Supplemental information

Targeted disruption of tumor vasculature via polyphenol nanoparticles to improve brain cancer treatment

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Materials
Quercetin (99%), iron (III) chloride hexahydrate (FeCl₃·6H₂O), europium (III) chloride hexahydrate (EuCl₃·6H₂O), sodium oleate, pyrene, IR-780 iodide, Folin–Ciocalteu reagent, MTT, Evans Blue, and F127 were purchased from Sigma-Aldrich. Cyanine 5 (Cy 5) amine and Cyanine 5.5 (Cy 5.5) NHS ester were purchased from Abcam (Cambridge, MA, USA). Cy 5.5 amine was obtained from Lumiprobe. DMEM medium and Fetal bovine serum were purchased from Invitrogen. EGM™-2 Endothelial Cell Growth Medium-2 with BulletKit™ Fetal bovine serum was obtained from Lonza. Matrigel matrix was purchased from Corning. All other reagents not mentioned above were obtained from Sigma-Aldrich.

Cell culture and cell viability assay
GL261, HBMECs, Normal Human Astrocytes (NHA) bEnd.3 and HUVEC cells were obtained from the American Type Culture Collection (ATCC). BCSC PS30 cells were derived from patient's tumor samples and cultured in Neurobasal-A medium (Invitrogen) supplemented with B27 (Invitrogen), fibroblast growth factor-2 (20 ng/mL, Peprotech), and epidermal growth factor (20 ng/mL, Peprotech) as previously reported. To determine the cytotoxicity of the NPs, GL261 and HUVEC cells were treated with various concentrations of NPs. Three days later, the cells viability was determined using the standard assay using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT).

Determinization of loading efficiency
To determine the quercetin loading efficiency, we synthesized Q-NPs following the same
procedure described above, except for the purification step. After stirred overnight, the resulting Q-NPs were centrifuged at 4000 rpm for 0.5 h using an ultrafiltration tube. The content of quercetin in the filter was detected using the Folin–Ciocalteu reagent. Briefly, 100 µl of sample supernatant, standard, or methanol blank was added to 1.5 mL microtubes. Then 200 µl diluted 10% (vol/vol) F–C reagent was added and vortexed vigorously. Afterward, 800 µl 700 mM Na2CO3 was added into each tube and incubated at room temperature for 2 h. Finally, 200 µl of the mixture was transferred to a clear 96-well micro test plate and the absorbance at 765 nm was recorded using a microplate reader. The loading efficiency of quercetin (LE) was calculated using Eq.: LE (%) = (Total quercetin − free quercetin)/Total Q-NPs×100.

**Determinization of critical micelle concentration (CMC)**

CMC was determined by a fluorescence probe technique as described previously, where pyrene was used as a hydrophobic probe. Briefly, pyrene was dissolved in methanol and added to a 1.5 mL centrifuge tubes. The solvent was then evaporated. After that, Q-NPs and F127 were added to tubes at serial of concentrations. The final pyrene concentration was maintained as 6.0×10⁻⁷ M. After incubation overnight in the dark, the concentration of encapsulated pyrene in Q-NPs and F127 was measured using a BioTek Synergy 2 Multi-Detection Microplate Reader (BioTek Instruments, Inc). The excitation wavelength was 334 nm and the range of fluorescence emission was between 350 nm and 500 nm. Fluorescence emission intensity at 372 (I372) and 383 nm (I383) was recorded. CMC was obtained by plotting the ratio of I372/I383 of the emission spectra profile versus the logarithm of the Q-NPs and F127 concentration.

**Synthesis of europium oleate.** First, 3.023 g EuCl₃·6H₂O and 6.5457 g Sodium oleate were added
to a mix solution containing 12.093 mL of water, 16.125 mL of ethanol, and 28.219 mL of Hexane. The mixture was stirred at room temperature for 30 min, then reflux at 70 °C. Four hours later, the mixture was transferred into a separatory funnel, and add 10 mL ethanol and 10 mL water each. Mix upside down several times, and then let stand for several minutes. Remove the liquid in the aqueous phase and repeat the wash process twice again. Finally, transfer the organic phase into flask and heat in a vacuum for more than two days with the temperature for the heating is about 50 °C.

**Synthesis of europium loaded Q-NPs**

In a typical synthesis, 106 mg F127 was dissolved in 8.8 mL water at room temperature. Then 0.2 mL FeCl₃ aqueous solution at a concentration of 100 mg/mL was added to the above F127 solution. After mixing for 1 h, 3.75 mL quercetin DMSO solution (20 mg/mL) was added to the above mixture. After stirring overnight, 0.375 ml EuCl₃ aqueous solution at a concentration of 20 mg/mL was added to the mixture and stirred for another 8 h. The resulting europium loaded Q-NPs were collected and dialyzed against deionized water using SnakeSkin Dialysis Tubing.

**Synthesis of IR780-labeled NPs**

In a typical synthesis, 106 mg F127 was dissolved in 8.8 mL water at room temperature with stirring. Then 0.2 mL FeCl₃ aqueous solution at a concentration of 100 mg/mL was added to the above F127 solution. After mixing for 1 h, 75 mg quercetin and 0.75 mg IR780 dissolved in 2 mL DMSO solution were added to the above mixture. After stirring overnight, the resulting IR780-labeled Q-NPs was collected and dialyzed against deionized water using SnakeSkin Dialysis Tubing. The same procedures were used to synthesize other IR780-labeled NPs, except that the
mass of the polyphenol was changed accordingly.

**Synthesis of Cy5 amine-Q-NPs and Cy5.5 amine-Q-NPs**

5 mg of Q-NPs was dissolved in 2 ml of Tris buffer (pH 8.8) under vigorous stirring. After 0.5 h of incubation, an amine-terminated Cy5 or amine-terminated Cy5.5 DMSO solution (10 µl, 10 mg/mL) was then added to the above solution and stirred overnight. The resulting Cy5 or Cy5.5 labeled Q-NPs were washed and concentrated using MWCO 10,000 Ultrafilters (Millipore), spun at 4000 rpm for 10 min to remove debris, filtered through a 0.22 µm membrane filter (Millipore), and stored at 4 °C. Cy5.5 labeled dendrimers were synthesized according to the literature 28.

**Synthesis of europium-loaded and IR780-labeled PLGA NPs**

PLGA NPs were synthesized according to our recent reported procedures with minor modifications 54. Briefly, for a typical synthesis of europium- loaded PLGA NPs, 50 mg PLGA, 2.5 mg DSPE-PEG3400, and 4 mg europium oleate were dissolved in 2 mL DCM, then added dropwise to the 2 ml 2.5% PVA solution and ultrasonicated 3 times. The solution was added dropwise into the beaker of 50 mL 0.3% PVA with stirring. After stirring overnight, NPs were collected by centrifugation at 18,000 g for 30 min and washed with water twice. The same procedures were used to synthesized IR780-labeled PLGA NPs except replacing 4 mg europium oleate with 0.5 mg IR780 during the synthesis.

**Flow cytometry**

Flow cytometry analysis was performed to identify the molecular targets accounting from NP uptake. Briefly, HUVECs or bEnd.3, which were transfected with plasmids for overexpression of
VEGFR2, Tie2, FGFR, IGFR, and EGFR, were treatment with Cy5-conjugated Q-NPs. Six hours later, the cells were collected, washed, fixed, and analyzed using a LSR II flow cytometer (BD Biosciences). For the competition study, the same experimental design was applied, except that cells in the competition group were pre-treated with 1 μg anti-VEGFR2 antibody prior to Q-NP incubation. The same experimental design was used for *in vitro* competition confocal study, except that each cell was pre-treated with free quercetin at different concentrations prior to Q-NPs administration.

**Western Blot**

The impacts of various treatment on the level of VEGFR2 expression and phosphorylation was determined using Western Blot analysis according to our previous report. Briefly, HUVECs were treated with DMSO (0.1%), ferric chloride (50 μM), quercetin (50 μM), or Q-NPs (50 μg ml⁻¹). After incubation for 120 min, cells were washed with PBS and lysed in RIPA buffer (Thermo Fisher Scientific) supplemented with cocktail protease inhibitor tablets (Roche). After electrophoresis on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, proteins were transferred onto nitrocellulose membranes (Bio-Rad). These membranes then incubated with primary antibodies, including anti-β-actin (CST, 58169S), anti-VEGFR2 (Abcam, ab42228), and phospho-specific anti-VEGFR2 (Tyr1175, CST, #2478) and then secondary antibody polyclonal goat antimouse/rabbit immunoglobulins/horseradish peroxidase (HRP) (Dako, P044701-2 and P044801-2). The colour reaction was completed with a detection reagent (Thermo Fisher Scientific).

**DCFH-DA Assay**

ROS formation was detected by measuring the change in fluorescence of a ROS-sensitive probe,
2′,7′-dichlorofluorescin diacetate (DCFH-DA, purchased from Sigma-Aldrich). GL261 cells were treated with Q-NPs at different concentrations prior to confocal imaging. After 24 hours, PBS supplemented with DCFH-DA (10 μM) was added. Then after 30 minutes, the cells were washed with PBS three times and subjected to confocal imaging.

**Quantification of quercetin in the brain by LC-MS**

Tumor-bearing mice were established through the intracranial injection of GL261 cells as described above. After 21 days, the mice were treated with Q-NPs (25 mg/kg). After additional 12 hours, the mice were euthanized. The tumors and brains were harvested and homogenized in 0.5 mL PBS. The tissue homogenates were mixed with 0.5 mL acetone, following with 1 mL ethyl acetate, and then vortexed for 30 minutes. After centrifugation at 5,000 g at 4°C for 10 min, the organic phase was collected, dried and redissolved in 50% methanol/50% water (total volume: 1mL) for LC-MS analysis. LC-MS analysis was performed on an API 4000 QTrap® mass spectrometer (Applied Biosystems Sciex, Toronto, Canada) coupled with Agilent HP1200 HPLC system (Agilent Technologies, Santa Clara, CA). Analyst 1.7 software was used for data acquisition and analysis. A targeted Multiple Reaction Monitoring (MRM) method was utilized to quantify the level of quercetin. An Agilent Eclipse C18 column (3.5 micros, 3.0 x 100 mm) coupled with Agilent C18 guard column was utilized for the liquid chromatography separation at 50 °C. The gradient started and maintained with 50% of 0.1% formic acid in acetonitrile (B) and 50% of 0.1% formic acid in water (A) for 0.5 minute, ramped up to 95% B in 1.5 minutes, and maintained 2.5 minutes, back to 50% B in 0.5 minute and maintained for 3 minutes. API 4000 Qtrap mass spectrometer was operated using an electrospray ionization (ESI) source in positive ion mode. MRM transition monitored for quercetin was 303.1/229.1, 303.1/153.1, 303.1/137.1 and
303.1/229.1 used as quantifier. The declustering, entrance, collision cell exit potentials were 103 eV, 10 eV, and 10 eV respectively. The collision energy settings were 37 eV, 43 eV and 41 eV for three MRM channels. The ion spray voltage was 5500 eV, source temperature was 450°C and ion source gases 1 and 2 pressures were both 50 psi, curtain gas and CAD gas was set as 15 psi and high mode.
SUPPLEMENTAL FIGURES

Figure S1. Representative images of nanoparticles formed by the indicated polyphenols in the presence of the listed polymers. Scale bar: 50 nm.
Figure S2. Characterization of NPs consisting of the indicated polyphenols. (A) Representative TEM images of NPs synthesized using the indicated polyphenols. (B) Representative images of HUVEC-formed tubes after treatment with PBS or the indicated polyphenols-NPs. Scale bar: 1,000 μm. (C) Quantification of tube formation after treatment with PBS or NPs synthesized using the indicated polyphenols. Data represent mean ± SEM; n=3.
Figure S3. Characterize the loading of quercetin in Q-NPs. (A) Linear correlation of the absorbance of quercetin at 760 nm with concentration. (B) Changes in loading efficiency with the feeding concentration of quercetin. Data represent mean ± SEM; n=3.
Figure S4. Representative DLS (A) and Zeta potential (B) analysis of Q-NPs.
Figure S5. Representative TEM images of F127-Quercetin-Fe (Q-NPs), F127-Quercetin, and Fe-Quercetin. Scale bar: 100 nm.
Figure S6. DLS analyses of Q-NP formation with indicated treatments.
Figure S7. Characterization of free quercetin in aqueous solution. (A) A representative image of quercetin precipitated out in PBS. 100 mg quercetin dissolved in 1 ml DMSO was added to 100 ml PBS to a final concentration of 1 mg/ml. Quercetin was immediately precipitated out. (B) A representative image of quercetin precipitated out in cell culture medium. Similar procedures as described in (A) were followed, except the final concentration was 125 μg/ml. (C) A representative image of HUVEC cells after treatment with free quercetin at 125 μg/ml. Cells were covered by precipitated quercetin. Scale bar: 100 μm.
Figure S8. Representative images of GL261 cells treated with Q-NPs and stained for ROS (green). Tert-Butyl hydroperoxide (TBHP) was used as a positive control. Scale bar: 50 μm.
Figure S9. Representative images of mice at the indicated time points after receiving intravenous administration of free IR780 or IR780-Q-NPs. All images were taken with the same instrumental settings: exposure time: 1s; fluorescence intensity range: 1000-10000.
Figure S10. Characterization of the delivery efficiency of Q-NPs. (A) Representative images of free Cy5.5, Cy5.5-labeled dendrimers NPs, and Cy5.5-labeled Q-NPs in the brain. n=3. (B) Quantification of free agents, dendrimers NPs, and Q-NPs, based on fluorescence intensity of Cy5.5 in brain tumors. Data represent mean ± SEM; n=3. Statistical analyses were performed using the unpaired, two-tailed Student’s t-test by Prism 8 (GraphPad). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure S11. Quantification of %ID/g of Q-NPs in major organs based on ICP-MS analysis.

Data represent mean ± SEM; n=3.
Figure S12. Characterization of the *in vivo* biodistribution of quercetin in the brain. (A) Linear correlation of the mass spectrometry signal intensity of quercetin with concentration. (B) Quantification of μg/g of quercetin in normal brain tissues and brain tumors in mice receiving treatment of Q-NPs. Data represent mean ± SEM; n=3. Statistical analyses were performed using the unpaired, two-tailed Student’s t-test by Prism 8 (GraphPad). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure S13. Representative images of Q-NPs (red) in brain tumors (upper panel) or normal brain tissues (bottom panel). Blue: DAPI. Yellow: CD31. GFP: tumor cells. Scale bar: 100 μm. n=3.
Figure S14. Characterization of the effects of Q-NPs and free quercetin on the normal brain and brain tumors. (A) Representative images of brain tissues immunostained for CD31 with the treatment of Q-NPs and free quercetin in normal brain tissues (upper panel) and brain tumors (bottom panel). Blue: DAPI. Green: CD31. (B) Representative H&E staining images of normal brain tissue isolated from mice with the treatment of Q-NPs. Scale bar: 50 μm. Free quercetin was given through i.p. injection.
Figure S15. Quantification of binding/uptake of Q-NPs by HUVEC cells (A) and bEnd.3 cells (B) engineered to express the indicated receptor molecules by flow cytometry. Statistical analyses were performed by comparing to the VEGFR2 group using the unpaired, two-tailed Student’s t-test by Prism 8 (GraphPad). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Data represent mean ± SEM; n=3.
Figure S16. Representative images (A) and quantification (B) of IR780-labeled Q-NPs in the indicated organs determined based on IR780 fluorescence. Data are presented as the means ± standard deviations (SDs). Statistical analyses were performed using the unpaired, two-tailed Student’s t-test by Prism 8 (GraphPad). Data represent mean ± SEM; n=3. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure S17. Representative images of Q-NPs uptake in bEnd.3 cells with and without pre-
treatment of quercetin. Scale bar: 50 μm.
Figure S18. Linear correlation of absorbance of Evans Blue at 600 nm with concentration.
Figure S19. Characterization of Q-NPs for treatment of GL261 gliomas. (A) Representative images of immunostaining by H&E, TUNEL, DAPI (blue), CD31 (green), and VEGFR2 (red) in tumors isolated from mice received the indicated treatments. Scale bar: 50 μm. (B) Representative images of H&E staining of the indicated organs isolated from mice treats with PBS or Q-NPs. Scale bar: 100 μm.
Figure S20. Hepatotoxicity assessment of Q-NPs based on ALT and AST tests.
Figure S21. Determinization of %ID/g values of Q-NPs and PLGA NPs after intravenous administration in mice bearing PS30 gliomas by ICP-MS. Data represent mean ± SEM; n=3. Statistical analyses were performed using the unpaired, two-tailed Student’s t-test by Prism 8 (GraphPad). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure S22. Representative images of immunostaining by H&E, TUNEL, DAPI (blue), CD31 (green), and VEGFR2 (red) in PS30 tumors isolated from mice received the indicated treatments. Scale bar: 50 μm.
Figure S23. Characterization of toxicity of Q-NPs in mice bearing PS30 gliomas. (A) Changes in body weight with time in mice received the indicated treatment. Data represent mean ± SEM; n=7. (B) Representative images of H&E staining of the indicated organs isolated from mice treats with PBS or Q-NPs. Scale bar: 100 μm.
Table S1. List of anti-angiogenic polyphenols tested in this study.

| Polyphenol   | Hydrophilic/hydrophobic | Reference |
|--------------|--------------------------|-----------|
| Naringenin   | hydrophobic              | 1-3       |
| Hesperidin   | hydrophobic              | 4,5       |
| Catechin     | hydrophobic              | 6         |
| Quercetin    | hydrophobic              | 7         |
| Silybin      | hydrophobic              | 8         |
| Ellagic acid | hydrophobic              | 9         |
| Curcumin     | hydrophobic              | 10        |
| Myricetin    | hydrophobic              | 11        |
| Luteolin     | hydrophobic              | 12        |
| Morin        | hydrophobic              | 13        |
| Caffeic acid | hydrophobic              | 14        |
| Chrysin      | hydrophobic              | 15,16     |
| Gallic acid  | hydrophilic              | 17,18     |
| Dopamine     | hydrophilic              | 19        |
| EGCG         | hydrophilic              | 20        |
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