Electronic Supplementary Information

Intracellular self-assembly of $Ru(bpy)_3^{2+}$ nanoparticles enables persistent phosphorescence imaging of tumor

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Contents:

1. General methods

All the starting materials were obtained from Adamas or Sangon Biotech. Commercially available reagents were used without further purification, unless noted otherwise. All chemicals were reagent grade or better. Ultrapure water (18.2 MΩ·cm) was used throughout the experiment. 2-cyano-6-aminobenzothiazole (CBT) was obtained from Shanghai Chemical Pharm-Intermediate Tech. Co.. The spectra of electrospray ionization-mass spectrometry (ESI-MS) were recorded on a LCQ Advantage MAX ion trap mass spectrometer (Thermo Fisher). Matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed with a time-of-flight Ultrflex Ⅱ mass spectrometer (Bruker Daltonics). HPLC analyses were performed on an Agilent 1200 HPLC system equipped with a G1322A pump and in-line diode array UV detector using an Agilent Zorbax 300SB-C18 RP column with CH_3CN (0.1% of trifluoroacetic acid (TFA)) and water (0.1% of TFA) as the eluent. ^{1}H NMR spectra were obtained on a Bruker AV-400 MHz spectrometer. ^{13}C NMR spectra were obtained on a Bruker AV-300 MHz spectrometer. Transmission electron micrograph (TEM) images were obtained on a JEM-2100F field emission transmission electron micrograph operated at an acceleration voltage of 200 kV. Phosphorescence emission spectra were obtained on a Hitachi F-4600 fluorescence spectrophotometer. Phosphorescence lifetimes were obtained on an Edinburgh FLS920 full-featured fluorescence spectrometer. Phosphorescence microscopic images were taken under a fluorescence microscopic OLMPUS IX71. The fluorescent intensity of Alamar Blue assay was read by a Thermo Scientific Varioskan Flash 3001 instruments. HepG2 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, Hycolon) supplemented with 10% fetal bovine serum at

37 °C, 5% CO_2, and humid atmosphere. 5-week-old (weighting 20 g) BALB/c nude mice were used for animal experiments. All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. The procedures were approved by the University of Science and Technology of China Animal Care and Use Committee.

Alamar blue cell viability assay

The cytotoxicity of **1** and **1C** was tested using a standard Alamar Blue assay. The assay is based on the ability of living cells to convert blue redox dye (resazurin) into bright red resorufin, which can be read by a spectrophotometric reader. Nonviable cells rapidly lose metabolic capacity and thus do not generate a color signal. Thus, the intensity of the color is proportional to cell viability. HepG2 cells growing in log phase were seeded into a black 96-well cell culture plate at 1×10^4 cells per well and incubated for 24 h at 37 °C under 5% CO_2. The solutions of **1** together with 50 μM **CKCBT**, or **1C** (100 μL/well) at concentrations of 10, 20, or 40 μM in 100 μL of medium were added to the wells, respectively. The cells were incubated for 6, 12, or 24 h at 37 °C under 5% CO_2. Next, culture medium was removed and fresh medium containing 10% Alamar Blue dye was added (100 μL). The cells were then incubated for 4 h. The data were obtained using a Thermo Scientific Varioskan Flash 3001 to detect fluorescence with the excitation and emission wavelengths set at 560 and 590 nm, respectively. The following formula was used to calculate the viability of cell growth: Cell Viability = (sample − background) / (untreated − background) × 100%.

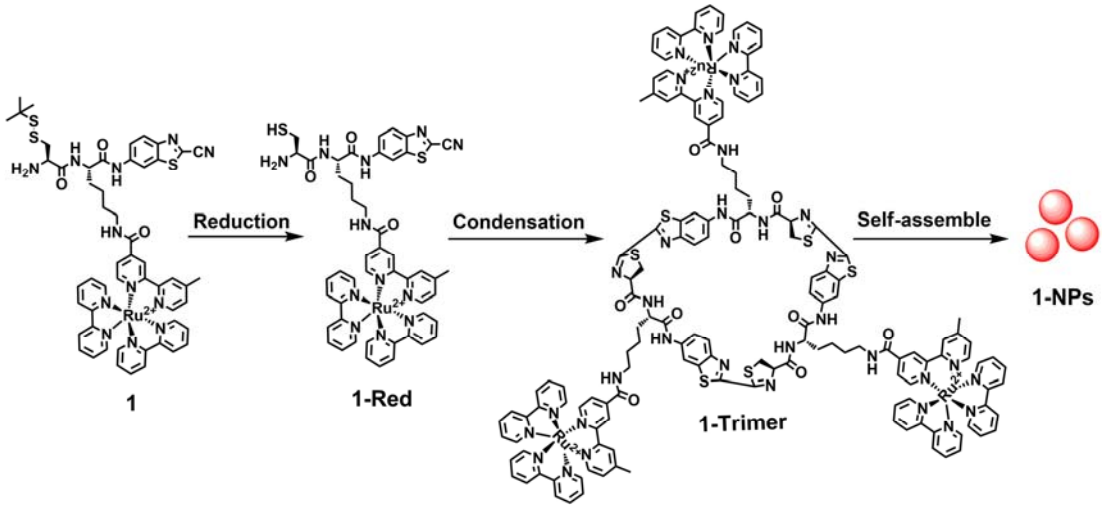
Phosphorescence microscopic imaging of cells

The hepatocellular carcinoma HepG2 cells growing in log phase were seeded into cell culture

dish (3.5 cm) at 2×10^5 cells and incubated at 37 °C in a CO_2 incubator for 24 h. The HepG2 cells then were washed for three times with phosphate-buffered saline (PBS, pH 7.4) and incubated with 20 μM **1** together with 50 μM **CKCBT**, or 20 μM **1C** in serum-free DMEM at 37 °C for 1 h in a CO_2 incubator, respectively. Next, the HepG2 cells were again washed for three times with PBS to remove the free compounds and treated with 10% Hoechst 33342 in serum-free DMEM (1 mL) at 37 °C for 20 min in a CO_2 incubator. Finally, the HepG2 cells were incubated with fresh culture medium and washed for three times with PBS prior to imaging at 0, 1, 2, 4, 8, and 16 h.

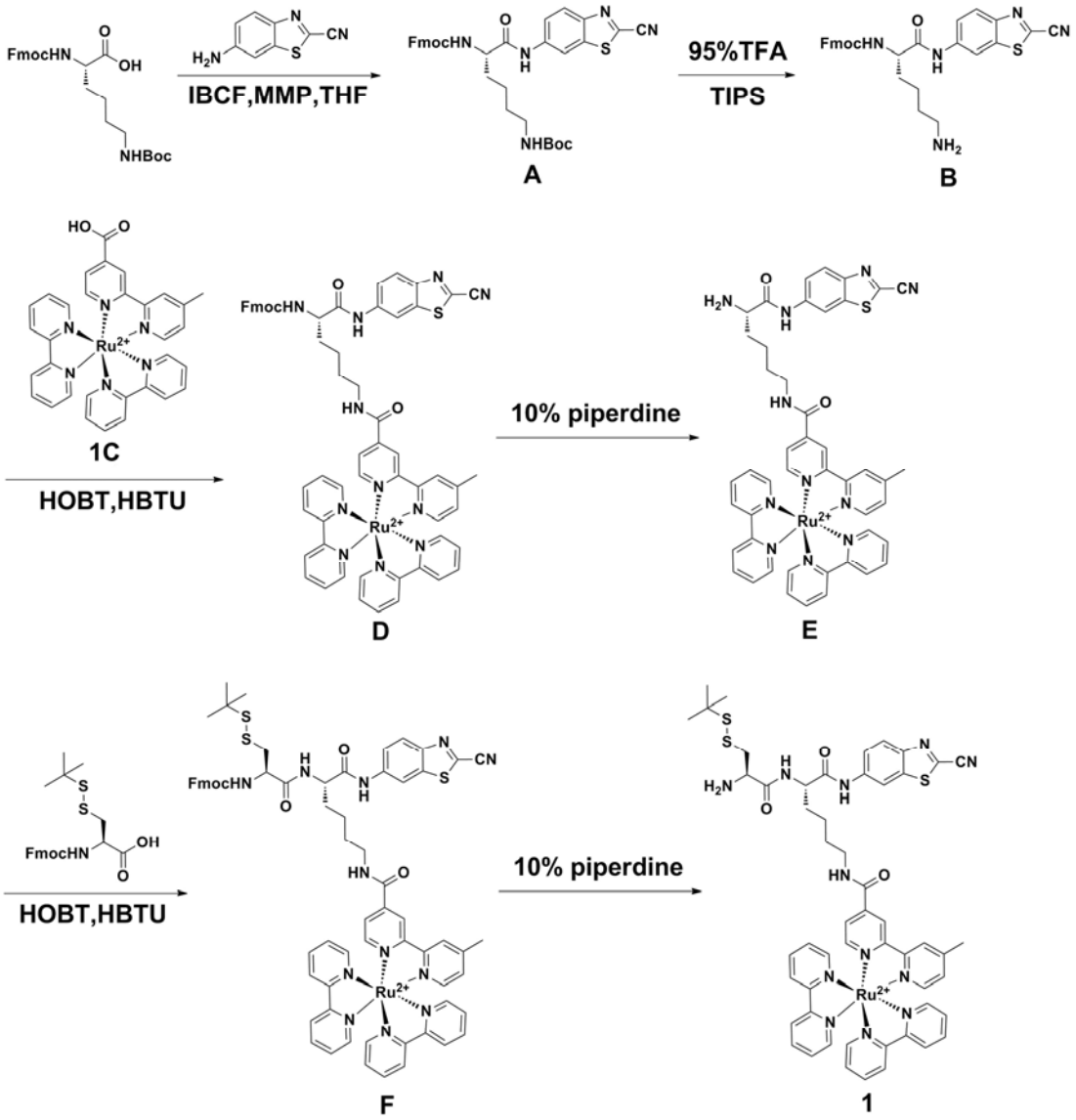
2. Syntheses and Characterizations of 1C and 1

Scheme S1. Detailed schematic illustration of reduction-controlled condensation of **1** to self-assemble into the nanoparticles **1-NPs**.



*Preparation of Ru(Ⅱ)(bpy)$_2$(4-methyl-4'-carboxyl-2,2'-bipyridine)bishexafiuorophosphate (**1C**) and Cys(StBu)-Lys(Ru(bpy)$_3^{2+}$)-CBT (**1**) (CBT = 2-cyano-6-aminobenzothiazole).*

Scheme S2. Synthetic route for **1C** and **1**.



*Synthesis of **A**:* The isobutyl chloroformate (IBCF, 77 μL, 0.6 mmol) was added to a mixture of Fmoc-Lys(Boc)-OH (281 mg, 0.6 mmol) and 4-methylmorpholine (MMP, 124 μL, 1.1 mmol) in THF (3 mL) at 0 °C and the reaction mixture was stirred for 30 min. The solution of 2-cyano-6-aminobenzothiazole (CBT, 87.5 mg, 0.5 mmol) was added to the reaction mixture and further stirred for 1 h at 0 °C. Then the mixture was stirred overnight at room temperature.

Compound **A** (250 mg, yield 80%) which was obtained after HPLC purification.

*Synthesis of **B**:* The Boc protecting group of compound **A** (250 mg, 0.4 mmol) was removed with dichloromethane (DCM, 1 mL) and triisopropylsilane (TIPS, 200 μL) in TFA (19 mL) for 3 h, then compound **B** (190 mg, yield 90%) which was obtained after HPLC purification.

*Synthesis of **1C**: **1C*** (210 mg, total yield 10%) which was synthesized following the literature method.[1-3] ^{1}H NMR of compound **1C** (d_4-CD_3OD, 400 MHz, Figure S1) δ (ppm): 8.95 (d, J = 1.69 Hz, 1 H), 8.72 (d, J = 8.20 Hz, 4 H), 8.55 (s, 1 H), 8.12 (m, 4 H), 7.82 (m, 6 H), 7.62 (d, J = 5.84 Hz, 1 H), 7.49 (m, 4 H), 7.35 (d, J = 5.84 Hz, 1 H), 2.59 (s, 3 H). ^{13}C NMR of **1C** (d_4-CD_3OD, 75 MHz, Figure S2) δ (ppm): 172.5, 161.2, 161.1, 161.0, 161.0, 160.4, 155.2, 155.2, 155.1, 155.1, 155.1, 155.1, 154.7, 154.2, 151.0, 141.7, 141.7, 141.7, 141.7, 132.4, 131.4, 131.4, 131.4, 131.4, 130.4, 128.9, 128.1, 128.1, 128.1, 128.1, 126.9, 23.8.

*Synthesis of **D**:* Compound **1C** (55 mg, 0.06 mmol), HBTU (22.8 mg, 0.06 mmol), and HOBt (8.1 mg, 0.06 mmol) in DMF (2 mL) were stirred for 30 min in presence of DIPEA (15 μL, 0.09 mmol). Then Compound **B** (38 mg, 0.072 mmol) was added and stirred for 24 hours in the dark at room temperature. Compound **D** (56 mg, yield 82%) which was obtained after HPLC purification. MS: calculated for **D** [M^{2+}/2]: 567.64, obsvd. ESI-MS: m/z 567.64.

*Synthesis of **E**:* The Fmoc protecting group of compound **D** (56 mg, 0.05 mmol) was cleaved with 10% piperidine in DMF (2 mL) for 5 min at 0 °C, then 240 μL TFA was added to neutralize the alkaline. Compound **E** (40 mg, yield 88%) which was obtained after HPLC purification. MS: calculated for **E** [M^{2+}/2]: 456.61, obsvd. ESI-MS: m/z 456.64.

*Synthesis of **F**:* Fmoc-Cys(StBu)-OH (10.5 mg, 0.024 mmol), HBTU (9.1 mg, 0.024 mmol), and HOBt (3.2 mg, 0.024 mmol) in DMF (2 mL) were stirred for 30 min in presence of

DIPEA (5.2 μL, 0.03 mmol). Then Compound **E** (18 mg, 0.02 mmol) was added and stirred for 24 hours in the dark at room temperature. Pure compound **F** (20 mg, yield 75%) which was obtained after HPLC purification. MS: calculated for **F** [M^{2+}/2]: 663.17, obsvd. ESI-MS: *m*/*z* 663.18.

*Synthesis of **1**:* The Fmoc protecting group of compound **F** (26.5 mg, 0.02 mmol) was cleaved with 10% piperidine in DMF (2 mL) for 5 min at 0 °C, then 240 μL TFA was added to neutralize the alkaline. Pure compound **1** (18 mg, yield 82%) which was obtained after HPLC purification. ^{1}H NMR of compound **1** (d_4-CD_3OD, 400 MHz, Figure S3) δ (ppm): 8.97 (d, J = 3.24 Hz, 1 H), 8.71 (d, J = 8.32 Hz, 4 H), 8.64 (d, J = 4.76 Hz, 1 H), 8.58 (d, J = 11.52 Hz, 1 H), 8.13 (t, J_1 = 6.72 Hz, J_2 = 7.44 Hz, 4 H), 8.00 (dd, J_1 = 3.28 Hz, J_2 = 9.04 Hz, 1 H), 7.91 (d, J = 5.92 Hz, 1 H), 7.80 (m, 4 H), 7.74 (d, J = 6.00 Hz, 1 H), 7.70 (d, J = 8.84 Hz, 1 H), 7.63 (d, J = 5.48 Hz, 1 H), 7.49 (m, 4 H), 7.37 (d, J = 5.32 Hz, 1 H), 4.60 (t, J_1 = 6.96 Hz, J_2 = 7.08 Hz, 1 H), 4.20 (m, 1 H), 3.45 (m, 2 H), 3.35 (m, 1 H), 3.12 (m, 1 H), 2.58 (s, 3 H), 1.97 (m, 1 H), 1.88 (m, 1 H), 1.72 (m, 2 H), 1.56 (m, 2 H), 1.30 (s, 9 H). ^{13}C NMR of **1** (d_4-CD_3OD, 75 MHz, Figure S4) δ (ppm): 172.2, 168.5, 165.7, 159.4, 158.6, 158.6, 158.5, 158.4, 157.5, 153.3, 152.7, 152.7, 152.7, 152.5, 152.4, 151.8, 149.8, 144.0, 140.5, 139.4, 139.4, 139.4, 139.3, 138.0, 136.8, 130.2, 129.0, 129.0, 129.0, 129.0, 126.8, 126.2, 125.9, 125.7, 125.7, 125.7, 125.7, 122.8, 122.3, 114.0, 112.9, 55.8, 53.8, 42.6, 41.0, 32.9, 30.0, 30.0, 30.0, 29.9, 24.1, 21.3, 21.3. MS: calculated for **1** [M^{2+}/2]: 552.13, obsvd. ESI-MS: *m*/*z* 552.04 (Figure S5).

3. Supporting figures and tables

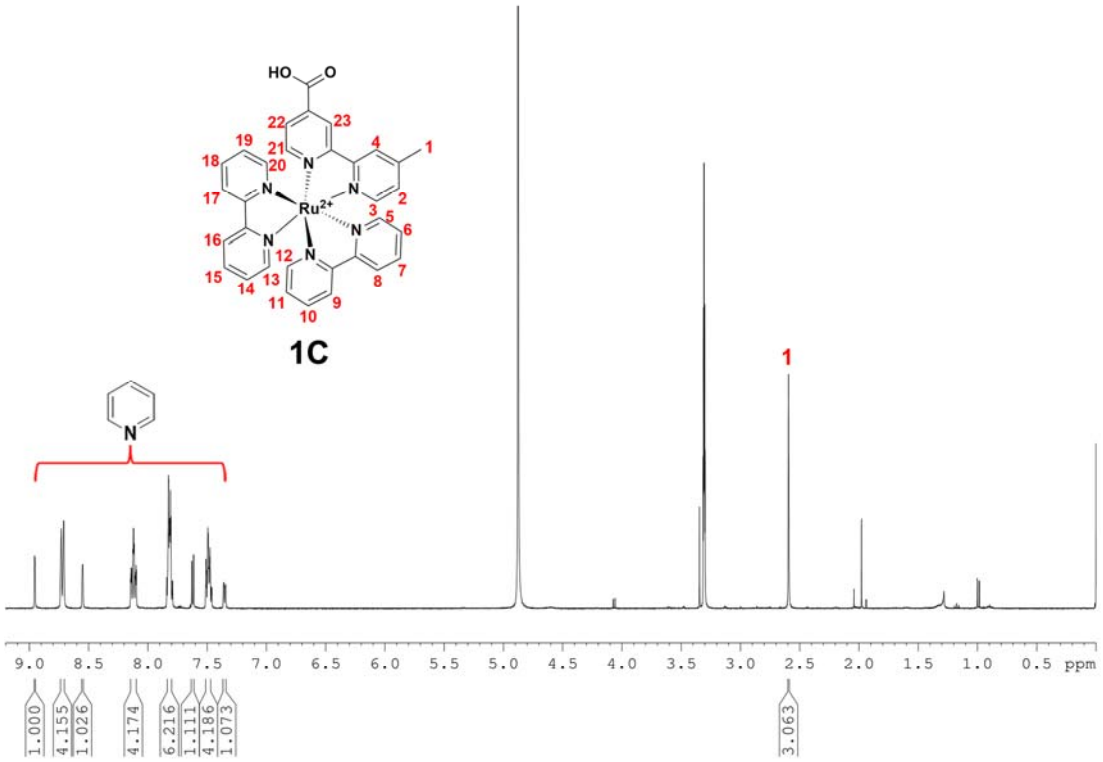


Figure S1. ^{1}H NMR spectrum of **1C** in d_4-CD_3OD.

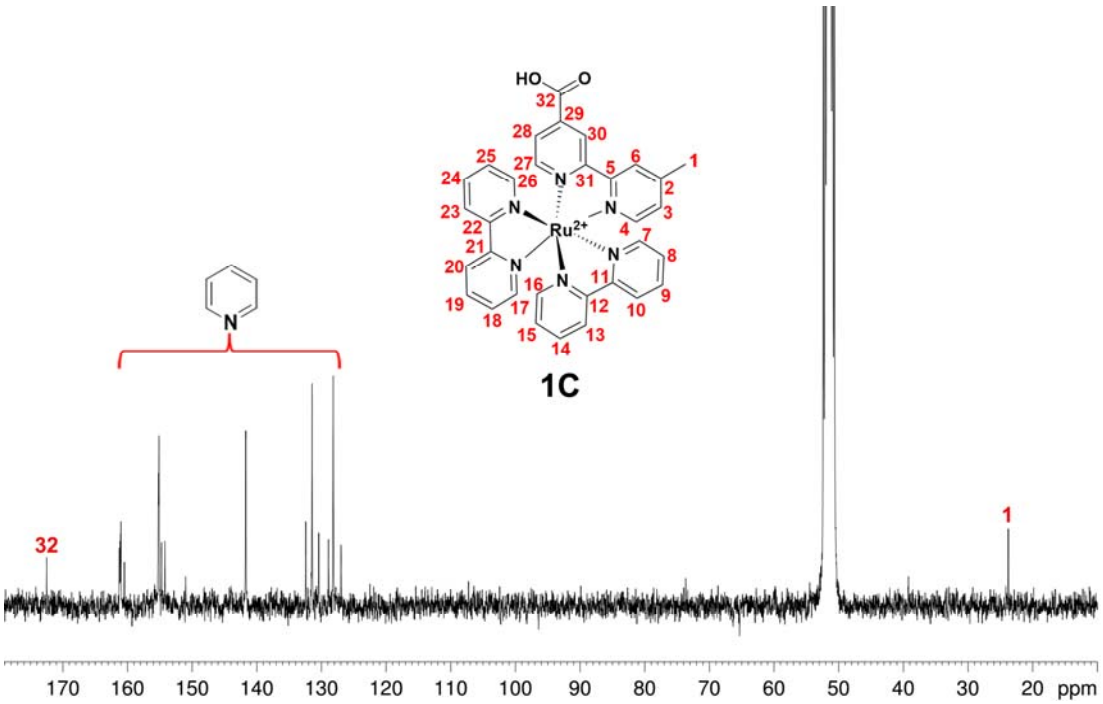


Figure S2. ^{13}C NMR spectrum of **1C** in d_4-CD_3OD.

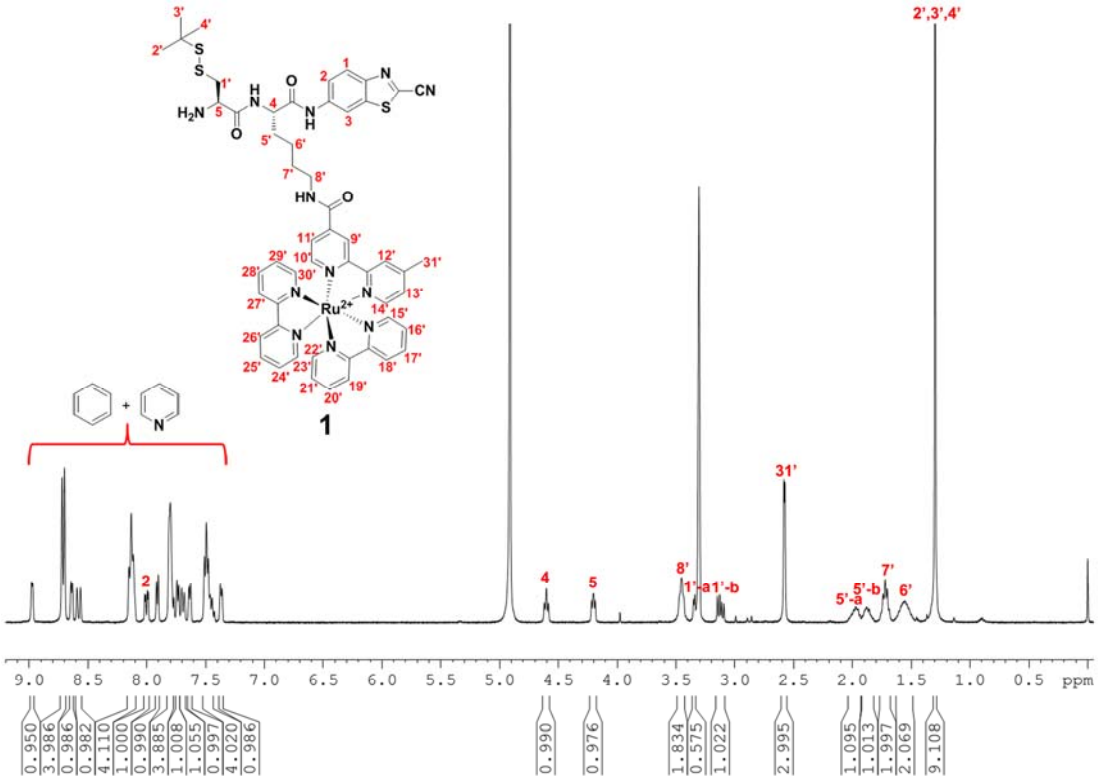


Figure S3. ^{1}H NMR spectrum of **1** in d_4-CD_3OD.

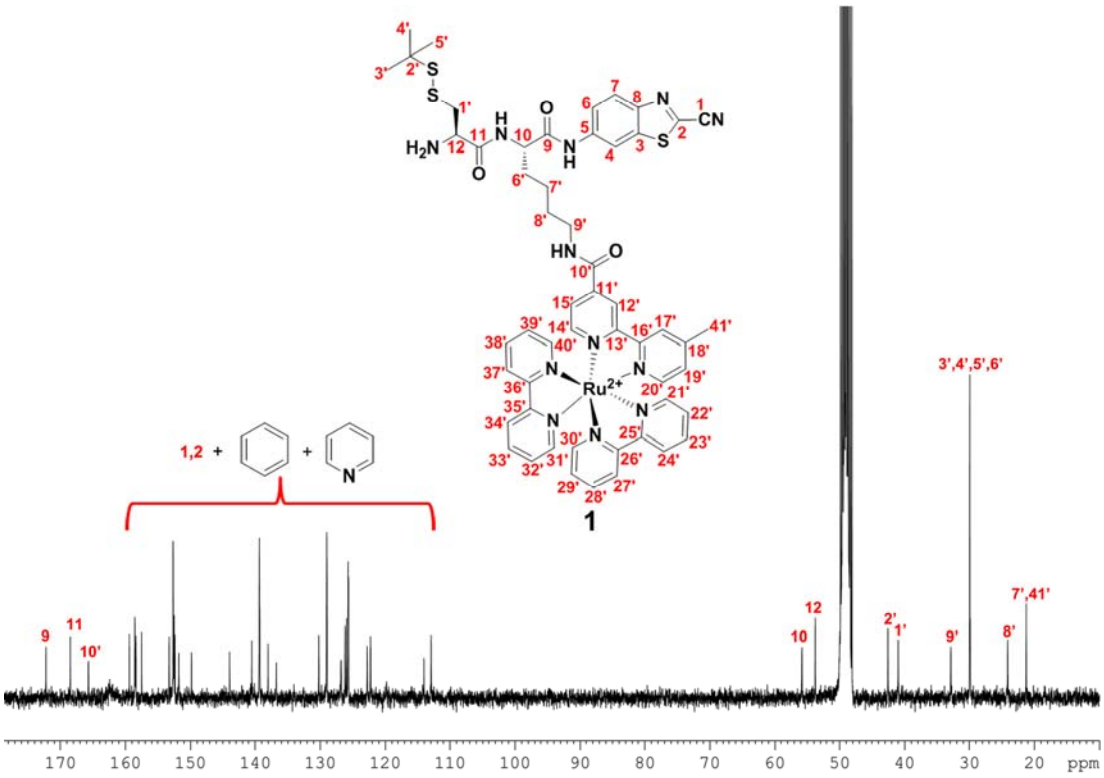


Figure S4. ^{13}C NMR spectrum of **1** in d_4-CD_3OD.

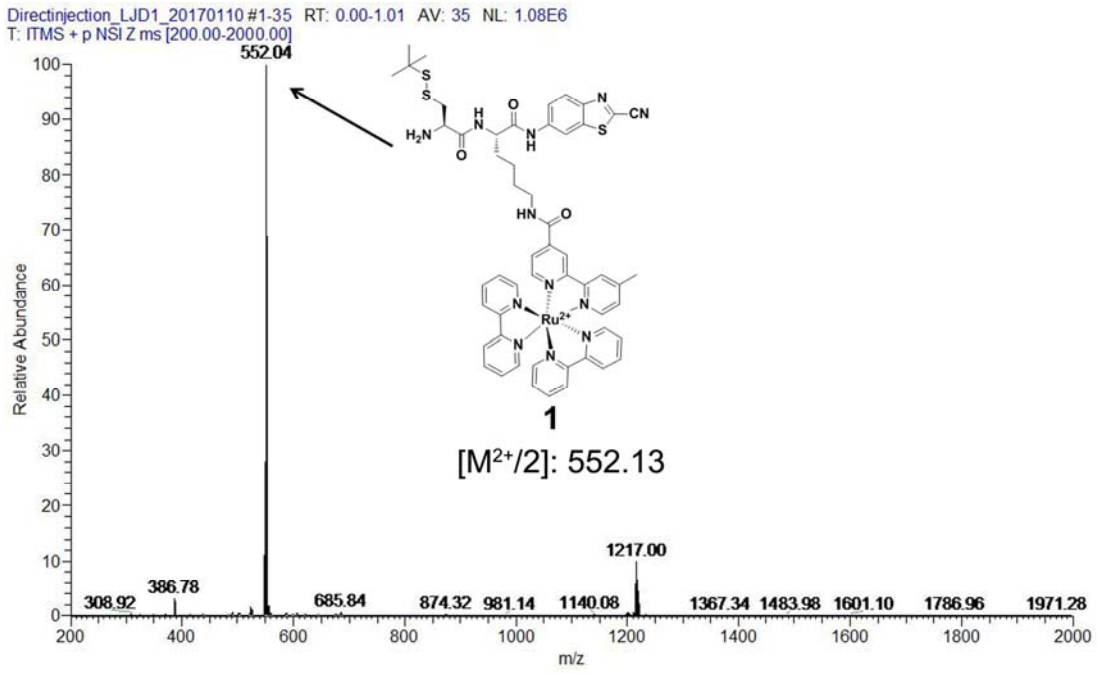


Figure S5. ESI-MS spectrum of **1**.

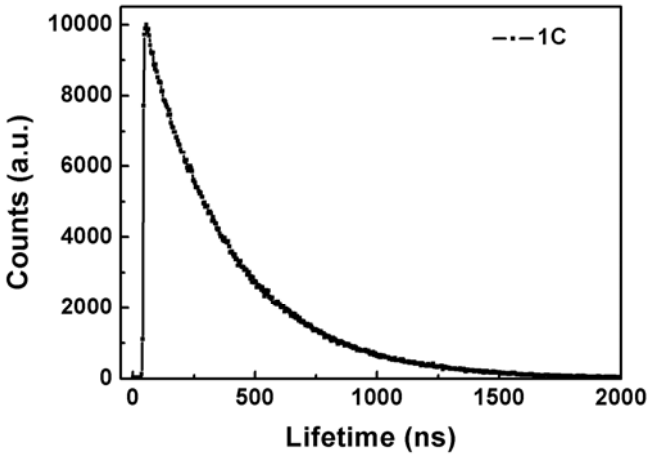


Figure S6. The lifetime decay curve of 100 μM **1C** at 37 °C for 2 h in PBS buffer.

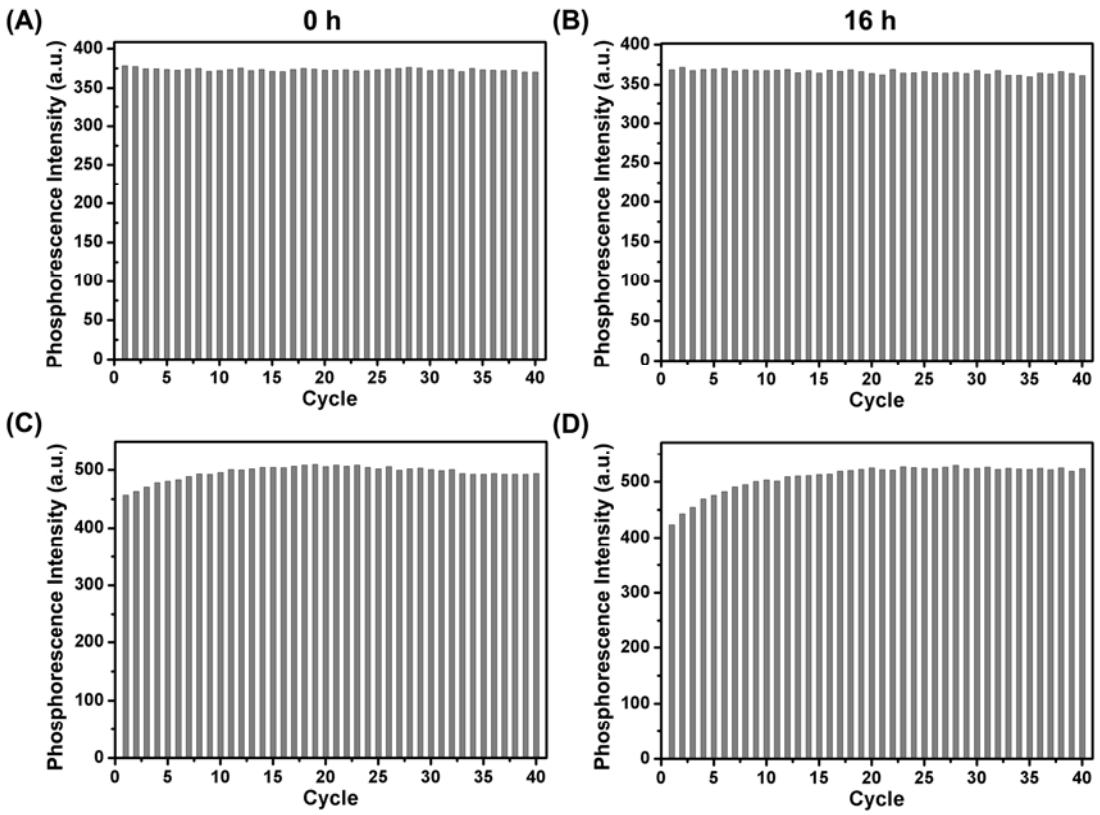


Figure S7. The phosphorescence intensity for 40 cycles of 100 μM **1** at 37 °C for 0 h (A) and 16 h (B) in PBS buffer. The phosphorescence intensity for 40 cycles of 33.3 μM **1-NPs** at 37 °C for 0 h (C) and 16 h (D) in PBS buffer. Excitation: 450 nm. Emission: 645 nm.

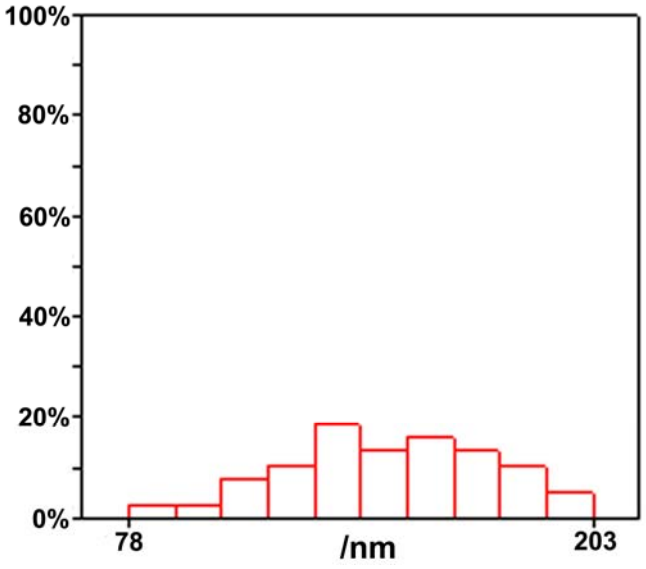


Figure S8. Histogram of diameters of **1-NPs** in the TEM image of Figure 2C.

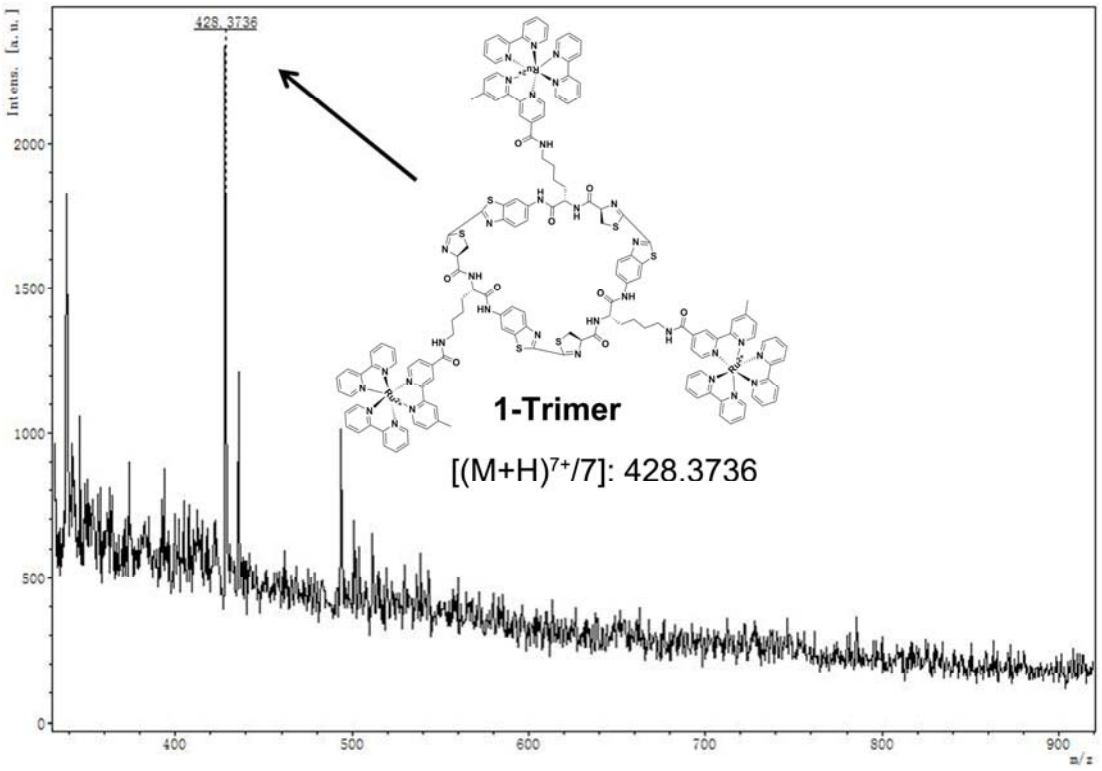


Figure S9. HR-MALDI-TOF/MS spectrum of HPLC peaks at 27.1 min (**1-Trimer**) in Figure 2D.

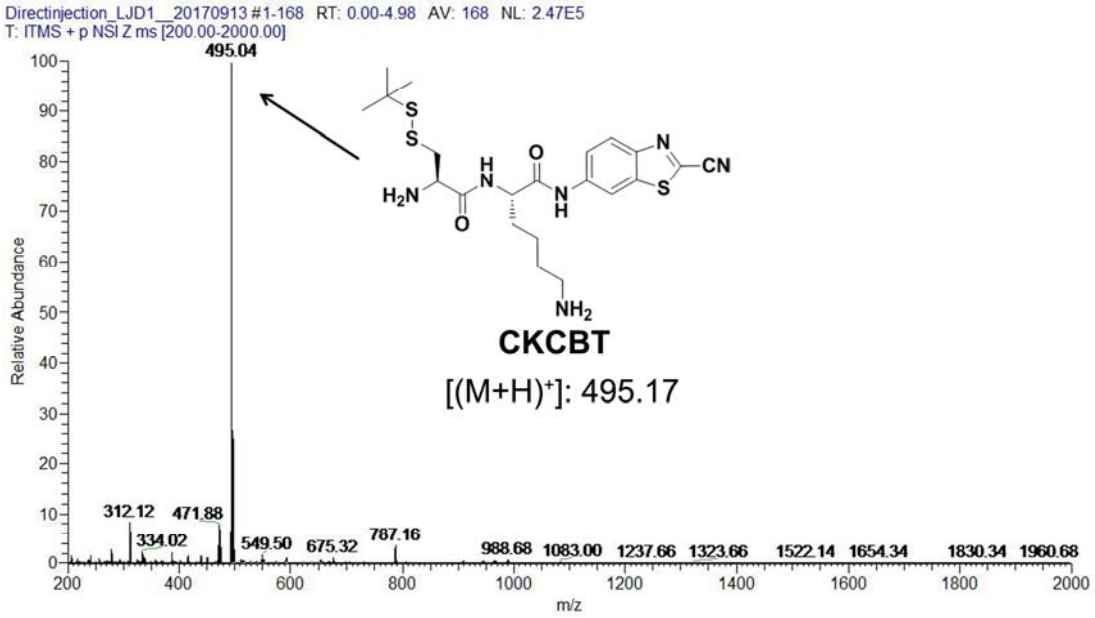


Figure S10. ESI-MS spectrum of **CKCBT**.

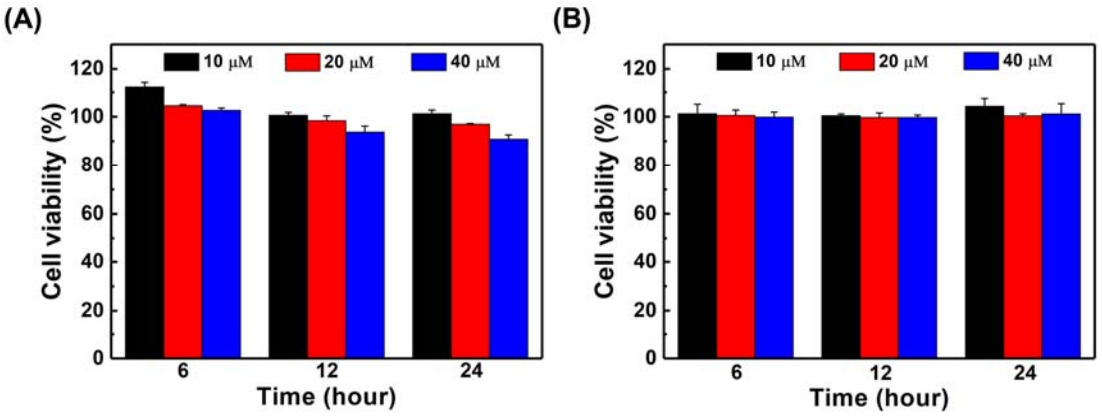


Figure S11. Cell viability of HepG2 cells incubated with **1** together with 50 μM **CKCBT** (A), or **1C** (B) at different concentrations for 6, 12, or 24 h. Each error bar represents the standard deviation of three independent experiments.

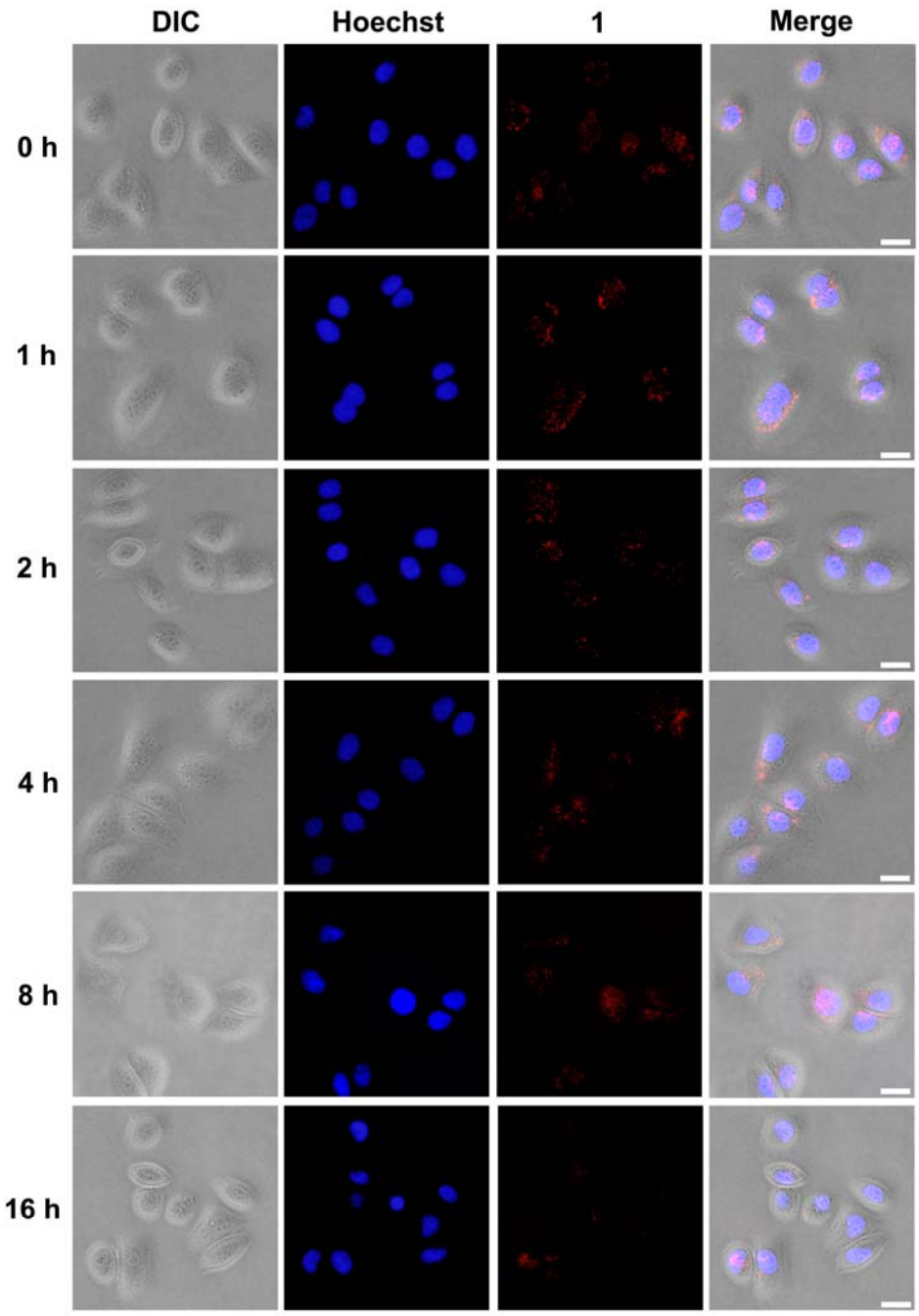


Figure S12. Time-course differential interference contrast (DIC), fluorescence, phosphorescence and overlay images of HepG2 cells incubated with 20 μM **1** together with 50 μM **CKCBT** in serum-free DMEM at 37 °C. Hoechst 33342 (blue fluorescence: excitation, 360 nm; emission, 420 nm) was used for nuclear counterstaining, and the red phosphorescence (excitation, 560 nm; emission, 610 nm) was from the chromophore $Ru(bpy)_3^{2+}$ in **1**. All images have the same scale bar: 20 μm.

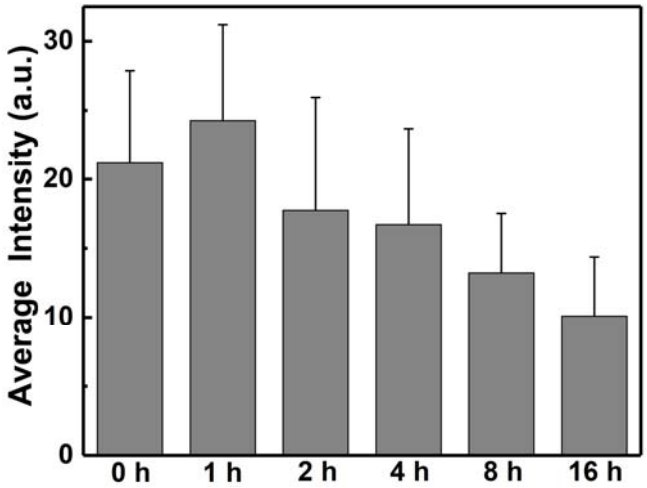


Figure S13. Average phosphorescence intensities of cell images from channel **1** in Figure S12.

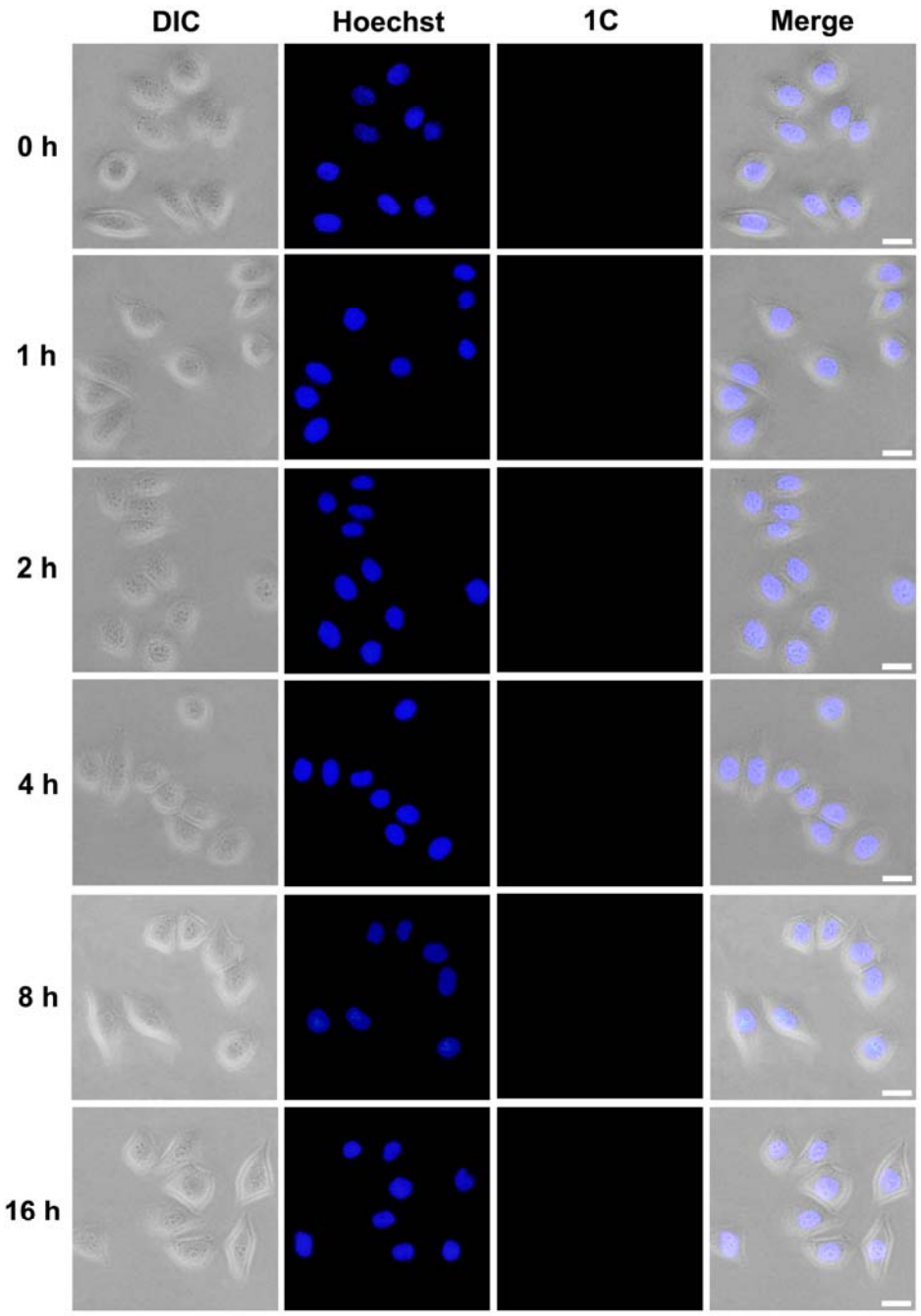


Figure S14. Time-course differential interference contrast (DIC), fluorescence, phosphorescence and overlay images of HepG2 cells incubated with 20 μM **1C** in serum-free DMEM at 37 °C. Hoechst 33342 (blue fluorescence: excitation, 360 nm; emission, 420 nm) was used for nuclear counterstaining, and the red phosphorescence (excitation, 560 nm; emission, 610 nm) was from the chromophore $Ru(bpy)_3^{2+}$ in **1C**. All images have the same scale bar: 20 μm.

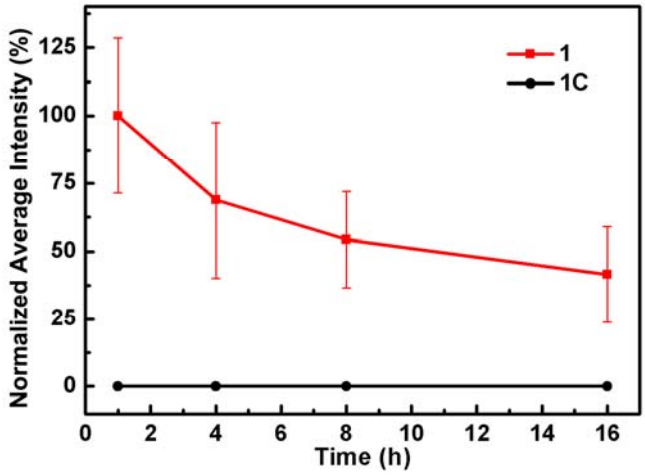


Figure S15. Normalized average phosphorescence intensities of cell images from channels **1** and **1C** in Figure 3.

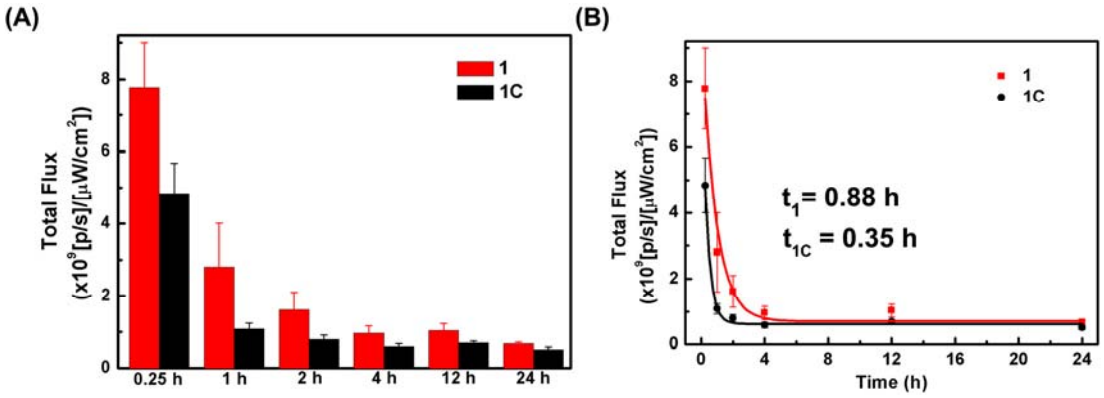


Figure S16. (A) Quantified total photon output from the tumor regions for the mouse images in Figure 4. (B) The exponential decay fitting plots of A.

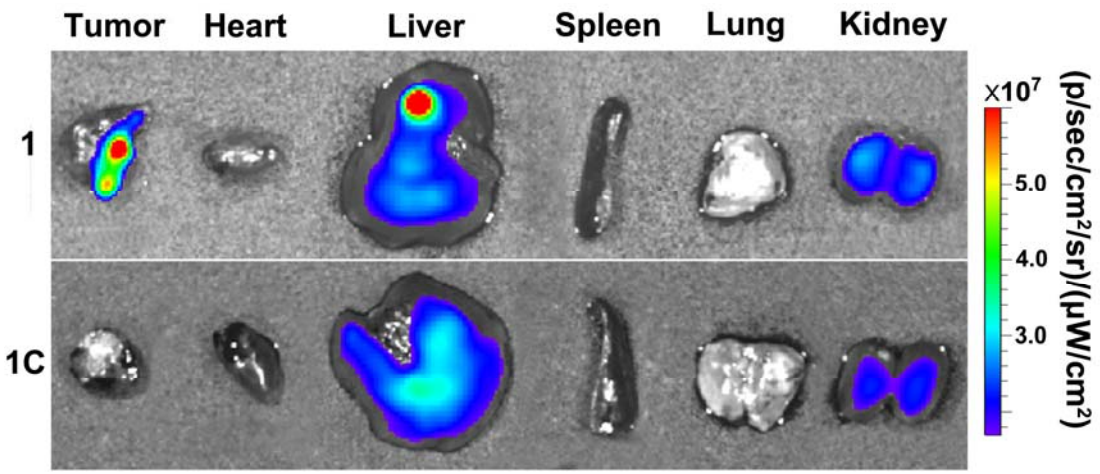


Figure S17. Ex vivo phosphorescence imaging of different organs from tumor-bearing nude mice after tumor-direct injection with 1.4 mg/kg **1** together with 1.5 mg/kg **CKCBT** (top row), or 0.8 mg/kg **1C** (bottom row) for 24 h in Figure 4. Excitation: 465 nm. Emission: 660 nm.

Table S1. HPLC condition for the HPLC traces in Figure 2D.

Time (min)	Flow (mL/min)	H_2O % (0.1 % TFA)	CH_3CN % (0.1 % TFA)
0	1.0	80	20
3	1.0	80	20
35	1.0	40	60
37	1.0	40	60
38	1.0	80	20
40	1.0	80	20

4. References

1. B. M. Peek, G. T. Ross, S. W. Edwards, G. J. Meyer, T. J. Meyer and B. W. Erickson, *Chem. Biol. Drug Des.*, 1991, **38**, 114-123.
2. D. M. Ryan, M. K. Coggins, J. J. Concepcion, D. L. Ashford, Z. Fang, L. Alibabaei, D. Ma, T. J. Meyer and M. L. Waters, *Inorg. Chem.*, 2014, **53**, 8120-8128.
3. D. G. Mccafferty, B. M. Bishop, C. G. Wall, S. G. Hughes, S. L. Mecklenberg, T. J. Meyer and B. W. Erickson, *Tetrahedron*, 1995, **51**, 1093-1106.