

Image-Guided Chemotherapy with Specifically Tuning Blood Brain Barrier Permeability in Glioma Margins

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1. Supplemental materials and methods:

Materials.

All chemical reagents were obtained from Aladdin Reagent (Shanghai, China) unless otherwise specified. Maleimide-PEG^{2k}-NHS and PEG^{2k}-NHS were purchased from JenKem Technology Co. Ltd (Beijing, China). PAMAM G5 dendrimer (MW: 28,826 Da) was purchased from Weihai CY Dendrimer Technology Co., Ltd. (Weihai, China). c(RGDyk) peptide was purchased from Chinapeptides Co. Ltd (Shanghai, China). Regadenosine, Reg-NH₂, Den-PEG and Den-Reg was prepared according to our previous work. Fetal bovine serum (FBS), trypsin and penicillin-streptomycin were purchased from Life Technologies Inc. (Carlsbad, USA). Rabbit anti-human ZO-1, A_{2A}R and β₃ integrin primary antibodies, mouse anti-human CD31 primary antibodies, Alexafluo488-labeled goat anti-rabbit secondary antibody, Alexafluo555-labeled goat anti-mouse secondary antibody were purchased from Abcam (Cambridge, USA). Radioactive ^{99m}Tc-DTPA were purchased from Shanghai Cancer Center (Shanghai, China).

Confocal fluorescence microscopic imaging

The immunofluorescence images were collected on a Zeiss LSM 710 META confocal laser scanning microscope (Carl Zeiss, Germany). DAPI was excited with a 405 nm laser and the emission was detected with a photomultiplier by a 420–480 nm band-pass filter. Alexa-fluo488 was excited with a 495 nm laser and its emission was detected by a second photomultiplier using a 505–550 nm band-pass filter. Alexa-fluo555 and was excited with a 560 nm laser and its emission was detected by a second photomultiplier using a 570–610 nm band-pass filter. The fluorescence images were processed by ZEN 2012 software. The fluorescence intensities of the images were quantified by ImageJ software (NIH). All data are mean ± SD.

Synthesis

Den-RGD-Reg was synthesized as follows: Mal-PEG^{2K}-NHS ester (12.4 mg, 6.2×10⁻⁶ mol) and c(RGDyK) (5 mg, 8.06×10⁻⁶ mol) were added into 500 μL anhydrous DMF in presence of 10 μL trimethylamine (TEA). After stirring at room temperature for 2 h, the mixture was added dropwise into PAMAM G5 dendrimer (17.9 mg, 6.2×10⁻⁷ mol) in 2.0 mL PBS (pH 7.4) to obtain **Den-RGD**. Similarly, Mal-PEG^{2K}-NHS ester (12.4 mg, 6.2×10⁻⁶ mol) and the reactive A_{2A}R ligand Reg-NH₂ (5.2 mg, 1.24×10⁻⁵ mol) were added into 500 μL anhydrous DMF in presence of 10 μL TEA. After stirring at room temperature for 2 h, the mixture was added dropwise into **Den-RGD**. The mixture was stirred for overnight to obtain **Den-RGD-Reg**, which was further purified by a centrifugal filter (Amicon ultra-15

centrifugal filter tube, MW 1,500 cut off) from Millipore (Bedford, MA, USA) to remove the unconjugated by-products.

Characterization

The molar ratios between dendrimer, PEG, c(RGDyk) and regadenosone in nanoagonists were quantified by ^1H NMR measured on a Varian Mercury 400 spectrometer. (Varian Inc., Palo Alto, USA).

Cell culture

Murine brain endothelial cells bEnd.3 and human glioblastoma cells U87MG were purchased from the American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin and streptomycin at 37 °C with 5% CO₂.

Actin stress fibres staining

After treatment with 10 μM of regadonson or nanoagonists, the cells were washed, fixed with 4% paraformaldehyde (PFA) and then permeabilized with 0.3% Triton X-100. After blocked with 1% BSA for 1 h, slides were stained with phalloidin-Rho (Cytoskeleton, CA, USA) followed DAPI and mounted with cover-slides. The immunofluorescence images were collected on a Zeiss LSM 710 META confocal laser scanning microscope by using a 60 \times oil lens.

Western blot studies

After the treatment with nanoagonists (10 μM) for 45 min, bEnd.3 cells were washed were homogenized in lysis buffer containing protease inhibitors. Total protein (80 μg) was loaded on an 8% SDS-PAGE. After the electrophoresis, the gel was transferred to nitrocellulose membrane that was blocked, cut and incubated with ZO-1, Occludin, β -actin, MLC or pMLC primary antibodies overnight. After washing thoroughly, the membranes were incubated with HRP-labeled goat anti-rabbit secondary antibody (1:20000), developed by using the Supersignal West Pico chemiluminescent substrate kit (Pierce Biotechnology, USA) and exposed to X-ray film.

Animal model

Mouse model bearing orthotropic glioma xenograft was developed according to previous work. Briefly, U87MG cells were inoculated into the right striatum of the nude mice (5×10^5 cells/mouse) using a stereotactic fixation device. The tumor sizes were monitored every other day by MRI and the animals were subjected to *in vivo* experiments when the diameter of the tumors reached 6–8 mm (typically 3-4 weeks after inoculation).

Immunofluorescence microscopic imaging studies

For cell culture studies, mouse brain capillary endothelial bEnd.3 cells were cultured to reach 100% confluence. After treatment with PBS, 10 μ M regadenoson or nanoagonist, the cells were washed, fixed, blocked, and stained with rabbit anti-mouse ZO-1 primary antibody followed by Alexa-Fluoro488-labeled goat anti-rabbit secondary antibody. The nuclei were stained with DAPI, and the slides were mounted for imaging. For ex vivo tissue studies, mouse brains were harvested, fixed, dehydrated, sectioned with a thickness of 10.0 μ m and immunostained with rabbit anti-mouse ZO-1 (1:50 dilution) and rat anti-mouse CD31 (1:100 dilution) primary antibody. Alexa-Fluro488 labeled goat anti-rabbit mouse (1:100 dilution) and Alexafluo555-labeled rat anti-mouse secondary antibody (1:100 dilution) for another 2 h followed the nucleus staining by DAPI (0.5 μ g/mL). Then the immunofluorescence images were collected on a Zeiss LSM 710 META confocal laser scanning microscope. The fluorescence images were processed by ZEN 2012 software. The fluorescence intensities of the images were quantified by ImageJ software (NIH). All data are presented as mean \pm SD.

Histological H&E staining.

The normal brain tissue or glioma margins were fixed in 4% PFA, dehydrated in ascending grades of ethanol and embedded in paraffin. The brain tissue were sectioned with a thicknesses of 5.0 μ m, stained with hematoxylin and eosin (H&E) and visualized under an optical microscope (Olympus BX51, Japan) equipped with a BIOPAD digital camera.

2. Supplementary Figures

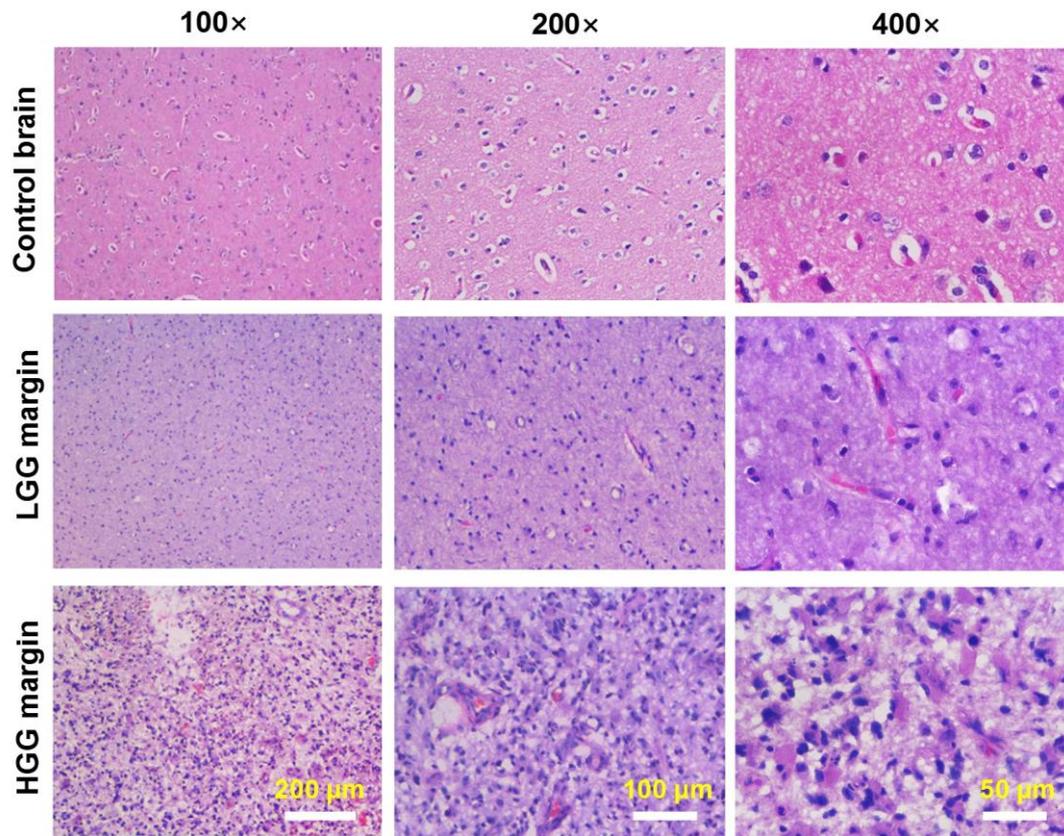


Figure S1. Representative H&E staining images of control brain tissue and glioma margins from patients. LGG: low grade glioma; HGG: high grade glioma.

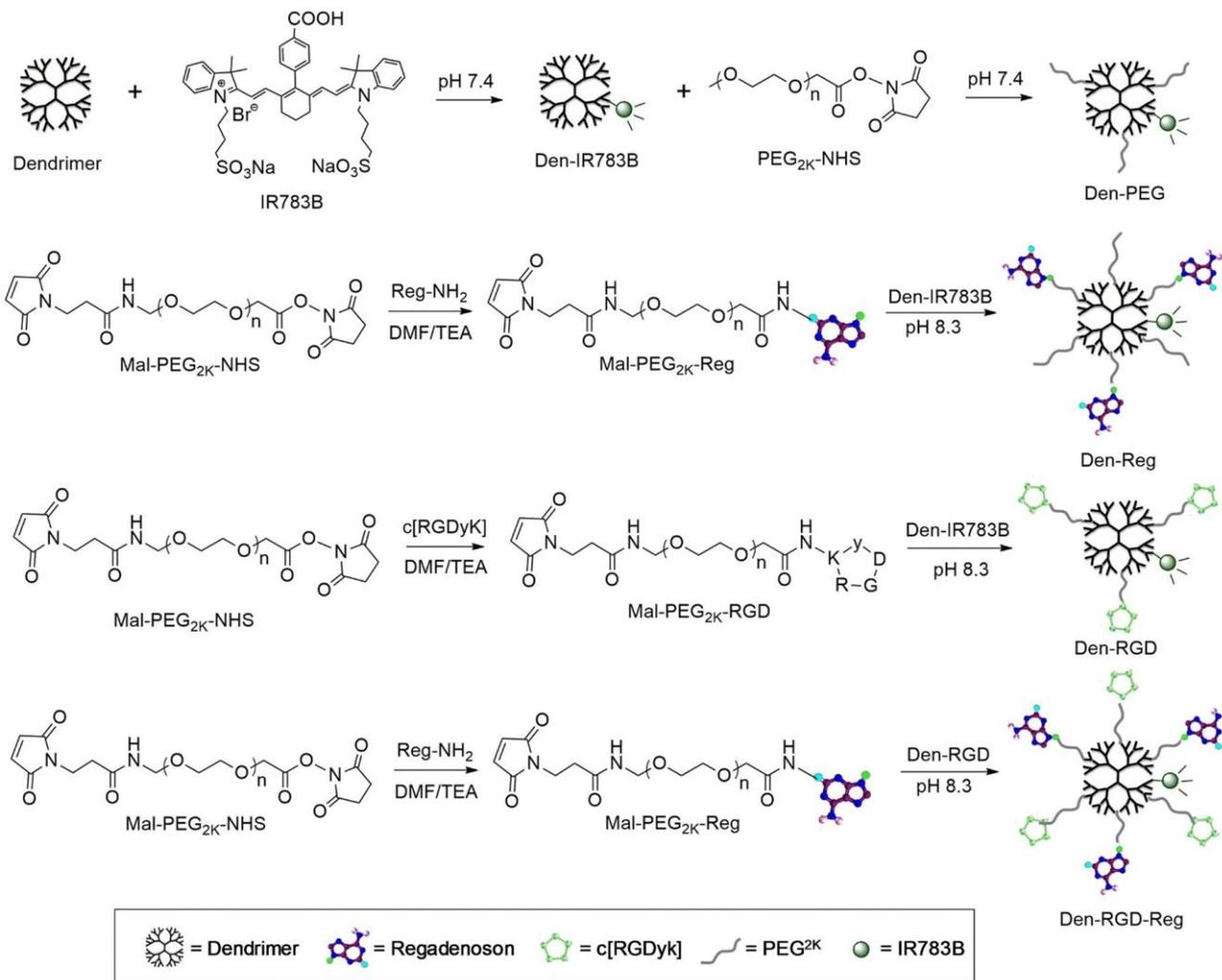


Figure S2. Synthetic steps of nanoagonist and control nanoparticles.

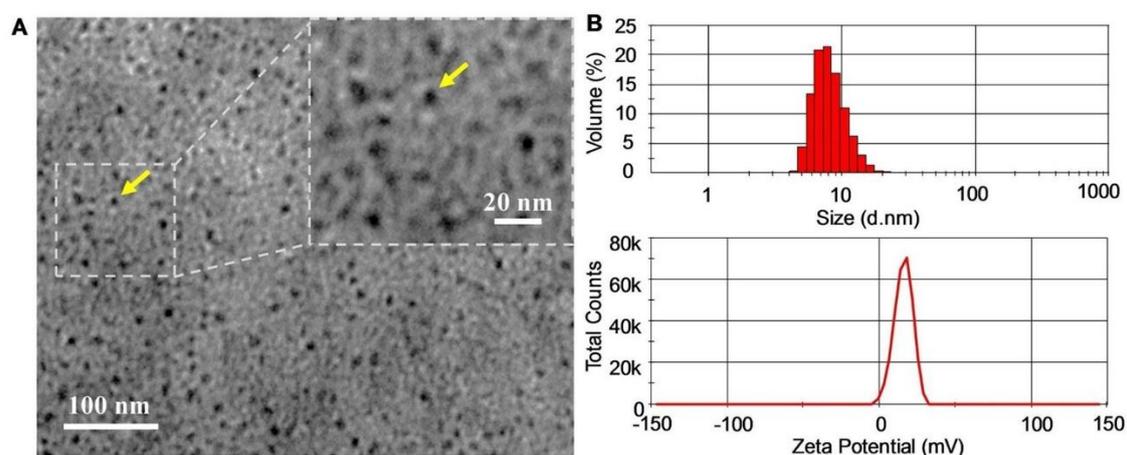


Figure S3. Characterization of the nanoagonist Den-RGD-Reg. (A) TEM image of Den-RGD-Reg. (B) Hydrodynamic diameter and zeta potential of Den-RGD-Reg.

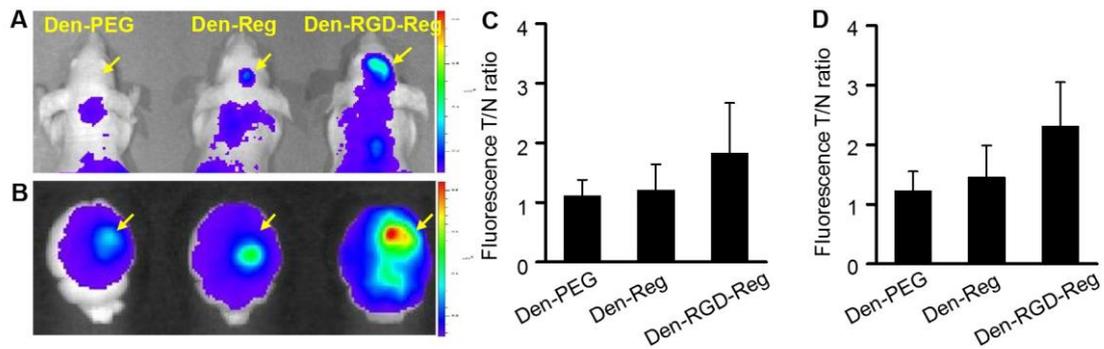


Figure S4. Nanoagonist shows targeting specificity to orthotopic glioblastoma xenograft. (A) Representative in vivo NIR fluorescence images of mice bearing orthotopic U87MG glioblastoma xenograft at 2 h PI of corresponding nanoparticle. (B) NIR fluorescence images of the excised mouse brain at 2 h PI of corresponding nanoparticle. (C) In vivo fluorescence intensity ratio between tumor and surrounding normal tissue (n = 3) at 2 h PI. (D) Ex vivo fluorescence intensity ratio between tumor and surrounding normal brain tissue (n = 3) at 2 h PI. PI: post injection.

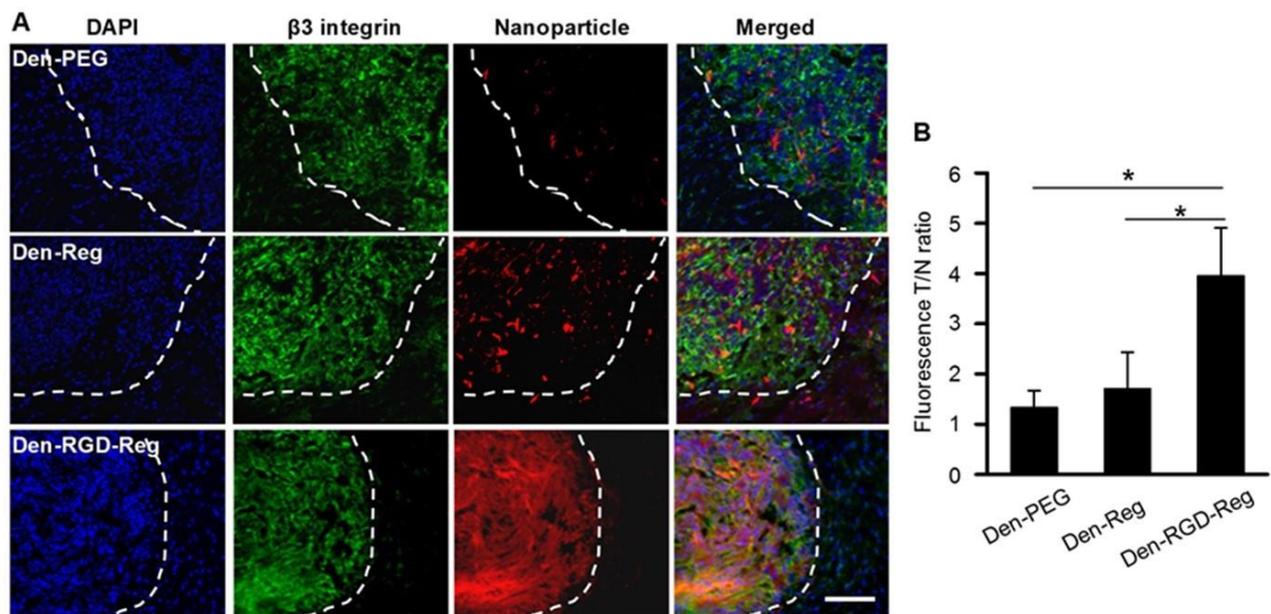


Figure S5. Nanoagonist shows targeting specificity to $\alpha v \beta 3$ integrin in glioma xenograft. (A) Representative fluorescence microscopic images of mouse brain sections immuno-stained with $\beta 3$ integrin antibody at 24 h PI of Den-PEG, Den-Reg or Den-RGD-Reg. $\beta 3$ integrin immunity was displayed in green and fluorophore labeled on nanoparticles was displayed in red. Nuclear stained with DAPI are shown in blue. Scale bar: 200 μ m. Tumor margin was delineated with dash line. (B) Fluorescence intensity ratio between tumor and surrounding normal brain tissue (T/B, n = 4). * $P \leq 0.05$ tumor vs normal brain tissue. PI: post injection.

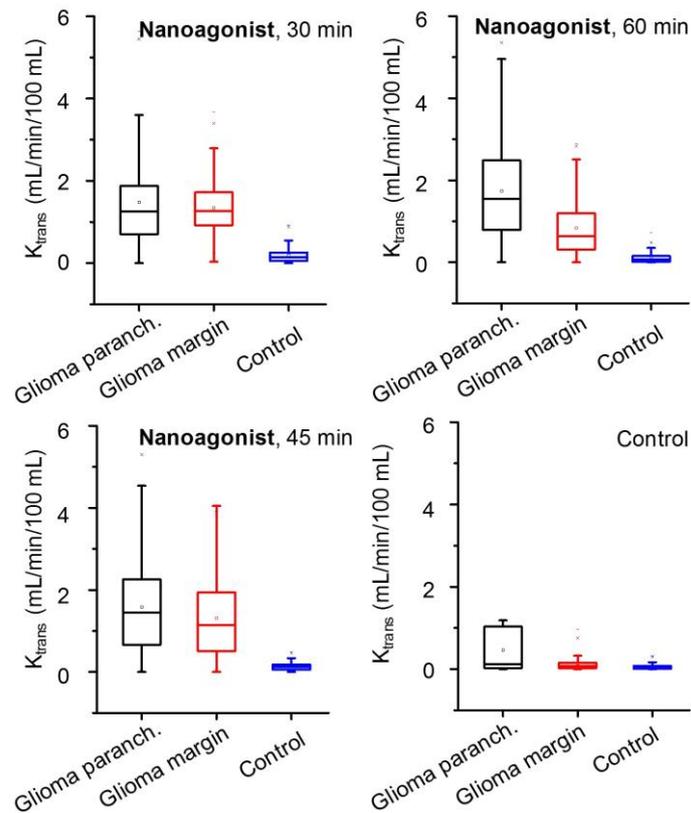


Figure S6. K_{trans} values in glioma parenchyma, glioma margin and control brain tissue when Gd^{3+} -DTPA was injected at 30, 45 and 60 min PI of nanoagonist ($n = 3$). In control experiment, K_{trans} values were obtained when Gd^{3+} -DTPA was injected at 45 min after PBS treatment.

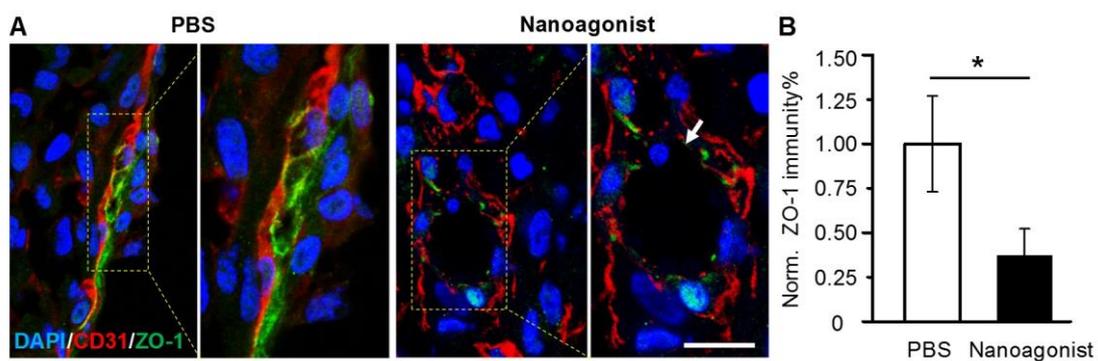


Figure S7. Nanoagonist attenuates ZO-1 expression in glioma margin. (A) Representative immunofluorescence images of glioma xenograft margin at 45 min post administration of PBS or nanoagonist. The immunities of vascular endothelium and ZO-1 are displayed in red and green respectively. Arrows point to the disrupted ZO-1 immunity. Scale bar: 30 μ m. (B) Normalized vascular ZO-1 immunities in control brain and glioma margins ($n = 4$). * $P \leq 0.05$ PBS vs Nanoagonist.

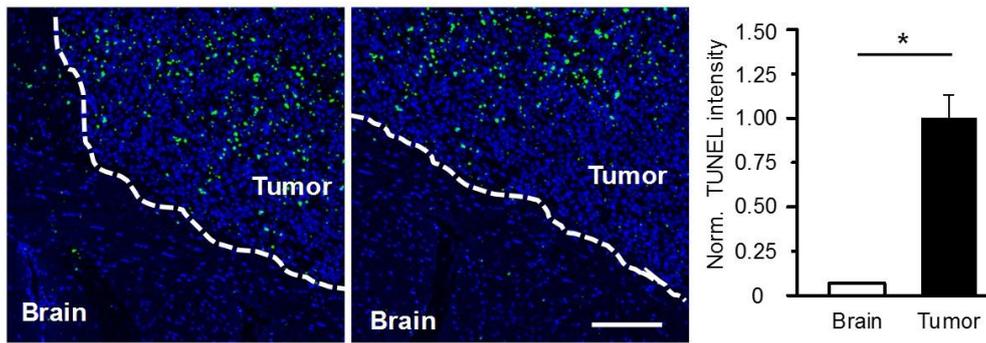
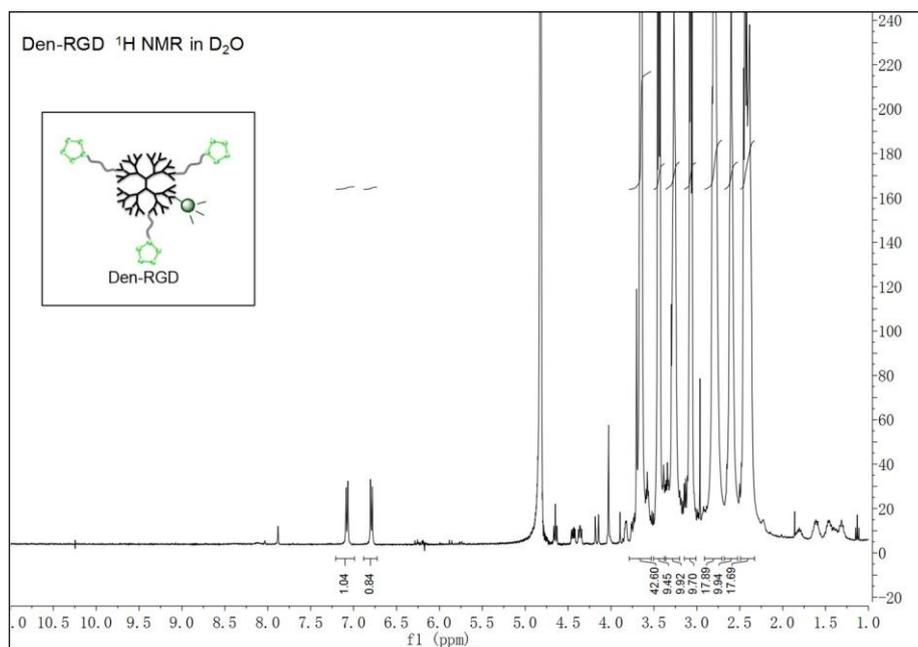
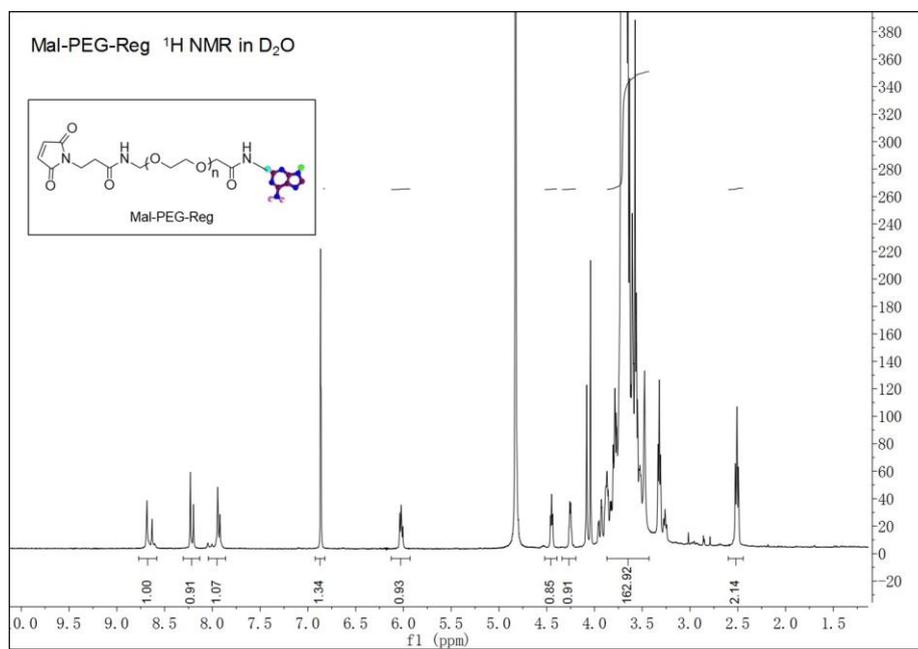


Figure S8. Image-guided drug delivery shows improved therapeutic response to glioma xenograft. (A) Representative TUNEL stained images of glioma xenograft margin. The mouse models were treated with nanoagonist plus paclitaxel for 14 days. Bar: 100 μm . (B) Normalized TUNEL intensity in brain and tumor (n=4). * $P \leq 0.05$ brain vs tumor.

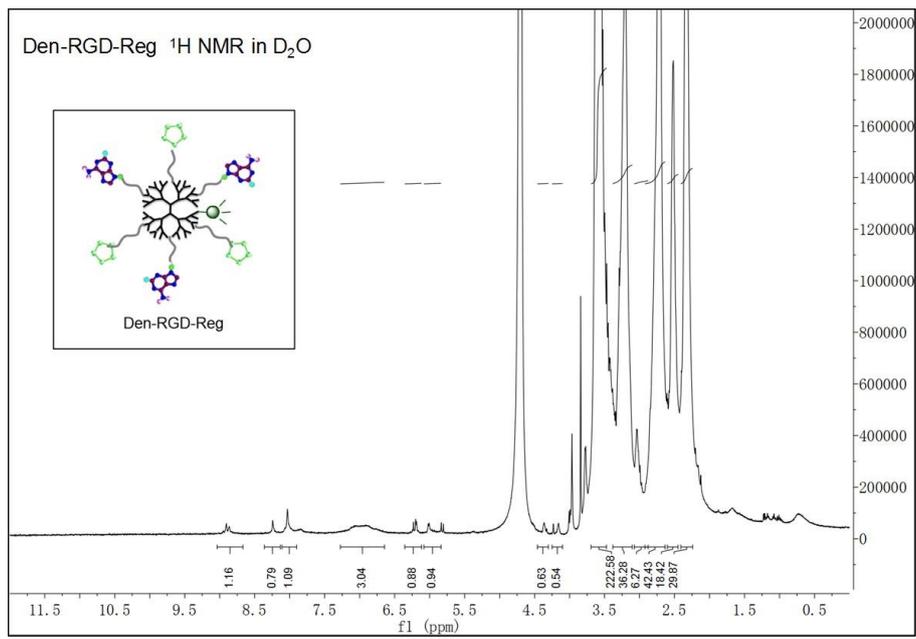
3. Supplemental Spectra



¹H NMR spectrum of Den-RGD



¹H NMR spectrum of Mal-PEG-Reg.



¹H NMR spectrum of Den-RGD-Reg.