Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Rationally designed Ru(II) Metallacycles with tunable imidazole ligands for Synergistical Chemo-Phototherapy of Cancer

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1. Materials and Apparatus

Materials. All chemicals were purchased from commercial sources. Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Australia). Cisplatin, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Annexin V-FITC apoptosis detection kit, crystal violet, caspase 3 activity assay kit, 2',7'-dichlorofluorescein diacetate (H_2-DCFH), and deoxyribonucleic acid sodium salt from calf thymus (ctDNA) were purchased from Sigma-Aldrich (Poole, UK). RNaseA was purchased from Thermo Fisher Scientific (USA). Transwell inserts with 8.0 μm-pore size filters were purchased from Corning (USA).

Instruments. NMR spectra were obtained using a Varian Inova 400 MHz NMR spectrometer. Mass spectra were recorded on a Micromass Quattro II triple-quadrupole mass spectrometer or Synapt G2-Si mass spectrometer using electrospray ionization with a MassLynx operating system (Waters, USA). Absorption spectra were measured on a UV−vis-NIR spectrophotometer (Shimadzu UV-3600, Japan). The lasers of 450-nm and 690-nm wavelengths were purchased from Beijing Hi-Tech Optoelectronic (China). Photothermal temperatures were monitored by an infrared thermal imaging camera (Fluke, Ti400, USA). Flow cytometry was performed on Fortessa X20 (BD Biosciences, USA).

2. Experimental Detail

Assessment of ROS generation

H_2-DCFH (1.0 mM, 0.8 mL) in DMSO was mixed with NaOH (0.01 M, 2 mL) to deacetylate into DCFH. Added the prepared DCFH (20 μM) into **Ru-M1**, **Ru-M2** and **Ru-M3** solution (20 μM) and then irradiated with 450 nm laser or 690 nm laser for 0, 1, 2, 3, 4, and 5min. The fluorescent spectra of DCF (λex = 488 nm, λem = 525 nm) were recorded.

Photo and Photothermal Stability Tests *In Vitro*

Ru-M1, **Ru-M2,** and **Ru-M3** solution (20 μM) weresuccessivelyirradiated with 450 nm laser (18.0 mW cm^{-2}) or 690 nm laser (0.9 W cm^{-2}) for 5 min, and data were recorded by an infrared thermal imaging camera. To further test the photothermal stability of the **Ru-M1**, **Ru-M2,** and **Ru-M3**, the **Ru-M1**, **Ru-M2,** and **Ru-M3** solution was irradiated with a 690 nm laser (0.9 W cm^{-2}) for 5 min and then naturally cooled for 5 min. The temperatures of five heating-cooling cycles were recorded using an infrared thermal imaging camera.

Stability Tests

Ru-M1, **Ru-M2,** and **Ru-M3** (50 μM) were incubated in Tris-HCl buffer solutions of different pH (pH = 4.5, 5.7, 6.5, 7.0, 7.4, and 8.0) for 1 h in 25 °C and the UV-Vis absorption of **Ru-M1**, **Ru-M2,** and **Ru-M3** was measured.

Cytotoxicity Assay

The cells (A549, A549cisR, 16HBE cell lines) were seeded in 96-well plates (4×10^3 cells in each well)

and incubated in a 37 °C cell incubator for 24 h. Then, the DMEM medium was replaced with different concentrations of **Ru-M1**, **Ru-M2,** and **Ru-M3** solutions in the medium without FBS for 24h, and the cells in the plates were irradiated with or without 450nm laser (18.0 mW cm^{-2}) and 690 nm laser (0.9 W cm^{-2}) for five minutes each. After incubation for another 24h, added MTT solution (5 mg/mL, 10 μL) in each well, and the cells were incubated for another 4 h. The formed formazan crystals were dissolved by DMSO (100 μL per well) and the absorbance at 570 nm was measured using a microplate reader.

Cytotoxicity Assay under Hypoxia

The A549 cells were seeded in 96-well plates (4×10^3 cells in each well) and incubated in a 37 °C cell incubator for 24 h. Placed the 96-well plates to a closedanoxic containing sealing anoxic bags (O_2 content < 1%), kept the cells in the anoxic environment for over 6 hours, then discarded the original culture solution. Added **Ru-M1**, **Ru-M2,** and **Ru-M3** with different concentrations into plates and incubated in an anoxicbox. The remaining procedures are consistent with the cytotoxicity assay procedures described above.

Interaction with ctDNA

Deoxyribonucleic acid sodium salt 5 mg was dissolved in 5 mL high purity water. **Ru-M1**, **Ru-M2,** and **Ru-M3** dissolved in the 5 mM Tris-HCl buffer solution (pH = 7.2) with 1 % DMSO. Titration of ctDNA (0-0.1 mM) into **Ru-M1**, **Ru-M2,** and **Ru-M3** (10 μM) was monitored by UV-vis spectroscopy, respectively.

Activation of Caspase 3/7

A549 cells were treated with serum-free medium (negative control), **Ru-M2** (5 μM), or **Ru-M2** (10 μM) respectively. After cells were incubated for 24h, cells were irradiated with 690 nm laser (0.9 W·cm^{-2}, 5 min) and 450 nm laser (18.0 mW·cm^{-2}, 5 min). Then the cells were incubated for another 24 h. Then cells were treated with a caspase 3/7 activity kit according to the manufacturer'sprotocol.

Apoptosis and Cell Cycle Analyses

For apoptosis tests, A549 cells were incubated with **Ru-M2** (5 μM) or serum-free medium for 24 h and then treated with or without 690 nm laser (0.9 W·cm^{-2}, 5 min) and 450 nm laser (18.0 mW·cm^{-2}, 5 min). After incubation for another 24 h, cells were stained with Annexin V-FITC and PI for 15 min and analyzed with flow cytometry. For cell cycle analysis, A549 cells were treated as above and then lysed by RNaseA (100 μg/mL) for 20 min. After that, cells were stained with PI (0.1 mg·mL^{-1}) for 15 min and subsequently analyzed cell cycle distribution by flow cytometry.

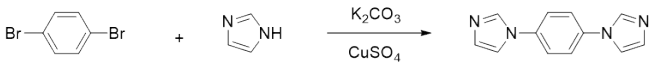
Migration and Invasion Inhibition

The anti-migration ability was performed by wound-healing assay. A549 cells were seeded into 6-well plates (1×10^6 cells/well). Cells were incubated with **Ru-M2** (10 μM), cisplatin (30 μM), or serum-free medium for 12 h and thentreated with or without laser illumination (450 nm, 18.0 mW·cm^{-2} and 690 nm, 0.9 W·cm^{-2}) for 5 min. Horizontal lines were drawn using micropipette tips in each well, and

subsequently, the wound was created. After further incubation for 24 and 48 h, cells were imaged by an invertfluorescence microscope. The wound area was measured by ImageJ, and the wound closure ratio was defined as [1-(wound area /original wound area)] × 100%. The anti-invasion capability was tested by Tanswell/Matrigel invasion assay. Transwell inserts were pretreated with Matrigel (200 μg·mL^{-1}, 100 μL/well). A549 cells were harvested and resuspended in **Ru-M2** (10 μM) or serum-free medium, and subsequently added to upper chambers. The upper chambers were placed into the receiver wells, which were supplemented with a complete medium. After further incubation for 48 h, the invaded cells were fixed with 4% paraformaldehyde, washed with PBS, and then stained with crystal violet. Transwell inserts visualization was performed on an invert fluorescence microscope. Cell invasion ratios were calculated according to OD_{590}.

3. Synthetic Procedures and Characterization Data

Synthesis of Compound **1**:



1

1,4-Dibromobenzene (2.0 g, 8.5 mmol), imidazole (2.4 g, 35.6 mmol), K_2CO_3 (3.75 g, 27.2 mmol) and $CuSO_4$ (0.027 g, 0.17 mmol) were heated and stirred at 180 °C for 12 h. After the reaction was completed, the mixture was cooled to room temperature, and water was added to wash the mixture. The crude product was dissolved in ethanol (3 × 30 mL). The organic layer was separated. The organic layer was evaporated to dryness to give a crude product. The residue was recrystallized from water and methanol to give a white solid (1.5 g, 86%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (s, 2H), 7.54 (s, 4H), 7.32 (s, 2H), 7.25 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 136.42, 135.53, 130.85, 118.19.

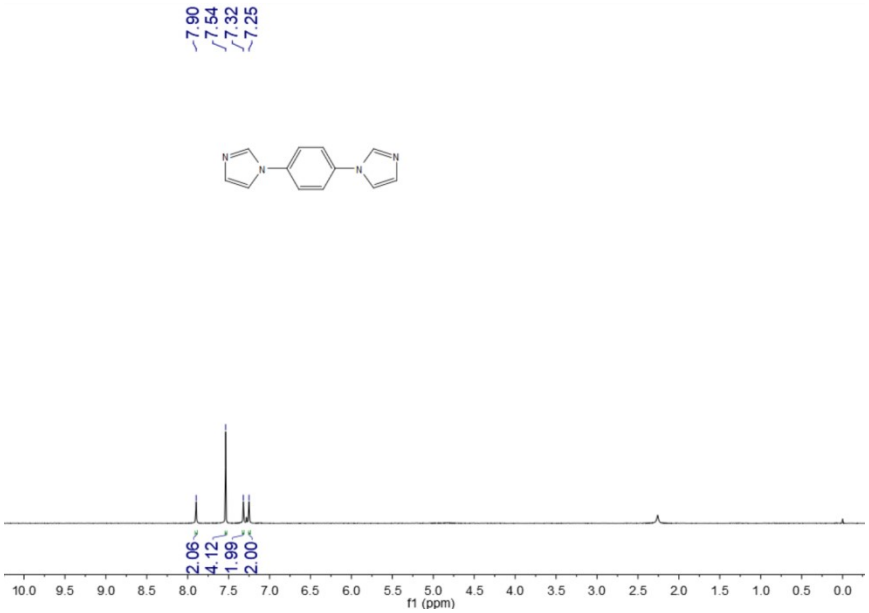


Figure S1. ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 298 K) of **1**.

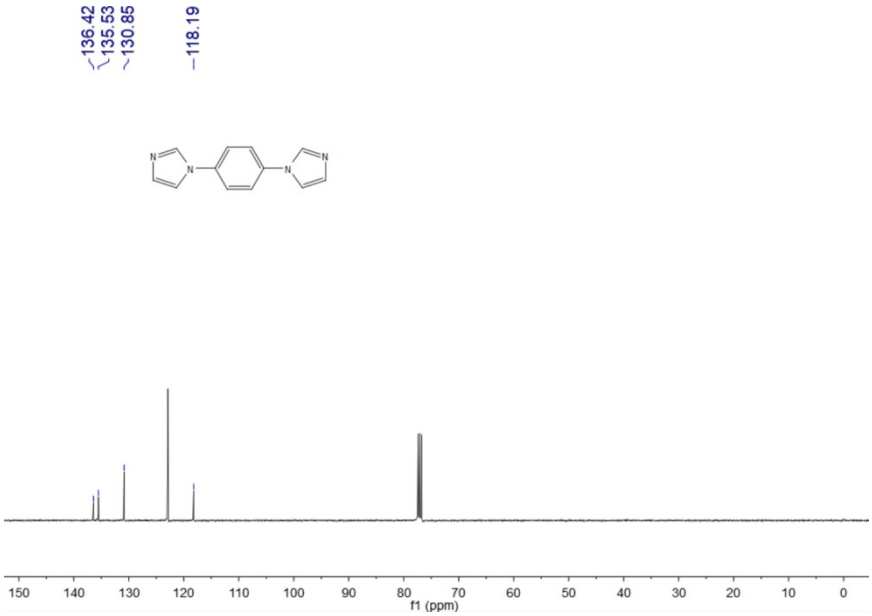
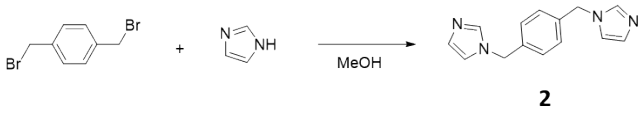


Figure S2. ^{13}C NMR spectrum (100 MHz, $CDCl_3$, 298 K) of **1**.

Synthesis of Compound **2**:



2

Imidazole (8.7 g, 127.5 mmol), 1,4-bis(bromomethyl)benzene (2 g, 8.4 mmol), and methanol (70 mL) were added to a three-necked flask. The mixed solution was stirred at 60°C for 18 h. Then, the mixture was cooled to room temperature and the ethanol was removed under vacuum. The resulting colorless solution was transferred to 2M K_2CO_3 solution to give a white needle-like solid (1.8 g, 92%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.55 (s, 2H), 7.15 (s, 4H), 7.09 (s, 2H), 6.90 (s, 2H), 5.13 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.37, 136.38, 129.83, 127.85, 119.26, 50.31.

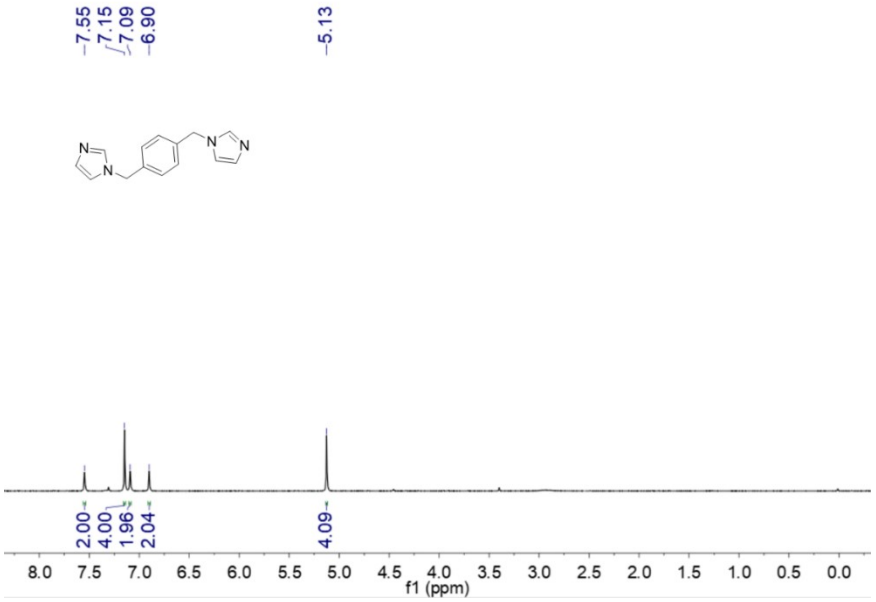


Figure S3. ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 298 K) of **2**.

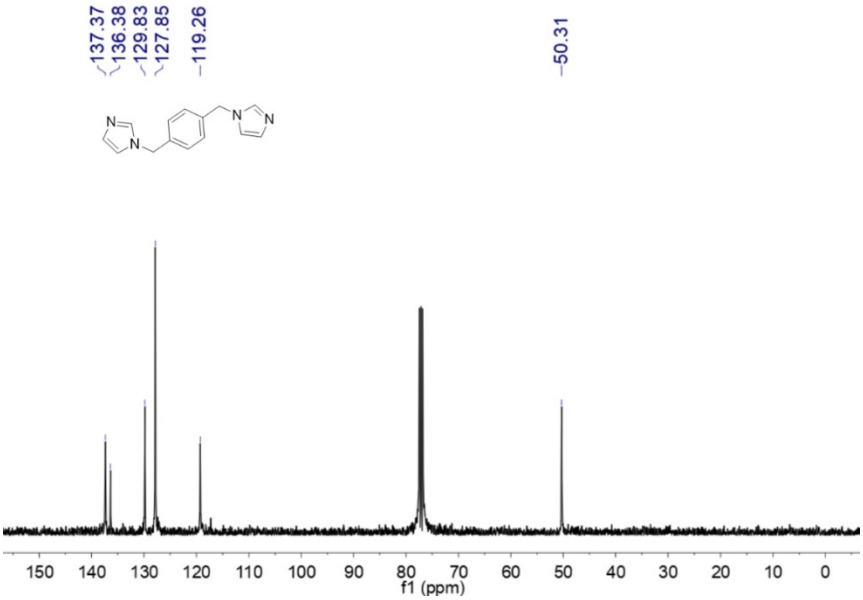
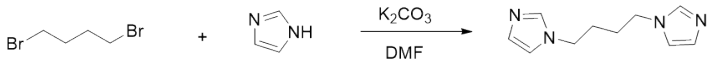


Figure S4. ^{13}C NMR spectrum (100 MHz, $CDCl_3$, 298 K) of **2**.

Synthesis of Compound **3**:



2

Dibromobutane (1.0 g, 4.6 mmol, 0.55 mL) was added to a mixture of imidazole (1.6 g, 23.2 mmol) and potassium carbonate (3.2 g, 23.2 mmol) in DMF (40 mL). The mixture was stirred at 80°C for 12 h. The suspension was cooled to room temperature. The reaction mixture was dissolved in CH_2Cl_2. The organic layer was washed three times with water and brine. The organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give the product a colorless oil (595 mg, 68%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 (s, 2H), 6.99 (s, 2H), 6.79 (s, 2H), 3.87 (s, 4H), 1.69 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.01, 129.81, 118.61, 46.37, 28.12.

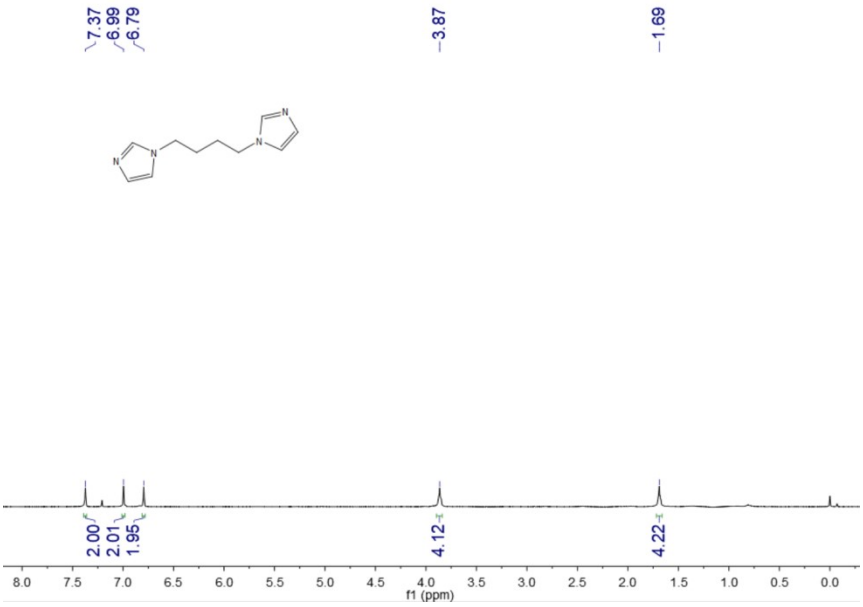


Figure S5. ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 298 K) of **3**.

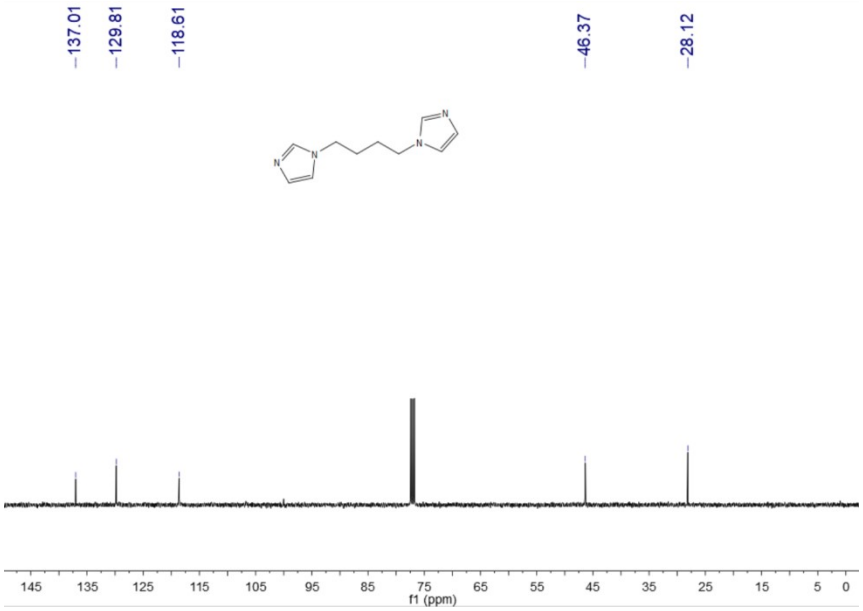
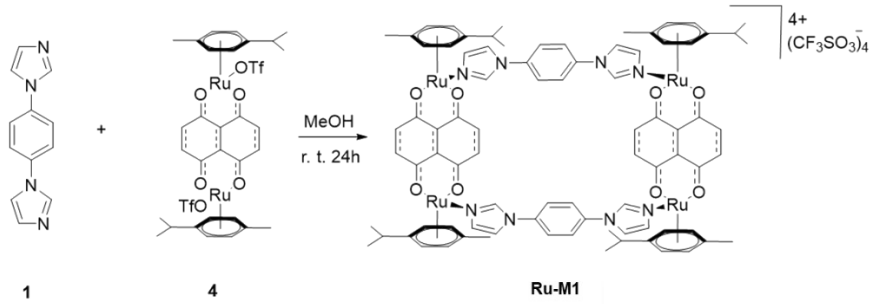


Figure S6. ^{13}C NMR spectrum (100 MHz, $CDCl_3$, 298 K) of **3**.

Synthesis of Compound **Ru-M1**:



In a 1:1 molar ratio, the ligand **1** (10 mg,0.048 mmol) and acceptor **4**[1] (47.52 mg, 0.048 mmol) were placed in a 5 mL of vial, followed by addition of CH_3OH (5 mL). After stirring at ambient temperature for 24 h, the solution was concentrated to 0.5 mL, self-assembly products were isolated via precipitation by addition of diethyl ether into concentrated solution, washed twice with diethyl ether and dried under

vacuum to obtain product **Ru-M1** (25 mg, 21.3%). ^{1}H NMR (600 MHz, CD_3OD) δ 8.29 (s, 4H), 7.49 (s, 8H), 7.47 (s, 4H), 7.09 (s, 8H), 6.91 (s, 4H), 5.75 (d, *J* = 5.9 Hz, 8H), 5.53 (d, *J* = 5.8 Hz, 8H), 2.75 – 2.71 (m, 4H), 2.04 (s, 12H), 1.23 (d, *J* = 6.8 Hz, 24H).

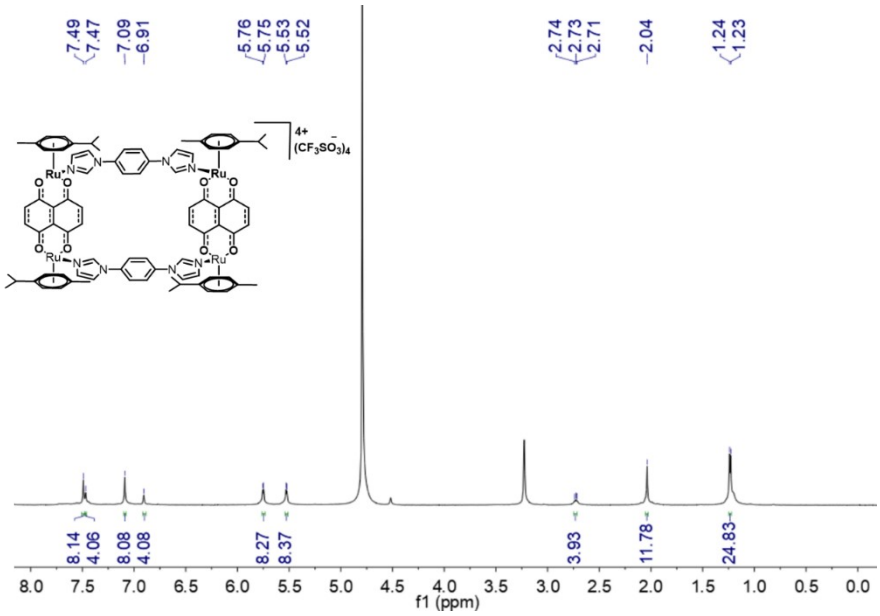
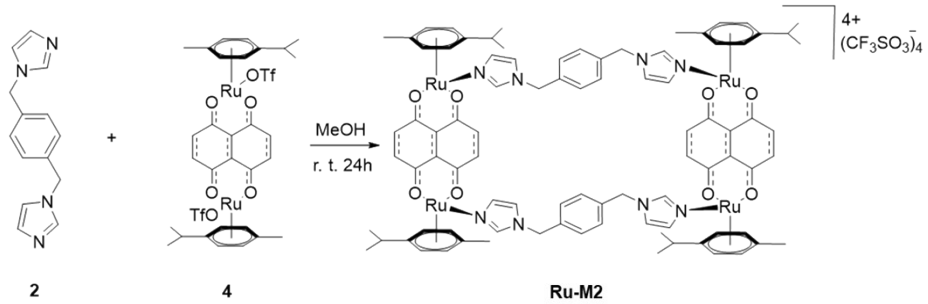


Figure S7. ^{1}H NMR spectrum (600 MHz, CD_3OD, 298 K) of **Ru-M1**.

Synthesis of Compound **Ru-M2**:



In a 1:1 molar ratio, the ligand **2** (10 mg, 0.042 mmol) and acceptor **4** (41.58 mg, 0.042 mmol) were placed in a 5 mL of vial, followed by addition of CH_3OH (1 mL) and $CHCl_3$ (1 mL). After stirring at ambient temperature for 24 h, the solution was concentrated to 0.5 mL, self-assembly products were isolated via precipitation by addition of diethyl ether into concentrated solution, washed twice with diethyl ether and dried under vacuum to obtain product **Ru-M2** (19 mg, 36.5%). ^{1}H NMR (600 MHz, CD_3OD) δ 7.83 (s, 4H), 7.11 (s, 8H), 7.08 (s, 4H), 6.94 (s, 4H), 6.76 (s, 8H), 5.78 (d, *J* = 6.0 Hz, 8H), 5.56 (d, *J* = 6.0 Hz, 8H), 5.11 (s, 8H), 2.82 – 2.79 (m, 4H), 2.13 (s, 12H), 1.32 (d, *J* = 6.9 Hz, 24H).

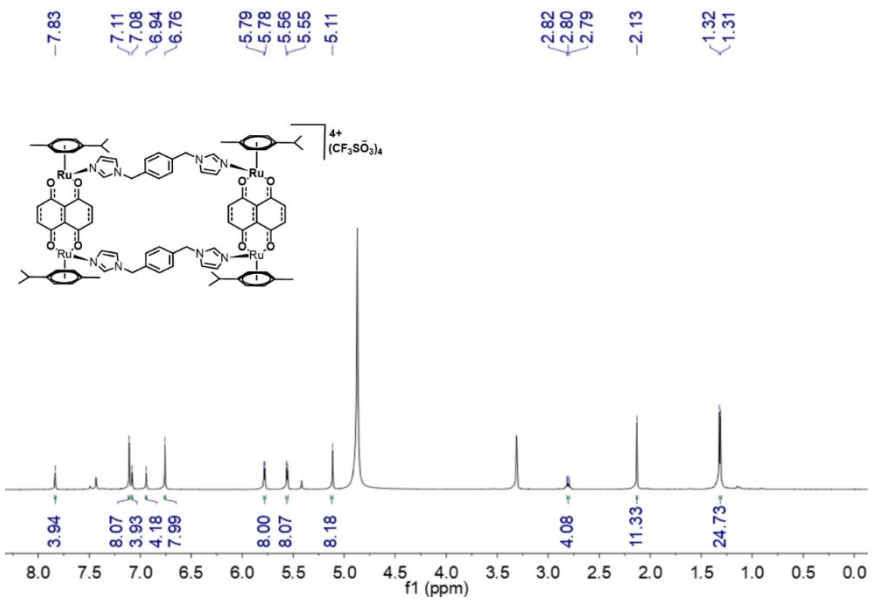
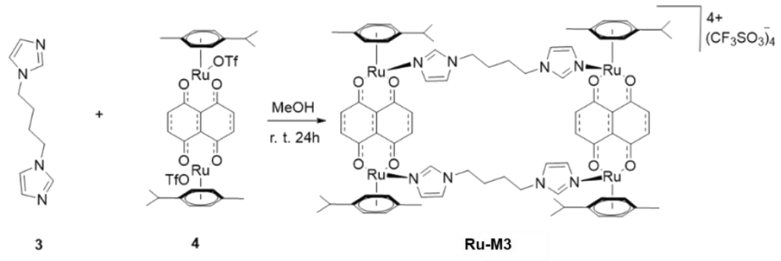


Figure S8. ^{1}H NMR spectrum (600 MHz, CD_3OD, 298 K) of **Ru-M2**.

Synthesis of Compound **Ru-M3**:



In a 1:1 molar ratio, the ligand **3** (10 mg, 0.052 mmol) and acceptor **4** (51.5 mg, 0.052 mmol) were placed in an 5 mL of vial, followed by addition of CH_3OH (1 mL). After stirring at ambient temperature for 24 h, the solution was concentrated to 0.5 mL, self-assembly products were isolated via precipitation by addition of diethyl ether into concentrated solution, washed twice with diethyl ether and dried under vacuum to obtain product **Ru-M3** (15 mg, 24.1%). ^{1}H NMR (600 MHz, CD_3OD) δ 7.83 (s, 4H), 7.11 (s, 8H), 7.08 (s, 4H), 6.94 (s, 4H), 6.76 (s, 8H), 5.78 (d, J = 6.0 Hz, 8H), 5.56 (d, J = 6.0 Hz, 8H), 5.11 (s, 8H), 2.82 – 2.79 (m, 4H), 2.13 (s, 12H), 1.32 (d, J = 6.9 Hz, 24H).

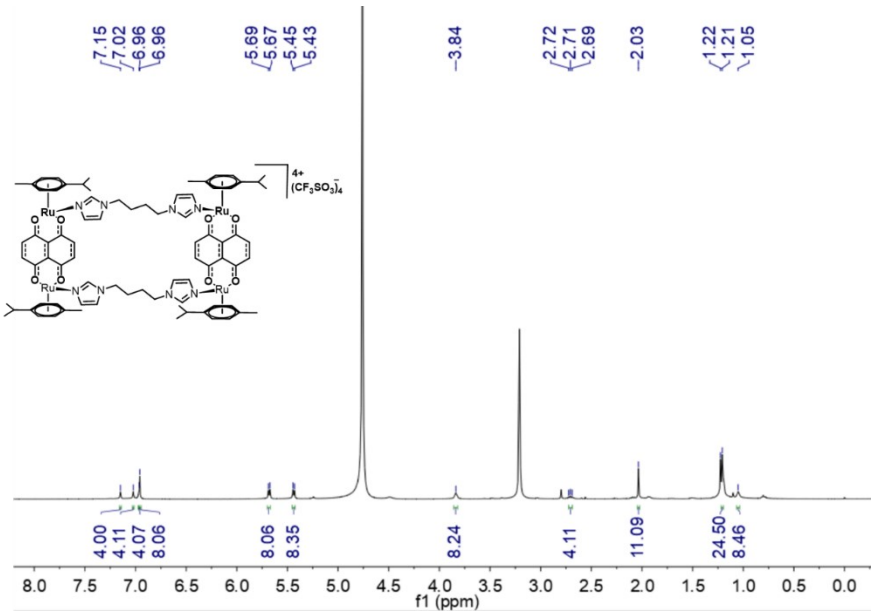
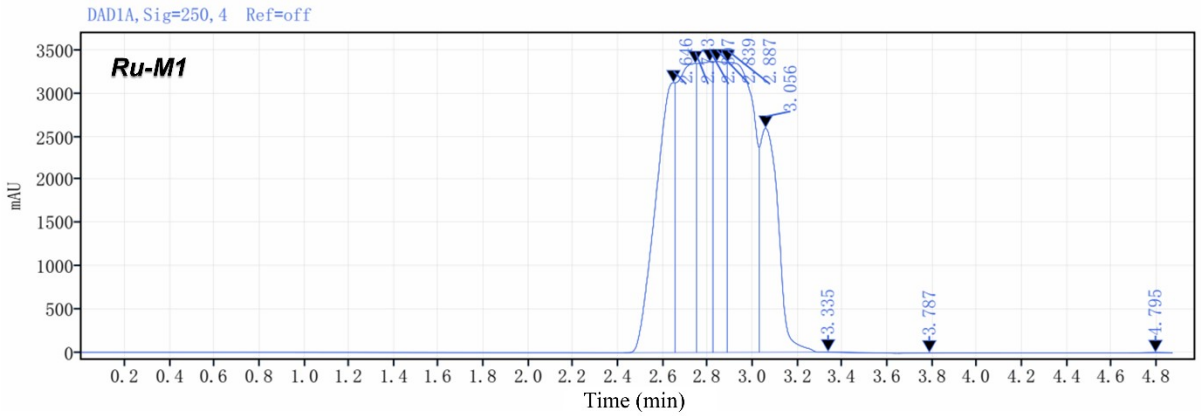


Figure S9. ^{1}H NMR spectrum (600 MHz, CD_3OD, 298 K) of **Ru-M3**.



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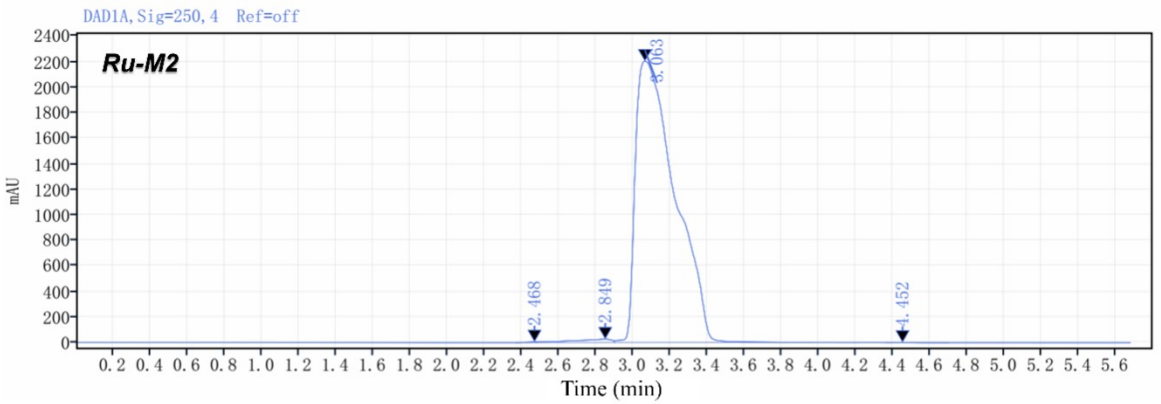
2.743	VV	0.10	18641.40	3348.80	17.62
2.807	VV	0.07	14719.24	3367.10	13.92
2.839	VV	0.06	12770.07	3370.40	12.07
2.887	VV	0.14	26714.68	3363.04	25.26
3.056	VB	0.26	14567.63	2598.51	13.77
3.335	BB	0.35	60.34	5.80	0.06
3.787	BV	0.46	67.47	3.88	0.06
4.795	BBA	0.19	22.40	3.75	0.02
		SUM	105770.78		



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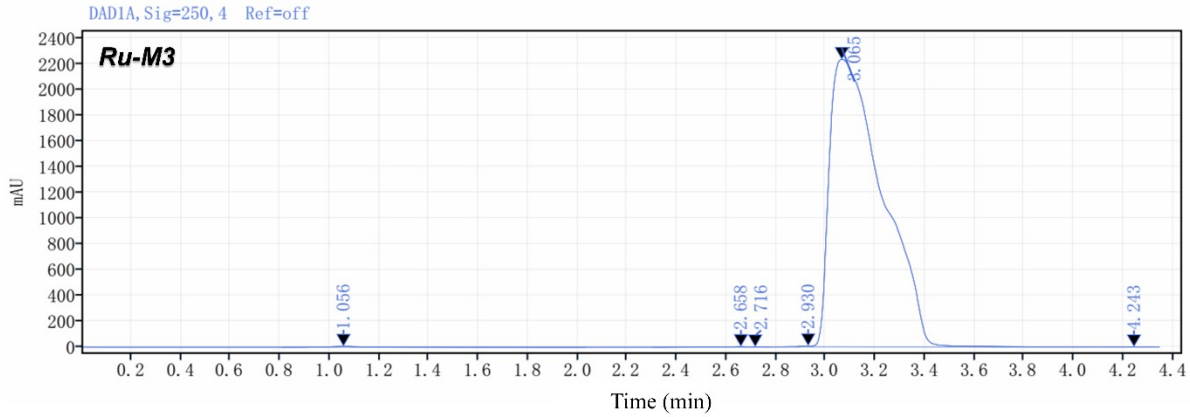
2.849	VV	0.42	415.21	27.74	1.25
3.063	VB	1.07	32818.42	2207.28	98.64
4.452	VB	0.34	12.35	2.12	0.04
		SUM	**33269.60**		



retention time [min] | type | peak width [min] | peak area | peak height | peak area%

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2.716	VB	0.07	5.05	2.31	0.02
2.930	BV	0.06	15.49	6.46	0.05
3.065	VB	1.16	33211.35	2235.75	99.56
4.243	BB	0.21	10.76	1.77	0.03
		SUM	**33356.52**		

Figure S10. HPLC analyses of **Ru-M1**, **Ru-M2** and **Ru-M3**.

4. Supplementary Tables

Compound	Dark IC_{50} (μM)	Light IC_{50}[a] (μM)	PI[b]
Ru-M1	2.95 ± 0.75	1.70 ± 0.86	1.74
Ru-M2	13.14 ± 1.87	2.93 ± 0.62	4.48
Ru-M3	3.25 ± 0.84	1.94 ± 0.81	1.68
Cisplatin	34.92 ± 1.24	-	-
1	> 50	> 50	-
2	> 50	> 50	-
3	> 50	> 50	-
4	7.27 ±0.89	-	-

[a]Light source: 450nm LED and 690 nm laser.

[b]PI (phototoxic index) = $IC_{50,\ dark}$ treated with compound/ $IC_{50,\ light}$ treated with compound.

Table S1. IC_{50} value of **Ru-M1**, **Ru-M2**, **Ru-M3**, Cisplatin, **1**, **2**, **3,** and **4** against A549 cell line by MTT assay.

5. Supplementary Figures

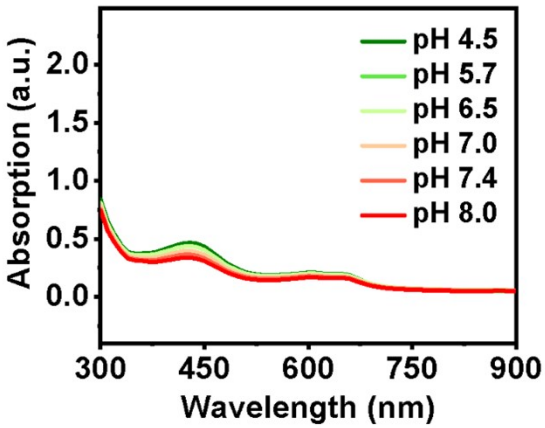

Figure S11. The stability tests of **Ru-M1** incubated at different pH values (pH = 4.5, 5.7, 6.5, 7.0, 7.4, and 8.0) for 1h.

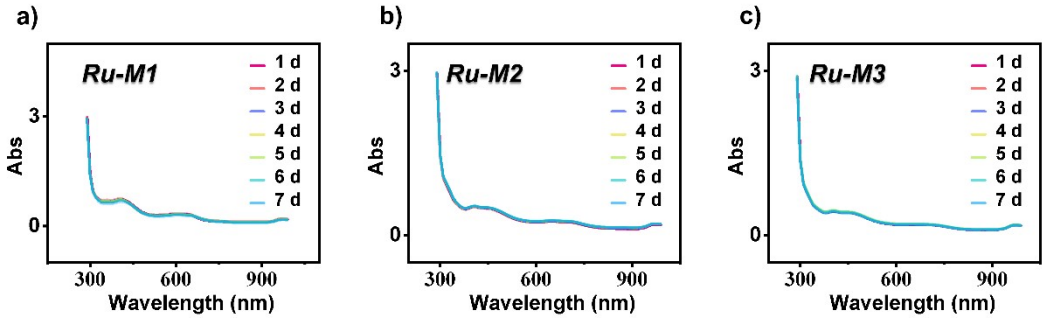

Figure S12. The stability tests of **Ru-M1**, **Ru-M2** and **Ru-M3** in 10% FBS for 7 days.

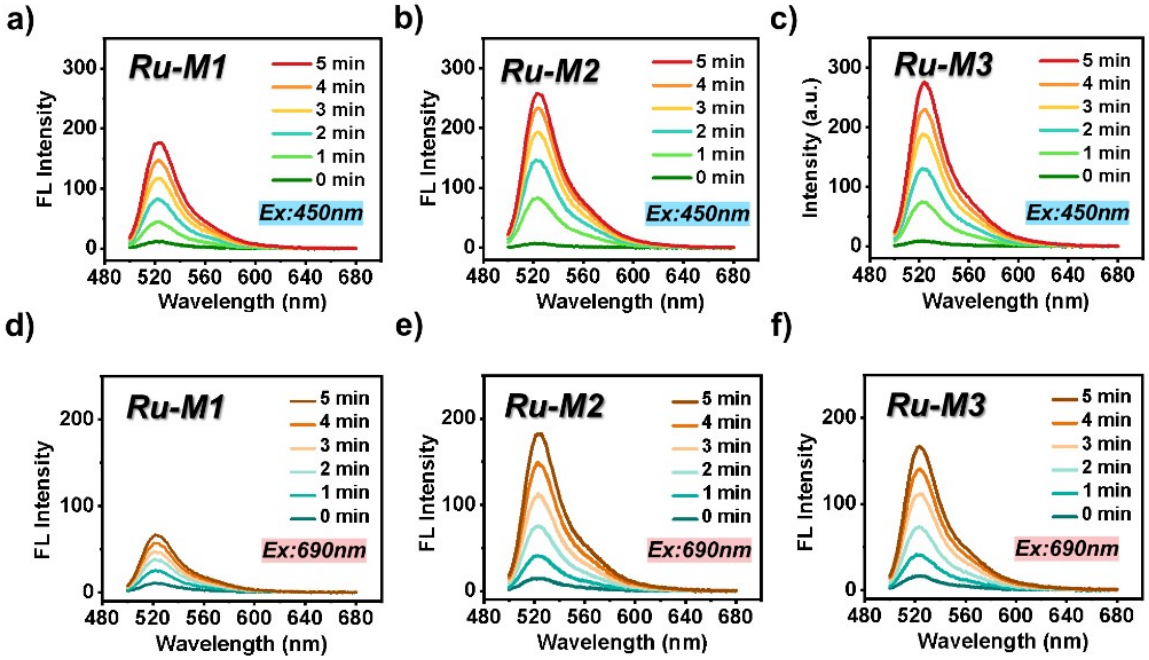

Figure S 13. ROS generation of a) **Ru-M1**; b) **Ru-M2**; c) **Ru-M3** illuminated by 450 nm laser (18.0 mW·cm^{-2}) and d) **Ru-M1**; e) **Ru-M2**; f) **Ru-M3** illuminated by 690 nm laser (0.9 W·cm^{-2}) using H_2-DCFH as an indicator.

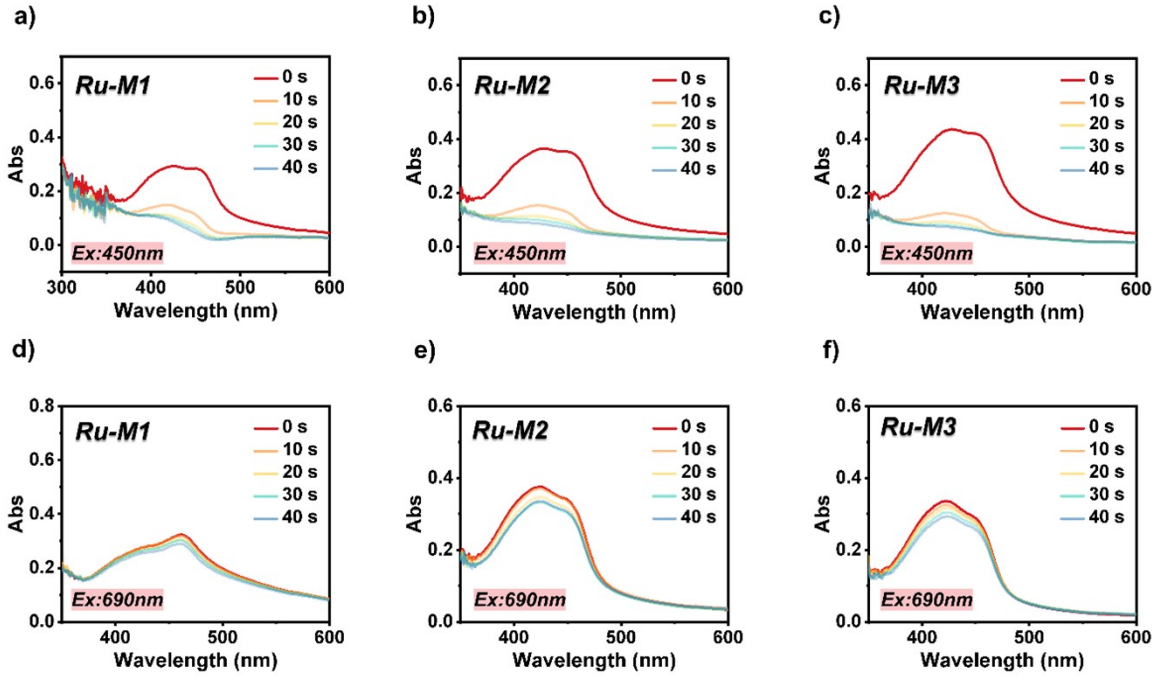

Figure S14. $^1O_2$ generation of a) **Ru-M1**; b) **Ru-M2**; c) **Ru-M3** illuminated by 450 nm laser (18.0 mW·cm^{-2}) and d) **Ru-M1**; e) **Ru-M2**; f) **Ru-M3** illuminated by 690 nm laser (0.9 W·cm^{-2}) using 1,3-diphenyliso-benzofuran (DPBF) as an indicator.

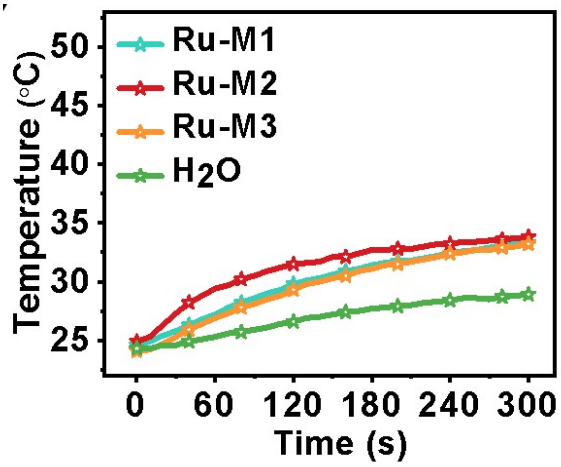

Figure S15. Photothermal curves of **Ru-M1**, **Ru-M2,** and **Ru-M3** with 450 nm laser illumination (18.0 mW·cm^{-2}) for 5 min.

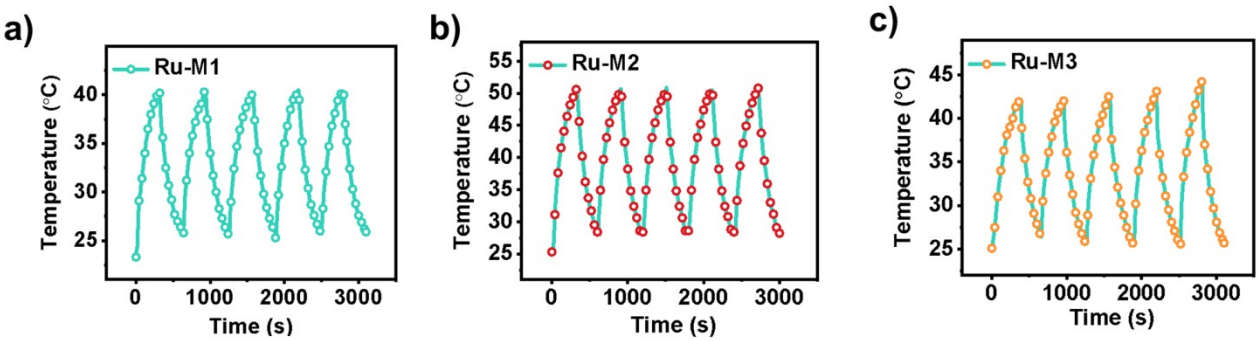

Figure S16. a) Photothermal heating/nature cooling cycles of **Ru-M1** (20 μM) under 690 nm laser irradiation (0.9 W·cm^{-2}); b) Photothermal heating/nature cooling cycles of **Ru-M2** (20 μM) under 690 nm laser irradiation (0.9 W·cm^{-2}); c) Photothermal heating/nature cooling cycles of **Ru-M3** (20 μM) under 690 nm laser irradiation (0.9 W·cm^{-2}).

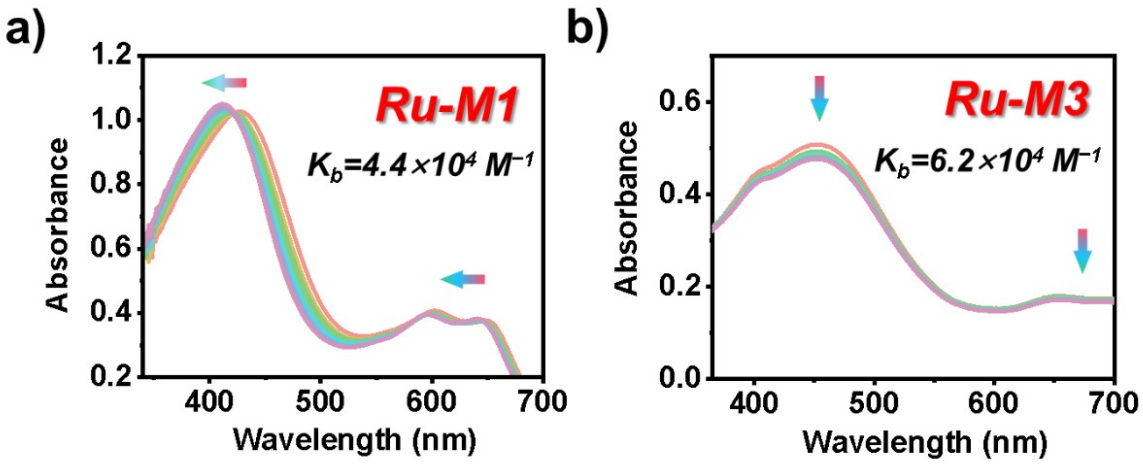

Figure S17. UV-vis spectra of **Ru-M1** a) and **Ru-M3** b) upon addition of ctDNA (0–0.1 mM) in 5 mM Tris·HCl buffer solution (pH = 7.2). The arrows show the change of absorbance upon the addition of ctDNA.

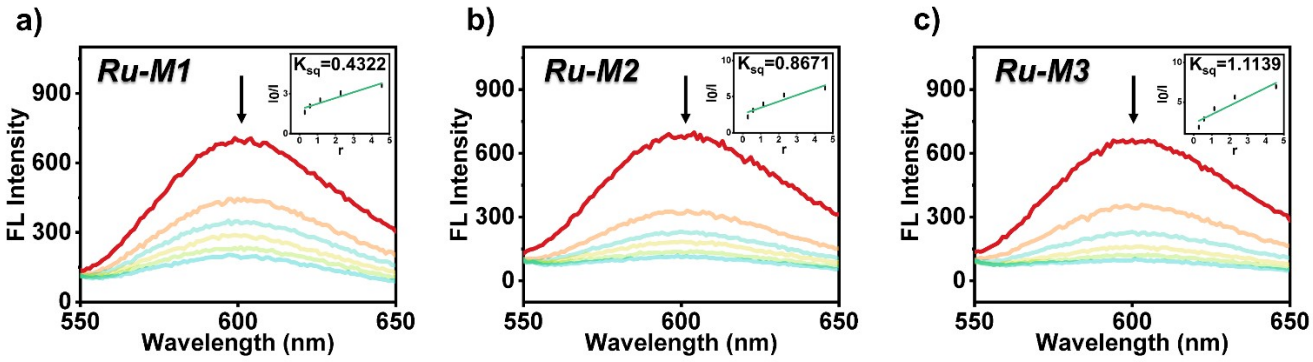

Figure S18. Fluorescence spectra resulted from the interaction of **Ru-M1** a), **Ru-M2** b) and **Ru-M3** c) with DNA (0–0.014 mM). The arrow direction represents the change of emission spectrum of EB-DNA with the increase of complex concentration. Insert means I_0/I plotted on $r(I_0/I = 1 + K_{sq}r)$.

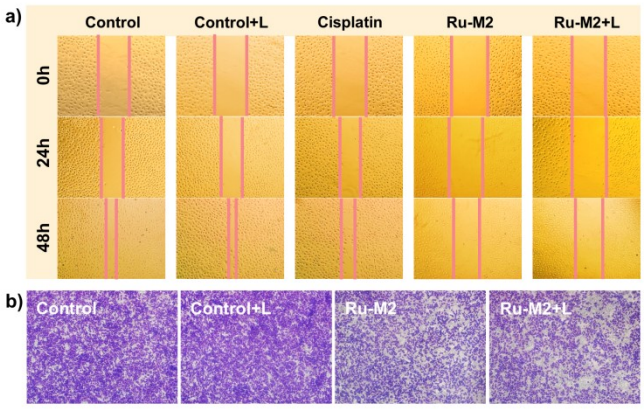

Figure S19. a) Anti-migration capability of **Ru-M2** by wound healing assay; b) Anti-invasion capability of **Ru-M2** by transwell invasion assay.

6. Supplementary References

1. Y. Zhao, L. Zhang, X. Li, Y. Shi, R. Ding, M. Teng, P. Zhang, C. Cao and P. J. Stang, *Proc. Natl. Acad. Sci. USA.*, 2019, 116, 4090.