Supporting Information For

A mitochondria-targeting dinuclear Ir-Ru complex as a synergistic photoactivated chemotherapy and photodynamic therapy agent against cisplatin-resistant tumour cells

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Supporting Experimental Procedures

Materials and Measurements

All starting materials were used as received from commercial sources unless otherwise stated. Ruthenium chloride hydrate (Alfa Aesar, USA), DPBF (1,3-diphenylisobenzofuran, Sigma Aldrich, USA), MB (methylene blue, Sigma Aldrich, USA), cisplatin (Sigma Aldrich, USA), Plasmid pBR322 DNA (MBI Fermentas, Canada), GeneFinder (Bai Wei Xin biotechnology, China), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich, USA), dimethyl sulfoxide (DMSO, Sigma Aldrich, USA), propidium iodide (PI, Sigma Aldrich, USA), 5,5′,6,6′-tetrachloro-1,1',3,3′-tetraethyl-benzimidazolyl carbocyanine iodide (JC-1, Life Technologies, USA), PicoGreen (Molecular Probes Inc., USA) were used as received. Annexin V-FITC apoptosis detection kit was purchased from Sigma Aldrich (USA). Caspase-3/7 activity kit and cellular ATP quantification assay kit were purchased from Promega (USA), Sigma GenElute mammalian genomic DNA miniprep kit, Elongase long range PCR enzyme kit from Invitrogen, Nucleus extraction kit and cytoplasm extraction kit were purchased from Thermo pierce. All primers were purchased from Sangon Biotech (China). All the compounds tested were dissolved in DMSO just before the experiments, and the final concentration of DMSO was kept at 1% (v/v).

Electrospray ionization mass spectrometry (ESI-MS) was recorded on a Thermo Finnigan LCQ DECA XP spectrometer (USA). The quoted m/z values represented the major peaks in the isotopic distribution. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 400 MHz spectrometer (Germany). Shifts were referenced relative to the internal solvent signals. The inductively coupled plasma mass spectrometry (ICP-MS) experiments were carried out on an Agilent's 7700x instrument. Microanalysis of elements (C, H, and N) was carried out using an Elemental Vario EL CHNS analyzer (Germany). UV/Vis spectra were recorded on a Varian Cary 300 spectrophotometer (USA). Cell imaging experiments were carried out on a confocal microscope (LSM 710, Carl Zeiss, Göttingen, Germany).

Quantification of singlet oxygen ($^1O_2$) generation

The $^1O_2$ quantum yields (Φ_Δ) were detected by monitoring the photooxidation of DPBF sensitized by **Ir**/**Ir-Ru** in MeOH.[1] DMSO solutions containing **Ir** or **Ir-Ru**

and DPBF (70 μM) were aerated for 10 min, and then excited at 405 nm or 450 nm, or 450 nm followed by 405 nm. The absorbance of DPBF at 418 nm was recorded every 2 s. MB was used as the reference of $^1O_2$ sensitization (Φ_Δ = 0.52). The absorbance of **Ir**, **Ir-Ru** and MB at 405 nm or 450 nm was kept at 0.10. The $^1O_2$ quantum yields of **Ir** and **Ir-Ru** were calculated according the following equation.

$$\Phi_{\Delta(x)} = \Phi_{\Delta(std)} \times (\frac{S_x}{S_{std}}) \times (\frac{F_{std}}{F_x})$$

The sample and standard are presented as *x* and std, respectively. *S* is the slope of plot of the absorption maxima of DPBF against the irradiation time (s). *F* ($1 - 10^{-OD}$; OD is the optical density of the samples and the standard at 405 nm or 450 nm) means the absorption correction factor.

MTT assay

The cytotoxicity of the complexes was determined by MTT assay. Briefly, the cells were seeded into 96-well microtiter plates at (1 × 104 cells per well), and grown for 24 h at 37 °C in a 5% CO_2 incubator conditions, and different concentrations of the complexes were added to the culture media. After incubated for 6 h, the original medium was removed and new medium was added. The plates were then incubated for 48 h in the dark or corresponding light condition. The MTT dye solution (10 μL, 5 mg/ml) was added to each well. After 4 h of incubation, the cultures were removed and 150 μL of DMSO solution was added to each well. The optical density of each well was measured on a microplate spectrophotometer at a wavelength of 595 nm. Data were reported as the means ± standard deviation (n = 3). Irradiation condition: (450 nm, 6 J • cm^{-2}, 10 min; 405 nm 3 J • cm^{-2}, 5 min).

DNA gel electrophoresis assay

pBR322 DNA (200 ng/mL) was incubated with **Ir-Ru** in Tris-HCl buffer B (20 mM Tris-HCl, 20 mM Na_2HPO_4, pH 7.4) at 37 °C for 1 h with or without 450 nm irradiation for 6 J · cm^{-2}. The DNA samples were analyzed by electrophoresis (98 V, 3 h) on a 1% agarose gel in 1 × TBE buffer (18 mM Tris-borate acid, 0.4 mM EDTA, pH 8.3). The gel was stained with 10 μL GeneFinder and the images were captured on Gel Imaging System (APLEGEN, San Francisco, CA).

DNA damage assay

A549R cells were seeded at 1×10^4 cells/well and allowed to adhere overnight. Cells were treated with 1 μM **Ir, Ir-Ru** or 100 μM **Cisplatin** for 12 h in the dark or light condition and harvested after trypsinization. DNA was isolated from cell pellets using the Sigma GenElute mammalian genomic DNA miniprep kit according to the manufacturer's instructions. Amplification of an 8.9 kb segment of mitochondrial DNA or a 13.5 kb segment of genomic DNA was performed using the Elongase long range PCR enzyme kit (Invitrogen) as described previously.[2] Quantitation of amplified product was performed by Pico Green staining and normalized to nontreated value.

mtDNA stained

A549R cells were pre-treated with **Ir** or **Ir-Ru** for 6 h, and then replaced the culture medium, cells were in the corresponding irradiation condition or in dark continued to incubate for 12 h in dark or light condition. After staining with PicoGreen for 1h, the green fluorescence was detected by confocal microscopy. λ_{ex} = 488 nm; λ_{em} = 520 ± 20 nm.

Cellular ROS detection

A549R cells plated into confocal dish treated with **Ir** or **Ir-Ru** at the indicated concentrations for 6 h and irradiated with a 450 nm LED light (6 J · cm^{-2}) or 405 nm LED light (3 J · cm^{-2}) array. Then cells were stained with H_2DCFDA (1 μM) for 20 min at 37 °C in the dark and washed twice with serum-free DMEM. The fluorescence intensity of DCF in A549R cells was measured by confocal microscopy. λ_{ex} = 488 nm; λ_{em} = 530 ± 20 nm.

Cell lines and culture conditions

A549, A549R, SGC-7901 and SGC-7901DDP cells were obtained from Experimental Animal Center of Sun Yat-Sen University (Guangzhou, China). Cells were routinely maintained in DMEM medium (Dulbecco's modified Eagle's medium, Gibco BRL), RPMI 1640 (Roswell Park Memorial Institute 1640, Gibco BRL) medium, Ham's F-12K (Kaighn's, Gibco BRL) medium and McCoy's 5A (Gibco

BRL) medium containing 10% FBS (fetal bovine serum, Gibco BRL), 100 μg/mL streptomycin, and 100 U/mL penicillin (Gibco BRL). Cells in tissue culture flasks were incubated in a humidified incubator (Atmosphere: 5% CO_2 and 95% air; Temperature: 37 °C). Cisplatin-resistant A549R cells were cultured in DMEM with cisplatin to maintain the resistance.

ICP-MS measurement

The cellular uptake capacity of complexes was measured by determination of intracellular iridium or ruthenium contents. Briefly, A549R cells were incubated in 100 mm dishes overnight. The medium was removed and replaced with medium/DMSO (v/v, 99:1) containing **Ir-Ru** (2.5 μM). After 6 h incubation, the cells were trypsinized and collected in PBS (3 mL). Mitochondria were isolated from Ir-Ru treated cells using the mitochondria isolation kit (Sangon Biotech, China) according to the manufacturer's instructions. Nuclear and cytosolic fractions were separated using a nucleoprotein extraction kit (Sangon Biotech, China) according to the manufacturer's instructions. The samples were digested with 50% HNO_3 and 10% H_2O_2 at RT for two days. Each sample was diluted with MilliQ H_2O to obtain 2% HNO_3 sample solutions. The iridium and ruthenium content were measured using inductively coupled plasma mass spectrometry (ICP-MS Thermo Elemental Co., Ltd.). Data were reported as the means ± standard deviation (n = 3).

Cell uptake mechanism

The cellular uptake mechanism was performed according to Ref. 3. For metabolic inhibition, the A549R cells were pre-treated with inhibitors (50 mM 2-deoxy-D-glucose and 5 μM oligomycin) for 1 h and then incubated with Ir-Ru (2.5 μM) for 2 h. For temperature dependent uptake study, HeLa cells were treated with 2.5 μM **Ir-Ru** for 1 h at 4 °C and 37 °C, respectively. NH_4Cl (50 mM) and chloroquine (100 μM) were used to inhibit endocytotic uptake, A549R cells pretreated with the indicated endocytotic inhibitors at 37 °C for 1 h were treated with 2.5 μM **Ir-Ru** for 2 h in the dark. Subsequently, all of A549R cells were washed with cold PBS for 3 times. After these, cells were detached and collected. The samples were digested with 60% HNO_3 at RT for one day. The cells were incubated with complexes for ICP-MS analysis. Data were reported as the means ± standard deviation (n = 3).

Analysis of MMP

A549 cells were seeded into confocal dish and treated with **Ir** or **Ir-Ru** for 6 h and irradiated with a 450 nm (6 J · cm^{-2}) or 405 nm (3 J · cm^{-2}) LED light array. The treated cell was incubated in 37 °C in the dark for another 12 h, then cells were stained with JC-1 and analyzed by confocal microscopy

Intracellular ATP level

Cellular ATP levels were measured using the CellTiter-Glo® Luminescent Cell Viability Assay kit (G7570, Promega, USA) according to the manufacturer's instructions. A549 cells were seeded in 96 well plates and treated with **Ir** or **Ir-Ru** for 6 h and irradiated with a 450 nm (6 J · cm^{-2}) or 405 nm (3 J · cm^{-2}) LED light array. These samples were equilibrated in PBS at room temperature for 30 min. The CellTiter-Glo® reagent was added and the plate was incubated for 10 min. The luminescence was recorded using a microplate reader (Infinite M200 Pro, Tecan, Switzerland).

Annexin V/PI staining assay

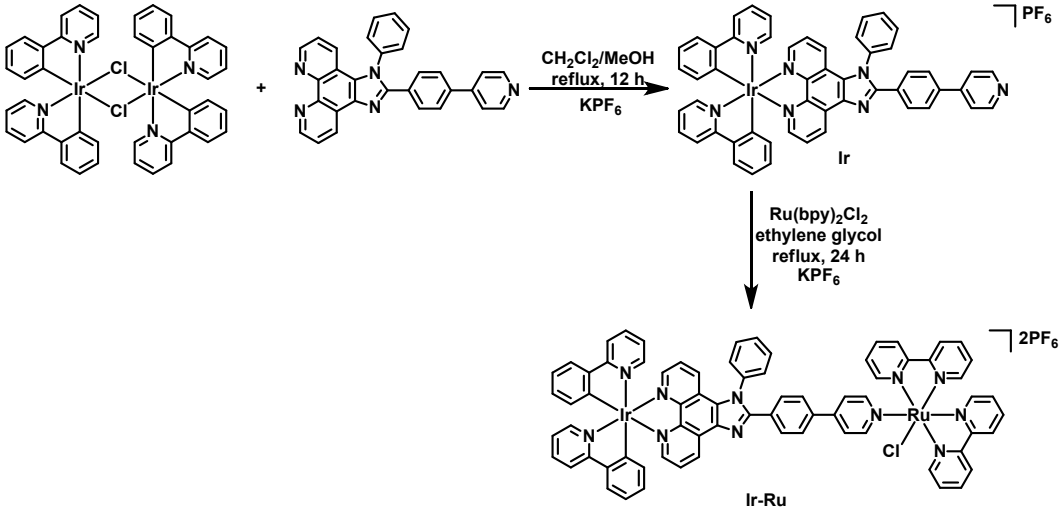
The assay was performed according to the manufacturer's (Sigma Aldrich, USA) protocol. Cells treated with **Ir** or **Ir-Ru** for 6 h were irradiated with a 450 nm (6 J · cm^{-2}) or 405 nm (3 J · cm^{-2}) LED light array and stained with annexin V reagent at room temperature for 15 min in the dark. The samples were immediately analyzed by confocal microscopy. λ_{ex} = 488 nm; λ_{em} = 530 ± 20 nm.

Caspase-3/7 activity assay

Caspase-3/7 activity was measured using the Caspase-Glo® Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Cells cultured in 96 well plates were treated with **Ir** or **Ir-Ru** for 6 h. After irradiation 450 nm (6 J · cm^{-2}) or 405 nm (3 J · cm^{-2}) LED light array, 100 µL of Caspase Glo® 3/7 reagent was added to each well containing 100 µL culture medium. The mixture was incubated at room temperature for 1 h and then luminescence was measured using a micro-plate reader (Infinite M200 Pro, Tecan, Switzerland).

Synthesis and characterization

$[Ir(ppy)_2Cl]_2$[4] was synthesized according to the published methods. The synthetic route used to access **Ir-Ru** is illustrated in Scheme S1.



Scheme S1. Synthesis of complex **Ir-Ru**.

Synthesis of **Ir**

A solution of $[(ppy)_2Ir(\mu\text{-}Cl)]_2$ (500 mg, 470 μmol, 1 eq) and 1-phenyl-2-(4-(pyridin-4-yl) (PIP, 230 mg, 1.00 mmol, 2.1 eq) in CH_2Cl_2/MeOH (2:1, v/v, 20 mL) was refluxed under nitrogen in the dark for 12 h. KPF_6 (1.01 g, 5.50 mmol, 12 eq) was added to the resulting red solution and cooled to room temperature with stirring for 2 h. After filtration of the insoluble inorganic salts, the filtrate was evaporated under reduced pressure. The residue was purified by silica gel column chromatography using acetone/CH_2Cl_2 (1:20, v/v) as the eluent to yield a fine yellow crystalline solid (854 mg, 780 μmol, 83%). Anal. Calcd for $C_{52}H_{35}F_6IrN_7P$ (%): C, 57.03; H, 3.22; N, 8.95; Found: C, 56.89; H, 3.10; N, 8.84. ESI-MS: m/z = 950 ($[M\text{-}PF_6]^+$). ^1H NMR (400 MHz, DMSO-d_6) δ 9.40 (d, J = 8.3 Hz, 1H), 8.62 (d, J = 6.3 Hz, 2H), 8.41 (d, J = 3.7 Hz, 1H), 8.27 (d, J = 3.7 Hz, 1H), 8.16 (m, 2H), 8.04 (m, 1H), 7.94 – 7.68 (m, 16H), 7.60 (m, 1H), 7.53 (t, J = 6.2 Hz, 2H), 7.10 (dt, J = 10.6, 7.0 Hz, 2H), 7.01 – 6.91 (m, 4H), 6.41 (m, 2H).

Synthesis of **Ir-Ru**

Upon refluxing a solution of **Ir** (100 mg, 110 μmol, 1 eq) and $Ru(bpy)_2Cl_2$ (140 mg, 110 μmol, 1 eq) in ethylene glycol (5 mL) in the dark for 24 h. After cooling to room temperature, saturated

KPF_6 solution (70 mL) was added and red solid was formed. The red solid was collected by filtration and purified by silica gel column chromatography using acetonitrile/CH_2Cl_2 (1:20, v/v) produce a fine dark red crystalline solid (109 mg, 70.4 μmol, 64%). Anal. Calcd for $C_{72}H_{51}ClF_6IrN_{11}PRu$ (%): C, 56.01; H, 3.33; N, 9.98; Found: C, 55.88; H, 3.40; N, 9.83. ESI-MS: m/z = 699 ($[M\text{-}PF_6]^{2+}$). ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.87 (d, *J* = 5.2 Hz, 1H), 9.32 (d, *J* = 7.1 Hz, 1H), 8.80 (d, *J* = 8.2 Hz, 1H), 8.68 (t, *J* = 7.1 Hz, 2H), 8.60 (d, *J* = 8.1 Hz, 1H), 8.54 (d, *J* = 5.5 Hz, 1H), 8.32 – 8.23 (m, 3H), 8.17 (m, 2H), 8.09 (d, *J* = 4.6 Hz, 1H), 8.00 – 7.69 (m, 23H), 7.58 (d, *J* = 5.2 Hz, 1H), 7.50 (d, *J* = 7.5 Hz, 3H), 7.37 (t, *J* = 6.3 Hz, 1H), 7.31 (t, *J* = 6.2 Hz, 1H), 7.10 – 6.92 (m, 7H), 6.28 (m, 2H).

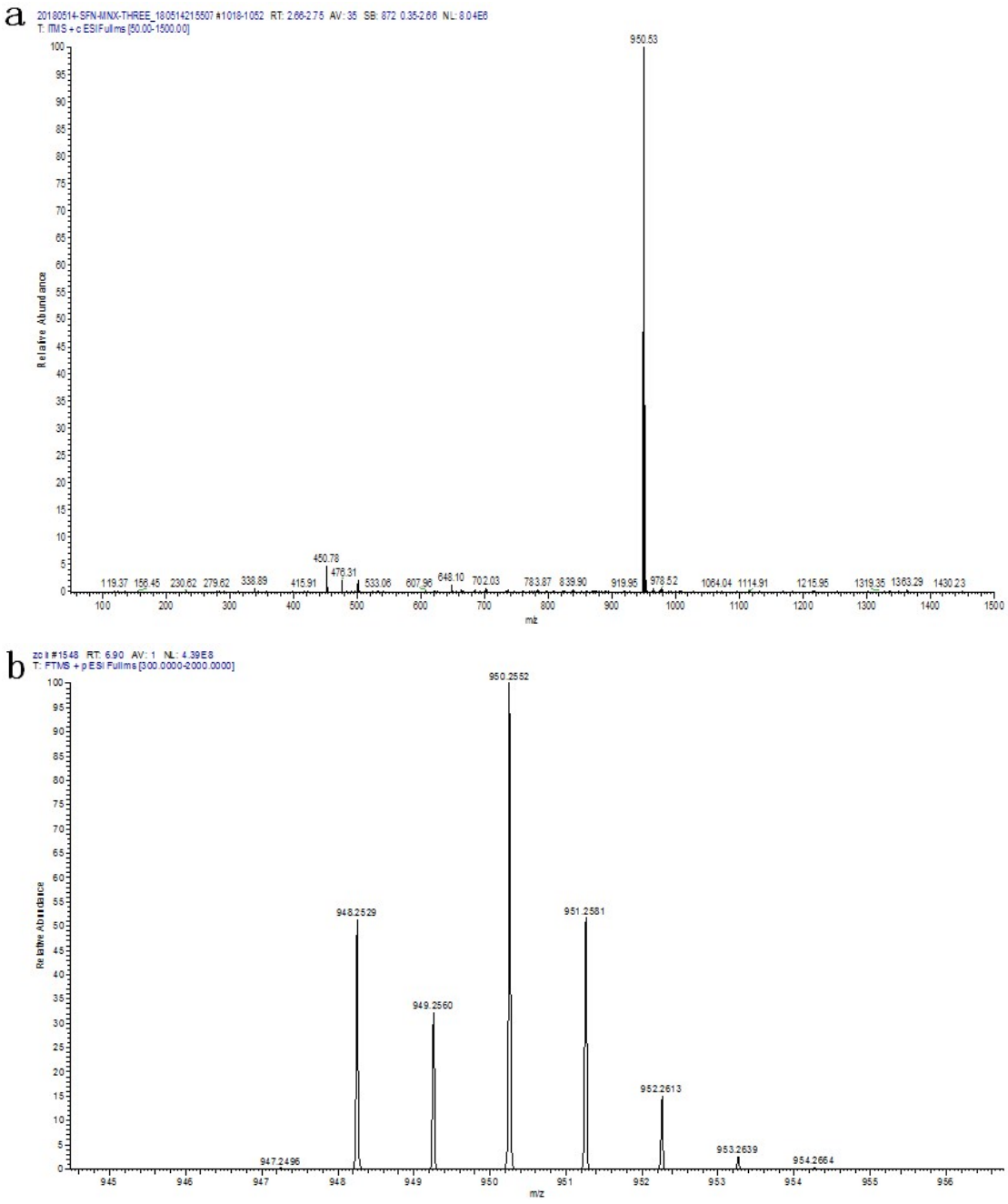


Figure S1. a) ESI-MS characterization of **Ir.** b) Zoom Scan MS spectra of **Ir** isotope peaks.

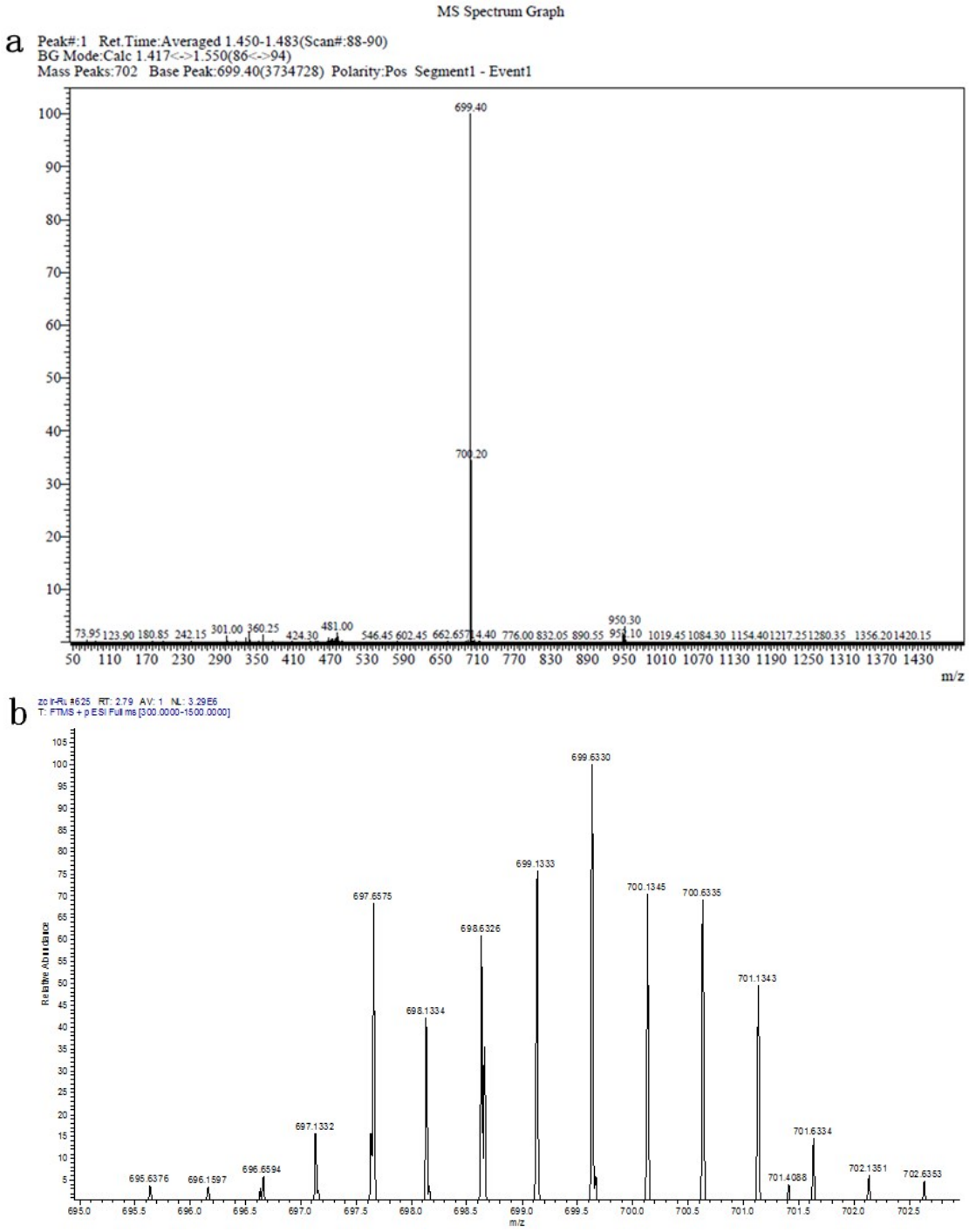


Figure S2. ESI-MS characterization of **Ir-Ru.** b) Zoom Scan MS spectra of **Ir-Ru** isotope peaks.

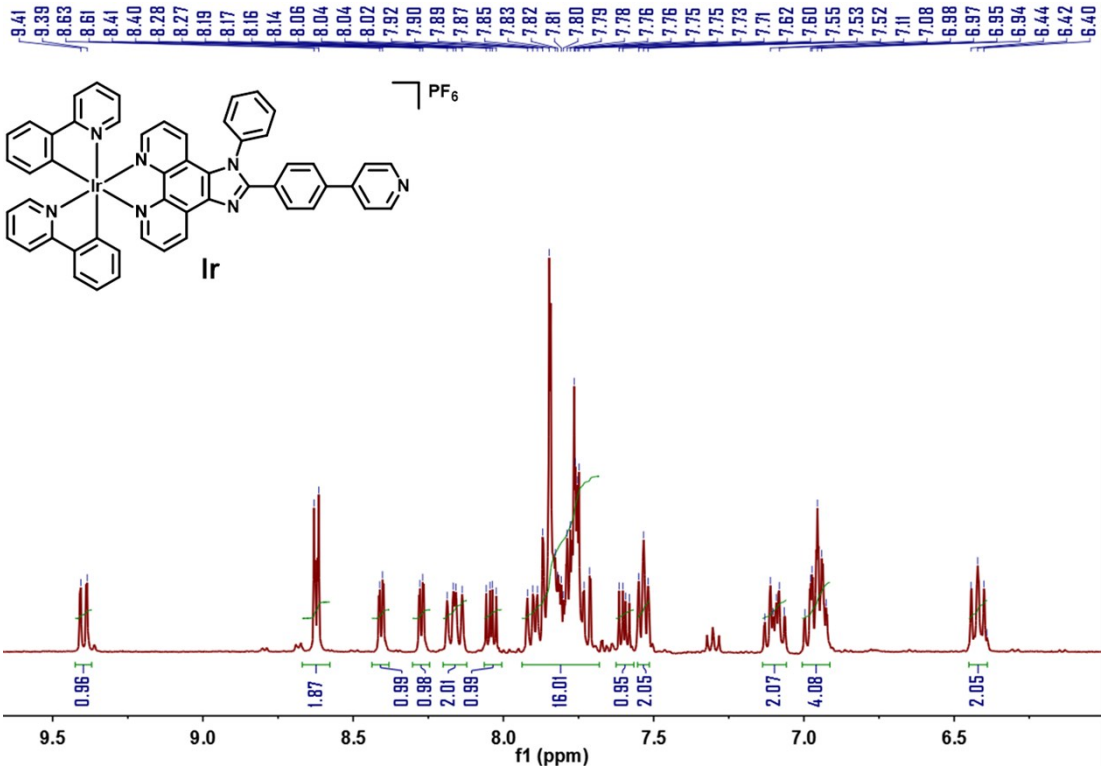


Figure S3. ^{1}H NMR spectrum of **Ir** in DMSO-d_6**.**

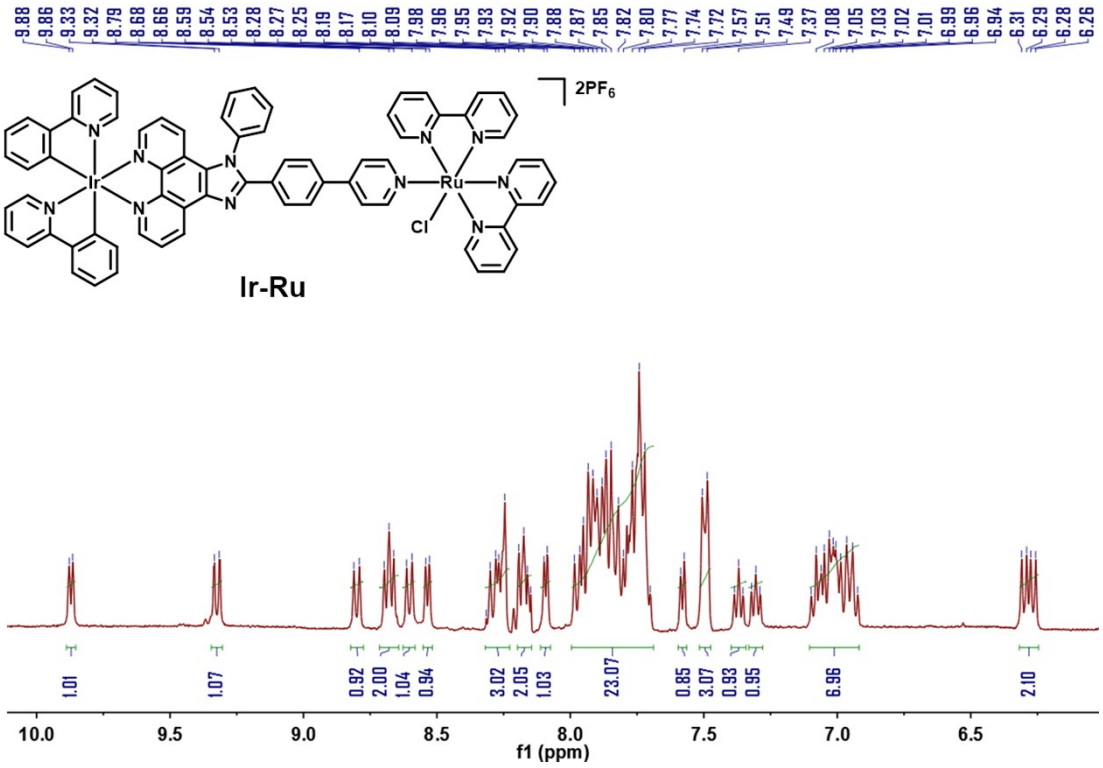


Figure S4. ^{1}H NMR spectrum of **Ir-Ru** in DMSO-d_6.

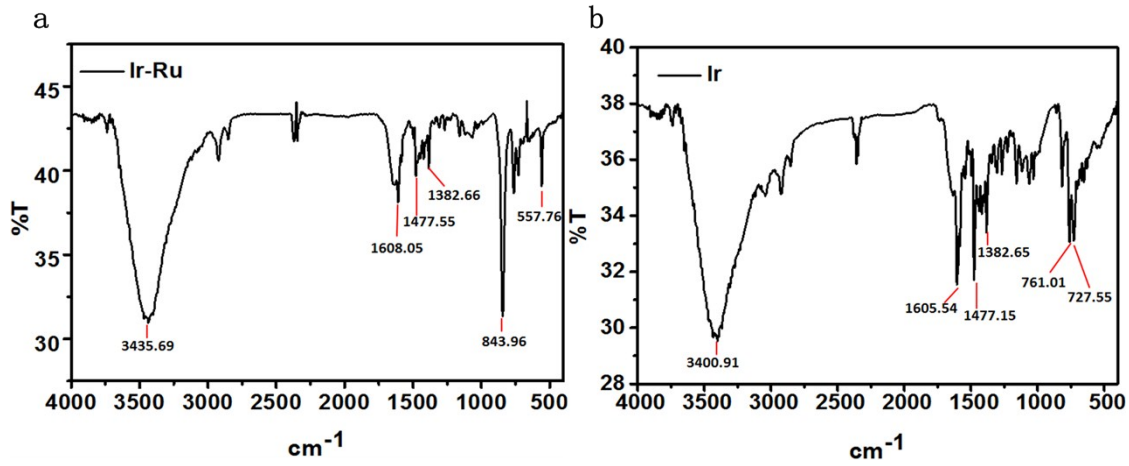


Figure S5. IR spectrum of **Ir-Ru** (a) and **Ir** (b).

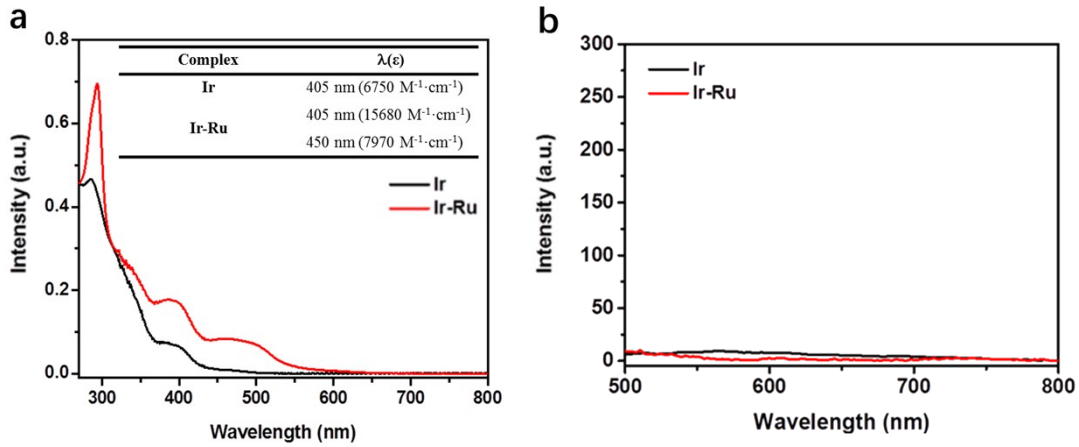


Figure S6. (a) UV-vis and (b) emission spectrum of **Ir** (10 μM) and **Ir-Ru** (10 μM) measured in PBS at 298 K.

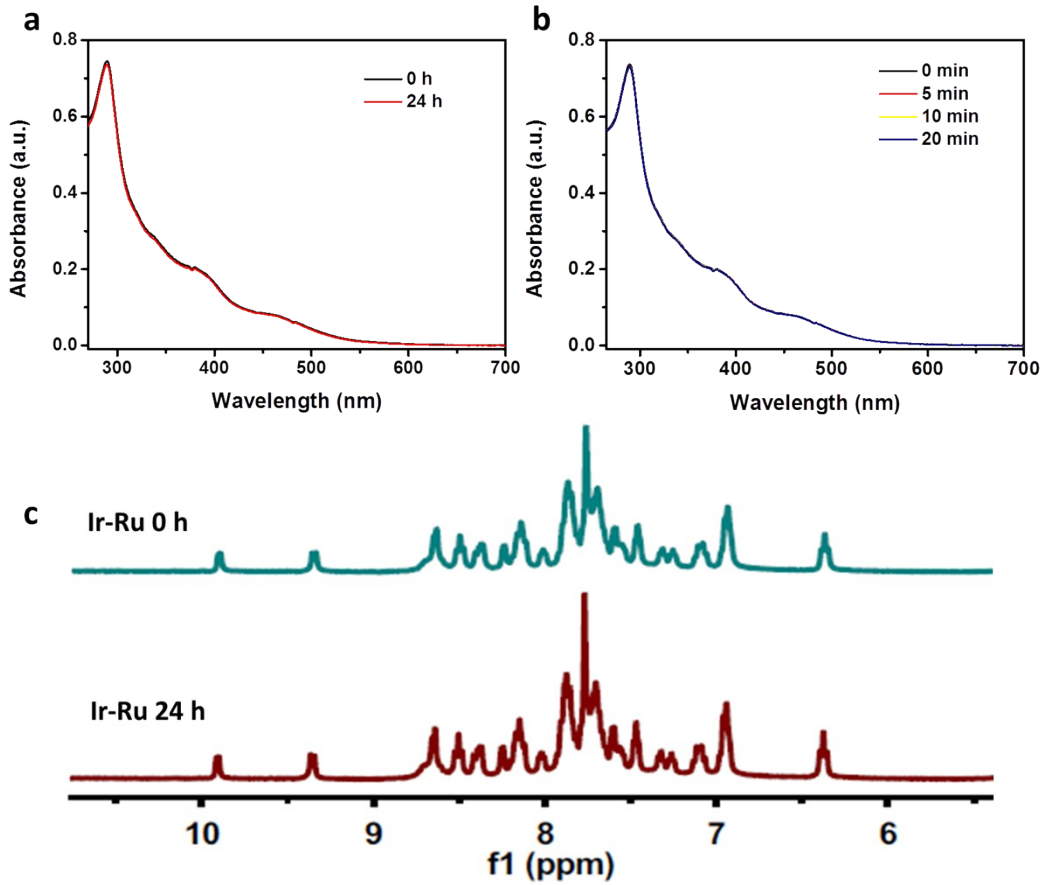
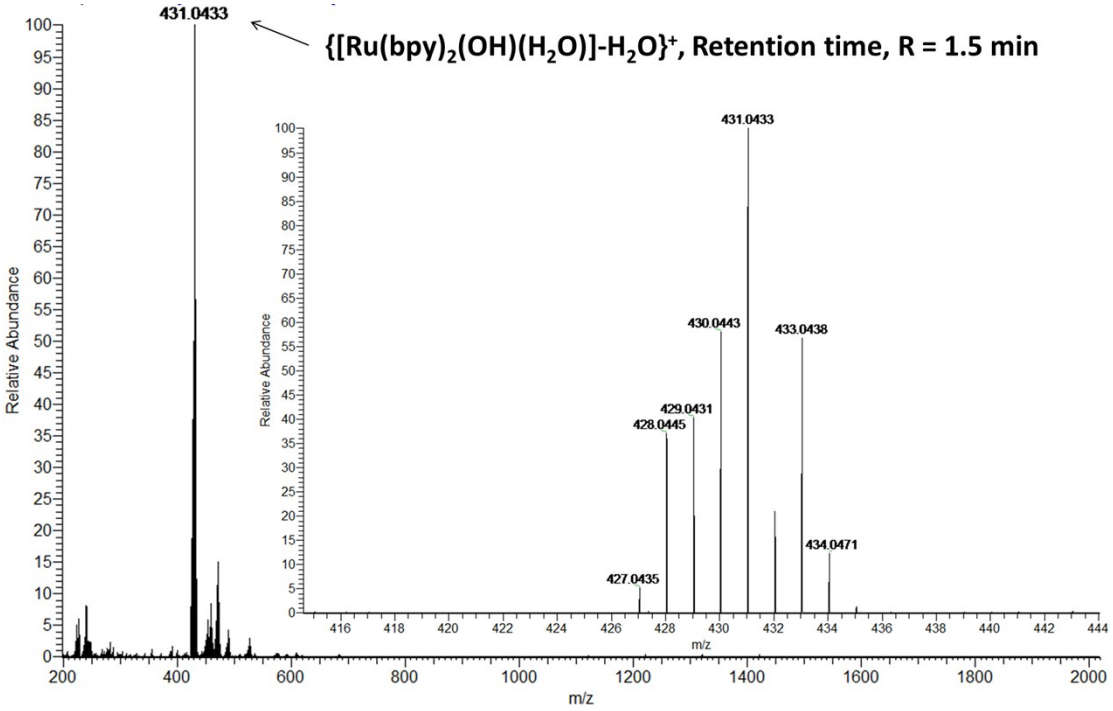
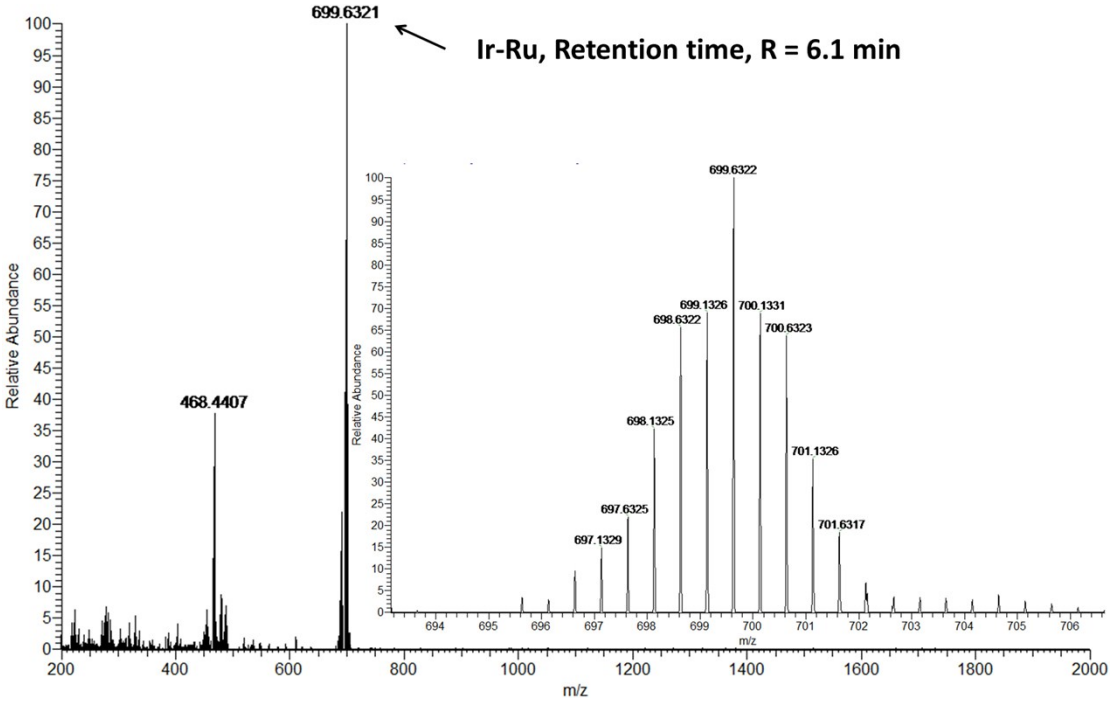


Figure S7. The stability of **Ir-Ru.** a) The UV Absorbance of 10 μM **Ir-Ru** in PBS at 0 h and 24 h in dark. b) Absorption spectral variation of **Ir-Ru** under 405 nm irradiation for 20 min (12 J • cm^{-2}). c) ^{1}H NMR spectrum of **Ir-Ru** in D_2O and methanol-d_4 (1:3, v/v) at 0 h and 24 h in the dark.


431.0433
{[Ru(bpy)2(OH)(H2O)]-H2O}+, Retention time, R = 1.5 min
Relative Abundance
m/z
431.0433
430.0443
433.0438
429.0431
428.0445
434.0471
427.0435


699.6321
Ir-Ru, Retention time, R = 6.1 min
468.4407
Relative Abundance
m/z
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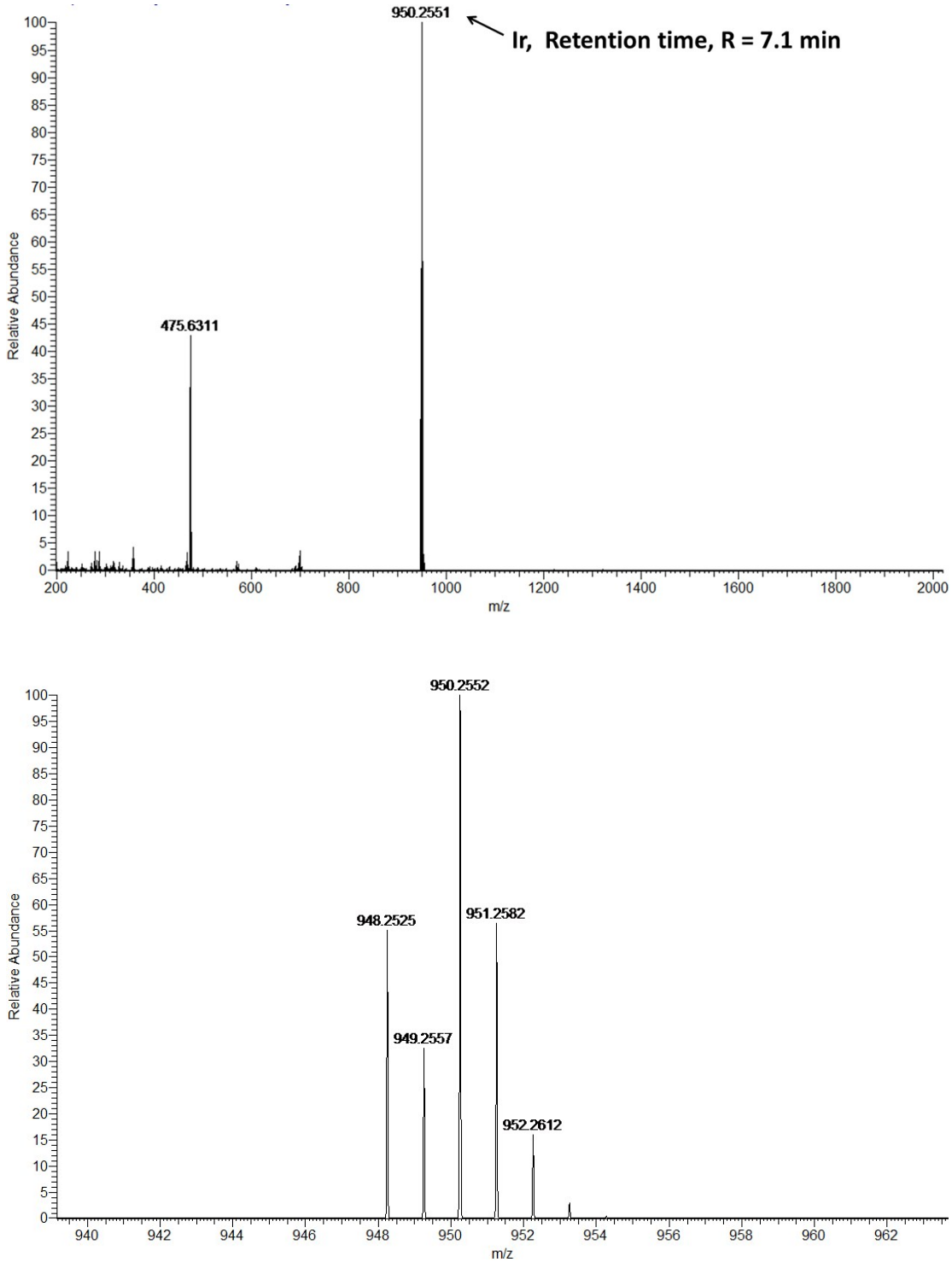

Figure S8. Corresponding components of the peaks after 450 nm irradiation for 10 min in the same retention time identified by HPLC-HRMS analysis.

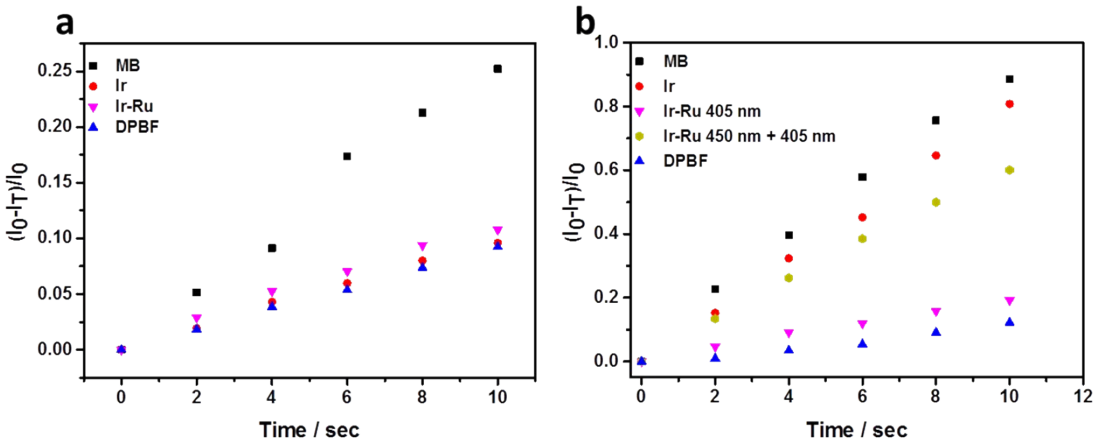

Figure S9. Photooxidation of DPBF (70 μM) by **Ir** and **Ir-Ru** in aerated CH_3OH. a) Changes in absorbance of DPBF at 417 nm upon irradiation at 450 nm or in the presence of **Ir-Ru** or **Ir** was monitored; b) Changes in absorbance of DPBF at 417 nm upon irradiation at 405 nm (or 450 nm + 405 nm) in the absence or presence of **Ir-Ru** or **Ir** was monitored. Methylene blue (**MB**) was used as the standard.

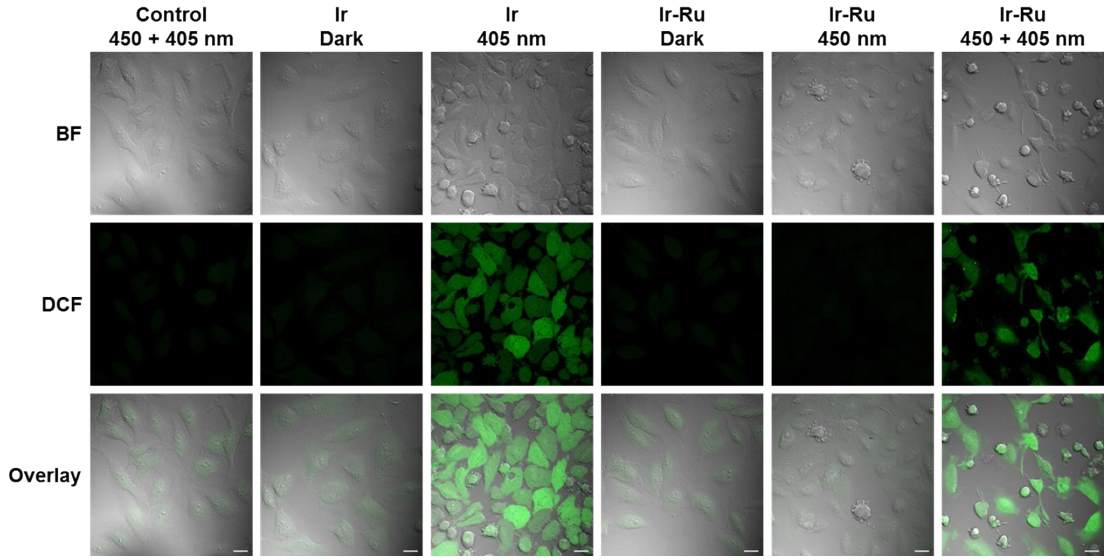

Figure S10. ROS generation assay. A549R cells were treated with 1 μM **Ir** or **Ir-Ru** for 6 h in the dark or with the listed irradiation, cells were then stained with DCFH-DA and incubated at 37 °C in the dark for another 20 min, the green fluorescence of DCF was measured by confocal microscopy. Scale bar: 20 μm.

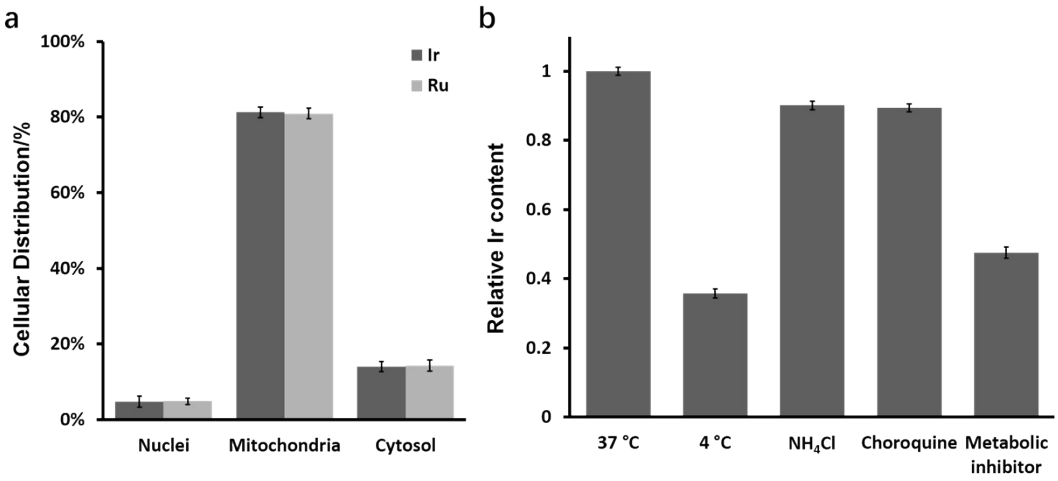

Figure S11. a) Subcellular distribution of **Ir-Ru** in A549R cells after treatment with 2.5 μM **Ir-Ru** for 6 h.; b) Investigation of the cellular uptake mechanism of **Ir-Ru.** A549R cells were incubated with **Ir-Ru** (2.5 μM) for 2 h followed by: control cells at 37 °C, cells pretreated at 4 °C, 50 μM chloroquine, 50 mM NH_4Cl, metabolic inhibitors.

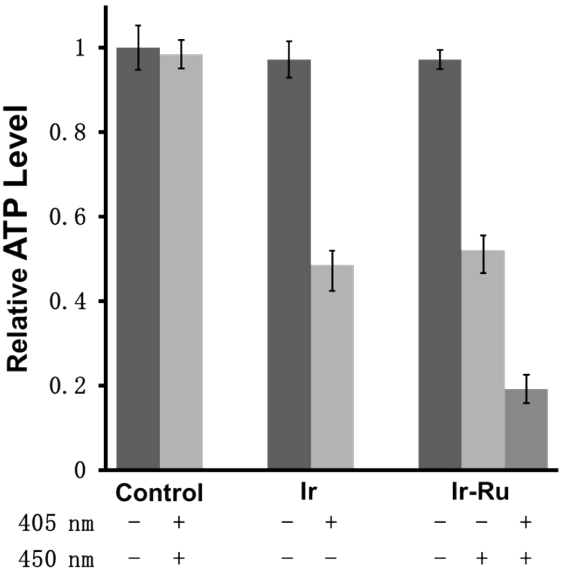

Figure S12. Intracellular ATP levels in A549R cells upon different irradiation treatments. The cells were treated with 1 μM **Ir** and **Ir-Ru** for 12 h at 37 °C, irradiation conditions: 450 nm, 6 J • cm^{-2}; 405 nm, 3 J • cm^{-2}; or 450 nm, 6 J • cm^{-2} followed by 405 nm, 3 J • cm^{-2}.

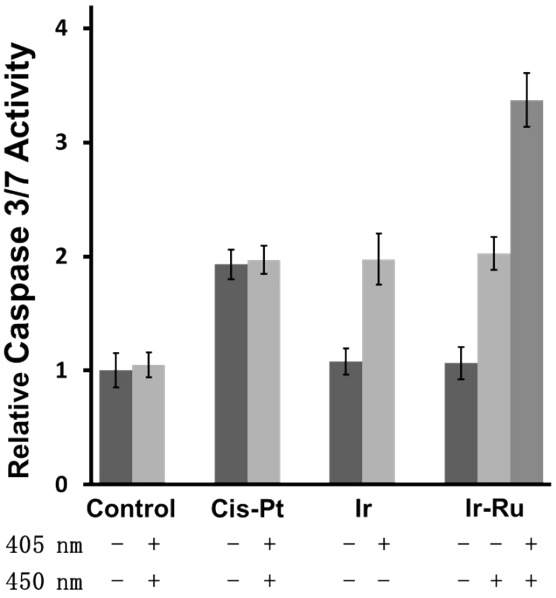

Figure S13. Detection of caspase-3/7 activity in A549R cells treated with 1 μM **Ir**, **Ir-Ru** or 100 μM **cisplatin** in the absence or presence of light at the indicated concentrations (Irradiation condition: 450 nm, 6 J • cm^{-2}; 405 nm, 3 J • cm^{-2}; or 450 nm, 6 J • cm^{-2} followed by 405 nm, 3 J • cm^{-2}).

Table S1. The sequences of primer pairs to amplify human target genes for Q-PCR-based DNA damage assay

Human	**sequences**	
β-Globin gene (nucleus, 13.5kb)	5' – TTG AGA CGC ATG AGA CGT GCA G – 3'	Sens
	5' – GCA CTG GCT TAG GAG TTG GAC T – 3'	Anti
Mitochondria long fragment (8.9kb)	5' – TCT AAG CCT CCT TAT TCG AGC CGA – 3'	Sens
	5' – TTT CAT CAT GCG GAG ATG TTG GAT GG – 3'	Anti

Table S2. IC_{50} values (48 h, μM) towards normal cells

	Ir-Ru Dark	**Ir-Ru** 450 nm	**Ir-Ru** 450+405 nm	**Cis-Pt**
LO2	15.40 ± 2.1	6.45 ± 0.1	2.55 ± 0.9	18.40 ± 2.4

Supporting References

[1] Y. Li, C.-P. Tan, W. Zhang, L. He, L.-N. Ji and Z.-W. Mao, *Biomaterials*, 2015, **39**, 95.

[2] J. H. Santos, J. N. Meyer, B. S. Mandavilli and B. V. Houten, *Methods Mol. Biol.*, 2006, **314**, 183.

[3] J.P. Liu, C. Z. Jin, B. Yuan, X. G. Liu, Y Chen, L.N. Ji, and H. Chao, Chem. Commum., 2017,**53**, 9878-9881.

[4] C. Jin, J. Liu, Y. Chen, L. Zeng, R. Guan, C. Ouyang, L. Ji and H. Chao, Chem. Eur. J., 2015, 21, 12000