Tumor Targeting and Microenvironment Responsive Smart Nanoparticles for Combination Therapy of Anti-Angiogenesis and Apoptosis

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Supporting Information

MATERIALS AND METHODS

Materials. 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, sulfosuccinimidyl ester, sodium salt (Green BODIPY) and 6-(((4, 4-difluoro-5-(2-pyrrolyl)-4-bora-3a, 4a-diaza-s-indacene-3-yl)styryloxy)acetyl) aminohexanoic acid, succinimidyl ester (Red BODIPY) were purchased from Molecular Probes (Eugene, OR, USA).

Cellular Uptake of Nanocarriers. U-87 MG cells were seeded in 24-well culture plates (Corning-Coaster, Tokyo, Japan) at a density of 3×10^4 cells/well. When achieving 70-80% confluence, the cells were incubated with PEG-DGL, (-MMP)*dt*ACPPD, *dt*ACPPD, or CPPD at the concentration of 10 μg DGL in 200 μl/well of the serum-free medium under pH 7.4 or pH 6.0. After 30 min incubation, the medium were removed, and the cells were washed with PBS three time. Then visualized and photographed under an IX2-RFACA fluorescent microscope (Olympus, Osaka, Japan). DGL used here was pre-labeled by Green BODIPY which could emit Green fluorescence.

***In Vivo* Distribution of Nanocarriers.** At the day 18th after implantation, glioma-bearing mice were injected intravenously through the tail vein with 200 μl of PEG-DGL, *dt*ACPPD, or CPPD at a dose of 200 μg DGL respectively. 1, 12, 24 h after administration, the mice were anesthetized and visualized by Cambridge Research & Instrumentation *in vivo* imaging system (CRi, MA, USA). After that, mice were sacrificed and the glioma-bearing brains were excised carefully for comparing the relative accumulation. DGL used here was pre-labeled by Red BODIPY which could emit red fluorescence.

Western Blotting for Evaluating VEGF Protein. The glioma tissues from experimental and control mice were minced and incubated on ice for 30 min in 80 μl RIPA Lysis Buffer. After that, the tissue was homogenized and centrifuged at 12,000×g at 4°C for 10 min. The supernatant protein content were measured by the BCA Protein Assay Kit. Total protein (15 μg), determined by using a Bio-Rad assay, was boiled at 100 ℃ in SDS sample buffer for 5 min, electrophoresed

on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, and transferred to polyvinyldifluoridine membranes. After that, protein detection was performed overnight at 4 ℃ with two purified primary antibodies: mouse monoclonal anti-GADPH (1:1,000), or rabbit monoclonal anti-VEGF (1:1,000). Membranes were then washed with Tris-buffered saline and Tween 20 (TBST) buffer, and incubated with horseradish peroxidase-conjugated anti-mouse, or anti-rabbit secondary antibody (1:1,000) at room temperature for 2 h, then washed three times for 10 min. The bound antibodies were detected with an enhanced chemiluminescence (ECL) detection kit and Western Blot Imaging System (FluorChem Q, Alpha Innotech, USA). The intensity of western blotting bands was analyzed by Adobe Photoshop CS5 to quantify relative VEGF protein level.

Supplemental Figures

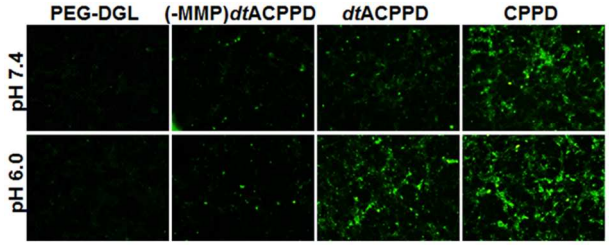

Figure S1. Cellular uptake of nanocarriers under pH 7.4 or pH 6.0. Green: Green BODIPY labeled DGL. Original magnification: 100×.

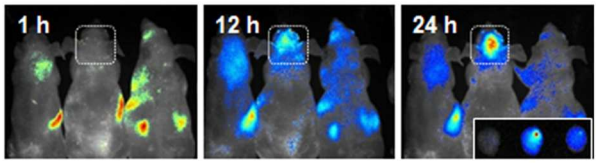

Figure S2. The real-time fluorescence imaging showed the *in vivo* distribution nanocarriers in the glioma-bearing mice treated with PEG-DGL (left in every image), *dt*ACPPD (midst in every image), or CPPD (right in every image). Images were taken at 1, 12, and 24 h after systemic administration. The illustration in the third image showed the corresponding exposed main organs that excised at 24 h after administration. DGL used here was labeled with Red BODIPY.

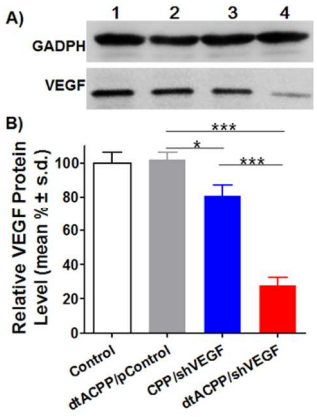

Figure S3. A) Inhibition of endogenous VEGF protein expression by transfection with shVEGF *in vivo*. Lane 1, Control (untreated); Lane 2, *dt*ACPPD/*p*Control (pGL2); Lane 3, CPPD/shVEGF; Lane 4, *dt*ACPPD/shVEGF. GADPH was used as an internal control. B) VEGF protein relative to GADPH were determined by Adobe Photoshop CS5. The results are expressed as means ± SD from three independent mean gray value analysis. Asterisks (*) denote statistically significant differences (*$p<0.05$, ***$p<0.001$) calculated by one-way ANOVA test.