in an ice water bath. Then, the brain homogenate was handled in the same way as the plasma, and the drug concentration at different time points was measured by HPLC assay.

The entire animal protocol was reviewed and approved by the ethics committee of the Animal Experiment Center (approval ZJAMS20180616) prior to conducting the experiments.

2.5. Tissue distribution

Tissue distribution studies were performed to quantitatively measure the concentrations of TMZ and β-elemene in different organs. Twelve ICR mice (20 ± 2 g) were divided into three groups: tail vein injection of β-E (40 mg/kg, iv.), intragastric administration of TMZ (30 mg/kg, po) and TMZ + β-E (30 + 40, po + iv). Mice were sacrificed at 1 h post-treatment to harvest the heart, liver, spleen, lung and kidney. Tissue samples (heart, liver, spleen, lung, kidney and brain) were processed and analyzed in the same way as the brain (see Section 2.4).

2.6. In vivo antitumor efficacy

To verify the antitumor effect in vivo, we used two brain tumor models, the U87 and GL261 in situ brain tumor models. Cultured U87MG-Luc cells were inoculated into the brains of BALB/c nude mice (male) and GL261-Luc cells were inoculated into the brains of C57 mice (male) to establish a brain glioma model. Briefly, anesthetized mice were fixed on a stereotaxic instrument. The top center scalp was cut in the middle and stripped to both sides. A dental microdrill was used to open a small hole in the skull at the right rear of the center. A 26G syringe needle connected to a microsyringe by a catheter was inserted to a depth of 3 mm. After 5 μL of cell suspension (1×10^5/μL) was infused slowly (1 μL/min) into the brain, the scalp wound was sutured. Bioluminescence imaging (BLI) obtained by an IVIS Lumina LT Series III (PerkinElmer, USA) was used to ensure the success of transplanted carcinoma one or two weeks after inoculation. The mice were randomly divided into four groups: normal saline, β-E (40 mg/kg, iv), TMZ (30 mg/kg, po) and TMZ + β-E (30 + 40, po + iv). Drugs were administered once a day for four days. The survival rate and body weight were recorded. Bioluminescence imaging was performed using the Xenogen IVIS Lumina LT system (Caliper Life Sicence, USA). Fifteen minutes after intraperitoneal injection of D-luciferin potassium salt (75 mg/kg), animals were imaged, and the same procedure was repeated at the specified time.

2.7. In vivo toxicity

The in vivo toxicity of different drugs was investigated in healthy male ICR mice (20 ± 2 g, 6–8 weeks, four groups, n = 10). ICR mice were administered normal saline, β-E (40 mg/kg, iv), TMZ (30 mg/kg, po) and TMZ + β-E (30 + 40, po + iv). Saline was used as the control. All mice received only one treatment. Their body weights and behaviors were recorded and monitored for one week; the mice were then sacrificed one week posttreatment. Blood was collected and centrifuged at 4000 rpm for 10 min to obtain the serum. Levels of alanine aminotransferase (ALT), total protein (TP), total bilirubin (T-BIL), γ-globulin (GLOB), serum albumin (ALB), ALB/GLOB (A/G), blood urea nitrogen (BUN) and uric acid (UA) were assayed as indicators of hepatic and renal function. Red blood cells (RBCs), white blood cells (WBCs), platelets (PLTs), hemoglobin (HGB) and hematocrit (HCT) were quantified for the detection of myelosuppression. Organs (liver, lung and kidney) were fixed and sectioned for H&E staining to evaluate organ-specific toxicity [25].

3. Results and discussion

3.1. Drugs traverse the BBB model in vitro

To investigate whether β-E possesses the ability to assist TMZ in traversing the BBB, we performed a BBB transcytosis assay in vitro. In the BBB transcytosis assay using in vitro models constructed of mouse brain endothelial cells (b.End3) and astrocytes (C6) (Fig. 1A), we found that β-E effectively assisted TMZ in traversing the BBB (Fig. 1B). Compared with the control group of TMZ, β-elemene assisted a much greater amount of TMZ to traverse the BBB. Without β-elemene, only 0.68% of TMZ had passed at 0.5 h. When the same amount of β-elemene was added, the penetration increased to 6.25%, and the traversed amount reached the highest value at 1 h.

To evaluate the ability of different ratios of β-elemene to assist TMZ in crossing the blood-brain barrier, we tried three ratios: 1:1, 1:2 and 1:3. When the mass ratio of TMZ and β-elemene was 1:1, there was already a notable promotion effect. When the ratio of β-elemene was increased, the amount of TMZ passing through the BBB also increased, but the increase was not significant. Together, these results demonstrated that β-elemene has the ability to assist TMZ in traverseing the BBB.

3.2. In vivo pharmacokinetic studies and the distribution of drugs in the brain

The pharmacokinetics of TMZ and β-elemene were investigated in healthy ICR mice. After intravenous administration of β-E (40 mg/kg) with intragastric administration of TMZ (30 mg/kg), the concentrations of TMZ and β-elemene in plasma and the brain were measured at 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h and 24 h posttreatment. There was a dose relationship between the concentration of TMZ in plasma and the brain (Fig. 2A and B), when the concentration of TMZ in brain was

decreased, the concentration of TMZ in plasma was increased. Compared with TMZ (30 mg/kg) alone, when TMZ (30 mg/kg) was used in combination with β-E (40 mg/kg), the concentration of TMZ in brain was reduced, and the metabolic rate was slightly accelerated. In contrast, following administration of the combination of β-E (40 mg/kg) and TMZ (30 mg/kg), the highest concentration of β-elemene in brain was increased, but the metabolic rate was also accelerated.

Table 1 summarizes the pharmacokinetic parameters of TMZ and β-elemene in plasma and the brain. The C_{max} value of TMZ in the brain decreased when TMZ was combined with β-elemene(T+β) (20.62 μg/g vs. 8.54 μg/g) but slightly increased in plasma (25.44 μg/mL vs. 20.96 μg/mL). However, the C_{max} value of β-elemene in the brain was increased in T+β group (49.08 μg/g vs. 82.06 μg/g). The AUC_{0-t} value in the brain of the TMZ group was much higher than that of the T+β group (53.30 μg/g*h vs. 28.93 μg/g*h), but that of β-elemene improved (53.63 μg/g*h vs. 68.24 μg/g*h). The $t_{1/2}$ of TMZ in the brains of T+β group was decreased from 4.20 h to 3.78 h, meanwhile the MRT values was increased from 3.35 h to 3.75 h. The $t_{1/2}$ and MRT values of β-elemene in T+β group were both decreased (2.18 h vs. 0.98 h, 2.12 h vs.0.93 h, respectively). The parameters of plasma showed that the AUC_{0-t} value of TMZ was increased from 39.85 μg/g*h to 64.61 μg/g*h and the $t_{1/2}$ was prolong from 4.04 h to 11.96 h in T+β group.

The results demonstrated that the combination could slow the metabolic rate of TMZ in plasma and prolong the MRT of TMZ in brain.

3.3. In vivo biodistribution

The tissue distribution reflected the location of the drug. To investigate whether the combination of TMZ and β-elemene affects their respective distributions in organ tissues, we examined the distribution in the main organs 1 h after administration of the drug. β-elemene (40 mg/kg) was administered by intravenous injection, and TMZ (30 mg/kg) was administered intragastrically. At 1 h postinjection, the heart, liver, spleen, lung, kidney and brain were harvested to measure the concentration of β-elemene and/or TMZ in each tissue. As shown in Fig. 2E, β-elemene had the advantage of penetrating the blood-brain barrier and was preferentially distributed to brain, as has been reported in previous literature [17,18]. Compared with that in the β-elemene and TMZ only groups, the distribution of total drugs in brain was highly improved. The comprehensive results indicate that the combination of TMZ and β-elemene promotes the distribution of drugs in the brain.

3.4. Anti-glioma effect

We used U-87MG orthotopic xenograft models to confirm the results of the combination treatment of TMZ and β-elemene (T+β) in vivo. TMZ at 30 mg/kg (T30) and β-E at 40 mg/kg (β40) were administered by tail vein injection and intragastric administration, respectively. At day 99 after treatment, mice were sacrificed for Western blot and immunohistochemistry assays. The T+β group exhibited visible regression of tumor growth (Fig. 3C) and thus an extended survival time (median survival 93
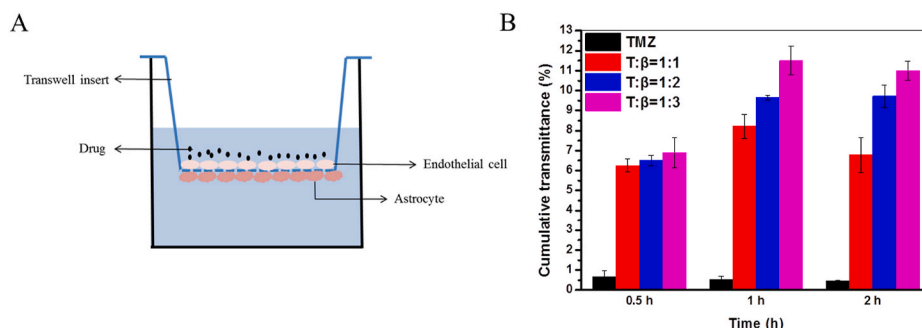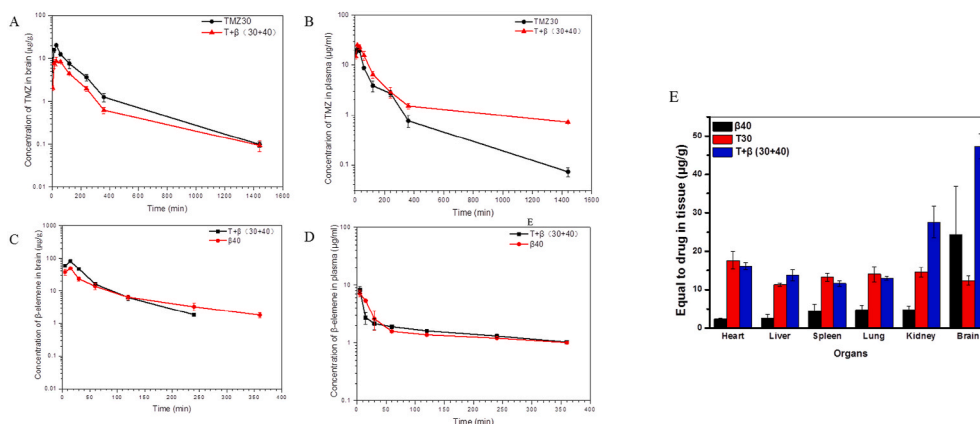

Fig. 1. In vitro evaluation in U-87MG cells. (A) Illustration of the in vitro BBB model. (B) Transcytosis of TMZ in an in vitro BBB model.

Fig. 2. Pharmacokinetics (A–D) and 1 h biodistribution (E) after administration TMZ and β-elemene were administered by oral and tail vein injection respectively. Drug concentrations of TMZ (A, B) and β-elemene (C, D) in the mouse brain and plasma.

Table 1
Pharmacokinetic parameters of TMZ and β-elemene in the brain and plasma.

Parameter of Brain	Unit	TMZ		β-elemene	
		T30	T+β (30+40)	β40	T+β (30+40)
$t_{1/2}$	h	4.20	3.78	2.18	0.98
T_{max}	h	0.50	0.50	0.25	0.25
C_{max}	μg/g	20.62	8.54	49.08	82.06
AUC_{0-t}	μg/g•h	53.30	28.93	53.63	68.24
MRT	h	3.35	3.75	2.12	0.93
Vz	(mg/kg)/(μg/g)	3.37	5.56	2.13	0.80
Cl	(mg/kg)/(μg/g)/h	0.56	1.02	0.67	0.56
Parameter of plasma	**Unit**	**TMZ**		**β-elemene**	
		T30	**T+β (30+40)**	**β40**	**T+β (30+40)**
$t_{1/2}$	h	4.04	11.96	9.09	6.16
T_{max}	h	0.25	0.25	0.08	0.08
C_{max}	μg/mL	20.96	25.44	7.19	8.35
AUC_{0-t}	μg/mL•h	39.85	64.61	10.01	10.53
MRT	h	2.96	10.68	11.84	8.12
Vz	(mg/kg)/(μg/mL)	4.34	6.71	22.52	18.06
Cl	(mg/kg)/(μg/mL)/h	0.74	0.39	1.72	2.03

days) (Fig. 3A), which was a significant improvement over those in the T30 (median survival 74 days) and β40 (median survival 33 days) groups. Fig. 3B shows the body weight changes of the tumor-bearing mice during the study. The mice experienced serious weight loss before death, and the T+β group showed delayed body weight loss compared to that in the other treatments, which might have been the combined result of effective tumor regression and lower toxicity. It has been reported in the literature that β-elemene can improve immunity [26].

Immunohistochemistry analyses demonstrated that the combination treatment significantly promoted the apoptosis of xenograft tumor cells (Fig. 3D). The low expression of Ki-67 and PCNA indicated suppression of proliferation following treatment with the combined drugs.

Many patients with malignant glioma experience no therapeutic effect from alkylated chemotherapy drugs. The main mechanism by which tumor cells resist alkylating drugs in gliomas is the DNA repair process mediated by MGMT [27,28].

Western blot assay were used to evaluate the expression level of MGMT in glioma. The results showed that β-elemene had no effect on the expression of MGMT (Fig. 3E).

Together, these results indicate that β-elemene enhances the

antitumor effect of TMZ and the chemosensitivity of GBM to TMZ without MGMT level changes.

3.5. Evaluation of systemic toxicity

To investigate the potential side effects of treatment with the combination of TMZ and β-elemene in mice, their biosafety was evaluated. Healthy ICR mice were treated with the therapeutic dose used in brain tumor bearing mouse therapy. As shown in Fig. S1A, the mice treated with the combination of TMZ and β-elemene showed no significant differences in body weight from healthy mice. The main biochemical indicators also showed no abnormalities. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum albumin (ALB), alkaline phosphatase (ALP), total bilirubin (TBIL), blood urea nitrogen (BUN), creatinine (CRE) and uric acid (UA) were all within the normal range (Fig. S1B). None of the main organs (liver, lung and kidney) exhibited any significant pathological changes (Fig. S1C).

Blood toxicity is one of the primary toxicities of chemotherapy drugs, so routine blood tests were also conducted. Compared to the PBS-treated group, there was no noticeable blood toxicity in the T+β-treated group (Table S1). Together, these results indicate low systemic toxicity of combined treatment with TMZ and β-elemene.

4. Conclusion

This study was carried out in vitro and in vivo with TMZ combined with β-elemene. An in vitro BBB model was used to evaluate BBB penetration, which proved that β-elemene enables TMZ to enter the brain. Pharmacokinetics demonstrated that TMZ combined with β-elemene could slow the metabolic rate of TMZ in plasma and prolong the MRT of TMZ in brain. The tissue distribution indicated that the drug combination could promote the distribution of β-elemene in the brain. Furthermore, the antitumor effect and toxicity analyses demonstrated that the combination of β-elemene and TMZ was well tolerated and significantly inhibited tumor growth in glioma xenografts. In summary, the study indicates a synergistic antitumor effect of β-elemene and TMZ in glioma.
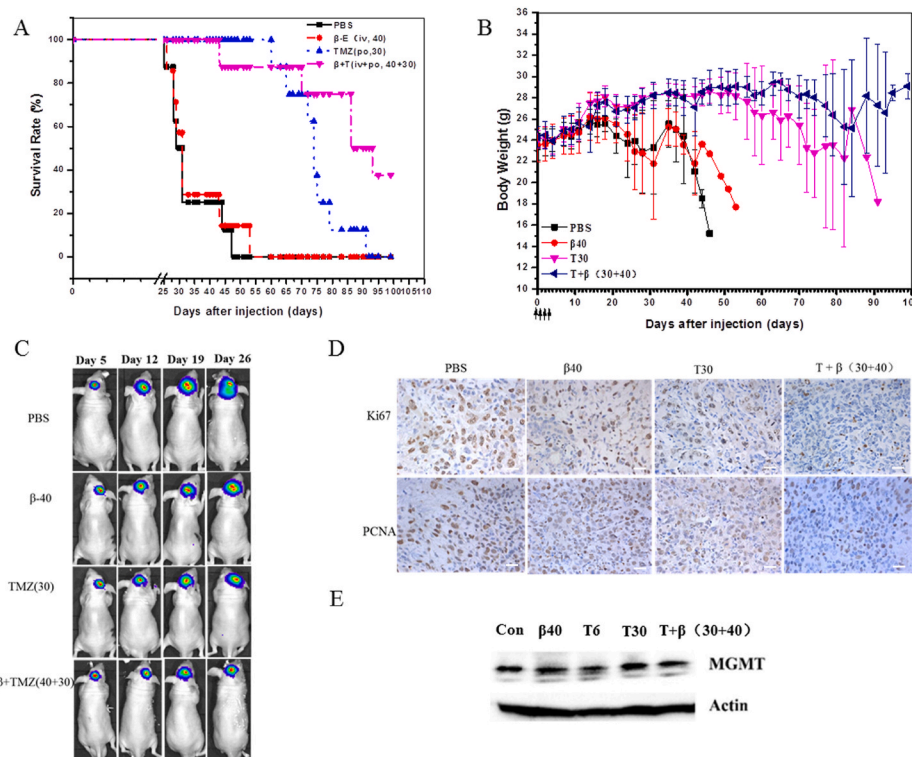
Fig. 3. In vivo antitumor effects (n = 10). (A) Survival curves in different groups. The arrows indicate drug administration times (B) Body weight changes in different groups (C) In vivo bioluminescence imaging images of GBM tumor cells in orthotopic mice. (D) Ki67 and PCNA-stained brain tissue sections after drug treatment (n = 3) (scale bar: 20 μm). (E) Western blot analysis of MGMT expression in brain tumors (MGMT 22 kDa, Actin 42 kDa).

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of the manuscript entitled.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi. org/10.1016/j.bbrep.2021.101144.

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