Supplemental Information

Mitochondrial Heat Shock Protein-Guided Photodynamic Therapy

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1. General Information: The reagents and materials for the synthesis were used as obtained from Sigma Aldrich and Alfa Aesar chemical suppliers. All solvents were used after drying by standard methods prior to use. The NMR solvents were used as received and the spectra were recorded in Agilent 400 MHz spectrometer. Spectra were referenced internally by using the residual solvent (^{1}H δ =3.34 and ^{13}C δ = 49.86 for CD_3OD-d4) resonances relative to $SiMe_4$. The ESI-MS spectra were recorded in Bruker, 1200 Series & HCT Basic System. The electronic absorption spectra and steady state fluorescence spectra were recorded in JASCO V-670 spectrophotometer and Hitachi F-7000 fluorescence spectrophotometer respectively.

2. Cell culture: Human cervical cancer HeLa cells and Human lung cancer NCI-H460 cells were cultured (using DMEM medium or RPMI medium) supplemented with 10% fetal bovine serum (FBS; Life Technologies), and 1% penicillin/ streptomycin (Life Technologies) at 37 °C in a humidified atmosphere of 5% CO_2. Primary hepatocytes were isolated from 8-week-old BALB/c mice. Briefly, mice were anesthetized, and the livers were perfused with collagenase solution, dissected, disrupted by pipetting of clumps, and filtered through a 100-μm cell strainer (BD Biosciences). Cells were washed several times by repeated centrifugation and resuspension in M199/EBSS medium (Hyclone). Cell debris and nonparenchymal cells were separated from hepatocytes by gradient centrifugation with Percoll (Sigma). After repeated washing, cell pellets were re-suspended and incubated in M199/EBSS medium containing 10% FBS at 37 °C in an atmosphere of 5% CO_2.

3. Co-localization study of IR-PU with Mito-tracker in HeLa cell line: HeLa cells were seeded in one well glass cover glass (Lab Tek II, Thermo Scientific) at a seeding density of 2 x 10^5 cells/well. After 24 h, cells were treated with 2.5 μM of **IR-PU** for a period of 4 h and replaced with fresh media followed by the treatment of Mito-tracker green. The cellular uptake

was monitored periodically using Carl Zeiss LSM 780 NLO multiphoton microscope connected to CO2 incubator setting the excitation at 720 nm and emission between 725-758 nm along with the co-localization analysis with Mito-tracker green FM setting excitation at 488 nm and emission between 500-550 nm.

4. MitoSox ROS generation analysis: HeLa cells were seeded on a Lab Tek II chamber cover glass at 90% confluence in DMEM media supplemented with 10% FBS, 100 μg mL^{-1} streptomycin, and 100 UmL^{-1} penicillin and incubated at 37 °C under 5% CO^2. After incubation with 2.5 μM of **IR-PU** for 4 hours, the cell culture medium was then replaced with media containing 5 μM MitoSox reagent working solution to cover the adherent cells following the manufacturer's protocol (MitoSox, M36008). The cells were then incubated for 10 minutes at 37 °C, protected from light and irradiated using 200 $mWcm^{-2}$. The cells were then analysed under a FV1000 laser confocal scanning microscope.

5. TMRM depolarization analysis: HeLa cell lines were incubated with **IR-PU** (2.5 μM) for 2 h. Media was replaced before adding TMRM (Tetramethylrhodamine methyl ester perchlorate) an indicator for measuring mitochondria depolarization. After incubating for another 30 minutes, medium was replaced again before irradiation with 808 nm laser for 2 minutes and analysed using an FV1000 laser confocal scanning microscope.

6. Analysis of cell viability: Cells (5×10^3 cells/well) were cultured in 96-well plates overnight and treated with **IR-PU** for 12 h in dark, then media was replaced with fresh media and the cells were irradiated for 2 minutes at 200$mWcm^{-2}$ using the 808 nm laser. Cells were incubated for another 12 h before checking the cell cytotoxicity using MTT assay. To determine cell viability, cells were exposed to 3(4,5-dimethyl-thyzoyl-2-yl)2,5 diphenyltetrazolium bromide

(MTT), and crystallized formazan was quantified by measuring the absorbance at 595 nm with SYNERGY NEO microplate reader (BioTek Instruments, Inc.). Absorbance data were compared with those of cells treated with vehicle control and expressed as percent viability. Control experiments were conducted without laser irradiation and using control molecule **IR-Pyr**.

7. Analysis of drug accumulation in cell: NCI-H460 cells (4 $\times 10^5$ cells/well) were cultured in 6-well plates overnight and treated with drugs. Cells were harvested as time course and lysed in RIPA buffer (50mM Tris-Hcl, pH 7.4, 150mM Sodium chloride, 1% NP-40, and 0.25% Na-deoxycholate). Drug accumulation was determined by fluorescence signal at 780nm/830nm (ex/em) wavelength with SYNERGY NEO microplate reader (BioTek Instruments, Inc.).

8. Accumulation study (imaging) of IR-PU in HeLa, NCI-H460 and hepatocyte cell line: HeLa, NCI-H460 and hepatocyte cells were seeded separately in one well glass cover glass (Lab Tek II, Thermo Scientific) at a seeding density of 20000 cells/well. After 24 h, cells were treated with 10 μM of **IR-PU** for a period of 6 h followed by Hoechst treatment. Finally, the cells were replaced with fresh media and **IR-PU** accumulation was monitored using Carl Zeiss LSM 780 NLO multiphoton microscope connected to CO_2 incubator setting the excitation at 720 nm and emission between 725-758 nm. [5]

9. Recombinant Protein Preparation and Fluorescence Polarization Assays: Recombinant TRAP1 and Hsp90 N-domain were prepared as described previously.[1] For fluorescence polarization experiments, the fluorescence probe PU-H71-FITC3 was synthesized as described previously,2 and 10 nM PU-H71-FITC3 (100nM with HSP90N) and 400 nM TRAP1 (600nM HSP90N) were incubated for 24 h at 4 °C with various concentrations of inhibitors in FP buffer

containing 135 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 1 mM DTT, 2 mM $MgCl_2$, 0.1 mg/mL BSA, and 0.05% NP40 (pH 7.3). Fluorescence polarization was measured using a SYNERGY NEO microplate reader (BioTek Instruments, Inc.).

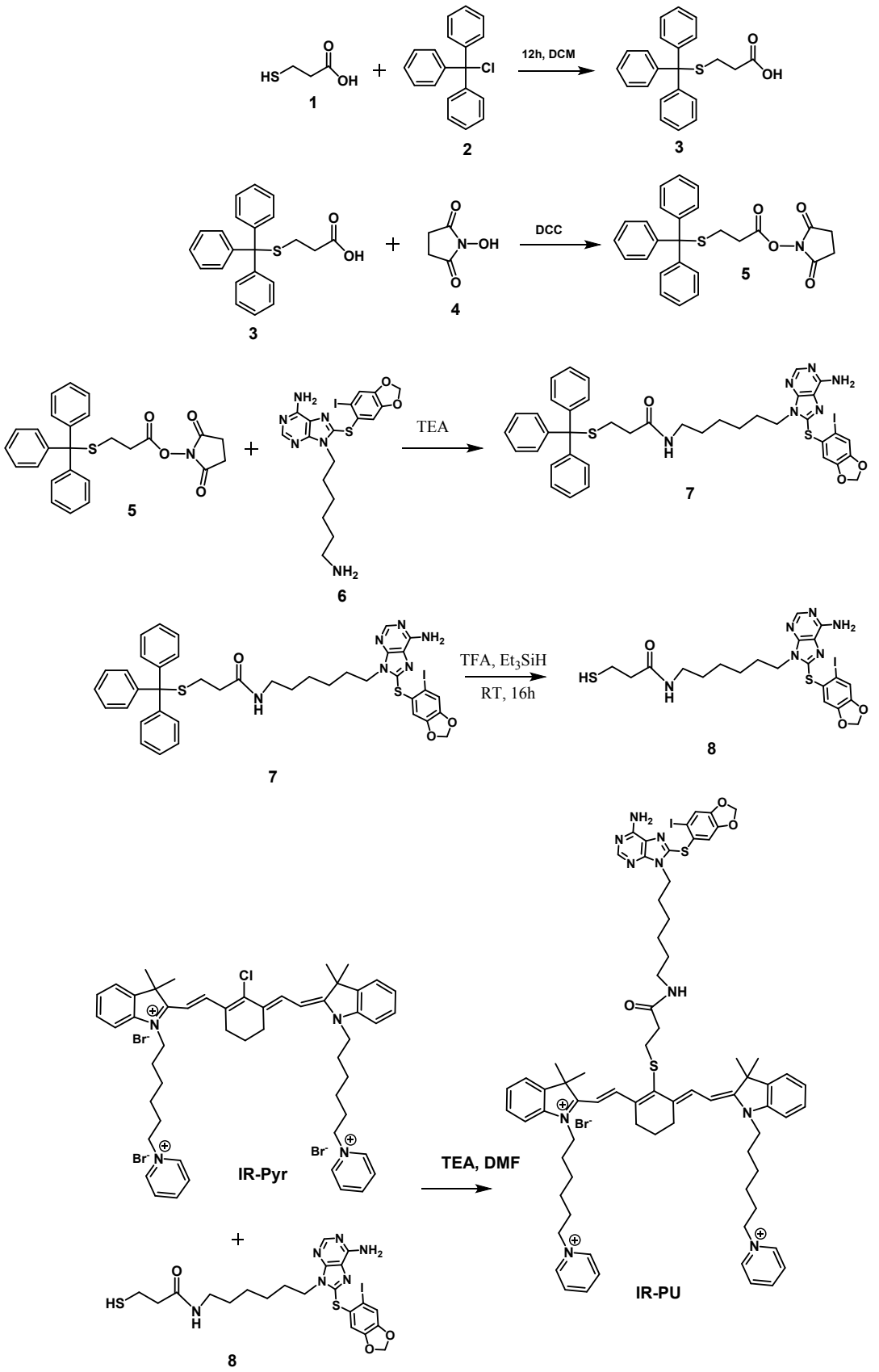
10. Apoptosis Induction: NCI-H460 cells (4×10^5 cells/well) were cultured in 6-well plates overnight and treated with drugs for 12 h. Cells were harvested another 12 h after laser irradiation for 2 minutes at 200 mWcm-2. To measure apoptosis induction, DNA contents (propidium iodide) and externalized phosphatidylserine (Annexin V) using apoptosis kit (Molecular Probe). Labeled cells were quantified using the FACS Calibur system (BD Biosciences). Data were processed using FlowJo software (TreeStar).

11. In vivo imaging with tumor xenografts: Balb/c nude female mice were purchased from the Orient bio, Korea and all animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Ulsan National Institute of Science and Technology (UNIST) and approved by the Animal Ethics Committee of UNIST. Nude mice bearing NCI-H460 cancer (tumor volume ~ 200 mm^3) were intraperitoneally injected with DMSO, **IR-Pyr**, and **IR-PU** (1 mg/mL with 20% Chremophor in PBS) as 10mg per kilogram. Then imaged using the in vivo optical imaging system (Bruker Xtreme model) by setting the excitation at 760 nm and 830 nm as emission, with standard X-ray background at periodic intervals. [6]

12. Cancer xenograft model establishment and PDT: NCI-H460 cells were injected subcutaneously into both flanks of 9-week-old BALB/c nu/nu male mice. Nude mice bearing NCI-H460 cancer (tumor volume ~ 200 mm^3) were intraperitoneally injected with DMSO, and **IR-PU** (10mg/kg) every day. Tumor volume was calculated by the formula volume = (tumor

length) x (tumor width)2/2. Three representative mice groups were selected DMSO, **IR-PU**, and **IR-PU** with laser. For PDT treatments, mice (+ laser) groups was irradiated using a NIR laser (808 nm, 200 mWcm^{-2}) for 2 min, after 24 h of I.P. injection. This pattern was repeated as to day 9.

13. **Synthetic procedure and spectral characterization:**



Scheme S1. Synthetic route for **IR-PU.**

Synthesis of IR-Pyr: IR-Pyr was synthesized by multi-step synthetic strategy as reported earlier.[1]

Synthesis of 3: 3-mercapto propanoic acid (**1**) (1g, 9.25 mmol) and trityl chloride (**2**) (2.57 g, 9.25 mmol) was dissolved in dry CH_2Cl_2 in argon atmosphere. The mixture was stirred at room temperature for 16h. The precipitate formed was washed with CH_2Cl_2 and hexane, followed by diethyl ether. Recrystallization from MeOH/H_2O gave the compound in 75% yield. ^{1}H NMR (400 MHz, $CDCl_3$, 298K): δ = 7.35 (d, 6H), 7.18 – 7.32 (m, 9H), 2.36 (t, 2H), 2.15 (t, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 174.21, 144.58, 129.46, 127.79, 126.58, 49.39, 49.18, 48.96, 48.75, 48.54, 33.24, 26.76. ESI-MS: m/z calculated for $C_{22}H_{20}O_2S$ = 348.46; found = 371.55 (M+Na).

Synthesis of 5: Solution of **3** (1 g, 2.87 mmol) and N-hydroxysuccinimide (0.363 g, 3.1 mmol) in CH_2Cl_2 was mixed with DCC (0.592, 2.87 mmol) and stirred at room temperature for 16 h. The solvent was evaporated and the crude mixture was purified by silica gel column to get the desired compound in 70 % yield. ^{1}H NMR (400 MHz, $CDCl_3$, 298K): δ = 7.44 (dd, 6H), 7.29 (t, 6H), 7.22-7.26 (m, 3H), 2.78 (s, 4H), 2.55 (t, 2H), 2.40 (t, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 168.85, 167.04, 144.31, 129.50, 128.04, 126.82, 49.29, 33.81, 30.50, 26.09, 25.57, 25.53, 24.89. ESI-MS: m/z calculated for $C_{26}H_{23}NO_4S$ = 445.53; found = 449.69).

Synthesis of 7: Compound **5** (0.5 g, 1.12 mmol) and **6** (0.575 g, 1.12 mmol) was dissolved in dry DMF, followed by slow addition of TEA (0.170 g, 1.68 mmol).[2] The mixture was stirred at room temperature for 16h. Solvent was removed in vacuum and the crude mixture was

purified by column chromatography using silica gel to get the desired product in 80% yield. ^{1}H NMR (400 MHz, CD_3OD, 298K): δ = 8.22 (s, 1H), 7.47 (s, 1H), 7.37-7.39 (m, 6H), 7.28 (s, 1H), 7.26 (t, 3H), 7.24 (t, 2H), 7.23 (t, 1H), 7.21 (t, 1H), 7.19 (t, 1H), 6.08 (s, 2H), 3.17 (t, 2H), 2.4 (t, 2H), 2.21 (t, 2H), 1.86 (t, 2H), 1.48-1.54 (m, 2H), 1.38-1.45 (m, 4H); ^{13}C NMR (100 MHz, CD_3OD): δ = 176.31, 155.72, 154.49, 154.37, 153.44, 152.80, 148.69, 133.27, 132.38, 131.45, 130.31, 128.19, 123.31, 118.65, 106.87, 99.53, 70.24, 47.79, 42.69, 38.42, 32.92, 32.71, 31.71, 29.85, 29.83. ESI-MS: m/z calculated for $C_{40}H_{39}IN_6O_3S_2$ = 842.82; found = 844.25.

Synthesis of 8: A solution of **7** (0.20 g, 0.24 mmol) in CH_2Cl_2 was stirred with TFA (0.067 g, 0.59 mmol) and triethylsilane (0.110 g, 0.95 mmol) at room temperature till the consumption of starting material. The reaction mixture was diluted with CH_2Cl_2 and washed with 5% $NaHCO_3$. Dried over anhydrous Na_2SO_4 and the solvent was evaporated. The crude mixture was purified in silica gel column using ethyl acetate/hexane (1:1) as eluent to yield the product in 70% yield. ^{1}H NMR (400 MHz, CD_3OD, 298K): δ = 8.22 (s, 1H), 7.38 (s, 1H), 7.16 (s, 1H), 5.99 (s, 2H), 4.22 (t, 2H), 3.07 (t, 2H), 2.61-2.64 (m, 2H), 2.34-2.37 (m, 2H), 1.76-1.79 (m, 2H), 1.39-1.41 (m, 2H), 1.29-1.32 (m, 6H); ^{13}C NMR (100 MHz, CD_3OD): δ = 172.30, 151.29, 150.67, 150.38, 149.53, 144.7, 124.51, 119.36, 118.7, 114.54, 102.91, 95.30, 39.31, 38.75, 28.97, 28.78, 25.97, 25.84, 19.77; ESI-MS: m/z calculated for $C_{21}H_{25}IN_6O_3S_2$ = 600.50; found = 601.39.

Synthesis of IR-PU: A solution of **8** (0.10 g, 0.16 mmol) and **IR-Pyr** (0.169 g, 0.16 mmol) in DMF was stirred with TEA (0.025 g, 0.25 mmol) at room temperature for 16 h. The solvent was evaporated and the crude mixture was purified by reverse phase HPLC (C18 column using methanol/water). The desired conjugate was isolated in 65% yield. ^{1}H NMR (400 MHz, CD_3OD, 298K): δ = 8.99 (dd, 4H), 8.88 (s, 1H), 8.40 (d, 1H), 8.58 (t, 2H), 8.28 (d, 1H), 8.08-8.12 (m, 4H), 7.47-7.50 (dd, 2H), 7.42 (d, 1H), 7. 40 (d, 1H), 7.38 (d, 1H), 7.25-7.29 (m, 3H),

7.22 (d, 1H), 6.28 (s, 1H), 6.24 (d, 1H), 6.07 (d, 2H), 4.61-4.66 (m, 4H), 4.26 (t, 2H), 4.15 (t, 4H), 3.14 (t, 2H), 3.05 (t, 2H), 2.65 (t, 4H), 2.52 (t, 2H), 2.65 (t, 4H), 2.52 (t, 2H), 2.00-2.06 (m, 4H), 1.85 (t, 6H), 1.74 (d, 11H), 1.47-1.55 (m, 10H), 1.38 (t, 4H), 1.28 (d, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ = 174.68, 173.61, 162.72, 162.35, 159.00, 147.73, 146.75, 145.94, 144.96, 143.24, 135.52, 130.69, 130.36, 127.16, 126.53, 124.34, 121.57, 120.84, 119.81, 116.93, 112.83, 105.17, 97.75, 63.81, 51.34, 46.02, 48.88, 41.19, 38.09, 35.62, 33.15, 31.17, 31.01, 29.31, 29.09, 28.37, 28.24, 28.10, 28.05, 27.88, 25.06, 22.98; ESI-MS: m/z calculated for $C_{73}H_{88}IN_{10}O_3S_2^{3+}$ = 447.85; found = 448.69.

14. NMR spectral analysis:

^{1}H NMR analysis

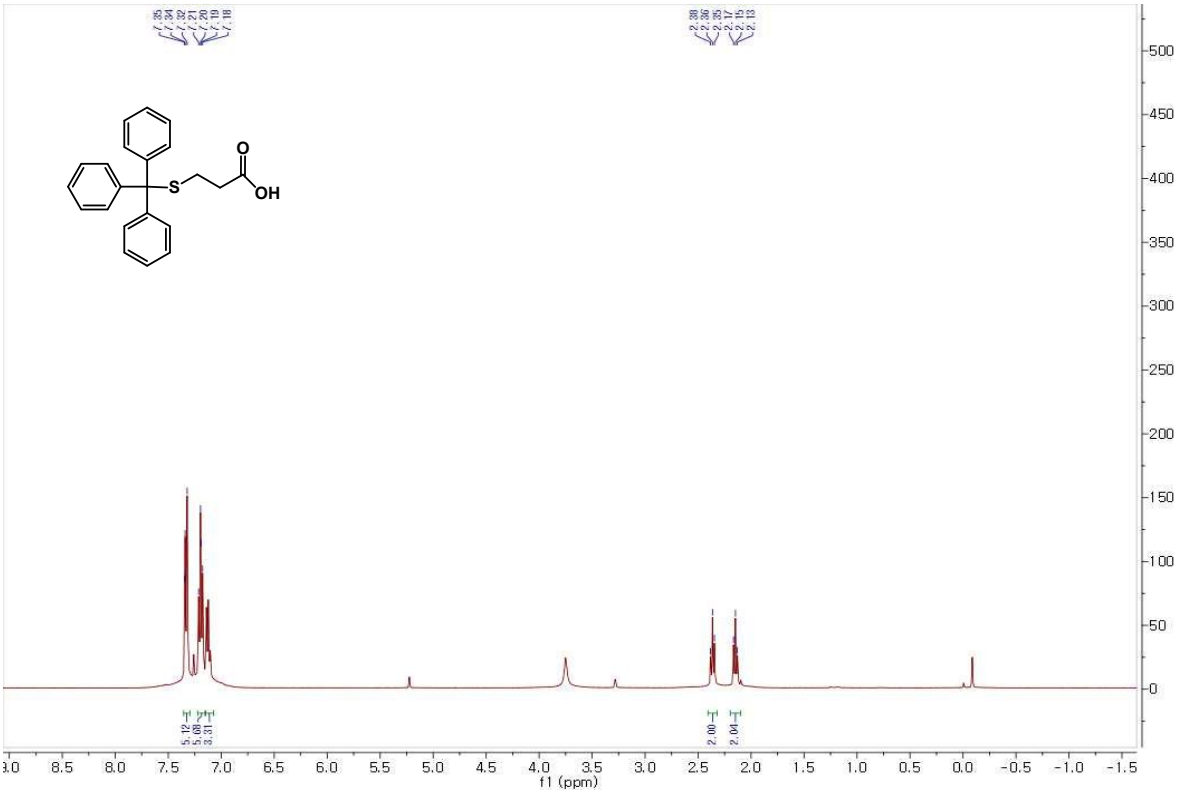


Figure S1. ^{1}H NMR spectrum of **3**.

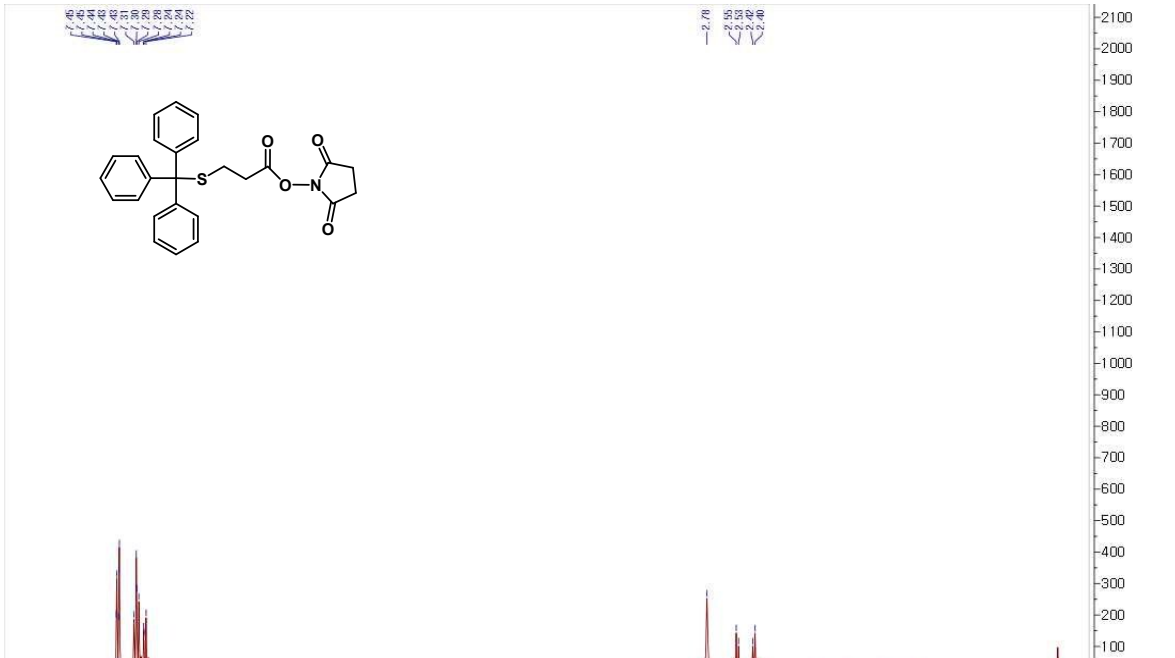


Figure S2. ^{1}H NMR spectrum of **5**.

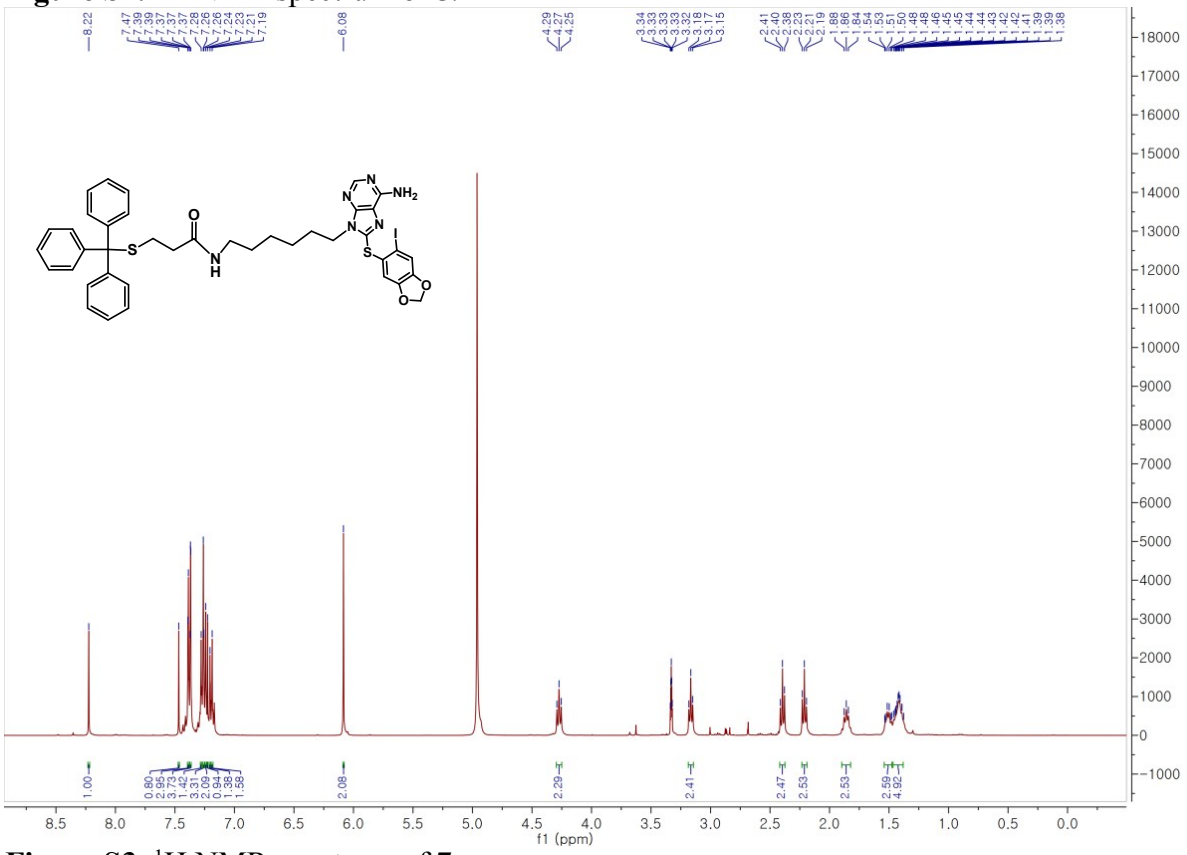


Figure S3. ^{1}H NMR spectrum of **7**.

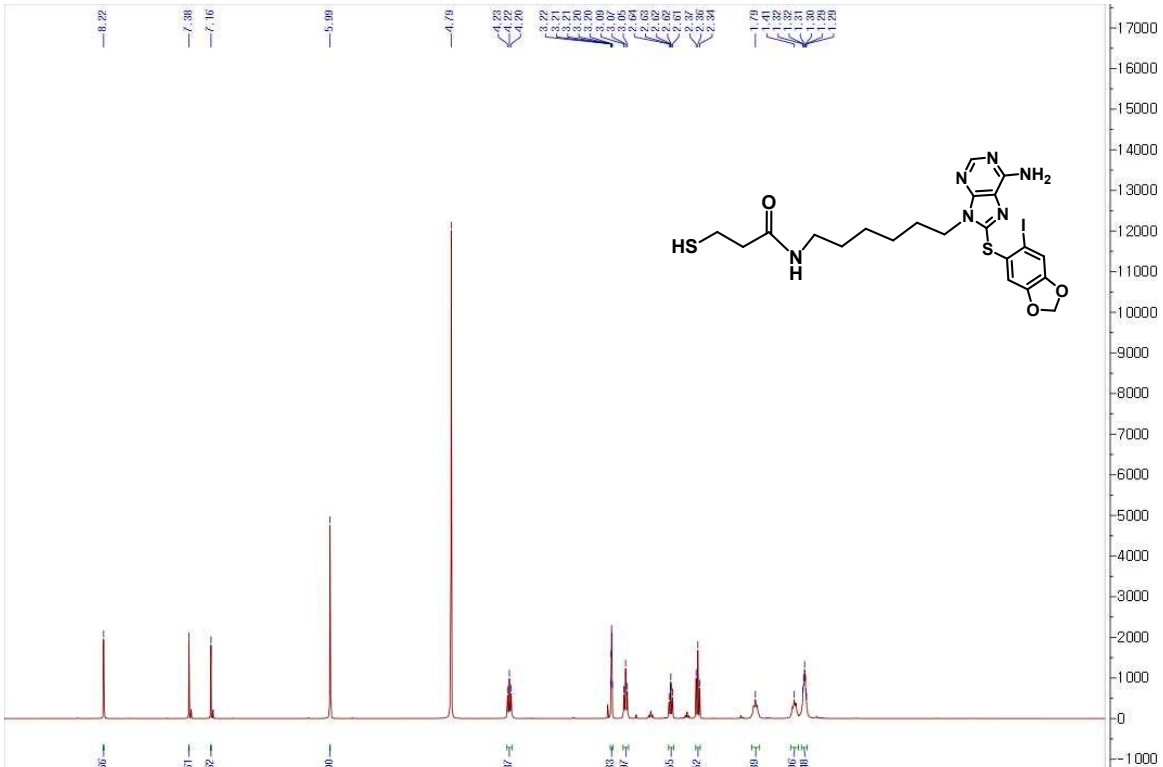


Figure S4. ^{1}H NMR spectrum of **8**.

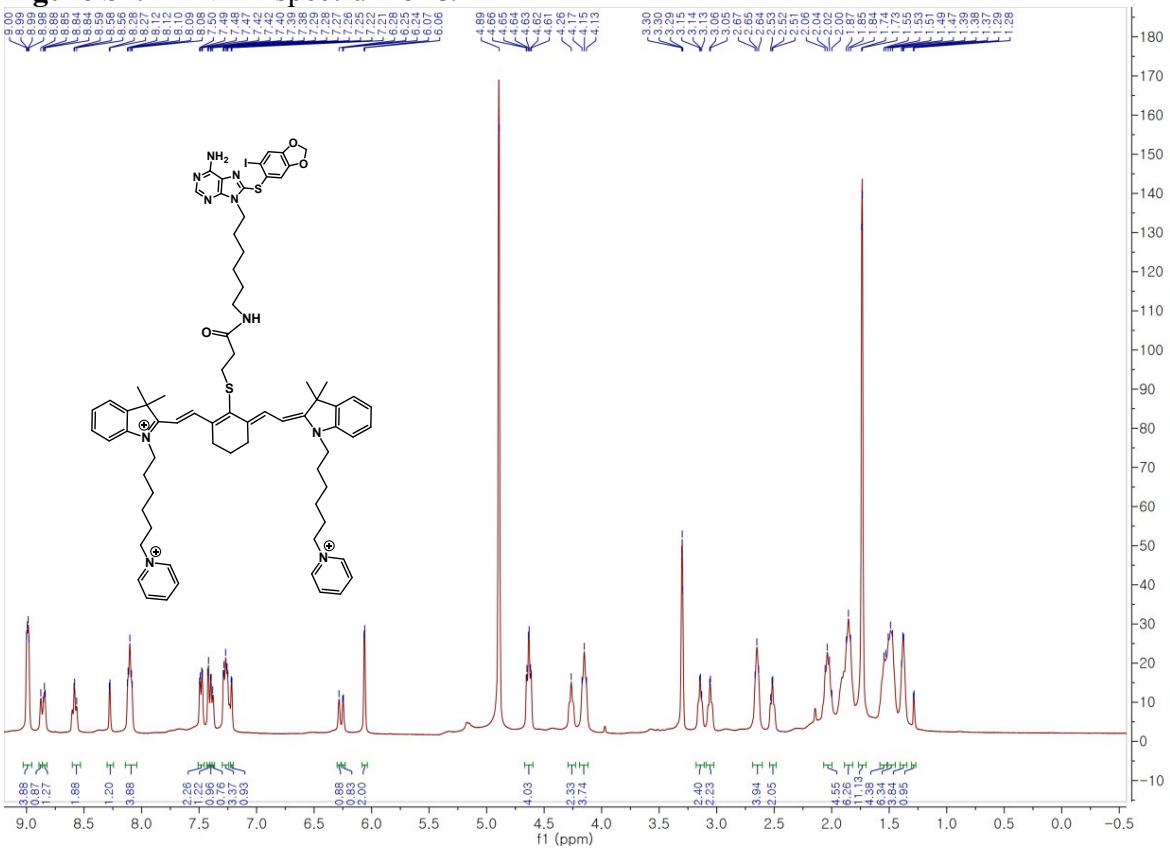


Figure S5. ^{1}H NMR spectrum of **IR-S-PU**.

15. ^{13}C-NMR

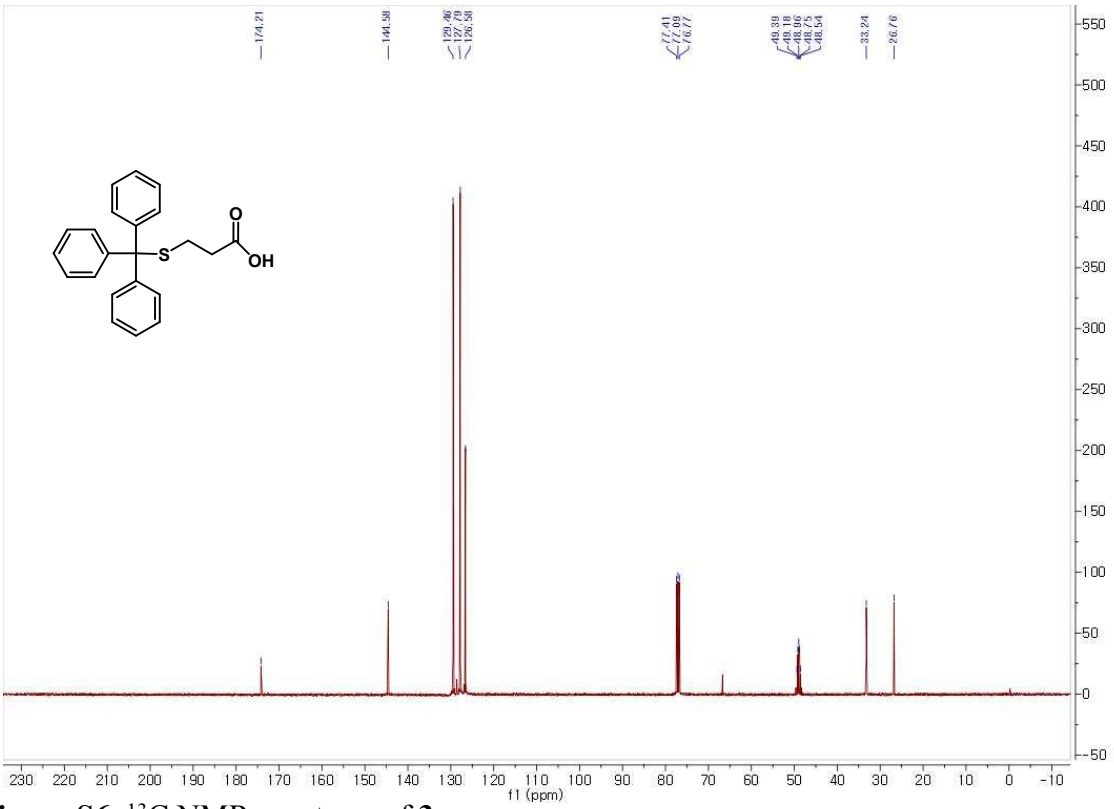


Figure S6. ^{13}C NMR spectrum of **3**.

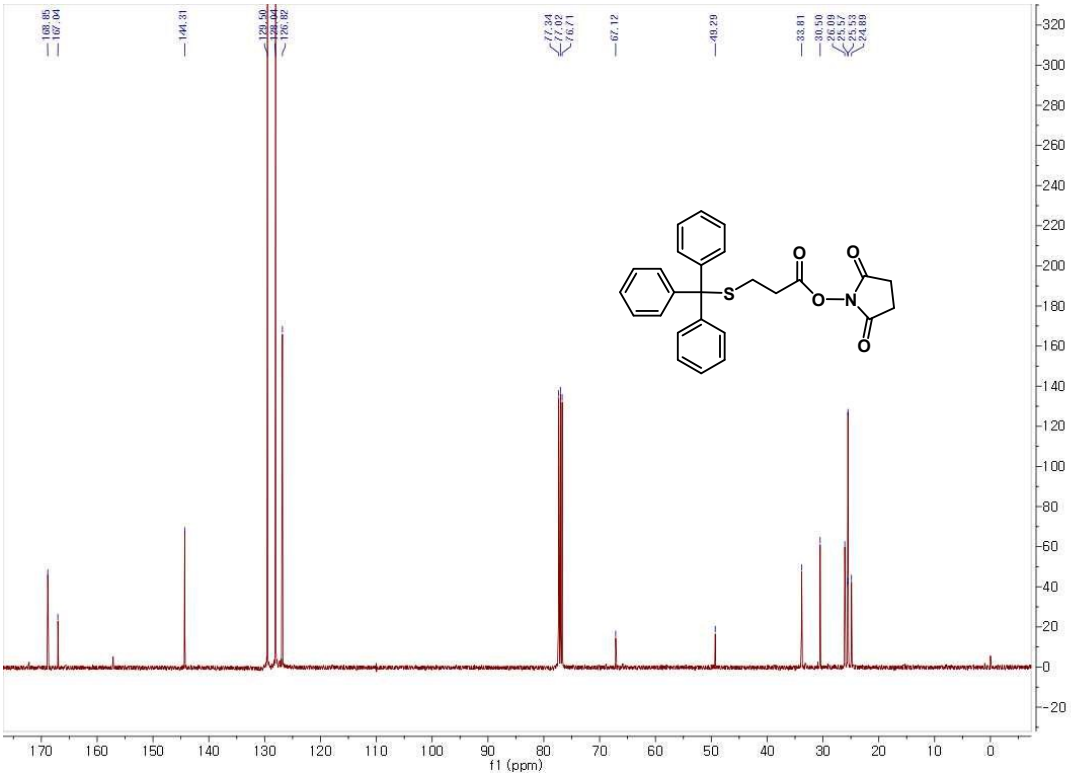


Figure S7. ^{13}C NMR spectrum of **5**.

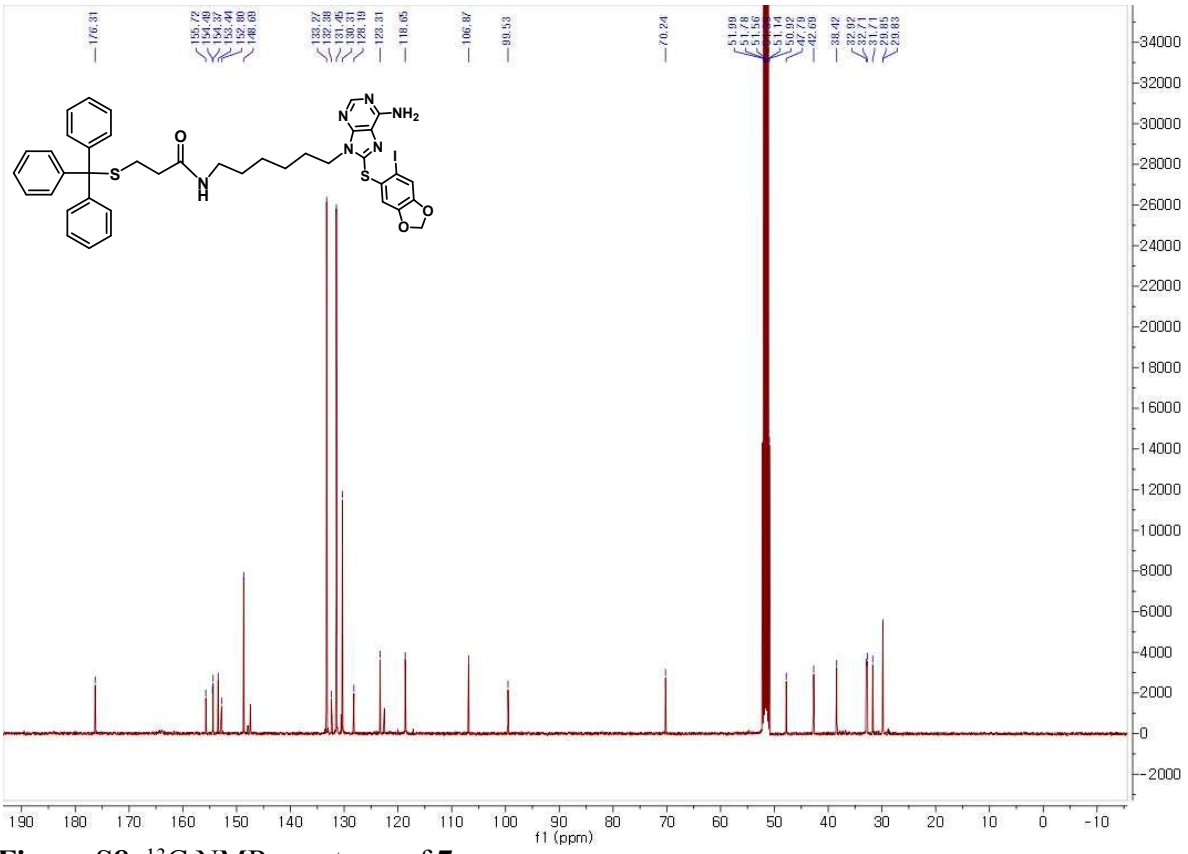


Figure S8. ^{13}C NMR spectrum of **7**.

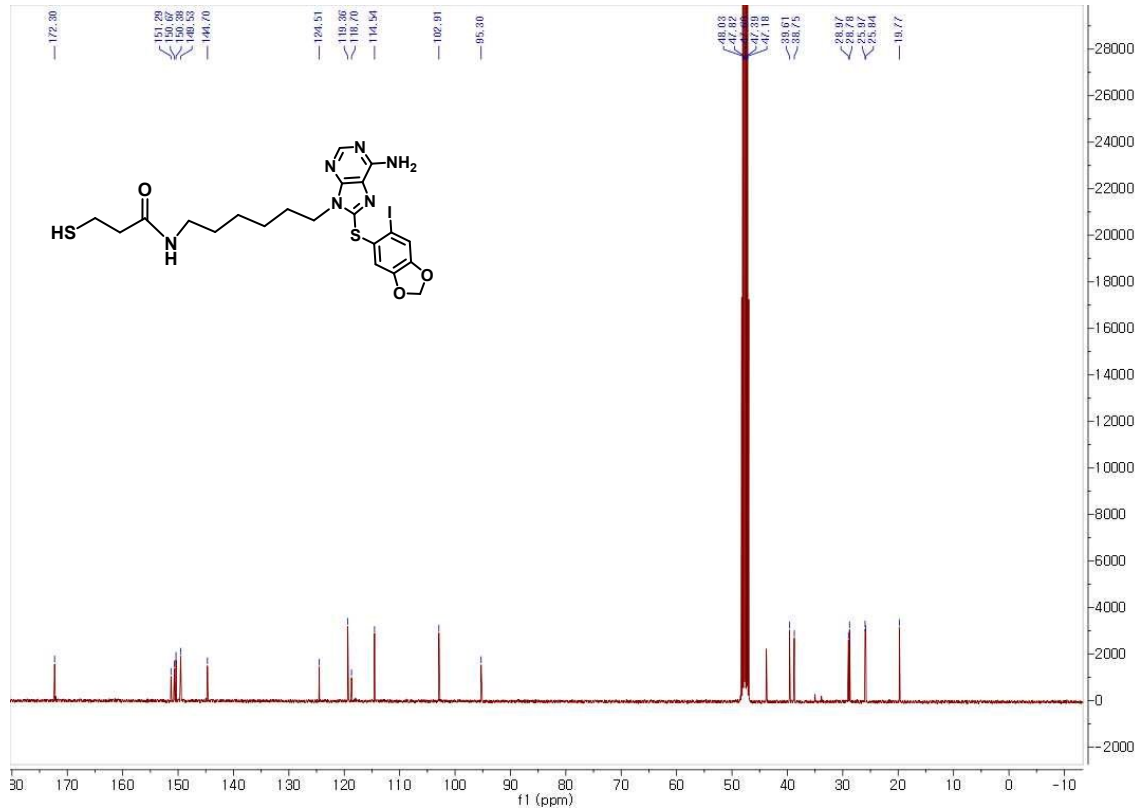


Figure S9. ^{13}C NMR spectrum of **8**.

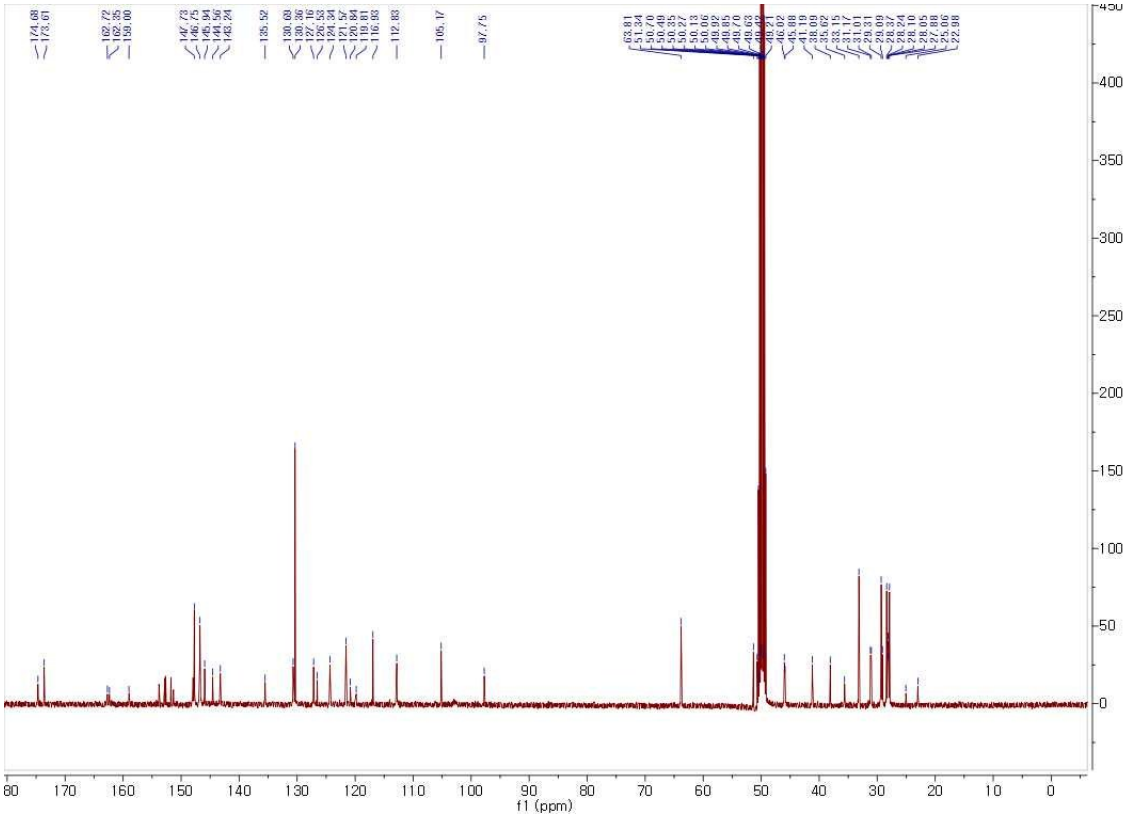
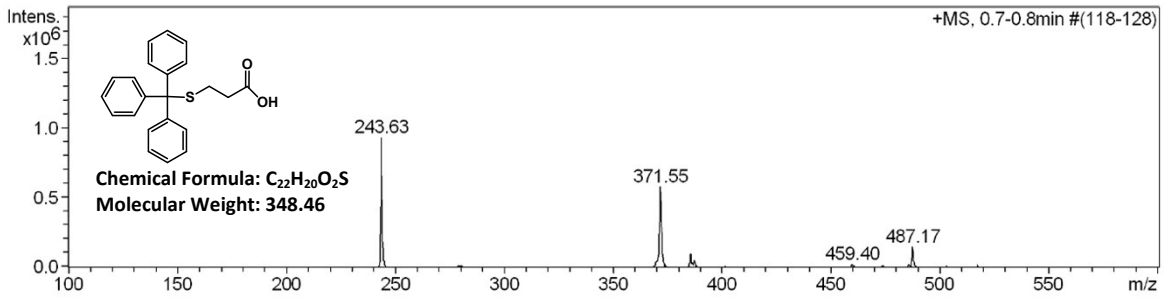


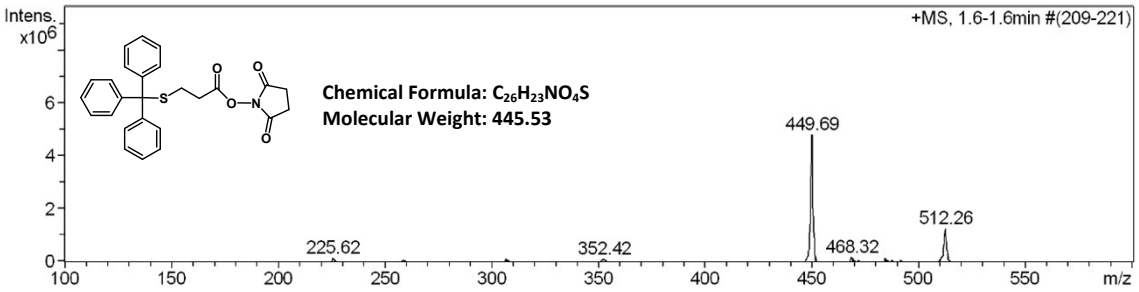
Figure S10. ^{13}C NMR spectrum of **IR-PU**.

16. ESI-MS



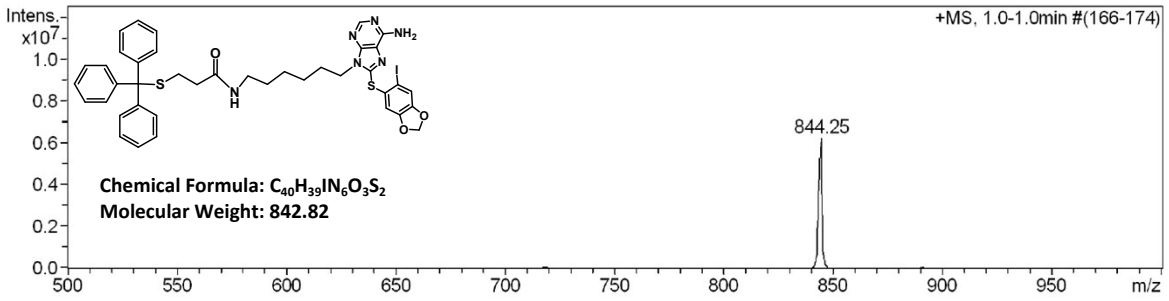
#	m/z	I
1	243.63	927370
2	371.55	577779
3	385.33	98485
4	386.21	34163
5	387.10	50395
6	388.04	12159
7	459.40	21254
8	485.51	21021
9	487.17	142752
10	517.03	15306

Figure S11. ESI-MS of **3**



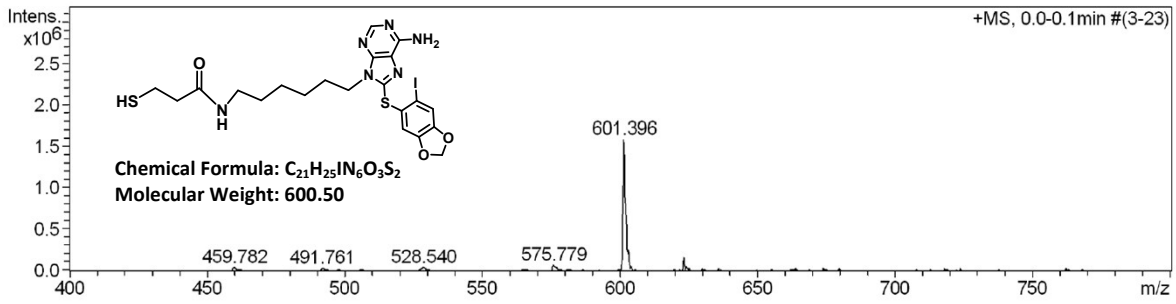
#	m/z	I
1	225.62	138120
2	258.78	65987
3	306.82	87595
4	352.42	103710
5	449.69	4796910
6	468.32	172383
7	484.29	115693
8	485.03	45093
9	487.35	55416
10	512.26	1251420

Figure S12. ESI-MS of **5**



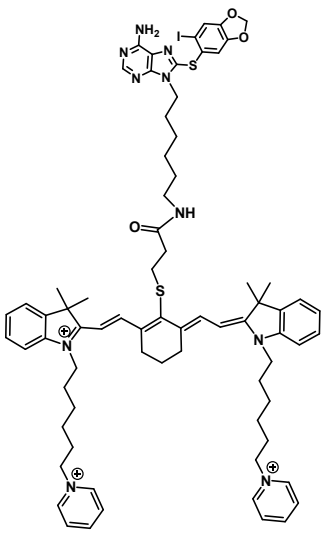
#	m/z	I
1	714.08	30490
2	717.68	72746
3	719.53	27458
4	758.30	27092
5	802.18	33099
6	844.25	6244698
7	890.19	41339
8	890.88	49338
9	934.10	37264
10	965.88	31503

Figure S13. ESI-MS of **7**

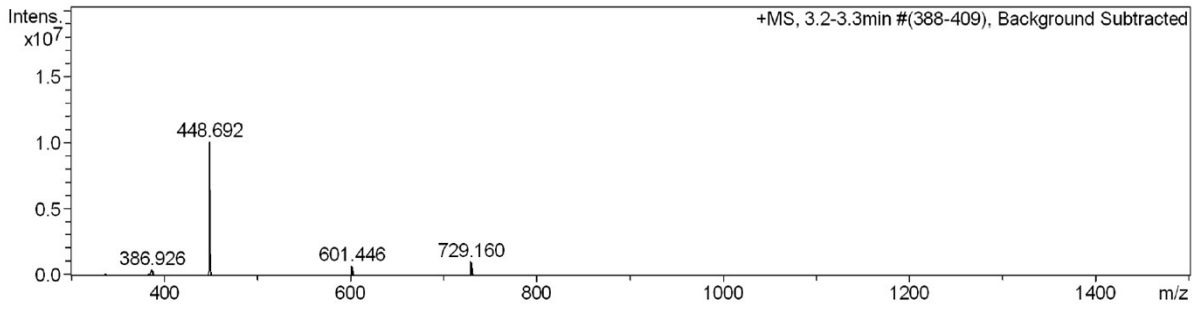


#	m/z	I
1	459.782	42537
2	491.761	32460
3	528.540	39479
4	575.779	70592
5	601.396	1583300
6	603.005	257812
7	604.064	53897
8	623.188	167283
9	624.158	59964
10	673.765	30025

Figure S14. ESI-MS of **8**



Chemical Formula: $C_{73}H_{88}IN_{10}O_3S_2^{3+}$
Exact Mass: 1343.55
Molecular Weight: 1344.58
m/z: 447.85



#	m/z	I
1	337.753	119939
2	345.797	54521
3	381.450	54957
4	382.512	61849
5	383.368	69664
6	386.926	426866
7	448.692	10086912
8	601.446	734717
9	603.143	128639
10	729.160	1043338

Figure S15. ESI-MS of **IR-PU**

17. Photo physical properties and cell experiments

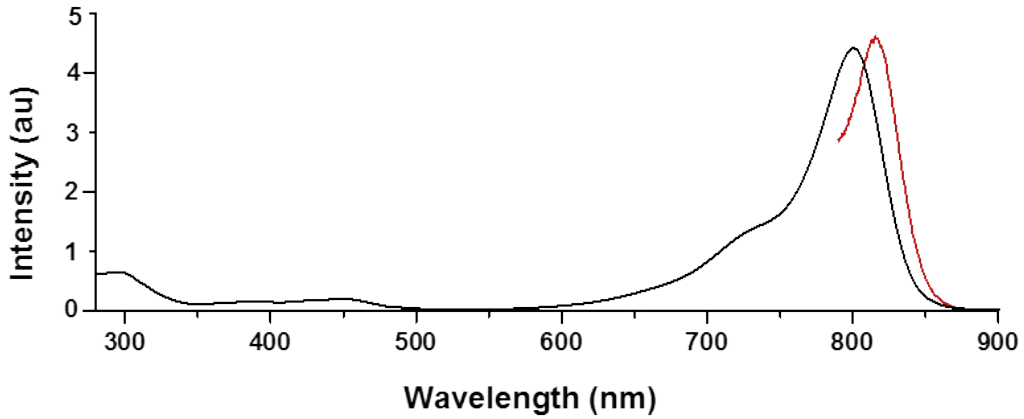


Figure S16. Absorption and emission spectra of **IR-PU**.

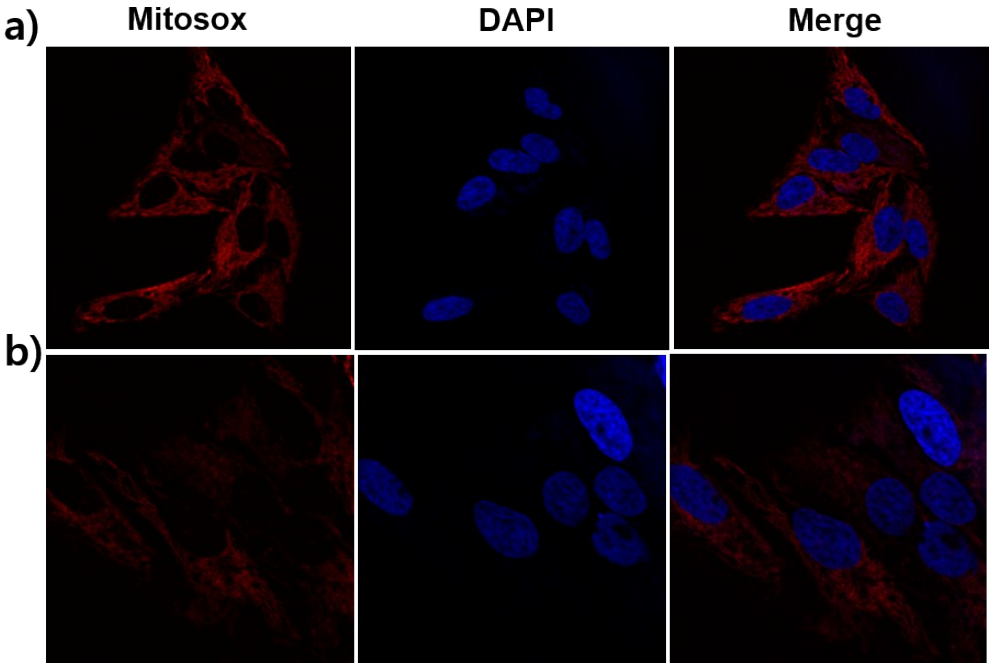


Figure S17. Mitosox fluorescence in HeLa cell lines (a) on incubation with **IR-PU** after 2 min irradiation using 808 nm (200 mW) (b) before irradiation with laser.

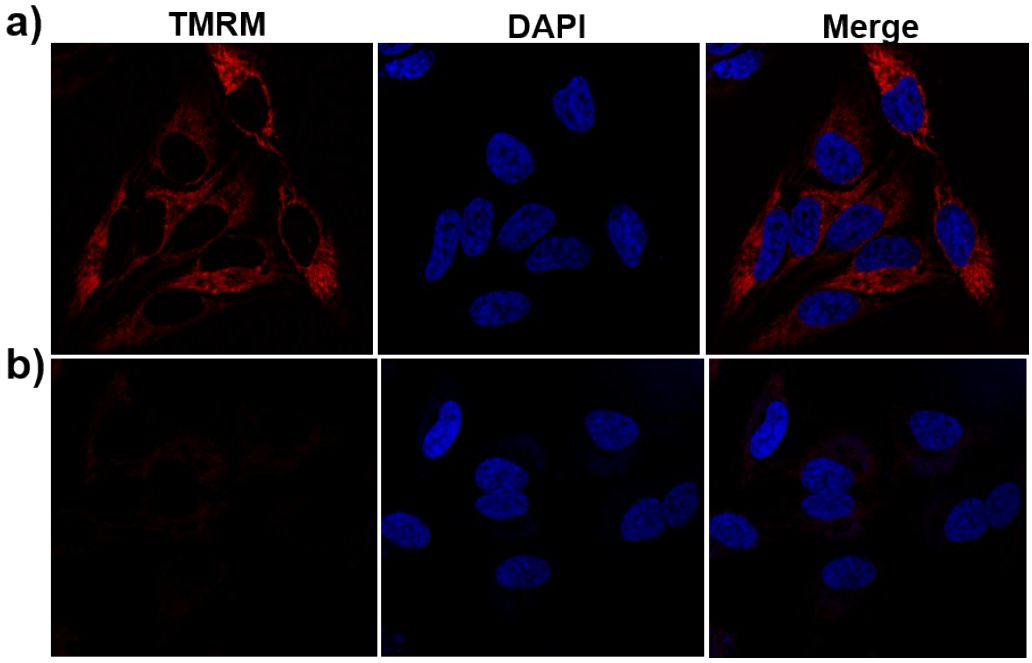


Figure S18. Mitochondria depolarization by **IR-PU** in HeLa cell lines monitored by the change in fluorescence of TMRM, a) before irradiation with laser, b) 2h after irradiating with 808 nm laser.

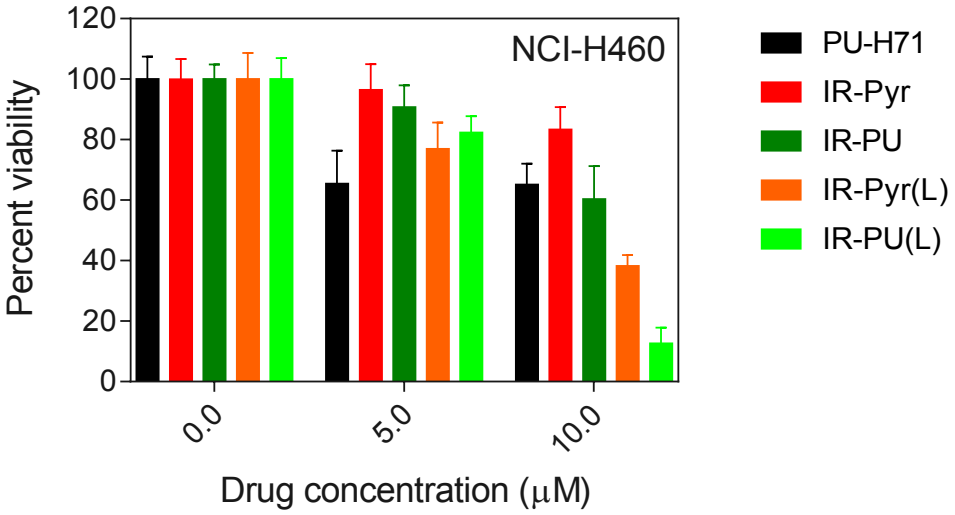


Figure S19. MTT assay to compare the phototoxicity and dark toxicity of **IR-PU** with control molecules

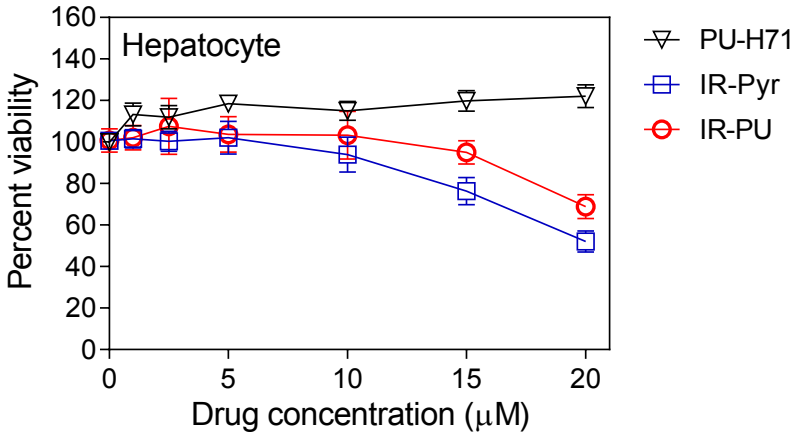


Figure S20. Cytotoxicity of **IR-PU** in Hepatocyte.

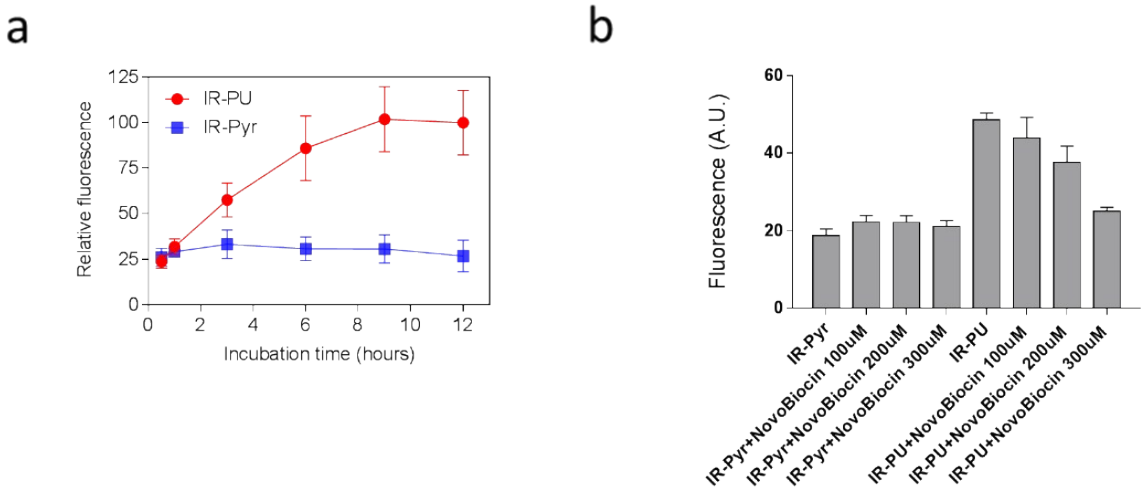


Figure S21. Cellular drug accumulation. (a) NCI-H460 cells were incubated with 10 μM **IR-PU** and **IR-Pyr** for indicated time, and analyzed with fluorescence spectrophotometer. The data are means ± SEM from duplicated two independent experiments. (b) NCI-H460 cells were incubated with novobiocin for 30min and 10 μM **IR-PU** and **IR-Pyr** was treated for 2 hours, and analyzed with fluorescence spectrophotometer.

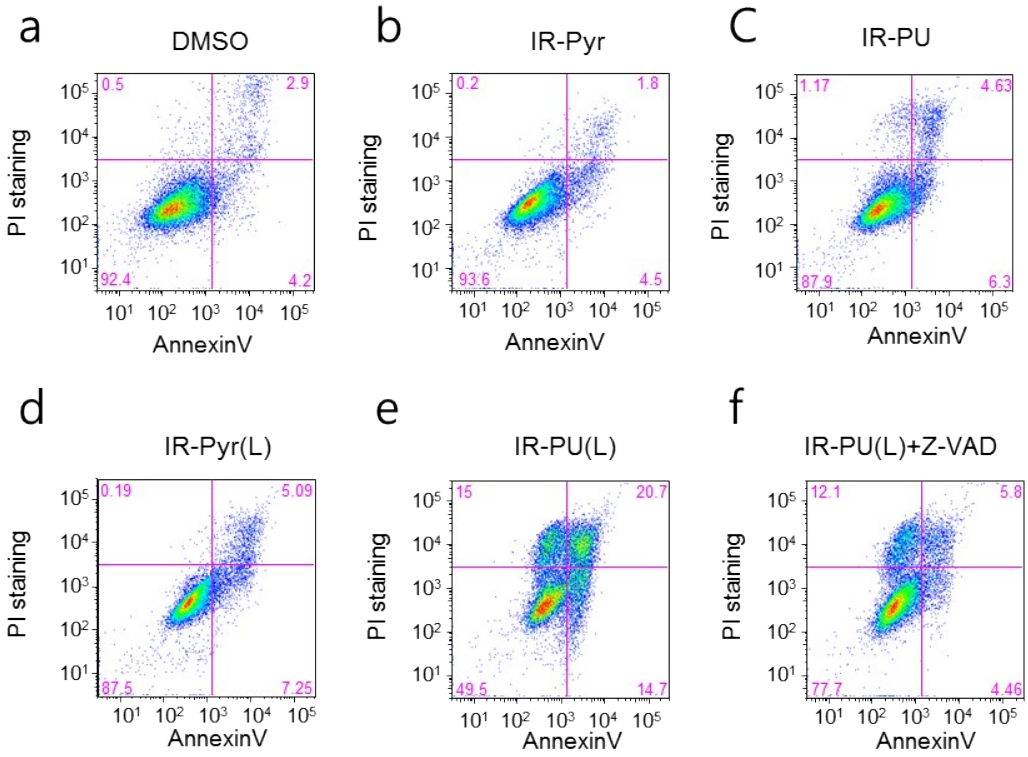


Figure S22. AnnexinV-PI staining**.** (a-c) NCI-H460 cells were incubated 24 h with DMSO (a), 10uM **IR-Pyr** (b), or 10uM **IR-PU** (c). (d-f) **IR-Pyr**, **IR-PU**, or **IR-PU** + 10uM Z-VAD were added to NCI-H460 cells, followed by 12 hours of IR for 2 minutes, followed by another 12

hours. Each samples was stained with Annexin V and propidium iodide (PI). AnnexinV-PI staining was analyzed by flow cytometry.

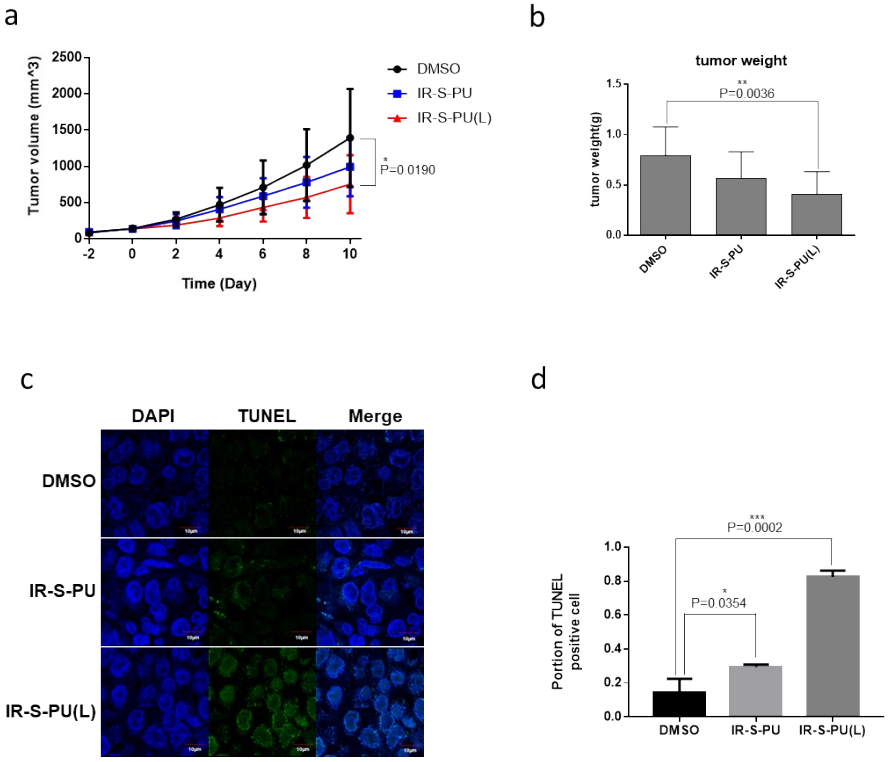


Figure S23. NCI-H460 cells were injected to 9w female nude mice. 10 mg/kg IR-PU was injected daily (i.p.). Mice were sacrificed after 7 days. Tumors, livers and lungs were isolated. (a) changes in tumor volume over time. (b) difference in tumor weight. (c) histologic analyses of the tumors. TUNLE staining shows apoptotic population in the tumor (d) TUNEL positive population.

18. Reference

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