Supplementary Information

Y_1 receptor ligand-based nanomicelle as a novel nanoprobe for glioma-targeted imaging and therapy

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Experimental Section

Regents: [Pro^{30}, Nle^{31}, Bpa^{32}, Leu^{34}]NPY (28-36) (sequence: Ile-Asn-Pro-Nle-Bpa-Arg-Leu-Arg-Try-NH_2) and [Asn^{6}, Pro^{34}]NPY (sequence: Tyr-Pro-Ser-Lys-Pro-Asn-Asn-Pro-Gly-Glu-Asn-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Pro-Arg-Tyr-NH_2) were synthesized by Dechi Biosciences Co, Ltd (Shanghai, China). 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (DSPE-PEG-COOH), 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG-NH_2), and l, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(Carbonyl-methoxypoly ethylene-glycol 2000) (DSPE-mPEG) were purchased from A.V.T. Pharmaceutical Ltd. (Shanghai, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and N-hydroxysuccinimide (NHS) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). HPLC grade acetonitrile and trifluoroacetic acid (TFA) were purchased from Aladdin Industrial Inc (Shanghai, China). Doxorubicin hydrochloride (DOX·HCl) was purchased from Hisun Pharmaceutical Co., Ltd (Zhejiang, China). The human U87MG glioma cell line was obtained from the American type culture collection (ATCC, USA). U87MG was stably transfected with the GFP-luciferase gene (U87-GFP-fLuc). Human brain microvascular endothelial cells (HBMEC) were purchased from Angio-Proteomie Co. Ltd. IRDye®800CW NHS Ester (IRDye) was bought from LiCor Biosciences.

Preparation of Y_1R ligand-modified NM: Y_1R ligand PNBL-NPY or AP-NPY-modified DOX or IRDye-loaded NM were prepared via a solvent evaporation method. Acetone (3 mL) containing 1 mg DOX (or 0.5 mg IRDye) and 25 mg DSPE-mPEG (or DSPE-mPEG:DSPE-PEG-NPY, 80:1, *w/w*) was added drop-wise into 2 mL of water with magnetic stirring for 5 min. The organic solvent was removed by evaporation under vacuum at 35 °C to obtain a micelle solution. The unencapsulated free DOX was removed by filtration (0.22 μm). The particle size, polydispersity index (PDI), and zeta potential were determined in double-distilled water using a Nano-ZS Zeta particle size analyzer (Malvern, England). The morphology of prepared NM was observed using a JEM-1230 transmission electron microscope (JEOL, Japan).

Cell culture: Human primary glioblastoma (U87MG) cells were cultured in minimum essential medium supplemented with 10 % fetal bovine serum, 1 % non-essential amino acids, 100 units mL^{-1} of penicillin, and 100 mg mL^{-1} of streptomycin. Human brain microvascular endothelial cells (HBMECs) were cultured in endothelial cell medium supplemented with 5 % FBS, 1 %

endothelial cell growth supplement, 100 units mL^{-1} of penicillin, and 100 mg mL^{-1} of streptomycin. U87-MG-fLuc-GFP cells were labeled with firefly D-luciferin, and green fluorescent protein was used as the standard for the stable transfection protocol. All cells were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2.

Cellular uptake investigation: U87MG cells were seeded at a density of 1×10^6 cells $well^{-1}$ into a 6-well culture plate and allowed to attach for 24 h until confluent. The medium was then changed with fresh free DOX, NM-DOX, PNBL-NPY-NM-DOX, or AP-NPY-NM-DOX (containing 5 μg mL^{-1} DOX). After a further 4 h of incubation, the cells were washed with PBS to remove any absorbed free NM. For laser scanning confocal microscopy (LSCM) analysis, the cells were then fixed with 4 % formaldehyde for 30 min, treated with 0.1 % triton for 5 min, and then treated with 1.0 % BSA for 30 min at 25 °C. Actin in the cells was stained with rhodamine-FITC for 30 min at 25 °C. The samples were simultaneously excited at 488 nm and the fluorescent images at emission wavelengths 488–540 and 555–625 nm were observed with a TCS SP8 LSCM (Leica, Germany). For flow cytometry analysis, the mean fluorescence intensity (MFI) of cells (1×10^4 counts) was analyzed using a FACSCalibur flow cytometer (BD Biosciences, USA), where the gate was arbitrarily set for the detection of red (EM: 570–610 nm) fluorescence. Forward and side scattering dot plots were used to discriminate cellular debris. All experiments were conducted in triplicate and data are presented as the means ± SEM.

Cell cytotoxicity assay: U87MG cells were seeded in 96-well culture plates at a density of 5000 cells $well^{-1}$ and grown for 24 h under a 5 % CO_2 atmosphere at 37 °C. Fresh medium containing free DOX, NM-DOX, PNBL-NPY-NM-DOX, or AP-NPY-NM-DOX in the DOX concentration range of 0.312–80 μg mL^{-1} was added, and the cells were incubated for 24 h at 37 °C. Then, 10 μL of MTT solution (5 mg mL^{-1} in PBS) was added into each well followed by a 4 h incubation at 37 °C in darkness, after which the medium was removed and 200 μL DMSO was added. A 168-1130iMark automated plate reader (BioRad, USA) was used to measure absorbance at 550 nm. Relative cell viability was calculated by comparing the absorbance intensity of groups treated with different drug-loaded micelles with the control group. The drug concentration of inhibition of 50 % cell growth (IC_{50}) was calculated using SPSS 18.0 (Chicago, USA). All experiments were performed in triplicate, and the results are presented as the means ± SEM.

In vitro drug release: DOX loaded NM (1 mL) were added to a dialysis membrane bag (MWCO: 2000 Da), which was incubated in 50 mL of phosphate buffered saline (PBS) at pH 7.4 or 5.0 at 37 °C with stirring at 100 rpm for 72 h. At the appropriate time intervals (2, 4, 6, 8, 12, 24, 36, 48, 60, and 72 h), 1 mL of the release medium was collected and replaced by an equal volume of fresh medium. The amount of DOX was determined using a T10CS UV-Vis spectrophotometer (Persee, China) at 480 nm and percent cumulative release was calculated per a previous method.[1]

Animal tumor model: All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Peking University and approved by the Animal Ethics Committee of Peking University. The experiments were carried out on 4–5-week-old male BALB/c athymic mice, weighing 15–18 g (Vital River Laboratory Animal Technology, China). Two types of glioma mouse models were established: subcutaneous and orthotopic tumors. Briefly,

100 µL of PBS (0.01 mol L^{-1}, pH 7.2) containing a suspension of ~10^6 U87-MG-GFP-fLuc cells was injected subcutaneously into the back of each subcutaneous tumor mouse. The glioma orthotopic model was established using a stereotaxic apparatus (RWD Life Science, China). After craniotomy, 10 µL U87-MG-fLuc-GFP cells were injected into the brains of the mice, at a flow rate of 1 µL min^{-1}. The tumors were detected and analyzed using the bioluminescence imaging (BLI) method until they reached full size.

In vivo fluorescence imaging: Fluorescence imaging was performed on the orthotopic and subcutaneous glioma-bearing mice using a small-animal optical molecular imaging IVIS Imaging Spectrum System (PerkinElmer, USA). IVIS Living Imaging 3.0 software (PerkinElmer) was used to analyze the data. The mice were anesthetized with 2 % isoflurane and administered an intraperitoneal injection of 80 µL D-luciferin solution (40 mg mL^{-1}) 10 min prior to the start of imaging. The mice were kept in a prostrate position. The parameters for the imaging system were binning, 0.5 s and exposure time, 2 s. The mice were injected with free IRDye, NM-IRDye, PNBL-NPY-NM-IRDye and AP-NPY-NM-IRDye (200 µL, equivalent to 50 µg mL^{-1} IRDye) for *in vivo* detection (n = 5 for each imaging probe).

In vivo antitumor studies: The subcutaneous glioma model was established for *in vivo* antitumor studies. At seven days after tumor cell implantation, the mice were randomly divided into five groups (n = 6) and were administrated PBS, free DOX, NM-DOX, PNBL-NPY-DOX, and AP-NPY-NM-DOX via tail vein injection with a dose of 5 mg kg^{-1} DOX every 2 days. Tumor volume and survival rate were evaluated. The major organs including the heart, liver, kidney, and spleen were stained with hematoxylin and eosin (H&E) and examined using a DMI3000 optical microscope (Leica, Germany).

Homology modeling and docking: Based on the 3D structure of human substance P receptor (PDB code: 2KS9) and OX2 orexin receptor (PDB code: 4s0V) elucidated by nuclear magnetic resonance (NMR) spectrometry, we conducted homology modeling using the MODELLER program in the Tianhe-2 supercomputer system (Guangzhou, China). We developed ten models based on the A chain structure, and the one with the highest value of DOPE-HR scoring function was chosen for docking analysis.[2,3] The docking program AUTODOCK was used for the preliminary protein-ligand docking, and the molecular dynamics simulation of PNBL-NPY or AP-NPY to Y_1Rs from 0 to 10 ns was further evaluated by the GROMACS program.

In vitro stability of Y_1R ligands: We incubated 100 µL of PNBL-NPY or AP-NPY solution (1 mg mL^{-1} in distilled water) with 0.9 mL 50 % FBS. After 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h shaking at 37 °C and 100 rpm, 200 µL acetonitrile (0.1 % TFA) was added to the 1 mL reaction mixture. The mixture was stored at 4 °C for 24 h, then centrifuged at 12000 rpm for 20 min. The supernatant (20 µL) was analyzed using an Agilent 1260 infinity liquid chromatography system to monitor and quantify peptide degradation.

References

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3 B. Xu, H. Fallmar, L. Boukharta, J. Pruner, I. Lundell, N. Mohell, H. Gutierrez-de-Teran, J. Aqvist and D. Larhammar, *Biochemistry*, 2013, **52**, 7987-7998.

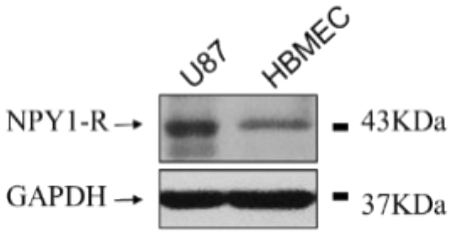

Fig. S1. Western blot analysis of Y_1R expression in human primary glioblastoma U87MG cell line and human brain microvascular endothelial cell line (HBMEC).

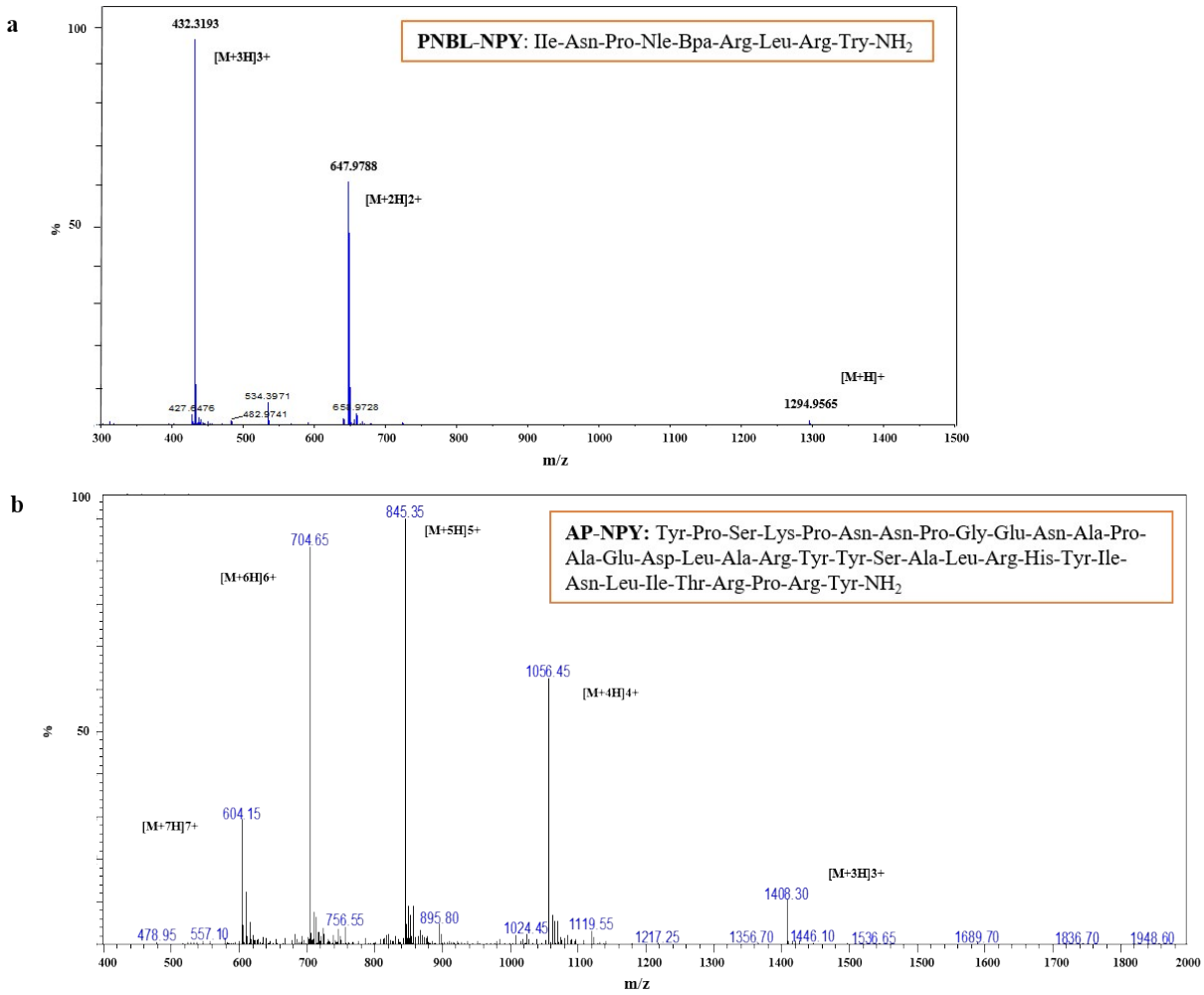

Fig. S2. Peptide Sequence and mass spectrum of a) PNBL-NPY and b) AP-NPY peptide (1 µM) in acetonitrile and water (1:1, *v/v*). Positive ionization mode was used for the MS detection.

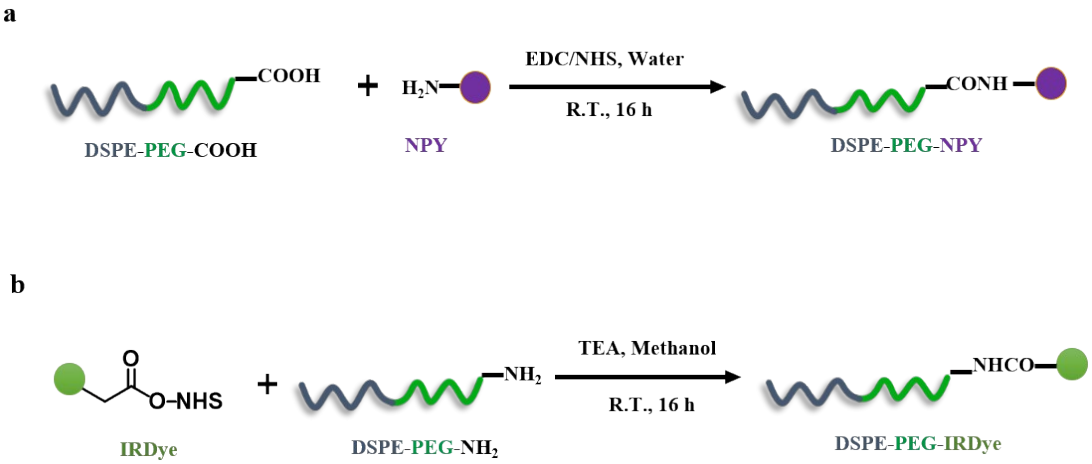

Fig. S3. Polymer synthesis of a) DSPE-PEG-NPY and b) DSPE-PEG-IRDye.

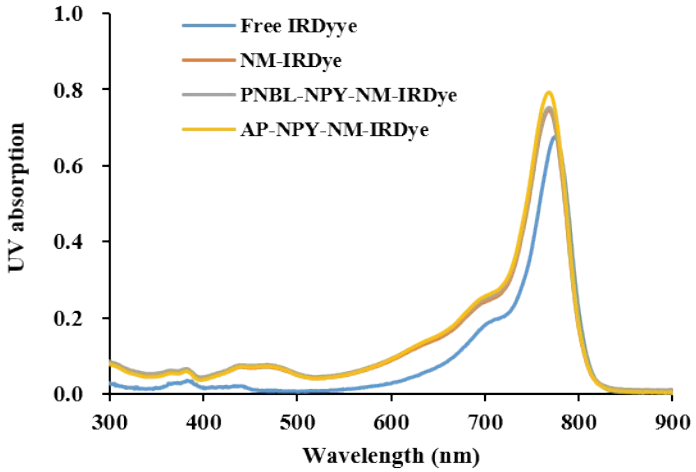

Fig. S4. UV-Vis spectrum of Y_1Rs ligand-modified NM-IRDye for the quantification of IRDye concentration in NM.

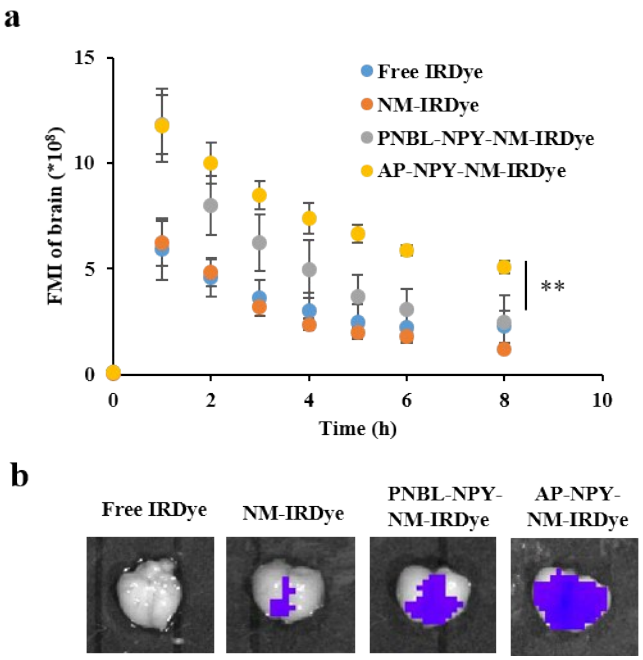

Fig. S5. Fluorescence imaging of normal mice brain after intravenous administration of Y_1R ligand-modified NM-IRDye. a) Fluorescence mean intensity (FMI) of mice brains at different time points (1, 2, 3, 4, 5, 6, and 8 h). Means ± SEM, n = 3, $^{**}P < 0.01$. b) *Ex vivo* brain imaging of mice 8 h after intravenous injection.

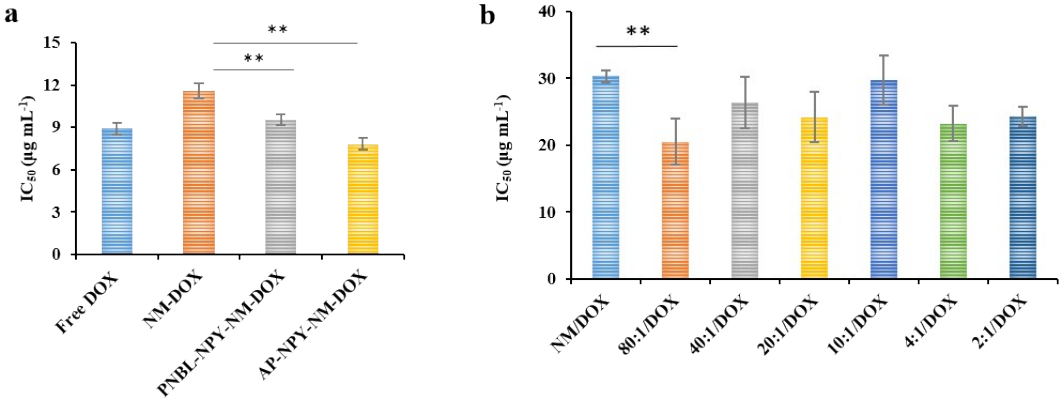

Fig. S6. a) Inhibitory effect of Y_1Rs ligand-modified NM-DOX on U87MG cell growth after 24 h incubation. b) Inhibitory effect of different proportions of DSPE-mPEG to DSPE-PEG-NPY (w/w) on U87MG cells. The proportion of 80:1 (DSPE-mPEG:DSPE-PEG-NPY, w/w) produced the highest inhibition rate and the lowest IC50 value. Means ± SEM, n = 3, $^{**}P < 0.01$.

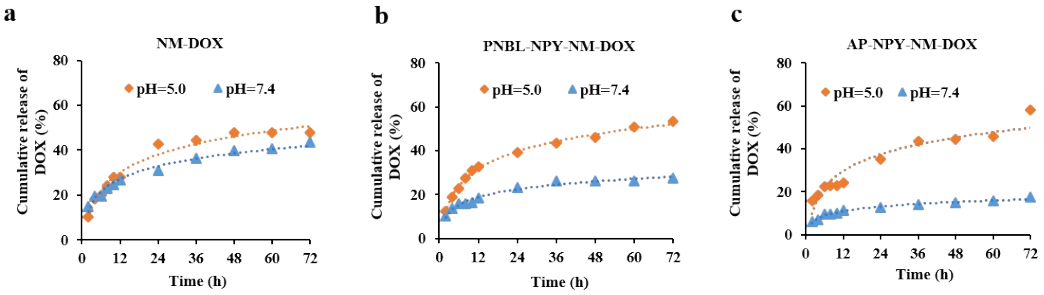

Fig. S7. *In vitro* release profile of DOX from a) NM-DOX, b) PNBL-NPY-NM-DOX and c) AP-NPY-NM-DOX in PBS (pH5.0 and 7.4) at 37 °C.

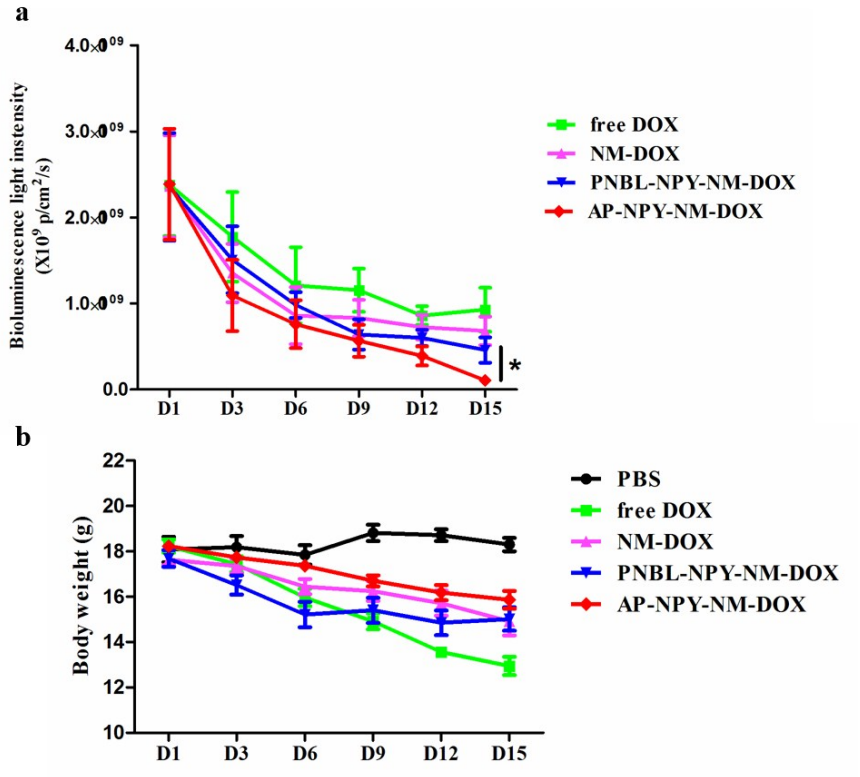

Fig. S8. Therapeutic efficacy of Y_1R ligand-modified NM-DOX in U87MG glioma-bearing mice. a) Bioluminescence light intensity of tumors. Means ± SEM, n = 6, *P < 0.05. b) Body weight changes of mice. Means ± SEM, n = 6.

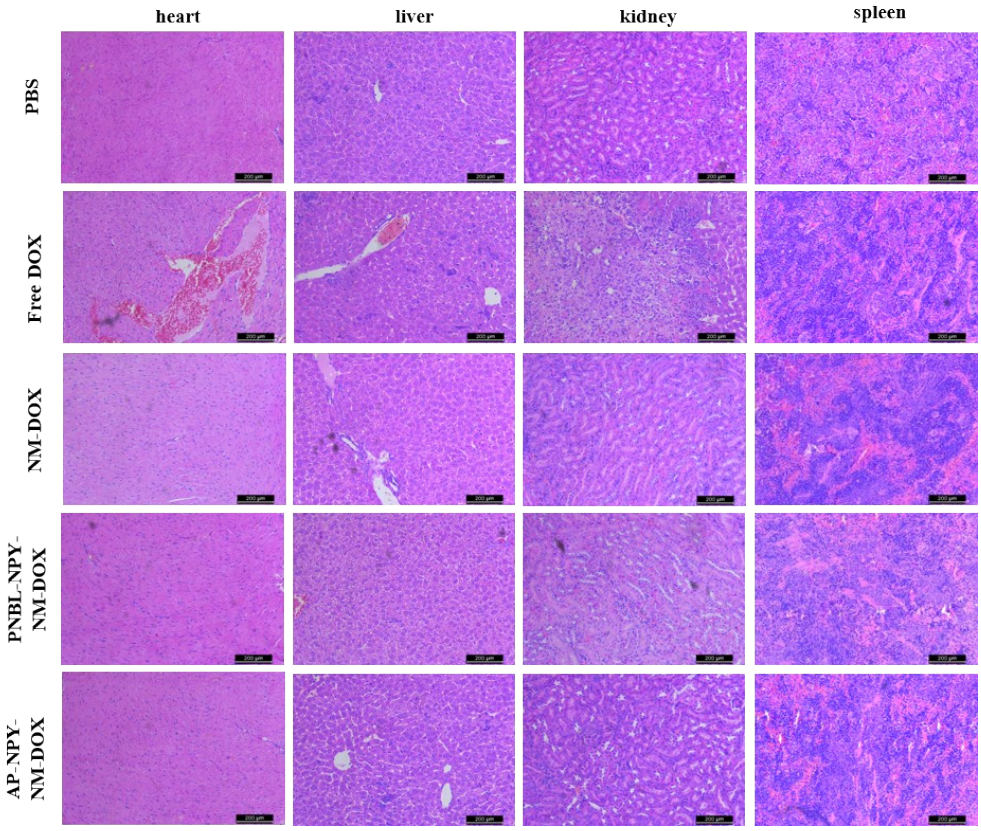

Fig. S9. Microscopic images of H&E-stained cross-sections of the heart, liver, kidney and spleen. Scale bar = 200 μm.

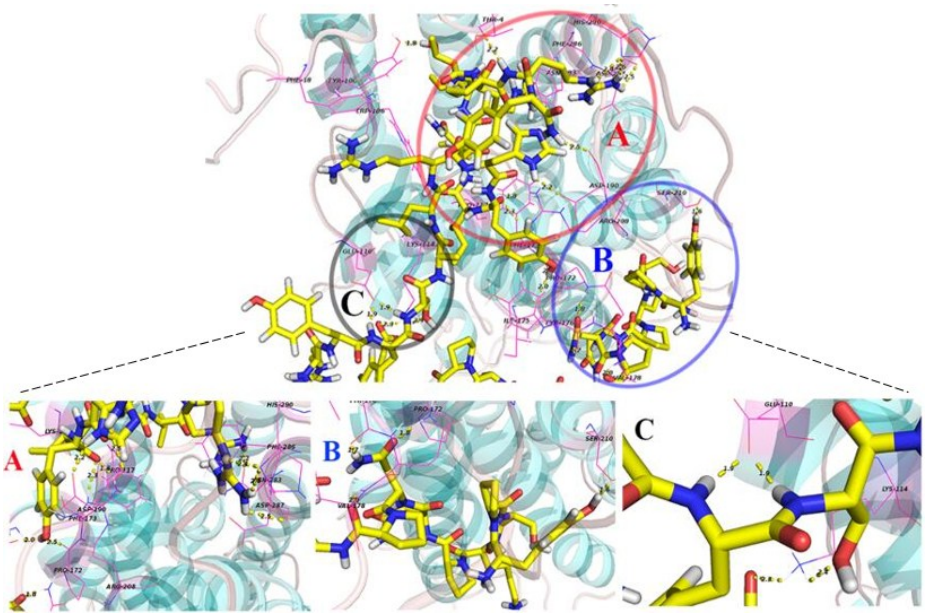

Fig. S10. Binding sites of AP-NPY with Y_1R are mainly located in regions A (red color), B (blue color) and C (black color).

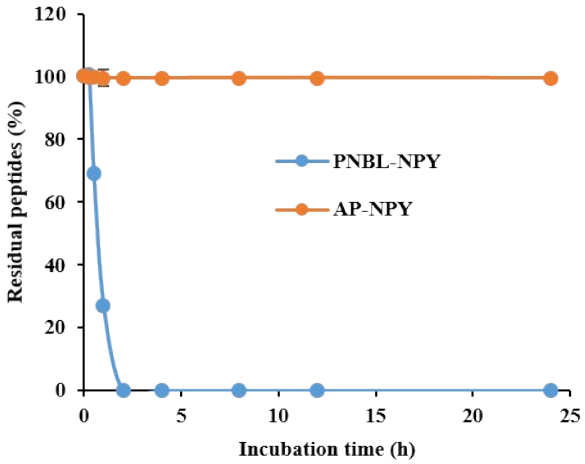

Fig. S11. Degradation of PNBL-NPY and AP-NPY in 50 % fetal bovine serum was determined with HPLC at 0, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h. Means ± SEM, n=3.