Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Iridium(III) complexes as mitochondrial topoisomerase inhibitors against cisplatin-resistant cancer cells

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Experimental section

Materials and reagents

All reagents were commercially available and used without further purification unless special noted. Cisplatin and camptothecin were purchased from Sigma Aldrich (USA). Plasmid pBR322 DNA and nucleus extraction kit were purchased from Thermo Scientific (USA). Topoisomerase I was purchased from Takara (Japan). Annexin V-FITC apoptosis detection kit was purchased from BD (USA). Caspase-3/7 activity kit quantification assay kit was 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).

Instruments

ESI-MS was carried out on an LCQ system (Finnigan MAT, USA). The quoted m/z values represented the major peaks in the isotopic distribution. The ^{1}H NMR spectra were recorded at 400 MHz on an AVANCE III 400 MHz nuclear magnetic resonance spectrometer. The chemical shifts δ are expressed as parts per million (ppm) compared with tetramethylsilane (TMS). UV-Vis spectra were recorded on a PerkinElmer Lambda 850 spectrometer. The Ir contents were measured using an Agilent inductively coupled plasma mass spectrometry (ICP-MS) 7700x. Flow cytometry was conducted by using a FACS Canto II flow cytometer (BD Biosciences, USA). Cell imaging experiments were carried out on a confocal microscope (LSM 880, Carl Zeiss, Göttingen, Germany).

Synthesis of the corresponding ligands and iridium(III) complexes

The synthetic route of the complexes is shown in Scheme S1. Benz[d]indeno[1,2-b]pyran-5,11-dione and 6-(3-aminopropyl)-5*H*-indeno[1,2-*c*]isoquinoline-5,11(6*H*)-dione were prepared according to previously published methods.[1]

6-(3-(1*H*-imidazol-1-yl)propyl)-5*H*-indeno[1,2-*c*]isoquinoline-5,11(6*H*)-dione (L1). N-(3-Aminopropyl)-imidazole was added to a stirred solution of benz[d]-indeno[1,2-b]pyran-5,11-dione (0.5 g, 2.0 mmol) in chloroform (50 mL), and the reaction mixture was heated at 60°C for 48 h. The reaction mixture was diluted with chloroform (150 mL) and washed with H_2O (3 x 50 mL) and saturated NaCl (aq.) (3 x 50 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated over *vacuum* to obtain an orange solid. The product was used for the next step without further purification.

6-(3-([2,2'-bipyridin]-4-ylamino)propyl)-5*H*-indeno[1,2-*c*]isoquinoline-5,11(6*H*)-dione(L2). 4-bromo-2,2'-bipyridine (235 mg, 1 mmol, 1eq.) , 6-(3-aminopropyl)-5*H*-indeno[1,2-*c*]isoquinoline-5,11(6*H*)-dione (304 mg, 1mmol, 1 eq.), BINAP (26.0 mg, 41.0 μmol, 4% eq.), $Pd(dba)_2$ (18.0 mg, 30.0 μmol, 3% eq.) and t-BuONa (480 mg, 5.00 mmol, 5 eq) were suspended in degassed toluene (50 mL) and refluxed for 48 h under argon atmosphere. After cooling to room temperature, the mixture was filtered and the filterate was evaporated under reduced pressure to yield orange solid. The product was used for the next step without further purification.

[Ir(ppy)$_2$L1]OTf (Ir1). A mixture of $[Ir(ppy)_2(CH_3CN)_2]OTf$ (81.62 mg, 0.14 mmol) and **L1** (200 mg, 0.56 mmol) was dissolved in 60mL dichloromethane and methanol mixture (v/v, 1:3) and heated at 65 °C for 24 h under argon in the dark. The solvent was evaporated under reduced pressure and the raw product was purified by recrystallization using methanol. Yield: 59%. ES-MS (CH_3OH): m/z = 1211.2 $[M-OTf]^+$. ^1H NMR (400 MHz, DMSO-d6) δ 8.80 (d, *J* = 5.7 Hz, 1H), 8.33 (d, *J* = 8.1 Hz, 1H), 8.25 (s, 1H), 8.03 (d, *J* = 8.2 Hz, 1H), 7.95 (d, *J* = 8.1 Hz, 1H), 7.89 (t, *J* = 7.8 Hz, 1H), 7.62 (d, *J* = 7.4 Hz, 1H), 7.56 (t, *J* = 7.6 Hz, 1H), 7.48 – 7.38 (m, 4H), 7.36 – 7.30 (m, 2H), 7.23 (t, *J* = 7.6 Hz, 2H), 6.82 (s, 1H), 6.79 (s, 1H), 6.29 (d, *J* = 7.0 Hz, 1H), 4.18 (s, 2H), 4.11 (d, *J* = 7.2 Hz, 2H), 2.11 (d, *J* = 5.0 Hz, 2H).

[Ir(ppy)$_2$L2]OTf (Ir2). A mixture of **L2** (100 mg, 0.21 mmol) and $[Ir(ppy)_2(CH_3CN)_2]OTf$ (162 mg, 0.28 mmol) was dissolved in 60mL mixed solvent

of chloroform and methanol (v/v, 1:3) and heated at 65 °C for 24 h under argon atmosphere in the dark. The solvent was evaporated under reduced pressure and the raw product was purified by alumina column chromatography using dichloromethane and methanol as the eluent to give an orange powder. Yield: 74%. ES-MS (CH_3OH): m/z = 959.7 $[M\text{-}OTf]^+$. ^1H NMR (400 MHz, DMSO-d6) δ 8.83 (s, 1H), 8.56 (d, *J* = 8.0 Hz, 1H), 8.28 – 8.16 (m, 4H), 7.97 – 7.88 (m, 4H), 7.86 – 7.78 (m, 4H), 7.74 (d, *J* = 7.5 Hz, 1H), 7.69 (d, *J* = 5.8 Hz, 1H), 7.63 (t, *J* = 6.1 Hz, 2H), 7.54 – 7.47 (m, 2H), 7.39 (t, *J* = 7.4 Hz, 1H), 7.30 (t, *J* = 7.5 Hz, 1H), 7.24 – 7.16 (m, 3H), 7.03 – 6.96 (m, 2H), 6.87 (q, *J* = 7.0 Hz, 2H), 6.76 (d, *J* = 4.5 Hz, 1H), 6.22 (d, *J* = 7.0 Hz, 1H), 6.17 (d, *J* = 6.9 Hz, 1H), 4.60 (t, *J* = 7.0 Hz, 2H), 3.53 (s, 2H), 2.20 – 1.99 (m, 2H).

$[Ir(ppy)_2bpy]PF_6$. A mixture of 2,2'-bipyridine (100 mg, 0.64 mmol) and $[Ir(ppy)_2Cl]_2$ (342 mg, 0.32 mmol) was dissolved in 60 mL mixed solvent of chloroform and methanol (v/v, 1:3) and heated at 65 °C for 24 h under argon atmosphere in the dark. The solvent was evaporated under reduced pressure, dissolved with saturated solutions of KPF_6. The product was purified by alumina column chromatography using dichloromethane and methanol as the eluent to give an orange powder. Yield: 95%. ES-MS (CH_3OH): m/z = 657.4 $[M\text{-}PF_6]$. ^1H NMR (400 MHz, DMSO-d_6) δ 8.90 (d, *J* = 8.2 Hz, 2H), 8.31 – 8.24 (m, 4H), 7.99 – 7.90 (m, 4H), 7.87 (d, *J* = 4.5 Hz, 2H), 7.73 – 7.68 (m, 2H), 7.62 (d, *J* = 5.1 Hz, 2H), 7.16 (t, *J* = 6.6 Hz, 2H), 7.02 (t, *J* = 7.0 Hz, 2H), 6.91 (t, *J* = 6.8 Hz, 2H), 6.20 (d, *J* = 6.7 Hz, 2H).

Topoisomerases inhibition assay

10 μL mixture of pBR322 plasmid DNA (25 ng/μL, Thermo, USA) and recombinant human DNA topo I (1 Unit; TaKaRa, Japan) was incubated with or without the test compounds at 37 °C for 30 min in relaxation buffer (50 mM Tris-HCl/18 mM NaCl, pH= 7.9). The reaction was terminated by the addition of 2 μL of 5× stop solution consisting of 0.25% bromophenol blue, 4.5% SDS, and 45% glycerol. The DNA samples were electrophoresed on 1% agarose gel at 90 V for 2 h with TBE (89 mM Tris-borate acid, 2 mM EDTA, pH 8.3) as the running buffer. The gel was

stained with 1 μg/mL EB and photographed on an OmegaLum C image system (Aplegen, USA).

Formation of topoisomerase-mediated DNA cleavage

The topoisomerases inhibition mechanism was measured by the formation of topoisomerase-mediated DNA cleavage according to the previously reported method with minor modifications.[2] Supercoiled DNA (pBR322, 100 ng; Thermo, USA) was reacted with 10 units topo I (TaKaRa, Japan) for 10 min before addition of the test compounds. After adding the complex, the mixture is incubated at 37°C for 20 minutes The reaction mixture was incubated at 37°C for 20 min. The reaction was stopped by the addition of 0.5% sodium dodecyl sulfonate, followed by digestion with proteinase K at 45°C for 30 min. After the addition of loading buffer, the reaction mixture was heated for 2 min at 70°C. The DNA samples were electrophoresed on 1% stained agarose gel at 100 V for 2 h with TBE (89 mM Tris-borate acid, 2 mM EDTA, pH 8.3) as the running buffer. The gel was photographed on an OmegaLum C image system (Aplegen, USA).

In vitro cytotoxicity

The *in vitro* cytotoxicity of complexes **Ir1-2** were determined by MTT assay. A549R, A549, HeLa, HepG2, and LO_2 cell lines were purchased from the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). All cells were seeded into 96-well plates at (1×10^4 cells per well) and incubated in DMEM supplemented with 10% FBS for 24 h at 37 °C under 5% CO_2. Then the cells were incubated with various concentrations of the complexes, after 44 h of incubation, MTT dye solution (10 μL, 5 mg/mL in 1× PBS) was added to each well and incubated for 4 h. 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).

ICP-MS assay

For the localization study, exponentially grown A549R cells were harvested, and the resulting single-cell suspension was plated into tissue plates (100 mm, Costar) at 1×10^6 cells/plate. After 24 h, the cells were treated with 2.5 μM Ir(III) complexes and incubated for 24 h at 37 °C. After digestion by trypsin, collected cells, were separated by nuclear extraction kits (Thermo Scientific) and mitochondria extraction kits (Thermo Scientific) respectively. The extractions were digested with 60% HNO_3 (500 μL) at room temperature for 2 days. Each sample was diluted with 9.5 mL double-distilled water. A standard curve was made for the quantitative determination. The Ir contents were measured by inductively coupled plasma mass spectrometry (ICP-MS Thermo Elemental Co., Ltd.). The ICP-MS parameters was: 1) selection of Sn and In as internal standard. 2) Acquisition parameters dwell time 0.02, the number of scans below 300. 3) Selecting In as interference correction.

DNA damage assay

A549R cells were seeded at 1×10^4 cells/well and allowed to adhere overnight. Cells were treated with 2.5 μM **Ir1-2**, or 60 μM cisplatin for 24 h in the dark 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.[3] Quantitation of amplified product was performed by PicoGreen staining and normalized to nontreated value.

PicoGreen staining

A549R cells were seeded in 35 mm glass-bottom dishes (Corning) for 24 h. Then the cells were pre-treated with **Ir1-2** (2.5 μM) for 24 h. After staining with PicoGreen for 1h, the cells were washed three times with PBS and the green fluorescence was detected by confocal microscopy.

ROS detection

A549R cells were seeded in 35 mm glass-bottom dishes (Corning) for 24 h. Then the cells were pre-treated with **Ir1-2** (2.5 μM) for 24 h. After staining with DCFH-DA for 30 min in 37°C, the cells were washed three times with PBS and the green fluorescence was detected by confocal microscopy. (λ_{ex} = 488 nm; λ_{em} = 520 ± 20 nm)

JC-1 staining assay

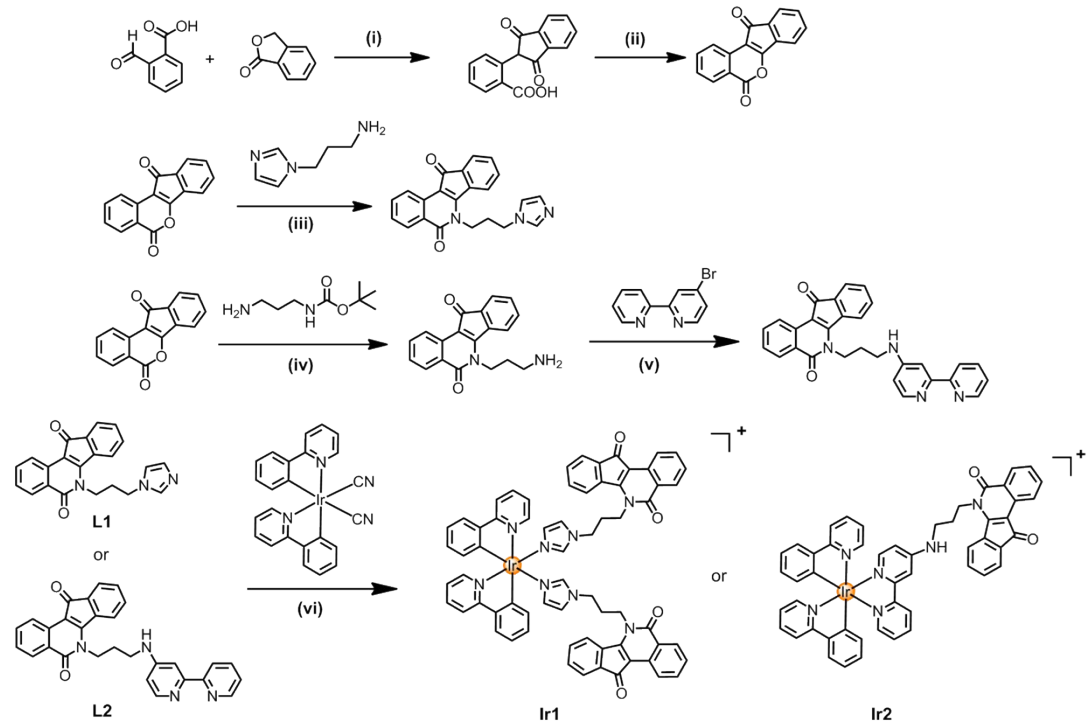
A549R cells were seeded in 35 mm glass-bottom dishes (Corning) for 24 h. Then the cells were pre-treated with **Ir1-2** (2.5 μM) for 24 h. After staining with JC-1 for 30 min in 37°C, the cells were washed three times with PBS and the green (λ_{ex} = 488 nm; λ_{em} = 530 ± 20 nm) and red fluorescence (λ_{ex} = 488 nm; λ_{em} = 590 ± 20 nm) was detected by confocal microscopy.

Caspase activation assay

Caspase-3/7 activity was assessed using the corresponding Caspase-Glo assay kit (Promega, USA). A549R cells were seeded in white-walled 96-well plates at a density of 8 × 10^3 cells for 24 h. The cells were incubated with **Ir1-2** (2.5 μM) or cisplatin (60.0 μM). After 24 h incubation, the caspase activations were detected according to the manufacturer's instructions. The luminescence intensity was measured by an Infinite M200 PRO (Tecan).

Annexin V-FITC/propidium iodide (AV/PI) dual staining

Tumour cell death induced by cisplatin and **Ir1-2** were quantified by flow cytometry using the annexin V-FITC kit (BD Pharmingen, USA) in accordance with the manufacturer's protocol. Briefly, A549R cells were seeded in a six-well plate at a density of 1 × 10^5 per well and incubated with or without the indicated tested compounds for 24 h. All the cells were collected. Washed with cool PBS for 3 times. The cell pellets were resuspended in 100 μL of 1× binding buffer at a concentration of 1 × 10^5 cells/mL. The 5 μL of annexin V-fluorescein isothiocyanate (FITC) and 5 μL of PI were added to the cell suspension. After incubation for 15 min at room temperature, 400 μL of 1× binding buffer was added, samples were examined using a FACSCaliber flow cytometer (BD Biosciences, USA). Analysis was performed by the BD Cell Quest Pro software supplied in the instrument.



(i) EtOAc/MeONa, 65°C, 18 hrs.(ii) PhH, 80°C, 7 hrs. (iii) $CHCl_3$, 60 °C, 48 hrs . (iv) $CHCl_3$, 60 °C, 72 hrs; 5M HCl, rt, 18 hrs . (v) Tol, BINAP, Pd(dba)2, t-BuONa, 60 °C, dega, 24 hrs. (vi) MeOH/$CHCl_3$, dark, Ar, 65 °C, 24 hrs.

Scheme S1 Synthetic route of **Ir1**-**Ir2**.

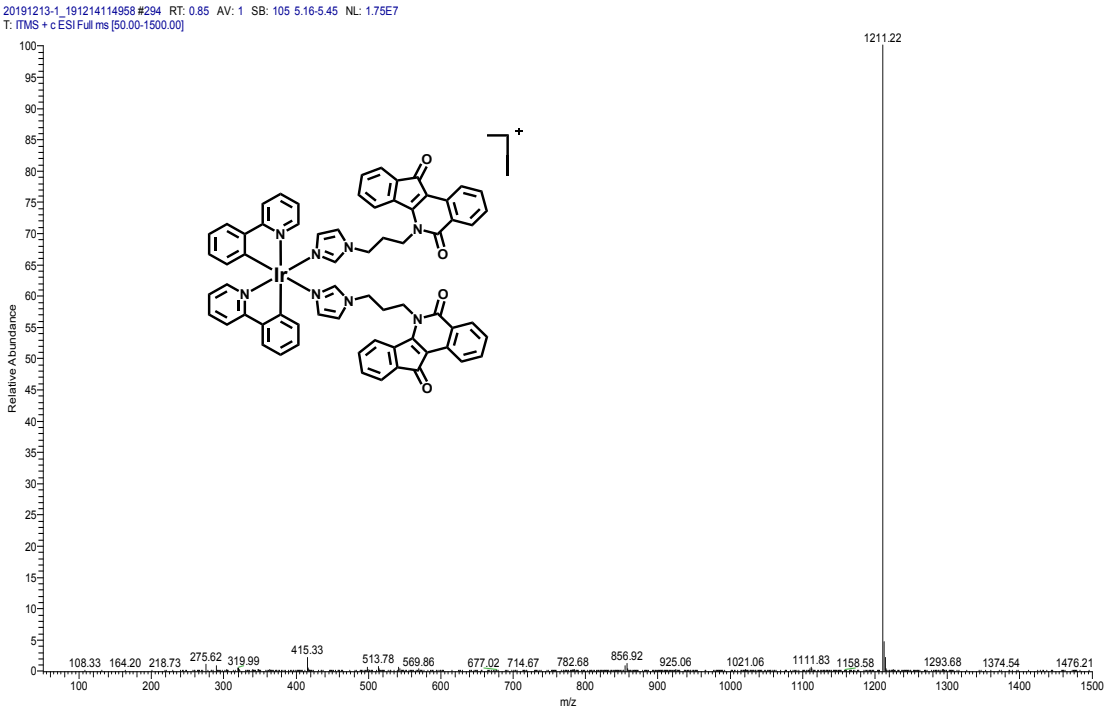


Fig. S1 ESI-MS spectra of **Ir1** in CH_3OH.

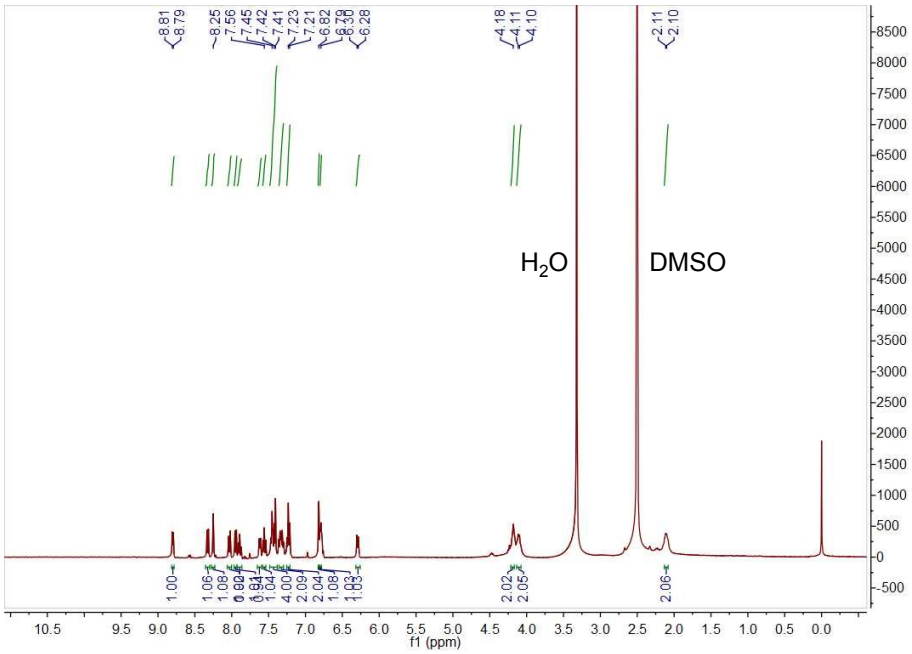


Fig. S2 ^{1}H NMR spectra of **Ir1** in DMSO-*d*$_6$.

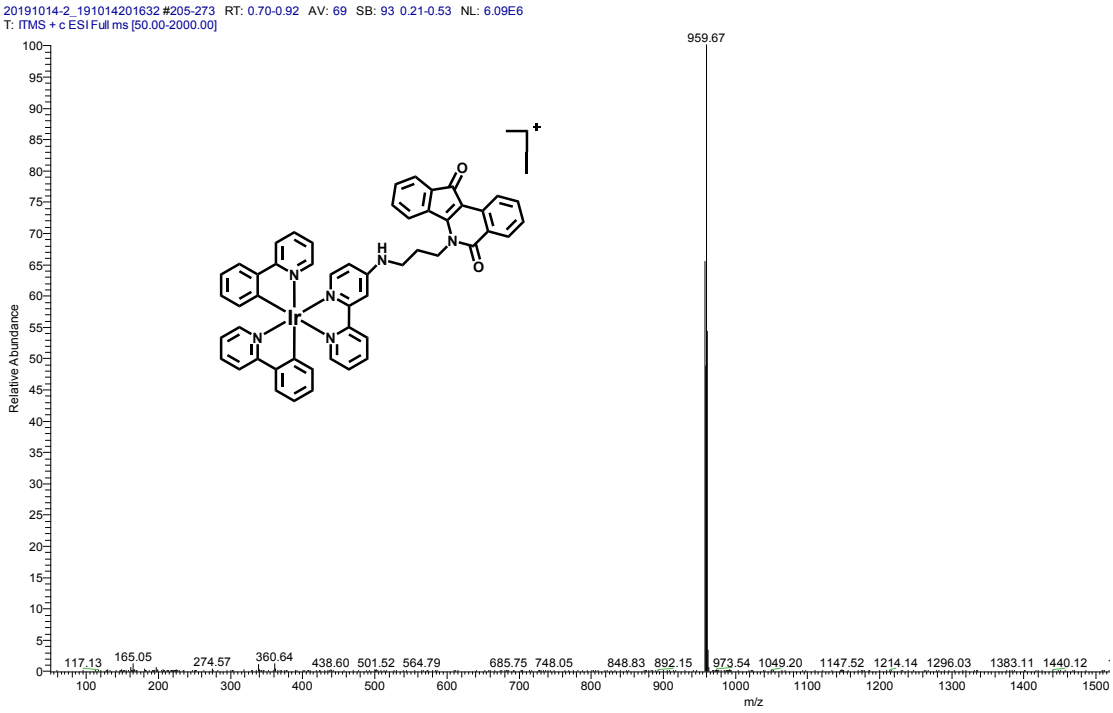


Fig. S3 ESI-MS spectra of **Ir2** in CH_3OH.

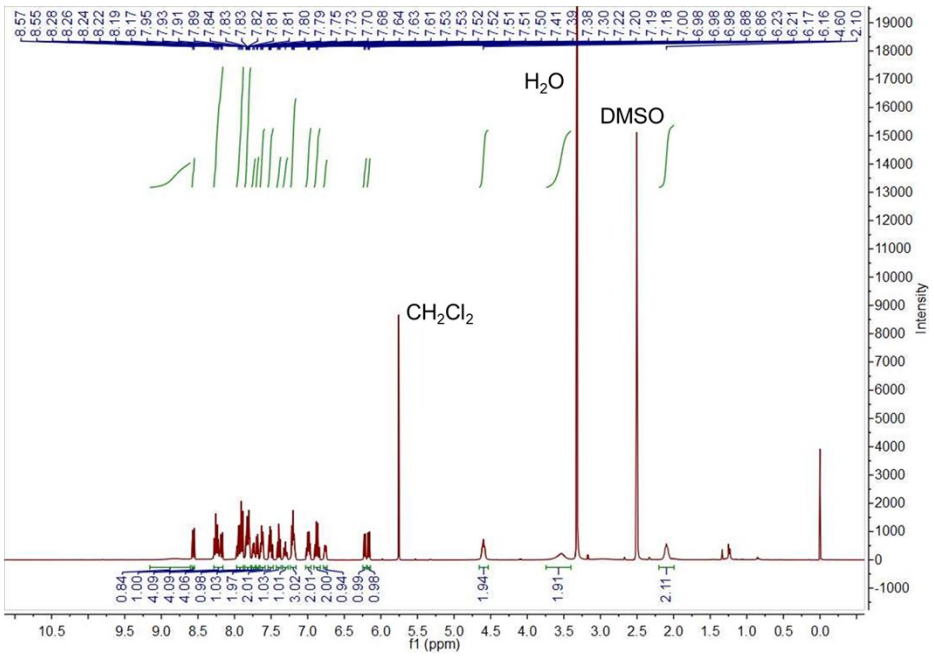


Fig. S4 ^{1}H NMR spectra of **Ir2** in DMSO-*d*$_6$.

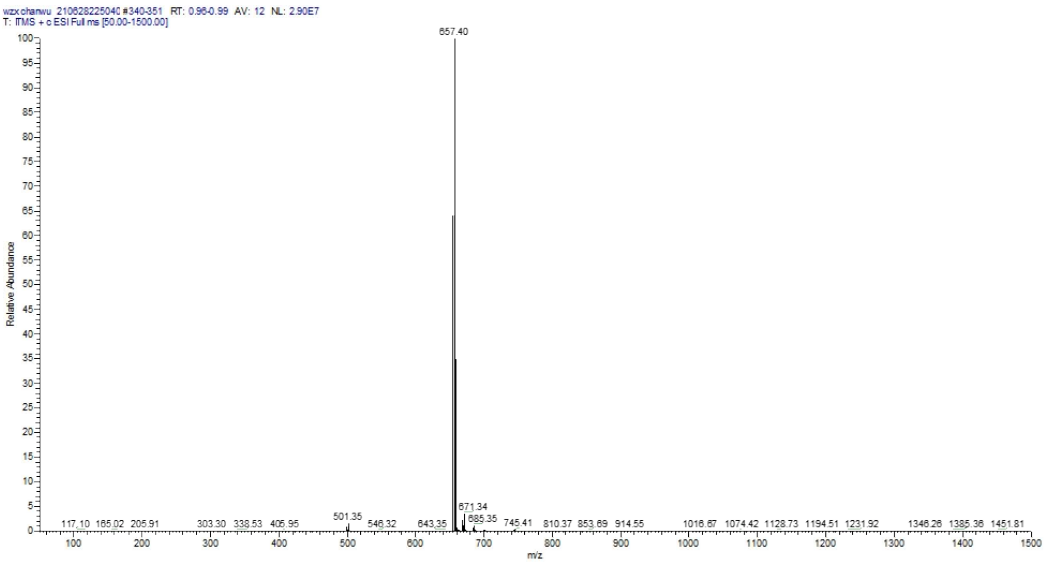


Fig. S5 ESI-MS spectra of $[Ir(ppy)_2bpy]PF_6$ in CH_3OH.

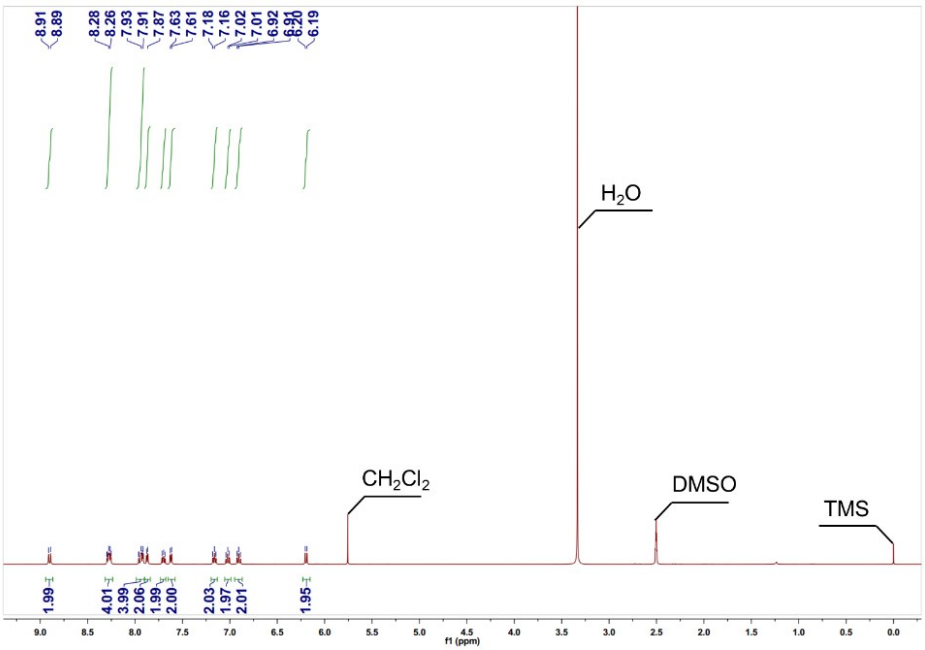


Fig. S6 ^1H NMR spectra of $[Ir(ppy)_2bpy]PF_6$ in DMSO-*d*6.

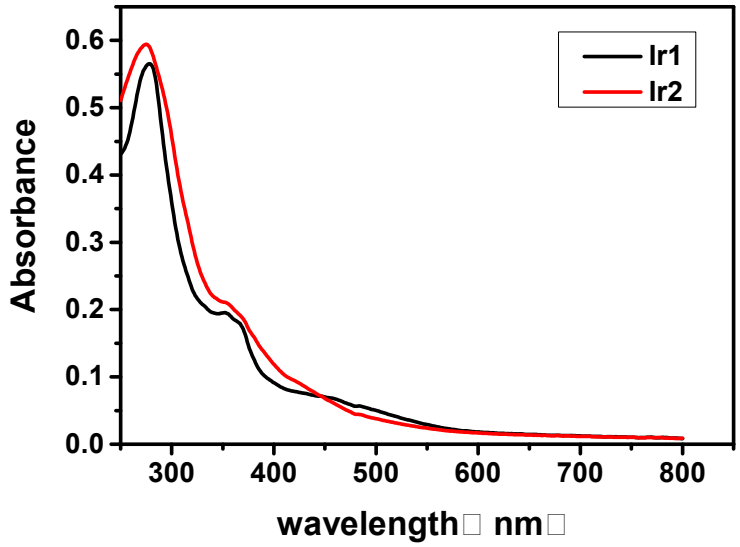


Fig. S7 The Absorption spectra of **Ir1-2** (10.0 μM) in PBS.

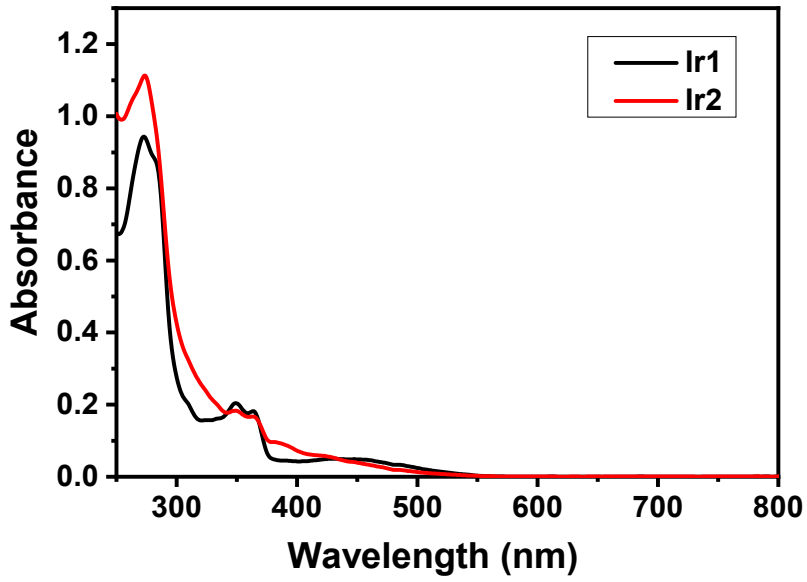


Fig. S8 The Absorption spectra of **Ir1-2** (10.0 μM) in CH_2Cl_2.

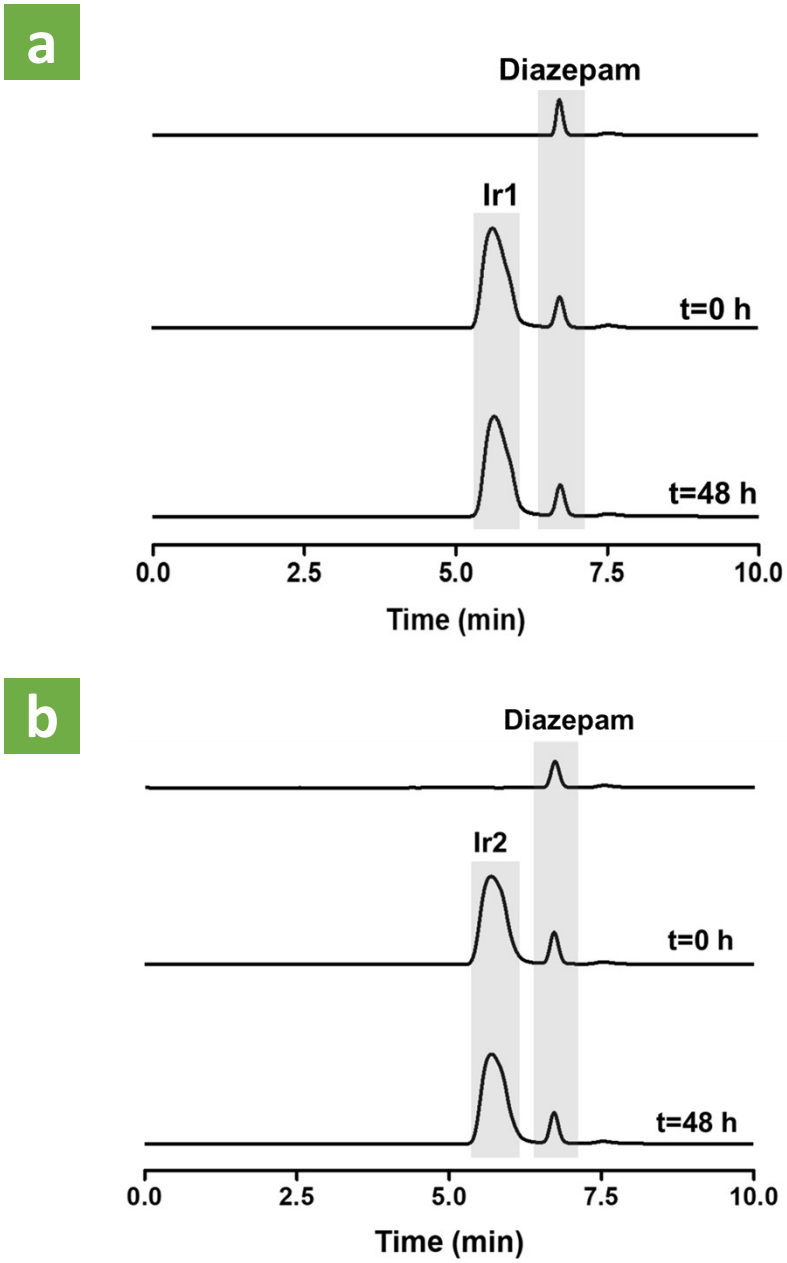


Fig. S9 Stability of **Ir1-Ir2** (10.0 μM) in H_2O with 1% DMSO by HPLC. (a) **Ir1**. (b) **Ir2**.

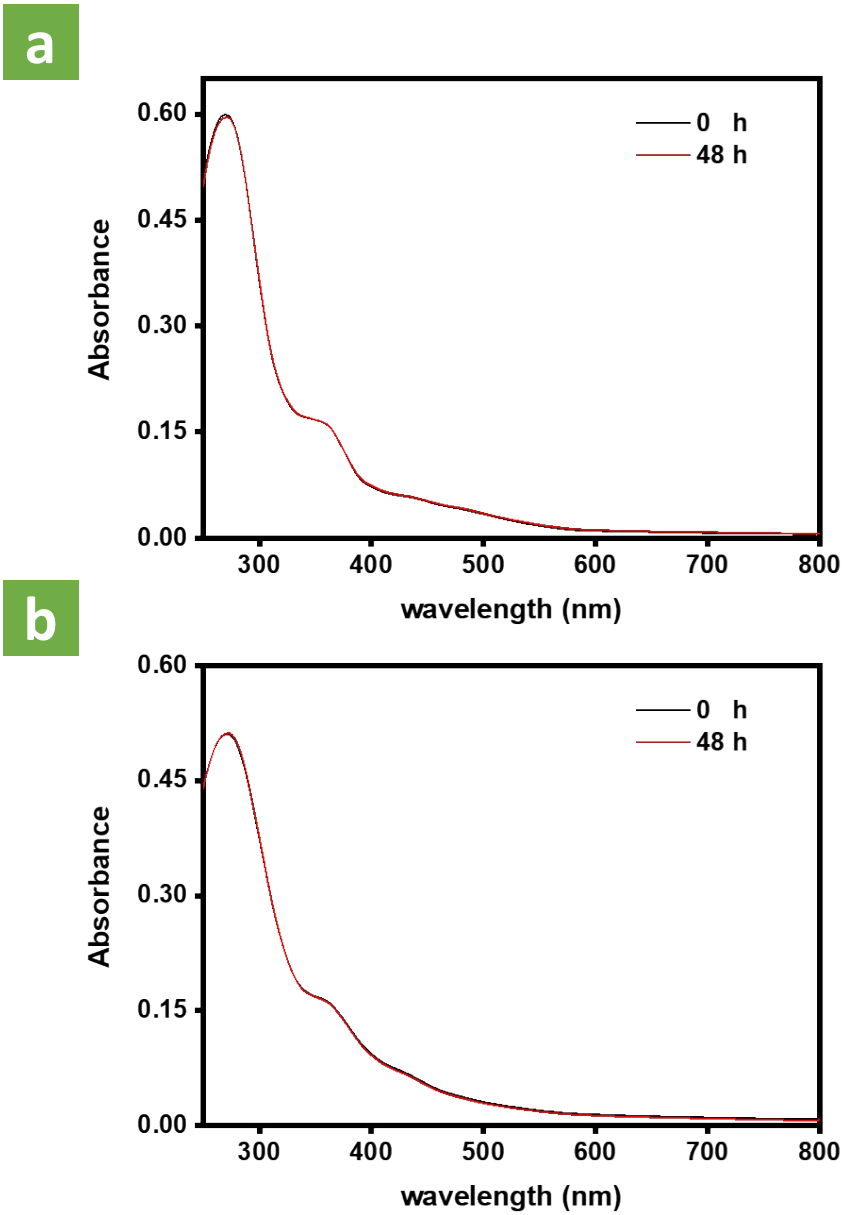


Fig. S10 Stability of **Ir1**-**Ir2** (10.0 μM) in PBS by the Absorption spectra. (a) **Ir1**. (b) **Ir2**.

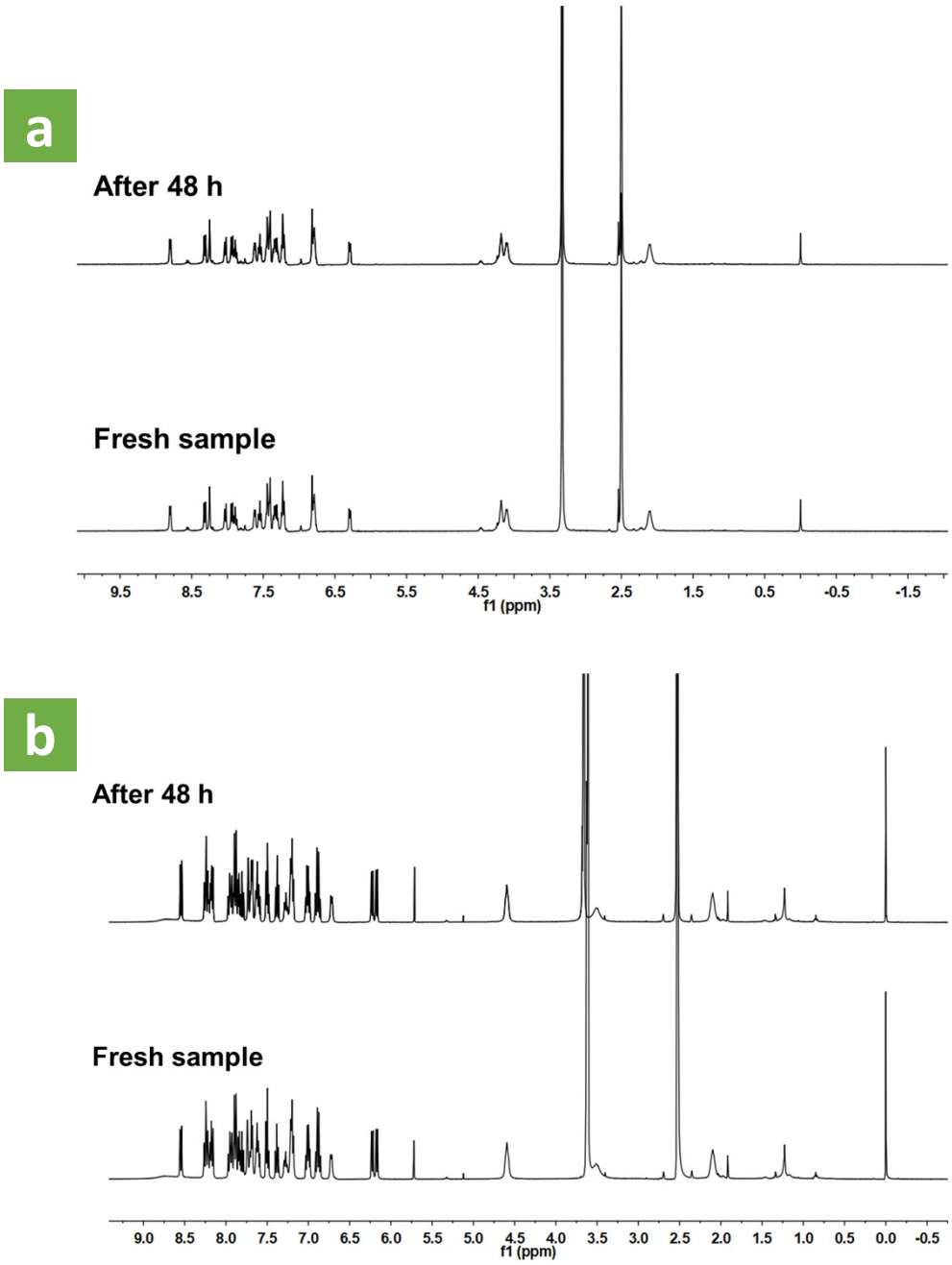


Fig. S11 Stability of Ir1-Ir2 (10.0 μM) in d_6-DMSO:D_2O = 6:1 by ^{1}H-NMR spectra.

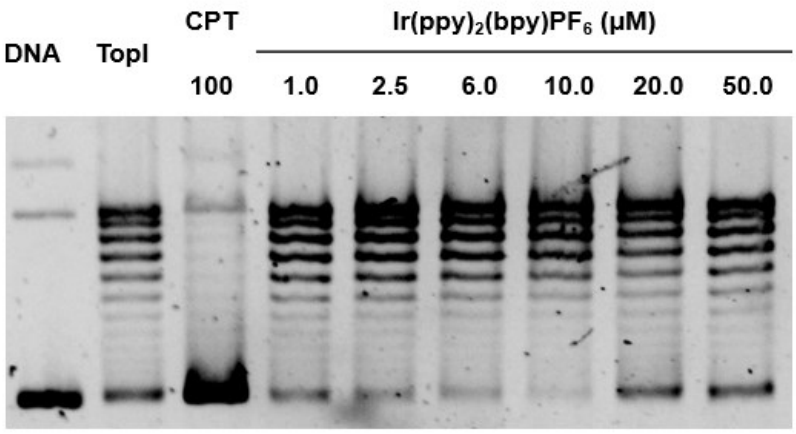


Fig. S12 Inhibitory effects of $[Ir(ppy)_2bpy]PF_6$ on the catalytic activity of topoisomerases I was determined with relaxation assays.

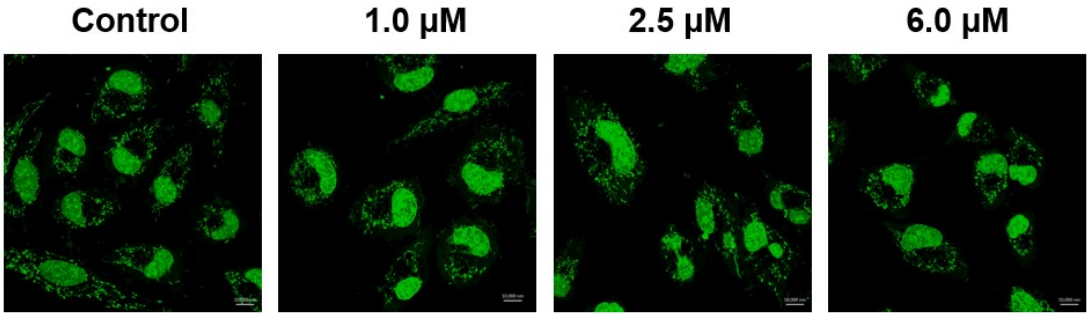


Fig. S13 PicoGreen staining of $[Ir(ppy)_2bpy]PF_6$.

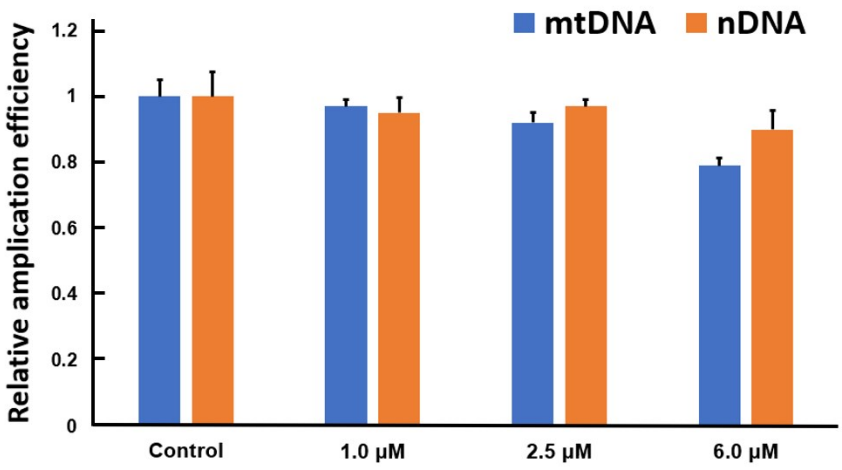


Fig. S14 mtDNA and nDNA damage analyzed by qPCR upon 24 h of treatment with $[Ir(ppy)_2bpy]PF_6$ in A549R cells. All values were normalized to the control.

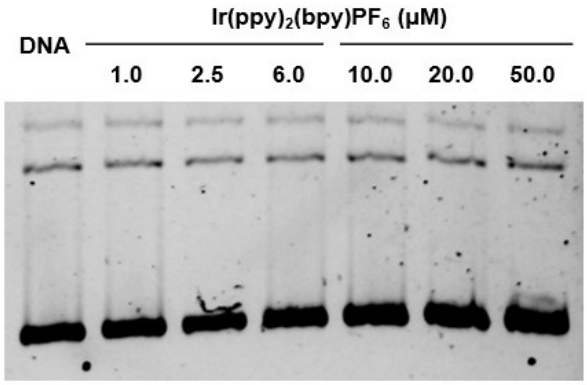


Fig. S15 DNA cleavage assay of $[Ir(ppy)_2bpy]PF_6$ (1.0, 2.5, 6.0, 10.0, 20.0 and 50.0 μM). No significant influence observed even at 50.0 μM.

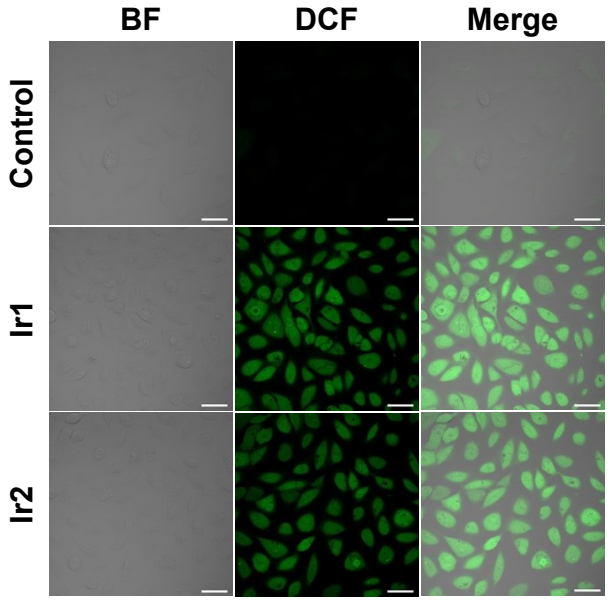


Fig. S16 ROS generation assay. A549R cells were treated with **Ir1**-**2** (2.5 μM) in the dark, then stained with DCFH-DA and incubated at 37 °C in the dark for another 20 min. The green fluorescence of DCF was measured by confocal microscopy. Scale bar: 50 μm.

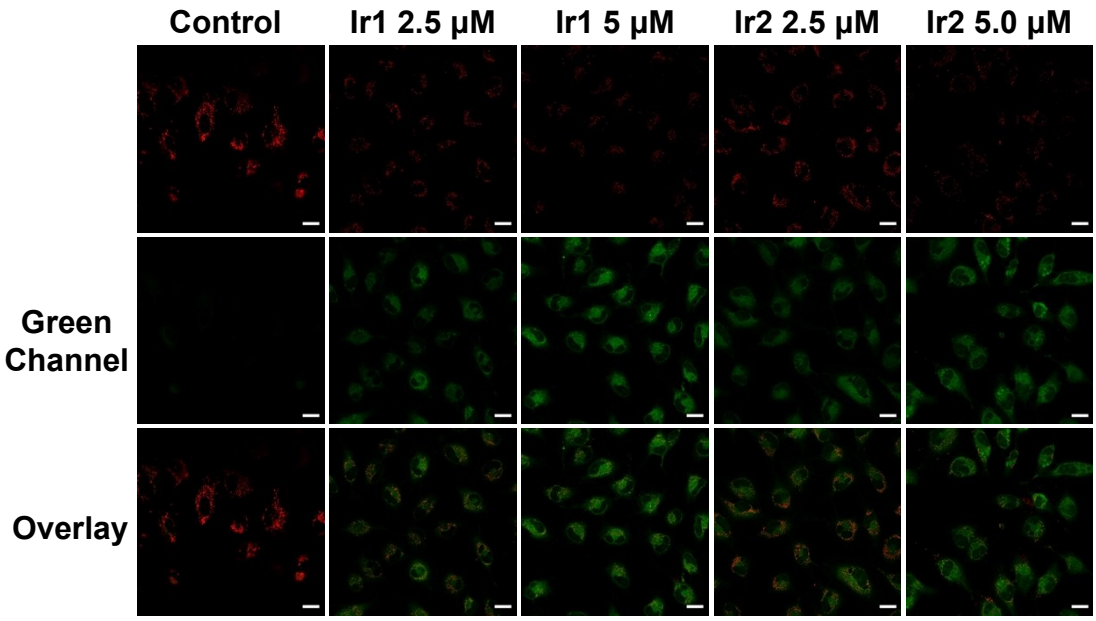


Fig. S17 Detection of MMP by JC-1 staining, in A549R cells after **Ir1-2** (2.5 μM or 5.0 μM) treatment for 24 h in the dark. Scale bar: 20 μm.

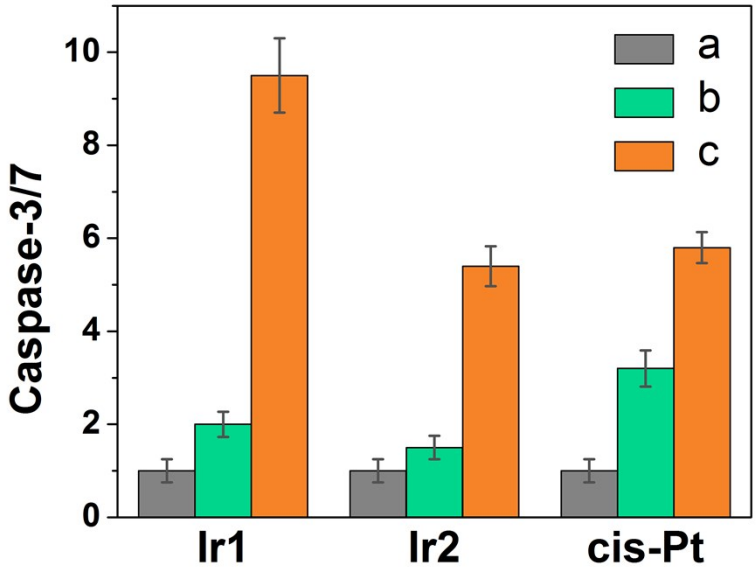


Fig. S18 Caspase-3/7 activity measurements. A549R cells were incubated with different concentrations of Ir complex or cisplatin (a: control; b: Ir1 2.5 μM, Ir2 2.5 μM, cis-Pt 40.0 μM; c: Ir1 5.0 μM, Ir2 5.0 μM, cis-Pt 60.0 μM) for 24 h.

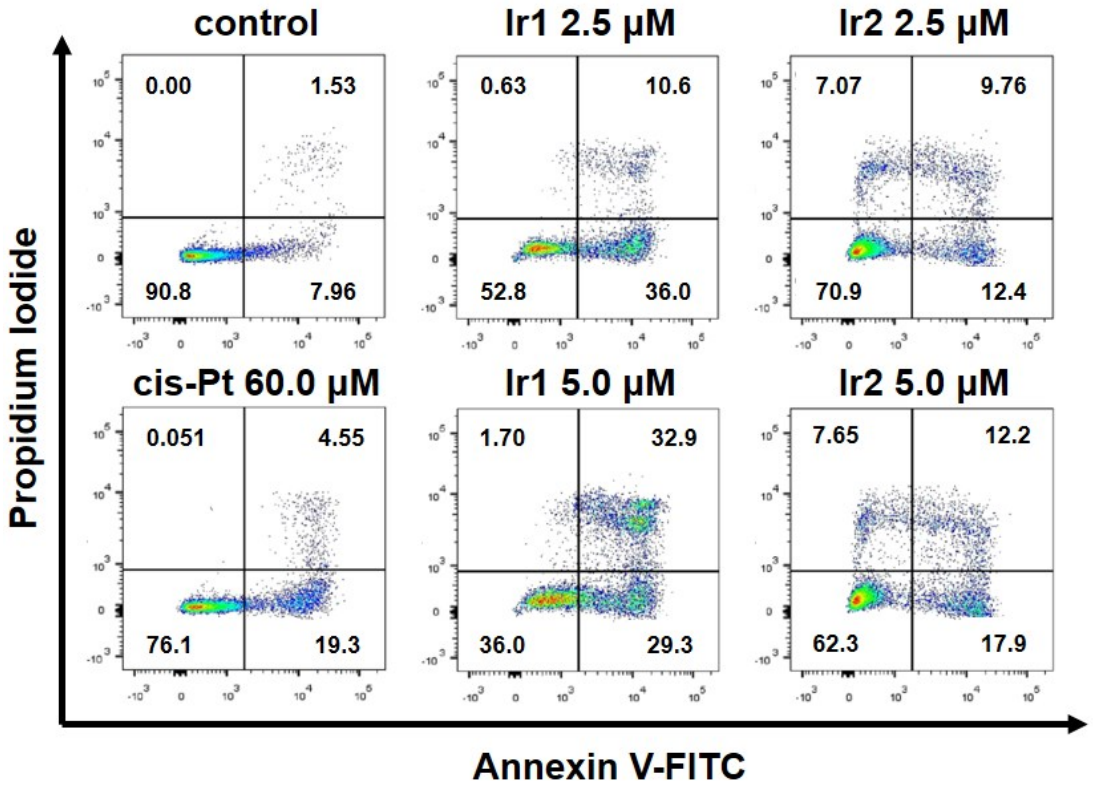


Fig. S19 Investigation of cell death by the Annexin V-FITC/propidium iodide co-staining assay in A549R cells after **Ir1-2** (2.5 μM or 5.0 μM) or cisplatin (60.0 μM) treatment for 24 h in the dark.

Table S1. IC_{50} values of **Ir1**-**Ir2**, **L1**-**L2**, and cisplatin towards various cancer cell lines and a non-tumour human cell line (μM, 48 h).

Complexes	A549R	A549	Hep G2	HeLa	LO2
Ir1	2.50 ± 0.23	7.32 ± 0.41	3.84 ± 0.37	6.61 ± 0.42	9.53 ± 0.37
Ir2	2.68 ± 0.27	2.27 ± 0.21	4.84 ± 0.34	5.87 ± 0.36	6.32 ± 0.28
L1	5.84 ± 0.31	13.16 ± 0.91	5.27 ± 0.53	8.71 ± 0.43	5.08 ± 0.21
L2	5.25 ± 1.02	7.64 ± 0.35	6.82 ± 0.57	8.35 ± 0.25	4.87 ± 0.23
cisplatin	75.60 ± 3.20	10.27 ± 0.82	12.18 ± 0.79	7.81 ± 0.42	8.84 ± 0.59
CPT [ref.4]	83.6 ± 5.2	78.2 ± 4.5	51.2 ± 7.3	46.5 ± 3.5	> 200

Table S2. The sequences of primer pairs to amplify human target genes for Q-PCR based DNA damage assay

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

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