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Supplementary Information

Artificial photosensitizer drug network for mitochondria-selective photodynamic therapy

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Materials and experimental design

Materials

Chlorin e6 (Ce6) was purchased from Frontier Scientific Inc (USA). Iodomethyltriphenylphosphonium (IMTP), triethylamine (TEA), N-(2-aminoethyl)maleimide (AEM), dimethylsulfoxide (DMSO), dichloromethane (DCM), 9,10-dimethylanthracene (DMA), dithiothreitol (DTT), N-hydroxysuccinimide (NHS), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), ethylenediamine, cystamine, and N,N′-dicyclohexylcarbodimide (DCC) were purchased from Sigma-Aldrich (USA). RPMI1640, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Welgene Inc (Korea). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies Inc (Japan). Mitotracker® was purchased from Invitrogen Inc (USA). Carboxylic poly(ethylene glycol) (PEG-COOH) and thiolated carboxylic poly(ethylene glycol) (HS-PEG-COOH) were purchased from Nanocs Inc (USA).

Synthesis of multimeric Ce6

Ce6 (10 mM) was pre-activated using DCC (40 mM) and NHS 50 mM) in DMSO at room temperature for 1 day, producing succinylated Ce6 (Ce6-NHS). Ce6-cystamine, as a counterpart reagent for preparing multimeric Ce6, was obtained after the reaction of Ce6-NHS (10 mM) and cystamine (100 mM) in DMSO with TEA (200 mM) at room temperature for 1 day. Next, Ce6-NHS (10 mM) and Ce6-cystamine (10 mM) in DMSO was mixed and stirred at room temperature for 1 day.

PEG-COOH (10 mM) or HS-PEG-COOH (10 mM) was pre-activated using DCC (12 mM) and NHS (15 mM) in DCM, producing succinylated PEG (PEG-NHS or HS-PEG-NHS). IMTP (10 mM) was coupled to terminal thiol group of HS-PEG-NHS (10 mM) in DMSO.

To tag IMTP on multimeric Ce6, the reaction between Ce6-NHS (10 mM) and Ce6-cystamine (10 mM) in DMSO was further persisted for 1 day, in the presence of PEG mixture (10 mM) [consisting of 10 wt.% of IMTP-PEG-NHS and 90 wt.% of PEG-NHS, for preparing IMTP-tagged multimeric Ce6: compound (**1**)] or [consisting of 100 wt.% of PEG-NHS, for preparing multimeric Ce6 without IMTP: compound (**2**)] . In order to remove non-reacted reagents, the solution was dialyzed using a dialysis membrane tube

(Spectra/Por MWCO 15K) against pure DMSO for 3 days and then against deionized water for 1 day, and lyophilized. In addition, to prepare non-cleavable multimeric Ce6 [compound (**3**)], ethylenediamine instead of cystamine was used as linker between Ce6 molecules.

Ce6 concentration in multimeric Ce6 conjugates was calculated after measuring the fluorescent intensity of each sample (10 μg/ml) in DMSO, based on the standard curve originated from the change in fluorescent intensity (at λ_{ex} 488 nm and λ_{em} 670 nm) in DMSO with changing concentrations of Ce6; an average of 0.70±0.01 g Ce6 per 1 g compound (**1**), 0.72±0.02 g Ce6 per 1 g compound (**2**), and 0.68±0.03 g Ce6 per 1 g compound (**3**) were embedded (n=3). IMTP concentration in compound (**1**) was calculated by ^{1}H-NMR peaks (see Supplementary Fig. 1) using the integration ratio of the peaks from δ 7.06 (-C**H**, IMTP part) and δ 1.32 [-C**H**$_3$, Ce6]; An average of 0.03 g IMTP per 1 g compound (**1**) was coupled.

Characterization of mulitimeric Ce6

The particle size distribution of multimeric Ce6 (0.1 mg/ml) treated with DTT[13] (0-500 mM) in PBS pH 8.0 (ionic strength: 0.15) at 45 °C for 45 min was measured with a Zetasizer 3000 (Malvern Instruments, USA) equipped with a He-Ne Laser beam at a wavelength of 633 nm and a fixed scattering angle of 90°. The morphology of multimeric Ce6 (0.1 mg/ml) treated with DTT was confirmed using a Field Emission Scanning Electron Microscope (FE-SEM, Hitachi s-4800, Japan). The self-photoquenching effect of multimeric Ce6 (0.1 mg/ml, PBS 150 mM, pH 7.4) with the DTT concentration (0-500 mM) was analyzed with a KODAK image station.

The generation of singlet oxygen of multimeric Ce6 (0.1 mg/ml) treated with DTT was confirmed using 9,10-dimethylanthracene (DMA)[4] as an extremely fast chemical trap for singlet oxygen. DMA (20 μmol) was mixed with multimeric Ce6 (0.1 mg/ml) in PBS (150 mM, pH 7.4) solution. The solution was irradiated at a light intensity of 5.2 mW/cm^2 using a 670 nm laser source for 10 min. When the DMA fluorescence intensity (measured using a Shimadzu RF-5301PC spectrofluorometer at λ_{ex} 360 nm and λ_{em} 380-550 nm) reached a plateau after 1 hour, the change in DMA fluorescence intensity was plotted after subtracting each sample fluorescence spectra from the pure DMA fluorescence spectra (without Ce6 conjugates), indicating no singlet oxygen.[4]

Cell culture

The human cervical carcinoma KB cells (from Korean Cell Line Bank) were maintained in RPMI1640 medium with 2 mM L-glutamine, 1% penicillin–streptomycin, and 10% FBS in a humidified standard incubator at 37°C and a 5% CO_2 atmosphere. Prior to testing, cells (1×10^5 cells/ml) grown as a monolayer were harvested via trypsinization using a 0.25 % (w/v) trypsin/0.03 % (w/v) EDTA solution. KB cells suspended in a RPMI1640 medium were seeded onto each well plate and cultured for 24 hours prior to *in vitro* cell testing.

Cellular localization study

KB cells were allowed to culture in Lab-Tek 8-well glass chamber slides for confocal microscope study. Multimeric Ce6 (20 μg/ml) was added into the culture media and incubated with KB cells for 4 hours. After incubation for the indicated time, the cells were washed three times with PBS (pH 7.4) solution, and then incubated with Mitotracker (50 nM) for 10 min and DAPI (5 μg/ml) for 5 min. The cells were again washed three times with PBS (pH 7.4) solution and then fixed using 3.7% formaldehyde in PBS. A cover slip was mounted on a microscope slide with a drop of anti-fade mounting media (5% *N*propyl galate, 47.5% glycerol and 47.5% Tris-HCl, pH 8.4) to reduce fluorescence photo-bleaching. The cells were examined using a confocal laser scanning microscope (CarlZeiss Meta LSM510, Germany).

Phototoxicity

Phototoxicity of multimeric Ce6 with light irradiation was tested for KB tumor cells. Multimeric Ce6 (equivalent Ce6 1-10 μg/ml) or free Ce6 (1-10 μg/ml) dissolved in RPMI1640 medium was administered to cells plated in 96-well plates. The cells were incubated with each sample for 4 hours and then washed three times with PBS (pH 7.4) solution. The cells were irradiated at a light intensity of 5.2 mW/cm^2 using a 670 nm laser source for 10 min and then further incubated for 12 hours. Cell viability was determined using a Cell Counting Kit-8 (CCK-8).[4,12]

Animal care

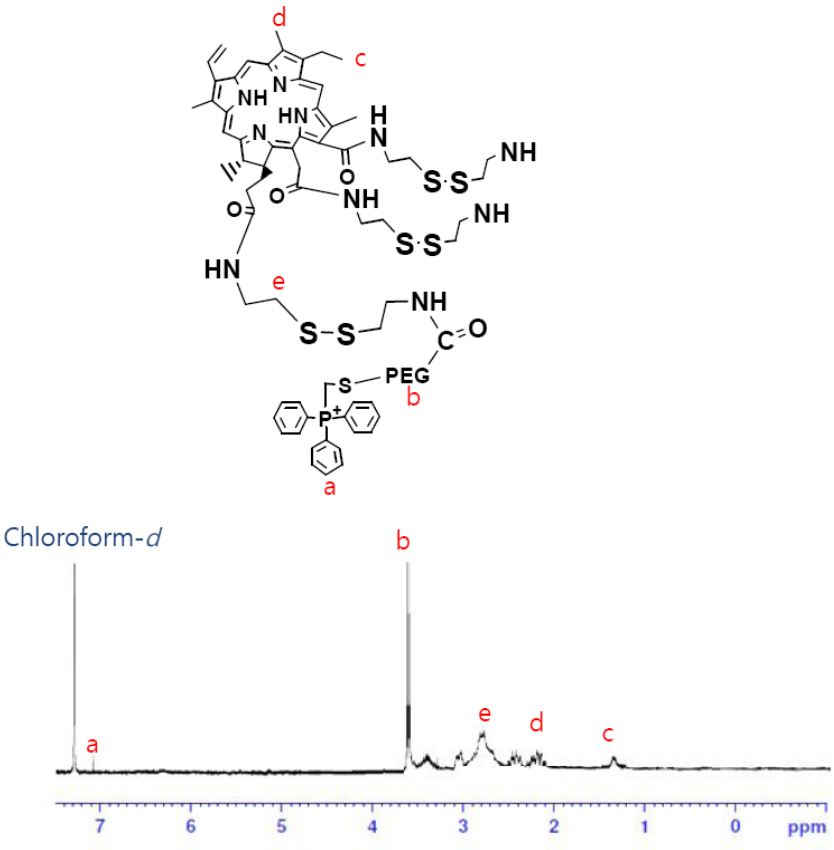
In vivo studies were conducted in 4–6 week old female nude mice (BALB/c nu/nu mice, Institute of Medical Science, Tokyo). Nude mice were maintained under the guidelines of an approved protocol from the Institutional Animal Care and Use Committee (IACUC) of the Catholic University of Korea (Republic of Korea).

In vivo fluorescence imaging and tumor inhibition

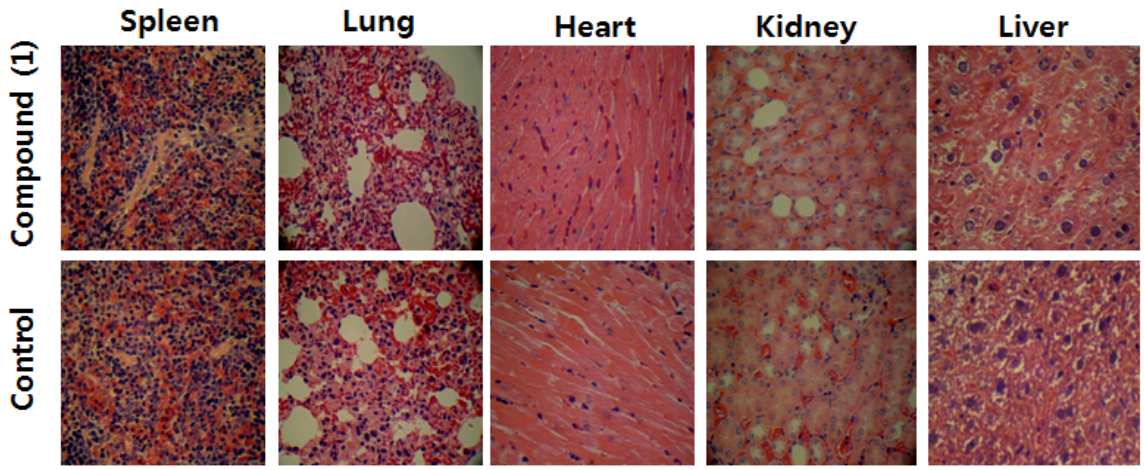
For the *in vivo* animal experiments, KB tumor cells were introduced into female nude mice via the subcutaneous injection of 1×10^4 cells suspended in PBS 7.4 (ion strength: 0.15) medium. When the tumor volume reached 25 mm^3, compound (1) (equivalent Ce6 0.5 mg/kg body), compound (2) (equivalent Ce6 0.5 mg/kg body), or free Ce6 (2.5 mg/kg) were injected (only once) intravenously into tumor-bearing nude mice through a tail vein. A 12-bit CCD camera (Image Station 4000 MM; Kodak, New Haven, CT) prepared with a special Cmount lens and a long wave emission filter (600-700 nm; Omega Optical, USA) were used to measure live fluorescence images of the nude mice.[4,10] At 24 hours' post-injection, the nude mice were sacrificed and the excised organs (tumor, liver, spleen, lung, kidney, heart) were also analyzed.

For the *in vivo* tumor inhibition test, drug-administered nude mice were locally illuminated for 40 min at a light intensity of 2.8 mW/cm^2 with a 670 nm laser source for photodynamic therapy.[4] The change in tumor volume was monitored over elapsed time. Tumor volume was calculated using the formula: tumor volume=length×$(width)^2/2$.[10] The change in the body weight of treated nude mice proved insignificant (data not shown), indicating no apparent toxicity over the whole body.

In addition, each tissue extracted at 24 hour after tumor-local irradiation was fixed with 10% formalin and embedded in paraffin. Cross-sliced tissue parts were stained with hematoxylin and eosin (H&E) and examined using a light microscope.



Supplementary Fig. 1. ^{1}H NMR peak of IMTP tagged mulimeric Ce6 [compound (**1**)].



Supplementary Fig. 2. Histology of each tissue extracted at 24 hour after tumor-local irradiation: tissues treated with compound (**1**) or untreated tissues (control).