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Photo-induced Synergistic Cytotoxicity towards Cancer Cells via Ru(II) Complexes

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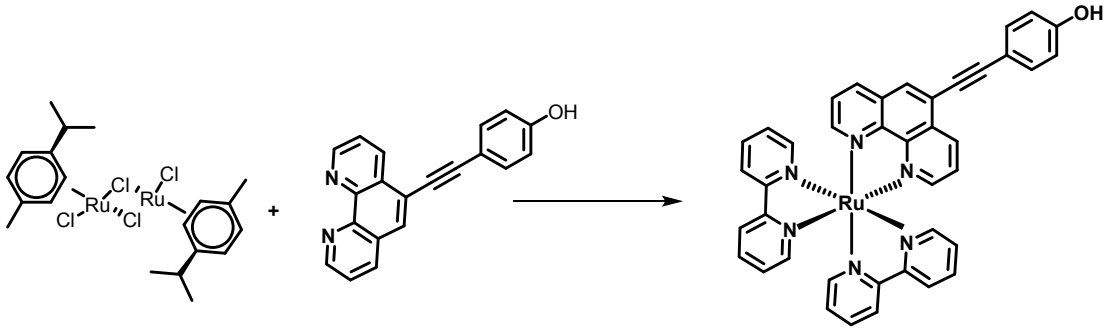
Materials and general methods

Materials: All reagents were purchased from commercial sources and used without further purification. All other chemical reagents of analytical grade were used directly without further purification. Deionized water was used to prepare all aqueous solutions. Cell culture reagents and fetal bovine serum (FBS) were purchased from Gibco. Control complexes **Ru(phen)** and **Ru(phen-NO_2)** were synthesized according to the reported literature.[1]

The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DRX-300 at 298 K using deuterated solvents. Chemical shifts are given in ppm, and are referenced against internal TMS. High resolution mass spectrometric data were determined using an Agilent LTQ Orbitrap XL mass spectrometer. Fluorescence spectra were determined using a FluoroMax-4 spectro-fluorometer with a 5 nm slit for both excitation and emission. Absorption spectra were recorded using a Shimadzu UV-3100 spectrophotometer.

Synthesis and characterization of Ru-1:



L1 **Ru-1**

Synthesis and characterization of Ru-1: Compound 1 was prepared according to the reported procedure.[2] $RuCl_2(cymene)_2$ (74 mg, 0.12 mmol) and **L1** (71 mg, 0.24 mmol) were suspended in ethanol. The mixture was stirred at room temperature under nitrogen atmosphere for ca. 2h until the solution become clear. Then a solution of 2,2'-bipyridine (75 mg, 0.48 mmol) in water was added and the mixture was refluxed for 22h. After cooling, the solution was treated with a saturated aqueous solution of KNO_3 and the solvent was evaporated under reduced pressure. The crude product was then subjected to column chromatography to get the product as a red solid (28 mg, 30%). ^{1}H NMR (300 MHz, CD_3CN): δ 9.01 (dd, 1H, J = 8.4, 1.1 Hz), 8.82 (s, 1H), 8.60–8.50 (m, 5H), 8.42 (s, 1H), 8.17–8.06 (m, 4H), 8.05–7.96 (m, 2H), 7.89–7.79 (m, 3H), 7.73 (dd, 1H, *J* = 8.3, 5.3 Hz), 7.65–7.53 (m, 4H), 7.50–7.42 (m, 2H), 7.27-7.21 (m, 2H), 7.01–6.93 (m, 2H). 13C NMR (75 MHz, CD_3CN) δ 159.56, 158.15, 157.87, 153.85, 153.59, 152.91, 148.59, 148.03, 138.78, 138.66, 137.24, 136.36, 134.72, 131.64, 131.53, 128.43, 128.31, 127.33, 125.19, 125.11, 123.10, 116.83, 113.49, 99.18, 83.53. HRMS (positive mode, *m/z*): Calcd. 355.0684, found 355.0686 for $[(M\text{-}NO_3^-)/2]^+$

Crystal structure analysis

Crystal structure was measured at 173 K on a Bruker CCD-APEX II diffractometer [λ(Mo-Kα) = 0.71073 Å], graphite monochromator. An absorption correction was applied with the SADABS program. The structures were solved by direct methods and all non-hydrogen atoms were subjected to anisotropic refinement by full-matrix least-squares on F^2 using the SHELXTL program. CCDC 1502447 contain the supplementary crystallographic data for this paper; these data can be obtained at http://www.ccdc.cam.ac.uk/conts/retrieving.html.

Singlet oxygen generation

Indirect method (singlet oxygen sensor): Singlet oxygen quantum yields (Φ_Δ) were detected through monitoring the oxidation of 1,3-diphenylisobenzofuran (DPBF). Briefly the oxygen-saturated acetonitrile solution of photosensitizer (10 μM) containing 10 μM DPBF was prepared in the dark and irradiated with 450 nm laser beam in an interval of 10 s. DPBF oxidation was monitored by UV-Vis spectrophotometer. Then equation (1) was used to calculate the singlet oxygen quantum yield of the sensitizer with respect to the reference.

$$\phi_\Delta(^1O_2)^s = \phi_\Delta(^1O_2)^r \frac{S^s F^r}{S^r F^s} \quad (1)$$

where $\phi_\Delta(^1O_2)$ is the quantum yield of singlet oxygen, superscripts " s " and " r " represent **Ru-1** and tris(2,2'-bipyridine)ruthenium dichloride ($^1O_2$ quantum yield of 0.56 in acetonitrile), respectively. "S" is the slope of a plot of difference in change in absorbance of DPBF (at 411 nm) with the irradiation time, and "F" is the absorption correction factor, which is given by $F = 1\text{-}10^{-OD}$ (OD at the irradiation wavelength).

Direct method (near-infrared luminescence): The Ru(II) complex whose singlet oxygen generation needed to be measured was diluted in D_2O to reach approximately 0.2 absorbance at the irradiation wavelength. This solution was then irradiated in fluorescence quartz cuvettes using combined fluorescence lifetime and steady state spectrometer (Edinburgh Instruments Ltd) with a 450 nm Xenon lamp. Singlet oxygen near-IR luminescence at 1270 nm was measured by recording spectra from 1050 to 1500 nm.

Cell lines and culture conditions

MCF-7 cells were cultured in RPMI (Roswell Park Memorial Institute)-1640 medium supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 ℃..

B16F10 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 ℃.

Intracellular Ru concent determination in MCF-7 cells

MCF-7 cells were seed in 35 mm dish for 24 h and then incubated in dark, respectively, with **Ru-1** (10 μM), **Ru(bpy)$_3$** (10 μM), **Ru(phen)** (10 μM) and **Ru(Phen-NO$_2$)** (10 μM) for 24 h. A short time photo-irradiation (5 min, 450 nm, 30 J·cm^{-2}) was given at the fourth hour of dark incubation, and the incubation without irradiation was also performed as the control. After the removal of the culture media and rinse with 1 mL of PBS buffer (1X), the cells were treated with 500 μL of 0.25% trypsin and centrifuged at 1000 rpm. The supernatant was then removed, and the cells were rinsed with PBS twice. After being counted by a cell counter, the cells were treated with concentrated nitric acid (65%, 50 μL) at 95 °C for 2 h, hydrogen peroxide (30%, 20 μL) at 95 °C for 1.5 h, and concentrated hydrochloric acid (37%, 20 μL) at 37 °C for 0.5 h in sequence. The resulting solution was diluted to 1 mL with water from Milli-Q system (>18 MΩ), and Ru content was determined directly by the inductively coupled plasma mass spectrometer (ICP-MS; VG Elemental). The experiment was performed in triplicate, and the average of the data was obtained. The Ru content in MCF-7 cells induced by **Ru-1** incubation was also determined with adding the ROS scavenger ascorbic acid (2 mM) 10 min before the short time photo-irradiation.

Confocal phosphorescence imaging of MCF-7 Cells

MCF-7 cells were planted on confocal petri dish and allowed to adhere for 24 h and then the cells were incubated with RPMI-1640 medium containing **Ru-1** (10 μM) for 4 h. For the experimental group, photo-irradiation (5 min, 450 nm, 30 J·cm^{-2}) was given at the fourth hour of dark incubation and confocal imaging was conducted at different time points (0 h, 2 h and 4 h after irradiation). For the control group, confocal imaging was conducted at 4 h, 6 h and 8 h of incubation. The band path is 550-650 nm upon excitation at 488 nm.

Antitumor cytotoxicity determination

The *in vitro* dark cytotoxicity of the four Ru complexes towards MCF-7 and B16F10 cell lines was determined by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Cells were seeded into a 96-well cell culture plate at 1×10^4 per well, under 100% humidity, and were cultured at 37 ℃ with 5% CO_2 for 24h. Ru complexes in different concentrations were added into the wells. The cells were subsequently incubated for 24 h at 37 ℃ under 5% CO_2 and photo-irradiation (450 nm, 5 min, 30 J/cm^2) was given at the 4th hour of incubation for the experimental group. After that, MTT (50 μL/well, 5 mg/mL) was added to each well and the plate was incubated for an additional 4 h at 37 °C under 5% CO_2. The medium was then replaced with 200 μL DMSO per well, and OD570 was monitored by an enzyme-linked immunosorbent assay reader (Thermo Scientific, Varioskan Flash).

Antitumor action mode study

MCF-7 and B16F10 cells were seeded into a 96-well cell culture plate at 1×10^4 per well, under 100% humidity, and were cultured at 37 ℃ with 5% CO_2 for 24h. Ru complexes and MB in different concentrations were added into the wells. Different actions were conducted for the cells.

1) MCF-7 or B16F10 cells were incubated with **Ru-1** or MB for 24 h in dark;

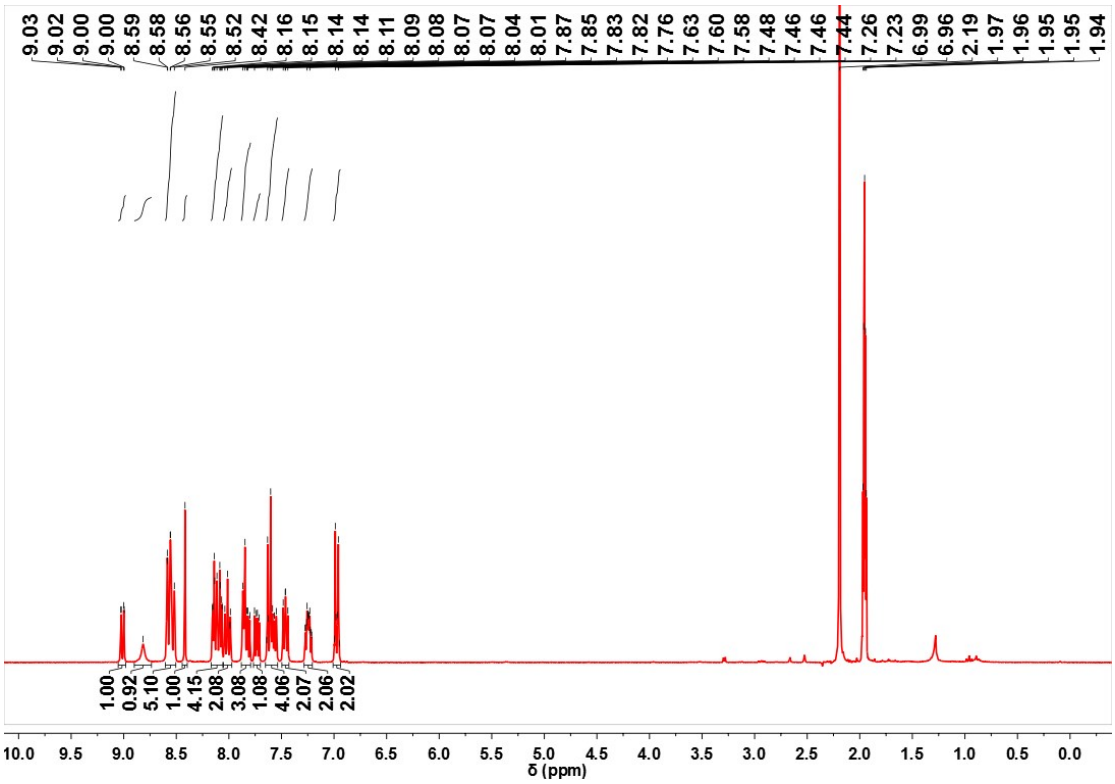
2) light irradiation (450 nm for **Ru-1** and 635 nm for MB, 30 J/cm^2) was employed at the fourth hour of incubation of the cells with **Ru-1** or MB and the cells were further incubated for 20 h;

3) light irradiation (450 nm for **Ru-1** and 635 nm for MB, 5 min, 30 J/cm^2) was employed at the fourth hour of incubation with **Ru-1** or MB. After that, the cells were washed three times with PBS and then the culture medium was replaced with new medium without **Ru-1** or MB for further incubation of 20 h;

4) the cells were washed three times with PBS and the culture medium was replaced with new medium without **Ru-1** or MB at the fourth hour. After that, light irradiation (450 nm for **Ru-1** and 635 nm for MB, 5 min, 30 J/cm^2) was employed and the cells were further incubated for 20 h;

5) MCF-7 cells were incubated with cisplatin for 24 h in dark;

6) light irradiation (450 nm for **Ru-1** and 635 nm for MB) was employed at the fourth hour of incubation of cells with cisplatin and the cells were further incubated for 20 h.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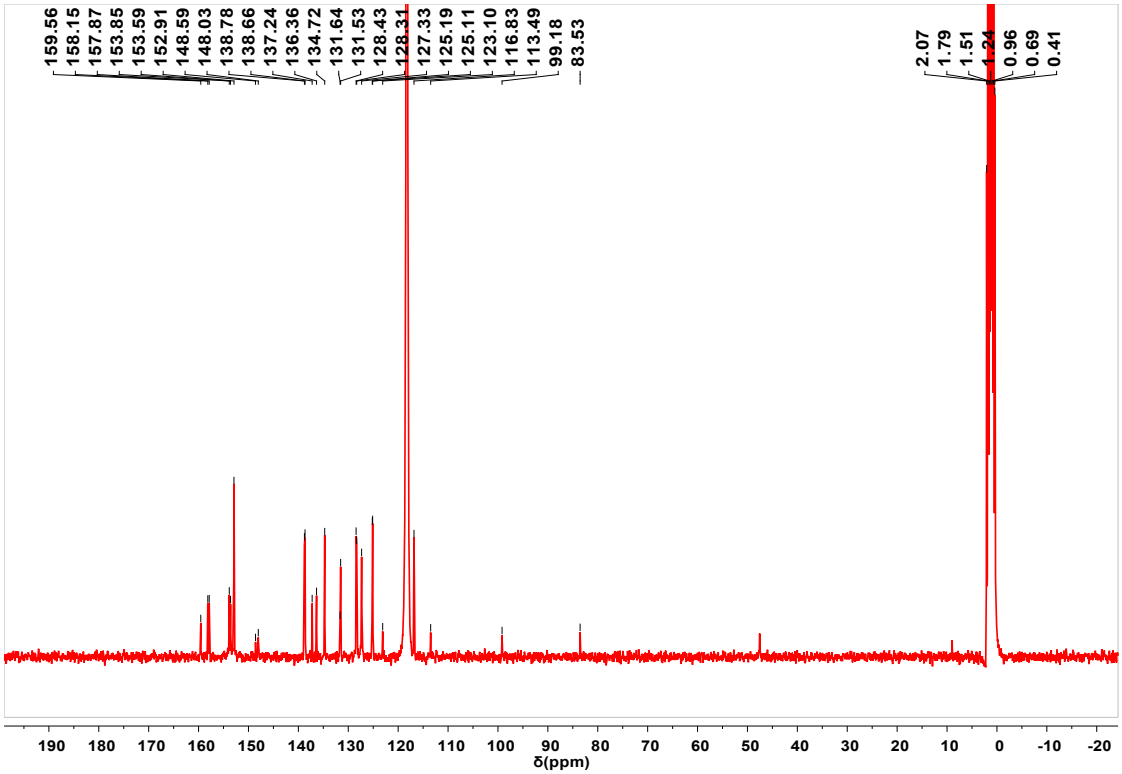
1.00 0.92 5.10 1.00 4.15 2.08 3.08 1.08 4.06 2.07 2.06 2.02
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
δ (ppm)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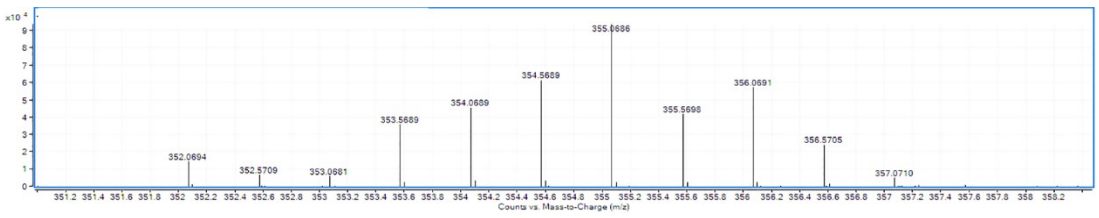
2.07 1.79 1.51 1.24 0.96 0.69 0.41
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20
δ(ppm)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354.5689
354.0689
356.0691
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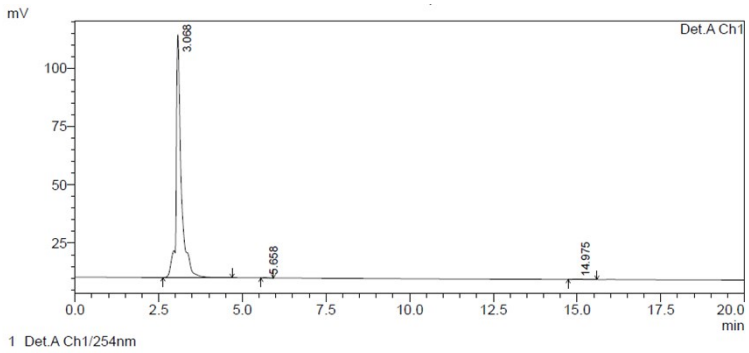
357.0710
Counts vs. Mass-to-Charge (m/z)



PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.068	1114583	104232	99.649	99.764
2	5.658	2182	179	0.195	0.171
3	14.975	1746	68	0.156	0.065
Total		1118511	104479	100.000	100.000

Figure S1. ^{1}H, ^{13}C NMR in CD_3CN-d_3, HR-MS and HPLC spectra of **Ru-1**.

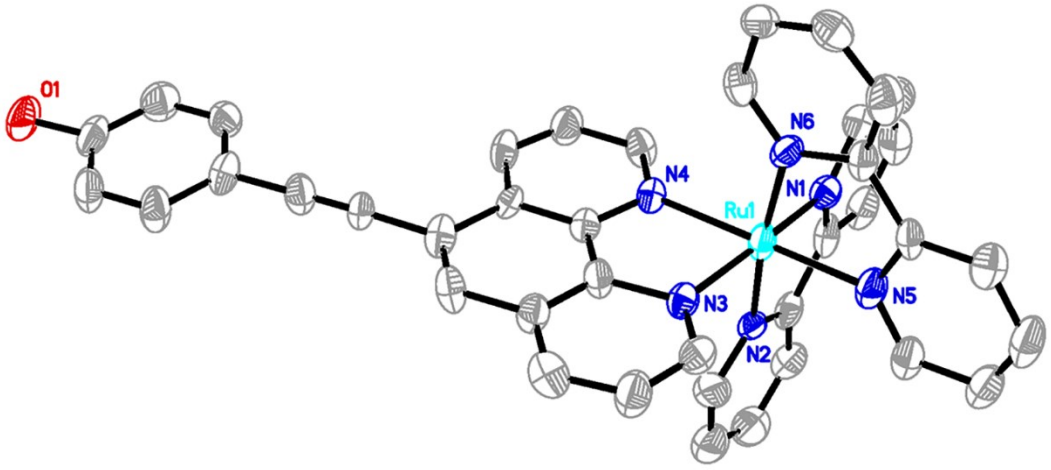


Figure S2. Molecular structure of **Ru-1**. All of the hydrogen atoms are omitted for clarity.

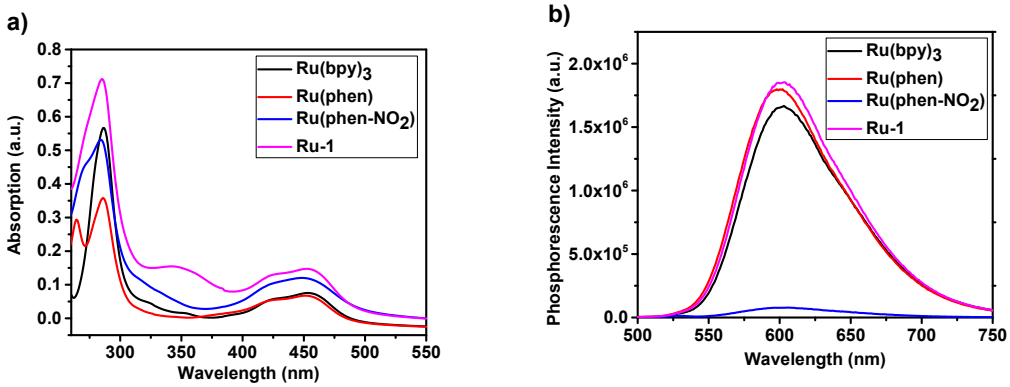


Figure S3. UV-Vis (a) and phosphorescence emission (b) spectra of the Ru complexes (10 μM) in HEPES buffer (50 mM, 100 mM KNO_3, pH=7.4)

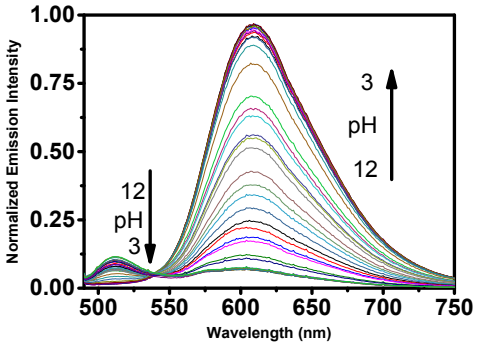


Figure S4. Phosphorescent emission spectra of 10 μM **Ru-1** in response to different pH (3 to 12) in HEPES buffer. λex= 480 nm.

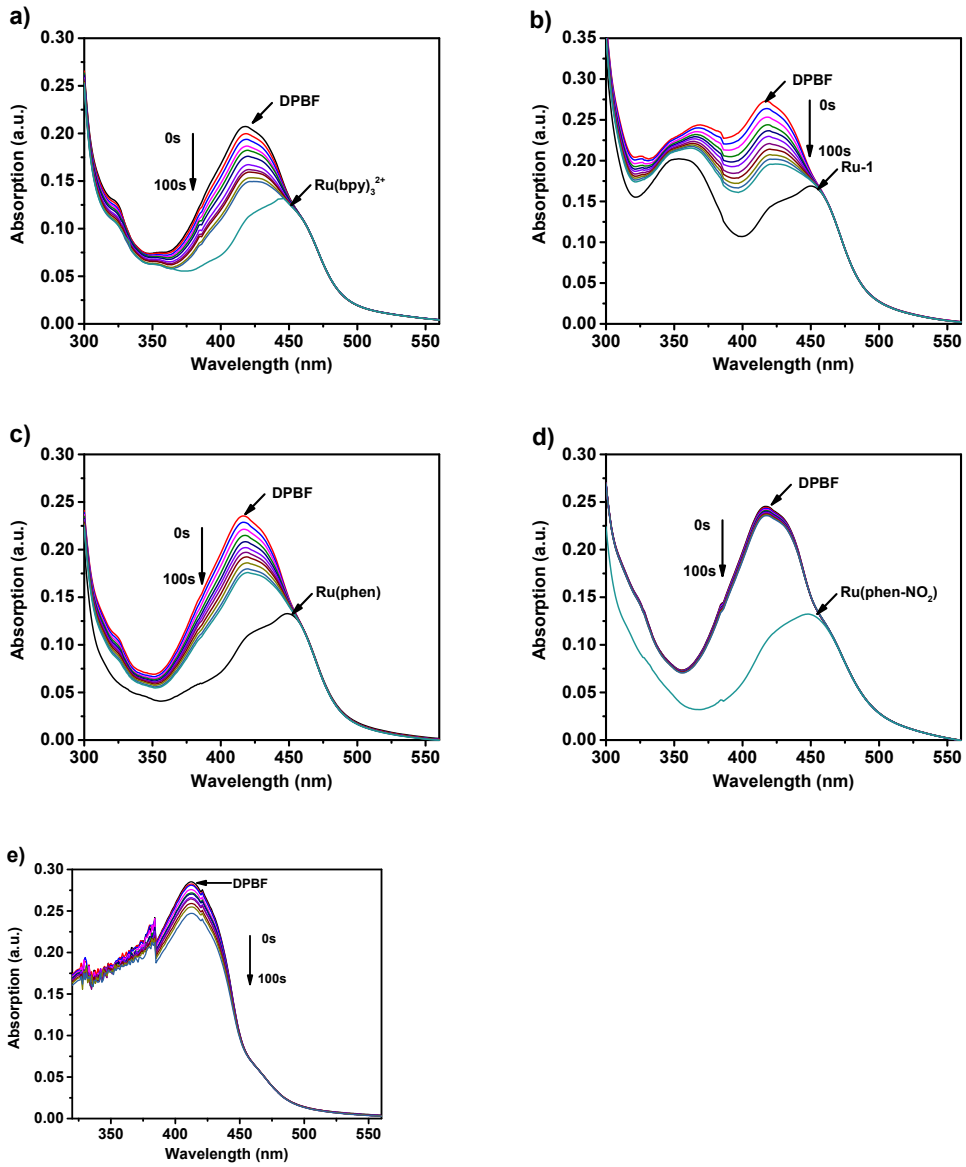


Figure S5. Changes in the absorption spectra of DPBF upon irradiation (λ= 450 nm) with 10s interval in the presence of **Ru(phen)**, **Ru(Phen-NO$_2$)**, **Ru-1** and **Ru-1** (with 2 mM ascorbic acid). The absorbance of **Ru-1** and the control complexes at the irradiation wavelength was adjusted to 0.1-0.3.

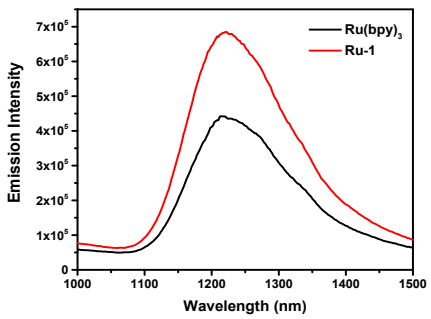


Figure S6. The $^1O_2$ phosphorescence spectra of I (red), II (green) in D_2O (λ_{ex}=450 nm)

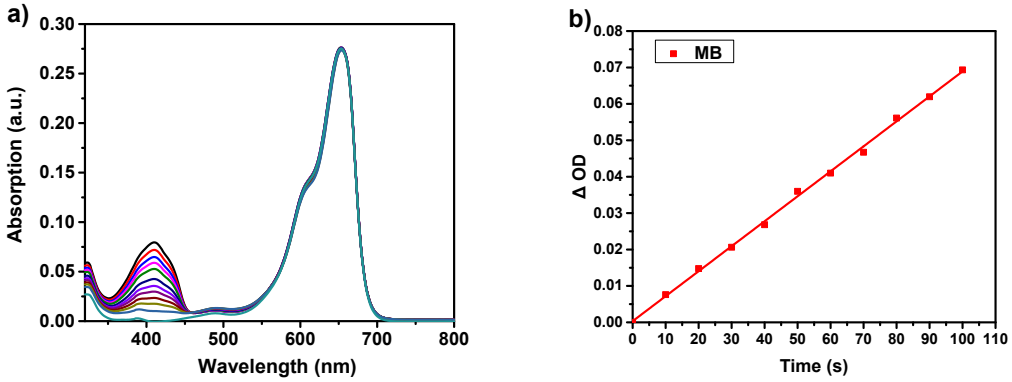


Figure S7. a) Changes in the absorption spectra of DPBF upon irradiation (λ= 635 nm) with 10s interval in the presence of **MB**. b) $^1O_2$ production from a plot of changes in absorbance by DPBF at 411 nm against irradiation time (λ_{ex} = 450 nm) in the presence of **MB**.

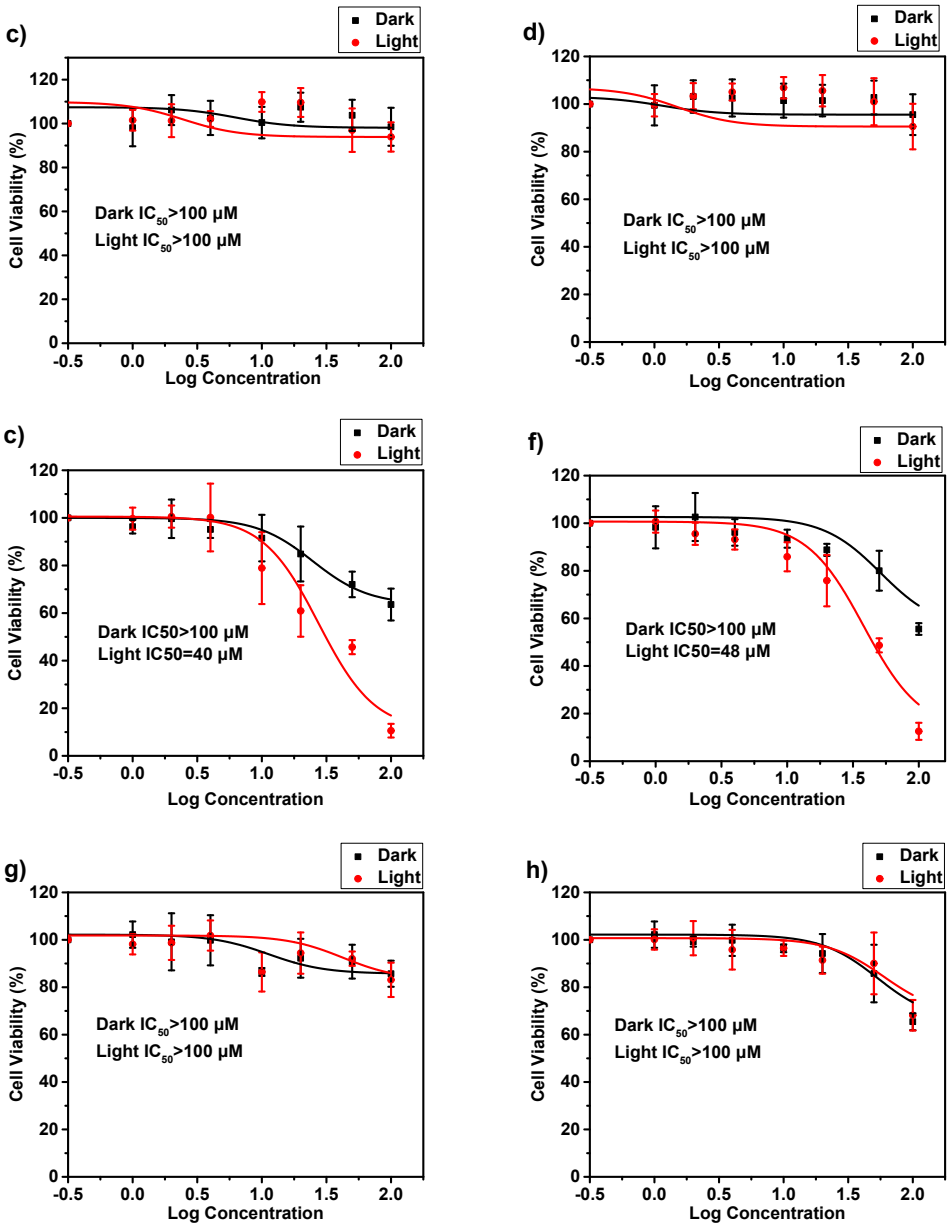


Figure S8. Cytotoxicities of **Ru(bpy)$_3^{2+}$**, **Ru(phen)** and **Ru(phen-NO$_2$)** against MCF-7 (a, c, e) and B16F10 (b, d, f) cell lines determined after 24 h of dark incubation with (red) or without (black) photo-irradiation (450 nm, 5 min, 30 J/cm^2) at the 4th hour of incubation.

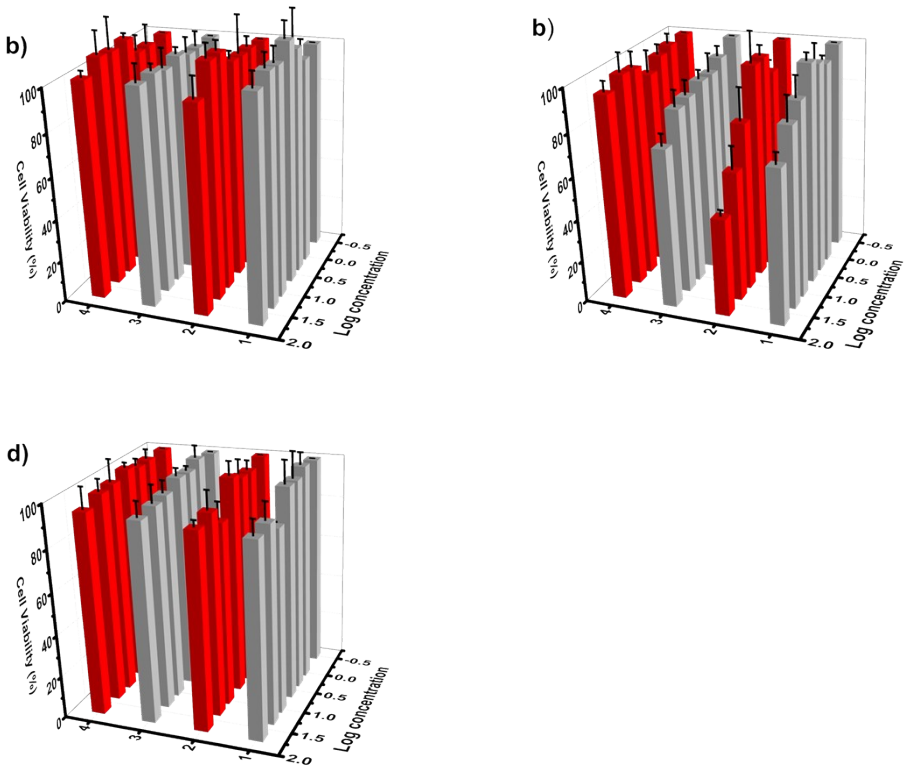


Figure S9. Cytotoxicities of **Ru(bpy)$_3^{2+}$** (a), **Ru(phen)** (b), **Ru(phen-NO$_2$)** (c) against MCF-7 cell lines under different treatment conditions. 1) MCF-7 cells were incubated with different Ru complexes for 24 h in dark; 2) light irradiation (450 nm, 5 min, 30 J/cm^2) was employed at the fourth hour of incubation of MCF-7 cells with different Ru complexes and the cells were further incubated for 20 h; 3) light irradiation (450 nm, 5 min, 30 J/cm^2) was employed at the fourth hour of incubation with different Ru complexes. After that, the cells were washed three times with PBS and then the culture medium was replaced with new medium without different Ru complexes for further incubation of 20 h; 4) the cells were washed three times with PBS and the culture medium was replaced with new medium without different Ru complexes at the fourth hour. After that, light irradiation (450 nm, 5 min, 30 J/cm^2) was employed and the cells were further incubated for 20 h.

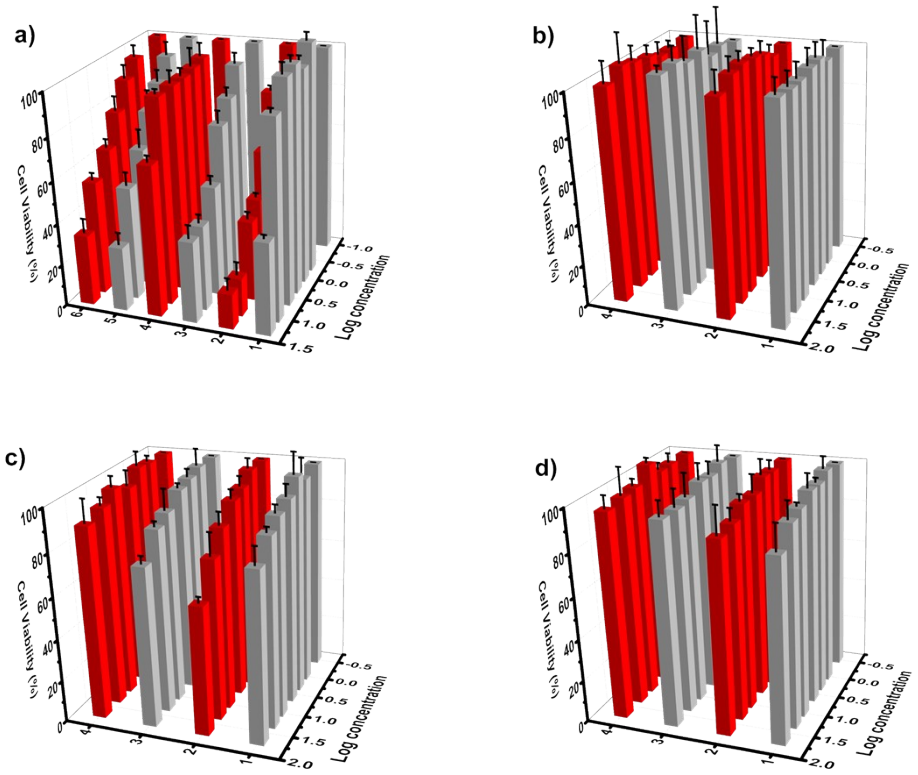


Figure S10. Cytotoxicities of **Ru-1** (a, 1-4), cisplatin (a, 5-6) and MB (b) against B16F10 cell lines under different treatment conditions. 1) B16F10 cells were incubated with **Ru-1** or MB for 24 h in dark; 2) light irradiation (450 nm for **Ru-1** and 635 nm for MB, 5 min, 30 J/cm^2) was employed at the fourth hour of incubation of B16F10 cells with **Ru-1** or MB and the cells were further incubated for 20 h; 3) light irradiation (450 nm for **Ru-1** and 635 nm for MB, 5 min, 30 J/cm^2) was employed at the fourth hour of incubation with **Ru-1** or MB. After that, the cells were washed three times with PBS and then the culture medium was replaced with new medium without **Ru-1** or MB for further incubation of 20 h; 4) the cells were washed three times with PBS and the culture medium was replaced with new medium without **Ru-1** or MB at the fourth hour. After that, light irradiation (450 nm for **Ru-1** and 635 nm for MB, 5 min, 30 J/cm^2) was employed and the cells were further incubated for 20 h; 5) B16F10 cells were incubated with cisplatin for 24 h in dark; 6) light irradiation (450 nm, 5 min, 30 J/cm^2) was employed at the fourth hour of incubation of B16F10 cells with cisplatin and the cells were further incubated for 20 h.

Table S1. Photo-physical data of the Ru complexes

	λ_{abs} (nm)[a]	logε_{max}[a]	λ_{em} (nm)[a]	Φ (%)[b]	τ (ns)[c]	$\Phi_{\Delta}(^1O_2)$[d]
Ru-1	450	4.22	602	1.8	300	0.81
$Ru(bpy)_3^{2+}$	450	3.82	602	1.8	-	0.56
Ru(phen)	450	3.87	602	-	-	0.57
Ru(phen-NO_2)	450	4.07	-	-	-	0.08

[a] Absorption and emission spectra were recorded in HEPES buffer (pH 7.4, 50 mM, 100 mM KNO_3) at room temperature; [b] Φ refers to the phosphorescence quantum yield in air-saturated acetonitrile, the standard used was $Ru(bpy)_3Cl_2$ (Φ=0.018, λ=450 nm, air-saturated acetonitrile); [c] τ refers to the lifetime and was evaluated in HEPES buffer (pH 7.4, 50 mM, 100 mM KNO_3); [d] $\Phi_{\Delta}(^1O_2)$ refers to the singlet oxygen quantum yield in air-saturated acetonitrile.

Table S2. X-ray crystallographic data for **Ru-1**

Formula	$C_{40}H_{27}N_7O_4Ru$
Crystal	red block
crystal size (mm)	0.34×0.25×0.16
formula weight	770.75
crystal system	triclinic
space group	P-1
a (Å)	13.31(5)
b (Å)	14.41(5)
c (Å)	14.49(6)
α (°)	97.92(7)
β (°)	102.57(7)
γ (°)	117.39(5)
V(Å^3), Z	2316(15), 2
ρ_{calc} (g·cm^{-3})	1.105
absorption coefficient, mm^{-1}	0.379
F(000)	784.0
θ range (°)	0.974 to 25.020
limiting indices	-15≤h≤15,-17≤k≤16, 0≤l≤17
independent reflections	7971
reflections observed	8182
refinement method	full-matrix least-squares on F^2
data/restraints/parameters	8182/118/457
goodness-of-fit on F^2	0.847
final R indices [$I>2\sigma(I)$]	R_1 = 0.0990, wR_2 = 0.2661
R indices (all data)	R_1 = 0.2097
largest diff. peak and hole	1.66 and -0.96 eÅ^{-3}

Table S3. Selected bond lengths (Å) and angles (°) for **Ru-1**

Ru(1)-N(1)	2.131(10)	Ru(1)-N(2)	2.110(10)
Ru(1)-N(3)	2.128(11)	Ru(1)-N(4)	2.158(9)
Ru(1)-N(5)	2.132(9)	Ru(1)-N(6)	2.113(10)
N(1)-Ru(1)-N(5)	90.8(3)	N(1)-Ru(1)-N(4)	95.0(3)
N(2)-Ru(1)-N(1)	77.6(4)	N(2)-Ru(1)-N(5)	96.5(4)
N(2)-Ru(1)-N(6)	173.5(3)	N(2)-Ru(1)-N(4)	88.5(4)
N(2)-Ru(1)-N(3)	96.0(4)	N(4)-Ru(1)-N(5)	173.0(3)
N(1)-Ru(1)-N(6)	97.7(4)	N(6)-Ru(1)-N(5)	78.9(4)
N(6)-Ru(1)-N(4)	96.5(4)	N(6)-Ru(1)-N(3)	89.0(4)
N(1)-Ru(1)-N(3)	172.0(3)	N(3)-Ru(1)-N(5)	94.8(3)
N(3)-Ru(1)-N(4)	79.8(3)		

References

[1] E. T. Luis, G. E. Ball, A. Gilbert, H. Iranmanesh, C. W. Newdick, J. E. Beves, *J. Coord. Chem.* 2016, **69**, 1686.
[2] Z. Han, Y. Wang, Y. Chen, H. Fang, H. Yuan, X. Shi, Y. Bai, Z. Chen, W. He, Z. Guo, *Chem. Commun.* 2020, **56**, 8055