Electronic Supplementary Information

Smart dual-functional warhead for folate receptor-specific activatable imaging and photodynamic therapy

Jisu Kim[a], Ching-Hsuan Tung[b] and Yongdoo Choi[a,*]

[a] Molecular Imaging & Therapy Branch, National Cancer Center, 323 Ilsan-ro, Ilsandong-gu, Goyang, Gyeonggi-do, Republic of Korea

[b] Molecular Imaging Innovations Institute, Department of Radiology, Weil Cornell Medical College, New York, NY 10065, United States

[* Corresponding author]
Yongdoo Choi, Ph. D.
Tel: +82-31-920-2512
Fax: +82-31-920-2630
E-mail: ydchoi@ncc.re.kr

Materials and methods

Materials

Chlorin e4 (Ce4) was purchased from Frontier Scientific, Inc. (Logan, UT). E64 and E64d (cathepsin B inhibitors) were purchased from Sigma-Aldrich (St. Louis, MO). Singlet Oxygen Sensor Green (SOSG), which is a singlet-oxygen-detecting reagent, was obtained from Invitrogen Corp. (Grand Island, NY). Cathepsin B (from human liver), cathepsin S (from human spleen), and cathepsin L (from human liver) were obtained from Calbiochem (La Jolla, CA).

Methods

Synthesis and characterization of folic acid-RRK-Ce4 (FRC) conjugates

Peptides (Scheme 1) were synthesized by Fmoc SPPS (Solid Phase Peptide Synthesis) using ASP48S (Peptron Inc. Korea):

NH_2-Lys(Dde)-2-chloro-Trityl Resin (Anaspec, USA) was added 8 eq. protected amino acid (Fmoc-Arg(Pbf)-OH, Fmoc-Glu(OH)-OtBu) and 8 eq. 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/8 eq. N-Hydroxybenzotriazole (HOBt)/16 eq. 4-Methylmorpholine (NMM) as coupling agent in dimethylformamide (DMF). The mixture was reacted for 2 h at room temperature and washed with DMF, MeOH, and DMF. For Fmoc deprotection, 20% piperidine in DMF is added the reactant, which reacted for 5 min two more times and washed with DMF, MeOH, and DMF. Peptide backbone attached resin (NH_2-gamma-E(OtBu)-R(Pbf)-R(Pbf)-K(Dde)-2-chloro-Trityl Resin) was prepared by repeating the above procedure. 4 eq. pteroic acid and 4 eq. HBTU/4 eq. HOBt/8 eq. NMM in dimethylsulfoxide (DMSO) were added peptide backbone attched resin. The mixture was reacted for 12 h, suctioned, and washed resin with DMF, methanol, and DMF. The protected group Dde of C-terminal Lys was removed by treatment with 2% hydrazine in DMF. Ce4 and HBTU/HOBt/NMM in DMF were added above peptide attached resin. The mixture was reacted for 12 h, suctioned, and washed resin with DMF, MeOH, and DMF. Synthesized peptide was cleaved from the resin by treatment with trifluoroacetic acid (TFA)/EDT/Thioanisole/triisopropylsilane (TIS)/H_2O (90/2.5/2.5/2.5/2.5). Synthesized FRC conjugates were purified by the reverse phase HPLC using a Vydac Everest C18 column (250 mm x 22 mm, 10 μm). Elution was carried out with a water-acetonitrile linear gradient (10~75% (v/v) of acetonitrile) containing 0.1% (v/v) TFA.

Molecular weights of the purified FRC conjugates were confirmed using LC/MSD (Agilent Hewlett Packard 1100 series, California, USA). UV/Vis spectra of free FA, Ce4 and FRC conjugate were measured with an UV/Vis spectrometer (DU730, Beckman Coulter, USA). Absorbance peaks at 285 nm and 403 nm in the UV/Vis spectrum of FRC conjugates (1 μM), confirmed successful conjugation of FRC conjugates.

Enzymatic activation of fluorescence emission and SOG

Enzymatic activation of FRC conjugate by cathepsin B was tested as follows: Firstly, fluorescence recovery of FRC conjugates was evaluated. The FRC (4 nmol) dissolved in 194 μL sodium acetate butter (20 mM sodium acetate, 1 mM EDTA, pH 5.0) was mixed with 97 pmol cathepsin B (6 μL in sodium acetate buffer) for the enzyme-treated sample, or equal volume of sodium acetate buffer for the buffer-treated sample. The mixture (200 μL) was then incubated for 4 h at 37 ℃. To test for enzymatic activation of FRC by other cathepsins, identical molar amounts of cathepsin L or cathepsin S were incubated with the conjugate using identical conditions as described above. For samples treated with a specific cathepsin B inhibitor (E64), 97 pmol cathepsin B (6 μL) was incubated with 0.1 mM E64 (20 μL) in 20 mM sodium acetate buffer (pH 5.0), at room temperature and for 30 min. Then, FRC (4 nmol) dissolved in 174 μL sodium acetate butter was applied to E64-pretreated cathepsin B solution and incubated for 4 h at 37 ℃. Before measuring the fluorescence spectra of the samples, the samples (50 μL) were diluted with 950 μL phosphate buffered saline (PBS, 6.7 mM, pH 7.4, 154 mM NaCl). Fluorescence spectra ($\lambda_{ex.}$ 400 nm) of the diluted samples were obtained on a multifunctional microplate reader (Safire 2; Tecan, Männedorf, Switzerland). All experiments were performed in quadruplicate. Data are expressed as the mean (SD).

Next, recovery of SOG after enzyme treatment was analyzed. The FRC (4 nmol) dissolved in 194 μL sodium acetate butter (20 mM sodium acetate, 1 mM EDTA, pH 5.0) was mixed with 97 pmol cathepsin B (6 μL in sodium acetate buffer), for the enzyme-treated sample, or equal volume of sodium acetate buffer for the buffer-treated sample. The mixture (200 μL) was then incubated for 4 h at 37 ℃. To test enzymatic activation of FRC by other cathepsins, identical molar amounts of cathepsin L or cathepsin S were incubated with the conjugate, using identical conditions as described above. For samples treated with a specific cathepsin B inhibitor (E64), 97 pmol cathepsin B (6 μL) was incubated with 0.1 mM E64 (20 μL) in 20 mM sodium acetate

buffer (pH 5.0) at room temperature for 30 min. Then, FRC (4 nmol) dissolved in 174 μL sodium acetate butter was applied to E64-pretreated cathepsin B solution, and incubated for 4 h at 37 °C. The samples (50 μL) were then mixed with an oxygen-saturated PBS containing concentrated SOSG. The final concentration of SOSG was adjusted to 1 μM. SOSG fluorescence changes were measured periodically during light illumination with a 670-nm continuous wave (CW) laser (irradiation dose rate 50 mW/cm^2). All experiments were performed in quadruplicate. Data are expressed as the mean (SD).

Fluorescence spectrum and SOG of free Ce4 at the same concentration were also tested and served as a control.

Stability of FRC in serum conditions

FRC conjugates were dispersed in PBS containing 10% fetal bovine serum (FBS, Gibco®, USA). The final concentration of FRC conjugates in the solution was adjusted to 1 μM. The solution was maintained at room temperature, and its fluorescence spectrum ($\lambda_{ex.}$ 400 nm) was measured at 0 min, 15 min, 1 h, and 4 h.

Live cell imaging studies

Folate receptor-overexpressing KB human epidermoid carcinoma cells were obtained from the American Type Culture Collection (ATCC, USA). The cells were maintained in folic acid-deficient RPMI1640 medium (Gibco) with 1% penicillin/streptomycin and 10% FBS at 37 ℃, and in a 5% CO_2 atmosphere. The cells were then plated on a 12-well plate at a density of 5×10^5 cells per well, and incubated for 24 h to allow for cell attachment. FRC conjugate and free Ce4 were dissolved and diluted with cell culture medium to achieve a concentration of 2 μM. The existing culture medium was replaced with 1 mL of fresh medium containing FRC or free Ce4, and then, without washing the cells, NIR fluorescence images were obtained every 15 min for 2 h ($\lambda_{ex.}$ 640 ± 15 nm and $\lambda_{em.}$ 690 ± 25 nm) using the Live Cell Imaging System (Axio observer Z1, 20 ×, Carl Zeiss, Germany). All the images were acquired at the same microscope settings in order to ensure reproducibility.

***In vitro* cell studies for cell-targeting and fluorescence activation**

KB cells were maintained in folic acid-deficient RPMI1640 medium (Gibco) with 1% penicillin/streptomycin and 10% FBS at 37 ℃, in a 5% CO_2 atmosphere. The cells were then plated on an 8-well Labtek chamber slide (Nalge Nunc International, USA) at a density of 1 ×

10^5 cells per well, and incubated for 24 h to allow for cell attachment. FRC conjugate and free Ce4 were dissolved and diluted with cell culture medium, to achieve a concentration of 2 μM. The existing culture medium was replaced with 200 μL of fresh medium containing FRC or free Ce4, and the cells were incubated for 4 h. In free FA competition studies, KB cells were incubated with FRC or free Ce4 for 4 h in the presence of 1 mM FA. In enzyme inhibition studies, KB cells were pre-incubated with cell-permeable cathepsin B inhibitor E64d (100 μM) for 30 min and then co-incubated with FRC or free Ce4 for a further 4 h. Untreated control cells were incubated for 4 h in the absence of photosensitizers. Finally, all the cells were washed 3 times with fresh culture medium. Fluorescence images ($\lambda_{ex.}$ 405 nm and $\lambda_{em.}$ 625–754 nm) of the cells were obtained using confocal laser scanning microscopy (CLSM, ZEISS LSM 510 META, Germany).

Next, localization of FRC in lysosomes was evaluated by labeling the lysosomes with LysoTracker® Blue DND-22 (Invitrogen, USA). KB cells were plated on an 8-well Labtek chamber at a density of 1×10^5 cells per well, and incubated for 24 h to allow for cell attachment. The cells were incubated with 2 μM FRC conjugate for 4 h and washed 3 times. Afterwards, fresh culture medium containing 100 nM LysoTracker® Blue DND-22 was added to the cells for 45 min and then fluorescence images of FRC ($\lambda_{ex.}$ 405 nm and $\lambda_{em.}$ 625–754 nm) and LysoTracker ($\lambda_{ex.}$ 405 nm, $\lambda_{em.}$ 411–497 nm) were acquired using CLSM.

***In vitro* cytotoxicity testing**

KB cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and incubated for 24 h for cell attachment. Free FRC conjugate and Ce4 were diluted in cell culture medium containing 10% FBS, to obtain equivalent concentrations of 1 μM, 2 μM, 5 μM, and 10 μM Ce4. The existing culture medium was replaced with 100 μL fresh medium containing various concentrations of FRC. After incubation for 24 h, the cells were washed 3 times, and fresh cell culture medium was added. The viability of the cells was then analyzed using a CCK-8 solution (Dojindo, Japan). The absorbance was measured at 450 nm (reference 650 nm) using a microplate reader. The student's t-test was examined for statistical analysis. Untreated control cells were used as a reference for 100% viable cells, and their medium served as the background. Data are expressed as the mean (SD) of 4 data samples.

***In vitro* phototoxicity testing**

To examine the cell viability after laser-induced PDT, cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and incubated for 24 h to allow for cell attachment. The existing medium was then replaced with 100 μL of fresh cell culture medium, containing FRC at 2 μM or 5 μM, and the cells were then incubated for 4 h. For competition assays, KB cells were incubated with FRC for 4 h in the presence of 1 mM FA. In enzyme inhibition studies, KB cells were pre-incubated with cell-permeable cathepsin B inhibitor E64d (100 μM) for 30 min, and then co-incubated with FRC for further 4 h. Finally, all cells were washed 3 times, and fresh culture medium was added. Thereafter, the cells were irradiated with a 670-nm CW laser at 20 J/cm^2 (irradiation dose rate 50 mW/cm^2). After further incubating the cells overnight, viability of KB cells was analyzed using a CCK-8 solution. Untreated control cells were used as a reference for 100% viable cells, and their medium served as the background. Data are expressed as the mean (SD) of four data samples. The student's t-test was examined for statistical analysis.

***In vivo* studies in a xenograft tumor model**

The Institutional Animal Care and Use Committee approved all animal studies. Female athymic nude mice (Balb-c/nude, 5 weeks old) were used for the *in vivo* experiments. KB cells (5×10^6 cells/50 μL of RPMI) were implanted subcutaneously into the hind flank of each mouse, and tumor size was measured periodically.[1] The animals were chosen for *in vivo* studies when their tumor sizes reached approximately 50 mm^3.

For *in vivo* NIR fluorescence imaging, 3 mice in the FRC-treated group and 3 mice in the free Ce4-treated group received intravenous injections of the sample solution at a dose of 1 mg Ce4 eq./kg at day 0. Three mice in the control group received intravenous injections of sterilized PBS solution (100 μL/mouse) at day 0. Drug solutions were prepared by dissolving FRC conjugate and the free Ce4 in sterilized 6.7 mM PBS (pH 7.4; 154 mM NaCl). NIR fluorescence images were obtained using an IVIS Lumina XR (Xenogen Corporation-Caliper, CA, USA, $\lambda_{ex.}$ 660 ± 10 nm, $\lambda_{em.}$ 710 ± 20 nm) at 3 h, 5 h, and 24 h after injection.

To investigate *in vivo* tumor therapeutic effects upon light illumination, 11 mice were tested. Briefly, 5 mice in the control group received intravenous injection with sterilized PBS solution (100 μL/mouse) on day 0, while mice in the free Ce4-treated group received intravenous injections of free Ce4 solution (1 mg Ce4 eq./kg). Mice in the FRC-treated group

received intravenous injections of FRC conjugate solution (1 mg Ce4 eq./kg). All the tumor sites in the mice were then irradiated with a 670-nm CW laser for PDT (100 mW/cm^2, 30 J/cm^2) 24 h after injection. Thereafter, tumor volumes were measured. When the tumor sizes of the control group exceeded 800 mm^3 (day 8), the measurements were stopped in all groups.

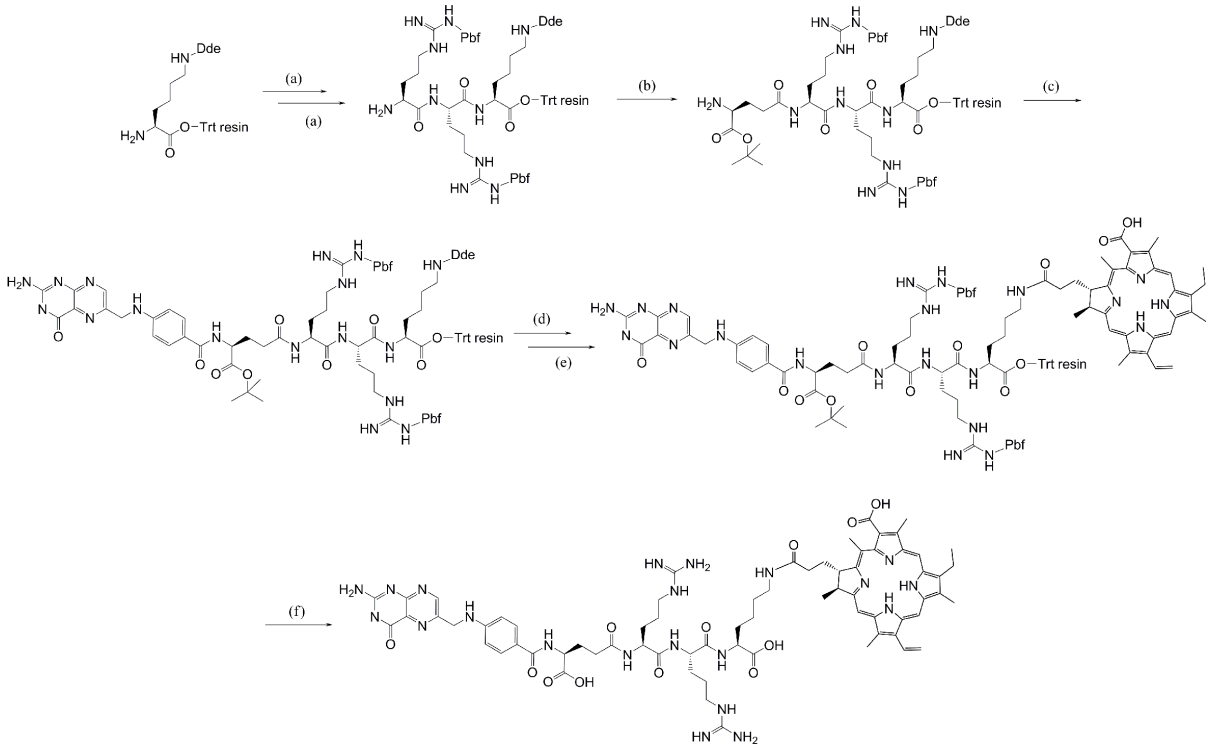
The illumination conditions used in this study were milder than that used in a previous study, in which no phototoxic effects to the cell were observed.[2]

Statistical Analysis

Data are expressed as mean ± standard deviation. Student's t-test was performed for statistical analyses.

References

1. M. M. Tomayko and C. P. Reynolds, *Cancer Chemother. Pharmacol.*, 1989, **24**, 148.
2. B. Jang, J. Y. Park, C. H. Tung, I. H. Kim and Y. Choi, *ACS Nano*, 2011, **5**, 1086.



Scheme 1. Synthesis of dual-targeted theranostic agent, FRC conjugate. (a) (1) Fmoc-Arg(Pbf)-OH, HBTU, HOBt, NMM, DMF (2) 20% piperidine, DMF (b) (1) Fmoc-Glu(OH)-OtBu, HBTU, HOBt, NMM, DMF (2) 20% piperidine, DMF (c) pteroic acid, HBTU, HOBt, NMM, DMSO (d) 2% hydrazine, DMF (e) Ce4, HBTU, HOBt, NMM, DMF (f) TFA, EDT, thioanisole, TIS, H_2O.

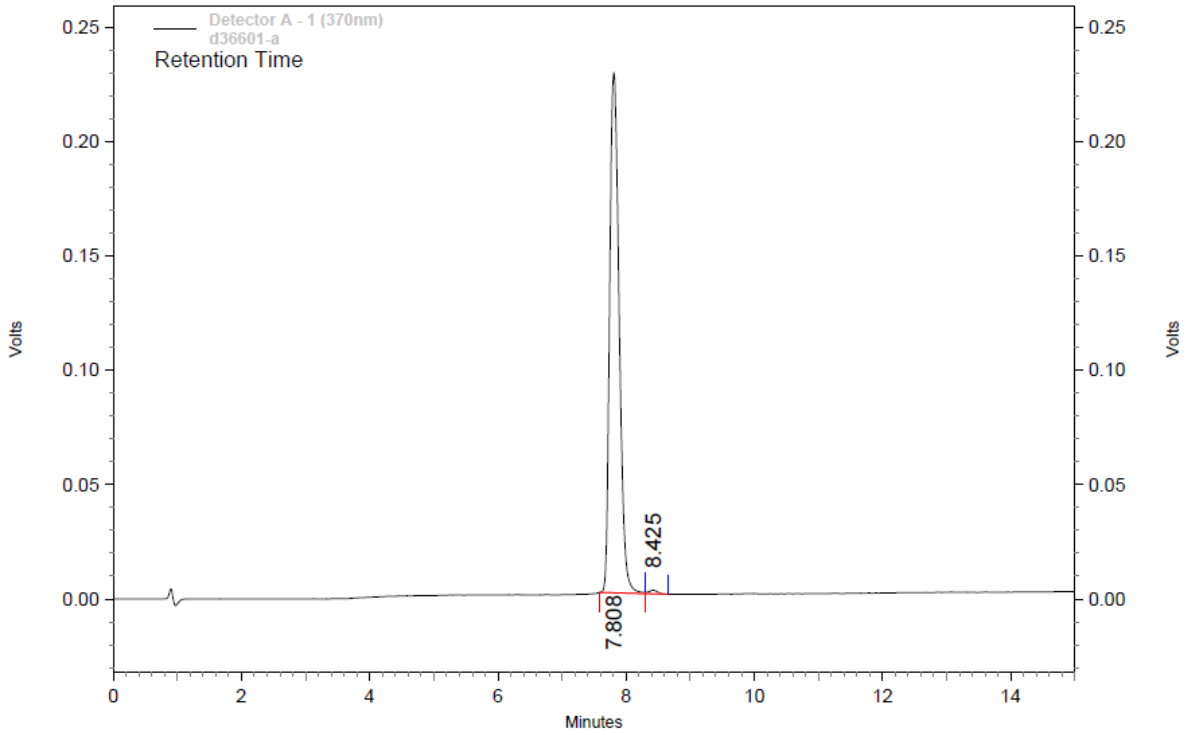


Fig. S1. HPLC chromatogram of synthesized FRC conjugate. The purified FRC conjugate was analyzed by reverse-phase HPLC (Shimadzu prominence HPLC, Kyoto, Japan) using a Vydac Everest C18 column (250 mm × 22 mm, 10 µm). Elution was carried out with a water–acetonitrile linear gradient containing 0.1% (v/v) trifluoroacetic acid. The flow rate was 1 mL/min. The composition of the mobile phase was as follows: 100% water and 0% acetonitrile at 0 min, 70% water and 30% acetonitrile at 2 min, 40% water and 60% acetonitrile at 12 min, and 10% water and 90% acetonitrile at 15 min. FRC conjugate was detected at 370 nm with a retention time of 7.808 min and a measured purity of 99.379%.

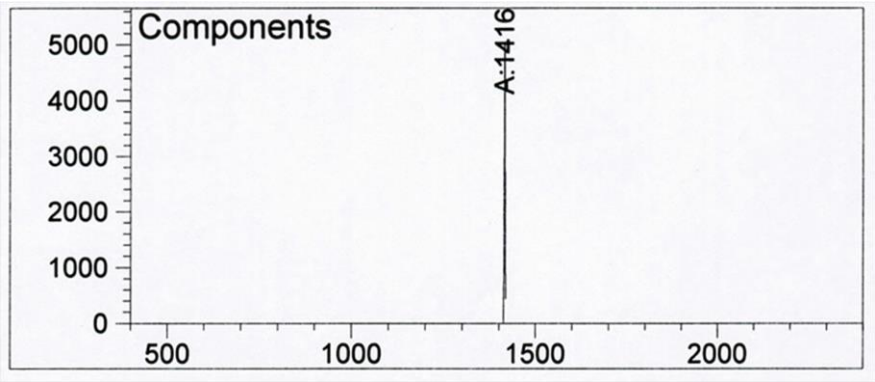


Fig. S2 Mass spectrum of the FRC conjugate.

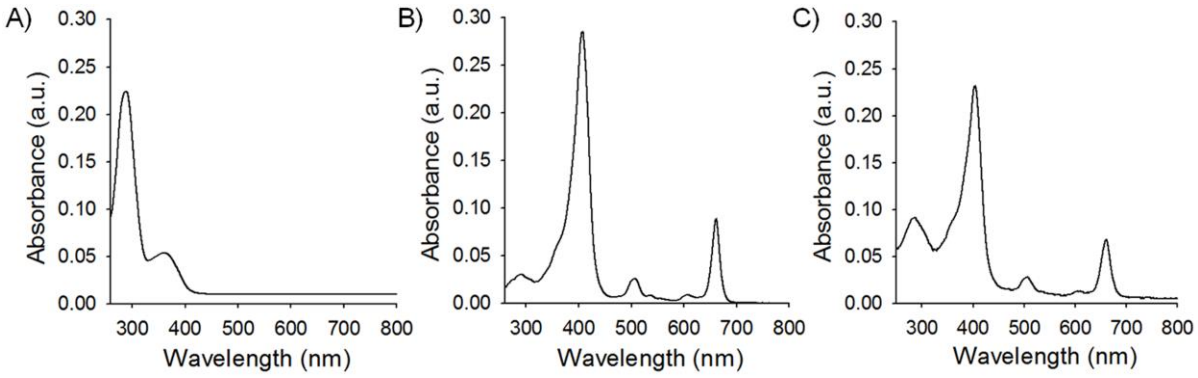


Fig. S3 UV/Vis spectra of (a) FA, (b) Ce4, and (c) FRC conjugate.

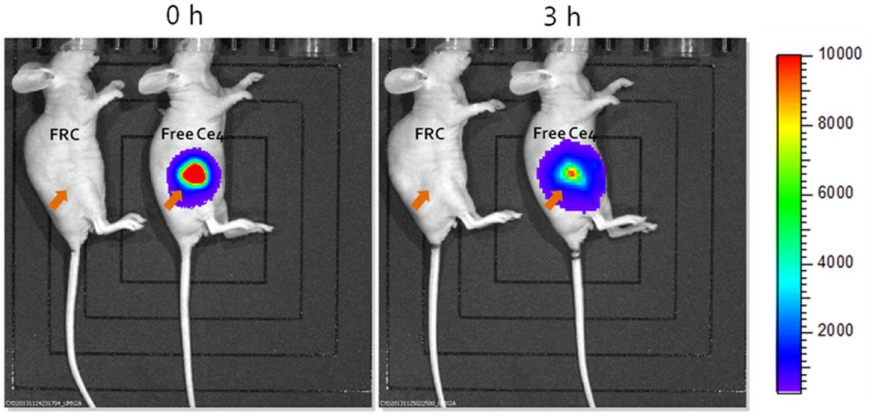


Fig. S4 NIR fluorescence image of athymic nude mice (Balb/c-nude), which received subcutaneous injection of the samples (30 µL each, 1 µM Ce4 eq./kg). The arrows indicate the injection sites of the samples. The fluorescence of the FRC conjugate was quenched *in vivo*, and its quenching was maintained for at least 3 h.

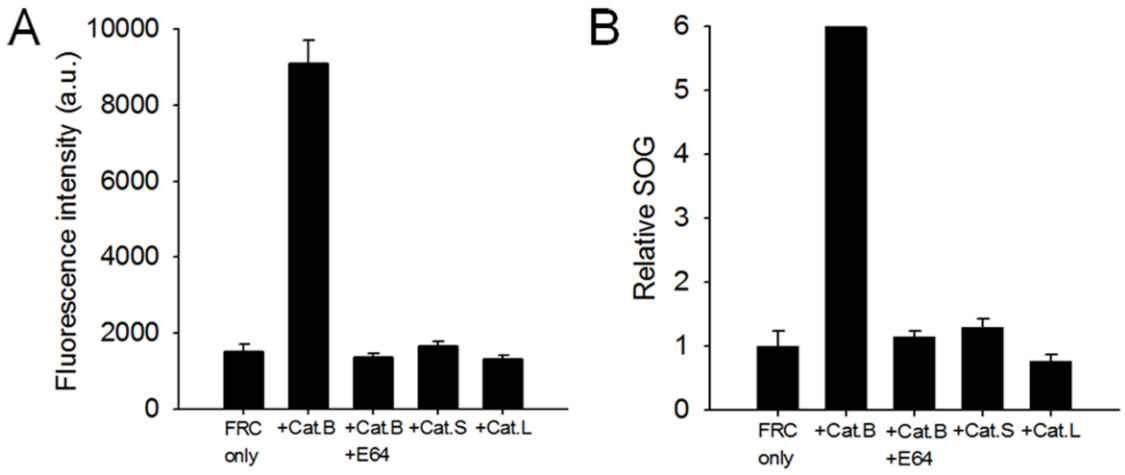


Fig. S5 (a) Changes in fluorescence intensity of buffer-treated FRC conjugate and FRC conjugates incubated with cathepsin B (Cat. B), E64-pretreated Cat. B, cathepsin S (Cat. S), and cathepsin L(Cat. L) ($\lambda_{ex.}$ 400 nm, $\lambda_{em.}$ 665 nm) of the FRC under various conditions (n = 4). (b) Relative SOG of FRC samples under various conditions.

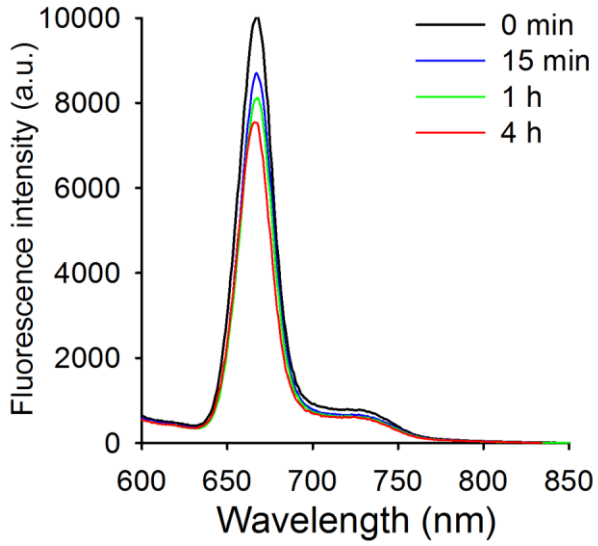


Fig. S6 Stability test of FRC conjugate in serum conditions. The conjugate was dissolved in PBS containing 10% fetal bovine serum (FBS), and the fluorescence changes were monitored for 4 h. No increase in FRC fluorescence was observed during the 4 h incubation in the presence of FBS, indicating that serum proteins do not interfere with the activation of FRC conjugate through nonspecific binding.

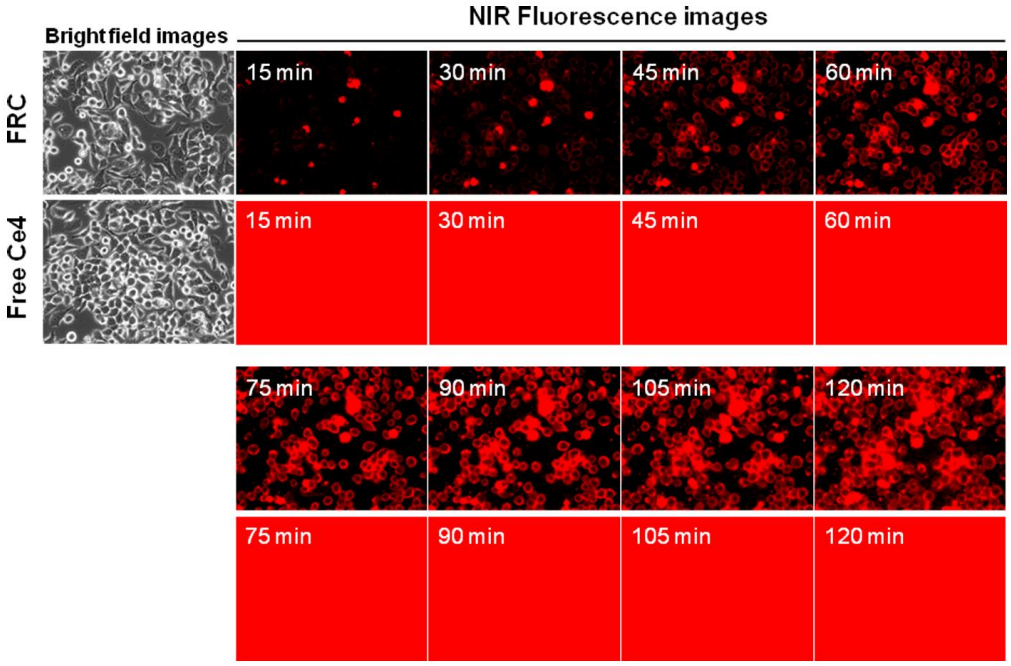


Fig. S7 NIR fluorescence images obtained from live cell fluorescence microscopy. KB cells were incubated with the FRC conjugate and free Ce4. Without washing the cells, NIR fluorescence images were obtained every 15 min for 2 h. The red color indicates the fluorescence signals generated from Ce4 ($\lambda_{ex.}$ 640 ± 15 nm, $\lambda_{em.}$ 690 ± 25 nm). The fluorescence emission from the free Ce4 inside the cancer cells could not be discriminated owing to the high background fluorescence generated from the free photosensitizers in the culture medium. In contrast, we could see the location of the cancer cells when KB cells were treated with FRC owing to the low background signals from FRC in the cell culture medium.

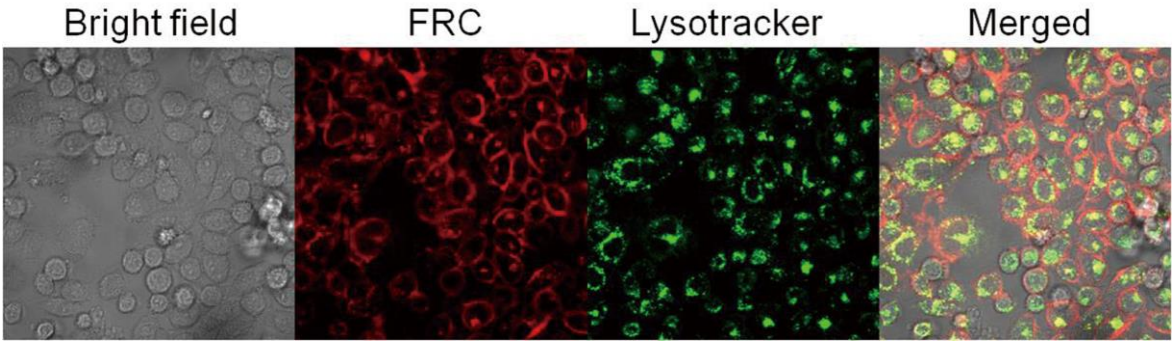


Fig. S8 Confocal microscopy images of KB cells. Cells were treated with FRC conjugates for 4 h and co-stained with LysoTracker. Yellow regions in the merged images represent co-localization of FRC (red) and LysoTracker (green) fluorescence, which indicates localization of FRC to lysosomes.

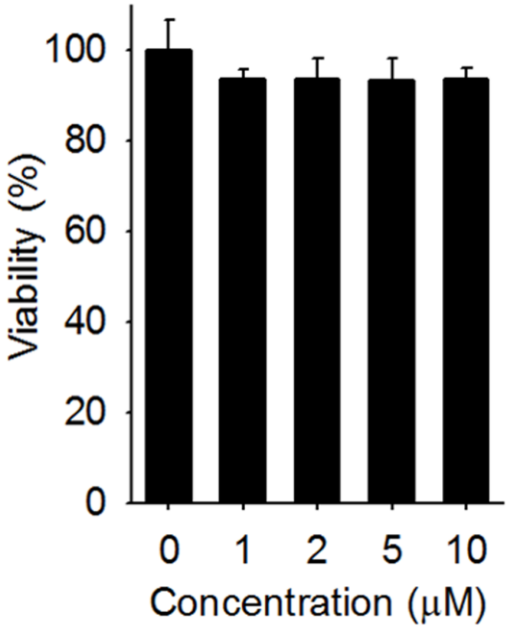


Fig. S9 Cell viability of KB cells treated for 24 h with the FRC conjugate at various concentrations.

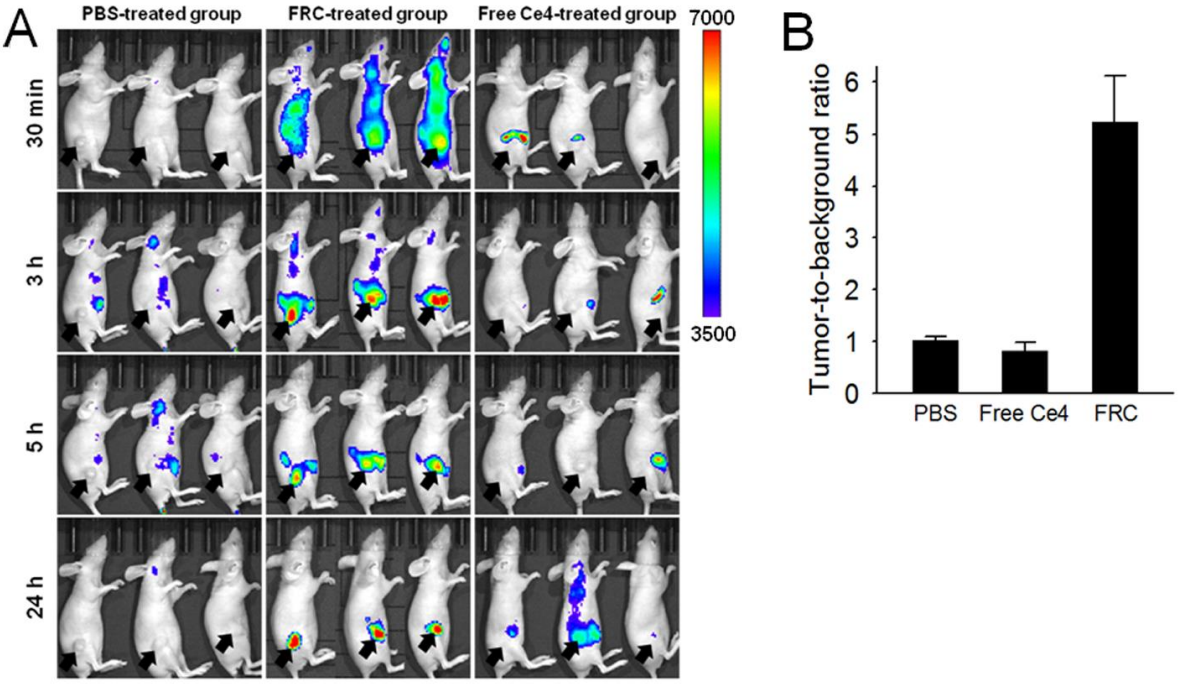


Fig. S10 (a) NIR fluorescence images ($\lambda_{ex.}$ 660 ± 10 nm, $\lambda_{em.}$ 710 ± 20 nm) of the PBS (left), FRC-treated (middle), and free Ce4-treated (right) mice were obtained 3 h, 5 h, and 24 h after injection, respectively. The arrows indicate tumor sites. (b) Tumor-to-background ratio analyzed from the NIR fluorescence images at 24 h post-injection.

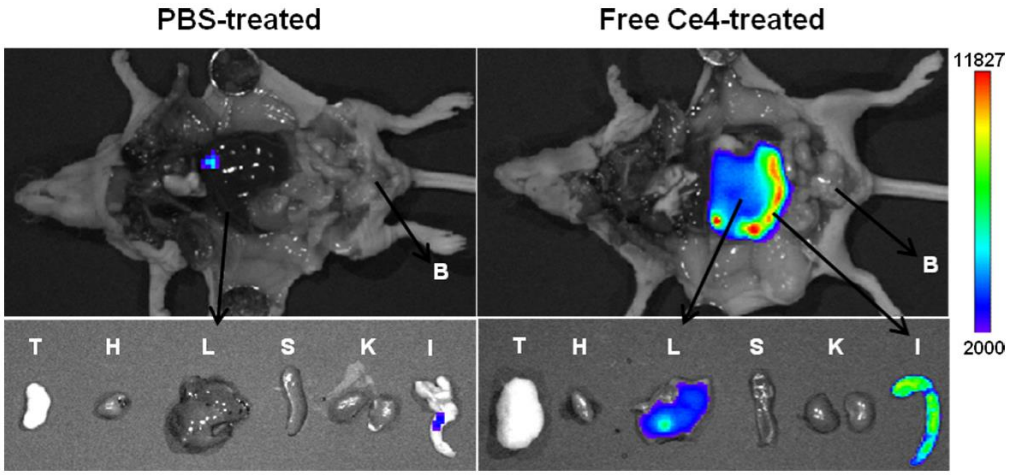


Fig. S11 *Ex vivo* imaging of internal organs and tumors at 30 min post-injection of PBS solution or free Ce4s (T: Tumor, H: Heart, L: Liver, S: Spleen, K: Kidneys, I: Intestine, and B: Bladder). High fluorescence signals shown in the Liver and intestine of free Ce4-treated mice suggest that most of the hydrophobic free Ce4s were rapidly eliminated from the blood circulation *via* hepatobiliary excretion.

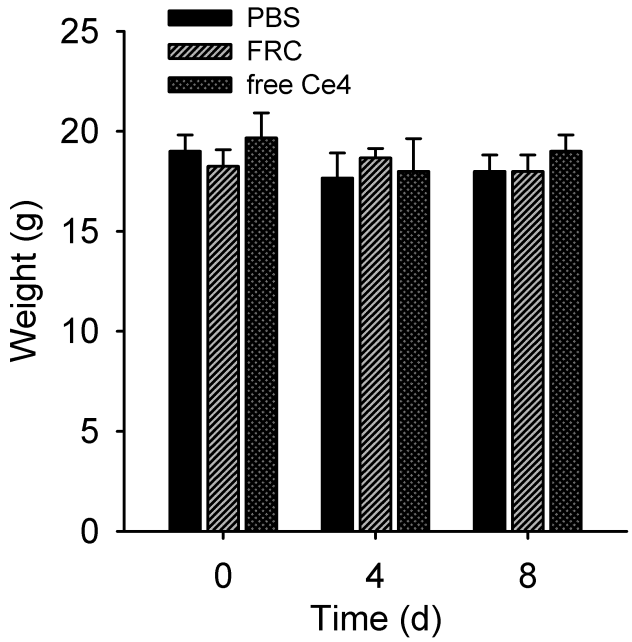


Fig. S12 Body weight of PBS as well as FRC- and free Ce4-treated mice. No significant differences in body weight were observed in FRC- and free Ce4-treated groups compared with that of the control group.