Supporting Information

Mustard-inspired delivery shuttle for enhanced blood-brain barrier penetration and effective drug delivery in glioma therapy

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1. Experimental section:

1.1. Methods:

1.1.1. Cell culture

C6 (rat glioma cells) and CaCo-2 (human colon carcinoma cell line) were purchased from Chinese Academy of Sciences Institute of Cell Resource Center (Shanghai, China). C6 cells were cultured in the medium of DMEM/F12k (1:1) with 10% FBS and 1%

penicillin-streptomycin. CaCo-2 cells were cultured in the medium of DMEM-high glucose with 20% FBS and 1% penicillin-streptomycin. Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Rat brain-derived microvascular endothelial cells (RBMEC) and corresponding complete medium were all purchased from CHI Scientific, Inc., Jiangyin, China. Cells were cultured at 37 °C with 5% CO_2.

1.1.2. Animals

BalB/C mice, BalB/C nude mice and KM mice were purchased from Shanghai SLAC Laboratory Animal CO. LTD. (Shanghai, China). The animals were housed in the specific pathogen-free animal facility with free access to food and water. All animal experiments were conducted in agreement with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University and the IACUC of Shanghai Jiao Tong University were conducted in agreement with the guidelines of Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The protocol of animal experiments was approved by the Animal Experimentation Ethics Committee of Shanghai Jiao Tong University School of Pharmacy.

1.2. *In vitro* cytotoxicity of the materials

The cytotoxicity of delivery system was assessed using MTT assay. C6 cells and RBMEC cells were seeded in a 96-well plate at a cell density of 1.0×10^4 cells per well and incubated for 24 h at 37 °C and 5% CO_2. All samples were incubated with the cells for 24 h. Before the MTT assay, each well was refilled with fresh medium. Then, 20.0 μL of 5.0 $mg \cdot mL^{-1}$ MTT (Beyotime Institute of Biotechnology, Shanghai, China) solution was added to each well and incubated for 4 h at 37 °C. After dissolving the formed

fomazan with 150.0 μL DMSO in each well, the plates were blended with shaker for 5 min. The absorbance (OD value) was measured at 490 nm with microplate reader.

1.3. Possible mechanism exploration

1.3.1. Cellular uptake under different conditions

Cellular uptake at different temperatures was performed to check whether the uptake process is energy-dependent. C6 glioma cells were seeded in 6-well plates at a density of 3×10^5 cells/well at 37 °C and in 5% CO_2. After cultured for 24 h and rinsed once with DMEM/ F12k (1:1) without FBS, C6 cells were treated with free SA (200 μM final concentration) and FITC labeled nBSA-SA nanoparticles, for 0, 5, 15, 30, 45 and 60 min at 4 °C and 37 °C, respectively. For free SA treated cells, the medium was collected for the detection of absorption of free SA at 313 nm with a Thermo Electron-EV300 UV-vis spectrophotometer at room temperature. For the nBSA-SA treated cells, they were rinsed with PBS twice and the cellular uptake was collected with a flow cytometer Accuri C6 (Becton, Dickinson and Company, Shanghai, China).

Confocal laser scanning microcopy (CLSM) was chosen to study the cellular uptake of FITC labeled nBSA-SA nanoparticles by C6 cells at 37 °C. C6 cells were seeded in glass-bottom 6-well plates at a density of 5.0×10^4 cells per well in 2.0 mL of complete DMEM/ F12k (1:1) medium and cultured for 24 h. Then the medium was replaced with 2.0 mL fresh medium containing 200 μL FITC labeled nBSA-SA nanogel (0.5 mg· mL^{-1}). The cells were incubated at 37 °C for 2 h. Then the culture medium was removed and the cells were rinsed with cold PBS three times and fixed with 4% paraformaldehyde for 30 min at room temperature. Subsequently, the cells were treated with 0.5% Triton X-100 in

ice bath for 15 min. Finally, the cells were stained with the nuclear dye DAPI as a positive control. Images of cells were acquired using a Zeiss LSM 5 Pascal Confocal laser scanning microscope (Carl Zeiss AG, Oberkochen, Germany) imaging system.

1.3.2. Expression of MDR1 in CaCo-2 cells with QRT-PCR

Total RNA was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was eluted in 60 μL nuclease-free water and the concentration was measured spectrophotometrically (Infinite® 200 NanoQuant, Tecan, Crailsheim, Germany). Isolated hypothalamic RNA was reverse-transcribed into cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen; Hilden, Germany). Primer sequences for MDR1 were designed using the Primer 3 algorithm (SDSC Biology Workbench, San Diego, CA, USA): forward, 50- CCCATCATTGCAATAGCAGG -30; reverse, 50- GTTCAAACTTCTGCTCCTGA-30. QRT-PCR was performed using Sensimix SYBR NoRox (Bioline, Luckenwalde, Germany) on 96-well format (Mastercycler® ep realplex epgradient S, Eppendorf, Hamburg, Germany). Gene expression levels of the samples were calculated from a standard curve generated from pooled cDNA of all samples (n = 6) and diluted by 2n in 8 steps. Sample cDNA were diluted 1:50 and measured in triplicates. Standards were measured in duplicates.

1.3.3. Western blot studies

After incubation with different concentrations of SA for 24 h with CaCo-2 cells and RBMEC cells with different concentrations of SA for 24 h and 200 μM for different time, the cells were washed twice with cold PBS and lysed with RIPA buffer (Solarbio, China) containing 1 mM phenylmethanesulfonyl fluoride (PMSF) (Solarbio, China). Protein

samples (40-80 μg) were electrophoresed on 10% sodium dodecyl sulfate–polyacrylamide gels. The proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane. Loading and transfer were then confirmed by Ponceau red staining. After preincubation in blocking solution at room temperature for 1 hour, the PVDF membrane was incubated with primary monoclonal antibody for P-gp (1:1,000) (Abcam, UK) with CaCo-2 cells and primary monoclonal antibody for Claudin-5 (1:1,000) (Abcam, UK) with RBMEC cells for 2.5 hours at room temperature. After being washed 3 times for 5 min with Tris-buffered saline (TBS) containing 0.5% Tween-20 (Solarbio, China), the membrane was incubated with a secondary antibody linked to 7 horseradish peroxidase (IgG, 1:10,000) (Santa cruz, USA) for 50 min at room temperature. Immunoreactive proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, USA).

1.3.4. Animal imaging

Whole-body *in vivo* biodistribution and brain accumulation in healthy BalB/C nude mice was examined using eXplore Optix MX animal optical molecular imaging system (General Electric Company, USA, Ex.=580 nm, Em. =692 nm). Tail vein injection of Cy5.5 labeled 0.5 $mg \cdot mL^{-1}$ (concentration of BSA) nBSA and nBSA-SA, 200.0 μL for each mouse. Then the mice were given general anesthesia with glutaraldehyde (2%). At time points of post-injection 0.5, 1, 2, 6 and 24 h, the mice were fixed with tapes and taken pictures from the back.

1.3.5. Fluorescence microscopic examination of traversing the BBB

Tail vein injection of FITC labeled 0.5 mg·mL^{-1} nBSA-SA of 5-6weeks healthy male BalB/C mice, 200.0 μL per mouse (n=5). The mice for fluorescence intensity test were sacrificed by cervical dislocation after i.p. injection for 1, 3, 9, 24, 48 and 72 h, respectively. Brain tissues of each mouse were separated, weighed and washed once with PBS. The brains were ultrasonic decomposition at a concentration of 100.0 mg weight tissue per milliliter of PBS. After centrifugation, the supernatant was collected and the fluorescence intensity of each sample was tested by Fluorescence spectrophotometer.

To further confirm the intracerebral location of the nanoparticles and the relationship with the blood vessels, FITC-labeled nanoparticles (200.0 μL injection volume; 0.5 mg·mL^{-1} nanoparticles in solution) were injected into the tail vein of 5-6 weeks healthy male BalB/C mice, 2 for each group. 1 h post treatment, the mice were sacrificed by cervical dislocation and their brains were separated and immersed in 4% paraformaldehyde overnight. The brain tissues were washed with PBS for 3 times, each for 30 min, and immersed in 20% sucrose solution overnight and kept in 4 °C. The brain slices were taken by freezing microtome section for 10.0 μm in thickness and kept in every 20 slices (Cryotome E, Thermo Fisher Scientific, USA). The air-dried slices were fixed with cold acetone for 10 min and washed with PBS for 3 times, 5 min for each time. Use EDTA antigen repair buffer (pH= 8.0) to repair the slices within microwave for 10 min. After sir-dried, the slices were washed with PBS for 3 times, 5 min for each. After blocking with 3% BSA in PBS for 30 min at room temperature, the slices were incubated with an antibody against PECAM- 1 (CD31) (BD Biosciences) overnight at 4 °C. Cy-3 conjugated secondary antibody (Invitrogen Carlsbad, CA) was applied for 1 h at room

temperature, together with DAPI. Samples were analyzed with a fluorescence microscope (Nikon Eclipse TI-SR, Japan).

1.3.6. Pharmacokinetics study

The pharmacokinetic properties of free SA, nBSA and nBSA-SA nanoparticles were investigated in a healthy mouse model using KM male mice (~40 g). The free SA and FITC labeded nBSA-SA nanoparticles were injected into the tail vein intravenously at a dose of 3.0 mg/kg (concentration of BSA) with three mice in each group (n=3). Time points selected for the nanoparticles were 0, 0.25, 0.5, 1, 1.5, 2, 3, 6, 10, 24, 48, and 72 h post-injection. At specified time points, mice were sacrificed and the blood samples were collected in coagulating tubes to sediment the blood cells. The samples were stored at 4 °C until analysis. The serums of free SA treated samples were analyzed using UV-vis. The absorbance of each sample at 313 nm was recorded. The residual content in plasma at each time point was calculated as follows:

$$\text{Residual content (\%)} = Abs_{\text{time point}} / Abs_{0h} \times 100$$

For the FITC labeled nBSA-SA treated group, the fluorescence intensity of each sample was tested by Fluorescence spectrophotometer.

1.3.7. Biodistribution in tissues

Tail vein injection of FITC labeled 0.5 $mg \cdot mL^{-1}$ nBSA and nBSA-SA of healthy 5-6 weeks male BalB/C mice, five mice for each group, 200.0 μL for each mouse. The mice treated with same amount of saline were used as a blank control. The mice for biodistribution test were sacrificed by cervical dislocation after bloodletting from the

retinal vein plexus 1 h and 24 h post-injection. The blood was collected in pro-coagulation tubes for getting the serum. Brain tissues, hearts, livers, spleens, lungs, kidneys and testis of each mouse were separated, weighed and washed three times with ice-cold PBS. The tissues were ultrasonic decomposition by Ultrasonic Processor (Scientz-IID, Ningbo Scientz Biotechnology CO,. LTD, Ningbo, China) at a concentration of 100.0 mg weight tissue per milliliter of PBS. After centrifugation, the supernatant was collected and the fluorescence intensity (FI) of each sample was tested by Fluorescence spectrophotometer. The fluorescence intensity of each experimental sample was corrected by subtracting the fluorescence intensity value of the blank control. The final results were expressed as the percentage of FI of each tissue to the summary of all the organs of each mouse.

1.4. *In vitro* drug release ability

In vitro TMZ release study was performed with a dialysis method. PB solution (pH 7.4 and 5.0) containing 0.1% (v/v) Tween 80 was used as the release media. TMZ-loaded nanoparticles AA-nBSA-SA-TMZ were placed into dialysis tubes (MWCO: 8000 Da) and tightly sealed. The dialysis tubes were added into 50 ml release media and incubated at 37 °C with gently oscillating for 48 h. At predetermined time points, 1.0 ml release media was sampled and replaced with equal volume of fresh release media. The samples were then analyzed by HPLC to determine the concentrations of TMZ.

1.5. Anti-tumor efficacy study *in vitro*

The anti-tumor efficacy of TMZ-loaded nanoparticles and free TMZ were measured with MTT assay. C6 cells were plated in 96-well plates at a density of 8×10^3 cells per

well and cultured for 24 h. AA-nBSA-TMZ, AA-nBSA-SA-TMZ and free-TMZ were diluted to predetermined concentrations with PBS, and added into each well for 24 h incubation. The final concentrations of TMZ were in the range of 0.4~250 $\mu g \cdot mL^{-1}$. Then 20 μL MTT (5.0 $mg \cdot mL^{-1}$ in PBS) was added into each well and incubated for 4 h under 37 °C. Finally the medium was removed and replaced by 150 μL DMSO. Then the absorbance was measured by a microplate reader at 490 nm. The cells treated with medium were evaluated as controls. Cell viability was calculated by the following formula:

Cell viability (%) = $Abs_{treated}/Abs_{control} \times 100$, in which $Abs_{treated}$ and $Abs_{control}$ represented the absorbance of treated cells and control cells, respectively. And the IC_{50} for each sample was collected as well, in which IC_{50} stood for half maximal inhibitory concentration.

1.6. Cellular uptake studies of TMZ-loaded nanoparticles

CLSM was chosen to study the cellular uptake of TMZ-loaded nanogel by C6 cells. C6 cells were seeded in glass-bottom 6-well plates at a density of 1.0×10^5 cells per well in 2.0 mL of complete medium and cultured for 24 h. Then the medium was replaced with 2.0 mL fresh medium containing 200 μL FITC labeled AA-nBSA-TMZ and AA-nBSA-SA-TMZ nanoparticles (0.5 $mg \cdot mL^{-1}$). The cells were incubated at 37 °C for 1 h. Then the culture medium was removed and the cells were rinsed with cold PBS three times and fixed with 4% paraformaldehyde for 30 min at room temperature. Subsequently, the cells were treated with 0.5% Triton X-100 in ice bath for 15 min. Finally, the cells were stained with the nuclear dye DAPI as a positive control. Images of cells were acquired

using a Zeiss LSM 5 Pascal Confocal laser scanning microscope (Carl Zeiss AG, Oberkochen, Germany) imaging system.

For flow cytometry, C6 cells were seeded in 6-well plates at a density of 2.0×10^5 cells per well in 2.0 mL complete medium and cultured for 24 h. Then the medium was replaced with 2.0 mL fresh medium containing 200 μL FITC-labeled AA-nBSA-TMZ and AA-nBSA-SA-TMZ nanoparticles (0.5 mg· mL^{-1}). Then, the cells were incubated at 37 °C for predetermined intervals, 0.25, 0.5, 1, 2 and 3 h. Thereafter, the culture medium was removed and the cells were washed with cold PBS three times and then harvested. The amount of intracellular fluorescent signal of FLTC was quantified using BD Accuri™ C6 flow cytometer, indicating the amount of nanoparticles internalized by C6 cells.

1.7. Construction of orthotopic glioma-bearing mice models

Orthotopic glioma-bearing mice models were constructed using C6 glioma cells. Briefly, 5 μL C6 cells (concentration: 5×10^5 cells·mL^{-1}) were slowly implanted into the right striatum (1.8 mm lateral to the bregma and 3 mm of depth) of BalB/C nude mice using a stereotactic fixation device with a mouse adaptor. After surgery, mice were further maintained under standard housing conditions.

1.8. Anti-glioma efficacy *in vivo*

Orthotropic transplantation C6 glioma bearing BalB/C mice were divided into 4 groups randomly (n =5). The mice were intravenously injected with free TMZ, AA-nBSA-TMZ and AA-nBSA-SA-TMZ (TMZ dose of 10.0 mg·kg^{-1}) at 5, 7, 9, 11, 13, 15, 17 and 19 days after implantation. Saline treated group was used as negative control. The survival

time and body weight of each group was recorded and analyzed. At the 20th day, mice were sacrificed, and the tissues (hearts, livers, spleens, lungs and kidneys) and brains were removed and cut into small pieces, fixed in 4% paraformaldehyde for 8 h. Then the tissues were embedded in paraffin and cut into 5 μm sections and placed on poly-L-lysine-coated slides for hematoxylin and eosin (H&E) staining and immunohistochemical analysis, respectively. Duplicate slices were placed on glass microscope slides and stained for histopathological analysis. Every second section was processed for immunohistochemistry. Brain slides were stained with DAPI and then subjected to TUNEL apoptosis detection kit to detect nuclear DNA fragments according to the manufacturer's instruction. Finally, the sections were counterstained with hematoxylin and prepared for mounting. The images were taken by using a Nikon ECLIPSE Ti microscope.

1.9. Statistical analysis

The results were analyzed using the Tukey's honestly significant difference test for multiple comparisons followed by John Wilder Tukey's test to identify significant differences between groups. Family-wise significance and confidence level was $p<0.05$. All results were expressed as means ± standard error of the mean (SEM).

2. Supplementary figures:

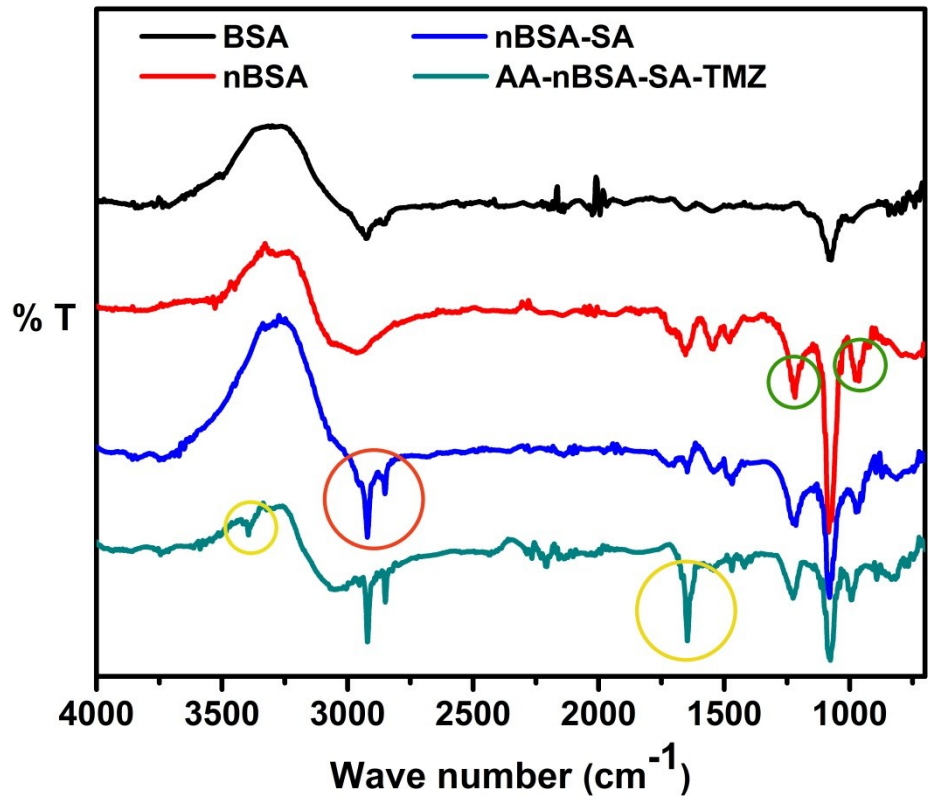

Figure S1. FT-IR spectra of the resulted nanoparticles. The green circles show the emergence of two new peaks of –C-N-C- at 1080 cm^{-1} and P=O at 1223.7 cm^{-1}, respectively, which confirms the successful modification of PMPC on BSA. Meanwhile, the red circle shows the emergence of a new peaks of SA at 2839 cm^{-1}, which confirms that SA have been successfully grafted onto nBSA nanoparticles. The yellow circles show the emergence of two new peaks of $–NH_2$ at 3378 cm^{-1} and C=O at 1678 cm^{-1}, respectively, which confirms the successful loading of TMZ on nanoparticles.

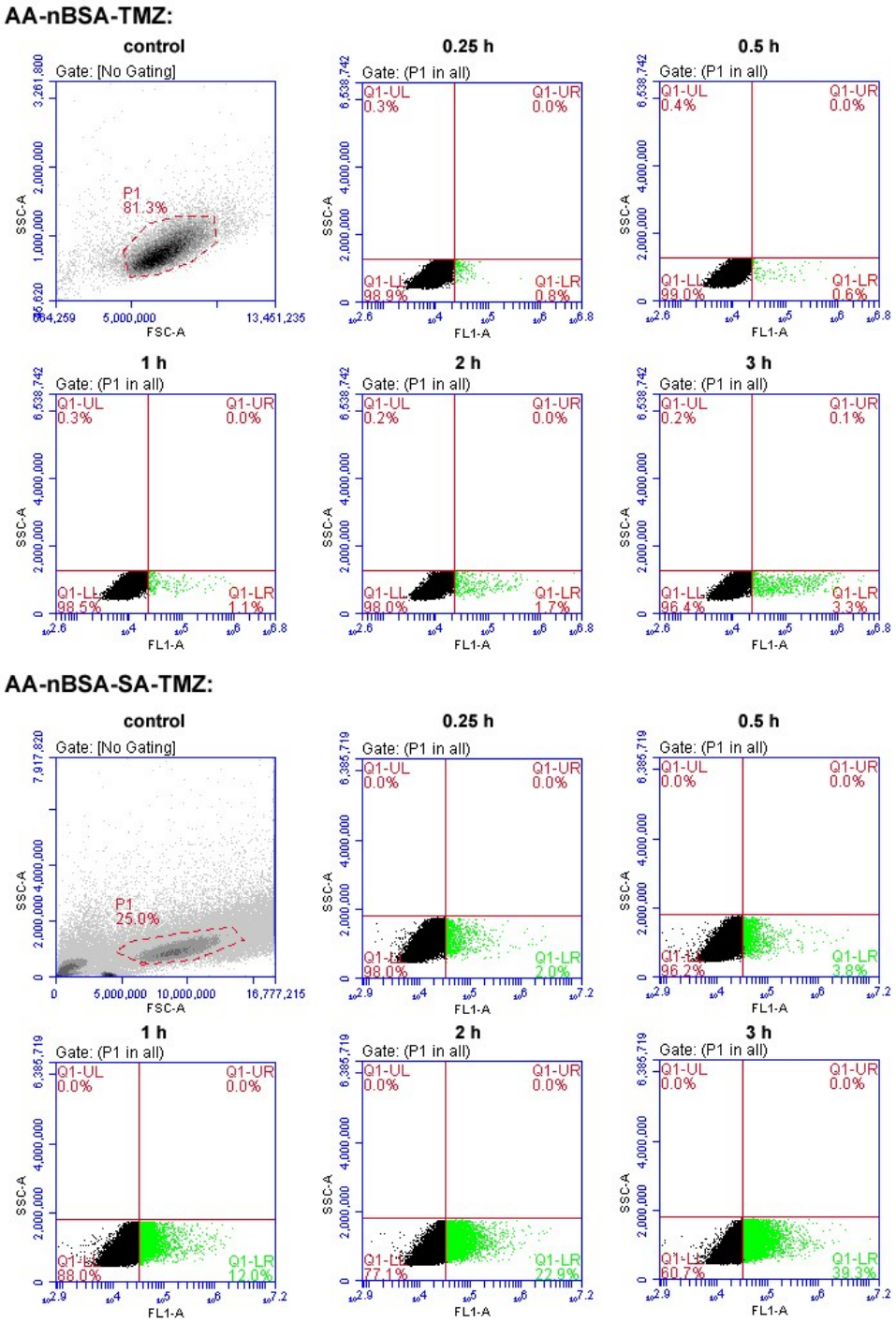

Figure S2. Raw flow cytometry data for AA-nBSA and AA-nBSA-SA nanoparticles co-culturing with cells from 0.25 h to 3 h. The data used in Figure 6c is the percentage number in the bottom-right quadrant for each time-point.

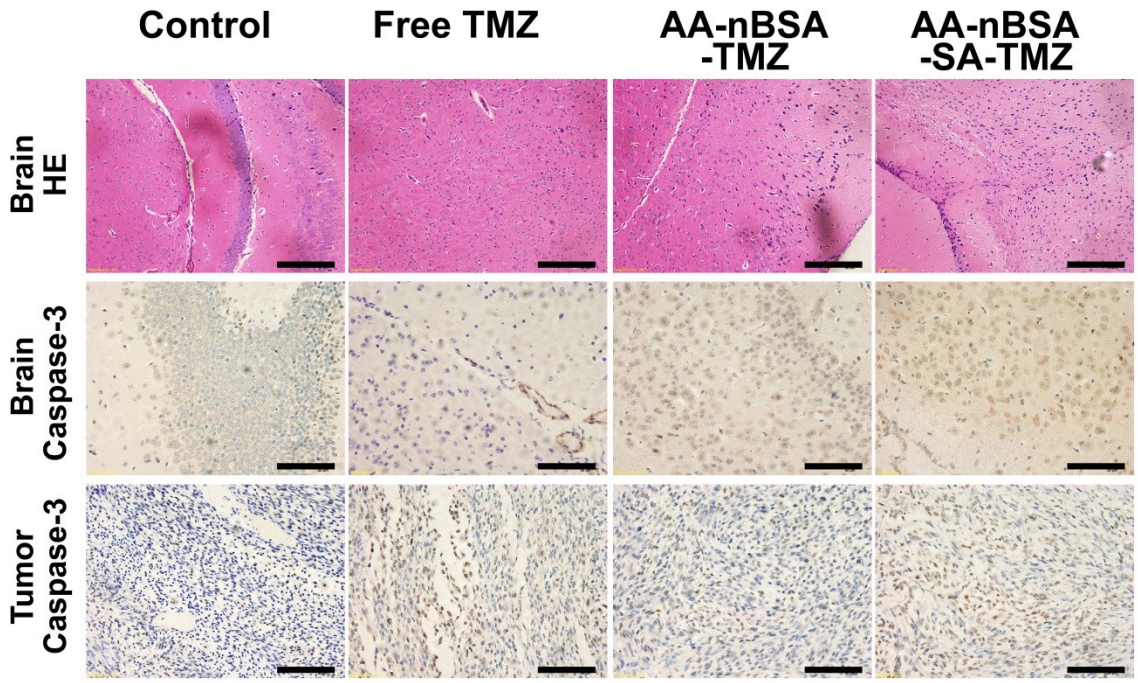

Figure S3. Histologic analyses of harvest brain tissues and tumor sections after treatment were made with: H&E staining (first row, scale bar is 100 μm), and caspase-3 immuno-histogram (positive: brown, scale bar is 50 μm).