Electronic Supplementary Informations

for

Selenium-containing ruthenium complex as cancer radiosensitizer, rational design and important role of ROS-mediated signalling

Experimental section

Material

4-Acetylpyridine, benzaldehyde, ruthenium trichloride and selenium dioxide were purchased from Shanghai Aladin Reagent Company. 5,6-diamino-1,10-phenanthroline was purchased from Jinan Camolai Trading Company . All the chemicals and solvents were analytically pure. Thiazolyl blue tetrazolium bromide (MTT), propidium iodide (PI), 4,6-diamidino-2-phenylindole (DAPI) was purchased from Sigma-Aldrich. All antibodies used in this study were purchased from Cell Signaling Technology (Beverly, MA). The water used in cellular experiments was ultrapure, supplied by a Milli-Q water purification system from Millipore. All radiation experiments were performed by Elekta Precise linear accelerator was provided by Wu Jing Zong Dui Hospital of Guangdong Province, Guangzhou, China.

Cell culture, MTT assay and determination of growth inhibition

A375 human melanoma cells and HK-2 human kidney cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). All cells were cultured in DMEN with 10% FBS, penicillin (100 units/ml) and streptomycin (50 units/ml) at 37 ℃ in CO_2 incubator (95% relative humidity, 5% CO_2). The cell viability with and without radiation was examined by MTT assay.[1] Cells were seeded in 96-wells plate for 24 h (the density of A375 and HK-2 cells is 2×10^4 and 4×10^4, respectively), cultured with various doses of complexes for 6 h. After this, cells received a dose of 8 Gy (6 MeV) radiations, followed by incubation at 37 ℃ for another 66 h. After incubation, 20 μM per well of MTT solution (5 mg/ml) was added and then incubated for 5 further hours. The medium was replaced with 150 μl DMSO per well. The colour intensity of the solution, which reflects the cell growth condition, was measured at 570 nm by microplate spectrophotometer (Versamax). Cell growth inhibition (%) were determined by $(OD_{control}-OD_{treatment}-OD_{blank})/(OD_{control}-OD_{blank})\times100\%$. The half maximal inhibitory concentration (IC_{50}) was obtained from the plot of growth (%) vs. complex concentrations.

Determination of cellular uptake of Ru complexes.

Intracellular uptake of Ru complexes in A375 and HK-2 cells was quantified by determining Ru concentration using ICP-MS. Briefly, A375 and HK-2 cells were treated with different complexes (10 μM) for different time periods. The cell pellets were counted and digested using a hydrogen nitrate (HNO_3) and perchloric acid ($HClO_4$) mixture (3:1). Finally, the solution was diluted with water to a volume of 10 mL, and then subjected to ICP-MS analysis.[2]

Flow cytometric analysis

Cells were seeded in 60-mm dishes to culture for 24 h (the density of A375 and HK-2 cells is 2×10^4 and 4×10^4, respectively), then incubated with different concentrations of Ru complexes for 6 h. After this, cells received a dose of 8 Gy (6 MeV) radiations and incubated for further 24 h. Cells were trypsinized and washed with PBS, followed by fixing with 70% ethanol overnight at -20℃. The fixed cells were washed with PBS and stained with PI working solution (1.21 mg/ml Tris,700 U/ml RNase, 50.1 μg/ml PI, pH = 8.0) for 4 h in darkness at 4℃. Then cells were determined with Epics XL-MCL folw cytometer (Beckman Coulter, Miami, FL) to analyze cell cycle distribution. Afterwards, use Multicycle software (Phoenix Flow system, San Diego, CA).[3] The cell proportion at G0/G1, S and G2/M phase was represented as DNA histogram. Apoptotic cells were detected by quantifying the Sub-G1 peak in cell cycle pattern.10,000 cells per sample were recorded for each experiment.

Measurement of intracellular reactive oxygen species (ROS) generation

Change of cellular ROS level in A375 cells, which treated with radiation, different concentration of Ru complexes or Ru complexes in combination with radiation, were measured by staining cells with Dihydroethidium (DHE) as our previously reported[3]. More narrowly, A375 and HK-2 **c**ells (10^6 cells per ml) pre-treated with different concentrations of complex for 6 h were irradiated at a dosage of 8 Gy (6 MeV). After this, all cells were incubated with 10 μM DHE at 37 °C for 30 min. Then cells were rinsed with PBS twice and incubated in fresh PBS. The level of cellular ROS was determined by detecting fluorescence intensity of DHE conducted fluorescence microplate reader (excitation and emission wavelength set at 300 nm and 610 nm respectively).

DAPI staining assay

Location of Ru complexes in cells was analysed by DAPI staining assay as our previous report.[4] Briefly, cells were incubated with or Ru complex for 6 h, then washed with PBS for three times, followed by permeabilizing with 0.1% Triton X-100 in PBS at room temperature for 5 min. Then permeabilized

cells were stained with DAPI for 15 min at 37 ℃. After washing with PBS, coverslips were examined on fluorescence microscopy (Nikon Eclipse 80i).

Western blot analysis

The expression level of cellular proteins was examined by western bolt analysis.[6] In briefly, cells were cultured with lysis buffer to extract total cellar protein. The concentration of protein was detected by BSA assay. SDS-PAGE was performed in 10% tricine gels with equal amounts of protein loaded per lane. After electrophoresis, protein were transferred to nitrocellulose membrane and then blocked with 5% nonfat milk for 1 h, followed by incubating with primary corresponding antibodies overnight at 4℃. The membranes were then incubated with secondary antibodies for 1hour at room temperature and wash by TBST for three times. Protein bands were visualized on X-ray film using enhanced chemiluminescence detection regents (Kodak). β-Actin was used to confirm the equal loading and transfer of proteins.

Partition coefficients

The partition coefficient of each complex, defined as log*P* = lg ([solute]octanol / [solute]water), was experimentally examined by using the "shake-flask" method.[7] In briefly, each complex was dissolved in 10 mM PBS buffer (pH 7.4), previously saturated with octanol, to give about 1 mL of a solution with a final concentration of 20 μ M. The same volume of octanol (previously saturated with 10 mM PBS buffer) was then added and the solution was shaken 100 times and equilibrated for 4.5 h. The complex concentration in the aqueous phase was then evaluated by UV−vis spectroscopy, using standard curve method. The evaluation on each complex was repeated for three times

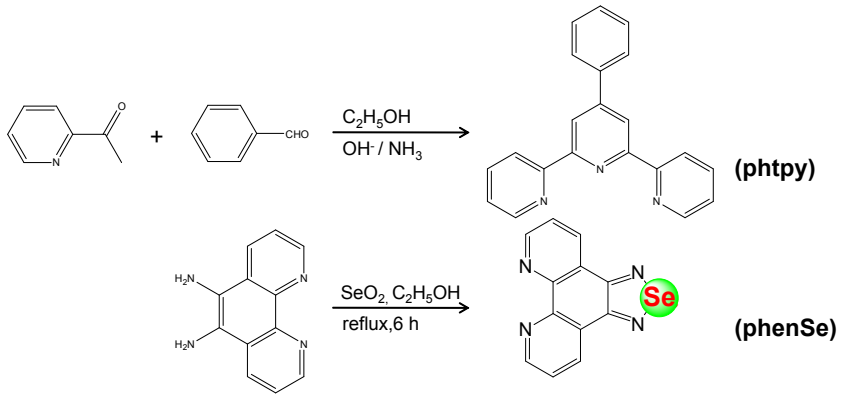
Synthesis of ligands

4-phenyl-2,2':6',2''-terpyridine (phtpy): The synthesis of phtpy were carried out as *Mallesh* et al described[8]. 4-Acetylpyridine (2.42 g, 20.0 mmol) and benzaldehyde (1.0 g, 9.4 mmol) were mixed together in 70 ml ethanol. NaOH (1.05 g, 26 mmol) and 30 ml ammonium hydroxide (30%) were added into the solution latter. After this, the mixture was stired at room temperature for 12 h. After reaction, the mixture was filtered to obtain precipitation. Then precipitation was purified by recrystallization with trichloromethane-MeOH, while phtpy was obtained as white crystal (1.33 g, 43% global yield).

2-selenicimidazole[4,5-f]1,10-phenanthroline (phenSe): The synthesis of phtpy were carried out as *Li* et al described[9]. 5,6-diamino-1,10-phenanthroline (0.210 g, 0.1 mmol) and Selenium dioxide (0.113 g, 0.1 mmol) were added in 50 ml ethanol to reflux for 6 h. After reaction, the solvent was removed by evaporation and the crude

product was purified by neutral alumina column chromatography, eluting with ethanol. Ethanol was removed and the solid filtered, washed, and dried to give a pink solid.

Synthetic route was shown in **Scheme S1**.



Scheme S1. Synthetic route of ligand phtpy and phenSe.

Synthetic routes of complexes

The synthesis process was carried out as our pervious description[10].

Ru(phtpy)Cl_3 (**1**): phtpy (0.288 g, 1 mmol) and $RuCl_3 \cdot 3H_2O$ (0.228 g, 1.1 mmol) were dissolved in 45 ml ethanol. The solution was refluxed under N_2 for 4 h. Methanol was removed and the solid filtered, washed by cold methanol and water, and dried to give a brown solid (0.78 mmol, 78% global yield). The ESI-MS and NMR spectra are consisted with literature reported[11]. Anal. Calc. for $C_{21}H_{15}Cl_3N_3Ru$ (%): C 48.81, H 2.93, N 8.13%. Found (%): C 48.92, H 3.11, N 8.24. ESI-MS (in DMSO): m/z =519.9 $[M + 3H^+]$.

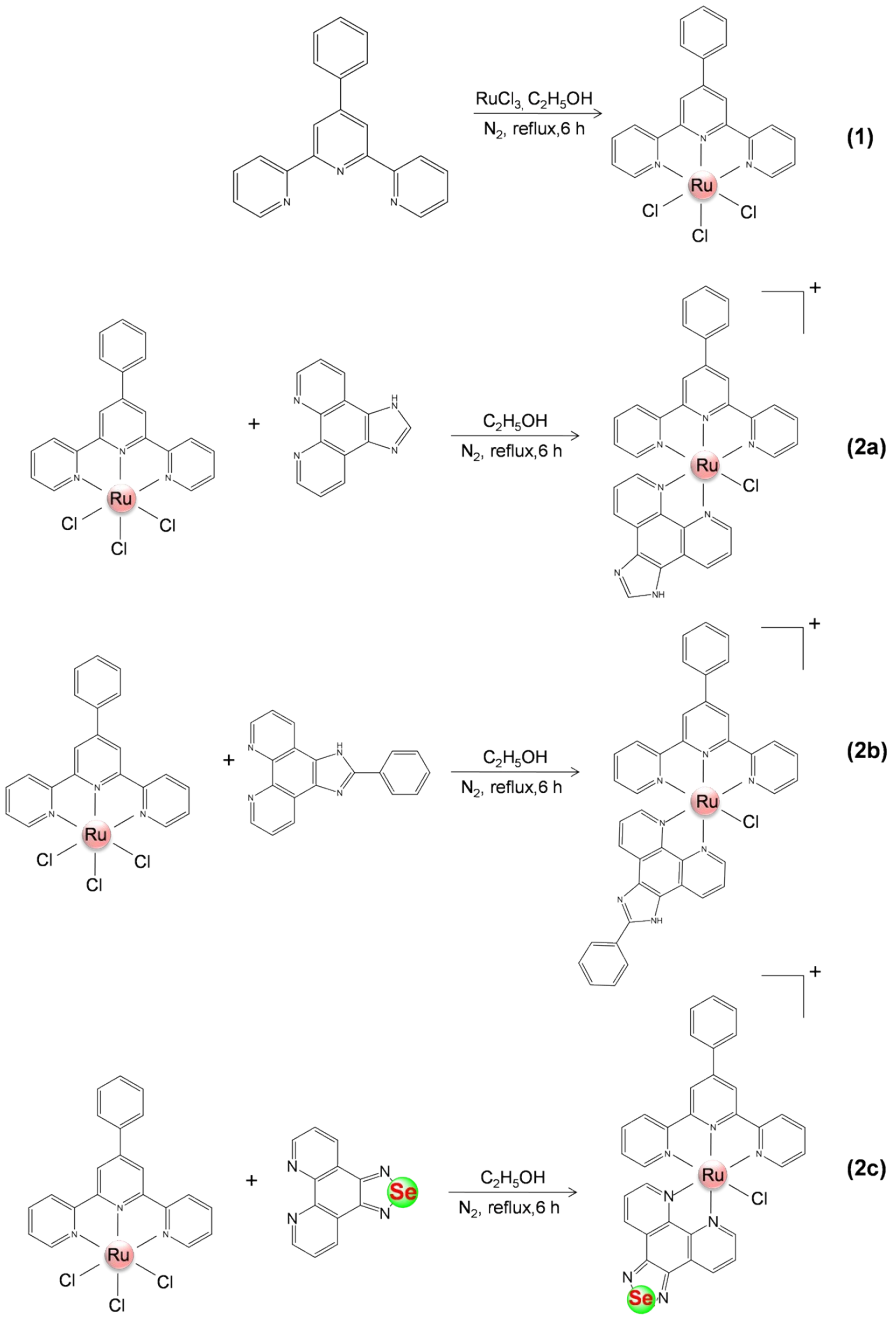
Ru(phtpy)(ip)Cl(ClO_4) (**2a**): Ru(phtpy)Cl_3 (1 mmol), ip (1 mmol) and a few drops of triethylamine were added into 45 ml ethanol to reflux for 6 h at N_2 atmosphere. After reaction, cool the solution to room temperature, add sodium perchlorate into the mixture, removed the solvent by suction filtration, then the crude product was purified by neutral alumina column chromatography, with methylbenzene/acetonitrile as eluent. Solvent was removed and the solid filtered, washed, and dried to give an atropurpureus solid. ^{1}H NMR (DMSO-d6 б ppm) б: 10.31 (1 H, d), 9.38 (1 H, s), 9.25 (2 H, s), 9.12 (1 H, t), 8.97 (2 H, d), 8.68 (2 H, s), 8.47 (2 H, d), 8.37 (2 H, m), 8.12 (1 H, t), 7.98 (2 H, t), 7.74 (4 H, d), 7.64 (1 H, d), 7.54 (2 H, d), 7.46 (1 , d), 7.37 (1 H, m), 7.25 (2 H, d). Anal. Calc. for $C_{34}H_{23}Cl_2N_7O_4Ru$ (%): C 53.34, H 3.03, N 12.81. Found (%): C 53.62, H 3.24, N 12.76. ESI-MS (in DMSO): m/z = 666 $[M - ClO_4^-]^+$.

Ru(phtpy)(pip)Cl(ClO_4) (**2b**): Ru(phtpy)Cl_3 (1 mmol), pip (1 mmol) and a few drops of triethylamine were added into 45 ml ethanol to reflux for 6 h at N_2 atmosphere. After reaction, cool the solution to room temperature,

add sodium perchlorate into the mixture to get precipitation, removed the solvent by suction filtration, then the crude product was purified by neutral alumina column chromatography, with methylbenzene/acetonitrile as eluent. Solvent was removed and the solid filtered, washed, and dried to give a brown solid. ^{1}H NMR (DMSO-d6 б ppm) б: 10.32 (1 H, d), 9.30 (1 H, d), 9.24 (2 H, s), 8.95 (2 H, d), 8.71 (1 H, d), 8.50 (1 H, s), 8.35 (4 H, m), 7.99 (2 H, d), 7.74 (3 H, t), 7.66 (3 H, m), 7.54 (3 H, m), 7.48 (1 H, s), 7.25 (2 H, t). Anal. Calc. for $C_{40}H_{27}Cl_2N_7O_4Ru$ (%): C 57.08, H 3.23, N 11.65. Found (%): C 57.19, H 3.42, N 11.74. ESI-MS (in DMSO): m/z = 742 $[M - ClO_4^-]^+$.

Ru(phtpy)(phenSe)Cl(ClO$_4$) (**2c**): $Ru(phtpy)Cl_3$ (1 mmol), phenSe (1 mmol) and a few drops of triethylamine were added into 45 ml ethanol to reflux for 6 h at N_2 atmosphere. After reaction, cool the solution to room temperature, add sodium perchlorate into the mixture, removed the solvent by suction filtration, then the crude product was purified by neutral alumina column chromatography, with methylbenzene/acetonitrile as eluent. Solvent was removed and the solid filtered, washed, and dried to give a red solid. ^{1}H NMR (DMSO-d6 б ppm) б: 10.13 (1H, d), 9.31 (1H, d), 9.24 (2H, s), 8.97 (2H, d), 8.73 (1H, d), 8.35 (3H, m), 7.98 (2H, t), 7.73 (2H, t), 7.65 (5H, m), 7.54 (2H, d), 7.48 (1H, t), 7.26 (2H, t). Anal. Calc. for $C_{33}H_{21}Cl_3N_7O_4RuSe$ (%): C 47.72, H 2.55, N 11.81. Found (%): C 47.65, H 2.61, N 11.79. ESI-MS (in DMSO): m/z = 732 $[M - ClO_4^-]^+$.

The synthetic route of the complexes was shown in **Scheme S2.**



Scheme 2. Synthetic route of complex **1**, **2a**, **2b** and **2c**.

Results

Table S1. Growth inhibition of **1-2c** and corresponding ligands treatment on A375 cells [a]

Cell line	IC_{50} (μM)							
	phentpy	ip	pip	phenSe	**1**	**2a**	**2b**	**2c**
A375	65.2±6.7	72.5±9.2	88.8±4.6	20.7±3.4	59.6±2.4	52.4±2.4	102.1±3.2	9.7 ± 2.9

[a] Cell viability was determined by MTT assay after treatment for 72 h.

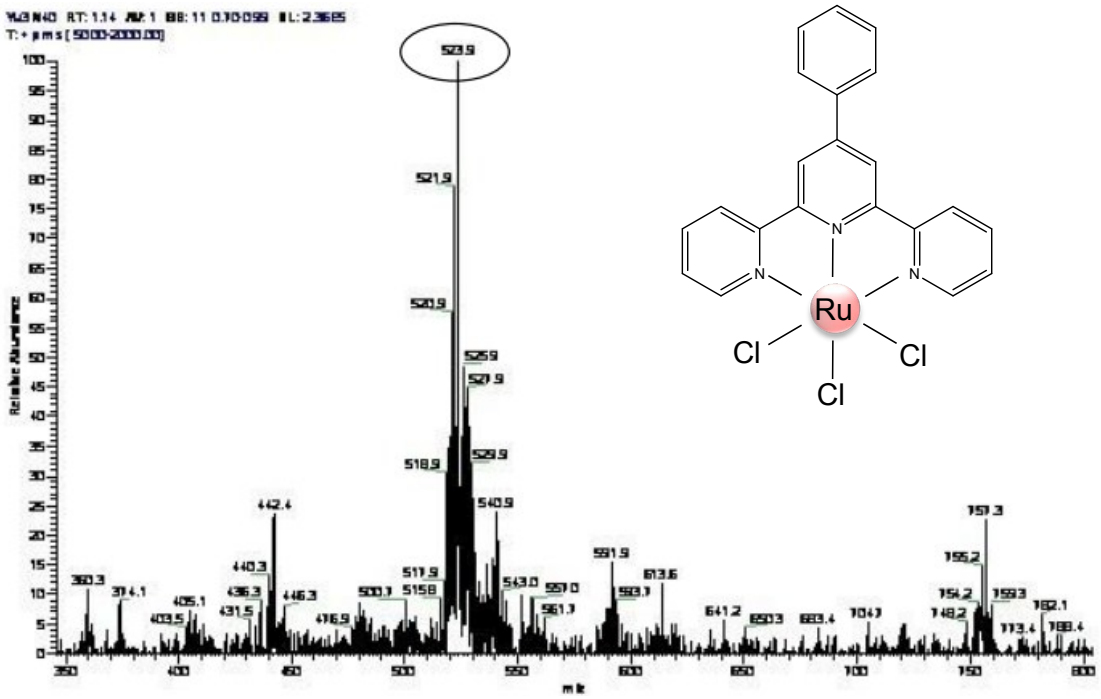


Figure S1. The ESI-MS spectra of complex **1**.

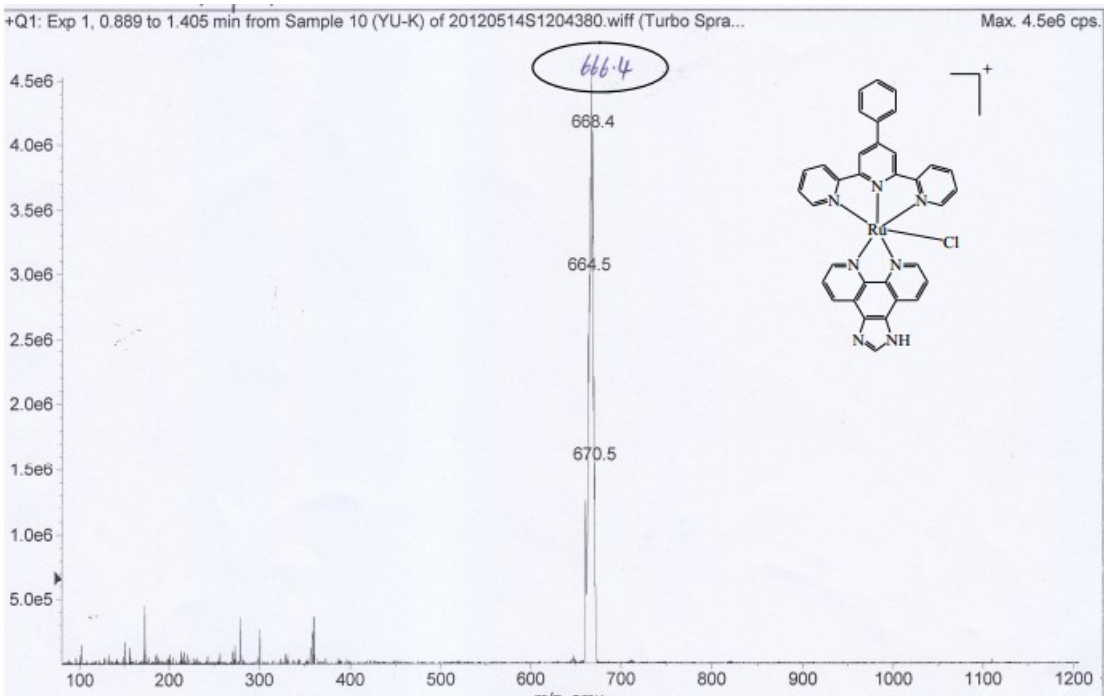


Figure S2. The ESI-MS spectra of complex **2a.**

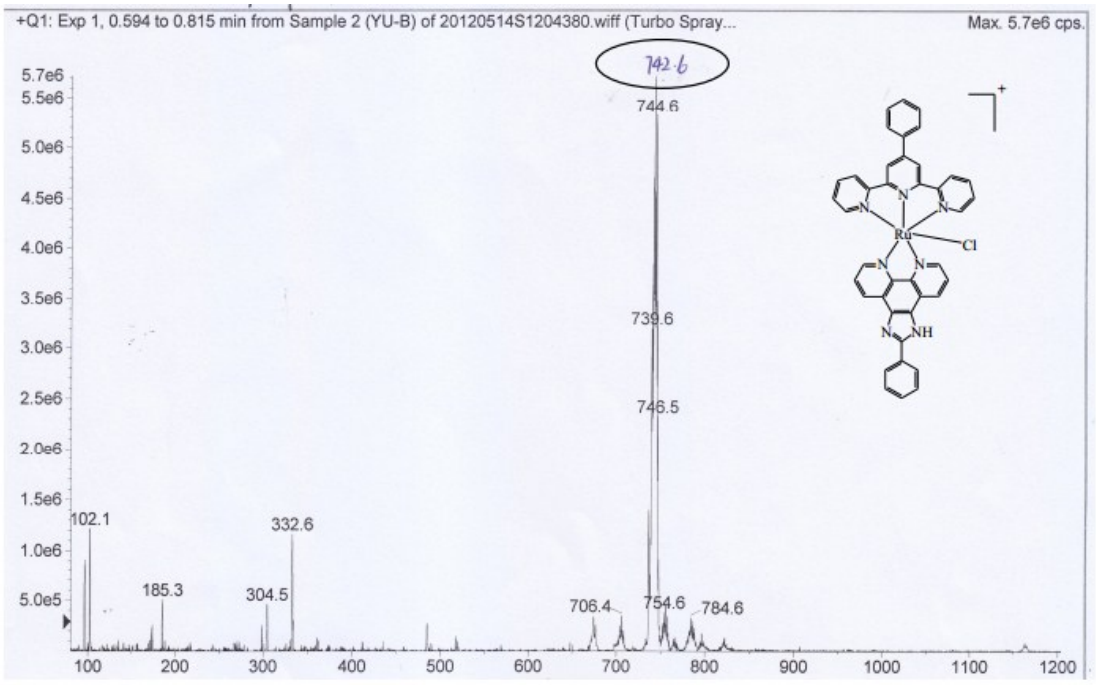


Figure S3. The ESI-MS spectra of complex **2b.**

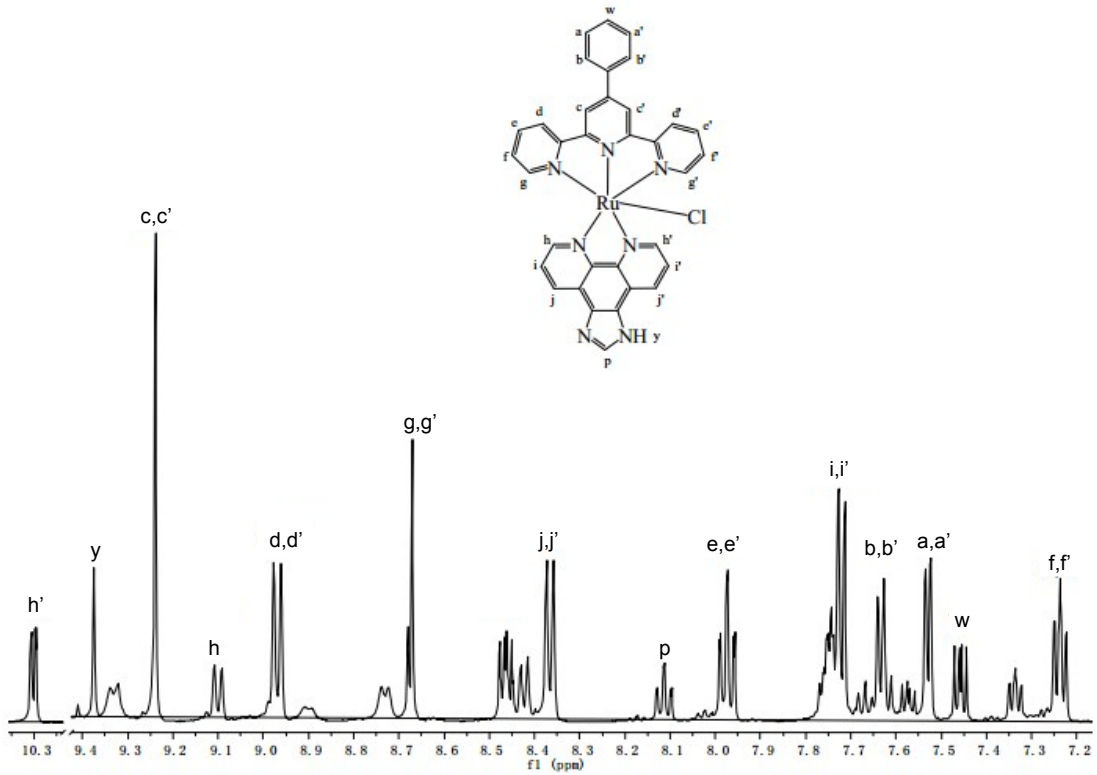


Figure S4. The ^{1}H NMR spectra of complex **2a**.

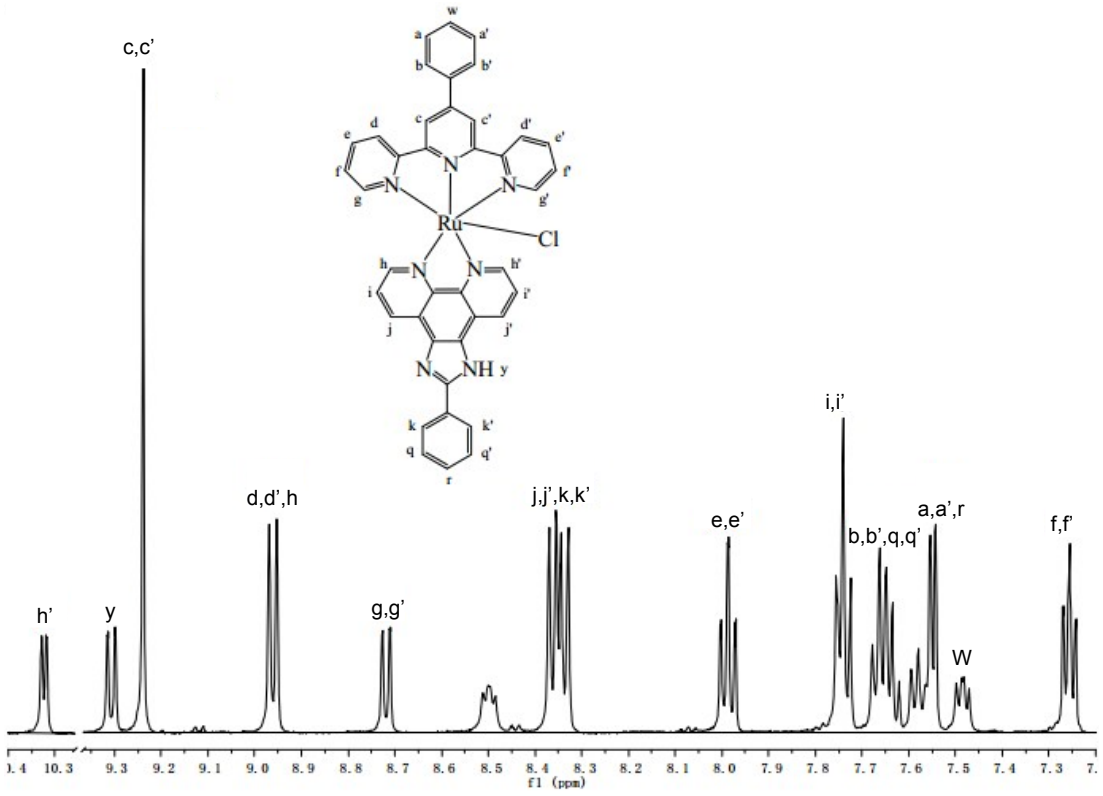


Figure S5. The ^{1}H NMR spectra of complex **2b**.

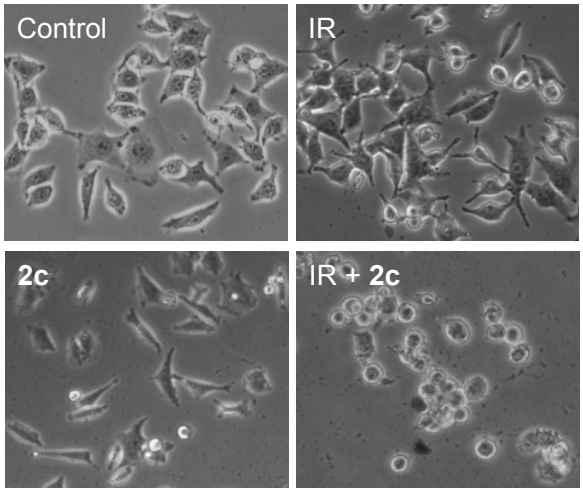


Figure S6. Changes in the morphology of A375 cells after incubated with different treatments for 72 h, as examined by phasecontrast microscopy (magnification, 200×). The concentration of **2c** was 10 μM and the dose of radiation was 8Gy.

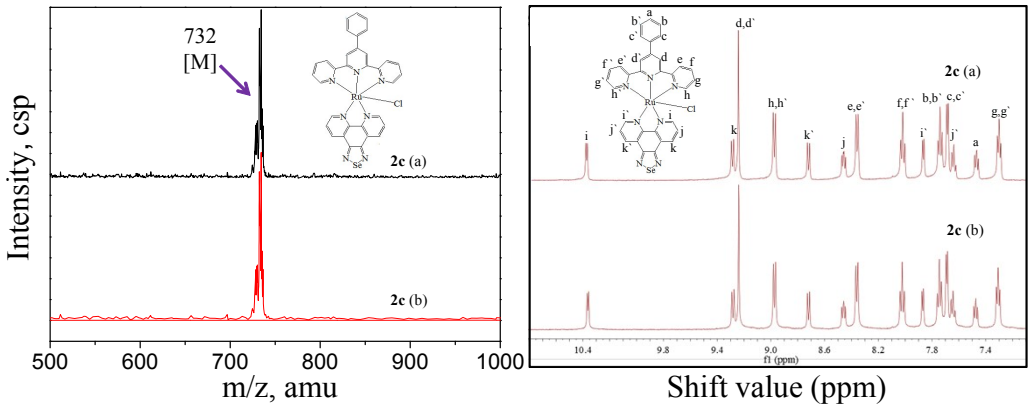


Figure S7. The ESI-MS and ^{1}H NMR spectrum of **2c** before (a) and after (b) radiation (8Gy).

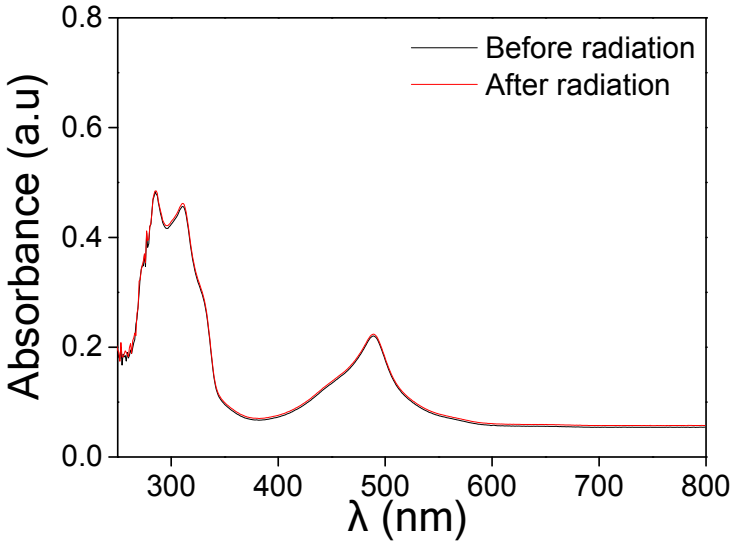


Figure S8. The UV-Vis spectra of complex **2c** before and after radiation (8 Gy).

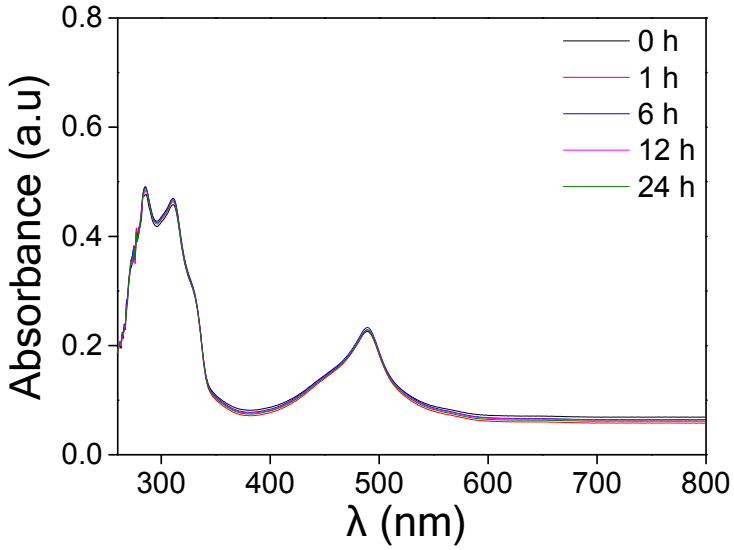


Figure S9. The UV-Vis spectra of complex **2c** incubated in aqueous solution for 24 h.

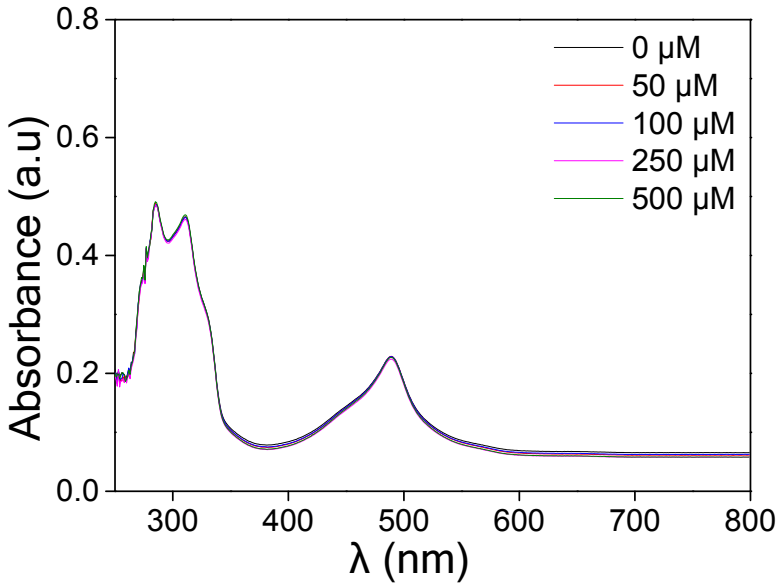


Figure S10. UV-Vis spectra of **2c** (10 μM) reacted with different concentrations of H_2O_2 (50-500 μM) for 30 min at room temperature.

References

1 B. Yu, Y. Zhang, W. Zheng, C. Fan and T. Chen, *Inorg. Chem.*, 2012, **51**, 8956.

2 Y. Huang, Y. Luo, W. Zheng and T. Chen, *Acs Appl. Mater. Interfaces,* 2014, **6**, 19217–19228.

3 Y. Huang, L. He, W. Liu, C. Fan, W. Zheng, Y.-S. Wong and T. Chen*, Biomaterials,* 2013, **34**, 7106.

4 L. Li, W. Cao, W. Zheng, C. Fan and T. Chen, *Dalton. Trans.*, 2012, **41**, 12766.

5 T. Chen, Y.-S. Wong, W. Zheng and J. Liu, *Chem-biol. Interact.*, 2009, **180**, 54.

6 J. Su, H. Lai, J. Chen, L. Li, Y.-S. Wong, T. Chen and X. Li, *PloS One*, 2013, **8**.

7 Z. Zhao, Z. Luo, Q. Wu, W. Zheng, Y. Feng and T. Chen, *Dalton. Trans.*, 2014 **43**, 17017-17028.

8 K. Mallesh and N. Mukunda, *J. Phys. A: Math. Gen.,* 1997, **30**, 2417.

9 Q. Li, D. Sun, Y. Zhou, D. Liu, Q. Zhang and J. Liu, *Inorg. Chem. Commun.*, 2012, **20**, 142.

10 (a) L. Li, W. Cao, W. Zheng, C. Fan and T. Chen, *Dalton. Trans.*, 2012, **41**, 12766; (b) L. Li, Y.-S. Wong, T. Chen, C. Fan and W. Zheng, *Dalton. Trans.*, 2012, **41**, 1138.

11 E. C. Constable, A. Thompson, D. A. Tocher and M. A. M. Daniels, *New J. Chem.*, 1992, **16**, 855.