

A Lipid Droplets Targeted Fluorescent Probe for High-efficiency Image-Guided Photodynamic Therapy of Renal Cell Carcinoma

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Note added after first publication: This Supplementary Information file replaces that originally published on 07 January 2021. There was an error in the caption of figure S5, in which "rose bengal" and "TTIE" had been incorrectly swapped. The caption has now been corrected.

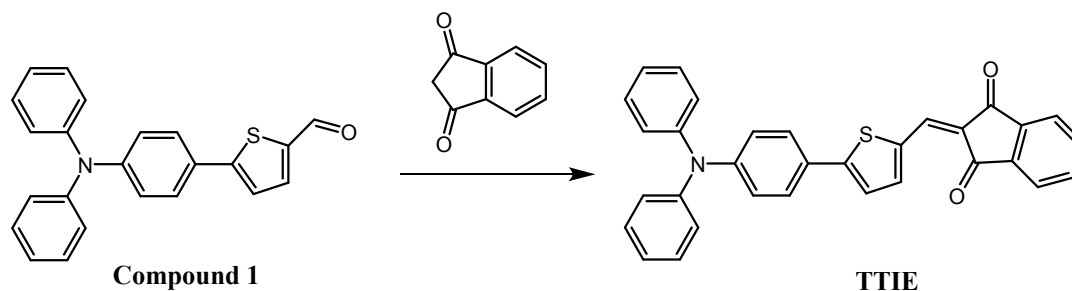
Experimental Section

Materials

1, 3-indandione, 9, 10-anthracenediyl-bis-(methylene)-dimalonic acid (ABDA), dihydrorhodamine 123 (DHR 123), vitamin C and rose Bengal were obtained from Sigma-Aldrich. 5-(4-(diphenylamino) phenyl) thiophene-2-carbaldehyde (compound 1) was prepared according to precious work.¹ All the chemicals used as received without further purification. Antibodies to PARP and cleaved PARP (9542) and Phospho-Histone H2A.X (Ser139) (9718) were purchased from Cell Signaling Technology; β -actin was from ZENBIO (340042).

Synthesis of TTIE

Under argon atmosphere, compound 1 (0.50 g, 1.41 mmol), 1, 3-indandione (0.29 g, 1.98 mmol), methanol (7 mL) and toluene (7 mL) were added to a Schlenk tube equipped with a magnetic stirring bar. A drop of piperidine was added and the mixture was refluxed at 120 °C for 1.5 h. After cooling down to room temperature, the solvent was removed under reduced pressure. **TTIE** was purified via silica gel chromatography (petroleum ether/ CH_2Cl_2 as eluent) with the yield of 82%. ¹H-NMR (400 MHz, DMSO-*d*₆, δ): 8.25 (d, *J* = 4.0 Hz, 1H), 8.05 (s, 1H), 7.89-7.96 (m, 4H), 7.76 (m, 2H), 7.69 (d, *J* = 4.0 Hz, 1H), 7.35-7.41 (m, 4H), 7.11-7.19 (m, 6H), 7.00 (m, 2H) ppm. ¹³C-NMR (100 MHz, CDCl₃, δ): 190.47, 189.66, 157.85, 149.28, 146.90, 143.94, 142.02, 140.44, 136.20, 135.78, 134.84, 134.62, 129.53, 127.47, 125.32, 125.29, 124.00, 123.73, 123.20, 122.86, 122.71, 122.17. HRMS (ESI⁺): calcd. for C₃₂H₂₁NO₂S [M + H]⁺ 484.1371, found 484.1375.



Scheme S1. Synthetic route of **TTIE**.

Characterization

NMR spectra were obtained on a Bruker AV II-400. Fluorescence emission spectra were obtained using a Hitachi F-4700 fluorescence spectrometer. Absorption spectra were obtained on a HITACHI U-2910 spectrometer. The ESI-TOF mass spectra were recorded with a Waters Q-ToF premier instrument. Laser confocal scanning microscope images were collected on Nikon laser scanning confocal microscope (A1R MP⁺).

Theoretical calculations

Theoretical calculations were carried out with the GAUSSIAN 09 series of programs. DFT method B3-LYP with a standard 6-31G* basis set was used for all atoms. All calculations were based on optimized (B3LYP/6-31G*) geometry of the presented **TTIE**.

Cell Culture

786-O, T24, PC3 and Hela cells were cultured in the RPMI 1640 or DMEM medium with 10% FBS and 1% antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin) as recommended by ATCC. Cells were incubated in 5% CO₂ humidity incubator at 37 °C.

Human ccRCC Samples and Primary Tumor Cells

The study was approved by the Ethical Research Committee of the West China Hospital (2020-701). Written informed consent was obtained from all patients involved. All patients had a confirmed pathologic diagnosis of ccRCC and underwent partial or radical nephrectomy at West China Hospital, Sichuan University. The tumor tissues were collected immediately after surgery, and primary tumor cells were developed using explant-cell culture system.² Briefly, fresh tumor tissues were minced into small pieces (~1mm³), and seeded into 6-well plates that have been coated with fetal bovine serum. After incubating for 2 h, 300 µL RPMI 1640 medium supplemented with 10% FBS were added. When the outgrowth of tumor cells forms a halo, the explants were taken out and transferred into a new dish to subculture. The outgrowth tumor cells were expanded and maintained as the same way of cell lines.

H&E staining and Oil Red Staining

H&E staining was performed according to the standard protocol with haematoxylin

staining for 2 min and Eosin for 1 min. For Oil Red O staining, Oil Red O stock solution was purchased from Solarbio (G1260) and the working solution was prepared by diluting 30 mL of the stock stain with 20 mL distilled water. 786-O cells were cultured in 6-well plates. Fresh ccRCC tumor tissues were frozen with O.C.T. compound and cut into 8 μm sections. Cells and slices were fixed with 4% PFA for 5 min and rinse in PBS for two times. Rinse with 60% isopropanol and then stain with freshly prepared Oil Red O working solution for 15 min. After staining, the samples were rinsed with 60% isopropanol and wash with PBS for two times, followed by staining nuclei with haematoxylin for 3 min and the samples were washed with PBS for three times before being observed.

Cytotoxicity Study

CCK8 was used to evaluate the cytotoxicity of **TTIE**. 786-O cells and ccRCC primary tumor cells from two patients were employed and seeded in 96-well plates at a density of 3000-4000 cells per well. To assess the biocompatibility of **TTIE** in living cells, the cells were incubated with medium containing different concentrations of **TTIE** for 24 h at dark. Then the medium of each well was replaced by 100 μL fresh medium containing 10 μL CCK8 and incubating for 2 h, and their OD values were read at 450 nm. The cell viability rates were calculated by $(\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}) \times 100\%$. To evaluate the cytotoxicity of **TTIE** under light irradiation, the cells were incubated with different concentrations of **TTIE** for 2 h as mentioned above and then exposed to white light ($\sim 10 \text{ mW cm}^{-2}$) for 30 min, and then the cells were stayed in incubator for 2 h before assessed by CCK8. T24 cells, a bladder cancer cell line containing rare LDs, and PC3 cells, a prostate cancer cell line with rich LDs as well as Hela cells with few LDs were selected as the controls of 786-O cells to explore the differences of the cytotoxic effect of **TTIE** among cells with different levels of LDs.

Cell Imaging

786-O cells and primary ccRCC tumor cells were co-stained with BODIPY493/503 and **TTIE**. Cells were first incubated with Hoechst 33342 (C1028, Beyotime) for 15 min, then incubated with different concentrations of **TTIE** (0.2 to 5 μM) for 1 h and BODIPY493/503 (100 nM) for 30 min. After finishing staining, the cells were washed with PBS for three times and fixed with 4% PFA. Cells were imaged under confocal microscope (Nikon A1R MP⁺).

Human Tumor Tissue staining and imaging

Fresh human ccRCC tissues were cut into slices and divided into 48-well plate and incubated with 500 μL Hoechst 33342 for 30 min, and then stained with **TTIE** (5 μM) for 1 h at room temperature followed by BODIPY493/503 (100 nM) for 1 h. After staining, tumor slices were washed with PBS for three times, and then imaged using a laser scanning confocal microscope. For **TTIE**, the excitation filter was 488 nm, and the emission filter was 570-620 nm; for BODIPY493/503, the excitation was 488 nm, and the emission filter was 500-550 nm.

Photostability

The dye-labeled 786-O cells (**TTIE** 5 μM ; BODIPY 493/503 100 nM) were continuously imaged by a confocal microscope. For **TTIE**, the excitation wavelength was 488 nm; for BODIPY 493/503, excitation wavelength was 488 nm (both **TTIE** and BODIPY493/503 were imaged under 1% laser power). The scanning rate was 6.1 s per frame.

Measurement of MDA Levels

The level of malondialdehyde (MDA), a production of lipid peroxidation, in the 786-O cells with or without PDT treatment was determined using a commercial kit (S0131S, Beyotime). The proteins were extracted as we described below. The MDA levels were quantified and measured according to the standard protocol recommended. MDA levels were expressed as nmol per milligram of protein.

Immunoblotting

The 786-O cells were seeded in 6-well plates at a density of 2×10^5 per well and incubated overnight. Then the medium in each well was replaced by 2 ml fresh medium containing 7.5 μM **TTIE**. After 1 h incubating, the plates were exposed to white light ($\sim 10 \text{ mW cm}^{-2}$) for 30 min, and then the proteins were extracted using RIPA buffer containing Protease inhibitors and Phosphatase inhibitors. Electrophoresis were performed according to standard protocols using 10% SDS-polyacrylamide gels and transferring to Immobilon-P (Millipore). Membranes were incubated with primary antibodies (1:1000-1:20000 dilution in 5% nonfat milk or BSA in TBST) overnight at 4 $^{\circ}\text{C}$, after TBST washing for three times, membranes were incubated with HRP-conjugated secondary antibodies (1:20000) for 1 h at room temperature, and finally visualized using Immobilon[®] Western Chemiluminescent HRP Substrate (Millipore, WBKLS0500).

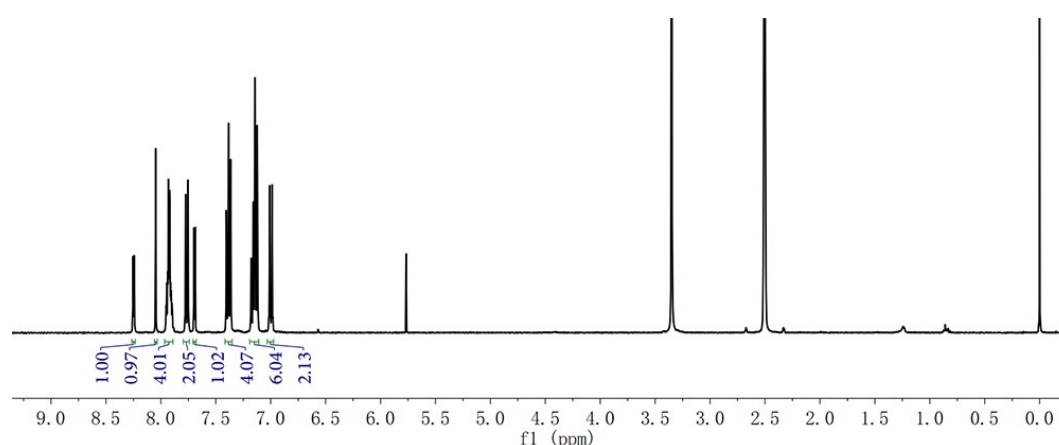


Fig. S1. ¹H NMR spectrum of **TTIE** in DMSO-*d*₆.

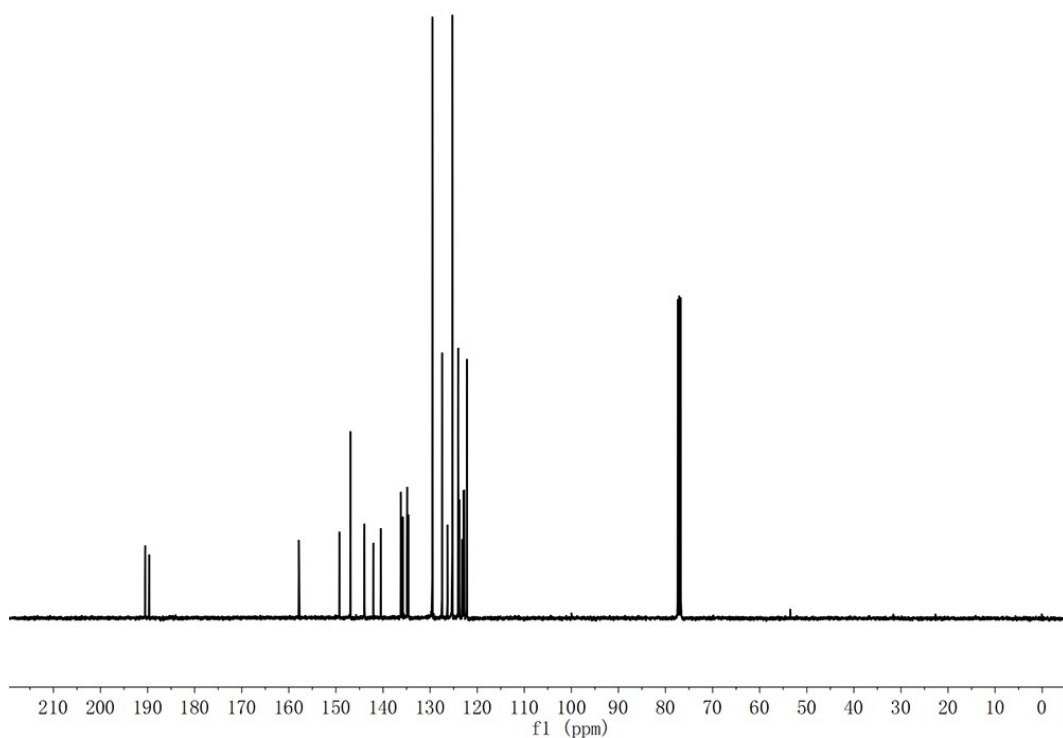


Fig. S2. ^{13}C NMR spectrum of TTIE in CDCl_3 .

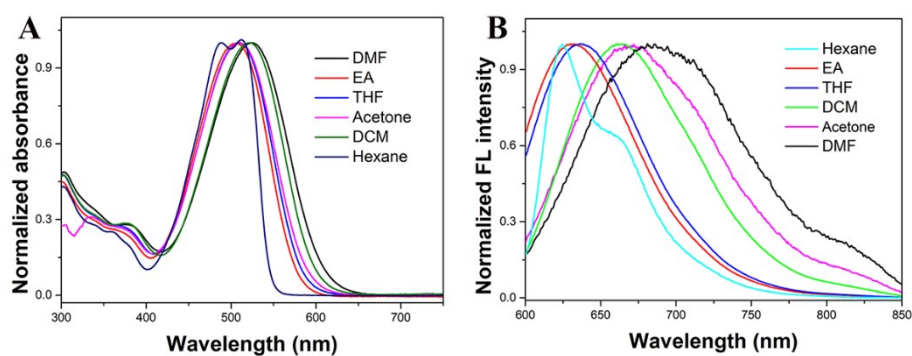


Fig. S3. Normalized UV-vis absorption spectra (A) and normalized PL spectra (B) of TTIE in different solvents with various polarity.

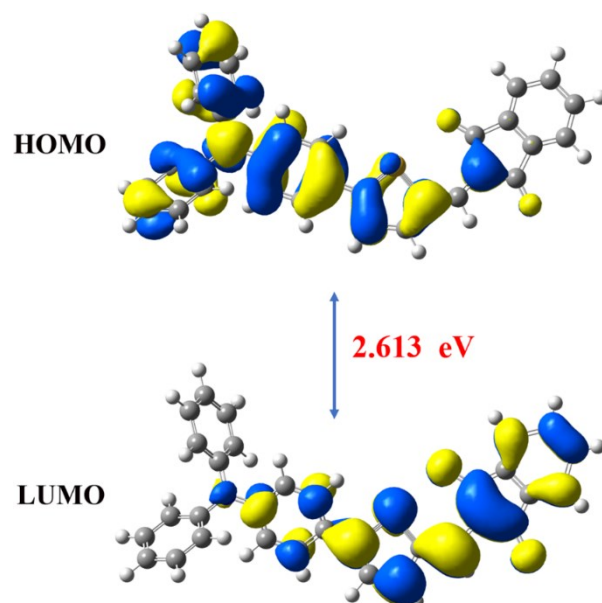


Fig. S4. DFT simulation of HOMO and LUMO energy levels of **TTIE**.

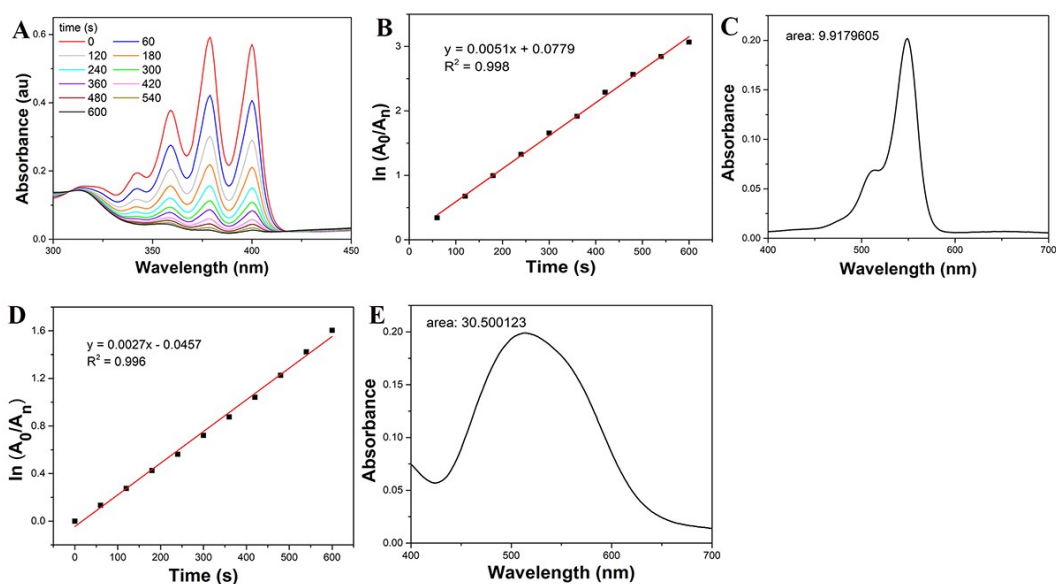


Fig. S5. UV-vis spectra of ABDA (50 μM) in water under white light irradiation in the presence of rose bengal (A). The absorbance decay of the ABDA at 378 nm under white light Irradiation ($\sim 10 \text{ mW cm}^{-2}$) in the presence of rose bengal (B). The absorption spectrum of rose bengal ranged in 400–700 nm (C). The absorbance decay of the ABDA at 378 nm under white light irradiation ($\sim 10 \text{ mW cm}^{-2}$) in the presence of **TTIE** (D). The absorption spectrum of **TTIE** ranged in 400–700 nm (E).

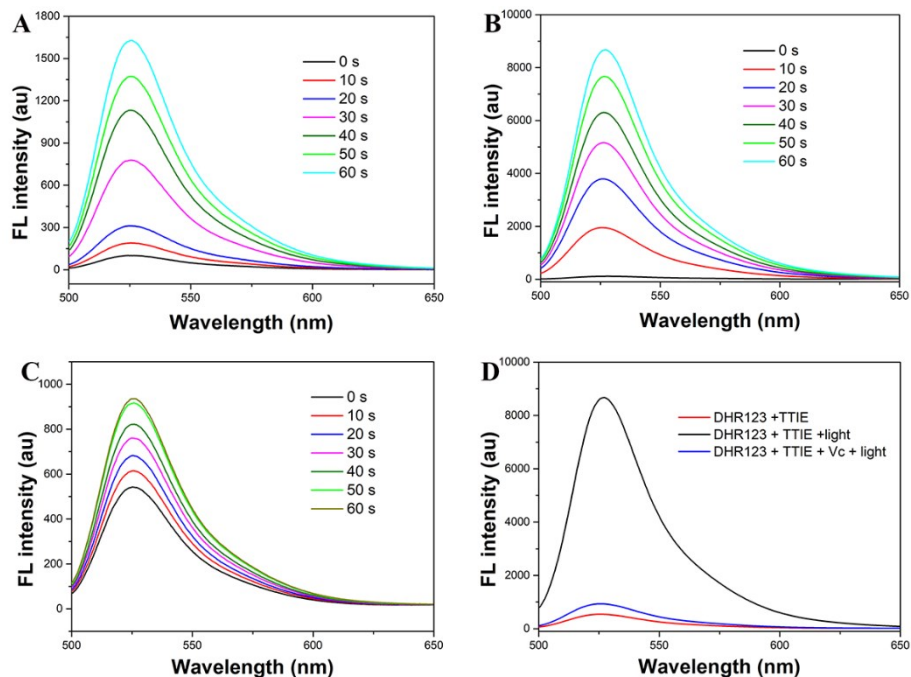


Fig .S6. PL spectra of DHR 123 (10 μ M) under white light irradiation ($\sim 10 \text{ mW cm}^{-2}$) with different time in DMSO/PBS (v/v) = 1/100 (A), in the presence of **TTIE** (10 μ M) (B), in the presence of **TTIE** (10 μ M) and vitamin C (Vc). Fluorescence spectra of DHR 123 probe for free radical ROS detection in the presence of Vc and **TTIE** with or without white light irradiation for 60 s.

Table S1 Theoretically calculated energy level and energy gap of **TTIE**.

S_n	Energy (eV)	T_n	Energy (eV)	S_1/T_n	S_1/T_n Energy gap
S_1	2.37	T_1	1.69	S_1/T_1	0.68
S_2	2.92	T_2	2.48	S_1/T_2	-0.11
S_3	2.97	T_3	2.66	S_1/T_3	-0.29
S_4	3.27	T_4	2.79	S_1/T_4	-0.42
S_5	3.30	T_5	2.82	S_1/T_5	-0.45
S_6	3.74	T_6	3.05	S_1/T_6	-0.68
S_7	3.75	T_7	3.20	S_1/T_7	-0.83
S_8	3.77	T_8	3.22	S_1/T_8	-0.85

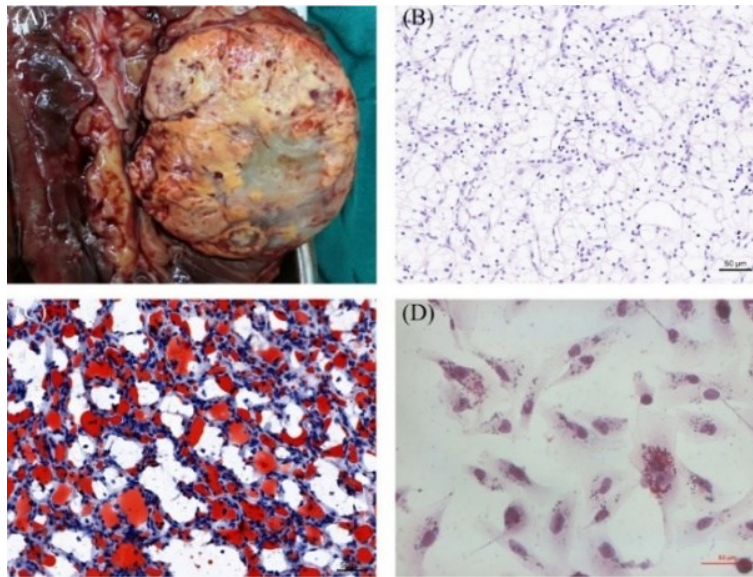


Fig. S7. (A) The representative gross image of human ccRCC tumors. (B) The representative image of H&E staining of human ccRCC tumor tissue (40X). Scale bar, 40 μ m. (C) Images of Oil Red O stained human ccRCC tumor tissue. Scale bar, 50 μ m. (D) Images of Oil Red O stained 786-O cells. Scale bar, 50 μ m.

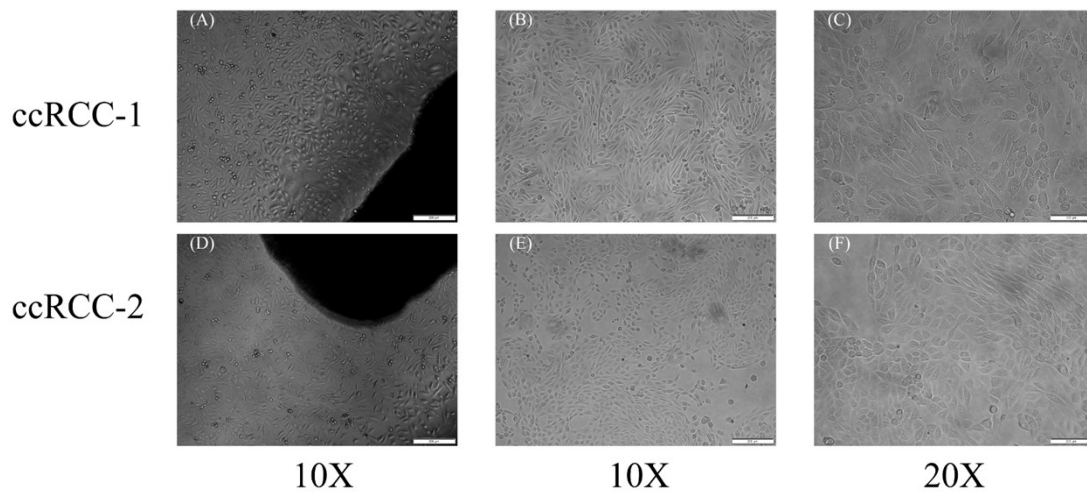


Fig. S8. Human primary ccRCC tumor cells derived from (A-C) patient 1 and (D-F) patient 2. Scale bar, 200 μ m. (A, D) The explant-cell culture system to develop primary tumor cells. The representative images of human primary ccRCC tumor cells at first passage (B, E) (10X) and (C, F) (20X).

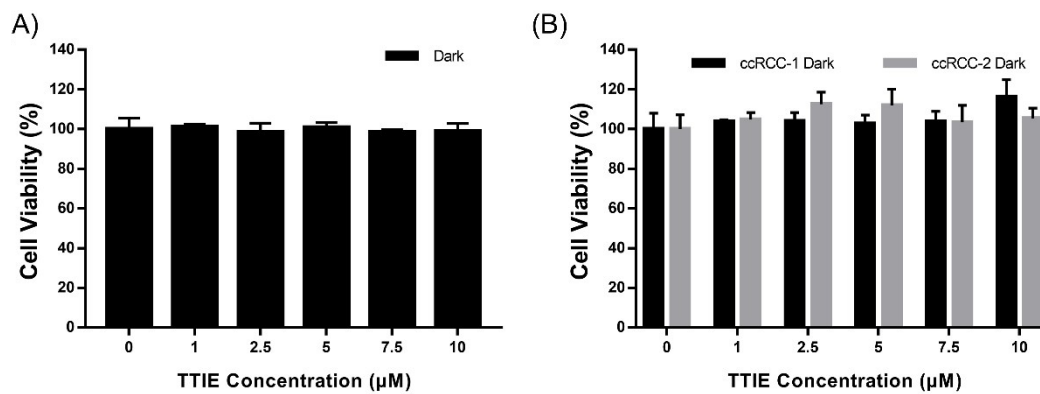


Fig. S9. Cell viability of (A) 786-O cells and (B) human primary ccRCC tumor cells incubated with different **TTIE** concentrations for 24 h.

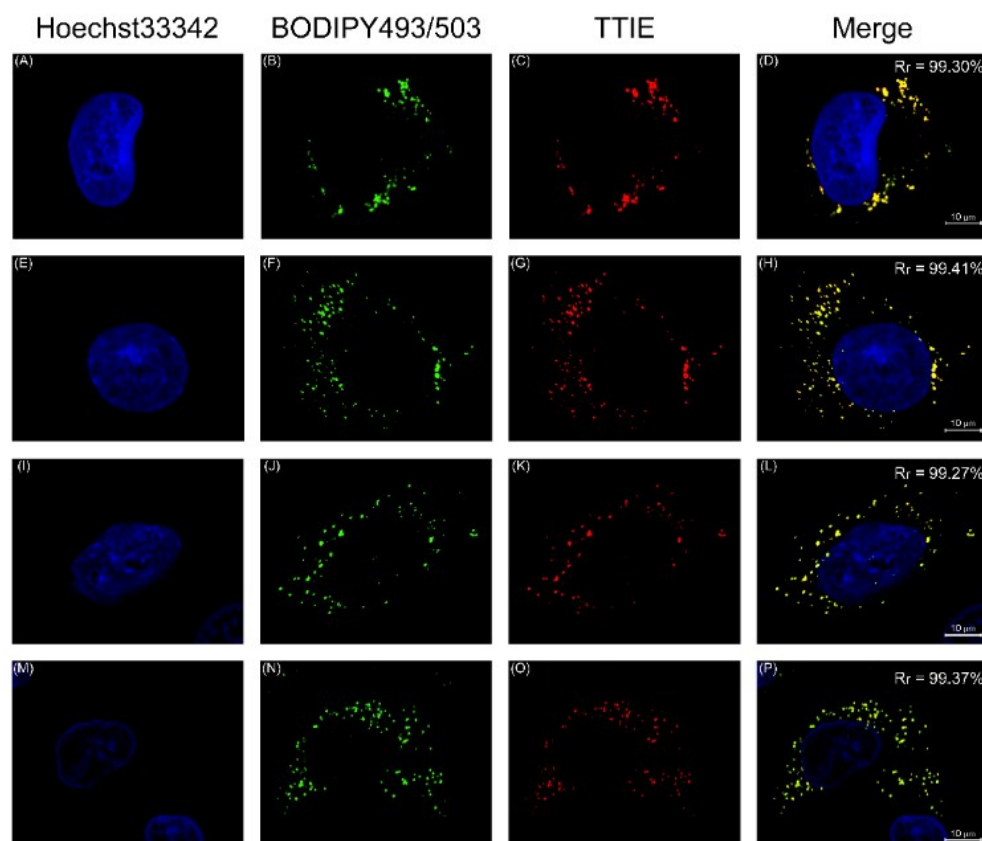


Fig. S10. Colocalization imaging of 786-O cells stained with Hoechst 33342 ($\lambda_{ex} = 405$ nm), BODIPY 493/503 ($\lambda_{ex} = 488$ nm, 100 nM) and different concentrations of **TTIE** ($\lambda_{ex} = 488$ nm) (A-D) 2 μM, (E-H) 1.0 μM, (I-L) 500 nM, and (M-P) 200 nM. Scale bar, 10 μm. $\lambda_{ex} = 488$ nm. Pearson's coefficients (R_r) of Fig. S5D, 5H, 5L and 5P were calculated as 99.30%, 99.41%, 99.27% and 99.37%, respectively. Manders' coefficients (m_1 and m_2) of Fig. S5D, 5H, 5L and 5P were calculated as 0.97 and 0.95, 0.95 and 0.94, 0.94 and 0.94, 0.95 and 0.94, respectively.

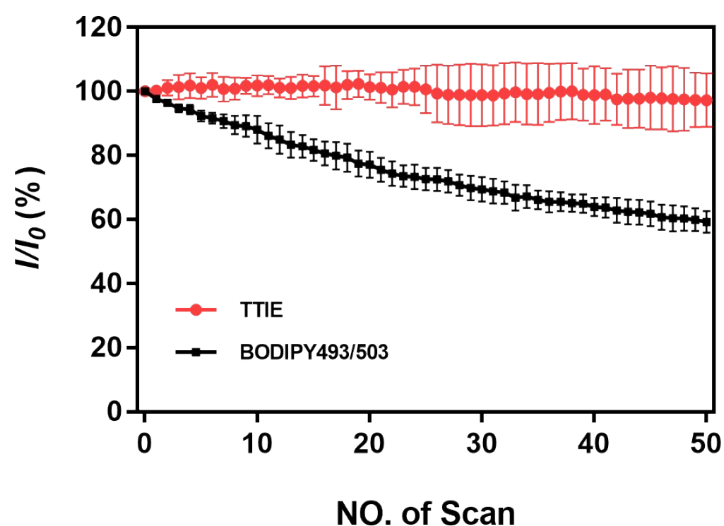


Fig. S11. Loss of emission intensity (I/I_0) of **TTIE** (5 μ M) and **BODIPY 493/503** (100 nM) in 786-O cells with the increasing irradiation time (scanning time: 6.1 s per scan). λ_{ex} = 488 nm.

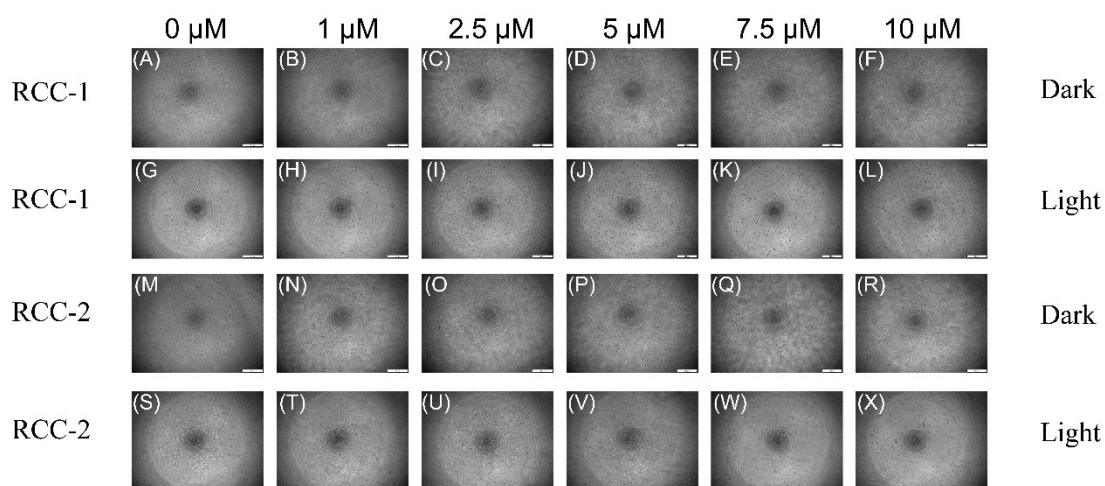


Fig. S12. Cytotoxicity of human primary ccRCC tumor cells from (A-L) patient 1 and (M-X) patient 2 stained by different concentrations of **TTIE** (G-L, S-X) with or (A-F, M-R) without white radiation for 30 min. Scale bar, 200 μ m.

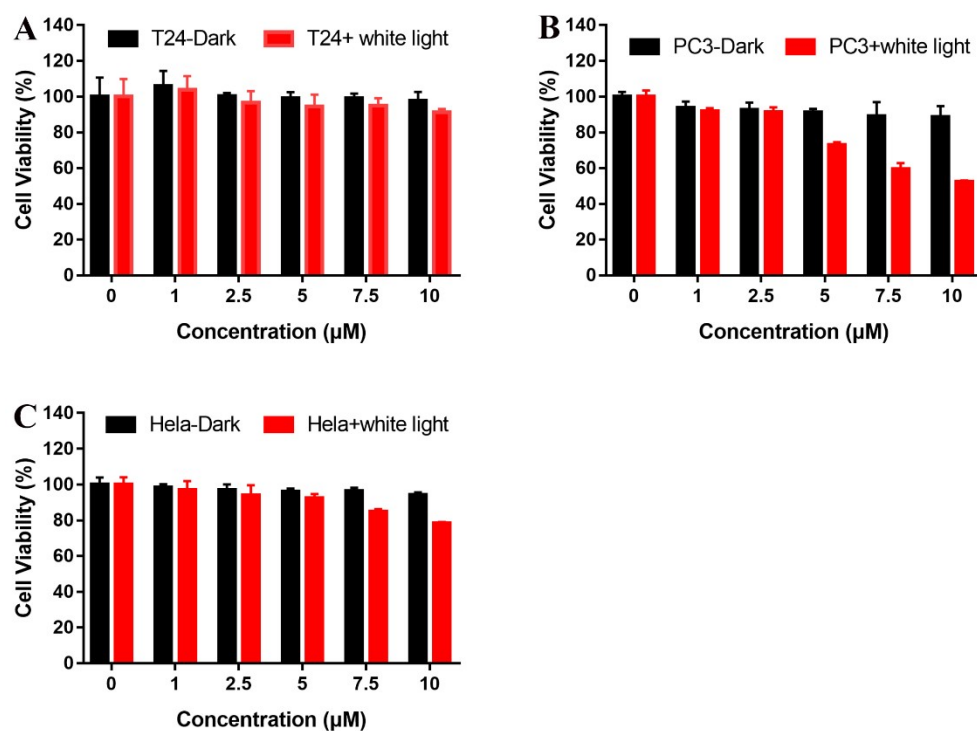


Fig.S13. Cell viability of (A) T24 cells, (B) PC3 cells and (C) HeLa cells incubated with different concentrations of **TTIE** in the absence or presence of white light irradiation (10 mW cm^{-2}) for 30 min.

References

- 1 Y. Li, T. Ren and W. J. Dong, *J. Photoch. Photobio. Chem.*, 2013, **251**, 1-9.
- 2 A. Mitra, L. Mishra and S. Li, *Trends Biotechnol.*, 2013, **31**, 347-357.