Electronic Supplementary Material (ESI) for ChemComm.
This journal is © The Royal Society of Chemistry 2015

Supplementary Information

Platinum Prodrug Conjugated with Photosensitizer with Aggregation-Induced Emission (AIE) Characteristics for Drug Activation Monitoring and Combinatorial Photodynamic–Chemotherapy against Cisplatin Resistant Cancer Cells

Youyong Yuan,[a] Chong-Jing Zhang,[a] and Bin Liu[*,a,b]*

[a]Department of Chemical and Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4, Singapore, 117585

[b]Institute of Materials Research and Engineering, Agency for Science, Technology and Research (A*STAR), 3 Research Link, Singapore, 117602

This journal is © The Royal Society of Chemistry 2013

Experimental Section:

General information. Glutathione (GSH), *tert*-butyl *N*-(3-bromopropyl)carbamate, *N*, *N*-diisopropylethylamine (DIPEA), 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA), 4-hydroxybenzaldehyde, vitamin C (VC), anhydrous dimethyl sulfate (DMSO), buthionine-sulfoximine (BSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), folic acid, glutamic acid, bovine serum albumin (BSA), lysozyme, pepsin and other chemicals were all purchased from Sigma-Aldrich and used as received. Deuterated solvents with tetramethylsilane (TMS) as internal reference were purchased from Cambridge Isotope Laboratories, Inc.. Peptide with sequence of Asp-Asp-Asp-Asp-Asp-cyclic(Arg-Gly-Asp-D-Phe-Lys) (D5-cRGD) was customized from GL Biochem Ltd (Shanghai). Compound **2**[1] and *N*-hydroxysuccinimide (NHS) activated *cis*, *cis*, *trans*-diamminedichlorodisuccinatoplatinum(IV) complex[2] (NHS-Pt-NHS) were synthesized following our previous methods.

Dulbecco's modified essential medium (DMEM) was purchased from Invitrogen. Milli-Q water (18.2 MΩ) was used to prepare the buffer solutions from 10× phosphate buffered saline (PBS) stock buffer (1st Base, Singapore). 1× PBS (pH = 7.4) contains KCl (2.7 mM), NaCl (137 mM), Na_2HPO_4 (10 mM) and KH_2PO_4 (1.8 mM). Hoechst 33342, Annexin V-FITC, trypsin-EDTA and fetal bovine serum (FBS) were purchased from Life Technologies. 2′,7′-Dichlorodihydrofluorescein diacetate (DCF-DA) cellular reactive oxygen species assay kit was purchased from Abcam.

Characterization. NMR spectra were measured on a Bruker ARX 400 NMR spectrometer. Chemical shifts were reported in parts per million (ppm) referenced

This journal is © The Royal Society of Chemistry 2013

with respect to residual solvent. Particle size and size distribution were determined by laser light scattering (LLS) with a particle size analyzer (90 Plus, Brookhaven Instruments Co., United States) at a fixed angle of 90° at room temperature. A 0.1% trifluoroacetic acid (TFA) in H_2O and acetonitrile were used as eluents for the HPLC experiments. Electrospray ionization mass spectrometry (ESI-MS) was performed on Proteome X-LTQ (Thermo Fisher Scientific, United States). Freeze drying was performed using Martin Christ Model Alpha 1-2/LD. UV-vis absorption spectra were taken on a Shimadzu Model UV-1700 spectrometer. Photoluminescence (PL) spectra were measured on a Perkin-Elmer LS 55 spectrofluorometer. All UV and PL spectra were collected at 24 ± 1 °C.

Synthesis of compound 1. To the solution of 4-hydroxybenzaldehyde (360 mg, 2.95 mmol) in acetonitrile (10 mL) was added *tert*-butyl *N*-(3-bromopropyl)carbamate 980 mg, 4.11 mmol) and K_2CO_3 (480 mg, 3.48 mmol). The resulting mixture was stirred at reflux overnight. After the mixture was cooled down to room temperature, the mixture was filtered and the filtrate was concentrated and purified with chromatography (hexane:ethyl acetate v/v = 3:1) to give the desired product (colorless oil, 390 mg, 47.4%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.85 (s, 1H), 7.81 (dd, J_1 = 1.6 Hz, J_2 = 5.6 Hz, 2H), 6.98 (dd, J_1 = 1.6 Hz, J_2 = 5.6 Hz, 2H), 4.78 (brs, 1H), 4.08 (t, J = 4.8 Hz, 2H), 3.32 (m, 2H), 2.02 (m, 2H), 1.41 (s, 9H).

Synthesis of TPECB-NH_2 (3). To the solution of compound **2** (40 mg, 0.083 mmol) in isopropanol (5 mL) was added compound **1** (30 mg, 0.11 mmol) and piperidine (0.68 mg, 0.008 mmol). The resulting solution was refluxed for 24 hours. Then the

solvent was removed under reduced pressure. The desired residue was purified with chromatograpy (hexane: ethyl acetate v/v = 5:1) to give a red oil. This oil was further treated with the mixture of dichloromethane (5 mL) and trifluoroacetic acid (1 mL) for 8 hours. Then the solvent was removed under reduced pressure. The residue was purified with reverse HPLC using acetonitrile and water as the mobile phase to give the desired product (yellow solid, 12 mg, 23.0%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.79 (brs, 2H), 7.63 (d, J = 8.8 Hz, 2H), 7.40 (d, J = 15.2 Hz, 1H), 7.27 (d, J = 8.4 Hz, 2H), 7.13-7.20 (m, 2H), 7.15 (m, 3H), 7.02-7.06 (m, 4H), 6.87-6.92 (m, 4H), 6.67-6.73 (m, 5H), 4.16 (d, J = 6.0 Hz, 2H), 3.68 (s, 6H), 2.95-3.00 (m, 2H), 2.00-2.04 (m, 2H). MS (ESI) calcd for $[M+H]^+$: 644.2913, found: 644.2926.

Synthesis and purification of the prodrug TPECB-Pt-D5-cRGD. In a typical reaction, TPECB-NH_2 (5.0 mg, 7.8 μmol) and amine-functionalized D5-cRGD (9.2 mg, 7.8 μmol) were dissolved in anhydrous DMSO (0.5 mL) with DIPEA (1.0 μL) and the mixture was stirred at room temperature for 10 min. Then NHS-Pt-NHS (5.6 mg, 7.8 μmol) in anhydrous DMSO (0.5 mL) was added quickly to the above solution. The reaction was continued with stirring at room temperature for another 24 h. The final product was purified by prep-HPLC (solvent A: water with 0.1% TFA, solvent B: CH_3CN with 0.1% TFA) and lyophilized under vacuum to yield the prodrug as yellow powders in 38% yield (6.6 mg).

General procedure for drug activation monitoring. DMSO stock solution of **TPECB-Pt-D5-cRGD** (1 mM) were diluted into a mixture solvent of DMSO and PBS (v/v = 1/199). Then the prodrug was incubated with GSH at room temperature and the fluorescence change was studied. The solution was excited at 405 nm, and the emission was collected from 525 to

775 nm.

ROS detection in solution. The ROS generation was studied by using ABDA as an indicator as the absorbance of ABDA decreases upon reaction with ROS.[3] For ROS detection, the stock solution of ABDA in DMSO was mixed with GSH pretreated **TPECB-Pt-D5-cRGD** (10 μM) and exposed to white light irradiation. The decomposition of ABDA was monitored by the absorbance decrease.

Cell culture. U87-MG human glioblastoma cancer cells, MDA-MB-231 and MCF-7 human breast cancer cells and 293T normal cells were provided by American Type Culture Collection (ATCC). The cells were cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated FBS (Invitrogen), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (Thermo Scientific) and maintained in a humidified incubator at 37 °C with 5% CO_2. Before experiments, the cells were precultured until confluence was reached.

Confocal imaging. U87-MG, MDA-MB-231, MCF-7 and 293T cells were cultured in the chambers (LAB-TEK, Chambered Coverglass System) at 37 °C. After 80% confluence, the culture medium was removed and washed twice with 1× PBS buffer. The prodrug in DMSO stock solution was diluted with DMEM medium and added to the chamber to reach a final concentration of 10 μM. In some experiments, the cells were pre-incubated with DMEM medium containing free cRGD (50 μM) or BSO (50 μM) prior to prodrug incubation. After incubation of the prodrug at 37 °C for different time, the cells were washed twice with ice-cold PBS and the cell nuclei were living stained with Hoechst 33342 following the standard protocol of the manufacturer (Life Technologies). Annexin V-FITC was used for the cell

apoptosis imaging following the standard protocol of the manufacturer (Life Technologies). The cells were then imaged immediately by confocal laser scanning microscope (CLSM, Zeiss LSM 410, Jena, Germany). For the prodrug detection, the excitation wavelength was 405 nm, and the emission was collected above 560 nm; for Hoechst detection, the excitation wavelength was 405 nm, and the emission filter was 430−470 nm; for Annexin V-FITC detection, the excitation wavelength was 488 nm, and the emission filter was 505−525 nm. The images were analyzed by Image J1.43 × program (developed by NIH, http://rsbweb.nih.gov/ij/).

Intracellular ROS detection. The ROS generation inside the cells upon light irradiation was studied using a cell permeable indicator DCF-DA. MDA-MB-231 cells in 8-well chambers were firstly incubation with the prodrug (10 μmol) for 4 h in the dark, then DCF-DA (1 μmol) was loaded into the cells. After 5 min incubation, the cells were washed with 1× PBS and exposed to white light irradiation (30 s, 0.25 W cm^{-2}). Then the cells were washed with 1× PBS and studied by confocal microscope (CLSM, Zeiss LSM 410, Jena, Germany). For DCF detection, the excitation was 488 nm, and the emission filter was 505−525 nm.

Flow cytometry study. MDA-MB-231, MCF-7 and 293T cells in 24-well plate (Costar, IL, USA) were precultured overnight and incubated with the prodrug for the designated time. After incubation, the cells were treated with trypsin, washed with medium twice and subjected to flow cytometric analysis using Cyan-LX (DakoCytomation). The mean fluorescence was determined by counting 10,000 events.

Cytotoxicity of the prodrug. 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were used to assess the metabolic activity of U87-MG, MDA-MB-231,

MCF-7 and 293T cells. The cells were seeded in 96-well plates (Costar, IL, United States) at an intensity of 4×10^4 cells mL^{-1} and incubated at 37 °C for 24 h. After incubating with the prodrug suspension at different concentrations for 4 h, each medium was replaced with the fresh one. After white light irradiation (0.25 W cm^{-2}) for different time duration, the cells were further incubated for 72 h. After that, the cells were washed twice with 1× PBS buffer, and 100 μL of freshly prepared MTT (0.5 mg mL^{-1}) solution in culture medium was added into each well. The MTT medium solution was carefully removed after 3 h incubation in the incubator at 37 °C. DMSO (100 μL) was then added into each well and the plate was gently shaken to dissolve all the precipitates formed. The absorbance of MTT at 570 nm was monitored by the microplate reader (Genios Tecan). Cell viability was expressed by the ratio of absorbance of the cells incubated with prodrug suspension to that of the cells incubated with culture medium only. The dose-effect profiles were obtained by sigmoidal logistic fitting using Origin 8.0 (OriginLab, Northampton, MA) and the half-maximal inhibitory concentration (IC_{50}) values were determined on the basis of the fitted data.

References:

(1) Yuan, Y. Y.; Zhang, C. J.; Gao, M.; Zhang, R. Y.; Tang, B. Z.; Liu, B. *Angew. Chem. Int. Ed.* **2015**, *54*, 1780.

(2) Yuan, Y. Y.; Kwok, R. T. K.; Tang, B. Z.; Liu, B. *J. Am. Chem. Soc.*, **2014**, *136*, 2546.

(3) Gomes, A., Fernandes, E., Lima, J. *J. Biochem. Biophys. Methods* **2005**, *65*, 45.

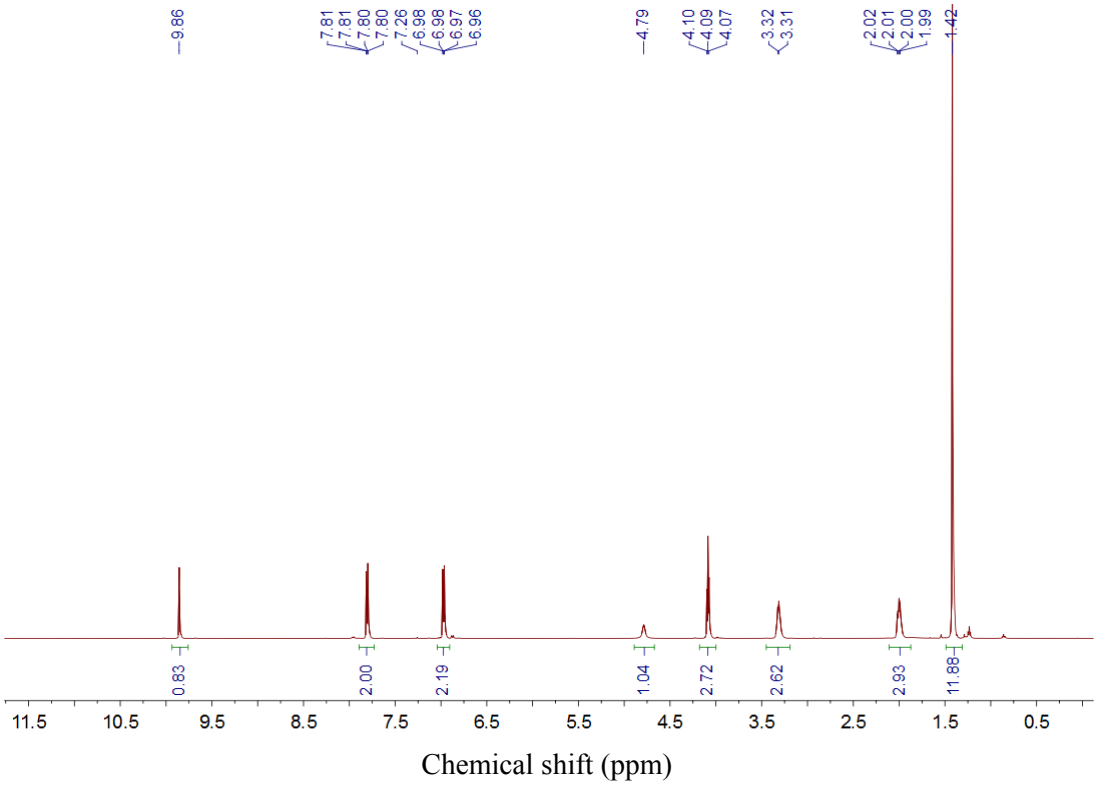
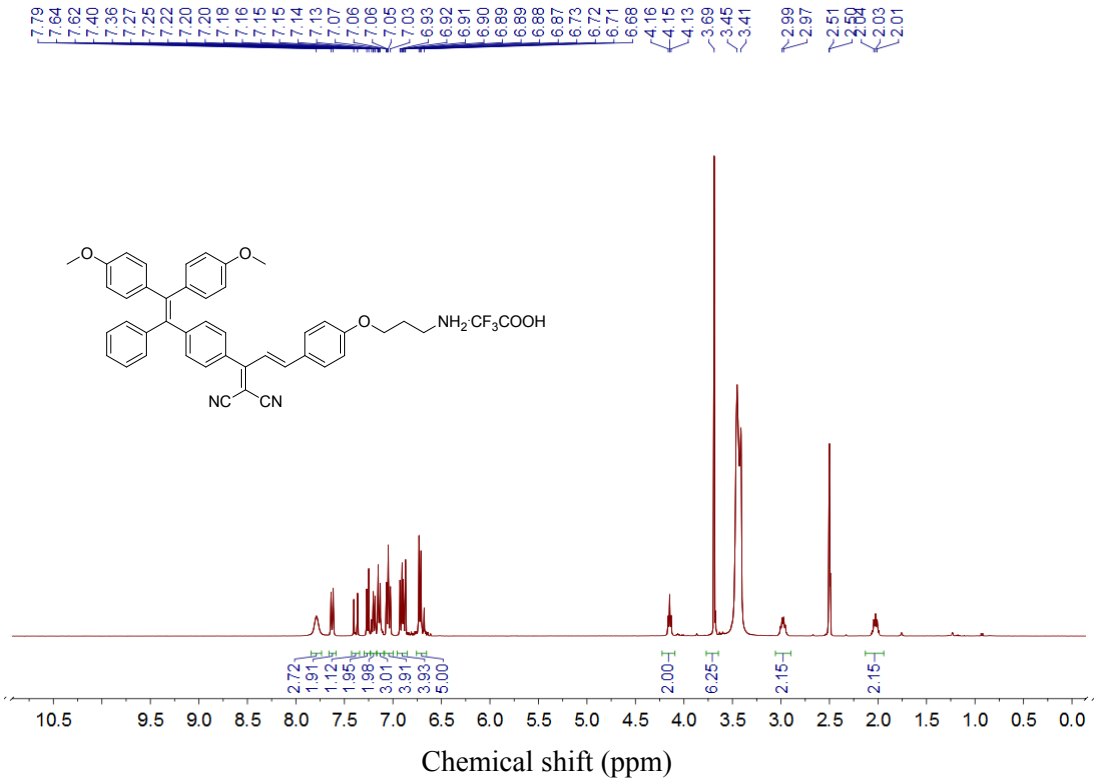


Figure S1. ^{1}H NMR spectrum of compound **1.**



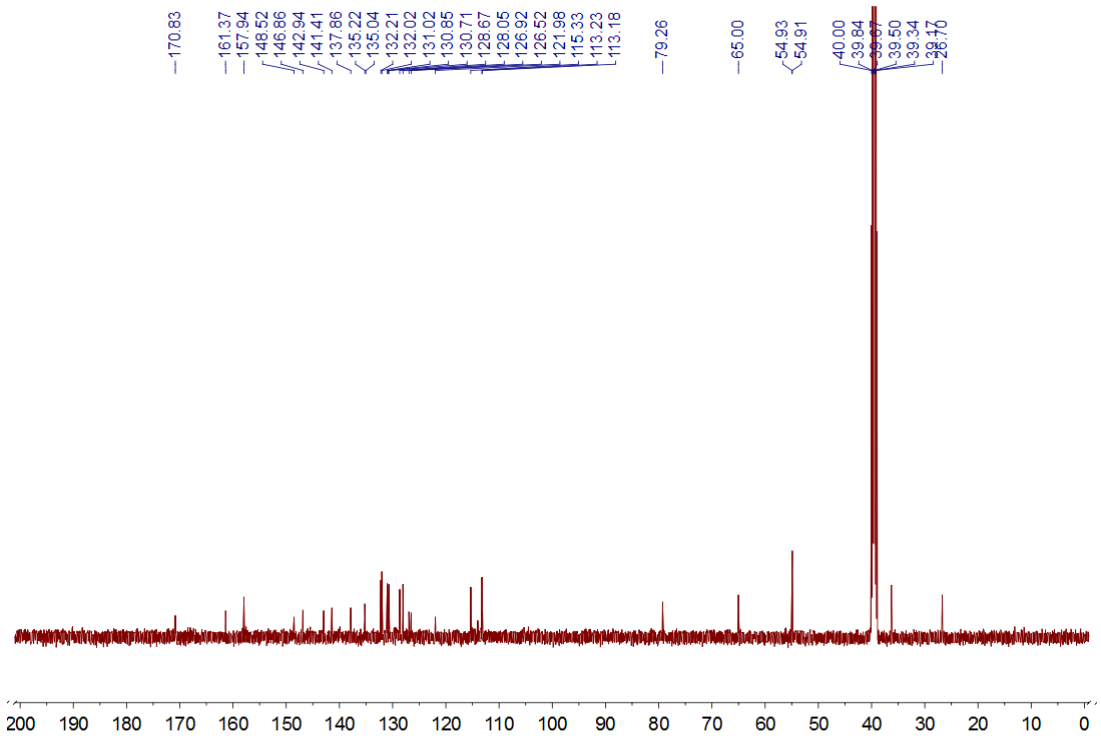


Figure S2. ^{1}H NMR (top) and ^{13}C NMR (bottom) spectrum of compound **3.**

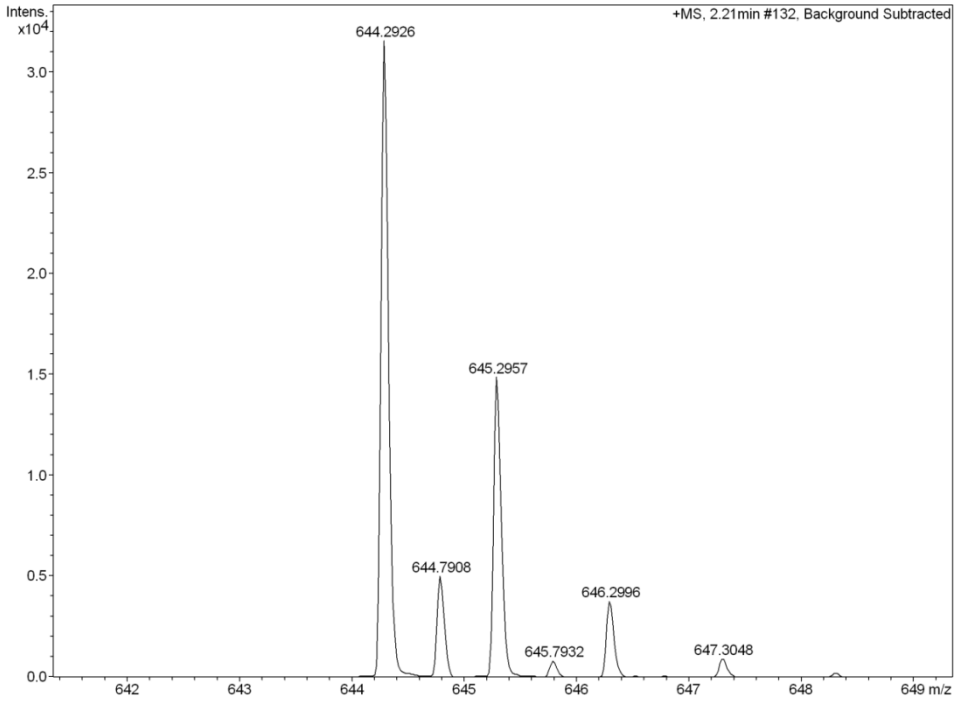


Figure S3. High resolution mass spectrum of compound **3**.

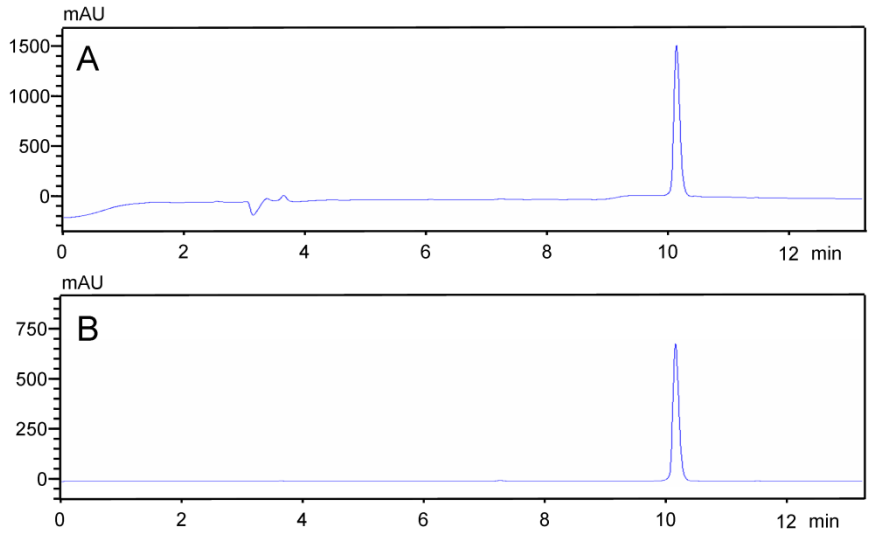


Figure S4. HPLC spectra of **TPECB-Pt-D5-cRGD** monitored with absorbance at 214 nm (A) and 405 nm (B).

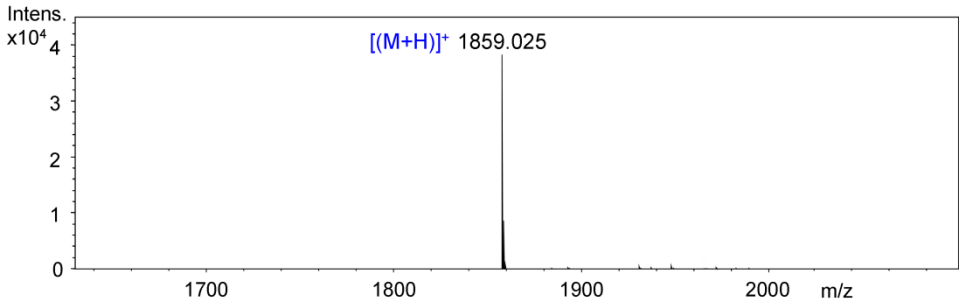


Figure S5. Mass spectrum (ESI) of **TPECB-Pt-D5-cRGD**.

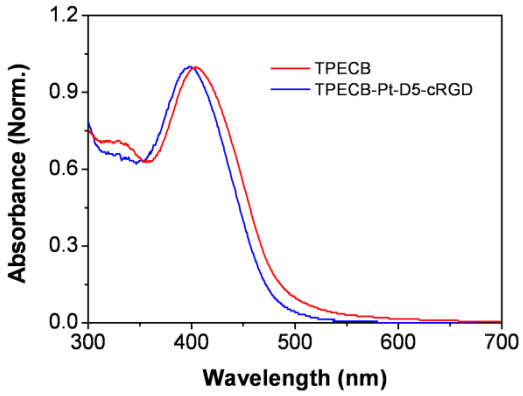


Figure S6. Normalized UV-vis absorption spectra of **TPECB** and **TPECB-Pt-D5-cRGD** in DMSO/PBS (v/v = 1/199).

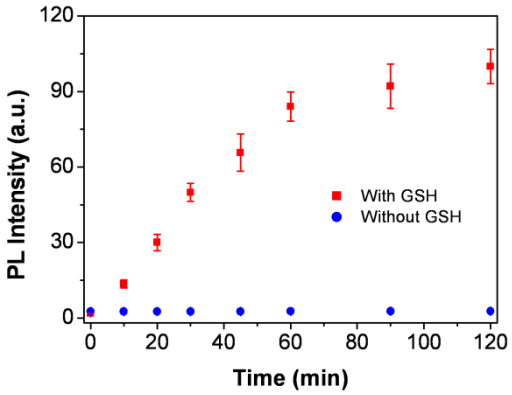


Figure S7. Time-dependent fluorescence intensity change at 640 nm of **TPECB-Pt-D5-cRGD** (10 μM) upon treatment with GSH (100 μM) in DMSO/PBS (v/v = 1/199).

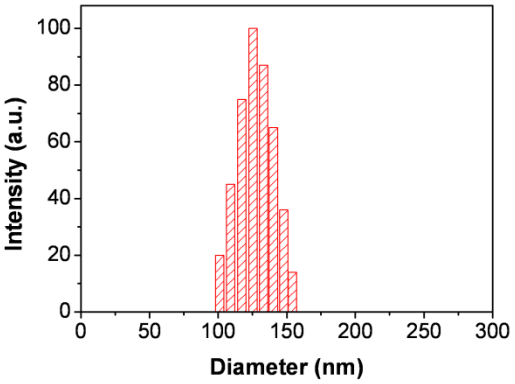


Figure S8. Hydrodynamic diameters obtained from LLS for the residue of **TPECB-Pt-D5-cRGD** (10 μM) after incubation with GSH (100 μM) in DMSO/PBS (v/v = 1/199)

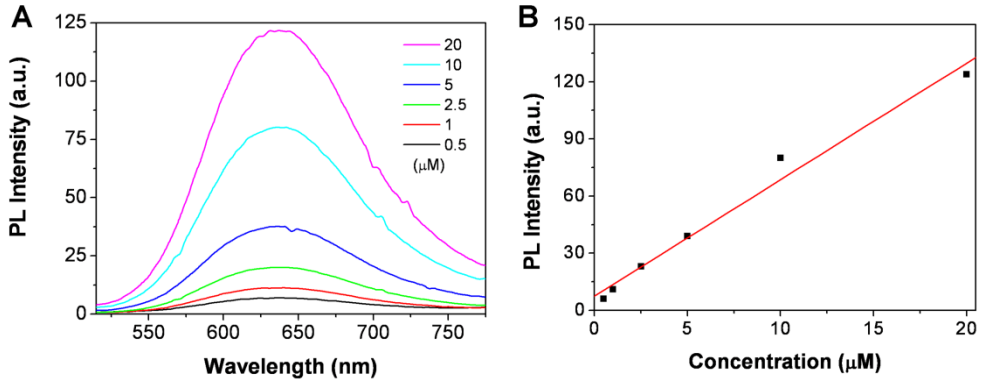


Figure S9. (A) PL spectra of different concentrations of **TPECB-Pt-D5-cRGD** upon incubation with GSH (100 μM) for 60 min. (B) Plot of PL intensity at 640 nm versus concentrations of **TPECB-Pt-D5-cRGD** upon incubation with GSH (100 μM) in DMSO/PBS (v/v = 1/199).

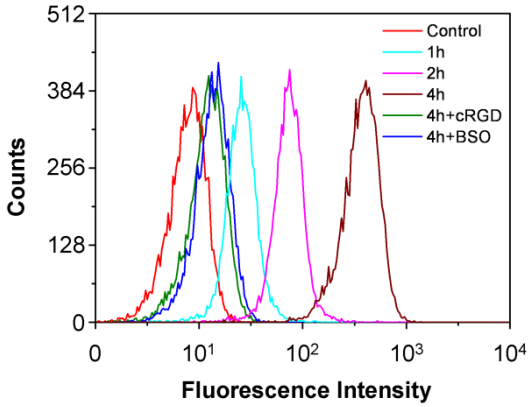


Figure S10. Flow cytometric analyses of MDA-MB-231 cells upon incubation with **TPECB-Pt-D5-cRGD** (10 μM) for different time duration or the cells were pretreated with cRGD/BSO (50 μM). The control samples refer to cells without any treatment.

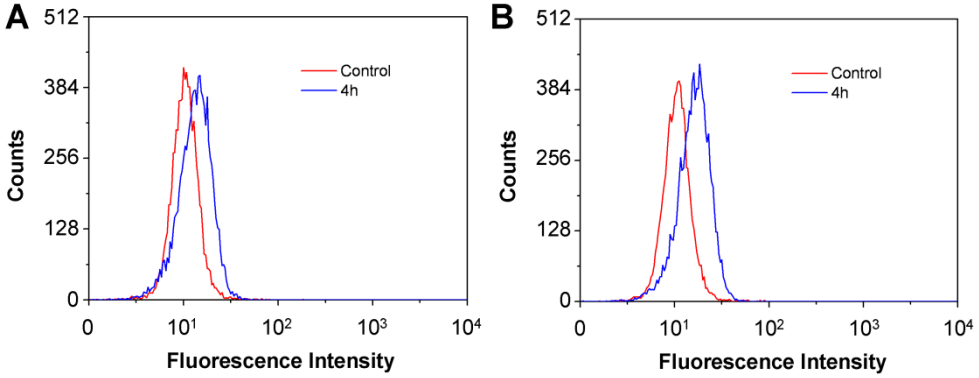


Figure S11. Flow cytometric analyses of MCF-7 (A) and 293T (B) cells upon incubation with **TPECB-Pt-D5-cRGD** (10 μM) for 4 h. The control samples refer to cells without any treatment.

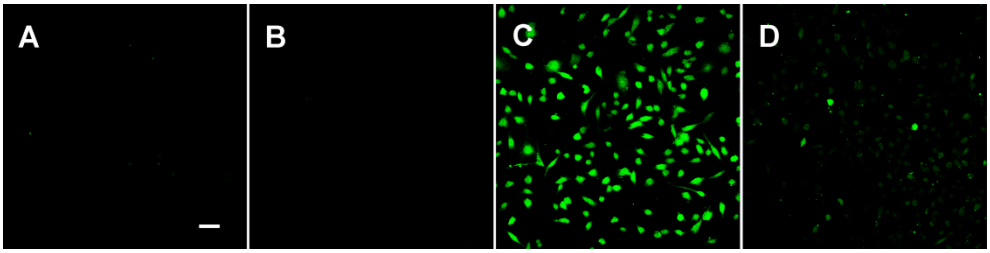


Figure S12. Intracellular ROS detection in MDA-MB-231 cells using DCF-DA as the indicator. MDA-MB-231 cells upon treatment with (A) DCF-DA with white light irradiation; (B) **TPECB-Pt-D5-cRGD** (10 μM) with white light irradiation; (C) **TPECB-Pt-D5-cRGD** (10 μM) and DCF-DA with light irradiation; (D) **TPECB-Pt-D5-cRGD** (10 μM) and DCF-DA in the presence of ROS scavenger vitamin C (VC, 100 μM) with light irradiation. The

green fluorescence is from DCF (E_x: 488 nm; E_m: 505−525 nm). The scale bar is 50 μm.

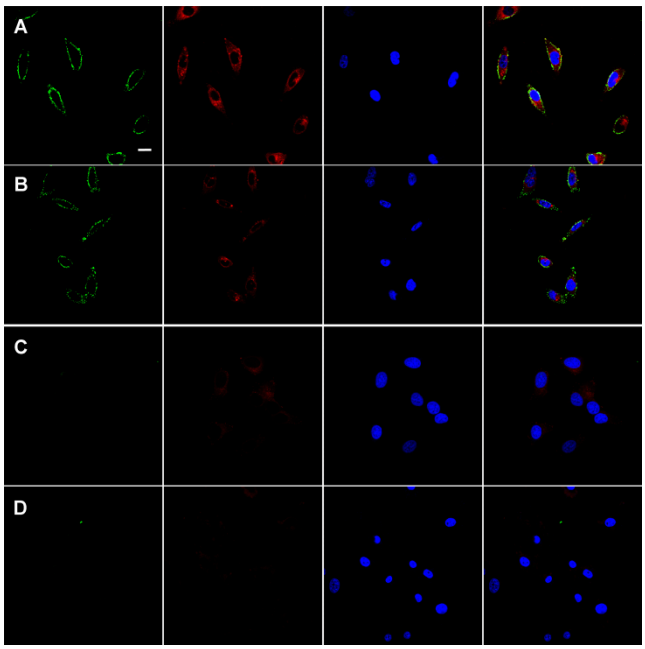


Figure S13. Cell apoptosis imaging using FITC-tagged Annexin V in MDA-MB-231 cells (A), U87-MG (B), MCF-7 cells (C) and 293T cells (D) after incubation of the cells with the prodrug (10 μM) for 4 h, followed by white light irradiation (0.25 W cm^{-2}). The nuclei were living stained with Hoechst (E_x: 405 nm; E_m: 430-470 nm); the green fluorescence is from FITC (E_x: 488 nm; E_m: 505−525 nm); the red fluorescence is from the prodrug (E_x: 405 nm; E_m: > 560). All images share the same scale bar (20 μm).

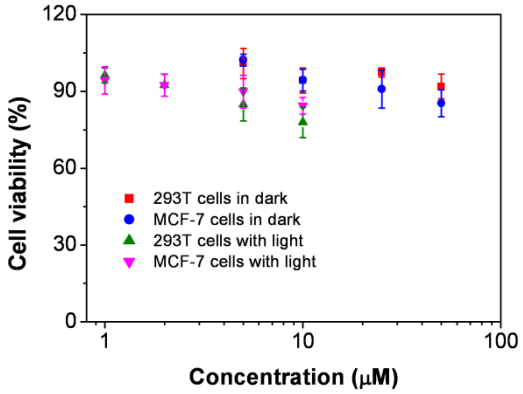


Figure S14. Cell viability of MCF-7 or 293T cells incubated with different concentrations of **TPECB-Pt-D5-cRGD** in dark conditions or with light irradiation (0.25 W cm^{-2}, 1 min) and further incubation for 72 h in fresh medium. Data represent mean values ± standard deviation, n = 3.