Supporting Information

Triphenylamine/carbazole-modified Ruthenium(II) Schiff base compounds: Synthesis, biological activity and organelle targeting

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Experimental section

NMR spectroscopy

^{1}H NMR spectra were acquired in 5 mm NMR tubes at 25 °C on Bruker DPX 500 (^{1}H = 500.13 MHz) spectrometers. ^{1}H NMR chemical shifts were internally referenced to $(CHD_2)(CD_3)SO$ (2.50 ppm) for DMSO-d_6, $CHCl_3$ (7.26 ppm) for chloroform-d_1. All data was carried out using XWIN-NMR version 3.6 (Bruker UK Ltd.).

UV-vis spectroscopy

The UV-vis spectra of these compounds were recorded by TU-1901 UV spectrophotometer with 1 cm path-length quartz cuvettes (3 mL). Spectra were processed using UV Winlab software. Experiments were carried out at 25 °C unless otherwise stated.

Stability study

Solutions of **Ru1-Ru4** with final concentrations of 1.5 mM in 60% DMSO-d_6/40% phosphate-buffered saline (PBS, pH ≈ 7.2, prepared from D_2O) were prepared by dissolution of the compounds in DMSO-d_6 followed by rapid dilution with D_2O, respectively. ^{1}H NMR spectra were recorded after various time intervals at 37 °C. The test was done by dissolving the **Ru1-Ru4** (1.5 mM) in 60% DMSO-d_6, and it was diluted with PBS buffer solution (pH ≈ 7.2). ^{1}H NMR spectra were recorded after various time intervals at 37 °C.

Cell culture

Both human tumor cells (cervical carcinoma HeLa cells and lung cancer A549 cells) were obtained from Shanghai Institute of Biochemistry and Cell Biology (SIBCB) and were grown in Dubelco's Modified Eagle Medium (DMEM). All media were supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin solution. All cells were grown at 37 °C in a humidified incubator under 5% CO_2 atmosphere.

Viability assay (MTT assay)

After plating 5000 cells per well in 96-well plates, the cells were preincubated in drug-free media at 37 °C for 24 h before adding different concentrations of the compounds to be tested. In order to prepare the stock solution of the drug, the solid compound was dissolved in DMSO. This stock was further diluted using cell culture medium until working concentrations were achieved. The drug exposure period was 24 h. Subsequently, 15 μL of 5 mg mL^{-1} MTT solution was added to form a purple formazan. Afterwards, 100 μL of dimethyl sulfoxide (DMSO) was transferred into each well to dissolve the purple formazan, and results were measured using a microplate reader (DNM-9606, Perlong Medical, Beijing, China) at an absorbance of 570 nm. Each well was triplicated and each experiment repeated at least three times. IC_{50} values quoted are mean ± SEM.

Cellular localization and uptake mechanism assay

Two Photon Laser Scanning Microscope (*/LSM/880NLO) is produced at Carl Zeiss AG, Germany. LTDR (Life Technologies, USA), MTDR (Life Technologies, USA), CCCP (Sigma Aldrich, USA), chloroquine (Sigma Aldrich, USA) were used as received. A549 cells were seeded into 35 mm dishes (Greiner, Germany) for confocal microscopy. After cultured overnight, the cells were incubated with **Ru3** ($1.0 \times IC_{50}$) for 1 h. The treated cells were observed immediately under a confocal microscope with excitation at 488 nm. For colocalization studies, the cells were incubated with **Ru3** ($1.0 \times IC_{50}$) for 1 h. Subsequently, the medium was replaced with staining medium containing MTDR/LDTR and stained for another 20 min. The cells were washed twice with PBS, and then viewed immediately under a confocal microscope. Investigation of drug entry pattern: Cells were incubated with **Ru3** ($1.0 \times IC_{50}$) at 37 °C and 4 °C for 1 h, after which media was replaced with staining medium containing CCCP (10.0 μM, 1 h) chloroquine (50.0 μM, 1 h) and re-stained for 15 min. The cells were washed twice with PBS, and then viewed immediately under a confocal microscope.

Lysosomal membrane permeabilization assay

A549 cells seeded into six-well plate (Corning) were exposed to **Ru3** at the indicated concentrations for 12 h. The cells were then washed twice with PBS and incubated with AO (5.0 μM) at 37 °C for 15 min. The cells were washed twice with PBS and visualized by confocal microscopy (LSM/880NLO). Emission was collected at 510 ± 20 nm (green) and 625 ± 20 nm (red) upon excitation at 488 nm.

Induction of apoptosis

Flow cytometry analysis of apoptotic populations of the cells caused by exposure to Ruthenium(II) compounds were carried out using the Annexin V-FITC Apoptosis Detection Kit (Beyotime Institute of Biotechnology, China) according to the supplier's instructions. Briefly, A549 cancer cells (1.5×10^6/2 mL per well) were seeded in a six-well plate. Cells were preincubated in drug-free media at 37 °C for 24 h, after which **Ru3** was added at concentrations of $1.0 \times IC_{50}$, $2.0 \times IC_{50}$ and $3.0 \times IC_{50}$ of **Ru3** against A549 cancer cells. After 24 h of exposure, cells were collected, washed once with PBS, and suspended in 195 μL of annexin V-FITC binding buffer which was then added to 5 μL of annexin V-FITC and 10 μL of PI, and then incubated at room temperature in the dark for 15 min. Subsequently, the buffer placed in an ice bath in the dark. The samples were analyzed by a flow cytometer (ACEA NovoCyte, Hangzhou, China).

Cell cycle analysis

A549 cells at 1.5×10^6 per well were seeded in a six-well plate. Cells were preincubated in drug-free media at 37 °C for 24 h, after which **Ru3** was added at concentrations of 1.0 ×

IC_{50}, 2.0× IC_{50} and 3.0 × IC_{50} of **Ru3** against A549 cancer cells. After 24 h of exposure, supernatants were removed by suction and cells were washed with PBS. Finally, cells were harvested using trypsin-EDTA and fixed for 24 h using cold 70% ethanol. DNA staining was achieved by suspending the cell pellets in PBS containing propidium iodide (PI) and RNAse. Cell pellets were washed and suspended in PBS before being analyzed in a flow cytometer (ACEA NovoCyte, Hangzhou, China) using excitation of DNA-bound PI at 488 nm, with emission at 585 nm. Data were processed using NovoExpress™ software. The cell cycle distribution is shown as the percentage of cells containing G_0/G_1, S and G_2/M DNA as identified by propidium iodide staining.

Mitochondrial membrane assay

Analysis of the changes of mitochondrial potential in cells after exposure to Ruthenium(II) compounds was carried out using the mitochondrial membrane potential assay kit with JC-1 (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. Briefly, 1.5×10^6 A549 cancer cells were seeded in six-well plates left to incubate for 24 h in drug-free medium at 37 °C in a humidified atmosphere. Drug solutions, with the concentration changed from 0.5×IC_{50} to 3.0×IC_{50} of **Ru3** against A549 cells, were added in triplicate, and the cells were left to incubate for a further 24 h under similar conditions. Supernatants were removed by suction, and each well was washed with PBS before detaching the cells using trypsin-EDTA. Staining of the samples was done in flow cytometry tubes protected from light, incubating for 30 min at ambient temperature. The samples were immediately analyzed by a flow cytometer (ACEA NovoCyte, Hangzhou, China). For positive controls, the cells were exposed to carbonyl cyanide 3-chlorophenylhydrazone, CCCP (5.0 μM), for 20 min. Data were processed using NovoExpress™ software.

ROS determination

Flow cytometry analysis of ROS generation in A549 cells caused by exposure to compounds was carried out using the Reactive Oxygen Species Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the supplier's instructions. Briefly, 1.5×10^6 A549 cells per well were seeded in a six-well plate. Cells were preincubated in drug-free media at 37 °C for 24 h in a 5% CO_2 humidified atmosphere, and then drugs were added at concentrations of 0.25×IC_{50} and 0.5×IC_{50}. After 24 h of exposure, cells were washed twice with PBS and then incubated with the DCFH-DA probe (10.0 μM) at 37 °C for 30 min, and then washed triple immediately with PBS. Fluorescence intensity was analyzed by flow cytometry (ACEA NovoCyte, Hangzhou, China). Data were processed using NovoExpress™ software.

Reaction with NADH

The reaction of **Ru1-Ru4** (ca. 1 μM) with NADH (ca. 100 μM) in 20% CH_3OH/80% H_2O (*v/v*) was monitored by UV-vis at 25 °C after various time intervals. TONS were calculated from the difference in NADH concentration after 8 h divided by the concentration of Ru catalyst. The concentration of NADH was obtained using the extinction coefficient $\varepsilon_{339} = 6220\ M^{-1}\ cm^{-1}$.

BSA binding experiments

The titration experiments including UV-vis absorption and fluorescence quenching were performed at constant concentration of BSA. BSA stock solution was prepared in Tris buffer (5 mM Tris–HCl/10 mM NaCl at pH = 7.2) and stored at 4 ºC. All spectra were recorded after each successive addition of the compounds and incubation at room temperature for 5 min to complete the interaction. In the UV–vis absorption titration experiment, BSA solution (2.5 mL, 1.0×10^{-5} M) was titrated by successive additions of the stock solutions of Ru compounds (1.0×10^{-6} M) and the changes in the BSA absorption were recorded after each addition. The fluorescence emission spectra of BSA in the absence and presence of Ru compound was also recorded with excitation at 285 nm. The concentrations of the Ru compounds were 0–10.0 μM, and the concentration of BSA was fixed at 10.0 μM. Synchronous fluorescence spectra of BSA with various concentrations (0–10.0 μM) were obtained from 240 to 340 nm when $\Delta\lambda$ = 60 nm and from 260 to 360nm when $\Delta\lambda$ = 15 nm.

The probable quenching mechanism can be interpreted by the classical *Stern-Volmer* equation:

$$F_0/F = 1 + K_{sv}[Q] = 1 + K_q\tau_0[Q] \qquad (1)$$

Where F_0 and F are the steady-state fluorescence intensities of BSA without and with quencher, respectively, $[Q]$ is the total concentration of quencher, K_q is the Stern-Volmer quenching rate constant, and τ_0 is the average lifetime of the fluorophore without the quencher (10^{-8} s). K_{sv} is calculated by the slope of F_0/F *vs* the concentration of compounds (Figure S13). The binding constant (K_b) and numbers of compound bound to BSA (n) are calculated using *Scatchard* equation:

$$\log\left[(F_0 - F)/F\right] = \log K_b + n\log[Q] \qquad (2)$$

As shown in Figure S14, K_b and n are the data after linear fitting of *log((F$_0$ - F)/F) vs log[Q]*. The values of n for these compounds are almost the same (~1), which are consistent with the results that the binding between BSA and Ru^{II} compounds can affect the conformation of tryptophan microregion. The binding constants (K_b) of compound **Ru3** is higher than other compounds, which are also consistent with the result of cytotoxicity test. Above all, BSA can be an excellent carrier for the delivery of these compounds in *vivo*.

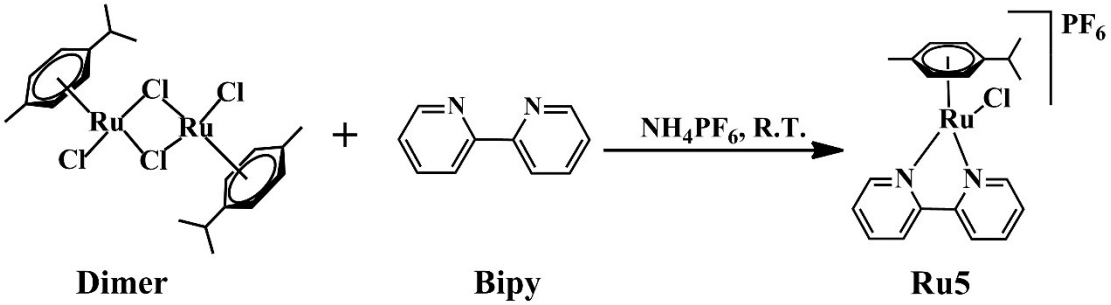
Synthesis of Schiff base pro-ligands (L1-L4)

The chelating pro-ligands were synthesized according to the literature[1]. The general synthesis process is as follows: Amino-substitued triphenylamine derivates (10 mmol) was dissolved in ethanol (60 mL). 2-Formyl pyridine/chinolin (15 mmol) was added dropwise under stirring, and subsequently added 2 drops formic acid, and a solid precipitated immediately. The mixture was heated under reflux for 5 h, and then cooled to room temperature, suction filtered, and the solid was recrystallized from ethanol to give products.

Synthesis of the $[(\eta^6\text{-}p\text{-cymene})RuCl_2]_2$ (Dimer)

Dimers were prepared according to literature methods[2]: Ruthenium chloride hydrate, *p*-cymene, ethanol were stirred at 80℃ for 6 h to obtain a crude product and filtered to obtain a final product.

Synthesis of the $[(\eta^6\text{-p-cymene})Ru(Bipy)Cl]PF_6$ (Ru5)



Scheme S1 Synthesis process of **Ru5** compound.

Ru5 was prepared according to literature methods[3]: $[(\eta^6\text{-}p\text{-cymene})RuCl_2]_2$ (**Dimer:** 48.9 mg, 0.08 mmol, 1 *equiv*), Bipy (0.16 mmol, 2 *equiv*) and ammonium hexafluorophosphate (130.4 mg, 0.7 mmol, 10 *equiv*) in methanol (50 mL) was stirred at room temperature (R.T.) overnight. The precipitate ammonium hexafluorophosphate was filtered through celite, and the solvent was concentrated to about 5.0 mL under reduced pressure and maintained at 253K for 12 h, filtered and washed with cold methanol and diethyl ether.

Reference

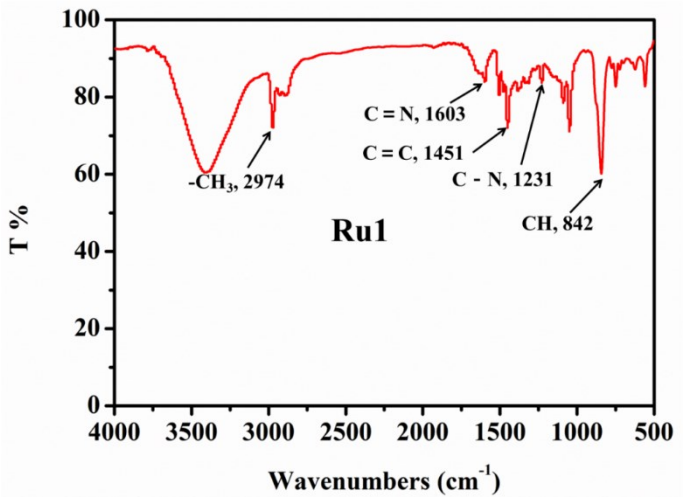
1. S. Chen, X. Liu, X. Ge, Q. Wang, Y. Xie, Y. Hao, Y. Zhang, L. Zhang, W. Shang, Z. Liu, Lysosome-targeted iridium(III) compounds with pyridine-triphenylamine Schiff base ligands: Syntheses, antitumor applications and mechanisms, *Inorg. Chem. Front.,* 2020, **7**, 91-100.

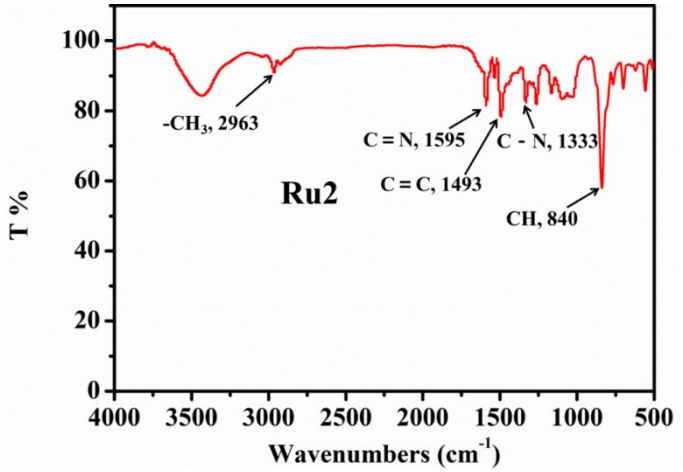
2. Y. Yang, L. Guo, Z. Tian, X. Liu, Y. Gong, H. Zheng, X. Ge, Z. Liu, Imine-N-heterocyclic carbene as versatile ligands in ruthenium(II) *p*-cymene anticancer complexes: A structure-activity relationship study, *Chem.-Asian J.,* 2018, **13**, 2923-2933.

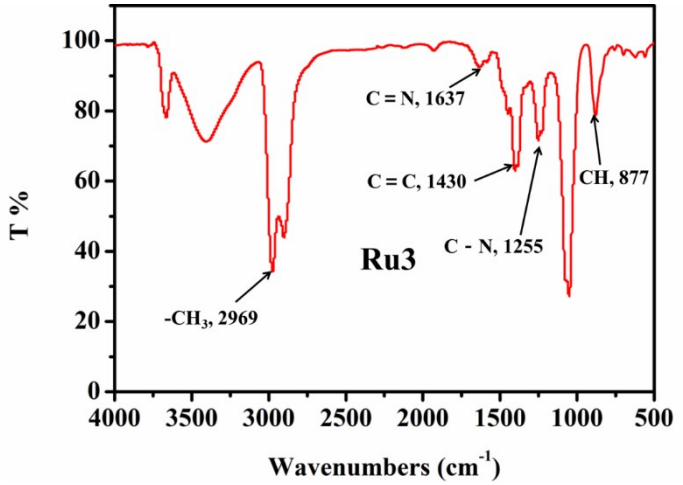
3. X. Ge, X. Liu, Z. Tian, S. Chen, X. Liu, L. Guo, P. Gong, B. Ling, X. Yuan, Z. Liu, Half-sandwich Ruthenium (II) complexes with triphenylamine modified dipyridine skeleton and application in biology/luminescence imaging, *Appl. Organomet. Chem.,* 2019, **33**, e5171.

Figures and tables

FT-IR spectra







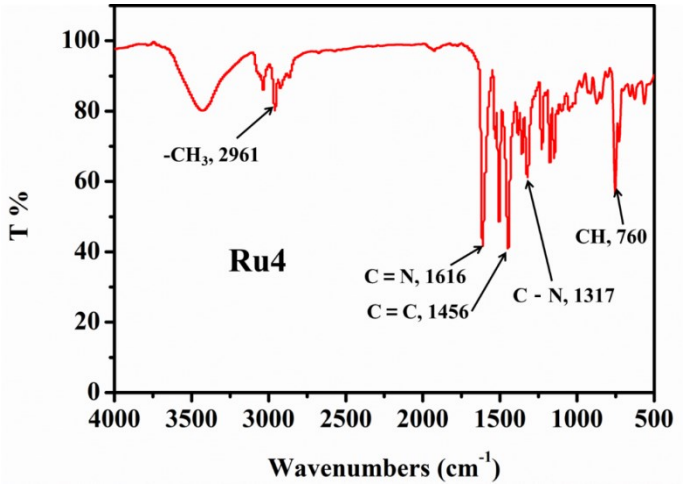
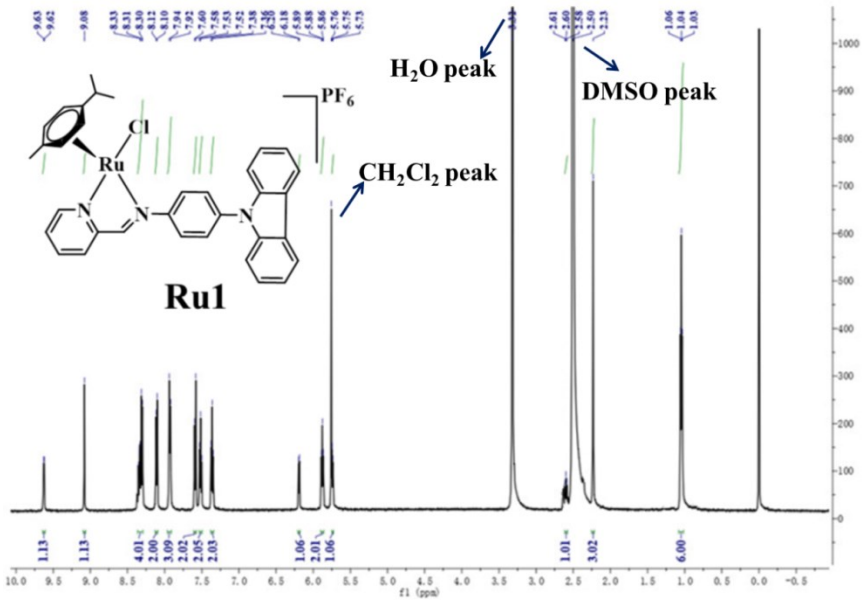
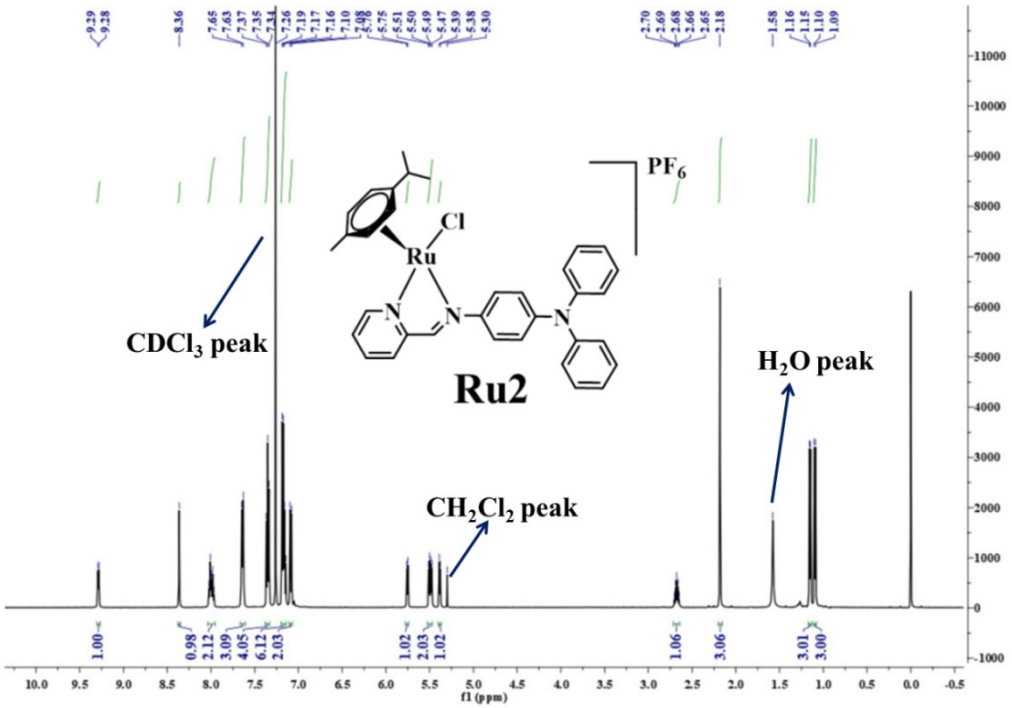


Figure S1. FT-IR spectra of **Ru1-Ru4.**

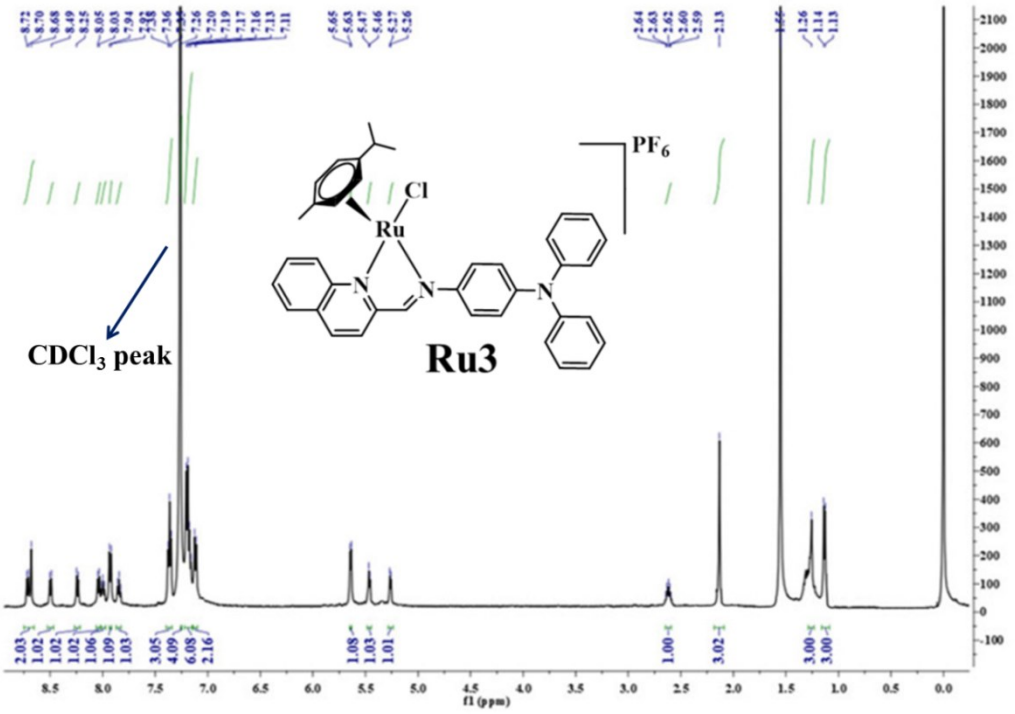
^{1}HNMR Spectra



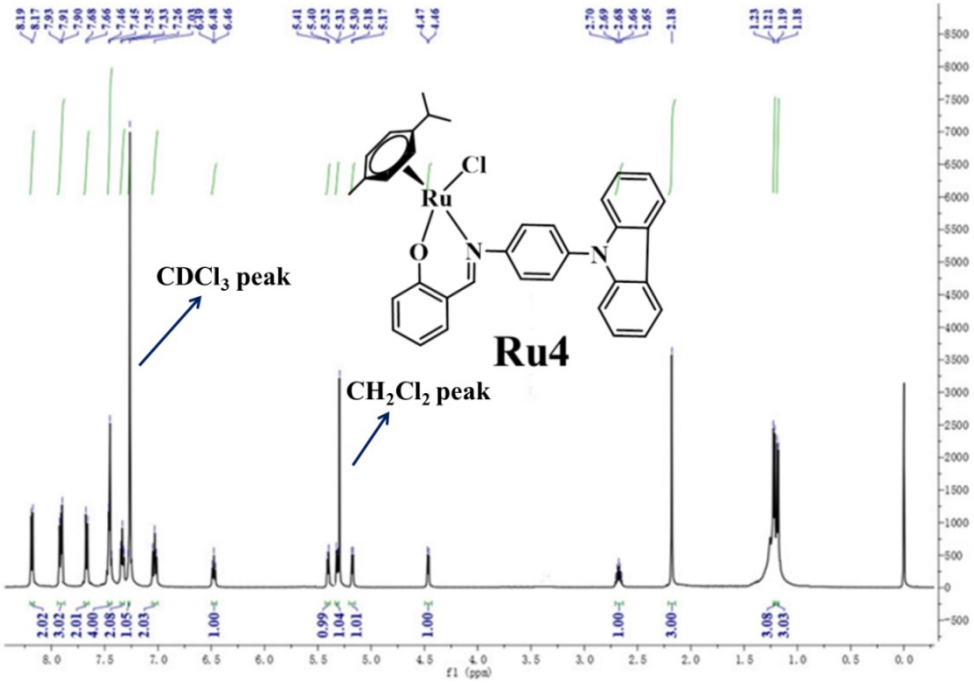
^{1}H NMR (500 MHz) spectrum of **Ru1** in DMSO.



^{1}H NMR (500 MHz) spectrum of **Ru2** in $CDCl_3$.



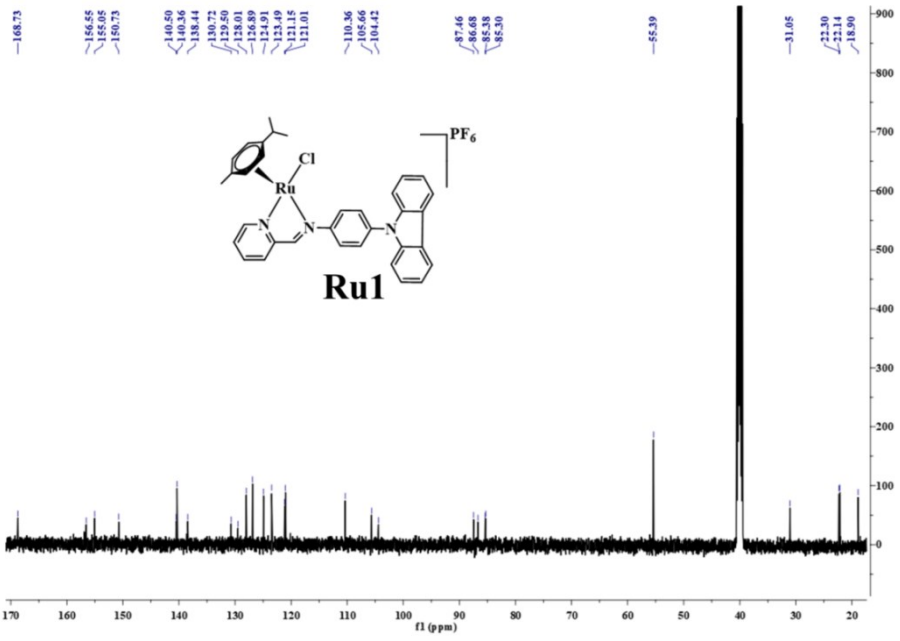
^{1}H NMR (500 MHz) spectrum of **Ru3** in $CDCl_3$.



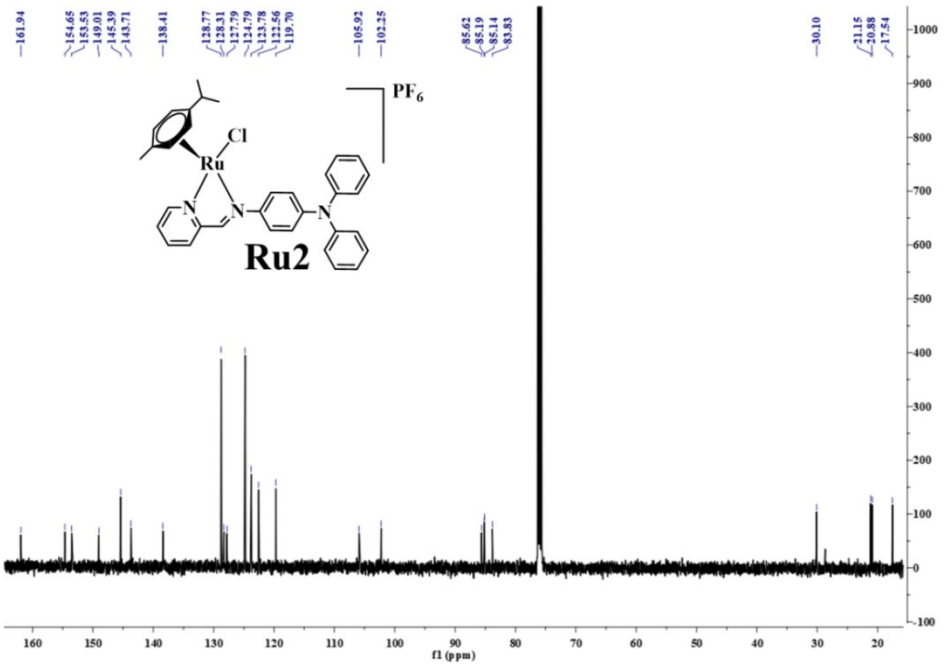
^{1}H NMR (500 MHz) spectrum of **Ru4** in $CDCl_3$.

Figure S2. ^{1}H NMR (500 MHz) of **Ru1- Ru4**

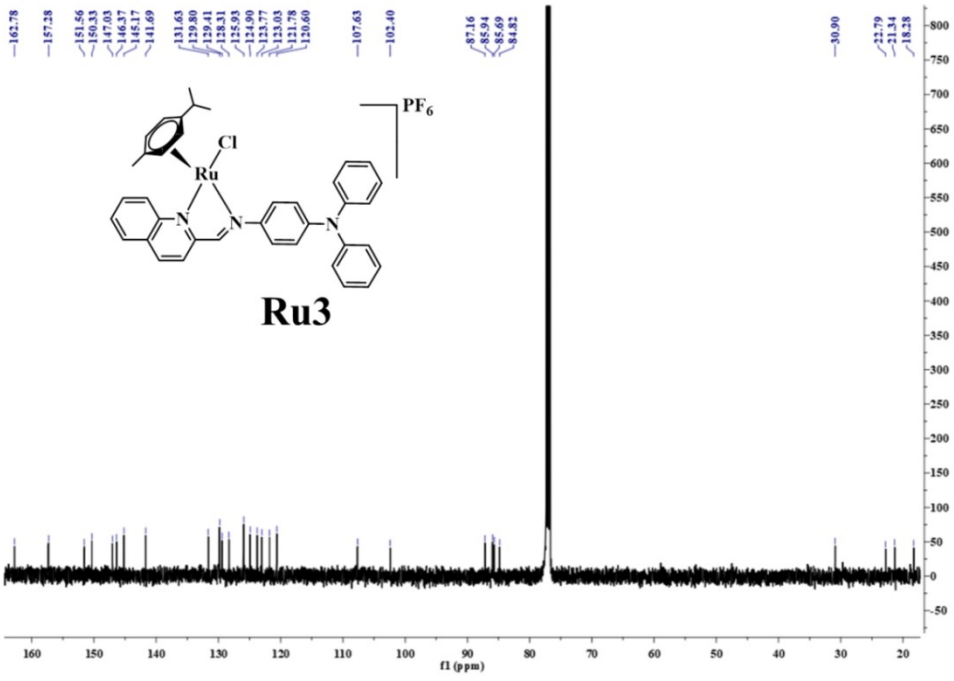
^{13}C NMR spectra



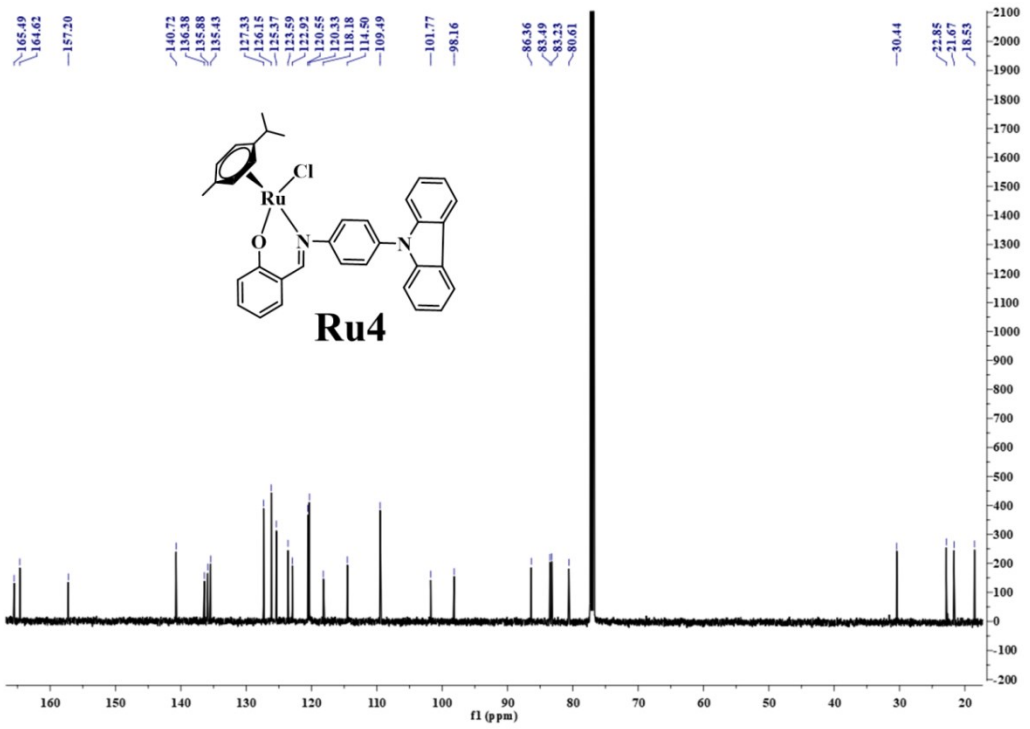
^{13}C NMR (126 MHz) spectrum of **Ru1** in DMSO.



^{13}C NMR (126 MHz) spectrum of **Ru2** in $CDCl_3$.



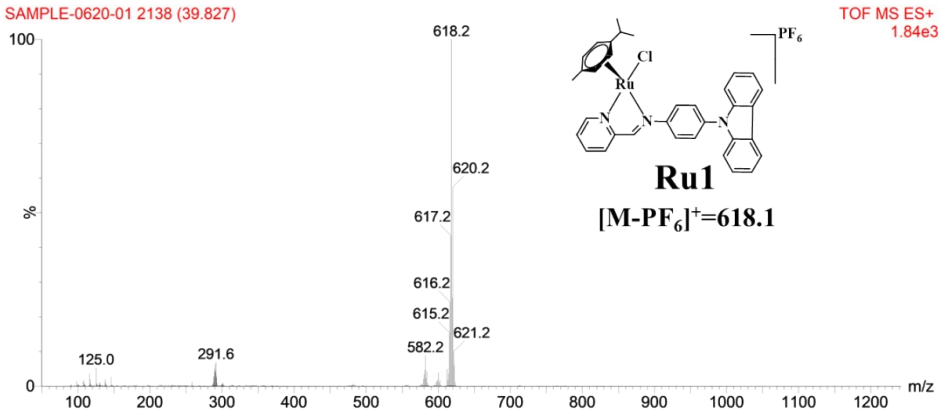
^{13}C NMR (126 MHz) spectrum of **Ru3** in $CDCl_3$.



^{13}C NMR (126 MHz) spectrum of **Ru4** in $CDCl_3$.

Figure S3. ^{13}C NMR (126 MHz) of **Ru1- Ru4**

ESI-MS



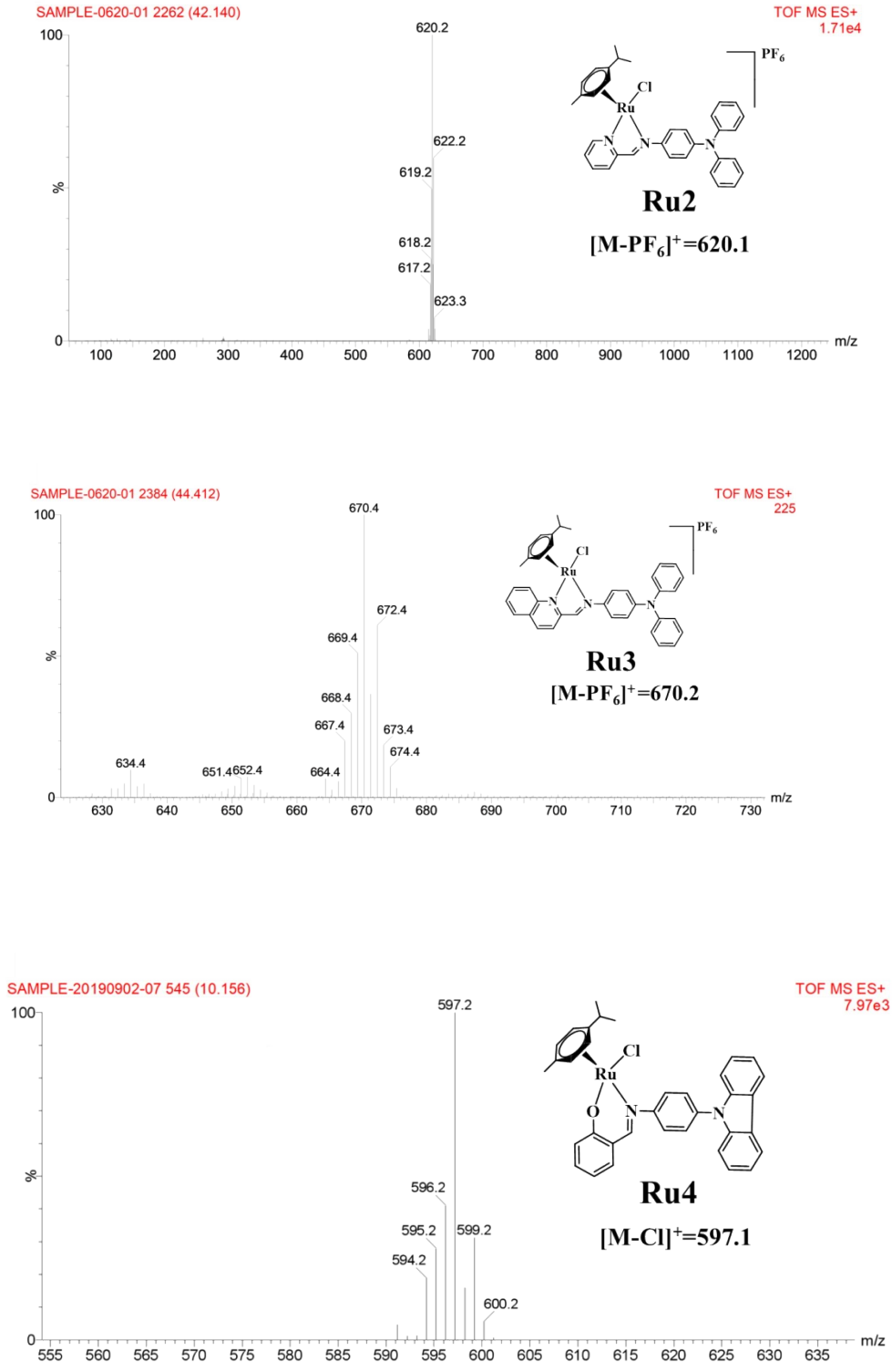


Figure S4. Mass spectra of **Ru1- Ru4**

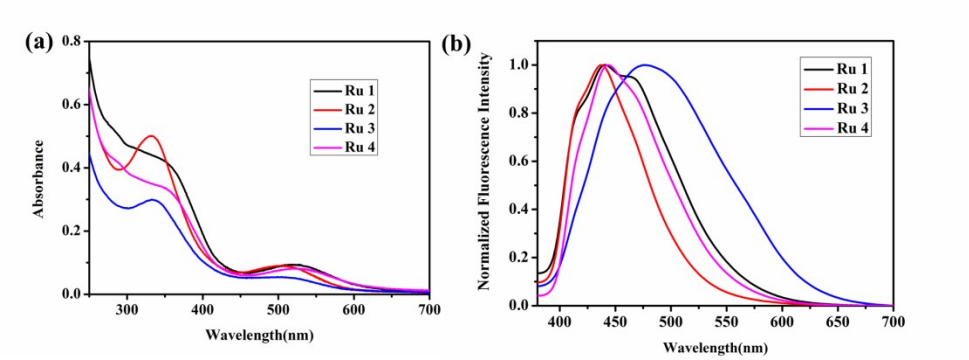
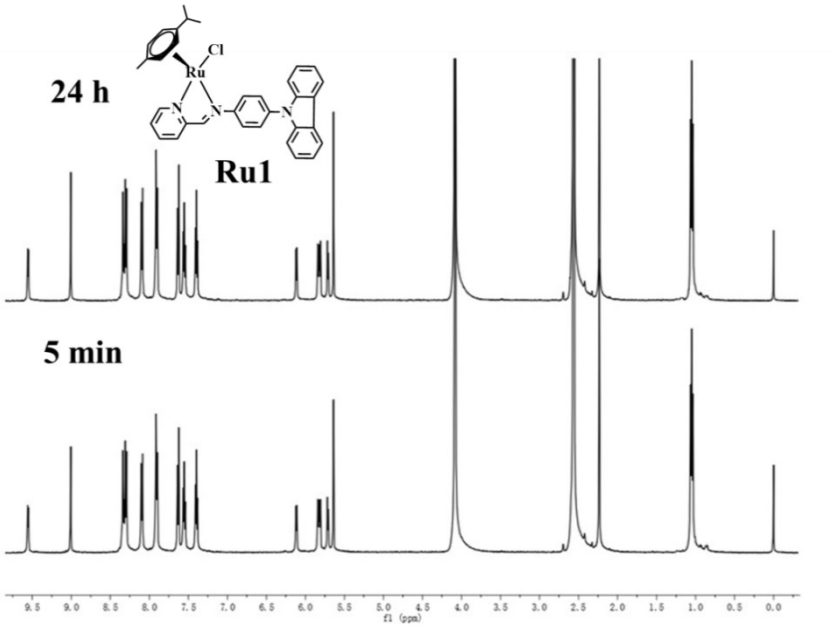
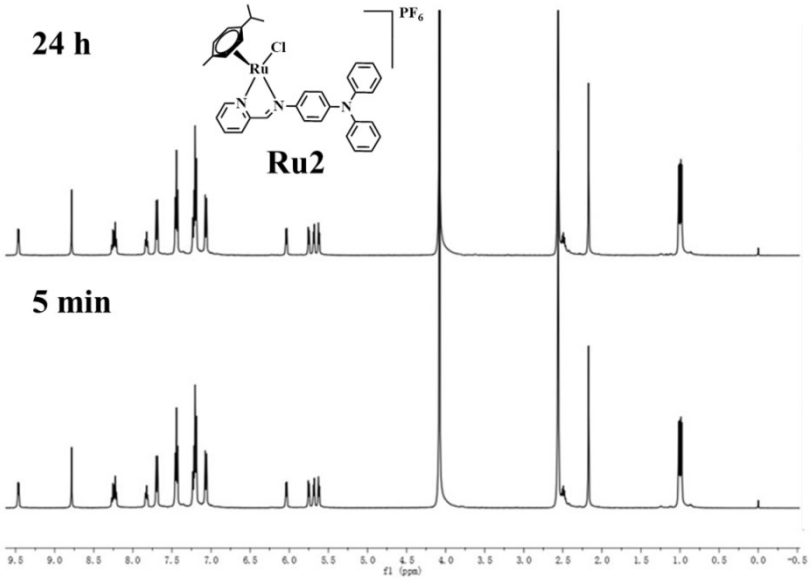


Figure S5. (a) UV-vis spectra of **Ru1-Ru4** (20.0 μM) in CH_2Cl_2 at 298 K. (b) Normalized emission spectra of **Ru1- Ru4** (20.0 μM) in CH_2Cl_2 at 298 K. (λ_{ex} = 350 nm).





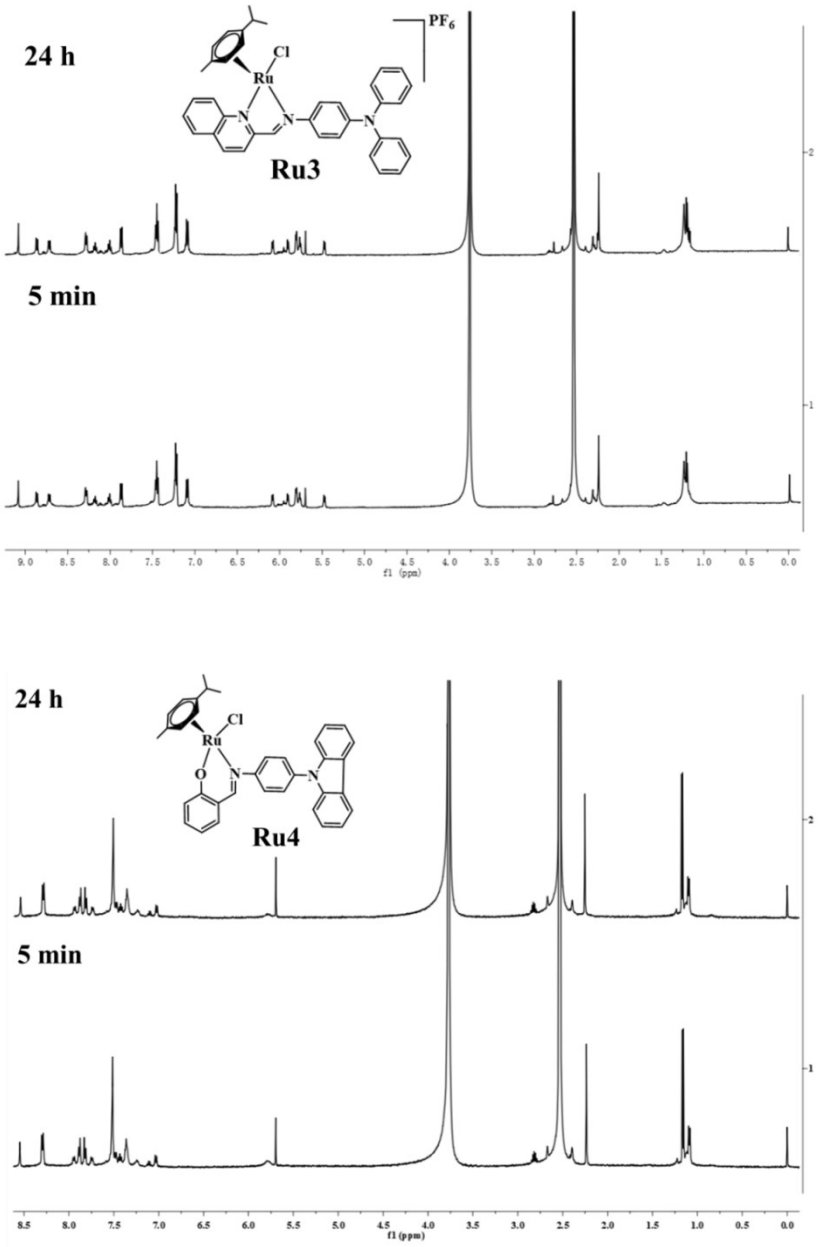


Figure S6. ^{1}H NMR spectra show the stability of **Ru1-Ru4** (1.5 mM) in 60% DMSO-d_6/40% PBS (*v*/*v*) at 37 °C (PBS: pH ≈ 7.2, PBS is prepared from D_2O).

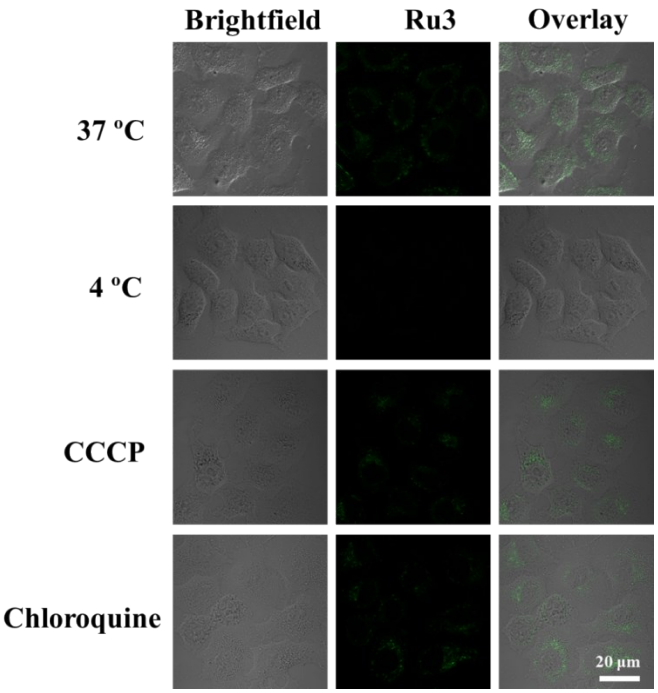


Figure S7. Confocal images of A549 cells after incubation with **Ru3** (1.0 × IC_{50}) under different conditions. Control cells without inhibitor at 37 °C; 4 °C; addition of CCCP (10.0 μM) at 37 °C; addition of chloroquine (50.0 μM) at 37 °C. **Ru3** was excited at 488 nm and emission was collected at 550 ± 30 nm. Scale bar: 20 μm.

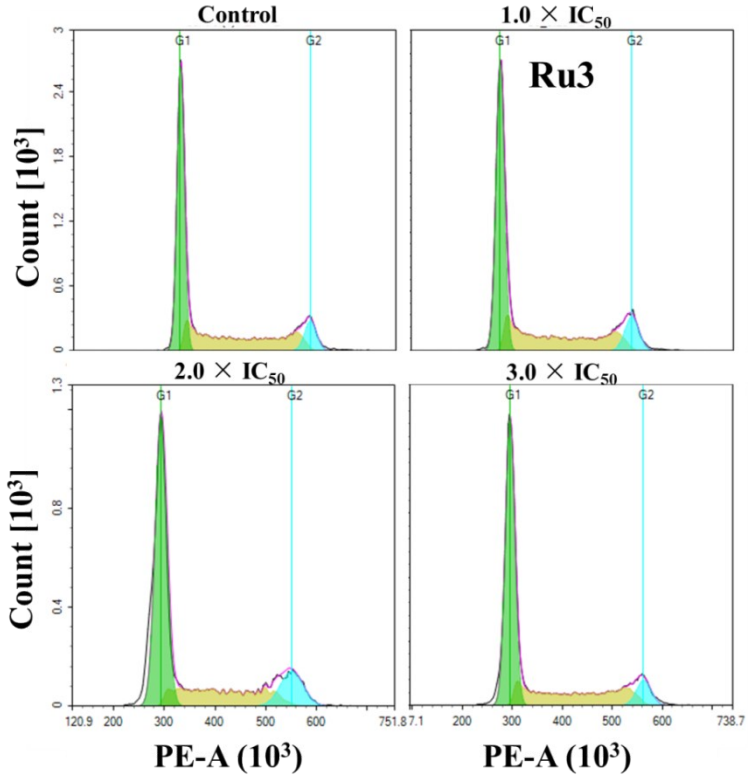


Figure S8. Flow cytometry data for cell cycle distribution of A549 cancer cells exposed to **Ru3** for 24 h with the concentration of 1.0 × IC_{50}, 2.0 × IC_{50} and 3.0 × IC_{50}. Cell staining for flow cytometry was carried out using PI/RNase. Cell populations in each cell cycle phase for control.

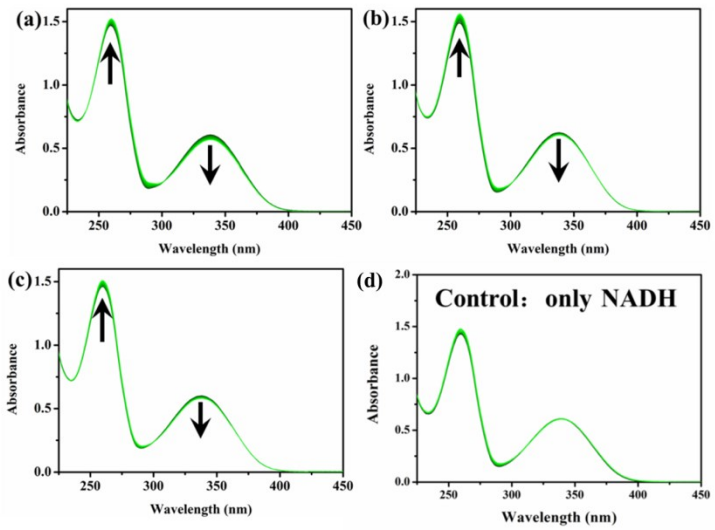


Figure S9. UV-vis spectra of NADH (100.0 μM) with **Ru1 (**a**)**, **Ru2 (**b**)**, **Ru4 (**c**)** and Control **(**d**)** (1.0 μM) in 20% CH_3OH/80% H_2O (*v:v*) at 298 K for 8 h.

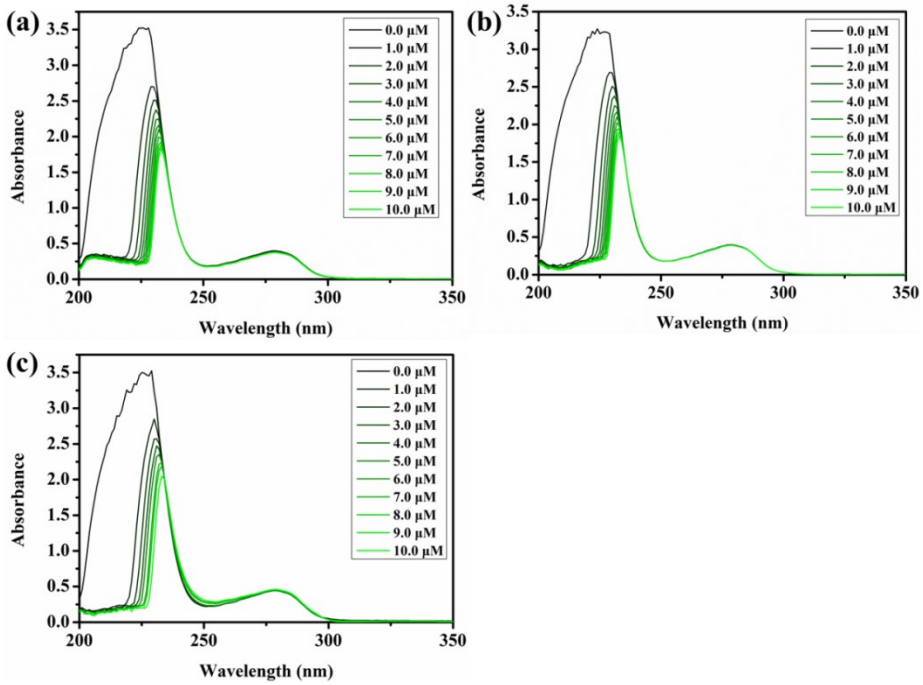


Figure S10. UV-vis spectra of BSA (10.0 μM) in 5 mM Tris-HCl/10 mM NaCl buffer solution (pH: 7.2) upon addition of the **Ru1** (a), **Ru2** (b) and **Ru4**(c) (0.0-10.0 μM).

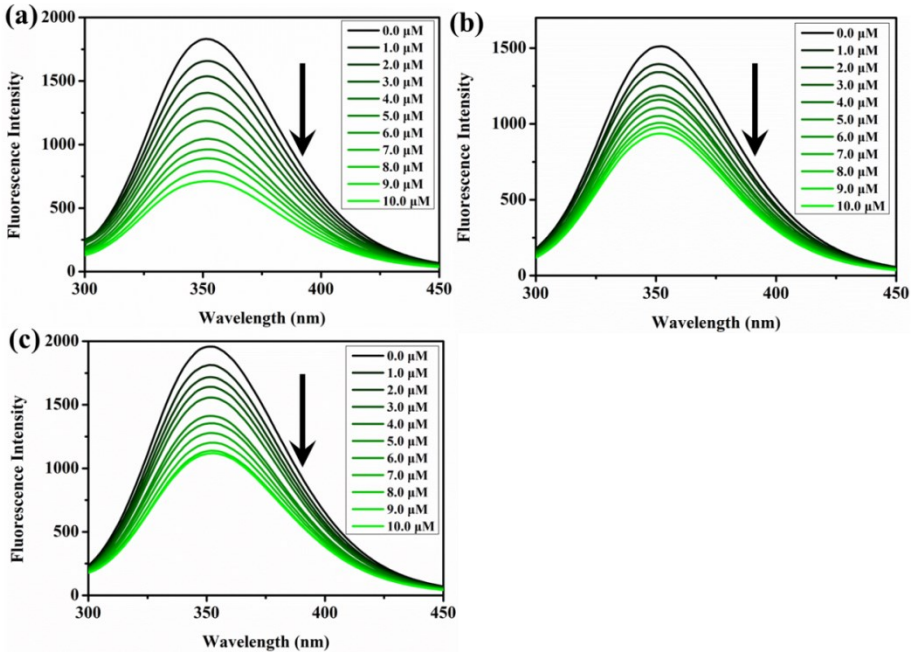


Figure S11. Fluorescence spectra of BSA (10.0 μM; λex = 280 nm; λem = 350 nm) in the absence and presence of **Ru1** (a), **Ru2** (b) and **Ru4** (c) (0-10.0 μM). The arrows show the intensity changes with the increase of Ru(II) compounds.

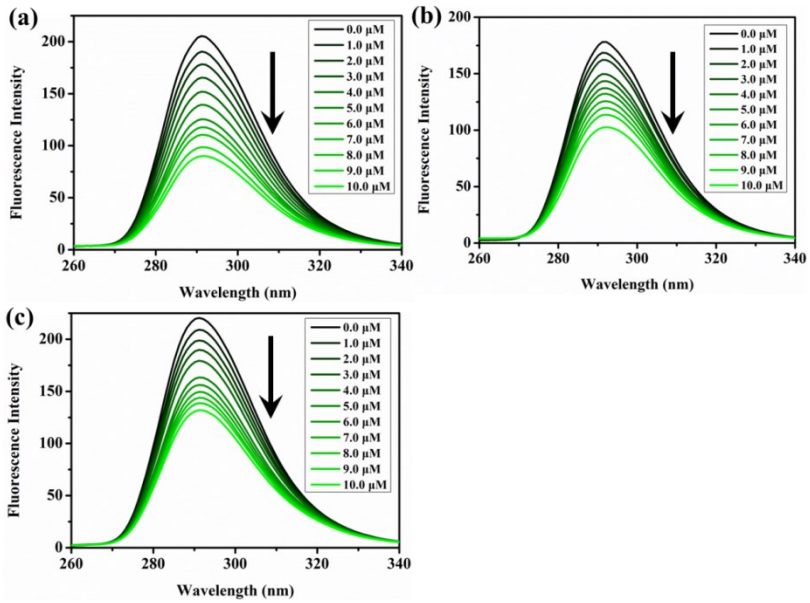


Figure S12. Synchronous spectra of BSA (10.0 μM, 50.0 mM Tris-HCl, 50.0 mM NaCl, pH = 7.2) with the increase of **Ru1** (a), **Ru2** (b) and **Ru4** (c) (0.0-10.0 μM) with a wavelength difference of $\Delta\lambda$ = 15 nm.

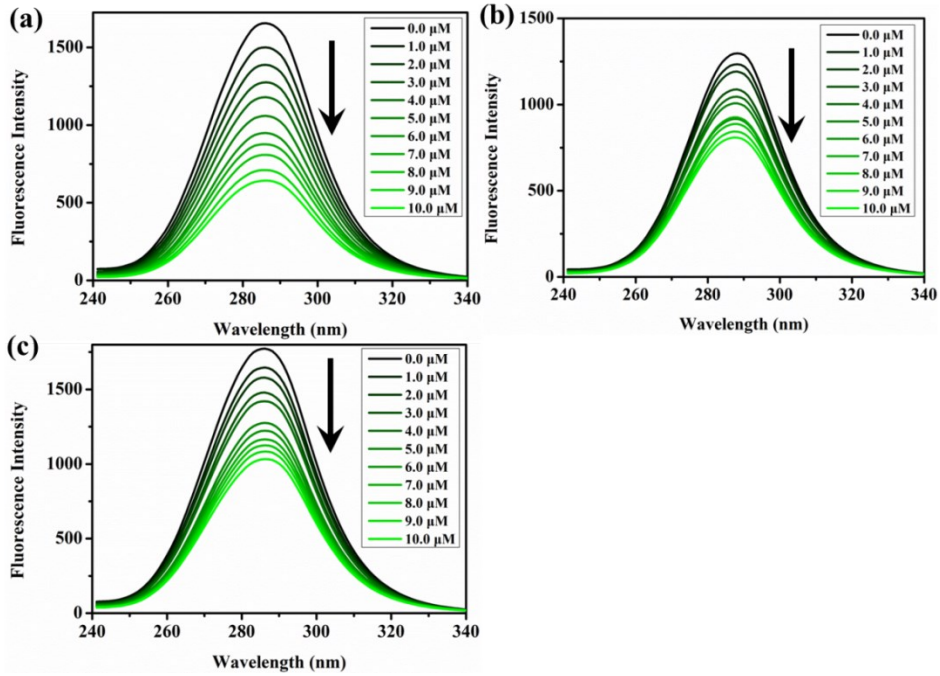


Figure S13. Synchronous spectra of BSA (10.0 μM, 50.0 mM Tris-HCl, 50.0 mM NaCl, pH = 7.2) with the increase of **Ru1** (a), **Ru2** (b) and **Ru4** (c) (0.0-10.0 μM) with a wavelength difference of $\Delta\lambda$ = 60 nm.

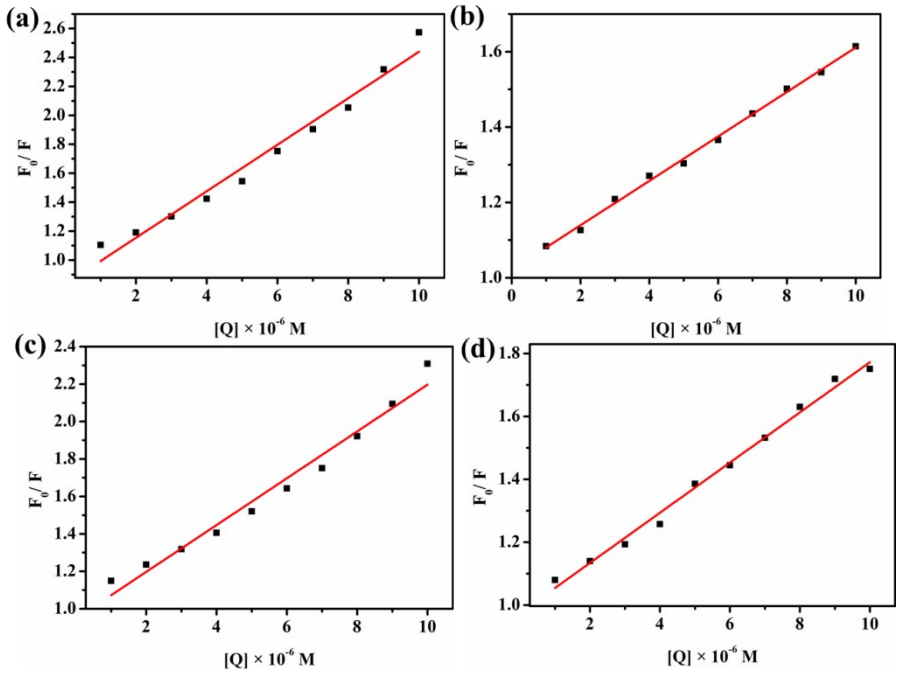


Figure S14. Stern–Volmer plots of F_0/F against the concentration of **Ru1** (a), **Ru2** (b), **Ru3** (c) **and Ru4** (d) (0-10.0 μM).

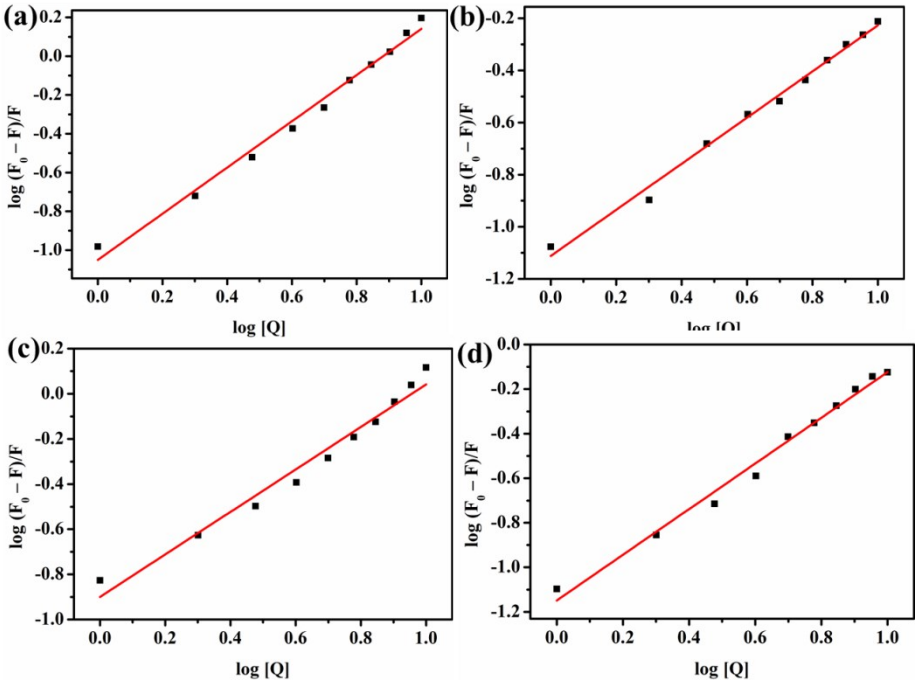


Figure S15. Plots of log[(F_0-F)/F] *vs* log[Q] for the interaction of BSA with compounds **Ru1** (a), **Ru2** (b), **Ru3** (c) and **Ru4** (d) (0-10.0 μM).

Table S1 Crystallographic data for [(η^6-*p*-cymene)Ru(L2)Cl]PF_6 (**Ru2**)

formula	$C_{34}H_{33}N_3ClF_6PRu$
MW	807.58
cryst size (mm)	0.40*0.13*0.05
λ(Å)	0.71073
temp (K)	298
cryst syst	Monoclinic
space group	P2(1)
a (Å)	17.9852(16)
b (Å)	9.8972(8)
c (Å)	19.7318(17)
α (°)	90
β (°)	92.578(2)
γ (°)	90
vol (Å^3)	3508.8(5)
Z	4
density (calc) (Mg·m^{-3})	1.529
abs coeff (mm^{-1})	0.706
F(000)	1636
θ range (deg)	2.26 to 26.24
index ranges	-19 ≤ h ≤ 21, -11 ≤ k ≤ 11, -23 ≤ l ≤ 19
reflns collected	18024
indep reflns	11872 [R(int) = 0.0590]

data / restraints / params	11872 / 485 / 892
final R indices [I > 2σ(I)]	R1 = 0.0598, wR2 = 0.1268
GOF	0.882
largest diff peak and hole	0.883 and -0.475

Table S2. Selected bond lengths (Å) and angles (°) for **Ru2**

Ru−C(benzene)	2.130(11)
	2.192(11)
	2.197(12)
	2.202(11)
	2.220(14)
	2.239(11)
Ru−C(centroid)	1.686
Ru-N_1	2.094(9)
Ru-N_2	2.088(9)
Ru-Cl	2.387(3)
N_2-Ru-N_1	76.6(4)
N_2-Ru-Cl	85.7(2)
N_1-Ru-Cl	85.4(3)

Table S3. Flow cytometry analysis to determine the percentages of apoptotic cells using Annexin V-FITC/PI staining after exposure to **Ru3**.

	Concentration	Population (%)			
		Viable	**Early apoptosis**	**Late apoptosis**	**Non-viable**
Control		99.93±6.33	0.00±0.09	0.06±0.11	0.01±0.06
Ru3	1.0 × IC_{50}	90.64±2.36	0.31±0.03	6.26±1.20	2.79±0.10
	2.0 × IC_{50}	88.73±1.93	0.98±0.32	8.55±1.30	1.73±1.03
	3.0 × IC_{50}	24.11±0.96	0.20±0.16	62.38±4.63	13.32±0.55

Table S4. Cell cycle analysis carried out by flow cytometry using PI staining after exposure to **Ru3**.

	Concentration	Population (%)			
		G_0/G_1 phase	**S phase**	**G_2/M phase**	**Sub-G1phase**
Control		52.43±1.55	36.57±1.09	8.87±0.17	0.20±0.02
Ru3	1.0 × IC_{50}	52.75±1.61	34.57±3.05	10.37±0.19	0.31±0.03
	2.0× IC_{50}	59.46±1.85	25.60±2.61	13.85±0.17	0.62±0.05
	3.0× IC_{50}	58.69±1.65	30.49±1.65	8.00±0.29	0.18±0.09

Table S5. The mitochondrial membrane polarization of A549 cells induced by **Ru3.**

	Concentration	Population (%)	
		JC-1 Aggregates	**JC-1 Monomers**
Ru3	0. 5 × IC_{50}	88.61±0.52	11.39±0.95
	1.0 × IC_{50}	87.83±0.30	12.17±0.73
	2.0 × IC_{50}	84.60±0.53	15.40±0.96
	3.0 × IC_{50}	67.54±0.99	32.46±0.56
	Negative Control	96.20±056	3.80±0.96
	Positive Control	21.84±0.22	78.16±0.68

Table S6. The values of K_{sv}, K_b , K_q and n for **Ru1**-**Ru4**.

	K_{sv} (10^5 M^{-1})	K_q (10^{13} M^{-1} s^{-1})	K_b (10^4 M^{-1})	n
Ru1	1.61±0.12	1.61	8.90	1.19
Ru2	0.59±0.09	0.59	7.73	0.89
Ru3	1.25±0.10	1.25	12.58	0.94
Ru4	0.80±0.11	0.80	7.10	1.03