Electronic Supplementary Information

Redox-responsive Theranostic Agent for Target-specific Fluorescence Imaging and Photodynamic Therapy of EGFR-overexpressing Triple-negative Breast Cancers

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Materials and methods

Materials

Chlorin e4 (Ce4) was purchased from Frontier Scientific, Inc. (Logan, UT, USA). Dithiothreitol (DTT) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Singlet Oxygen Sensor Green (SOSG), which is a singlet-oxygen-detecting reagent, was obtained from Invitrogen Corp. (Grand Island, NY, USA). Anti-EGFR antibodies were obtained from Santa Cruz Biotechnology, Inc. (sc-120, Santa Cruz, CA, USA). Bovine serum albumin (BSA) was obtained from Merck/Millipore (Darmstadt, Germany). Tween 20 was purchased from Biosesang (Seongnam, Korea). CCK-8 cell viability assay kit was obtained from Dojindo (Kumamoto, Japan).

Methods

Synthesis of photosensitizer-peptide conjugate (RedoxT)

RedoxT was synthesized by 9-fluorenylmethoxycarbonyl solid phase peptide synthesis (Fmoc-SPPS) using ASP48S (Peptron Inc. Daejeon, Korea): 8 eq. Fmoc-miniPEG2-OH 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) in dimethylformamide (DMF) were added to H-Cys(Trt)-2-chloro-Trityl resin (Anaspec, CA, USA). The mixture was reacted for 2 h at room temperature and washed with DMF, MeOH, and DMF. For Fmoc deprotection, 20% piperidine in DMF was added to the reaction mixture and reacted for 5 min. The previous step was repeated once more and then the product was washed with DMF, MeOH, and DMF. Ce4 (4 eq.) and HBTU (4 eq.)/HOBt (4 eq.)/NMM (8 eq.) in dimethyl sulfoxide (DMSO) were added to the miniPEG2-Cys(Trt)-2-chloro-Trityl resin. The mixture was reacted for 12 h, suctioned, and washed with DMF, MeOH, and DMF. Prepared Ce4-miniPEG2-Cys was cleaved from the resin by treatment with trifluoroacetic acid (TFA)/EDT/thioanisole/triisopropyl-silane (TIS)/H_2O (90/2.5/2.5/2.5/2.5). The reaction mixture was added to 10-fold cold diethyl ether for precipitation and centrifuged at 3000 rpm for 10 min. After removing supernatant, collected peptide was purified by prep-LC and lyophilized. Ce4-miniPEG2-Cys and Aldrithiol™-2 were reacted in acetic acid for 4 h. After confirming that the reaction was completed, Ce4-miniPEG2-Cys(Pys) was purified and lyophilized.

8 eq. Fmoc- amino acid and 8 eq. HBTU/8 eq. HOBt/16 eq. NMM in dimethylformamide (DMF) were added to H-Ile-2-chloro-Trityl Resin (Anaspec, CA, USA). The mixture was reacted for 2 h at room temperature and washed with DMF, MeOH, and DMF. For Fmoc deprotection, 20% piperidine in DMF was added to the reaction mixture and reacted for 5 min. The previous step was repeated once more and then the product washed with DMF, MeOH, and DMF. Peptide backbone attached resin (H-Cys(Trt)-Tyr(t-Bu)-His(Trt)-Trp(Boc)-Tyr(t-Bu)-Gly-Tyr(t-Bu)-Thr(t-Bu)-Pro-Gln(Trt)-Asn(Trt)-Val-Ile-2-chloro-Trityl Resin) was prepared by repeating the above procedure. Synthesized peptide was cleaved from the resin by treatment with TFA/EDT/thioanisole/TIS/H_2O (90/2.5/2.5/2.5/2.5). The reaction mixture was added to 10-fold cold diethyl ether for precipitation and centrifuged at 3000 rpm for 10 min. Supernatant was removed and the procedure was repeated 2 more times. Thereafter, collected peptide (CYHWYGYTPQNVI-NH_2) was purified using prep-LC and lyophilized.

Ce4-miniPEG2-Cys(Pys) and CYHWYGYTPQNVI-NH_2 were dissolved in water-acetonitrile (1:1) solution and reacted for 12 h at room temperature. When it is confirmed that the reaction was complete, synthesized RedoxT was purified by prep-LC, lyophilized and preserved in refrigerator before use.

Characterization of RedoxT

The molecular weight of the purified RedoxT was measured using LC/MSD (Agilent Hewlett Packard 1100 series, CA, USA). The UV/Vis spectra of free Ce4 and RedoxT were measured with a UV/Vis spectrometer (DU730, Beckman Coulter, CA, USA).

Redox-responsive recovery of fluorescence emission and singlet oxygen generation (SOG)

To analyze fluorescence quenching and recovery, both free Ce4 and RedoxT were dissolved in phosphate-buffered saline (PBS, 6.7 mM; pH 7.4; 154 mM NaCl) at a concentration of 2 μM, after which its fluorescence spectrum (λex. 400 nm) was obtained using a multifunctional microplate reader (Tecan, Männedorf, Switzerland). In addition, RedoxT in PBS solution (2 μM) was treated with 2 μM or 5 mM DTT for 4 h at 37 °C, after which the fluorescence spectra of these samples were also measured and compared. For analysis of time-dependent fluorescence changes, RedoxTs were treated with PBS or DTT (final concentration of DTT: 0, 2 μM and 5 mM), and thereafter fluorescence intensities ($\lambda_{ex.}$ 400 nm and $\lambda_{em.}$ 665 nm) of the samples were measured every 30 min for 4 h. All experiments were performed in quadruplicate. Data are expressed as the mean (SD).

To evaluate the inhibitory and recovery characteristics of SOG, RedoxT in PBS (2 μM) were treated with DTT (0, 2 μM and 5 mM) for 4 h, and then mixed with an oxygen-saturated PBS containing concentrated SOSG. The final concentration of SOSG was adjusted to 5 μM. Increase in SOSG fluorescence ($\lambda_{ex.}$ 488 nm and $\lambda_{em.}$ 525 nm) were measured periodically during light illumination with a 670-nm continuous wave (CW) laser (50 mW/cm^2). For comparison, SOG of free Ce4 at the same concentration was also measured. All experiments were performed in quadruplicate. Data are expressed as the mean (SD).

Stability of RedoxT in serum conditions

RedoxT was dispersed in PBS containing 10% fetal bovine serum (FBS; Gibco, OR, USA). The final concentration of RedoxT in the solution was adjusted to 2 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.

Cell culture

Primary coronary artery smooth muscle cells (PCA-SMCs) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Vascular Cell Basal Medium containing recombinant human (rh) insulin, ascorbic acid, L-glutamine, rh EGF, and FBS at 37 °C in a 5% CO_2 atmosphere. MDA-MB-231 (human breast adenocarcinoma) cells were maintained in RPMI1640 medium (Gibco) supplemented with 1% penicillin/streptomycin and 10% FBS. MDA-MB-468 (human breast adenocarcinoma) cells were cultured in DMEM (Dulbecco's Modified Eagle Medium, Gibco) containing 1% penicillin/streptomycin and 10% FBS.

Immunocytochemistry analysis of EGFR expression levels

PCA-SMC, MDA-MB-231, and MDA-MB-468 cells were fixed with 4% paraformaldehyde in PBS for 15 min, washed 2 times using ice cold PBS. Fixed cells were incubated in PBS containing 0.25% Triton X-100 for 10 min and washed 3 times for 5 min using PBS. For blocking unspecific binding of the antibodies, cells were incubated with 1% BSA in PBST (PBS containing 0.1% Tween 20) for 30 min. Primary antibodies in 1% BSA in PBST were added the cells in a humidified chamber for 1 h at room temperature. Decant the solution and wash the cells three times with PBS for 5 min

each. Incubate cells with the secondary antibodies in 1% BSA for 1 h at room temperature in the dark. Decant the solution and wash the cells three times with PBS for 5 min each in the dark. The cells were counterstained by using 4',6-diamidino-2-phenylindole (DAPI; 1:1000 dilution in 1% BSA in PBST) for 1 min and rinsed with PBS. And then, the cells were mounted with the mounting solution. Coverslips were sealed and stored in the dark at -20 °C.

Confocal fluorescence microscopy for cell-targeting and intracellular localization

MDA-MB-231 and MDA-MB-468 cells were plated on 8-well Labtek chamber slide (Nalgene Nunc International, OR, USA) at density of 5×10^4 cells per well. And PCA-SMCs were plated on 8-well Labtek chamber slide at a density of 5 × 103 cells per well, after which they were incubated for 24 h to allow for cell attachment. RedoxT was dissolved in DMSO and diluted with cell culture medium to a concentration of 2 μM. The existing culture medium was replaced with 200 μL of fresh medium containing RedoxT, and the cells were incubated for 30 min, 1 h, and 4 h, respectively. At each time points, cells were washed 3 times with fresh culture medium, and fluorescence images of the cells ($\lambda_{ex.}$ 405 nm and $\lambda_{em.}$ 625–754 nm) were obtained using confocal laser scanning microscopy (CLSM, ZEISS LSM 510 META, Carl Zeiss, Oberkochen, Germany).

For the analysis of intracellular localization of RedoxT, MDA-MB-468 cells were treated with 2 μM RedoxT for 4 h, washed 3 times, and then fresh culture medium containing 100 nM LysoTracker® Blue DND-22 (Invitrogen, OR, USA) were to the cells and incubated for 45 min. Finally, fluorescence images of RedoxT ($\lambda_{ex.}$ 405 nm and $\lambda_{em.}$ 625–754 nm) and LysoTracker ($\lambda_{ex.}$ 405 nm and $\lambda_{em.}$ 411–497 nm) in the cells were acquired using CLSM.

***In vitro* dark toxicity testing**

MDA-MB-468 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated for 24 h for cell attachment. RedoxT was diluted in cell culture medium containing 10% FBS to concentrations of 1 μM, 2 μM, 5 μM, and 10 μM. The existing culture medium of the cells was replaced with 100 μL of fresh medium containing various concentrations of RedoxT. After 24 h, the cells were washed 3 times, and fresh cell culture medium was added. The viability of the cells was analyzed using a CCK-8 assay kit. The absorbance was measured at 450 nm (reference 650 nm) using a microplate reader. 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.

Evaluation of target specific photodynamic therapy (PDT) of TNBCs

PCA-SMCs, MDA-MB-231, and MDA-MB-468 cells were seeded on 96-well plates at a density of 1×10^4 cells per well and incubated for 24 h to allow cell attachment. RedoxT was dissolved and diluted with cell culture medium at various concentrations (0.1 μM, 0.5 μM, 1 μM, 2 μM, 5 μM, or 10 μM). The existing medium was replaced with 100 μL of fresh cell culture medium containing RedoxT, and the cells were then incubated for 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, cell viability was analyzed using a CCK-8 solution. Untreated control cells were used as a reference for 100% viable cells, while their medium served as the background. Data are expressed as the mean (SD) of four data samples.

***In vivo* near-infrared (NIR) fluorescence imaging in a xenograft tumor model**

The Institutional Animal Care and Use Committee approved all animal studies. Female athymic nude mice (Balb-c/nude,

4 weeks old) were used for the *in vivo* experiments. MDA-MB-468 cells (5×10^6 cells/50 μL of DMEM) supplemented with Matrigel (40%) were implanted subcutaneously into the hind flank of each mouse, and tumor size was measured periodically. For *in vivo* NIR fluorescence imaging, 3 mice in the RedoxT-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. RedoxT and free Ce4 were dissolved in sterilized PBS (pH 7.4; 154 mM NaCl) to prepare the drug solutions. NIR fluorescence images were obtained using an IVIS Lumina XR (Xenogen Corporation-Caliper, CA, USA; $\lambda_{ex.}$ 660 ± 10 nm and $\lambda_{em.}$ 710 ± 20 nm) 24 h after injection.

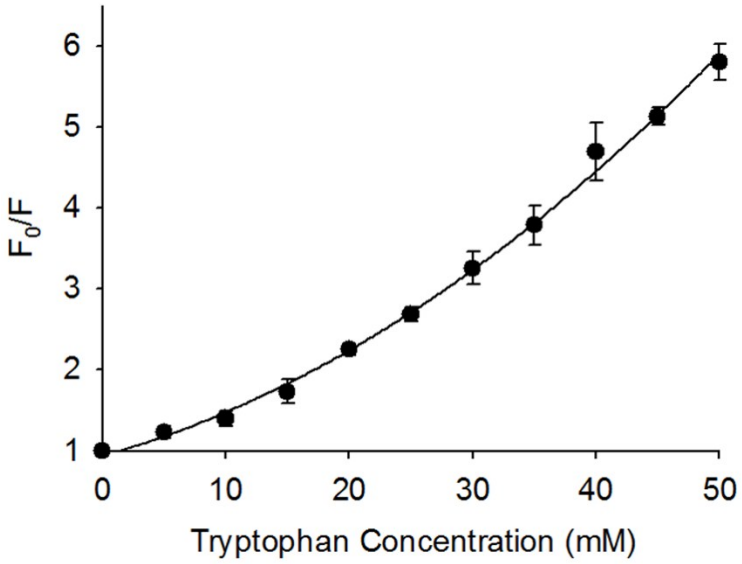

Figure S1 Fluorescence quenching of Ce4 by tryptophan. Fluorescence intensities of Ce4 were measured in the presence (F) and absence (F0) of tryptophan at various concentrations. As there is no overlap between the fluorescence of Ce4 (i.e., 665 nm) and the excitation wavelength of Trp (i.e., 282 nm), fluorescence resonance energy transfer from Ce4 to Trp can be ruled out. Photoinduced electron transfer (PET) is a potential quenching mechanism that is strongly dependent on the distance between an electron donor and acceptor [1].

Reference

[1] S. Doose, H. Neuweiler and M. Sauer, Fluorescence quenching by photoinduced electron transfer: A reporter for conformational dynamics of macromolecules. *Chem. Phys. Chem.* 2009, **10**, 1389.

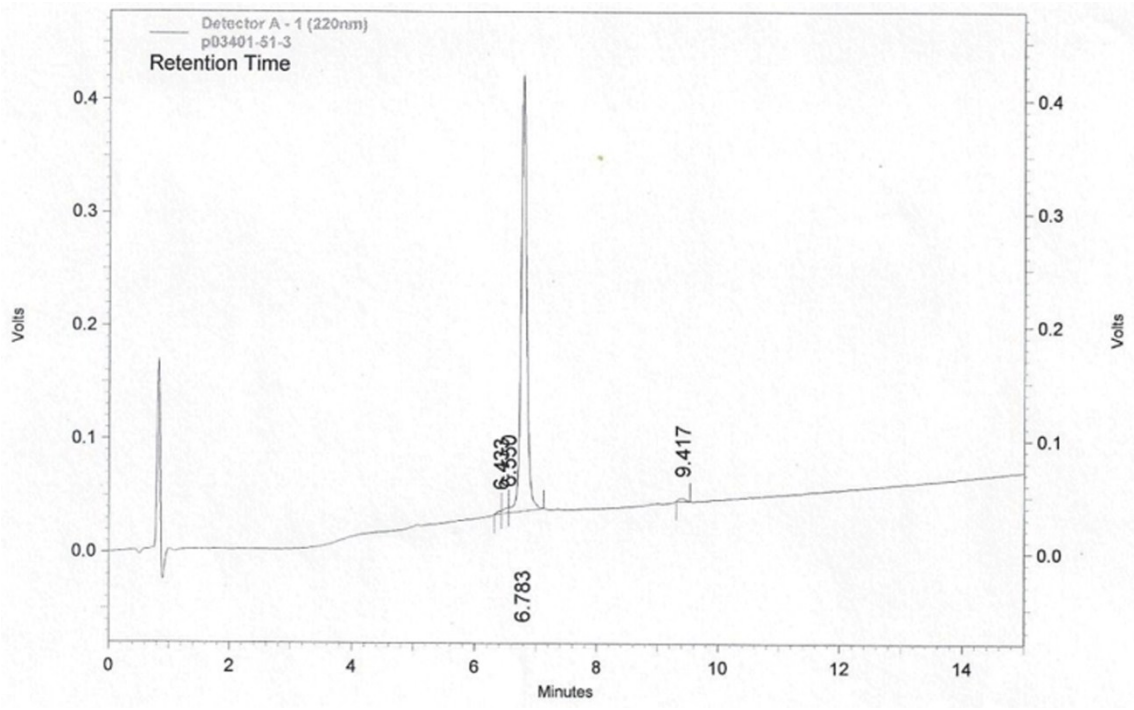

Figure S2 HPLC chromatogram of RedoxT. The purified RedoxT was analyzed by reverse-phase HPLC (Shimadzu prominence HPLC, Kyoto, Japan) using a Shiseido capcell pak C18 column. Elution was carried out with a water–acetonitrile linear gradient containing 0.1% (v/v) TFA. The flow rate was 1 mL/min. The composition of the mobile phase was as follows: 100% water and 0% acetonitrile at 0 min, 60% water and 40% acetonitrile at 2 min, 30% water and 70% acetonitrile at 12 min, and 10% water and 90% acetonitrile at 14 min. RedoxT was detected at 220 nm with a retention time of 6.783 min and a measured purity of 97.541%.

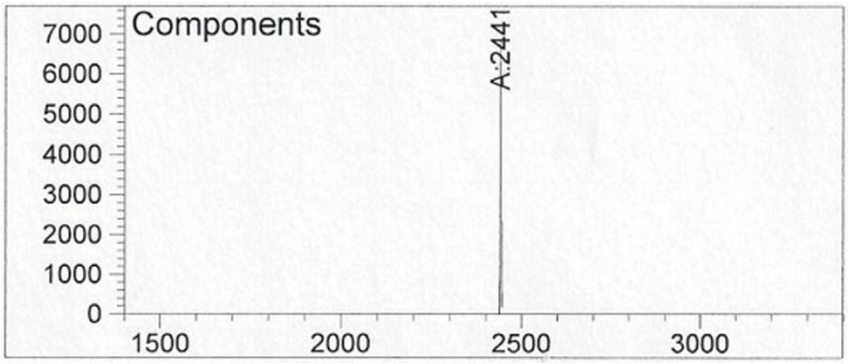

Figure S3 Mass spectrum of RedoxT.

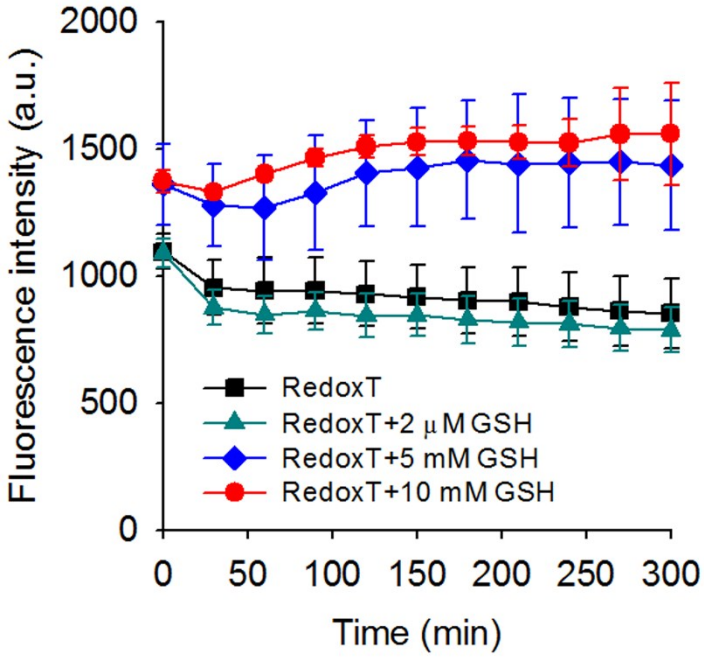

Figure S4 Time-dependent changes in RedoxT fluorescence upon glutathione (GSH) treatment. RedoxT was treated with various concentration of GSH (none, 2 μM, 5 mM, and 10 mM), and fluorescence intensity ($\lambda_{ex.}$ 405 nm and $\lambda_{em.}$ 665 nm) was measured every 30 min. A 1.8-fold increase in fluorescence intensity was obtained by exposing RedoxT to 10 mM GSH for 5 h, whereas no significant increase in florescence was observed in RedoxT samples treated with 2 μM GSH or buffer solution only.

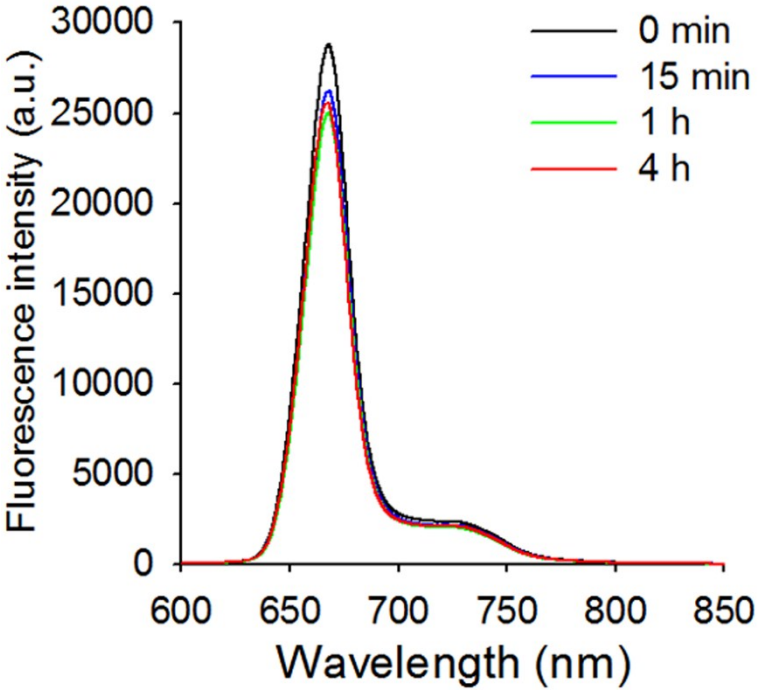

Figure S5 Stability test of RedoxT in serum conditions. RedoxT was dissolved in PBS containing 10% FBS, and the fluorescence changes were monitored for 4 h ($\lambda_{ex.}$ 400 nm). No increase in RedoxT fluorescence was observed during the 4 h incubation in the presence of FBS, indicating that serum proteins do not interfere with the RedoxT fluorescence through non-specific binding.

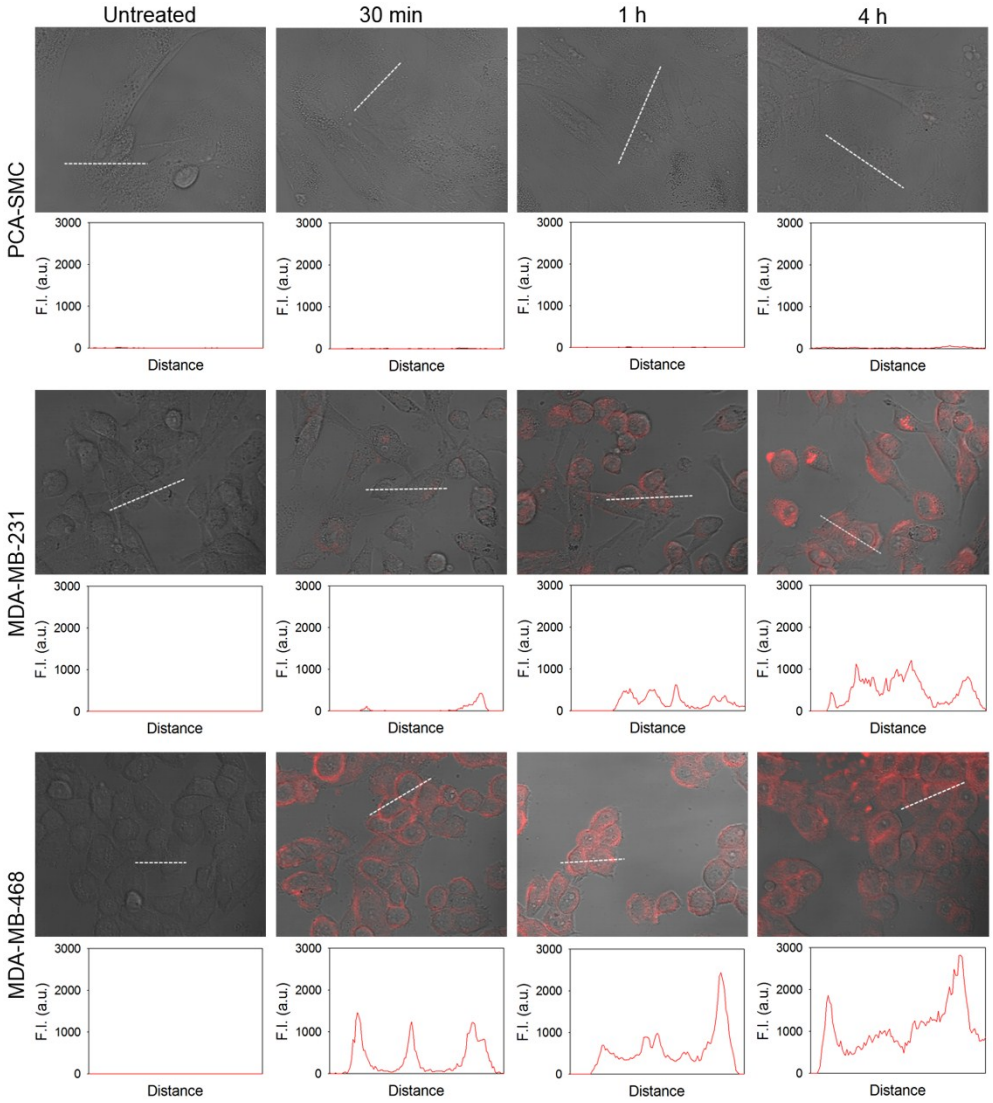

Figure S6 Fluorescence quenching and activation of RedoxT within target cells. Confocal microscopy images of PCA-SMC, MDA-MB-231, and MDA-MB-468 cells obtained using a CLSM ($\lambda_{ex.}$ 405 nm and $\lambda_{em.}$ 625–754 nm). The cells were incubated with 2 μM RedoxT and fluorescence images were obtained at 30 min, 1 h, and 4 h. The fluorescence intensity (F.I.) across the area indicated by white lines was analyzed.

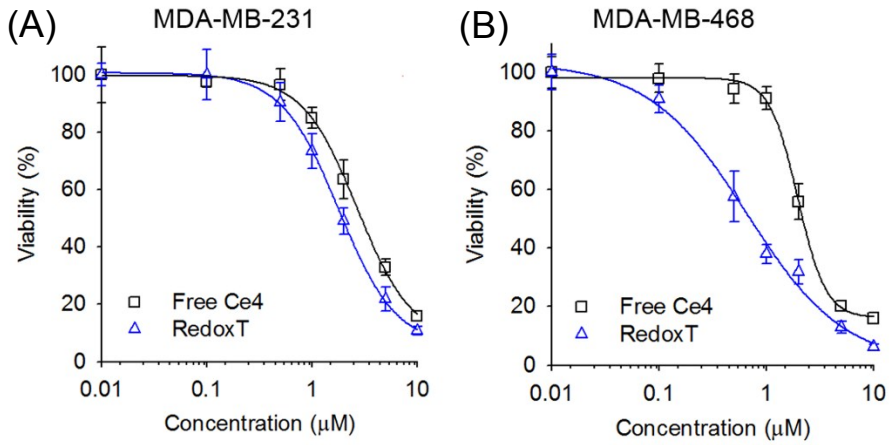

Figure S7 *In vitro* assessment of free Ce4 phototoxicity. (A) MDA-MB-231 and (B) MDA-MB-468 cells were treated with free Ce4 and RedoxT at various concentrations for 4 h, washed, and irradiated with a 670-nm CW laser (50 mW/cm^2 and 20 J/cm^2). IC_{50} of free Ce4 was calculated to be 2.98 μM in MDA-MB-231 cells and 2.22 μM in MDA-MB-468 cells.