Supporting information

NIR phosphorescent osmium(II) complex as lysosome tracking reagent and photodynamic therapeutics

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Contents

Experimental Section

Materials. $RuCl_3 \cdot nH_2O$, $(NH_4)_2[OsCl_6]$, 3,4-diaminobenzoic acid, pyridine-2-carbaldehyde, methylamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO-d_6 and cisplatin were purchased from Alfa Aesar. Human lung carcinoma (A549) cell line, cervical carcinoma (HeLa) cell line, liver hepatocellular carcinoma (Hep-G2) cell line and fetal lung fibroblast (MRC-5) cell line were purchased from Sigma-Aldrich. Roswell Park Memorial Institute medium (RPMI-1640), fetal calf serum (FCS), glutamine and penicillin/streptomycin were purchased from Sigma-Aldrich.

Instruments. ^{1}H NMR spectra were recorded on a BrukerAV-400 spectrometer. Elemental analysis was performed by Exeter Analytical using a CHN/O/S Elemental Analyser (CE440). Positive ion ESI-MS spectra were obtained using an Agilent 6130B single quad coupled to an automated sample delivery system. UV-visible absorption spectra were recorded on a Varian Cary 300UV-vis spectrophotometer fitted with an external Varian Cary temperature controller. The fluorescence spectra were recorded on a JASCO FP-6500 Fluorimeter. The complexes were tested against cancer cells using the blue and red lights irradiation (blue: λ_{irr} = 465 nm, 4.8 mW/cm^2, red: λ_{irr} = 633 nm, 11.1 mW/cm^2).

Synthesis of Ligand 1. To a mixture of a solution of 1.6 g of 3,4-diaminobenzoic acid in 15 ml of ethanol an a solution of 2.2 g of copper acetate in 25 ml of water were added 1.3 g of pyridine-2-carbaldehyde and 0.4 g of methylamine, the reaction mixture was heated for 2 h on a boiling water bath.[1] The black precipitate of the formed copper complex was filtered off and dispersed in 10 ml of ethanol. To decompose the complex 5.2 g of $Na_2S \cdot 9H_2O$ was added to the suspension, and the mixture was heated for 0.5 h on a water bath, copper sulfide was filtered off from the hot solution and washed with hot water on the filter. The filtrates were combined and strongly acidified with hydrochloric acid. H_2S was removed from the solution by heating on the water bath. After cooling, the precipitation was filtered off and recrystallized from ethanol. Yield: 63%. ^{1}H NMR (400 MHz, DMSO): δ 8.77 (d, *J* = 4.3 Hz, 1H), 8.32 (d, *J* = 12.7 Hz, 2H), 8.03 (t, *J* = 7.7 Hz, 1H), 7.95 (d, *J* = 8.5 Hz, 1H), 7.74 (d, *J* = 8.3 Hz, 1H), 7.56 (t, *J* = 6.0 Hz, 1H), 4.26 (s, 3H). ^{13}C NMR (75 MHz, DMSO): δ 168.25, 152.10, 150.07, 149.38, 142.01, 140.59, 137.98, 125.59, 125.13, 125.06, 124.70, 121.67, 111.22, 33.43.

Synthesis of Ru1 and Os1

Preparation of Os(bpy)$_2$Cl$_2$. A DMF solution of $(NH_4)_2[OsCl_6]$ (1mmol, 1 eq) and 2,2'-bipyridine (2.1 mmol, 2.1 eq) was refluxed under an N_2 atmosphere for 5 h. After cooling to room temperature, the solution was added to an aqueous solution containing $Na_2S_2O_4$ (6.8 g)

and then kept in a refrigerator overnight. The dark precipitate was collected by vacuum filtration, washed with water and diethyl ether, and directly used in the following reactions without further purification.

Preparation of [Ru/Os(bpy)$_2$(N^N)](PF$_6$)$_2$. $Ru(bpy)_2Cl_2$ or $Os(bpy)_2Cl_2$ (0.10 mmol, 1.0 eq) and the ligand (0.21 mmol, 0.21 eq) were suspended in ethylene glycol (10 mL) in flask equipped with a magnetic stir bar. The mixture was heated to 120 °C and stirred for 5 h under a N_2 atmosphere. Following the reaction, the mixture was cooled to room temperature, excess amount of NH_4PF_6 was added to precipitate the product as the hexafluorophosphate salt. The precipitate was washed with water and diethyl ether to give the crude product, which was purified by column chromatography on basic alumina using acetonitrile as the eluent.

Ru1: Yield: 82%. ^{1}H NMR (400 MHz, DMSO-d^6): δ 8.86 (d, *J* = 8.1 Hz, 3H), 8.78 (dd, *J* = 12.2, 8.3 Hz, 2H), 8.27 – 8.13 (m, 4H), 8.09 (t, *J* = 7.9 Hz, 1H), 8.01 (dd, *J* = 23.9, 8.9 Hz, 2H), 7.93 (d, *J* = 5.4 Hz, 1H), 7.85 (dd, *J* = 9.6, 5.4 Hz, 2H), 7.76 (d, *J* = 5.4 Hz, 2H), 7.57 (m, 4H), 7.49 – 7.42 (m, 1H), 6.37 (s, 1H), 4.46 (s, 3H).^{13}C NMR (101 MHz, DMSO-d^6): δ 166.95, 157.99, 157.71, 157.07, 157.05, 153.32, 153.05, 152.63, 152.32, 152.15, 151.87, 149.08, 140.03, 139.68, 138.69, 138.52, 138.32, 138.20, 138.09, 137.96, 128.37, 128.33, 128.25, 128.19, 128.09, 126.68, 126.59, 124.98, 124.82, 124.72, 124.43, 117.60, 113.10, 34.11. ESI-MS: 666.1[M–2PF$_6$–H]$^+$; Elemental analysis: Anal. Calcd for $C_{34}H_{27}N_9O_3RuP_6F_{12}$, C, 41.90%; H, 3.00%; N, 10.06%; Found: C, 41.76%; H, 3.02%; N, 10.01%.

Os1: Yield: 74%. ^{1}H NMR (400 MHz, DMSO-d^6): δ 8.84 (d, *J* = 8.3 Hz, 3H), 8.75 (t, J = 8.1 Hz, 2H), 8.07 – 7.92 (m, 6H), 7.85 (t, *J* = 6.3 Hz, 2H), 7.75 (t, *J* = 6.7 Hz, 2H), 7.68 – 7.60 (m, 2H), 7.53 – 7.48 (m, 1H), 7.43 (dd, *J* = 15.7, 9.8 Hz, 3H), 7.36 (t, *J* = 6.5 Hz, 1H), 6.43 (s, 1H), 4.55 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d^6): δ 166.03, 159.36, 159.32, 158.56, 158.52, 156.63, 151.80, 151.14, 150.91, 150.28, 150.13, 149.96, 149.16, 139.19, 138.41, 136.84, 136.52, 136.41, 136.28, 135.99, 127.86, 127.74, 127.64, 127.43, 125.95, 125.88, 124.12, 124.09, 123.92, 123.80, 123.48, 116.49, 111.98, 32.25. ESI-MS: 756.1([M–2PF$_6$–H]$^+$); Elemental analysis: Anal. Calcd for $C_{34}H_{27}N_9O_3RuP_6F_{12}$, C, 38.39%; H, 2.75%; N, 9.22%; Found: C, 38.46%; H, 2.81%; N, 9.30%.

Log P$_{o/w}$ Measurement. A shake-flask ultraviolet spectrophotometry method was used to determine the *n*-octanol/water partition coefficients of **Ru1**/**Os1**. First, the mixed solution of 50 mL of *n*-octanol and 50 mL of water was left shaking at 37 °C for 48 h. The **Ru1**/**Os1** standard solutions (50 μM in 5 mL) were prepared using the water phase and organic phase. Second, the **Ru1**/**Os1** detection solutions (50 μM) were prepared with mixed solvent containing 5 mL of *n*-octanol and 5 mL of water and shaking for 48 h. Third, the

concentration of **Ru1/Os1** in the water phase (C_w) and organic phase (C_o) of the detecting solution were determined separately using UV-Vis spectrophotometry. The *n*-octanol/water partition coefficient, namely, log P = log (C_o/C_w).

Quantum Yields of Singlet Oxygen. An air-saturated PBS buffer solution, containing the complexes (OD = 0.1 at irradiation wavelength), p-nitrosodimethyl aniline (RNO, 20 μM), histidine (10 mM) were irradiated with blue light in a quartz cuvette for different time intervals.[2] The absorbance of the solution was then recorded. Plots of variations in absorbance at 440 nm in PBS (A_0–A, where A_0 is the absorbance before irradiation) versus the irradiation times for each sample were prepared and the slope of the linear regression was calculated (S_{sample}). As a reference compound, $[Ru(bpy)_3]^{2+}$ (Φ_{ref} ($^1O_2$) = 0.22 in H_2O)[3] was used in both methods, to obtain S_{ref}. Equation (1) was applied to calculate the singlet oxygen quantum yields (Φ_{sample}) for every sample: I (the absorbance correction factor) was obtained using Equation (2), where I_0 is the light intensity of the irradiation source in the irradiation interval and A_λ is the absorbance of the sample at wavelength λ.[2]

$$\Phi_{sample} = \Phi_{ref} * S_{sample}/S_{ref} * I_{ref}/I_{sample} \quad (1)$$

$$I = I_0 * (1 - 10^{-A\lambda}) \quad (2)$$

Electron Paramagnetic Resonance (EPR). The EPR measurements were carried out at ambient temperature on a Bruker EMX spectrometer. Irradiation was carried out with 465 nm or 633 nm LEDs. The samples were contained in a flat-cell (WG812) positioned in a TM110 cavity (ER4103 TM). For kinetic measurements, the EPR parameters were: sweep width 8 mT, 1024 points, time constant 10.24 ms and conversion time 20.48 ms, giving a sweep time of ~30 s. Field modulation was applied at 100 kHz and 0.05 mT, and the microwave attenuation was 18 dB (~3.2 mW). The spin trap, 2,2,6,6-tetramethyl-piperidine (TEMP for trapping $^1O_2$, 20 mM), was used to verify the formation of $^1O_2$ generated by the complexes (100 μM).

Cell Culture. The cells were grown in RPMI-1640 with or without phenol red (for photoactivable experiment). All media were supplemented with 10% v/v of fetal calf serum (FCS), 1% v/v of 2 mM glutamine and 1% v/v penicillin/streptomycin. All cells were grown as adherent monolayers at 310 K in a 5% CO_2 humidified incubator and passaged regularly at approx. 80% confluence.

Cellular Localization. A549 cancer cells were incubated with 20 μM **Ru1/Os1** at 37 °C for 4 h and then co-stained with 500 nM Hoechst 33258, 100 nM LysoTracker® Green or MitoTracker® Green for 30 min. The Cells were then washed three times with PBS and

visualized using Zeiss 880 confocal microscopy (63×oil-immersion objective). The excitation wavelengths were 458 nm for **Ru1**, 561 nm for **Os1**, 405 nm for Hoechst 33258, 488 nm for MitoTracker® Green and LysoTracker® Green; the emission wavelengths were 600±30 nm for **Ru1**, 700±30 nm for **Os1**, 450±30 nm for Hoechst 33258, 520±30 nm for MitoTracker® Green and LysoTracker® Green.

Cell Uptake Assay. For the uptake studies, exponentially growing A549 cancer cells were harvested, and the resulting single-cell suspension was plated into 100 mm tissue culture plates. After 24 h at 37 °C, the cells were incubated with 20 μM of the complexes for 4 h at 37 °C. The cells were rinsed with PBS, detached with trypsin, counted and divided into four portions. In the first portion, the mitochondria fraction was extracted using a mitochondria isolation kit (Thermo Scientific) following the manufacturer's protocol. In the second portion, the lysosome fraction was extracted using a lysosome isolation kit (Thermo Scientific). In the third portion, the cell membrane fraction was extracted using a membrane isolation kit (Thermo Scientific). In the fourth portion, the nucleus fraction was extracted using a nucleus isolation kit (Thermo Scientific). The samples were digested with 60% HNO_3 for 48 h at 310 K. Each sample was diluted with Milli-Q H_2O to obtain 2% HNO_3 sample solutions. The Ru/Os content was measured using an Agilent technologies 7500 Series inductively coupled plasma mass spectrometry (ICP-MS). Data were reported as the means ± standard deviation (n = 3).

Phototoxicity Test. The photocytotoxicity test was determined towards the A549, Hep-G2, HeLa and MRC-5 cells. The cells were grown in RPMI-1640 with or without phenol red according to the protocol below. All media were supplemented with 10% v/v of foetal calf serum (FCS), 1% v/v of 2 mM glutamine and 1% v/v penicillin/streptomycin. All cells were grown as adherent monolayers at 310 K in a 5% CO_2 humidified incubator and passaged regularly at approx. 80% confluence. Approximately 5×10^3 cells/well were seeded into 96-well plates, followed by 24 h incubation for attachment. The cells were then exposed to the complex with different concentrations. For photocytotoxicity studies, after 4 h incubation with complex, each well of both dark and irradiated plates were washed with phosphate-buffered saline (PBS), and fresh medium was added into the wells. Cells of irradiated plate were then irradiated by 465 nm and 633 nm light (465 nm: 4.8 mW/cm^2, 1 h; 633 nm: 11.1 mW/cm^2, 3 h). After irradiation, the cells continue to be incubated for in total 48 h. The photocytotoxicity was measured by standard MTT method. The change in optical density (OD) at 540 nm was monitored using microplate reader (Promega).

Cellular $^1O_2$ Measurement. The intracellular ROS under irradiation was measured using the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA diffuses

through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to form the non-fluorescent compound DCFH, which is then rapidly oxidized to form the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. The DCF fluorescence intensity is thought to parallel the amount of ROS formed intracellularly. Firstly, the cultured cancer cells were treated with the complexes in the dark for 4 h, washed with PBS and then incubated with 10 μM of DCFH-DA for 30 min in the dark. The cells were subjected to 465 nm or 633 nm light irradiation and then imaged by confocal microscopy immediately. The excitation wavelength of intracellular ROS was set as 488 nm, and the fluorescence was collected at 530 ± 20 nm.

Table

Table S1. Dark, blue, and red light IC_{50} values for the compounds toward A549, Hep-G2, HeLa cancer cells and MRC-5 normal cells.

Cell lines	Compounds	IC_{50} (μM)		
		Dark	465 nm (PI_{465})	633 nm (PI_{633})
A549	**Ru1**	425 ± 27	12.3 ± 1.6 (37.8)	442 ± 6 (1.0)
	Os1	406 ± 34	345 ± 28 (1.2)	31.7 ± 2.8 (12.8)
	Cis-Pt	93.3 ± 8.7	90.9 ± 9.6 (1.0)	> 100
	5-ALA	> 100	> 100	> 100
Hep-G2	**Ru1**	456 ± 21	22.5 ± 0.9 (20.3)	436 ± 34 (1.0)
	Os1	454 ± 36	321 ± 15 (1.4)	34.3 ± 1.9 (13.1)
	Cis-Pt	> 100	> 100	> 100
	5-ALA	> 100	> 100	> 100
HeLa	**Ru1**	398 ± 23	11.5 ± 1.6 (34.6)	403 ± 13 (1.0)
	Os1	422 ± 15	356 ± 23 (1.2)	29.9 ± 2.4 (14.1)
	Cis-Pt	90.1 ± 6.3	> 100	> 100
	5-ALA	> 100	> 100	> 100
MRC-5	**Ru1**	488 ± 19	101 ± 9.6 (4.8)	484 ± 29 (1.0)
	Os1	476 ± 12	421 ± 26 (1.1)	135 ± 11 (3.5)
	Cis-Pt	98.5 ± 13	> 100	> 100
	5-ALA	> 100	> 100	> 100

The cells incubated with the compounds for 4 h, medium replaced in both 'dark' and 'light' plates with fresh non-drug medium, 'light' plate irradiated with blue (465 nm, 4.8 mW/cm^2, 1 h) or red light (633 nm, 11.1 mW/cm^2, 3 h). All plates incubated for another 44 h. PI_{465} and PI_{633} values in brackets are phototoxicity indexes for 465 nm and 633 nm light irradiation, respectively; PI = IC_{50} (dark) / IC_{50} (light); Cis-Pt = cisplatin; 5-ALA = 5-amino-levulinic acid.

Figures

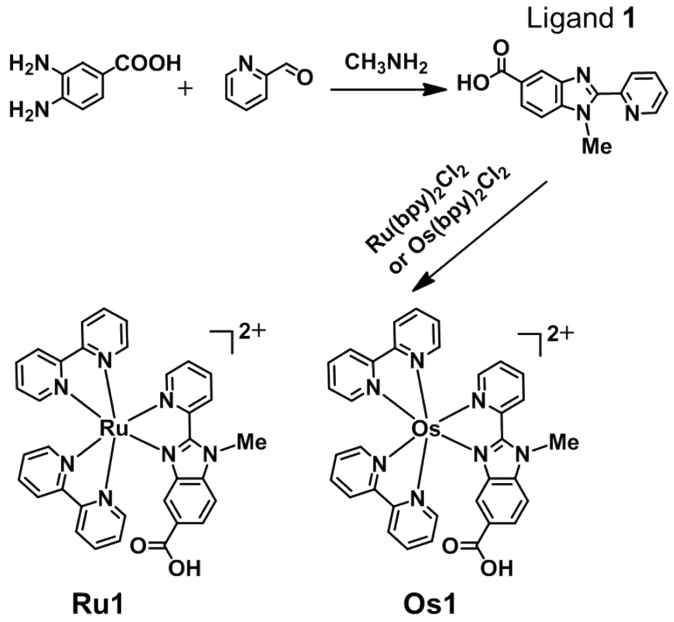

Fig. S1. The synthetic scheme of the ruthenium and osmium complexes.

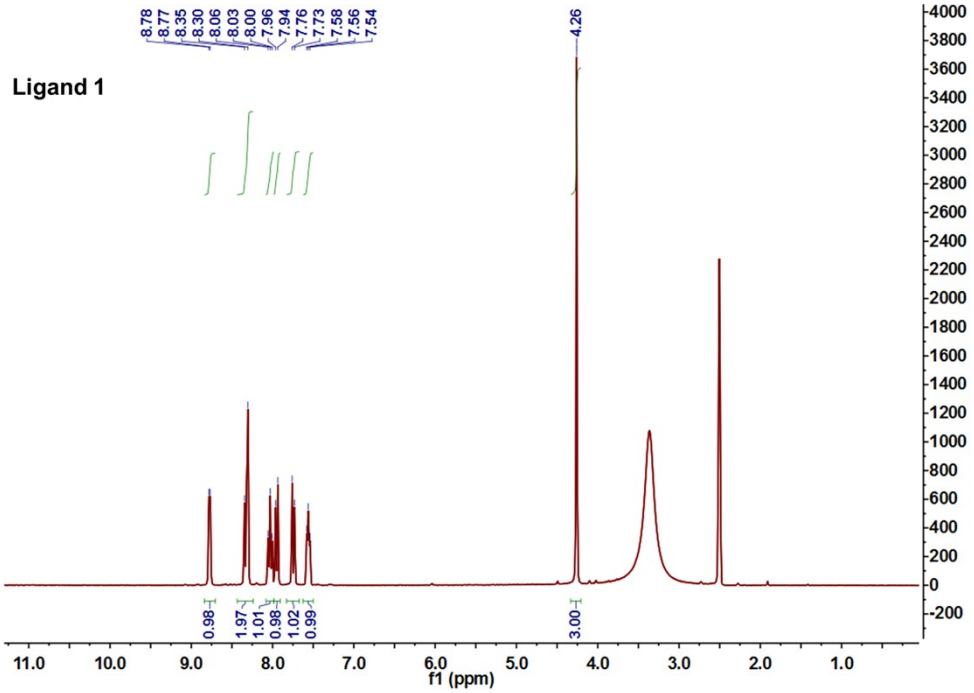

Fig. S2. The 400 MHz ^{1}H NMR spectrum of ligand **1** in DMSO-d^{6}.

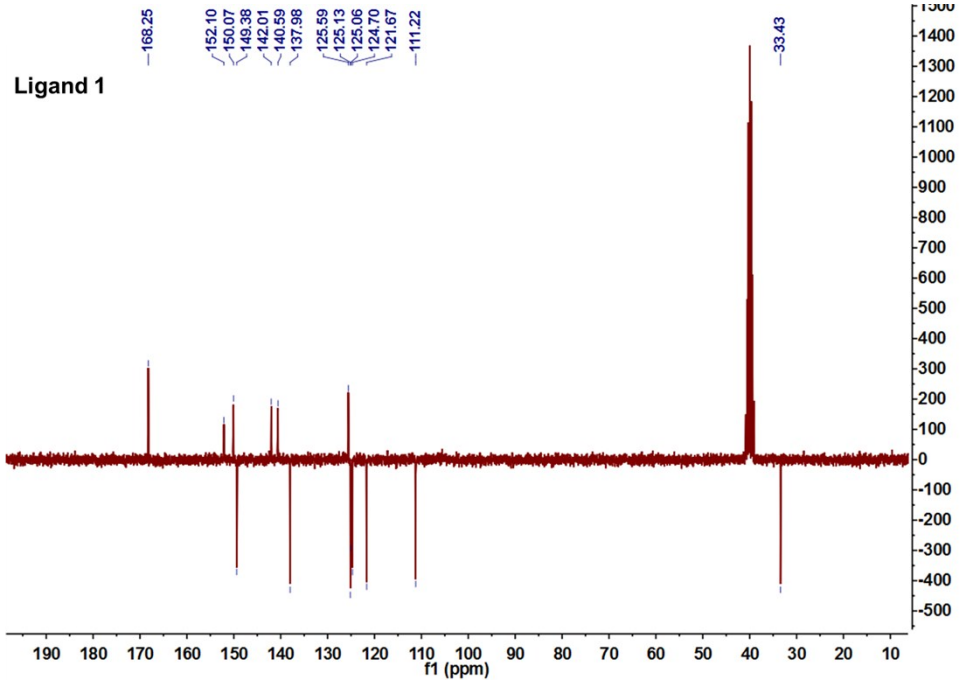

Fig. S3. The ^{13}C NMR spectrum of ligand **1** in DMSO-d^{6}.

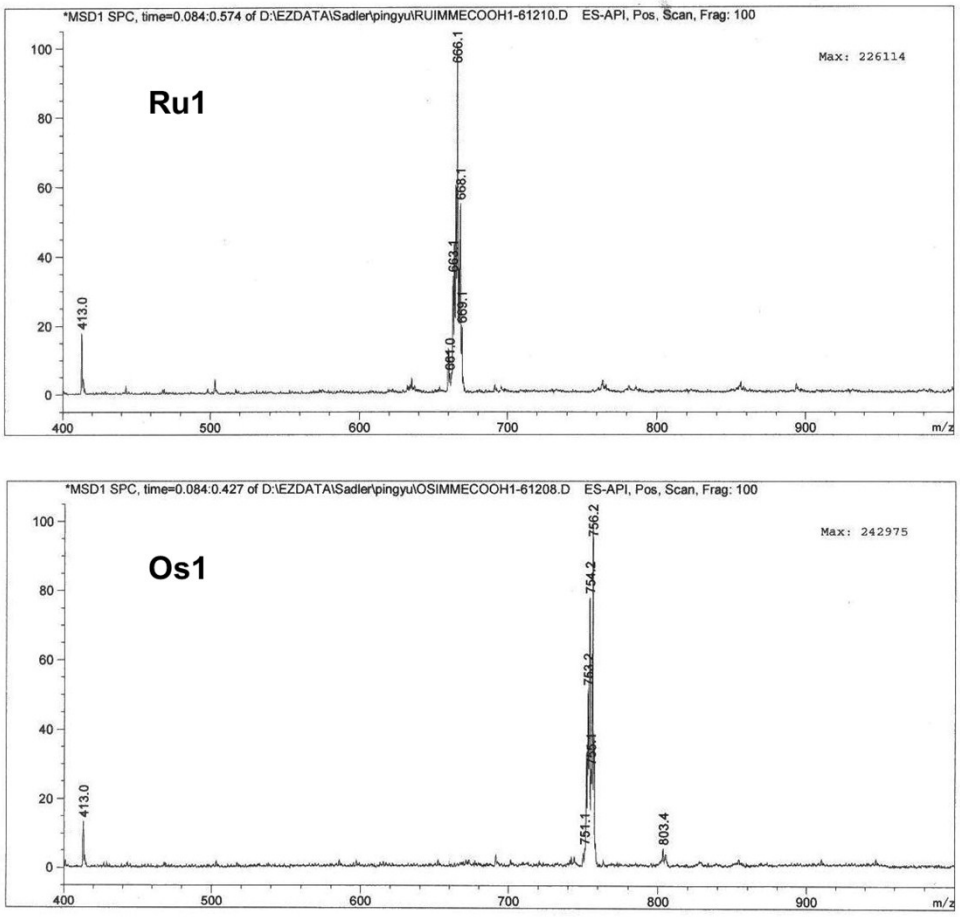

Fig. S4. The ESI-MS spectra of **Ru1** and **Os1** in CH_3OH.

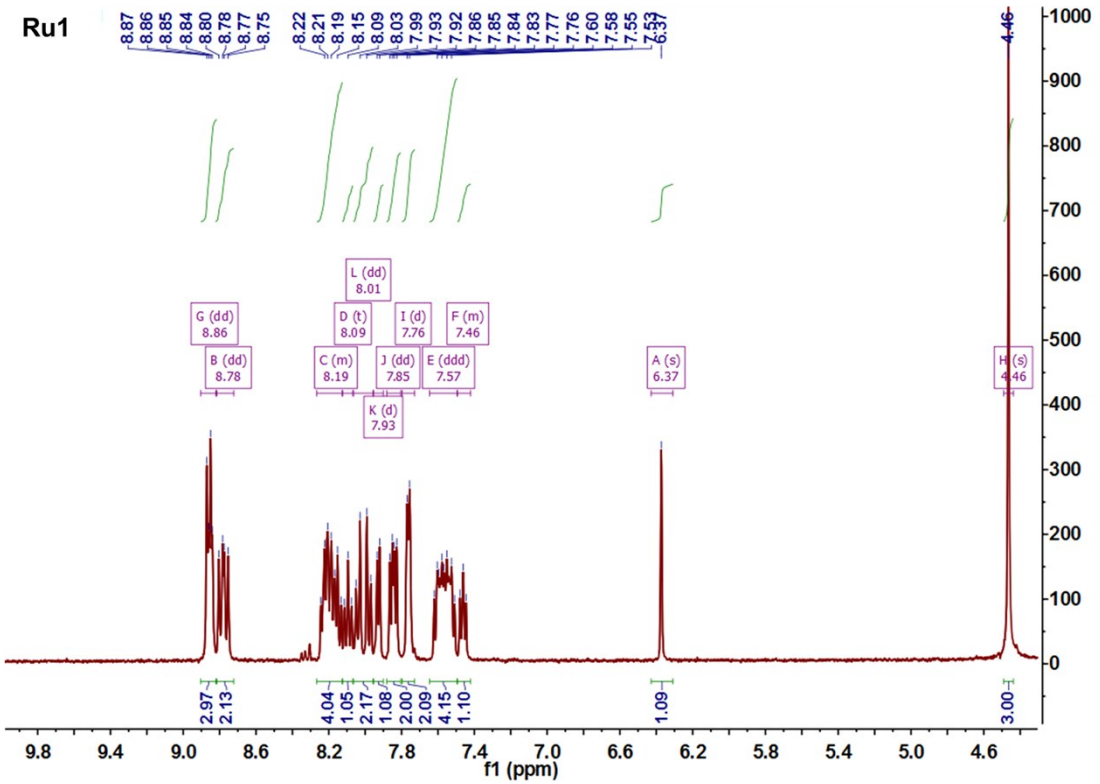

Fig. S5. The 400 MHz ^{1}H NMR spectrum of **Ru1** in DMSO-d^{6}.

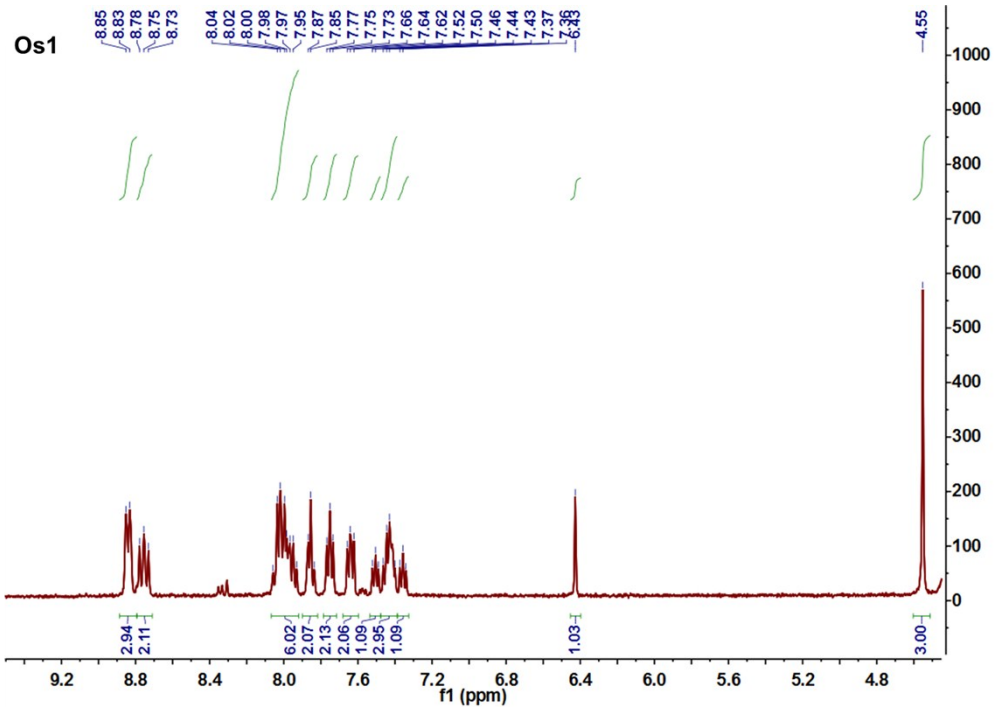

Fig. S6. The 400 MHz ^{1}H NMR spectrum of **Os1** in DMSO-d^{6}.

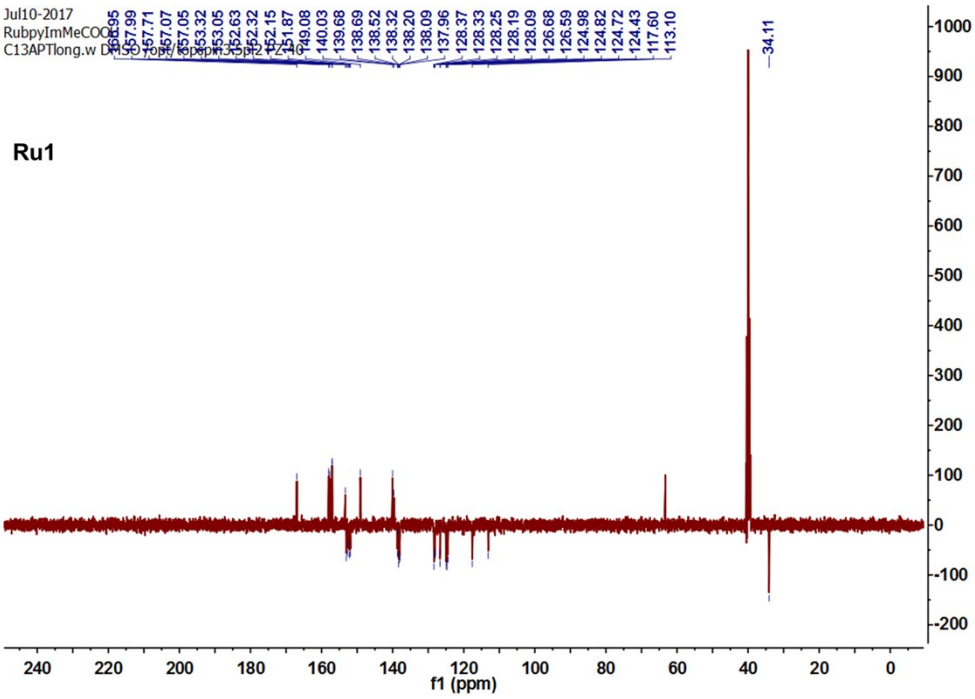

Fig. S7. The ^{13}C NMR spectrum of **Ru1** in DMSO-d^6.

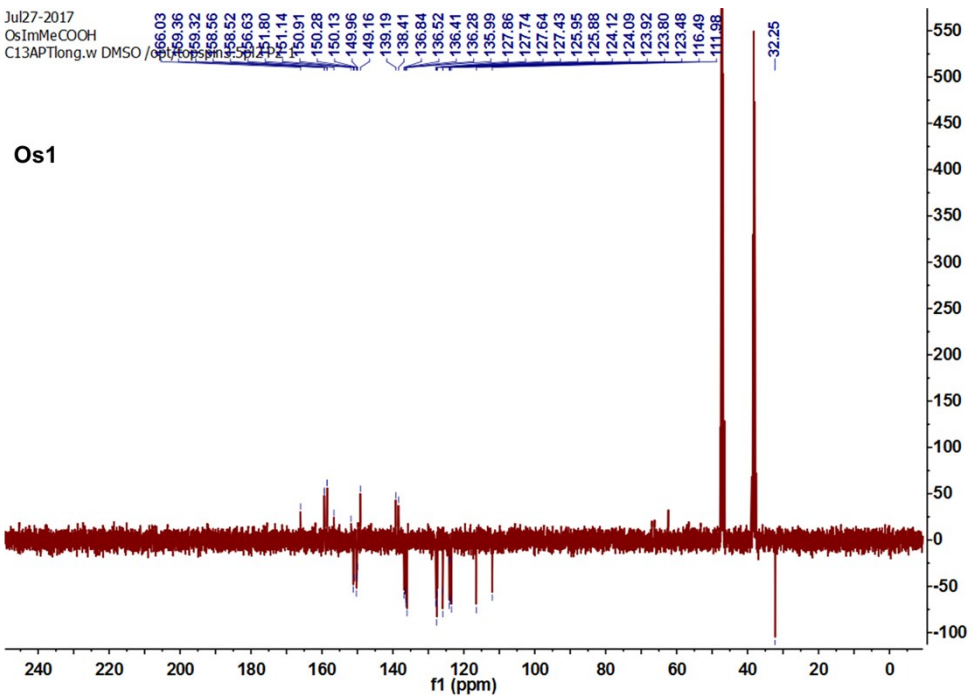

Fig. S8. The ^{13}C NMR spectrum of **Os1** in DMSO-d^6.

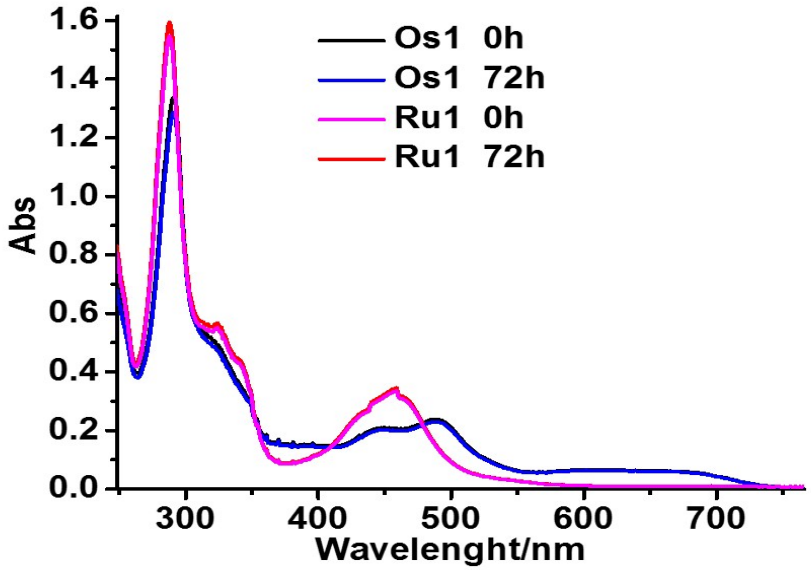

Fig. S9. The stabilities of **Ru1** and **Os1** in the cell culture medium (RPMI-1640, with 1% DMSO) for 72h.

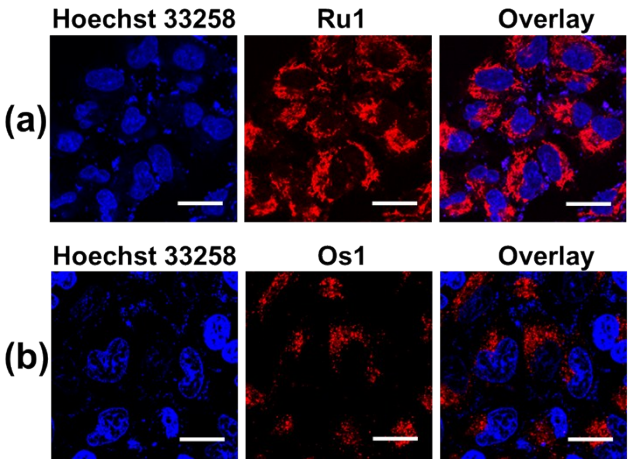

Fig. S10. Confocal images of the A549 cells colabeled with (a) **Ru1** or (b) **Os1** (20 μM, 4 h) and Hoechst 33258 (500 nM, 30 min). **Ru1**: λ_{ex} = 458 nm, λ_{em} = 600±30 nm; **Os1**: λ_{ex} = 561 nm, λ_{em} = 720±30 nm; Hoechst 33258: λ_{ex} = 405 nm, λ_{em} = 450 ± 30 nm; Scale bar: 20 μm.

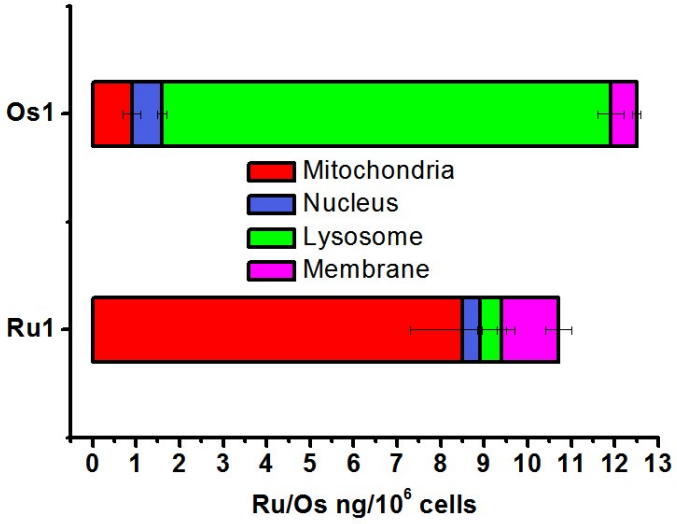

Fig. S11. ICP-MS assay for Ru/Os content of the nucleus, cytoplasm, mitochondria and membrane fractions (ng/10^6 cells) of A549 cells after 4 h of exposure to 20 μM **Ru1/Os1**. Results are the means of two independent experiments in triplicate and are expressed as means (±SDs).

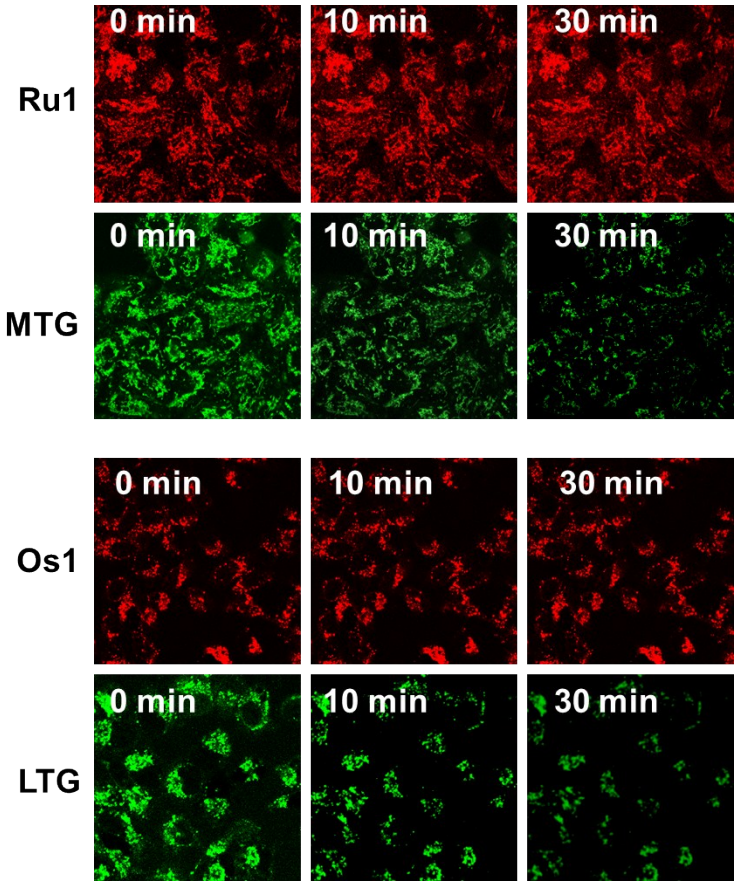

Fig. S12. Fluorescence (or phosphorescence) intensity decay of LTG, MTG, **Ru1** and **Os1** in living A549 cancer cells with irradiation by 458 nm light irradiation (0 min, 10 min and 30 min).

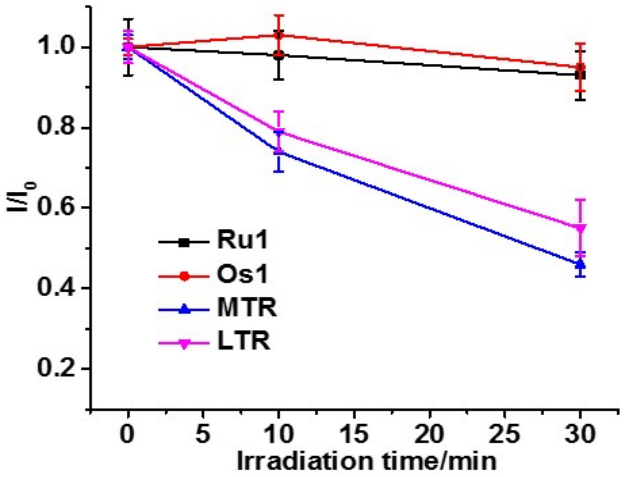

Fig. S13. Fluorescence (or phosphorescence) intensity decay of LTG, MTG, **Ru1** and **Os1** in water (with 1% DMSO, 20 μM) with irradiation by 458 nm light. I_0 is the fluorescence (or phosphorescence) intensity before the irradiation. I is the fluorescence (or phosphorescence) intensity of the above compounds after a fixed time of irradiation.

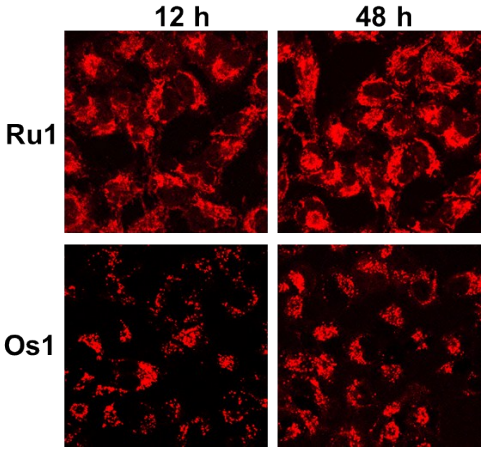

Fig. S14. Confocal images of A549 cells colabeled with **Ru1** or **Os1** (20 μM) for 12 h and 48 h incubation. **Ru1**: λ_{ex} = 458 nm, λ_{em} = 600±30 nm; **Os1**: λ_{ex} = 561 nm, λ_{em} = 720±30 nm.

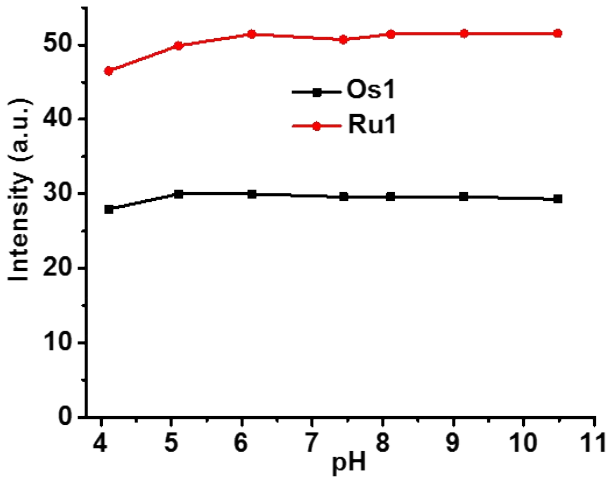

Fig. S15. pH-sensitive emission intensities at 619 nm (**Ru1**) and 736 nm (**Os1**) in PBS buffer solution.

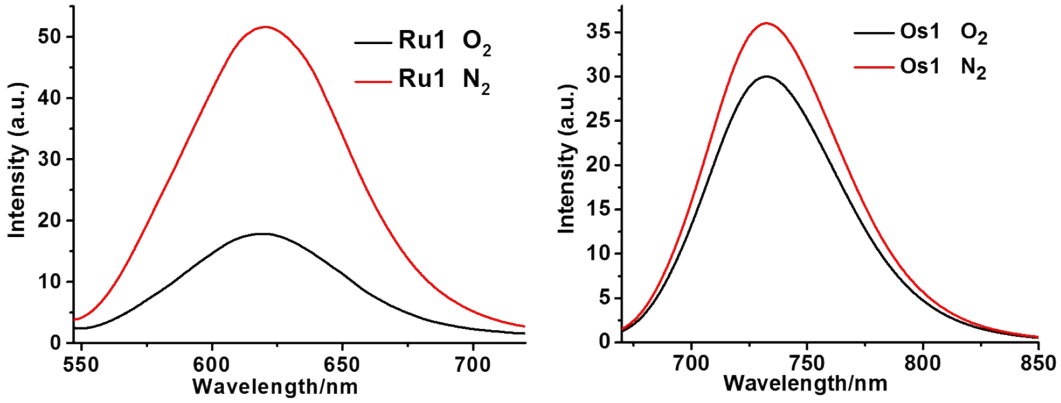

Fig. S16. The emission intensities of **Ru1** (λ_{ex} = 465 nm) and **Os1** (λ_{ex} = 633 nm) in the air and under N_2 in the PBS solution (with 1% DMSO).

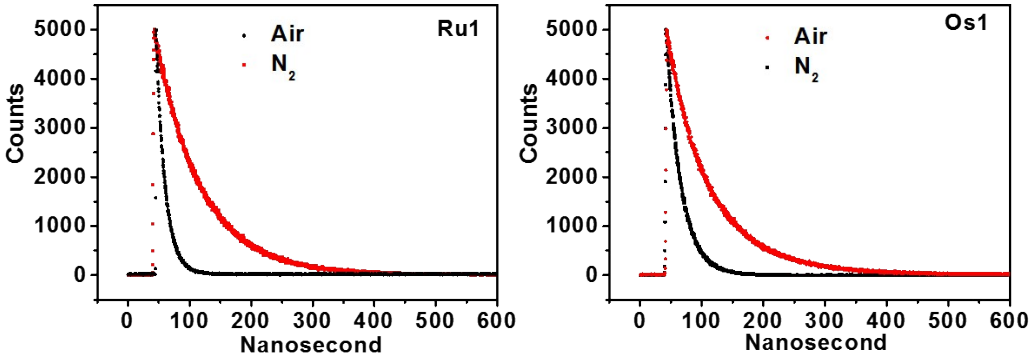

Fig. S17. The phosphorescence lifetimes of **Ru1** and **Os1** in the PBS solution (1% DMSO) in the air and under N_2 environment.

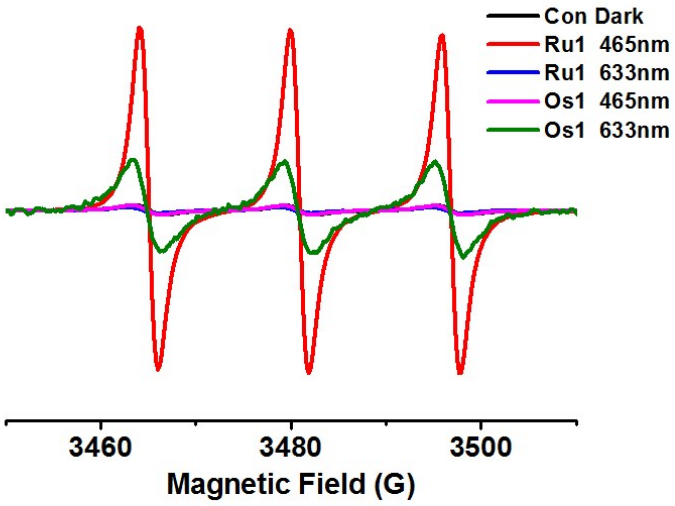

Fig. S18. EPR spectrum of **Ru1** and **Os1** (50 μM) with TEMP as spin trap (20 mM) after 465 nm (4.8 mW/cm^2, 1 h) and 633 nm light (11.1 mW/cm^2, 3 h) irradiation.

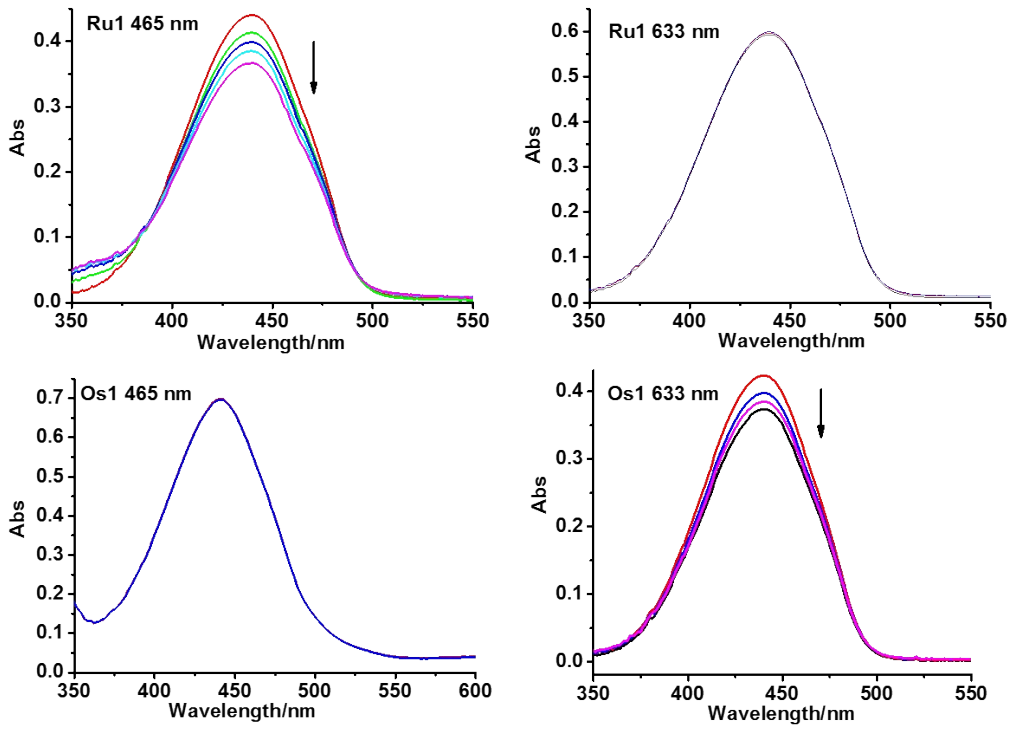

Fig. S19. The quenching of the absorbance of RNO in the presence of **Ru1** and **Os1** (20 μM) in PBS solution upon 465 nm (0-1 h) and 633 nm (0-3 h) light irradiation on dependence of irradiation time.

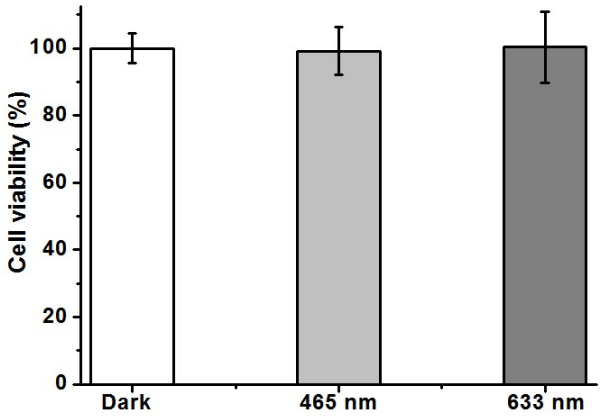

Fig. S20. The control A549 cancer cells were irradiated with 465 nm (4.8 mW/cm^2, 1 h) and 633 nm lights (11.1 mW/cm^2, 3 h).

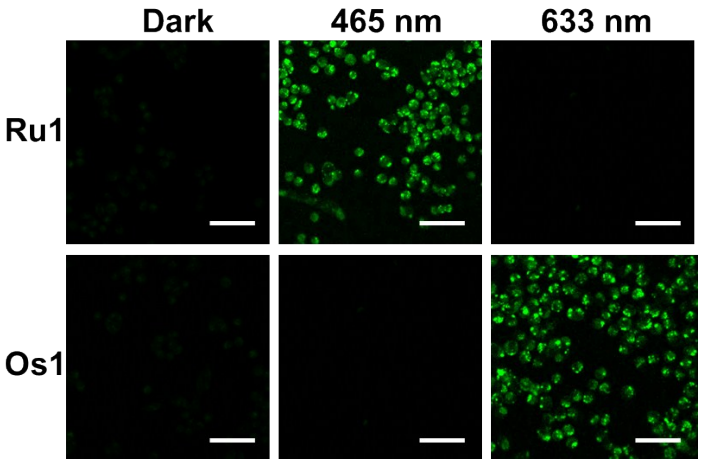

Fig. S21. A549 cells treated with the **Ru1** or **Os1** (20 μM) and DCFH-DA (10 μM) in the dark or upon 465 nm or 633 nm lights irradiation; scar bar: 100 μm.

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