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Electronic Supplementary Information (ESI) for

Mitochondrial DNA-targeting and impairment by a dinuclear Ir-Pt complex that overcomes cisplatin resistance

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Supporting Experimental Procedures

Materials and Measurements

All starting materials were used as received from commercial sources unless otherwise stated. Iridium chloride hydrate (Alfa Aesar, USA), 4-(2,2':6',2"-terpyridin-4'-yl)benzaldehyde (Energy Chemical China), 1,10-phenanthroline-5,6-dione (Energy Chemical China), Aniline (Aladdin China), 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), MitoSox reagent kit, 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 500 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).

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. The plates were then incubated for 48 h in the dark. 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).

DNA titration assay

The DNA-binding and cleavage experiments were performed at room temperature. The absorption titrations of **Ir-Pt** in buffer (5 mM Tris-HCl, 50 mM NaCl, pH = 7.2) were performed by using a fixed complex concentration to which increments of the DNA stock solution were added. **Ir-Pt** employed were 2 μM in concentration and calf thymus DNA was added in concentration of 0-16.1 μM. Complex-DNA solutions were allowed to incubate for 10 min before the absorption spectra were recorded. The intrinsic binding constants K_b of **Ir-Pt** to DNA were calculated from equation (1) [25].

$$[DNA]/(\varepsilon_a - \varepsilon_f) = [DNA]/(\varepsilon_b - \varepsilon_f) + 1/[K_b(\varepsilon_b - \varepsilon_f)] \quad (1)$$

where [DNA] is the concentration of DNA in base pairs, ε_a, ε_f and ε_b are correspond to the apparent absorption coefficient A_{obsd}/[Ru], the extinction coefficient for the free complex and the extinction coefficient for the complex in the fully bound form, respectively. In plots of [DNA]/(ε_a - ε_f) versus [DNA], K_b is given by the ratio of slope to the intercept.

DNA gel electrophoresis assay

pBR322 DNA (200 ng/mL) was incubated with **Ir-Pt** in Tris-HCl buffer B (20 mM Tris-HCl, 20 mM Na_2HPO_4, pH 7.4) at 37 °C for 30 min. 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 3 μM **Ir-Pt** or 80 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). Quantitation of amplified product was performed by Pico Green staining and normalized to nontreated value.

MtDNA staining

A549R cells were seeded in the confocal dish in in the right amounts, which continue to incubate for 24 h. And then the cells were treated with two different concentration of **Ir-Pt** for 24 h. 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-Pt** at the indicated concentrations for 24 h. 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.

Mitochondrial Superoxide Detection

A549R cells were treated with the indicated concentrations of **Ir-Pt** (1.5 μM and 3 μM) and incubated for 24 h. The medium was removed and cells were washed with PBS and then incubated with 5 mM MitoSox reagent (Invitrogen) in DMEM for 30 min in the absence of light. Cells were washed three times with PBS, observed under confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany).

Cell lines and culture conditions

A549, A549R, HepG2 and Hela 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-Pt** (3 μM). After 24 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 previous procedure.[1] 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-Pt (3 μM) for 2 h. For temperature dependent uptake study, A549R cells were treated with 3 μM **Ir-Pt** for 2 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 2 h were treated with 3 μM **Ir-Pt** 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

A549R cells were seeded into confocal dish and treated with **Ir-Pt** (1.5 μM and 3 μM) for 24 h. After incubation, the treated cells were stained with JC-1 for 30 min protected from light and immediately wash with the JC-buffer. The cells, collected in flow cytometry tubes, were immediately analyzed by a flow cytometer.

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. A549R cells were seeded in 96 well plates and treated with **Ir-Pt**. 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-Pt** for 24 h and harvested in tube stained with annexin V reagent at room temperature for 15 min in the dark. The samples were immediately analyzed by flow cytometer.

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-Pt** for 24 h. After incubation, 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).

Western blot

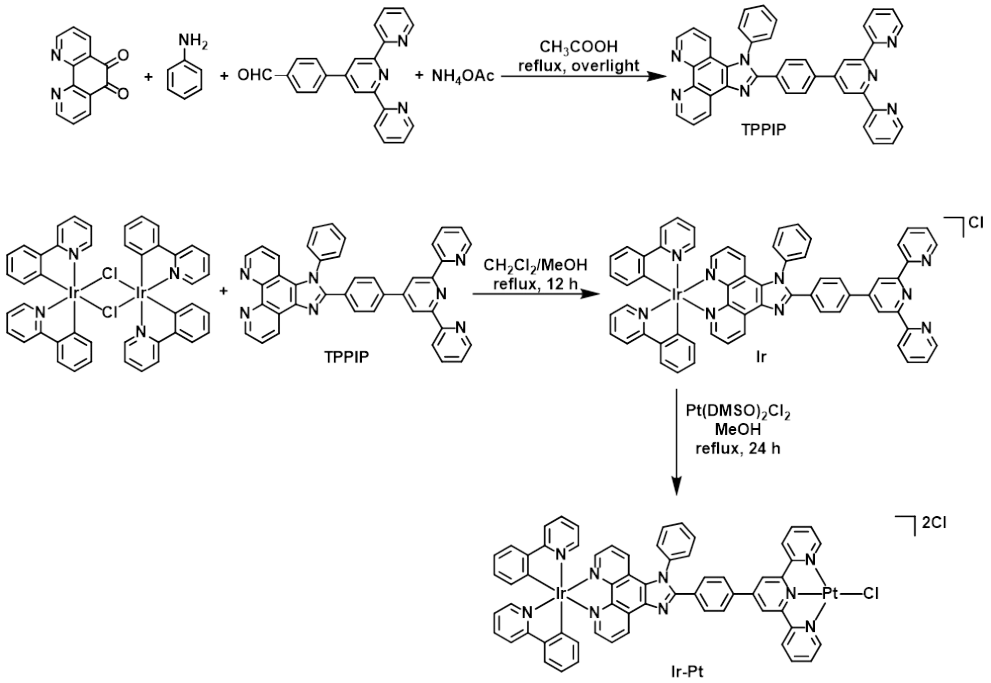
The A549R cells were seeded into 100 mm tissue culture plates (Costar), incubated for 24 h, and then treated with **Ir-Pt** (1.5 μM and 3 μM) for 24 h. The cells were washed with ice-cold PBS and lysed by incubation in radio immune precipitation assay buffer (RIPA) with a protease inhibitor cocktail (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)

LDH leakage assay

A549R cells incubated with **Ir-Pt** (1.5 μM and 3μM) for 24 h in black-walled glass-bottomed 96-well plates. Subsequently, the cells were treated with CytoTox-ONE™ Homogeneous Membrane Integrity Assay Technical Bulletin (Promega) according to the manufacturer's protocol and the fluorescence in RLUs was quantified by an Infinite M200 PRO (TECAN, Swiss).

Synthesis and characterization

$[Ir(ppy)_2Cl]_2$ was synthesized according to the published methods.[2] The synthetic route used to access **Ir-Pt** is illustrated in Scheme S1.



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

Synthesis of TPPIP

The ligand was synthesized according to our privious work.[1,2] A mixture of 4-(2,2':6',2''-terpyridin-4'-yl)benzaldehyde (250 mg, 0.74 mmol, 1 eq), ammonium acetate (680 mg, 8.9 mmol, 12 eq), 1,10-Phenanthroline-5,6-dione (150 mg, 0.74 mmol, 1 eq) and Aniline (75 mg, 0.81 mmol, 1.1 eq) was dissolved in glacial acetic acid (20 mL). The mixture was refluxed overnight under an argon. After cooling to room temperature, the solution was added with 50 mL water, adjusted to pH 6 using a 25% NH_3⓾H_2O solution. The precipitate filtered and dried under vacuum. The crude product was drectly used to next step sythesis without further purification. Anal. Calcd for $C_{40}H_{25}N_7$ (%): C, 79.58; H, 4.17; N, 16.24; Found: C, 79.21; H, 4.36; N, 16.08. ESI-MS: m/z = 604.57 ($[M+H]^+$)，667.54 ($[M+H+2CH_3OH]^+$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.22 (s, 2H), 9.14 – 9.05 (m, 2H), 8.72 (s, 2H), 8.67 (d, *J* = 7.9 Hz, 1H), 7.88 (d, *J* = 8.1 Hz, 2H), 7.83 – 7.63 (m, 7H), 7.61 – 7.41 (m, 6H), 7.39 – 7.28 (m, 3H).

Synthesis of Ir

A solution of $[(ppy)_2Ir(\mu\text{-}Cl)]_2$ (500 mg, 470 μmol, 1 eq) and 2-(4-([2,2':6',2''-terpyridin]-4'-yl)phenyl)-1-phenyl-1H-imidazo[4,5-f][1,10]phenanthroline (TPPIP, 603 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. The resulting yellow solution and cooled to room temperature. 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 (948 mg, 830 μmol, 86%). Anal. Calcd for $C_{62}H_{41}Cl_1IrN_9$ (%): C, 65.34; H, 3.63; N, 11.06; Found: C, 65.02; H, 3.81; N, 11.27. ESI-MS: m/z = 1104.39 ($[M\text{-}Cl+H]^+$)，553.59 ($[M\text{-}Cl+H]^{2+}$). ^1H NMR (500 MHz, DMSO-d_6) δ 9.31 (d, *J* = 8.2 Hz, 1H), 8.74 (d, *J* = 4.2 Hz, 2H), 8.68 (s, 2H), 8.59 (d, *J* = 7.8 Hz, 2H), 8.29 (d, *J* = 8.2 Hz, 1H), 8.27 – 8.20 (m, 2H), 8.13 – 8.07 (m, 2H), 8.03 – 7.84 (m, 10H), 7.82 – 7.72 (m, 6H), 7.55 – 7.42 (m, 5H), 7.05 (m, 7.3 Hz, 4H), 6.94 (m, 4.2 Hz, 2H), 6.28 (dd, *J* = 14.0, 7.3 Hz, 2H).

Synthesis of Ir-Pt

Upon refluxing a solution of **Ir** (125.34 mg, 110 μmol, 1 eq) and $Pt(DMSO)_2Cl_2$ (46 mg, 110 μmol, 1 eq) in MeOH (5 mL) in the dark for 24 h. After cooling to room temperature, the resulting yellow solution was filtrat and purified by silica gel column chromatography using acetonitrile/CH_2Cl_2 (1:20, v/v) produce a fine yellow solid. The target product is without further purification. (109 mg, 70.4 μmol, 64%). Anal. Calcd for $C_{62}H_{41}Cl_3IrN_9$ (%): C, 52.97; H, 2.94; N, 8.97; Found: C, 52.65; H, 3.12; N, 8.74. ESI-MS: m/z = 667.31 ($[M\text{-}2Cl]^{2+}$). ^1H NMR (500 MHz, DMSO-d_6) δ 9.35 (d, *J* = 7.1 Hz, 1H), 9.02 (s, 2H), 8.87 (d, *J* = 8.1 Hz, 2H), 8.81 (d, *J* = 5.1 Hz, 2H), 8.51 (t, *J* = 7.5 Hz, 2H), 8.31 – 8.24 (m, 5H), 8.18 (dd, *J* = 8.2, 5.1 Hz, 1H), 8.11 (d, *J* = 4.2 Hz, 1H), 7.99 – 7.78 (m, 14H), 7.57 – 7.47 (m, 3H), 7.09 – 7.01 (m, 4H), 6.98 – 6.93 (m, 2H), 6.29 (dd, *J* = 17.7, 7.5 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.35, 158.33, 154.65, 152.63, 151.42, 150.92, 150.53, 149.96, 149.61, 149.05, 145.34, 144.90, 144.45, 142.99, 139.27, 137.11, 136.76, 135.85, 132.98, 132.04, 131.67, 131.60, 130.79, 130.21, 130.01, 129.70, 129.18, 128.74, 128.61, 128.37, 127.17, 126.79, 126.42, 125.61, 124.39, 124.32, 122.92, 122.51, 121.87, 120.57, 120.51. ^{195}Pt NMR (500 MHz, DMSO-d_6): δ = -2954 (s, 1Pt).

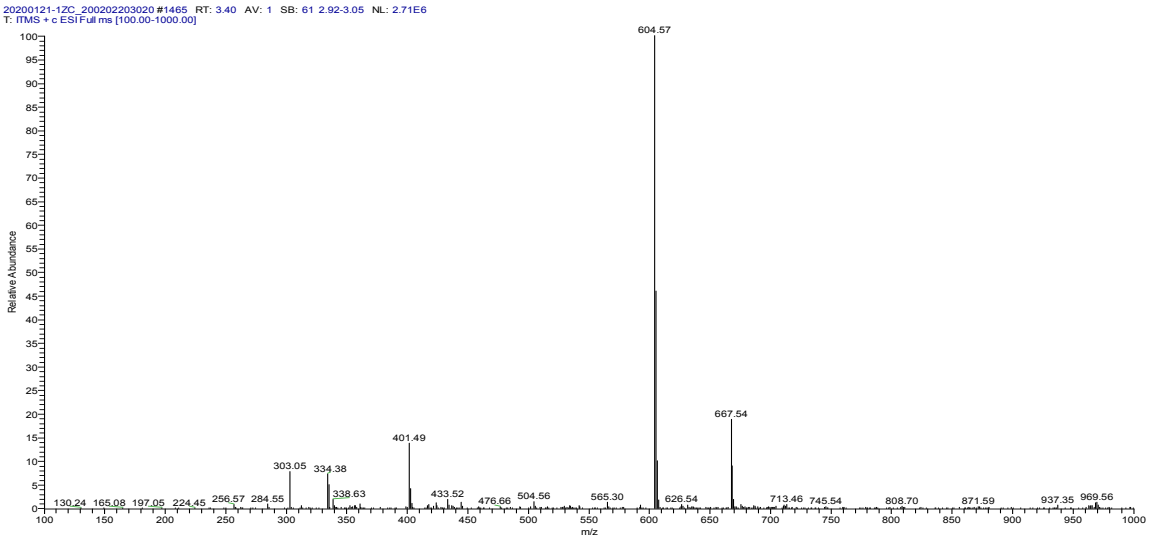


Figure S1. ESI-MS characterization of TPPIP.

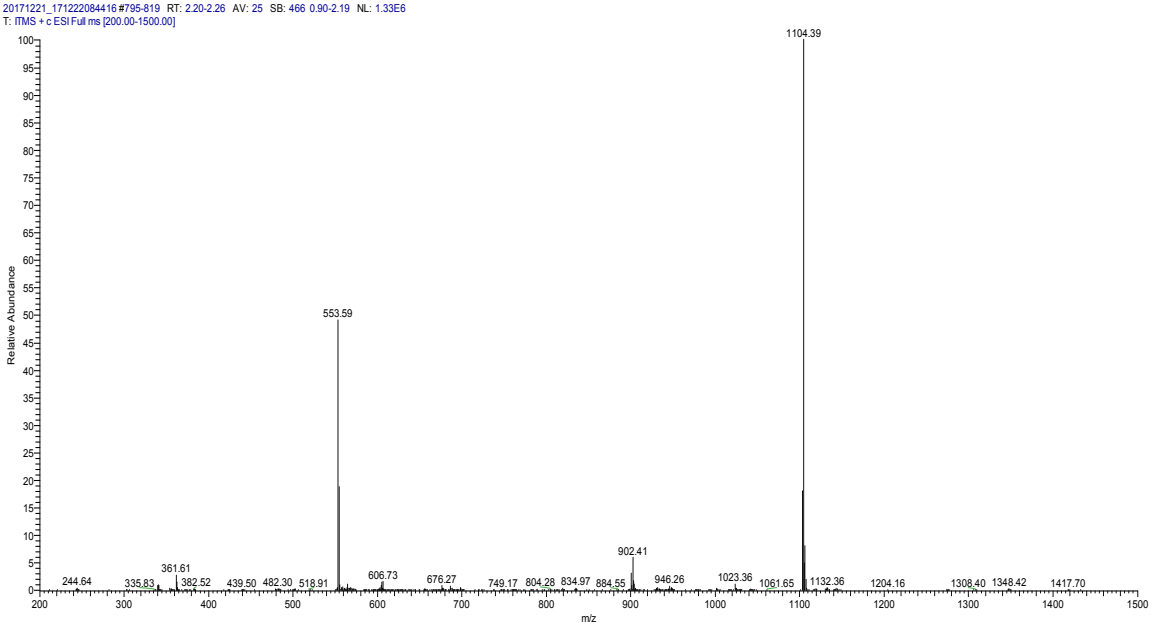


Figure S2. ESI-MS characterization of **Ir.**

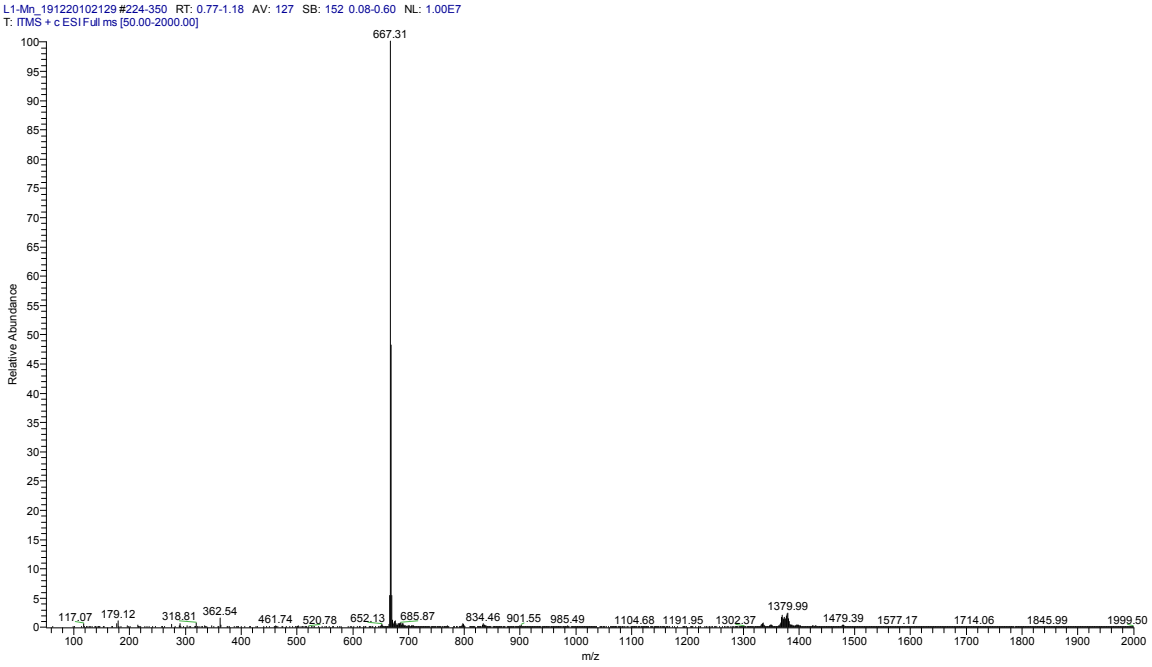


Figure S3. ESI-MS characterization of **Ir-Pt.**

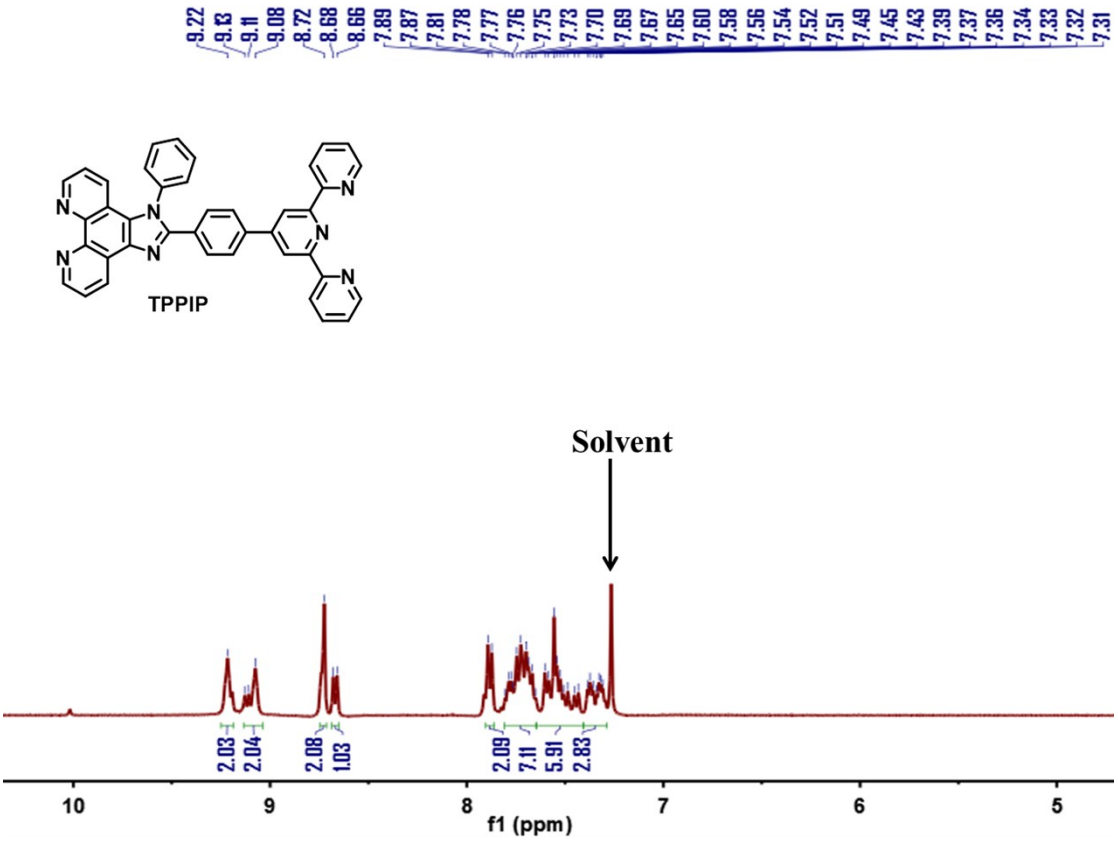


Figure S4. ^{1}H NMR spectrum of TPPIP in $CDCl_3$**.**

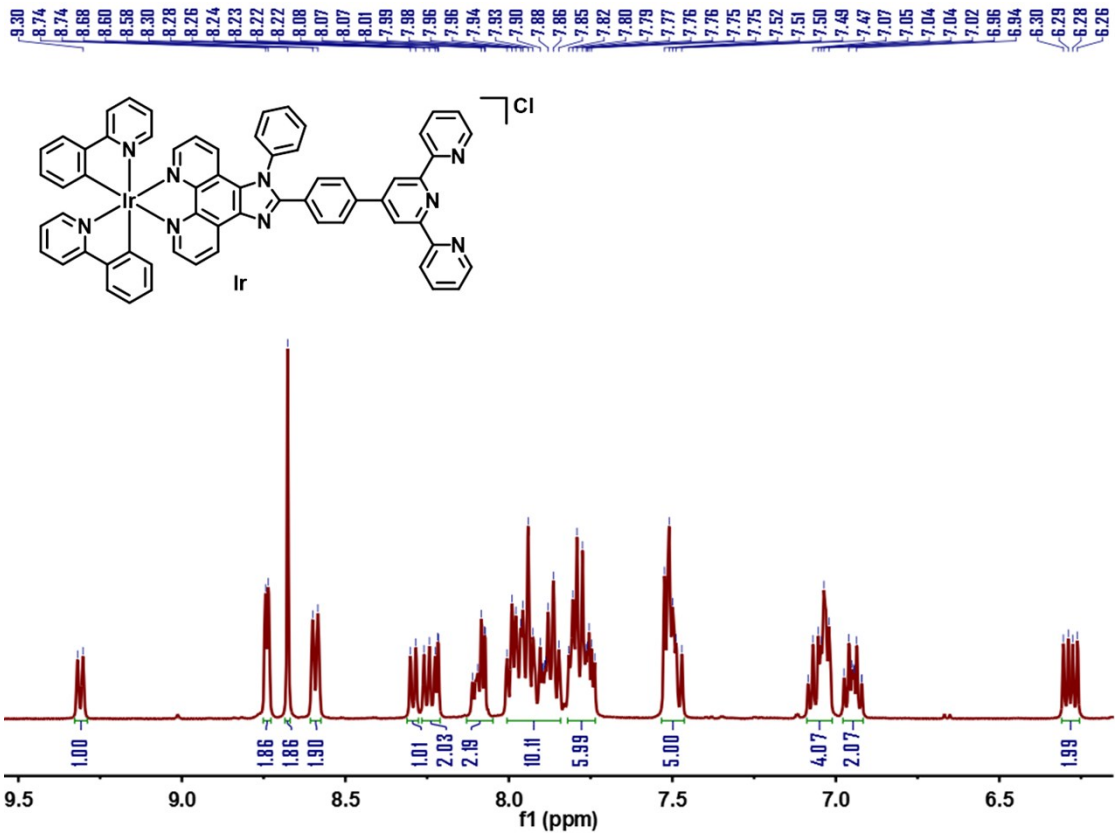


Figure S5. ^{1}H NMR spectrum of **Ir** in DMSO-d_6**.**

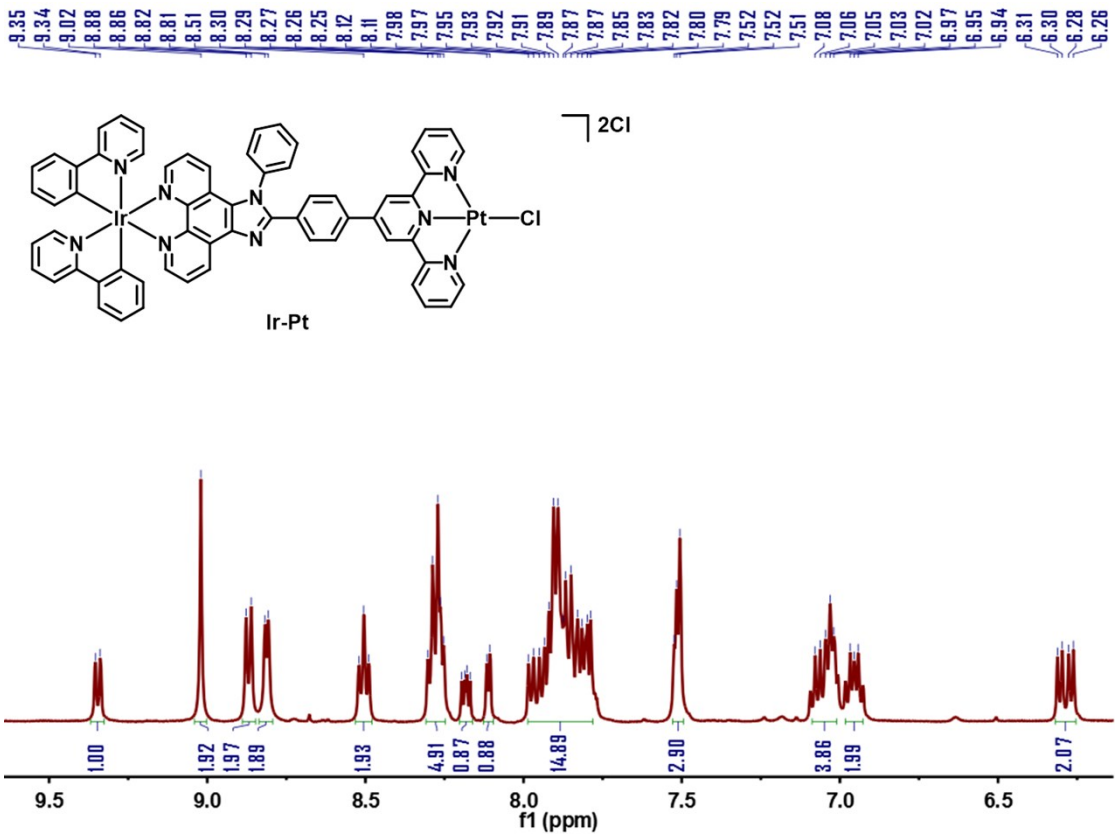


Figure S6. ^{1}H NMR spectrum of **Ir-Pt** in DMSO-d_6.

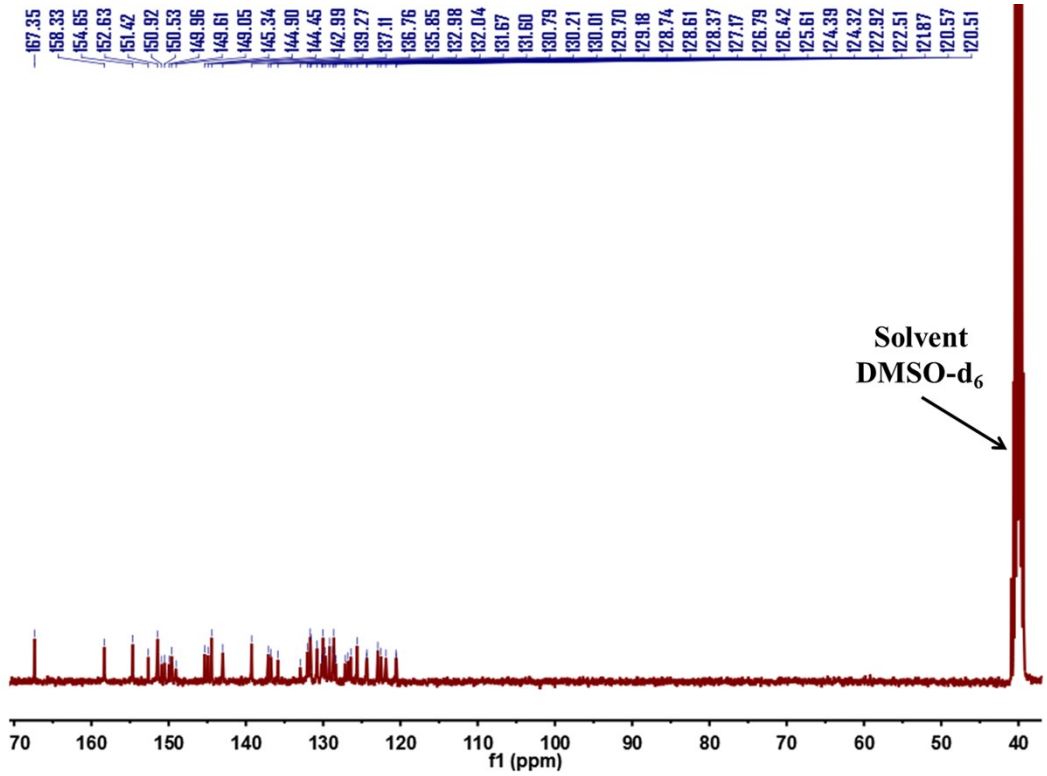


Figure S7. ^{13}C NMR spectrum of **Ir-Pt** in DMSO-d_6

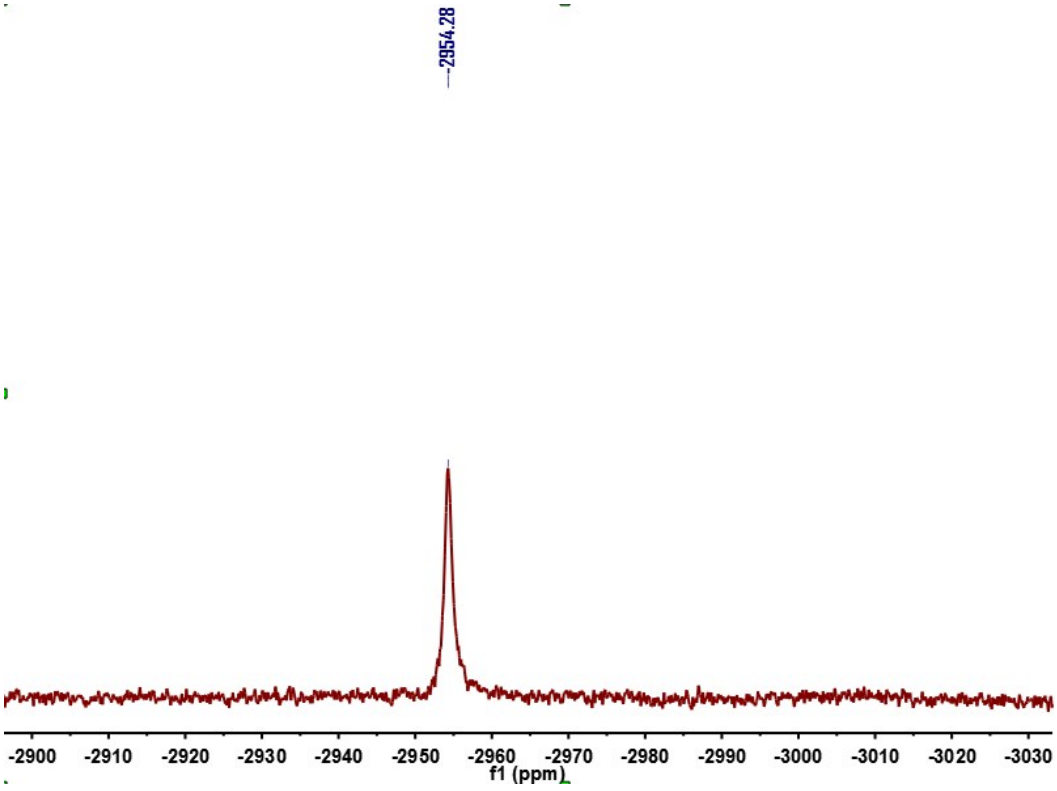


Figure S8. ^{195}Pt NMR spectrum of **Ir-Pt** in DMSO-d_6

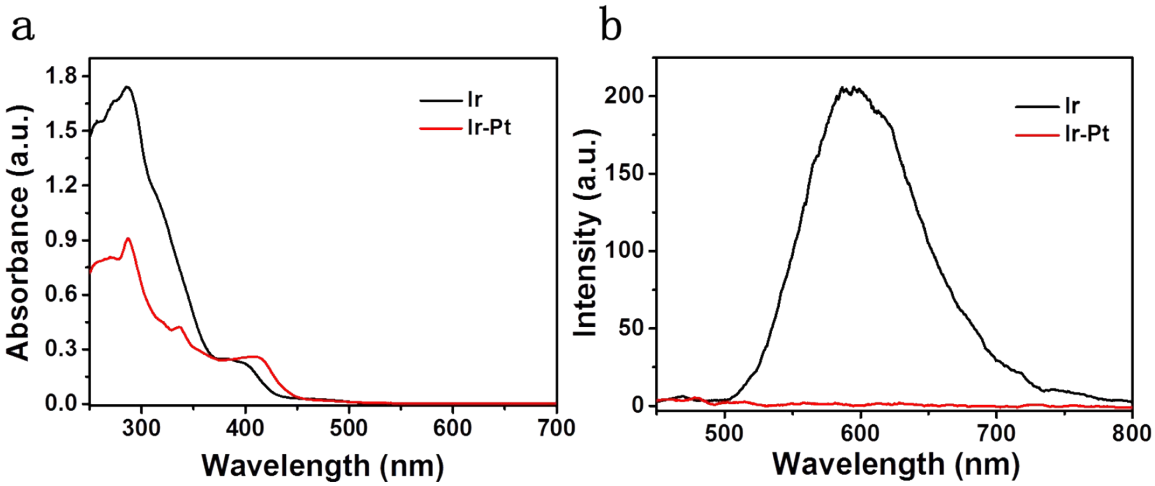


Figure S9. (a) UV-Vis and (b) emission spectrum of **Ir** (10 μM) and **Ir-Pt** (10 μM) measured in CH_3OH at 298 K.

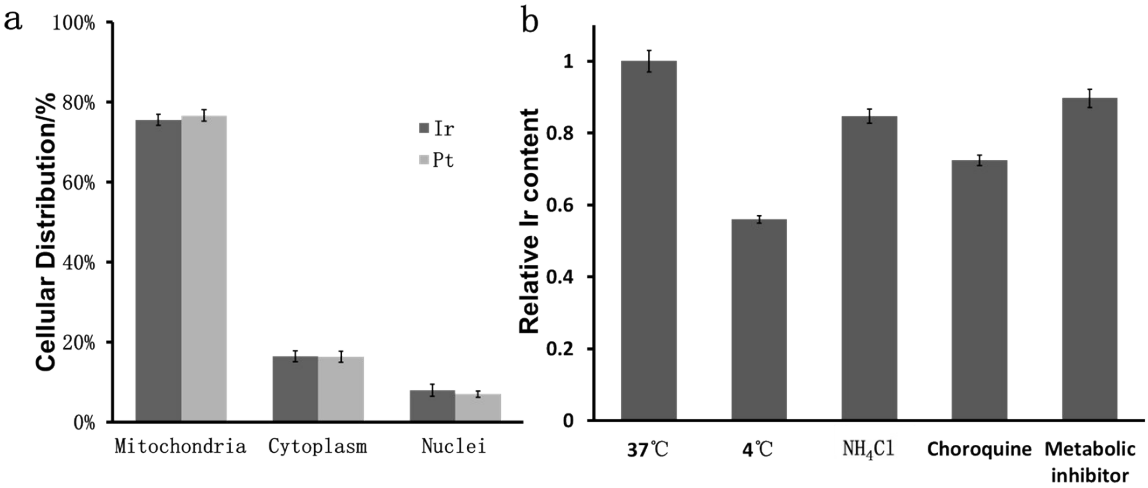


Figure S10. a) Subcellular distribution of **Ir-Pt** in A549R cells after treatment with 3 μM **Ir-Ru** for 24 h.; b) Investigation of the cellular uptake mechanism of **Ir-Pt.** A549R cells pretreated in: control cells at 37 °C for 30 min, cells pretreated at 4 °C for 30 min, 50 μM chloroquine for 30 min, 50 mM NH_4Cl for 30 min, and metabolic inhibitors for 1 h, before incubated with **Ir-Pt** (3 μM) in the aforementioned solution for 2 h respectively.

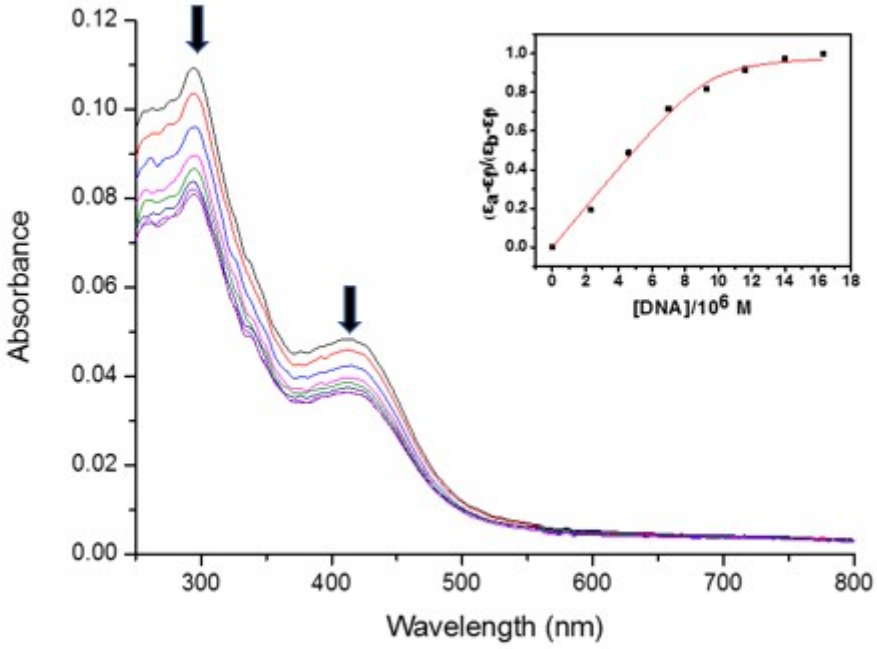


Figure S12. Absorption spectra of **Ir-Pt** in the presence of increasing amounts of DNA with subtraction of the DNA absorbance ([**Ir-Pt**] = 2 μM, [DNA] = 0-16.1 μM). The arrow shows the absorbance changes on increasing DNA concentration.

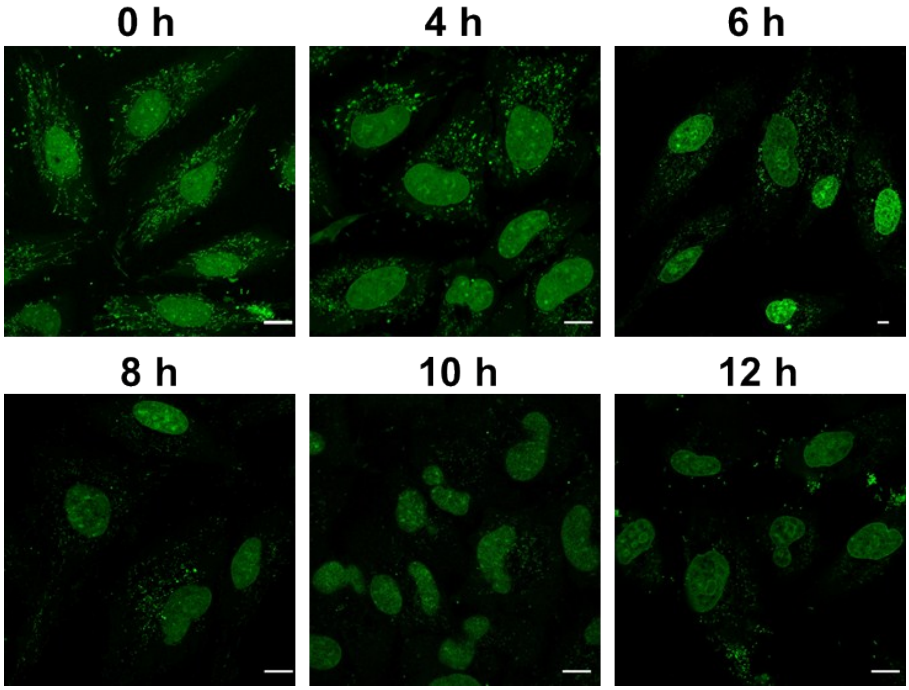


Figure S12. Observation of mtDNA stained with PicoGreen in A549R cells treated with **Ir-Pt** (6 μM) at the indicated time intervals.

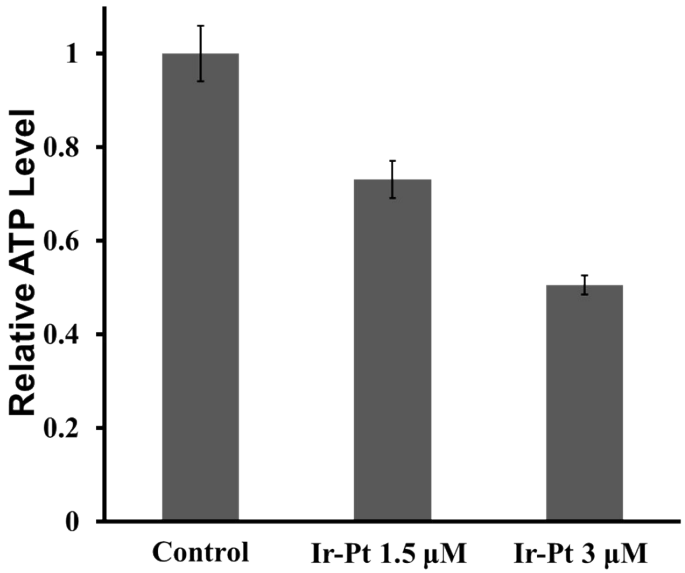


Figure S13. Intracellular ATP levels in A549R cells upon different irradiation treatments. The cells were treated with 1.5 μM and 3 μM **Ir-Pt** for 24 h at 37 °C.

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

Supporting References

1 J. P. Liu, C. Z. Jin, B. Yuan, X. G. Liu, Y. Chen, L. N. and H. Chao, Enhanced cancer therapy by the marriage of metabolic alteration and mitochondrial-targeted photodynamic therapy using cyclometalated Ir(III) complexes, *Chem. Commum.*, 2017, **53**: 9878-9881.

2 C. Z. Jin, J. P. Liu, Y. Chen, L. L, Zeng, R. L. Guan, C. Ouyang, L. N. and H. Chao, Cyclometalated iridium(III) complexes as two-photon phosphorescent probes for specific mitochondrial dynamics tracking in living cells, *Chem. Eur. J.*, 2015, **21**: 12000-120010.