Mitochondrial-Targeted Photosensitizer-Loaded Albumin Nanoparticles for the Treatment of Glioblastoma Multiforme

Supplementary Materials and Methods

Reagents

Bovine serum albumin (BSA) and folic acid (FA) were obtained from Sigma-Aldrich (St. Louis, Missouri), and pheophorbide-a (PheoA) was purchased from Frontier Scientific, Ltd. (Logan, UT). (4-Carboxy-butyl)-triphenylphosphonium bromide (TPP) was obtained from Tokyo Chemical Industry (Tokyo, Japan). All other reagents were purchased from Sigma-Aldrich or Tokyo Chemical Industry (Tokyo, Japan) unless otherwise stated.

Synthesis of mitochondrial-targeted photosensitizer (PS) Conjugate

PheoA-ethylenediamine (PheoA-EDA) was prepared according to a previous methodology [1]. Briefly, PheoA : ethylene-carbodiimide hydrochloric acid (EDC-HCI) : N-hydroxysuccinicimide (NHS) (molar ration 1:2:0.93, total 410 mg) in five mL of anhydrous chloroform and three drops of triethylamine (TEA) were mixed into solution under a nitrogen atmosphere in the dark at room temperature for 3 h. Ethylene diamine (EDA) (120 mg) protected as a tert-Butoxycarbonyl (Boc) was then added to the reaction mixture and incubated for 30 min. The reaction mixture was extracted with dichloromethane and the extract was washed twice with water of 100 mL. The organic layer was separated and dried over anhydrous sodium sulfate, and evaporated. Next, the residue was dissolved in a mixture of anhydrous dichloromethane (4 mL) and trifluoric acid (TFA) (4 mL) to remove the Boc group and stirred at room temperature for 20 min. After evaporation of solvent in a vacuum, the residue was rinsed with water and extracted with chloroform. The prepared PheoA-EDA (30 mg, 0.047 mmol) in 5 mL of dichloromethane (DCM) was subjected to reaction with N-hydroxysuccinimide (NHS; 5.4 mg, 0.047 mmol) and ethylene-carbodiimide hydrochloric acid (EDC-HCl) (14.6 mg, 0.094 mmol), in the presence of triethylamine (5 drops) for 6 h at

room temperature (RT). The reaction was monitored by thin-layer chromatography on a silica gel plate with methanol/DCM (1:5) as eluent. TPP (20.8 mg, 0.047 mmol) in DCM (1 mL) was slowly added under argon gas and stirred in the dark for 30 min. Thereafter, the solvent was removed under vacuum and the residue was purified on silica gel (230−400 mesh) and eluted with 10% acetone in dichloromethane to obtain the target compound. The product was recrystallized from dichloromethane-hexane and 80 % yield was obtained.

Synthesis of the Chol-BSA conjugate

Initially, cholesteryl bovine serum albumin (Chol-BSA) was prepared according to previous methods [2]. In brief, cholesterol (50 mg, 0.12 mmol) in 5 mL of anhydrous tetrahydrofuran (THF) was reacted with 91.5 mg (0.36 mmol) of N,N-disuccinimidyl carbonate (DSC) in 5 mL of THF in the presence of 42.9 mg (0.36 mmol) of 4-(dimethylamino) pyridine in 5 mL of THF for 24 h at room temperature (RT) under argon. Upon completion of reaction, the mixture was purified on a silica gel column using DCM/ethylacetate (98:2) as the eluent. The resulting succinated cholesterol (19 mg) in 1 mL of THF was added to 235 mg (0.012 mmol) of BSA in 12 mL of distilled water at a rate of 200 μL per 5 min, and stirred overnight under argon. The reaction product was passed through a Sephadex G25 column (DP-10; GE Healthcare, Little Chalfont, UK) to remove impurities and freeze-dried for 40 h by lyophilizer.

Synthesis of FA-chol-BSA conjugate

Initially, polyethylene-glycosylated (PEGylated) FA was prepared according to previous methods with slight modification [3]. Briefly, 100 mg (0.225 mmol) of FA was dissolved in 1 mL of dry dimethyl sulfoxide (DMSO) and added to 25.9 mg (0.225 mmol) of NHS and 92.5

mg (0.45 mmol) of dicyclohexylcarbodiimide in the presence of three drops of triethylamine. After stirring for 3 h at RT, 281 mg (0.14 mmol) of PEG2000–amine (1:1.6 molar ratio of NH_2–PEG/folate) was added to the activated FA, followed by 2 h of stirring at RT under nitrogen. The organic solvent was removed using a rotary evaporator, and the remainder was rehydrated with 6 mL of deionized water and centrifuged at 4000 rpm for 10 min to remove insoluble byproducts. Subsequently, the supernatant was lyophilized for 48 h. Further, FA-PEG-OH (12.5 mg, 0.0052 mmol) in 2 mL of anhydrous tetrahydrofuran (THF) was subjected to reaction with 6.56 mg (0.025 mmol) of *N,N*-disuccinimidyl carbonate in 2 mL of THF, in the presence of 2 mg (0.016 mmol) of 4-(dimethylamino) pyridine in 2 mL of THF for 3 h at RT under argon to activate the hydroxyl group. After the reaction was completed, the mixture was evaporated under vacuum. The resulting succinated FA-PEG in 0.4 mL of DMSO was added to 60 mg (0.00086 mmol) of chol-BSA pre-dissolved in 5 mL of 0.1 M sodium bicarbonate (pH 8.3) at a rate of 200 µL per 5 min and stirred for 1.5 h under argon. The reaction product was passed through a Sephadex G25 column to remove impurities and freeze-dried for 48 h using a lyophilizer. The conjugation was confirmed by UV-vis and FT-IR spectroscopy.

Preparation of Mitochondrial-targeted photosensitizer–Loaded Albumin Nanoparticle

Mitochondrial-targeted photosensitizer-loaded cholesteryl bovine serum albumin nanoparticles (PS@chol-BSA NPs) were prepared according to the previous method, with minor modifications [4, 5]. Briefly, 3 mg of FA-chol-BSA and chol-BSA at a ratio of 1:2 was dissolved in 2 mL of PBS. PS (1.2 mg in 100 µL of absolute ethanol) was slowly added to this solution to obtain an albumin/drug (w/w) ratio of 2.5:1 and stirred for 15 min. While it was stirred, the beaker was opened to allow ethanol evaporation. The resulting emulsion was

sonicated at 4 °C for 30 min by using a bath sonicator (Branson, Danbury, Connecticut). To remove the free PS, the nanoparticle solution was centrifuged at 2000 rpm for 3 min or filtered through a 0.45-μm membrane filter.

***In vivo* biodistribution and brain-tumor accumulation of PS@chol-BSA NPs by LC-MS/MS**

The orthotopic GBM-xenografted mice were randomly divided into 2 groups and intravenously injected with free PS and PS@chol-BSA NPs (100 μL, 1 mg/kg for PS), respectively. The free PS or PS@chol-BSA NPs were administered three times to each mice group at an interval of 1 h. One hour after last administration, organs including the brain, liver, lung, spleen, kidney and heart were excised and then the brains were divided into normal (left) and tumor side (right). Each organ and tumor was homogenized using Bioprep-24 homogenizer (Allsheng, China) and extracted by liquid-liquid extraction (LLE) [6] for quantification of PS. An internal standard (IS) of 10 μL (paclitaxel, 500 ng/mL) containing mobile phase and ethyl acetate of 250 μL to the homogenate of 10 μL. The mixture was vortexed for 3 min and centrifuged at 10,000 g, 4 °C for 10 min. The separated organic phase from the aqueous phase was transferred into new Ep tube of 0.6 mL and dried using a centrifugal vacuum evaporator. The residue was re-suspended with mobile phase of 20 μL and transferred to an insert with an auto sampler vial. The prepared samples were analyzed by LC-MS/MS. The LC-MS/MS instrumentation and analytical conditions were reported in a previous study [7]. Briefly, chromatographic separation was achieved by an analytical Sepax BR-C18 (120 Å 1.0 × 100 mm, 5 micron) column. 2 μL of sample solutions were injected and the analytes were eluted using acetonitrile and 0.1% formic acid in water (55: 45%, v/v) pumped at a constant flow of 0.05 mL/min. The isocratic separation run time was 5 min.

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Detection of the analytes were performed using multiple reactions monitoring (MRM) mode to monitor the precursor to product ion transitions of 979.5-474.3 *m/z* for PS (collision energy 56) and 876.2-308.0 *m/z* for paclitaxel (collision energy 28) in positive mode, respectively.

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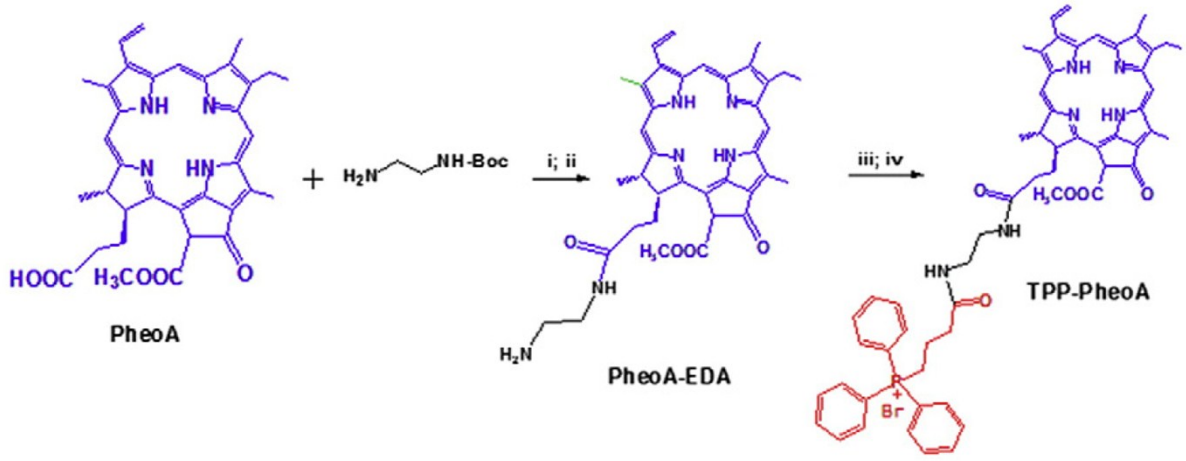
Supplementary Figure



Supplemental figure 1. A) Scheme for synthesis of TPP-PheoA conjugate. i) ethylene-carbodiimide hydrochloric acid (EDC-HCl) and 4-dimethylaminopyridine in dichloromethane (DCM); ii) trifluoroacetic acid in dichloromethane; iii) N-hydroxysuccinimide (NHS), EDC-HCl, and triethylamine in DCM, iv) (4-Carboxybutyl)triphenylphosphonium bromide in DCM [8].

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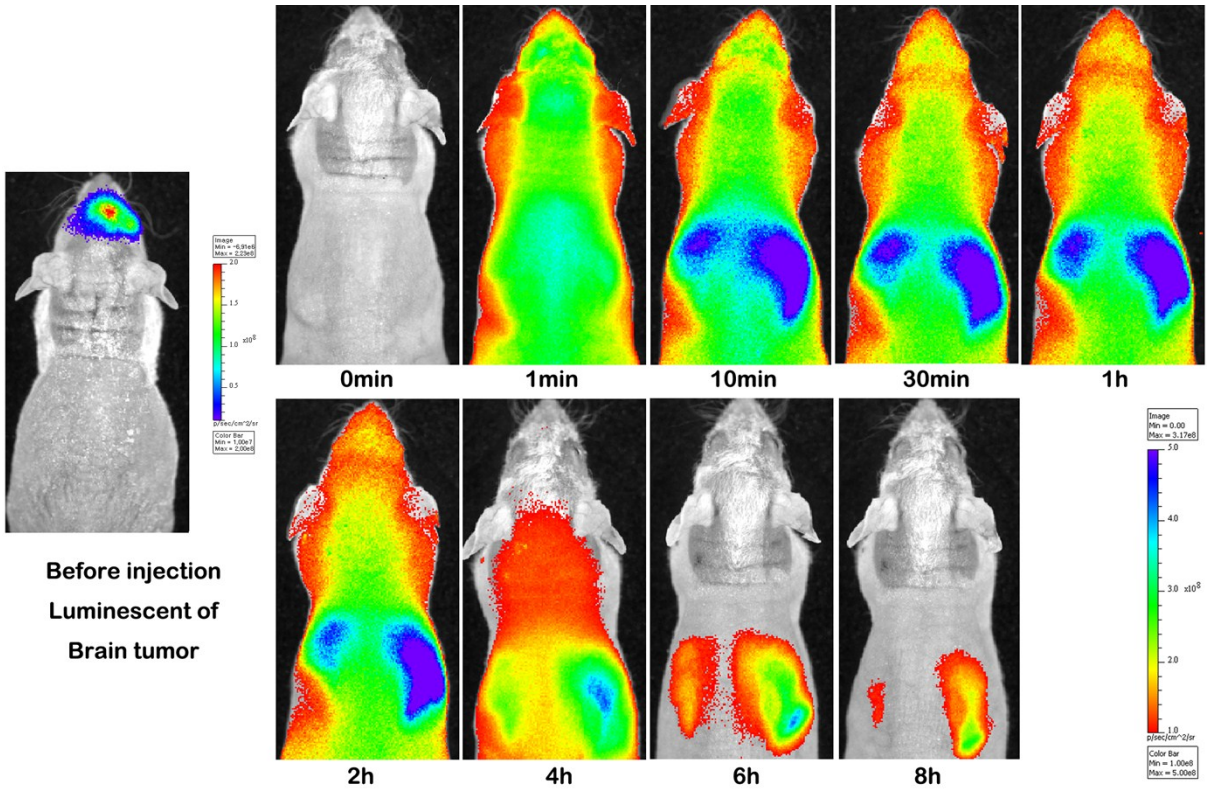

Supplemental figure 2. *In vivo* real-time biodistribution image from the orthotopic GBM-xenografted mouse following intravenous injection of free PS. Representative fluorescence images at different times following injection of free PS are shown. Fluorescence signals of free PS remained until 2 h post-treatment in the brain tumor and were cleared by 4 h post-treatment.

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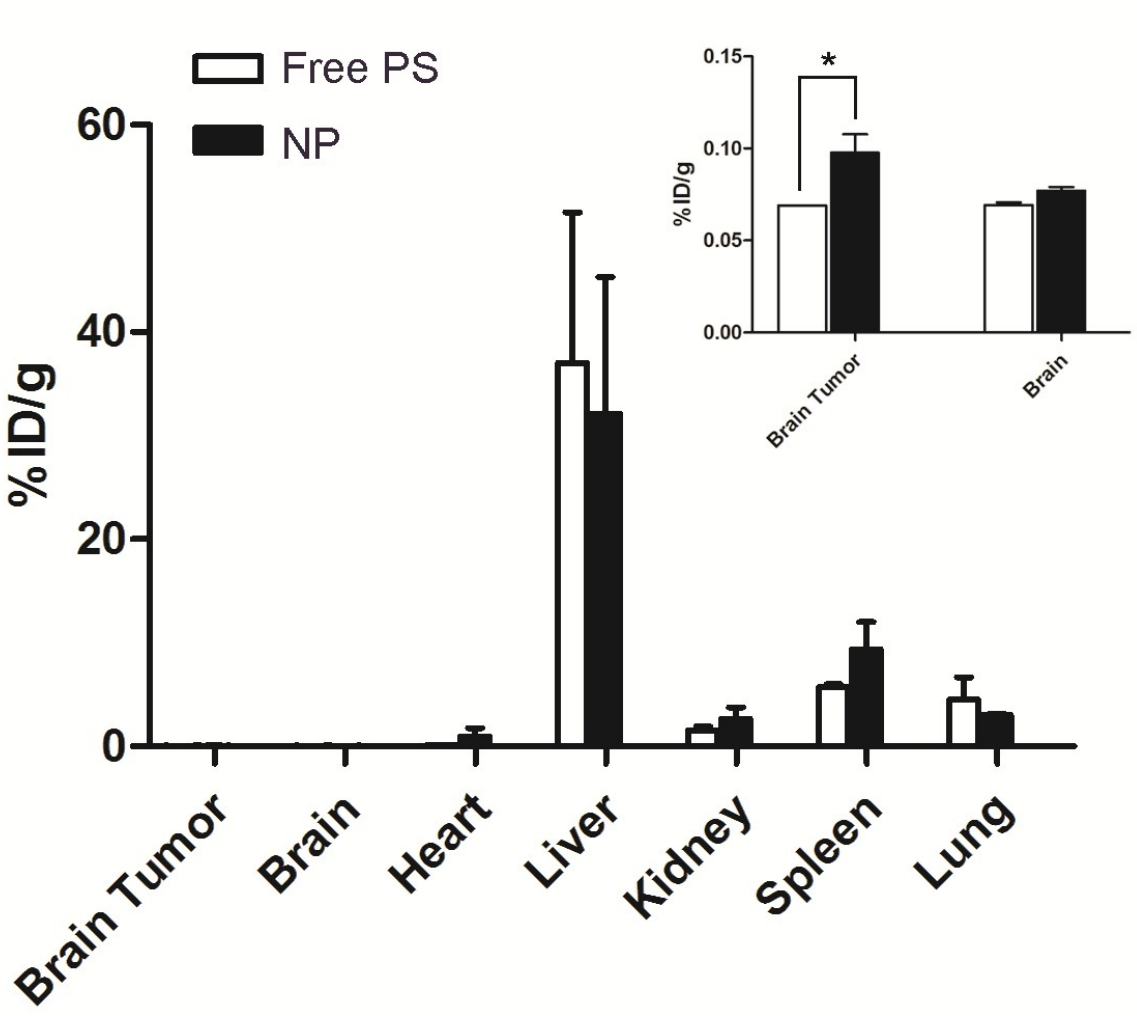

Supplemental figure 3. The %ID graph of PS contents in tissues following multiple i.v. administration. Higher accumulation of PS in brain tumor was observed in mice group treated with PS@chol-BSA NPs than that of free PS. * denotes $p<0.05$. (n=3)

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