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Supporting Information For

A mitochondria-targeting hetero-binuclear Ir(III)-Pt(II) complex induces necrosis in cisplatin-resistant tumor cells

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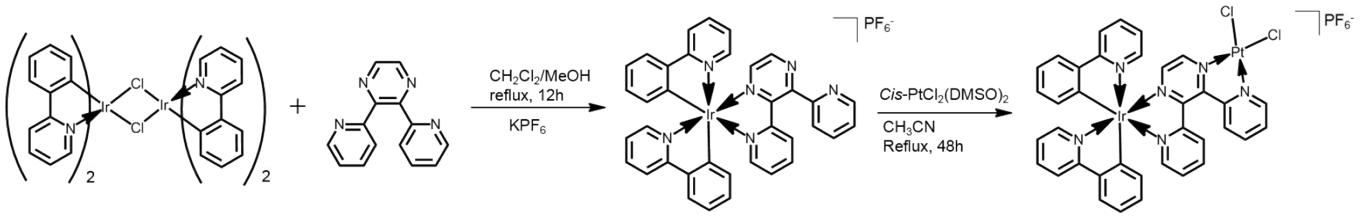
Materials and instruments

Unless otherwise noted, all chemical reagents and solvents were commercially available and used without further purification. Double distilled (DD) water was used throughout all of the experiments. $IrCl_3 \cdot xH_2O$, cisplatin, 2-phenylpyridine (ppy), 2,3-bis(2-pyridyl)pyrazine (dpp), Ir standard solution (1000 μg/mL), Pt standard solution (1000 μg/mL), PBS, Tris, Tween-20, Sodium hexafluorophosphate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Invitrogen. JC-1, propidium iodide (PI), Caspase-3/7 activity kit. Cytotoxicity Assay Kit were purchased from Thermo. MitoSox reagent kit, Sigma GenElute mammalian genomic DNA miniprep kit, Elongase long range PCR enzyme kit from Invitrogen. XF Cell Mito Stress Test Kit and XF Glycolysis Stress Test Kit were bought from Seahorse Bioscience. Nucleus extraction kit and cytoplasm extraction kit were purchased from Thermo pierce. BCA protein assay reagent kit (Novagen Inc, USA), rabbit monoclonal anti-RIP3 (Proteintech USA) and rabbit anti-GADPH (Cell Signaling Technology, USA), secondary antibodies (Cell Signaling), ECL prime Western blot detection reagent (Amersham Inc., USA). Cisplatin is dissolved in saline (1 mM) and be used immediately. Stock solutions of Ir-Pt in DMSO with different concentration were prepared to keep the final content of DMSO is 1% for all biological experiments.

Microananlysis (C, H and N) was performed using a Perkin-Elmer 240Q elemental analyzer. Electrospray ionization mass spectra (ES-MS) were recorded on a LCQ system (Finnigan MAT, USA). The ^{1}H NMR spectra and ^{195}Pt NMR spectra were recorded on a Varian INOVA500NB Superconducting Fourier Transform Nuclear. The inductively coupled plasma mass spectrometry (ICP-MS) experiments were carried out on an Agilent's 7700x instrument. Cell imaging was conducted on a LSM 710 (Carl Zeiss, Germany) Laser Scanning Confocal Microscope. OCR and ECAR were determined by a Seahorse XF-24 extracellular flux analyzer (Seahorse Bioscience). Cell membrane disruption, caspase-3/7 contents were measured by an infinite M200 PRO equipment (TECAN, Swiss). Kinetics of cytotoxicity responses for drugs were performed on an xCELLigence RTCA DP system real-time cell analyser (Roche Diagnostics GmbH, Germany).

Synthesis and characterization

$[Ir(ppy)_2Cl]_2$ was synthesized according to the published methods.[1] The synthetic route used to access **Ir-Pt** is illustrated in Scheme S1.



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

Synthesis of $[(ppy)_2Ir(dpp)](PF_6)$ (**Ir**)

A solution of $[(ppy)_2Ir(\mu\text{-}Cl)]_2$ (500 mg, 0.47 mmol) and 2,3-bis(2-pyridyl)pyrazine (dpp) (230 mg, 1.00 mmol) in 2 : 1 CH_2Cl_2/MeOH (20 mL) was refluxed under nitrogen in the dark for 12 h. A 10-fold excess of KPF_6 (1.01 g, 5.50 mmol) was added to the resulting red solution and cooled to room temperature with stirring for 2 h. After filtration of 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 red microcrystalline solid (Yield: 610 mg, 73%). Anal. Calcd for $C_{36}H_{26}F_6IrN_6P$: C, 49.15; H, 2.98; N, 9.55. Found: C, 48.88; H, 3.19; N, 9.43. ESI-MS: m/z 735 $[M]^+$. ^1H NMR (500 MHz, DMSO-d_6): δ = 8.89 (d, *J* = 3.0 Hz, 1H), 8.65 (d, *J* = 4.5 Hz, 1H), 8.307 (d, *J* = 8.0 Hz, 2H), 8.20 (t, *J* = 7.75 Hz, 1H), 8.09 (d, *J* = 8.0 Hz, 1H), 7.94 (m, 8H), 7.77 (d, *J* = 6.0 Hz, 1H), 7.69 (t, *J* = 6.75 Hz, 1H), 7.59 (t, *J* = 6.5 Hz, 1H), 7.29 (t, *J* = 7.25 Hz, 1H), 7.2 (m, 2H), 7.04 (dd, 2H), 6.94 (m, 2H), 6.18 (q, 2H).

Synthesis of $[(ppy)_2Ir(dpp)PtCl_2](PF_6)$ (**Ir-Pt**)

Upon refluxing a solution of $[(ppy)_2Ir(dpp)](PF_6)$ (100 mg, 0.11 mmol) and cis-$PtCl_2(DMSO)_2$ (140 mg, 0.33 mmol) in CH_3CN (5 mL) in the dark for 12 h, a red solid was precipitated. After cooling to room temperature, the red solid was collected by filtration and recrystallized from acetone to produce a fine bright orange microcrystals (Yield: 63 mg, 50%). Anal. Calcd for $C_{36}H_{26}Cl_2F_6IrN_6PPt$: C, 37.24; H, 2.29; N, 7.33. Found: C, 37.12; H, 2.41; N, 7.26. ESI-MS: m/z 501 $[M+H]^{2+}$. ^1H NMR (500 MHz, CD_3Cl): δ = 9.93 (d, *J* = 2.5 Hz, 2H), 9.82 (d, *J* = 2.5 Hz, 2H), 9.24 (d, *J* = 2.5 Hz, 1H), 7.89 (m, 8H), 7.81 (m, 2H), 7.48 (m, 1H), 7.34 (m, 1H), 7.28 (m, 1H), 6.92 (t, *J* = 5 Hz, 2H), 6.88 (t, *J* = 7.5 Hz, 2H), 6.81 (m, 2H), 6.77 (m, 2H), 6.49 (d, *J* = 5 Hz, 1H). ^{195}Pt NMR (500 MHz, CD_3Cl): δ = -2914 (s, 1Pt).

Stability under physiological conditions

The stability of Ir-Pt was performed according to the previous report.[2] Ir-Pt in DMSO and diazepam in actone were added to the hfetal bovine serum (FBS) to a total volume of 1000 μL. The resulting FBS was incubated for 48 h at 37 °C with continuous shaking. The reaction was stopped by adding 2 mL of acetonitrile, and the mixture was centrifuged for 45 min at 1000g at 4 °C. The supernatant was evaporated, and the residue was dissolved in ethanol and injected into an HPLC instrument. A C18 reverse phase column was used. The runs were performed with a linear gradient of ethanol in distilled water.

Cell lines and culture conditions

The A549R, A549R, SGC7901, SGC7901/DDP, SKOV3, SKOV3/DDP were obtained from the Experimental Animal Center of Sun Yat-Sen University (Guangzhou, China). The cells were maintained in DMEM (Dulbecco's modified Eagle's medium, Gibco BRL) medium supplemented with 10% FBS (fetal bovine serum, Gibco BRL), 100 μg/ml streptomycin, and 100 U/ml penicillin (Gibco BRL) in a humidified incubator at 37 °C with 5% CO_2. The human normal lung cells MRC-5 was purched from Shanghai Institutes for Biological Sciences. The culture condition is similar to that of A549 but replacing DMEM with MEM.

Cytotoxicity Test

The cytotoxicity of the complexes were determined by MTT assay. Briefly, the cells were seeded into 96-well microtiter plates at (1×10^4 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. The plates were then incubated for 48 h in the same conditions. The stock 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).

ICP-MS assay

Exponentially growing A549R cells were harvested, and the resulting single-cell suspension was plated into 100 mm tissue culture plates (Costar). After 24 h at 37 °C, the cells were incubated with 5 μM **Ir-Pt** for different time at 37 °C. The cells were rinsed with PBS, detached with trypsin, counted and divided into three portions. In the first portion, the nuclei were extracted using a nucleus extraction kit (Pierce, Thermo) following the manufacturer's protocol. In the second portion, the cytoplasm was extracted using a cytoplasm extraction kit (Pierce, Thermo). In the third portion, the mitochondria were extracted using a mitochondrial extraction kit (Pierce, Thermo). 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 platinum content was measured using inductively coupled plasma mass spectrometry (ICP-MS Thermo Elemental Co., Ltd.). Data were reported as the means ± standard deviation (n = 3).

Real-time cell growth and proliferation assay

These experiments were performed on an xCELLigence RTCA DP system real-time cell analyser (Roche Diagnostics GmbH, Germany), which was placed in a incubator at 37 °C under 5% CO_2. Cell growth curves were constructed using 16-well plates (E-plate 16, Roche Diagnostics GmbH, Germany). To evaluate the cell status based on the measured cell electrode impedance, the cell index (CI) parameter is introduced. CI is a relative value to estimate the number of cells attached to the electrodes. The slopes of CI curves reflect the growth speed of the cells.[2] Briefly, 50 μL of cell culture medium at room temperature was added to each well of an E-plate 16. Then, the E-plate 16 was connected to the system and placed in the cell culture incubator for proper electrical contact, and the background impedance was measured after 10 s. Next, a total of 100 μL of A549R cells at a density of 1×10^4 cells per well was added to each well of the plate. Approximately 24 hours after seeding, when the cells were in the exponentially growth phase, 50 μL of cell culture medium which contain various concentrations of the **Ir-Pt** were added, and the CI were automatically recorded every 15 min over 80 h by the xCELLigence system. Meanwhile, untreated cells were used as a control, and cisplatin was introduced as a positive contrast. Data analysis was performed using the RTCA software 1.2 supplied with the instrument.

Drug efflux assay

Exponentially growing A549R cells were harvested, and the resulting single-cell suspension was plated into 100 mm tissue culture plates (Costar). After 48 h at 37 °C, the cells were incubated with 5 μM **Ir-Pt** or cisplatin for 6 h at 37 °C. The cells were rinsed with PBS, detached with trypsin after incubation with fresh DMEM for different times. The samples were digested with 60% HNO_3 at RT for one day. The iridium and platinum content was measured by ICP-MS assay.

Cellular Uptake Mechanism

The cellular uptake mechanism was performed according to Ref. 4 and 5. For metabolic inhibition, the A549R cells were pre-treated with inhibitors (50 mM 2-deoxy-D-glucose, 5 μM oligomycin, 50 mM NH_4Cl or 100 μM chloroquine) or high K^+-HBSS (containing 170 mM K^+) for 1 h and then incubated with **Ir-Pt** (5.0 μM) for 2 h. Subsequently, culture medium (including inhibitors and **Ir-Pt**) was removed and the attached 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).

Mitochondrial Dysfunction

A549R cells were cultured in 6-well tissue culture plates for 24 h and then treated with **Ir-Pt** (5.0 μM) for 12 h. After the treatment, the cells were collected and resuspended at 1 × 10^6/mL in the pre-warmed staining working solution containing JC-1 (5 μg/ml), and then incubated for 15 min at 37 °C. Subsequently, the cells were washed twice with pre-warmed PBS and analyzed immediately on a FACS Canto II flow cytometer (BD Biosciences, USA). This change was also observed under confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany) with a 63×Scientific oil-immersion objective lens.

Cell cycle analysis

Briefly, A549R cells after treatment with 5 μM **Ir-Pt** or 100μM cisplatin for 12 h were washed in cold phosphate buffer solution (PBS) and fixed in 70% ethanol at −20 °C overnight. The fixed cells were washed twice with PBS, re-suspended in PBS (1 mL) that contained RNase A (0.25 mg), EDTA (2 mm), and propidium iodide (0.1 mg). This solution was incubated at 37 °C for 30 min, and cells were analyzed. The fluorescence of 10^4 cells was measured via flow cytometry on a FACS Canto II flow cytometer (BD Biosciences, USA).

DNA damage assay

A549R cells were seeded at 1 × 10^4 cells/well and allowed to adhere overnight. Cells were treated with **Ir-Pt** (5.0 μM) or 100 μM cisplatin for 12 h 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 Pico Green staining and normalized to nontreated value.

An alkaline single-cell gel electrophoresis (comet assay) was performed to examine the nDNA damage by **Ir-Pt**. The cell solutions were pipetted to produce single-cell suspensions, and the suspensions were combined with molten low-melting-point agarose at a ratio of 1:10 (v/v). The lowmelting-point agarose was then spread on slides precoated with 1% normal-melting agarose, and the slides were placed in the refrigerator at 4 °C for 15 min. The slides were then immersed in a prechilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% Triton X-100, and 10% DMSO, pH = 10) and returned to the refrigerator for approximately 2 h. The lysis buffer was then removed, and the slides were immersed in alkaline unwinding solution for 20 min in the dark at room temperature. The slides then were removed from the alkaline unwinding solution and electrophoresed at 20 V and 300 mA for 20 min in the dark. After electrophoresis, the slides were soaked three times in neutralizing buffer (0.4 M Tris, adjusted to pH = 7.5 with HCl) for 5 min each and then immersed in 100% ethanol for 5 min. Finally, the slides were allowed to dry before staining with PI solution (2 μg / mL) for 5 min in the dark and analyzed using a confocal microscopy (Carl Zeiss, LSM 710, Germany).

A549R cell metabolism test

The mitochondrial OXPHOS and glycolysis function of A549R cells was measured by determining the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) with a Seahorse XF24 extracellular flux analyzer. A549R cells were seeded at a density of 5 × 10^4 cells/well to the XF24-well culture microplates (Seahorse Bioscience) the day before Seahorse test. The sensor cartridge was hydrated by being immersed in calibration buffer at 37 °C in non-

CO_2 incubator overnight. For measuring OCR, the assay medium was consist of sodium pyruvate (2 mM), glucose (25 mM) and L-glutamine (2 mM) in unbuffered DMEM. For the glycolysis test, the assay medium consisted of L-glutamine (4 mM) in unbuffered DMEM. The as-prepared culture medium was adjusted to pH 7.4, filteredand stored at 4 °C. A549R cells were treated for 2h with culture medium, 5.0 μM cisplatin, 10.0 μM cisplatin, 5.0 μM **Ir-Pt** and 10.0 μM **Ir-Pt** respectively. Then the cell metabolism was assessed including OCR and ECAR by using XF Cell Mito Stress Test Kit and XF Glycolysis Stress Test Kit, respectively. Different OXPHOS parameters were calculated by subtracting the average respiration rates before and after the injection of electron transport inhibitor (oligomycin A, 1 μM), the electron chain transport (ETC) accelerator (FCCP, 500 nM) and a mixture of complex III inhibitor (antimycin A, 1 μM). The parameters calculated include: non-mitochondrial respiration (respiration after antimycin A/rotenone injection), ATP production(baseline respiration minusrespiration stimulated by Oligomycin), proton leak(respiration stimulated by Oligomycin minus non-mitochondrial respiration), basal respiration (baseline respiration minus non-mitochondrial respiration), maximal respiratory capacity (respiration stimulated by FCCP minus non-mitochondrial respiration), and spared respiratory capacity (maximal respiration minus basal respiration). Different glycolytic parameters were also calculated by subtracting the average acidification rates before and after the injection of glycolysis substrate (glucose, 10 mM), electron transport inhibitor (oligomycin A, 1 μM), and glycolysis inhibitor (2-dG, 50 mM). The glycolytic parameters were calculated including: non-glycolytic acidification (average acidification after 2-dG injection), glycolysis (post glucose injection acidification minus non-glycolytic acidification), glycolytic capacity (post oligomycin A injection acidification minus non-glycolytic acidification), and glycolytic reserve (glycolytic capacity minus glycolysis). All test results had four replicates.

Caspase-3/7 assay

A549R cells were seeded in white-walled nontransparent-bottomed 96-well micro-culture plates at a density of 1.0×10^4 cells/well and allowed to incubated overnight to adhere. The cells were then treated with culture medium, 100 μM cisplatin, 5.0 μM **Ir-Pt**, 10.0 μM **Ir-Pt,** respectively. The cells were incubated for 12 h, then treated with Caspase-3/7 activity kit according to the manufacturer's protocol and the luminescence in RLUs was quantified by an Infinite M200 PRO (TECAN, Swiss).

Intracellular SOX

A549R cells were treated with the indicated concentrations of **Ir-Pt** (5.0 μM) and incubated for 12 h. The medium was removed and cells were washed with Hank's buffered saline (HBS) and then incubated with 5 mM MitoSox reagent (Invitrogen) in HBS for 30 min in the absence of light. Cells were washed three times with HBS, observed under confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany).

Cell membrane disruption

Cytotoxicity Assay Kit (Thermo, USA) was employed to assess the cell membrane integrity. A549R cells were seeded in white 96-well plates at a density of 1×10^4 cells per mL and precultured for 24 h. The cells were treated with culture medium and **Ir-Pt** (5.0 μM, 10.0 μM) for 12 h at 37 °C. Cell membrane integrity was detected according to the manufacturer's instructions. The luminescence was recorded in a microplate reader (InfiniteM200 Pro, Tecan, Männedorf, Switzerland).

Western Blot

The A549R cells were seeded into 100 mm tissue culture plates (Costar), incubated for 24 h, and then treated with **Ir-Pt** (5.0 μM, 10.0 μM) for 24 h. The cells were washed with ice-cold PBS and lysed by incubation in radio immune precipitation assay buffer (RIPA) with a protease inhibitor cocktail (Sigma) for 30 min on ice. The lysates were centrifuged at 15000 rpm for 15 min at 4 °C, and the protein concentrations were quantified by a BCA protein assay reagent kit (Novagen Inc, USA). The proteins were fractionated by electrophoresis in a 10% acrylamide gel, transferred to PVDF membranes, and subjected to Western blot analysis. Rabbit monoclonal anti-RIP3 (abcam, USA) and rabbit anti-GADPH (abcam, USA) antibodies were diluted (1:1000 and 1:2000, respectively) in TBS containing 5% nonfat powdered milk and 0.1% Tween-20 and then incubated with the membrane overnight at 4 °C. Horseradish peroxidase conjugated secondary antibodies (Cell Signaling) were used. The bound immune complexes were detected using an ECL prime Western blot detection reagent (Amersham Inc., USA).

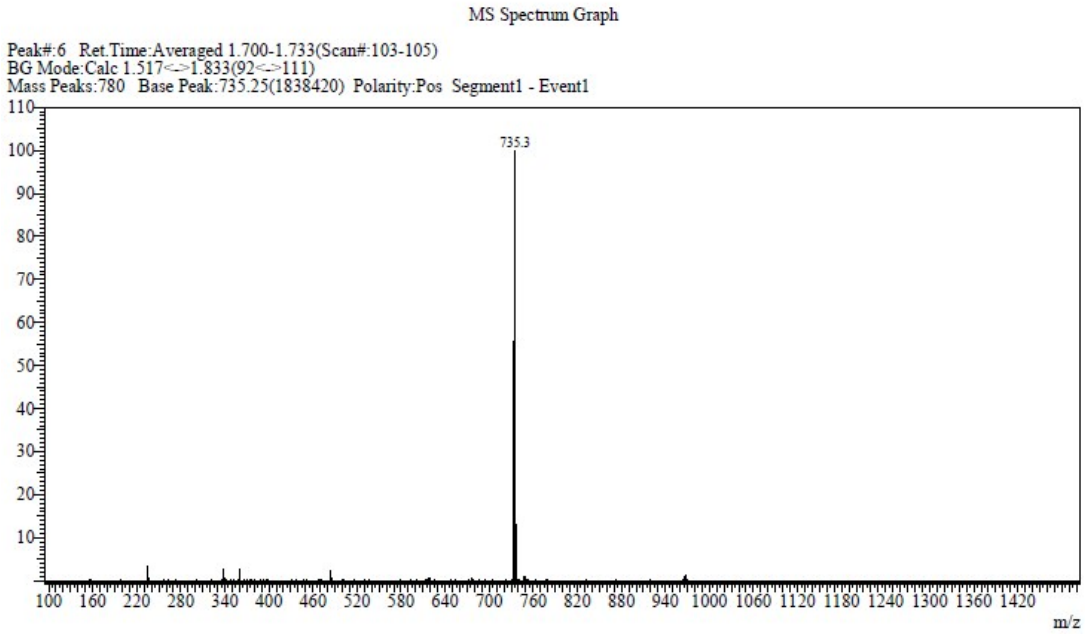


Figure S1. ESI-MS spectrum of **Ir**.

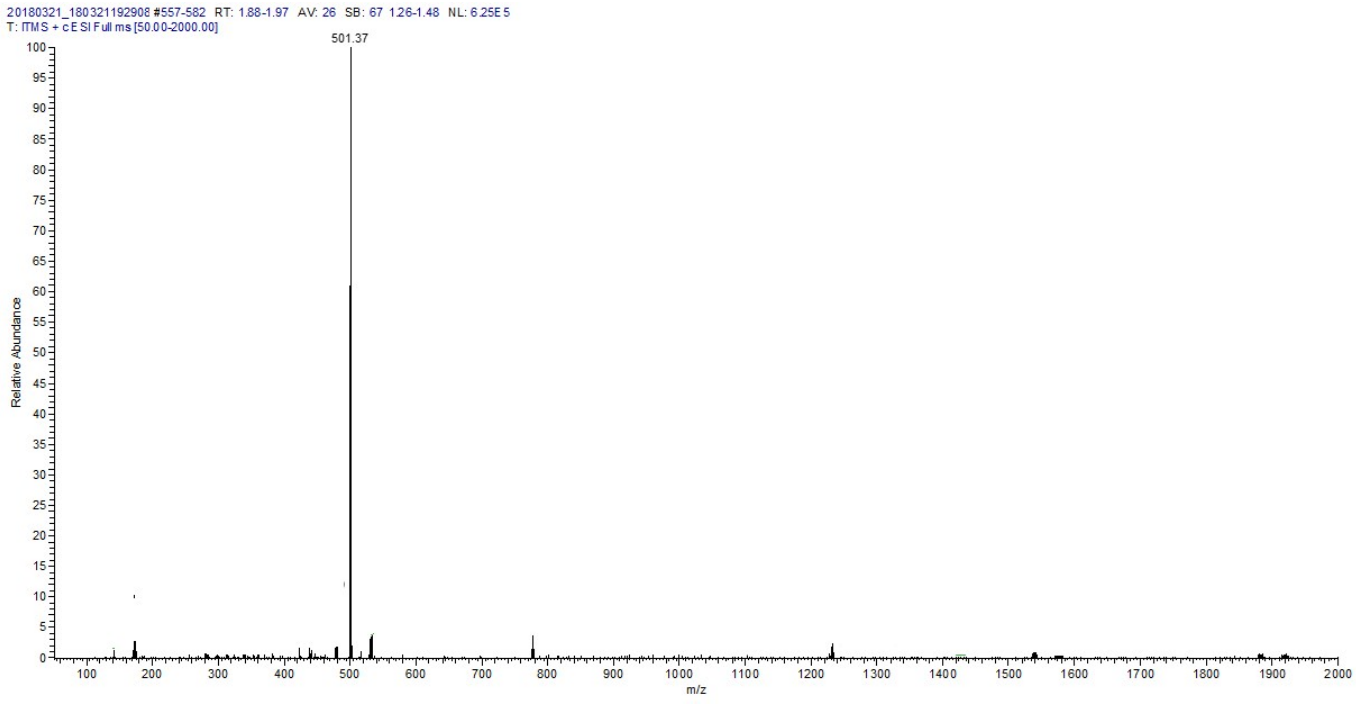


Figure S2. ESI-MS spectrum of **Ir-Pt**.

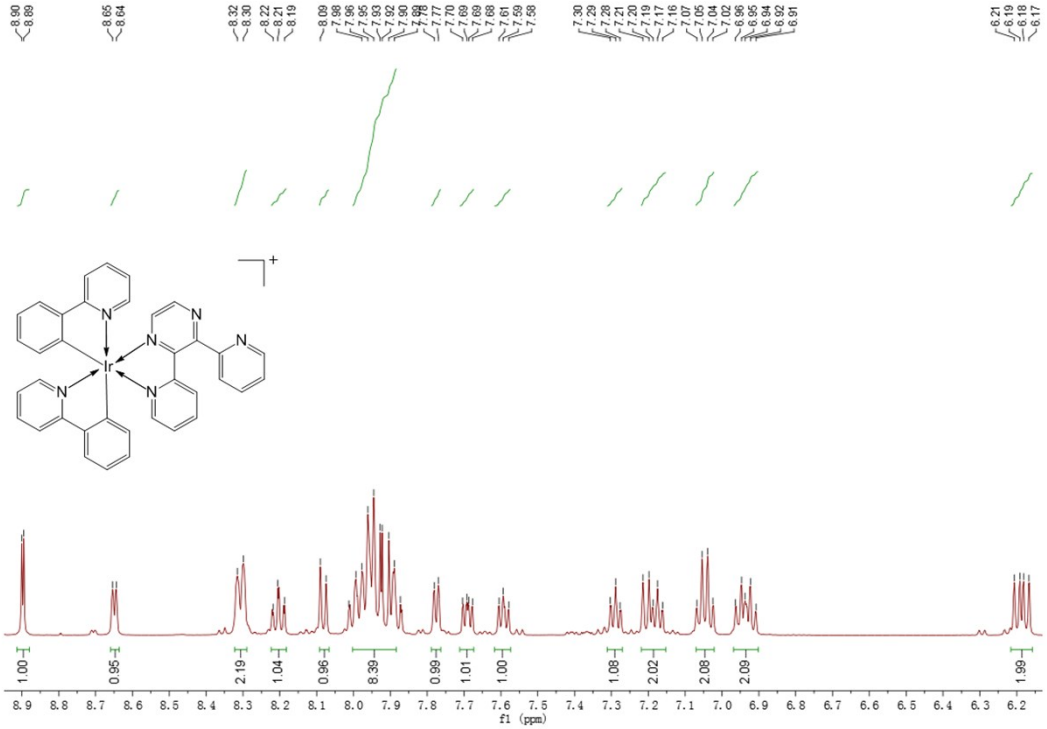


Figure S3. ^{1}H NMR spectrum of **Ir** in DMSO-d_6.

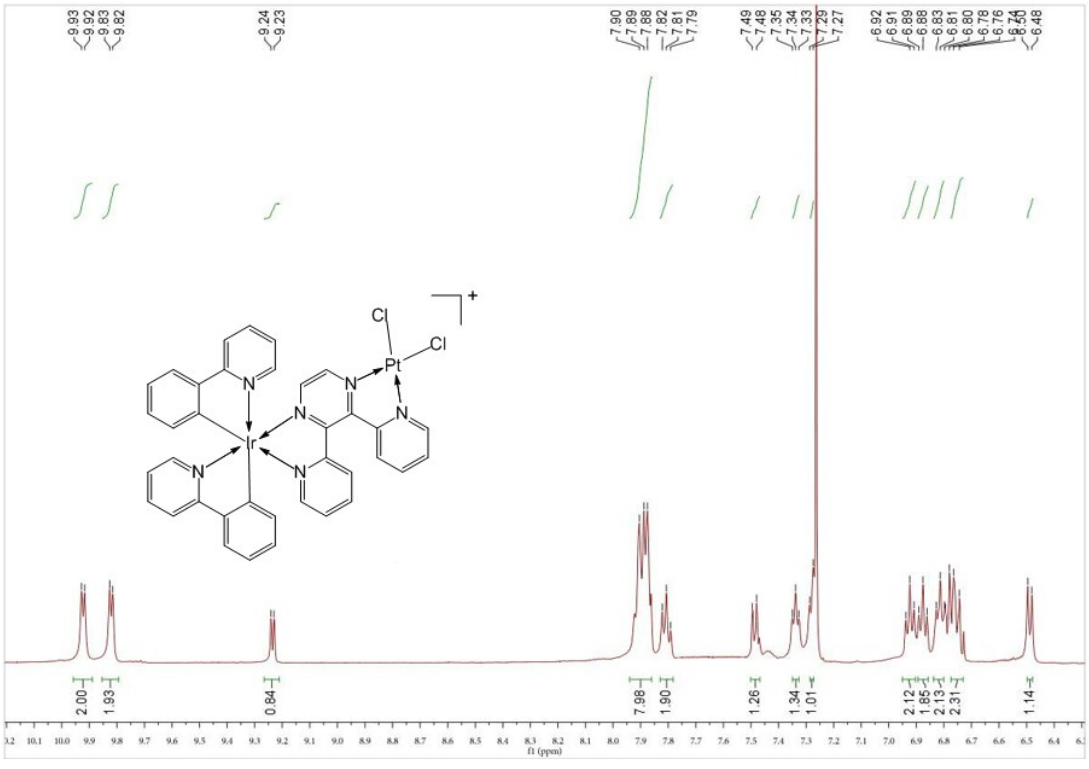


Figure S4. ^{1}H NMR spectrum of **Ir-Pt** in CD_3Cl.

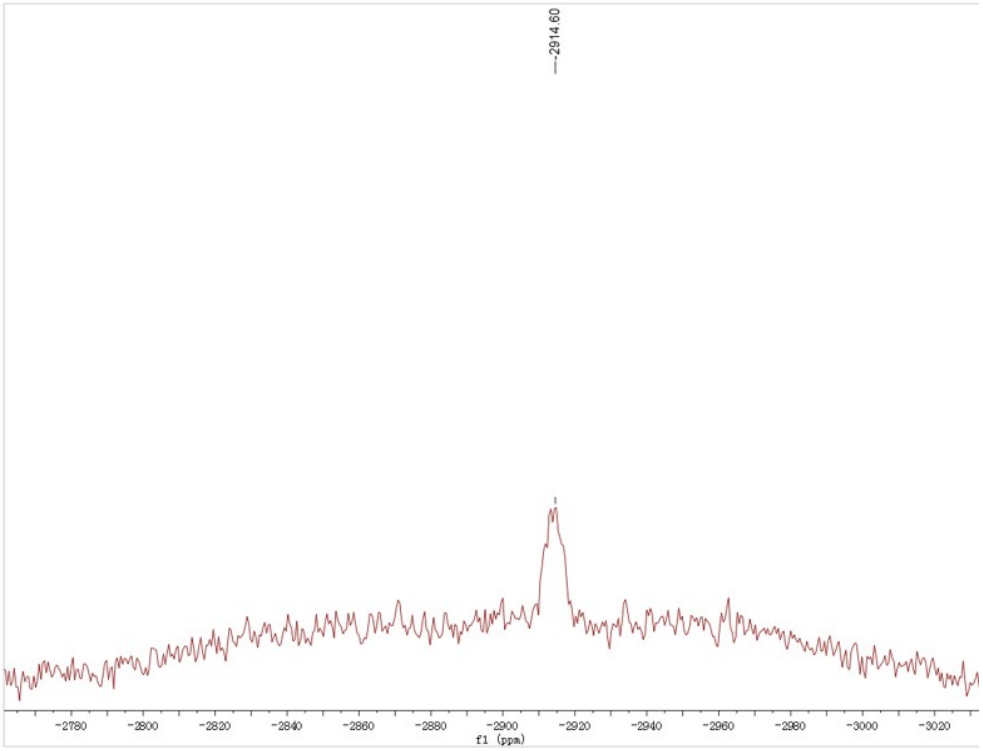


Figure S5. ^{195}Pt NMR spectrum of **Ir-Pt** in CD_3Cl.

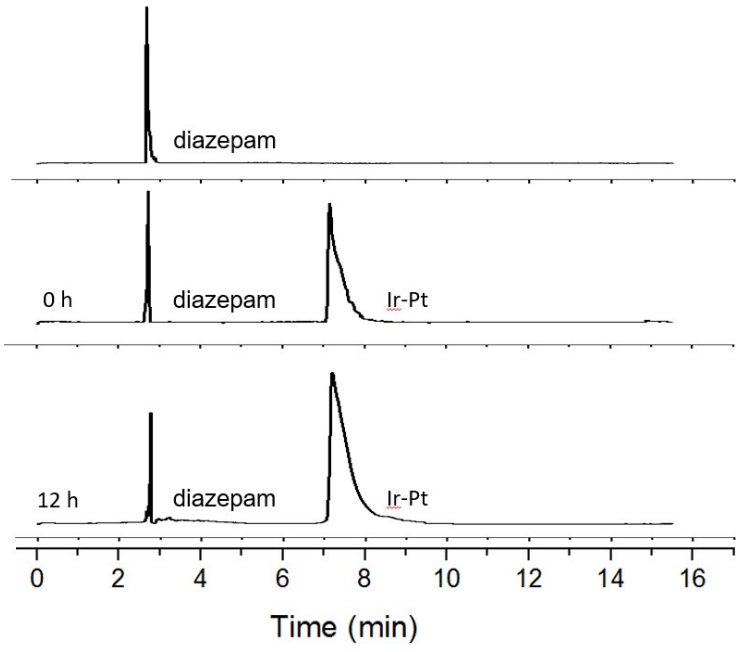


Figure S6. The stability of **Ir-Pt** under physiological conditions.

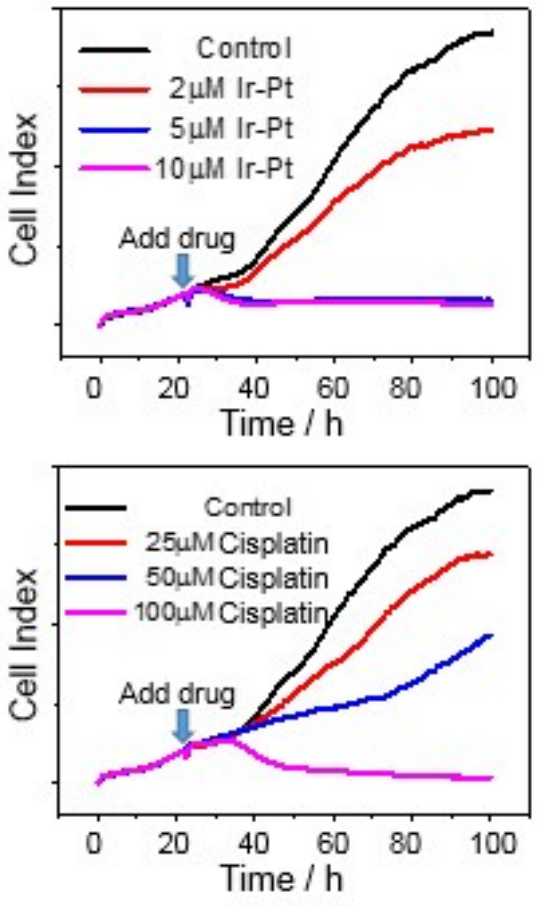


Figure S7. Kinetics of cytotoxicity responses for drug in A549R cells monitored by the xCELLigence RTCA system. The arrow signifies the time of the drug addition. Different concentrations of **Ir-Pt** and cisplatin in A549R cells.

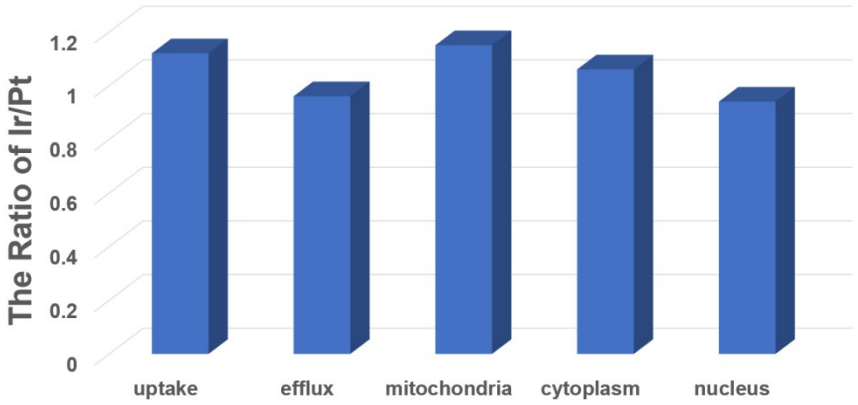


Figure S8. The molar ratio of Ir to Pt in the cellular uptake, efflux, and distribution

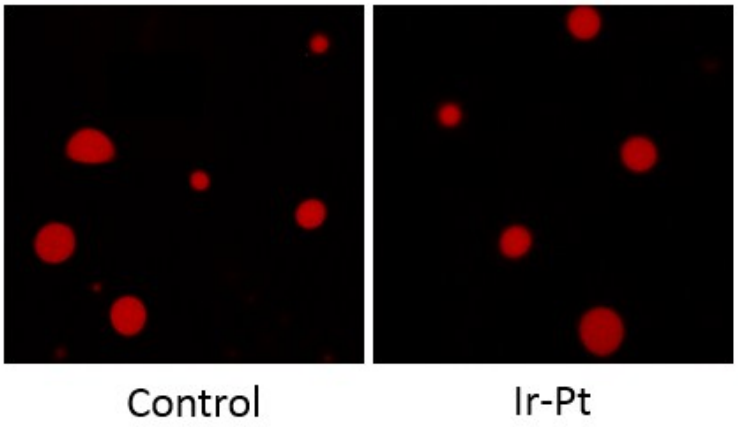


Figure S9. Chromosomal DNA strand breaks by Ir-Pt (5 μM, 12 h) in A549R cells as detected by comet assay.

Table S1 IC_{50} values (μM) towards cancer cells and their cisplatin-resistant cell lines

IC_{50} / μM	A549	A549R	SGC7901	SGC7901/DDP	SKOV3	SKOV3/DDP
Ir-Pt	1.83 ± 0.20	2.46 ± 0.28	1.37 ± 0.11	1.52 ± 0.1	3.61 ± 0.35	2.82 ± 0.17
cisplatin	10.5 ± 1.2	78.8 ± 8.9	5.46 ± 0.2	66.1 ± 1.4	4.23 ± 0.08	47.1 ± 2.4

Table S2 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'	Sense
	5' – GCA CTG GCT TAG GAG TTG GAC T – 3'	Antisense
Mitochondria long fragment (8.9kb)	5' – TCT AAG CCT CCT TAT TCG AGC CGA – 3'	Sense
	5' – TTT CAT CAT GCG GAG ATG TTG GAT GG – 3'	Antisense

Reference

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