Supporting information

Brominated cyclometalated iridium(III) complexes for mitochondrial immobilization as potentiated anticancer agents

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Table of Contents

Supporting Materials and Methods

Supporting Tables and Figures

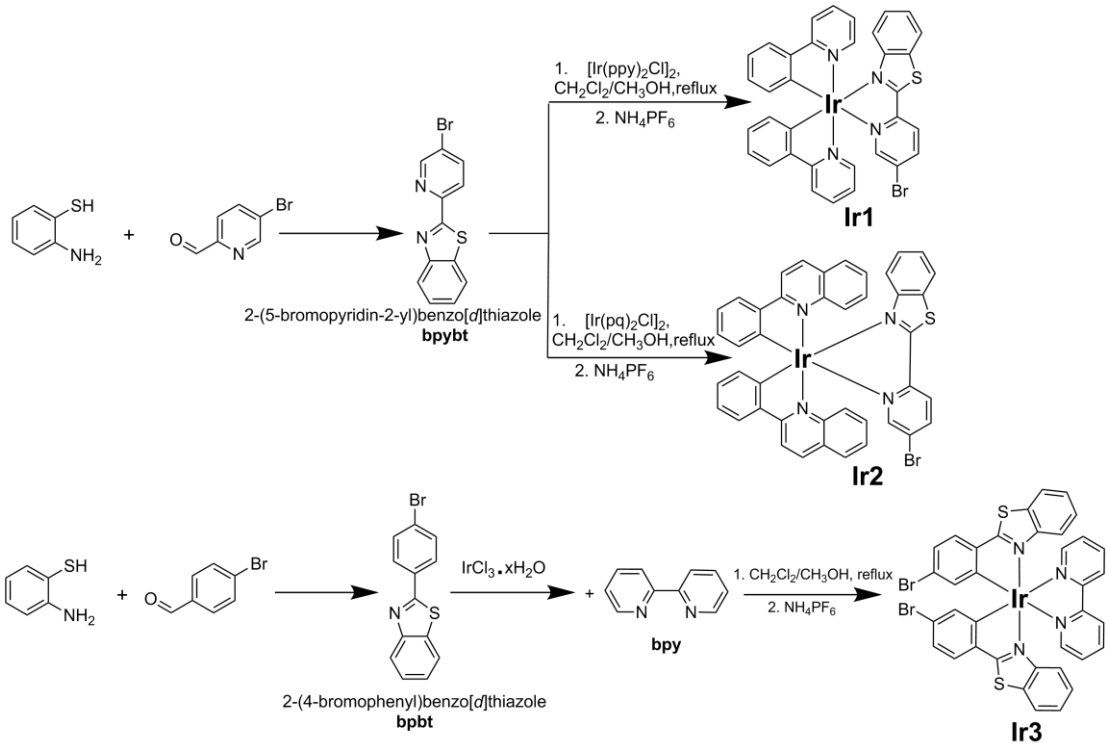
Experimental section

All solvents and chemicals were commercially received and used without further purification unless otherwise stated. The $IrCl_3•H_2O$, cisplatin 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), 2',7'-dichlorodihydro fluorescein diacetate (H2DCFDA) and 5,5',6,6'- tetrachloro-1,1',3,3'-tetraethyl benzimidazolylcarbocyanine iodide (JC-1) were purchased from Sigma Aldrich, MitoTracker Deep Red FM (MTDR) and LysoTracker Deep Red FM (LTDR) were obtained from Invitrogen Inc. Calcein-AM/PI cell double stain kit was purchased from Yeason Biotech. The tested compounds were dissolved in dimethylsulfoxide (DMSO) just before the experiments, and if no otherwise specified. All the cell lines were obtained from ATCC and cultured in DMEM (Gibco BRL) medium containing 10% fetal bovine serum (FBS, Gibco BRL), 100 IU/mL streptomycin, 100 IU/mL penicillin, 100 IU/mL streptomycin at 37 ℃ in 5% CO_2 and 95% air.

High Resolution Mass Spectrometer (HRMS) spectra were performed by a Bruker TIMSTOF. The quoted m/z values represent the major peaks in the isotopic distribution. NMR spectra were recorded on a Bruker Biospin Avance III 500 MHz spectrometer. Shifts were referenced relative to the internal solvent signals. Microanalysis (C, H, and N) was carried out using a Vario EL elemental analyzer. Fluorescence was recorded on an FLS 920 combined fluorescence lifetime and steady state spectrometer. UV-vis spectra were recorded on a Varian Cary 300 spectrophotometer.

Synthesis of the complexes

The ligand 2,2'-bipyridine (bpy), 2-phenylpyridine (ppy) and 2-phenylquinoline (pq) was purchased from (Sigma Aldrich). The ligand 2-(5-bromopyridin-2-yl)benzo [d]thiazole (bpybt)[1] and 2-(4-bromophenyl)benzo[d]thiazole (bpbt)[2] was synthesized according to literature procedures. The iridium(III) complexes **Ir1-Ir3** (**Scheme S1**) were synthesized according to previous methods with slight changes [3].



Scheme S1 Synthesis of ligands and complexes.

Synthetic procedure of the complexes: The iridium(III) precursor (0.2 mmol), bpybt or bpy (0.4 mmol) were added in methol/dichloromethane (2:1, v/v). The mixtures were then refluxed for 4 h under nitrogen atmosphere, followed by removing of CH_2Cl_2 under reduced pressure, and NH_4PF_6 (0.8 mmol) was dropped into this solution. The mixture was stirred for another 3 h and evaporated to dryness. The residue was dissolved in CH_2Cl_2, and purified by column chromatography on silica gel eluted with CH_2Cl_2/ CH_3OH (20:1, v/v).

$[Ir(ppy)_2(bpybt)]PF_6$ (**Ir1**): Complex **Ir1** was obtained as a red powder. Yield: 75.3%. ^{1}H NMR (500 MHz, DMSO) δ 8.79 (d, *J* = 8.6 Hz, 1H, H_9), 8.60 (dd, *J* = 8.6, 2.1 Hz, 1H, H_1), 8.41 (d, *J* = 8.1 Hz, 1H, $H_{1'}$), 8.31 (d, *J* = 8.2 Hz, 1H, H_{11}), 8.22 (d, *J* = 8.1 Hz, 1H, H_5), 7.98 (d, *J* = 7.6 Hz, 1H, $H_{5'}$), 7.97– 7.93 (m, 2H, H_{10} and H_{12}), 7.91 (t, *J* = 7.8 Hz, 1H, H_4), 7.82 (d, *J* = 5.7 Hz, 1H, $H_{4'}$), 7.76 (d, *J* = 5.2 Hz, 1H, H_{15}), 7.73 (d, *J* = 6.0 Hz, 1H, H_{14}), 7.57 (t, *J* = 8.2 Hz, 1H, H_{13}), 7.24 (t, *J* = 8.4 Hz, 1H, H_3), 7.15 (dd, *J* = 12.7, 7.0 Hz, 2H, H_6 and $H_{6'}$), 7.09 (q, *J* = 7.5 Hz, 2H, H_8 and $H_{8'}$), 6.97 (q, *J* = 6.2 Hz, 2H, H_7 and $H_{7'}$), 6.80 (d, *J* = 8.5 Hz, 1H, $H_{3'}$), 6.25 (d, *J* = 7.2 Hz, 1H, H_2), 6.14 (d, *J* = 7.1 Hz, 1H, $H_{2'}$). ^{13}C{^{1}H} NMR (126 MHz, DMSO) δ 169.63 (C),

166.59 (C), 166.06 (C), 150.39 (C), 150.32 (CH), 150.14 (C), 149.95 (CH), 149.84 (CH), 147.09 (CH), 146.75 (C), 144.29 (C), 143.79 (CH), 142.61 (CH), 139.01 (C), 138.86 (CH), 134.53 (CH), 131.49 (CH), 130.53 (CH), 130.44 (CH), 130.01 (CH), 128.27 (CH), 128.03 (CH), 125.33 (C), 125.17 (C), 125.06 (CH), 124.37 (CH), 123.95 (CH), 122.92 (CH), 122.55 (CH), 121.17 (CH), 120.23 (CH), 119.69 (C). HRMS: m/z 791.0433 $[M-PF_6]^+$. Elemental analysis: calcd (%) for $IrC_{34}H_{23}F_6N_4PSBr$: C, 43.60; H, 2.47; N, 5.98; found C, 43.43; H, 2.52; N, 5.85.

$[Ir(pq)_2(bpybt)]PF_6$ (**Ir2**): Complex **Ir2** was obtained as a red powder. Yield: 67.1%. ^1H NMR (500 MHz, DMSO) δ 8.67 – 8.61 (m, 2H, H_5 and $H_{5'}$), 8.49 (dd, *J* = 8.5, 2.0 Hz, 1H, H_{13}), 8.45 (s, 1H, H_{11}), 8.43 (d, *J* = 6.1 Hz, 1H, H_4), 8.41 (d, *J* = 10.1 Hz, 1H, $H_{4'}$), 8.38 – 8.32 (m, 1H, H_{12}), 8.30 (d, *J* = 8.2 Hz, 1H, H_1), 8.25 (d, *J* = 7.8 Hz, 1H, $H_{1'}$), 7.99 (d, *J* = 8.0 Hz, 1H, H_{14}), 7.96 (d, *J* = 1.9 Hz, 1H, H_2), 7.90 (d, *J* = 7.2 Hz, 1H, $H_{2'}$), 7.60 (t, *J* = 7.8 Hz, 1H, H_3), 7.55 (d, *J* = 8.9 Hz, 1H, $H_{3'}$), 7.46 (d, *J* = 7.4 Hz, 1H, H_{17}), 7.44 – 7.39 (m, 1H, H_{16}), 7.36 (t, *J* = 8.2 Hz, 1H, H_{15}), 7.21 (ddd, *J* = 9.0, 7.5, 5.1 Hz, 3H, H_9, H_7 and $H_{9'}$), 6.99 (d, *J* = 8.9 Hz, 1H, $H_{7'}$), 6.88 (ddd, *J* = 20.5, 14.7, 8.5 Hz, 4H, H_8 H_{10}, $H_{8'}$ and $H_{10'}$), 6.47 (d, *J* = 7.6 Hz, 1H, H_6), 6.38 (d, *J* = 7.5 Hz, 1H, $H_{6'}$). $^{13}C\{^1H\}$ NMR (126 MHz, DMSO) δ 170.75 (C), 169.42 (C), 169.07 (C), 150.16 (CH), 149.38 (C), 148.59 (C), 147.74 (CH), 147.39 (C), 146.93 (CH), 146.63 (C), 145.85 (CH), 143.51 (CH), 141.51 (CH), 141.09 (CH), 134.21 (C), 134.06 (C), 133.59 (CH), 131.96 (CH), 131.64 (CH), 131.48 (CH), 131.39 (C), 130.08 (CH), 130.04 (CH), 129.06 (CH), 128.76 (CH), 128.05 (C), 128.00 (C), 127.89 (CH), 127.56 (CH), 125.56 (CH), 125.26 (C), 124.88 (C), 123.96 (CH), 123.91 (CH), 123.64 (CH), 121.13 (CH), 118.63 (CH), 118.03 (C). HRMS: m/z 891.0755 $[M-PF_6]^+$. Elemental analysis: calcd (%) for $IrC_{42}H_{27}F_6N_4PSBr$: C, 48.65; H, 2.62; N, 5.40; found C, 48.51; H, 2.75; N, 5.31.

$[Ir((bpbt))_2(ppy)]PF_6$ (**Ir3**): Complex **Ir3** was obtained as a yellow powder. Yield: 52.4%. ^1H NMR (500 MHz, DMSO) δ 8.85 (d, *J* = 8.2 Hz, 2H, H_{11} and $H_{11'}$), 8.34 (td, *J* = 8.0, 1.5 Hz, 2H, H_8 and $H_{8'}$), 8.28 (d, *J* = 7.7 Hz, 2H, H_4 and $H_{4'}$), 8.11 (dd, *J* = 5.5, 0.8 Hz, 2H, H_7 and $H_{7'}$), 8.04 (d, *J* = 8.2 Hz, 2H, H_1 and $H_{1'}$), 7.80 (ddd,

J = 7.5, 5.6, 1.1 Hz, 2H, H_5 and $H_{5'}$), 7.49–7.44 (m, 2H, H_6 and $H_{6'}$), 7.38 (dd, *J* = 8.2, 1.9 Hz, 2H, H_{10} and $H_{10'}$), 7.24–7.18 (m, 2H, H_2 and $H_{2'}$), 6.30 (d, *J* = 1.9 Hz, 2H, H_3 and $H_{3'}$), 6.07 (d, *J* = 8.5 Hz, 2H, H_9 and $H_{9'}$). $^{13}C\{^1H\}$ NMR (126 MHz, DMSO) δ 180.28 (C), 155.83 (C), 152.11 (C), 150.67 (CH), 148.14 (C), 140.74 (CH), 139.27 (C), 134.46 (CH), 131.35 (CH), 129.36 (CH), 128.99 (C), 128.41 (CH), 126.43 (CH), 126.37 (CH), 126.27 (C), 124.96 (CH), 124.90 (CH), 116.61 (CH). HRMS: m/z 926.9246 $[M-PF_6]^+$. Elemental analysis: calcd (%) for $IrC_{36}H_{22}F_6N_4PS_2Br_2$: C, 40.35; H, 2.07; N, 5.23; found C, 40.16; H, 2.24; N, 5.11.

Protein binding assay [4]

Measure the fluorescence emission spectrum after BSA (10 μM) reacts with different concentrations (1-10 μM) of **Ir1-Ir3** for 30 min, λ_{ex} = 280 nm.

Stern-Volmer equation: $F_0 / F = 1 + K_{sv}[Q] = 1 + K_q\tau_0[Q]$

Scatchard equation: $\log[(F_0 - F) / F] = \log K_b + n \log[Q]$

F_0 and F are used to represent the fluorescence intensity of BSA in the absence and presence of compound [Q], K_{sv} is the quenching constant, K_q is the quenching rate constant, and the average lifetime of the fluorophore in the absence of a quencher is τ_0 (10^{-8} s).

Cell lines and culture conditions

Hela, 143B, MCF-7, A3475, C28/I2, BMSC and EA.hy926 were purchased from ATCC. Various cells were cultured in DMEM (Gibco BRL) or RPMI 1640 (Gibco BRL) medium containing 10% fetal bovine serum (FBS, Gibco BRL), 100 IU/mL penicillin, and 100 IU/mL streptomycin at 37 ℃ in 5% CO_2 and 95% air.

Confocal microscopy

HeLa cells are typically seeded at a density at the bottom of 8 well chamber slide at a density of 4×10^4 cells/mL for confocal microscope imaging. After incubating in a 5% CO_2/95% air incubator at 37℃ for 24 h. The Hela cells were co-stained with complexes **Ir1-Ir3** (10 μM) and commercial mitochondrial-specifc dye (MTG or MTDR: 100 nM) or lysosome-specifc dye (LTG or LTDR: 100 nM) for 20 min. Cells were washed with PBS for three times and visualized by confocal microscopy (Nikon

Eclipse Ti2) immediately. The iridium complexes excited at 405 nm, MTG or LTG excited at 488 nm, MTDR or LTDR excited at 630 nm. Emission was collected at 650 ± 20 nm (**Ir1** and **Ir2**), 570 ± 20 nm (**Ir3**), 520±15 nm (MTG or LTG) and 660±15 nm (MTDR or LTDR).

Cellular uptake

After the HeLa cells were incubated with CCCP (30 μM) and Chloroquine (50 μM) for 1 h, the culture medium was aspirated, and the culture medium containing **Ir1-Ir3** (10 μM) was incubated for 20 min. In addition, the other cells were pretreated at 4 ℃ and then incubated with **Ir1-Ir3** (10 μM) for 20 min. The cells were washed three times with PBS and visualized by confocal microscopy.

Inductively coupled plasma mass spectrometry (ICP-MS) analysis

The intracellular iridium contents of **Ir1-Ir3** were determined by a method reported in literature with slight modifications.[3] Briefly, HeLa cells were grown in 10 cm culture dishes and incubated for 24 h. The medium was removed and replaced with medium-DMSO (99:1 v/v) containing **Ir1-Ir3** (10 μM) under different conditions. After 1 h incubation, the cells were d collected, counted, and digested with HNO_3 (65%, 0.5 mL) at 60 ºC for 1 h. The solution was then diluted to a final volume of 10 mL with Milli-Q water. The concentration of iridium was measured using the ICP-MS.

Live−Dead cell staining

After the HeLa cells were incubated with **Ir1-Ir3** (10 μM) and cisplatin (50 μM) for 5 h, they were stained with Calcein-AM and PI, observed and photographed through a fluorescence microscope.

MMP analysis

HeLa cells are incubated with the designated **Ir1-Ir3** for 5 h, cells were harvested and stained with 5 μg/mL JC-1 for 20 min at 37 ºC in the dark, and analyzed immediately by flow cytometry. Data were analyzed with FlowJo software.

ROS analysis

HeLa cells are incubated with the designated **Ir1-Ir3** for 5 h, cells were

harvested and stained with 10 μM H_2DCFDA for 30 min at 37 ºC in the dark. and analyzed immediately by flow cytometry.

Annexin V/PI assay

HeLa cells were incubated with the designated iridium **Ir1-Ir3** for 5 h, cells were harvested and stained with 5 μL Annexin V-FITC and 10 μL PI for 30 min in the dark, and analyzed immediately by flow cytometry.

Tumor cell clone inhibition test

HeLa cells seeded in a 6-well plate for 24 h at 37℃ and 5% CO_2 environment, complexes **Ir1-Ir3** (50 nM) was then added to the experimental group, and continued to incubate for 24 h, changed to adding fresh medium and continued incubating for 7 days. Use 4% paraformaldehyde solution to fix the cells at 4℃ for 1 h, then used crystal violet solution to stain cell for 15 min.

Antiangiogenic activity assay

Mix the Matrigel with FBS-free DMEM (*v*/*v*=1:1), place the mixture in the 48-well plate with a pipette, and place the 48-well plate in 37 ºC incubator for 30-40 min. Prepare EA.hy926 cell suspension for FBS-free DMEM and adjust the cell suspension to a concentration of 10×10^4 cells/well. Cells were incubated with a certain concentration of iridium complexs for 5 h, and control cells were incubated with 1%DMSO (*v*/*v*).

Inhibition of TrxR

The TrxR enzyme activity was performed as previously described [5], and determined using a specific kit (BC1150; Solarbio Life Sciences, Beijing, China).

Western blotting analysis

Western blotting was performed as previously described [6]. Briefly, HeLa cells seeded in 6 cm culture dishes were exposed to **Ir2** at 2 μM, 4 μM or 8 μM for 12 h. Cells were lysed in radio-immunoprecipitation assay (RIPA) buffer. The proteins were separated on SDS-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes. The membrane was blocked and incubated with the primary antibodies at 4 ºC overnight. After a subsequent washing step, the

membrane was incubated with the secondary antibody for 30 min. The immunoreactivity was detected using the enhanced chemiluminescence detection kit. Images were captured using a CLINX 6300 imaging station and analyzed manually with Alpha Innotech software.

Immunofluorescence staining analysis

HeLa cells were treated with **Ir2** (4 μM) for 24 h, and then 4% paraformaldehyde was added for 20 min. The cells were washed with PBS three times, treated with Triton X-100 (0.2%) to break the membrane for 15 min, and washed with PBS three times. Then, 10% goat serum was used to block for 1 h, and added with primary antibody overnight at 4 ℃. After a subsequent washing step, the fluorescent secondary antibody was added for 40 min at room temperature, and then stained nucleus with DAPI and analysed with confocal microscopy.

Cytotoxicity test on MCTSs

HeLa MCTSs (diameter .400 mm) were treated by carefully replacing 50% of the medium with drug-supplemented standard medium by using an eight-channel pipette. In parallel, 50% of the solvent-containing medium were replaced by solvent-free medium for the unt reated MCTSs. Three MCTSs were incubated with **Ir2** at different concentrations (2/4/8 μM), and cisplatin (10 μM), and the DMSO volume was less than 1% (v/v). Take pictures and record every 24 h.

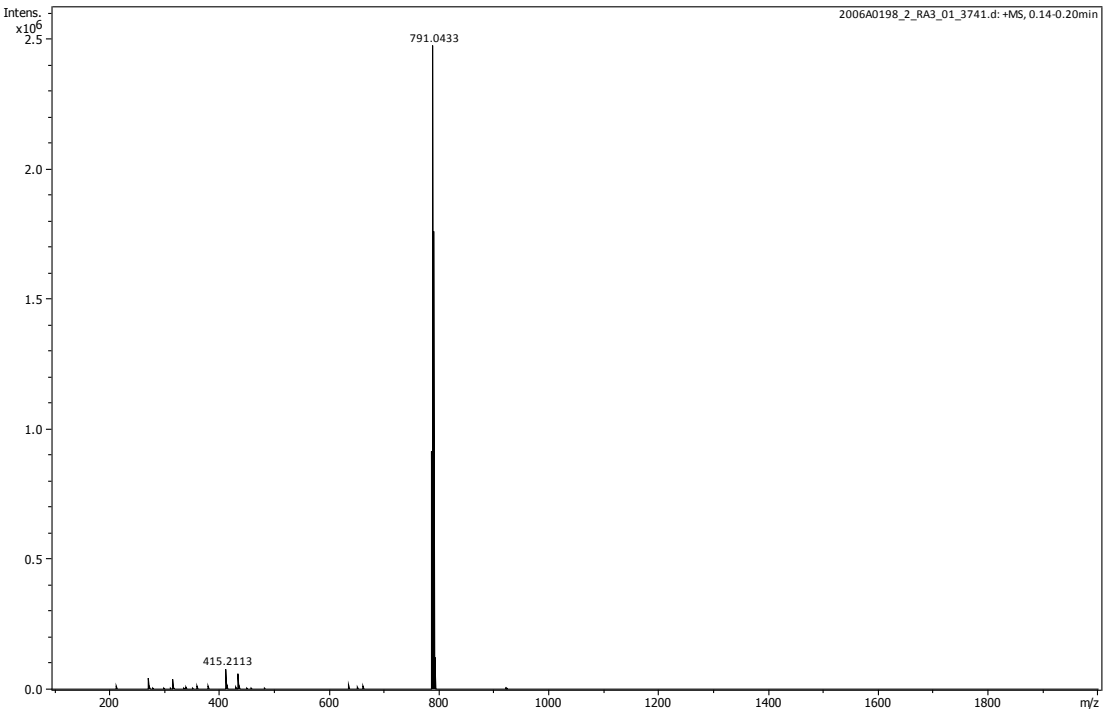


Fig. S1 HRMS spectrum of complex **Ir1**.

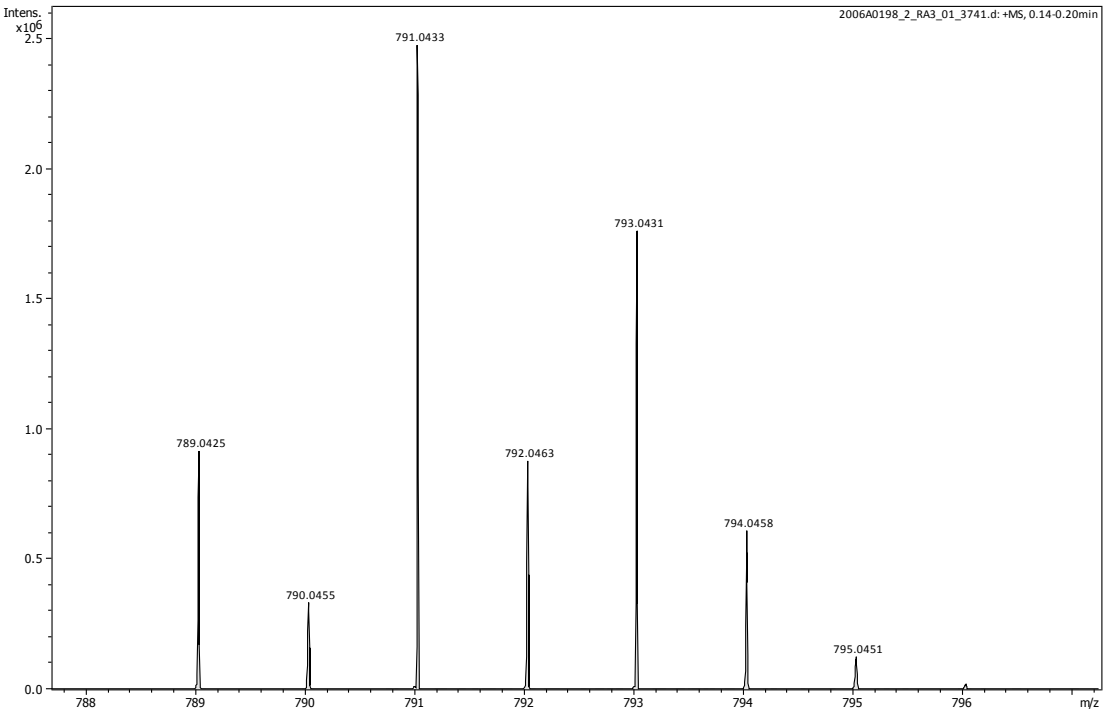


Fig. S2 Ion isotopes spectrum of complex **Ir1**.

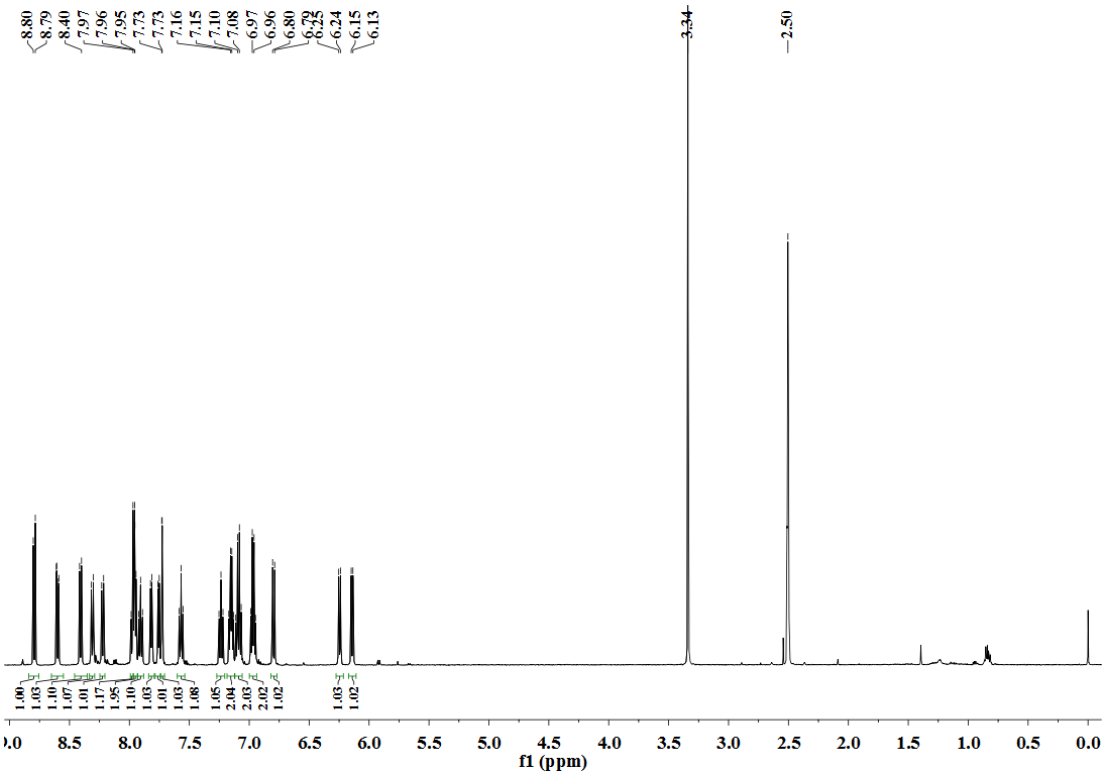


Fig. S3 ^{1}H NMR spectrum of complex **Ir1**.

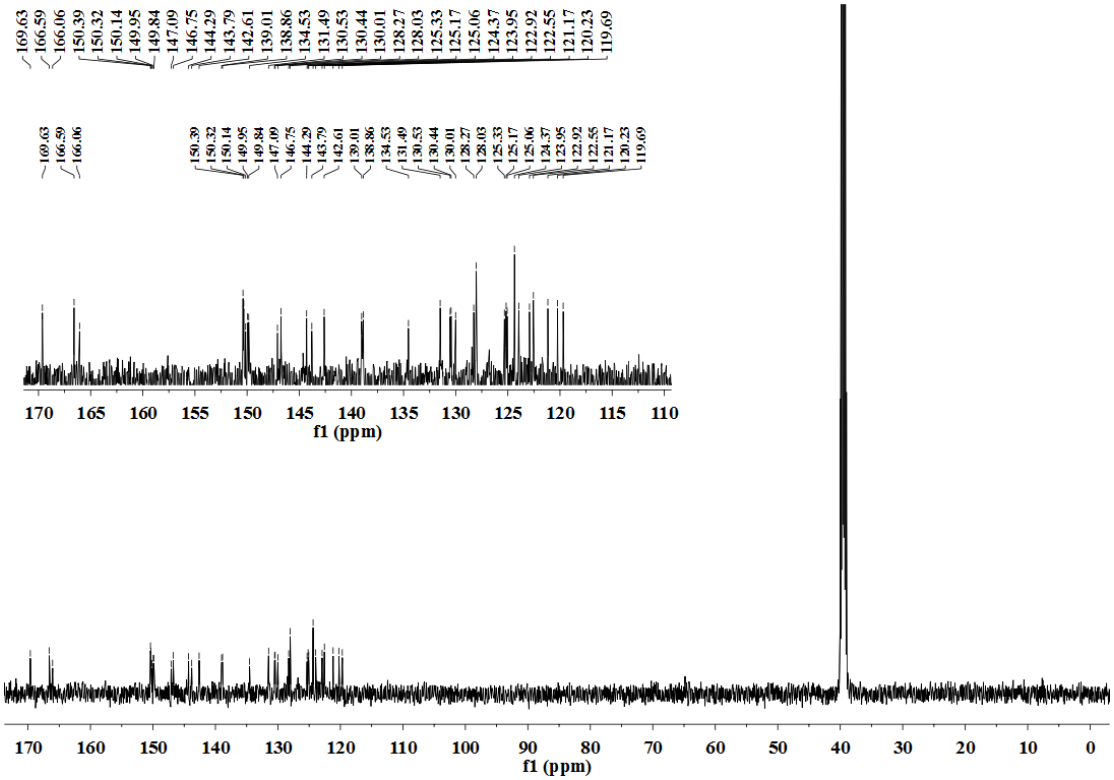


Fig. S4 ^{13}C NMR spectrum of complex **Ir1**.

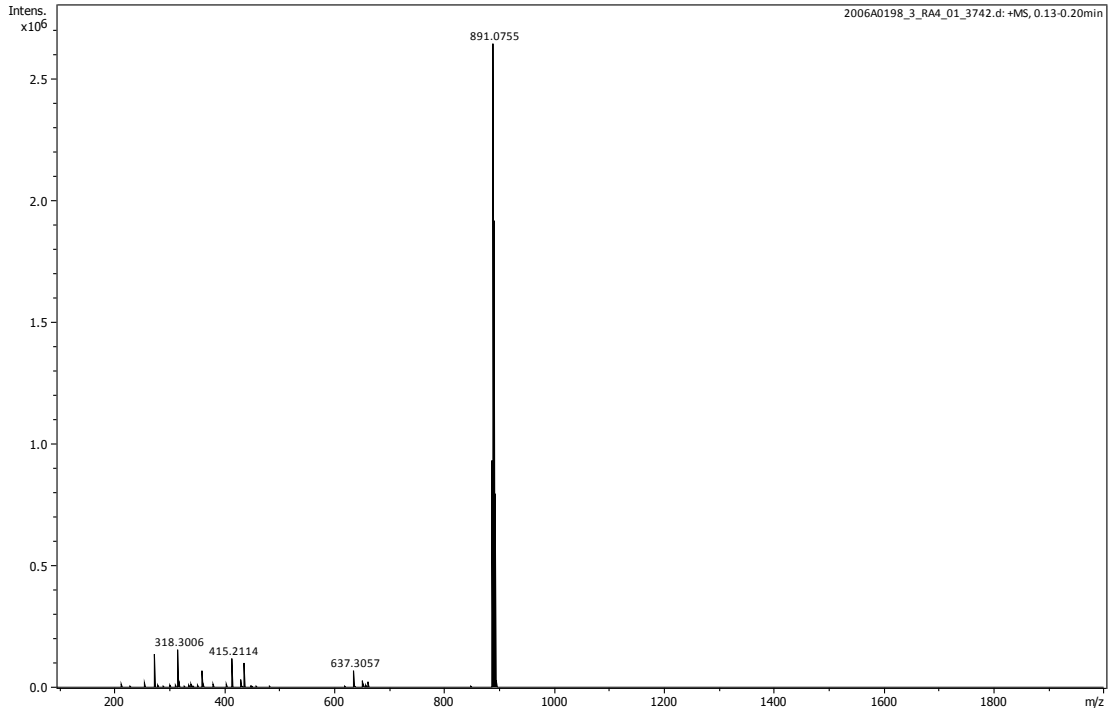


Fig. S5 HRMS spectrum of complex **Ir2**.

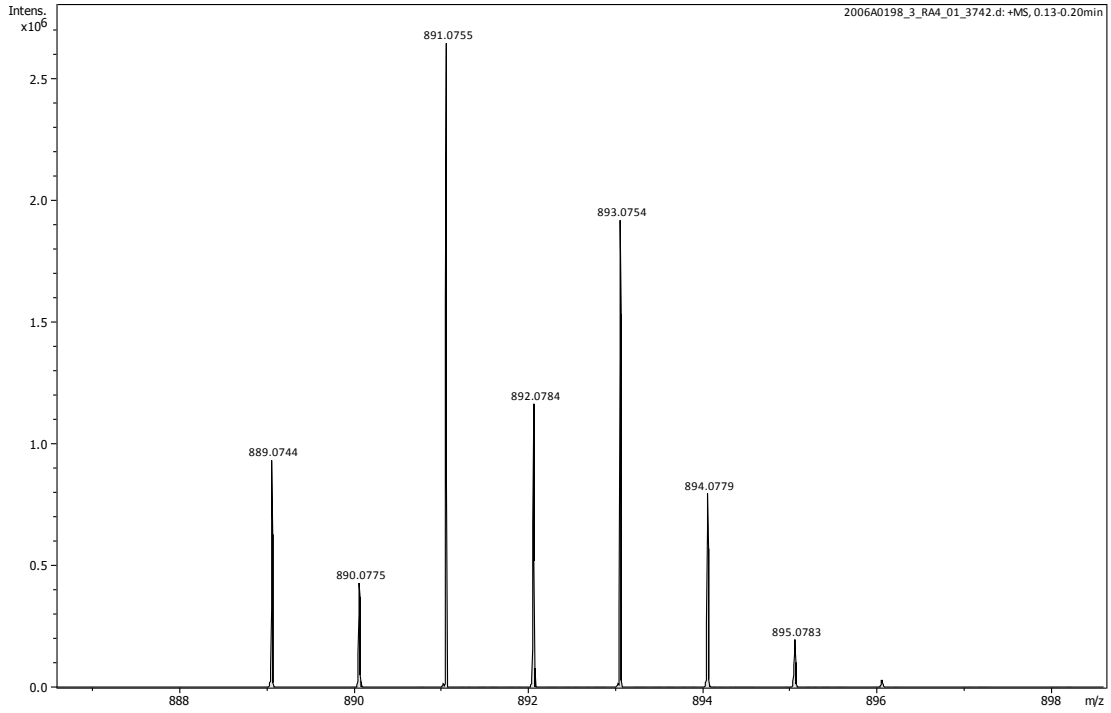


Fig. S6 Ion isotopes spectrum of complex **Ir2**.

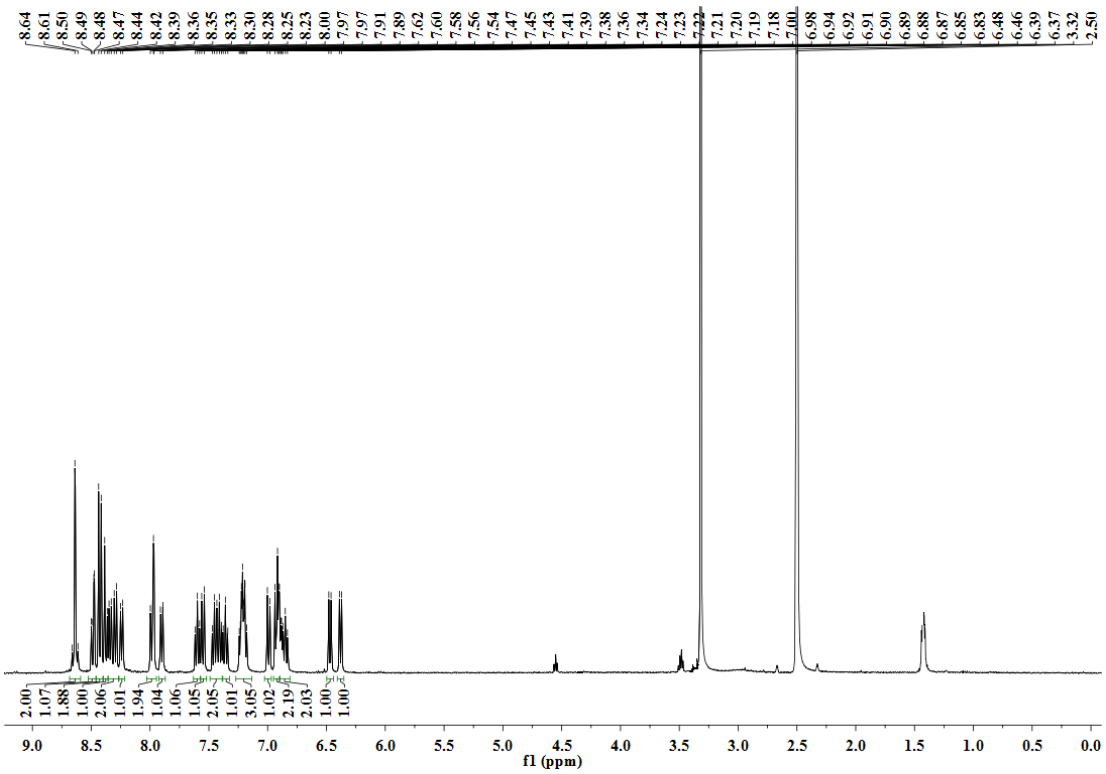


Fig. S7 ^{1}H NMR spectrum of complex **Ir2**.

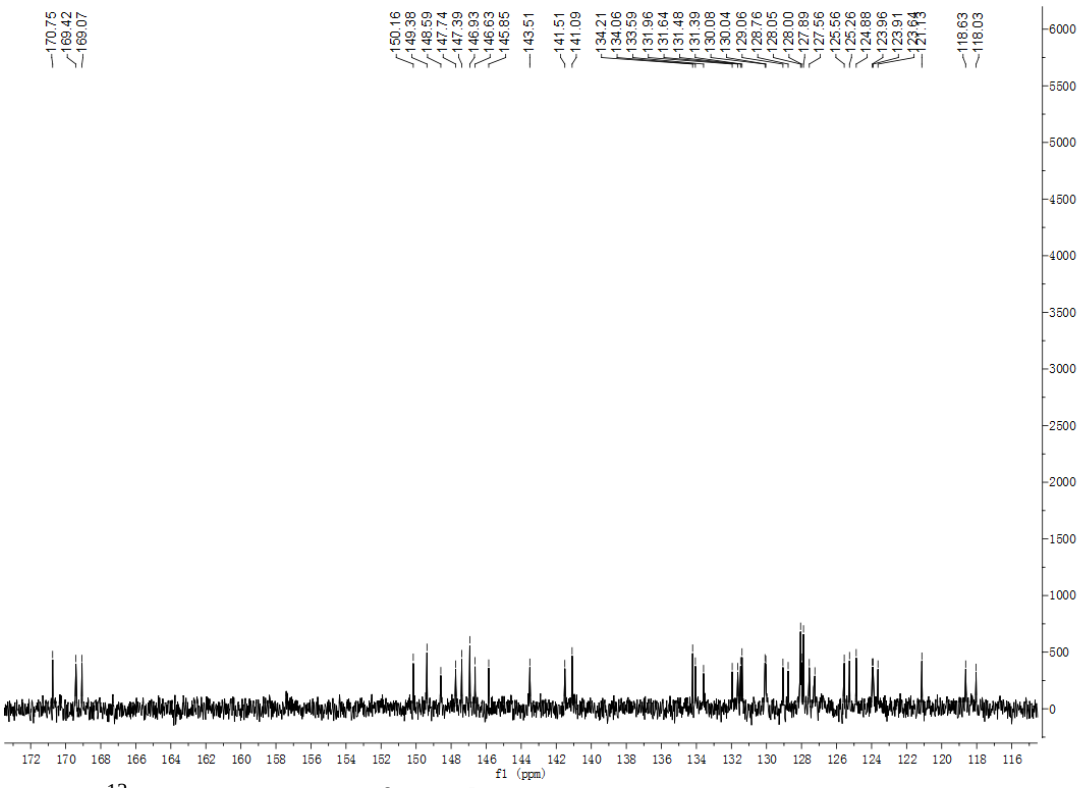


Fig. S8 ^{13}C NMR spectrum of complex **Ir2**.

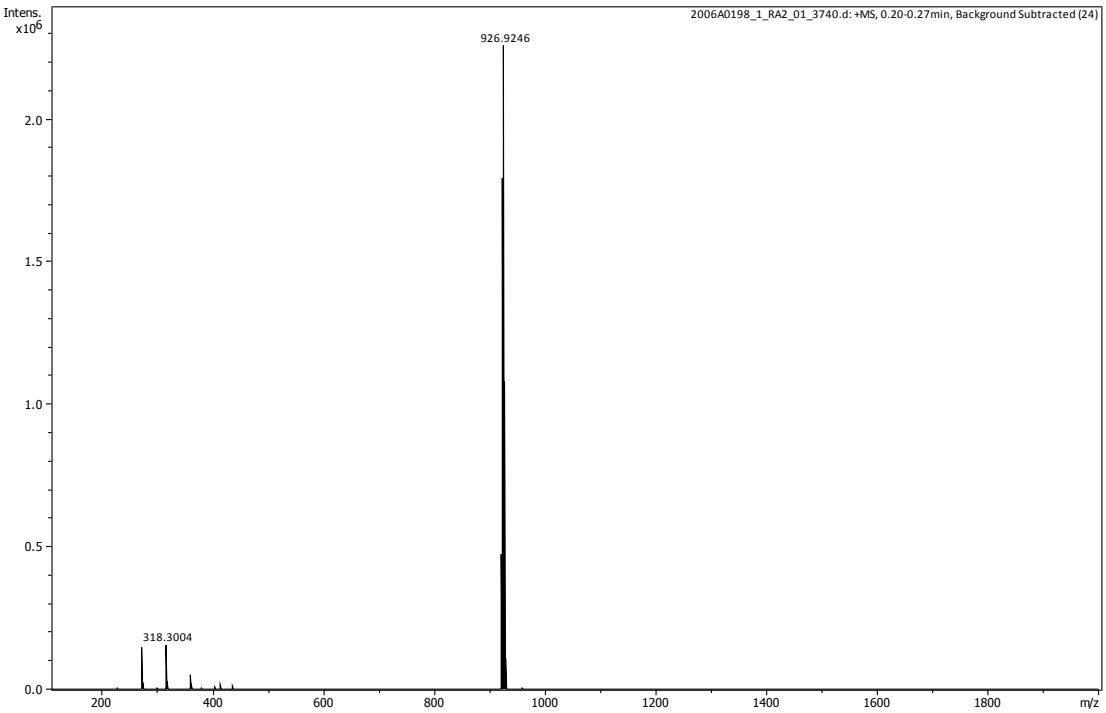


Fig. S9 HRMS spectrum of complex **Ir3**.

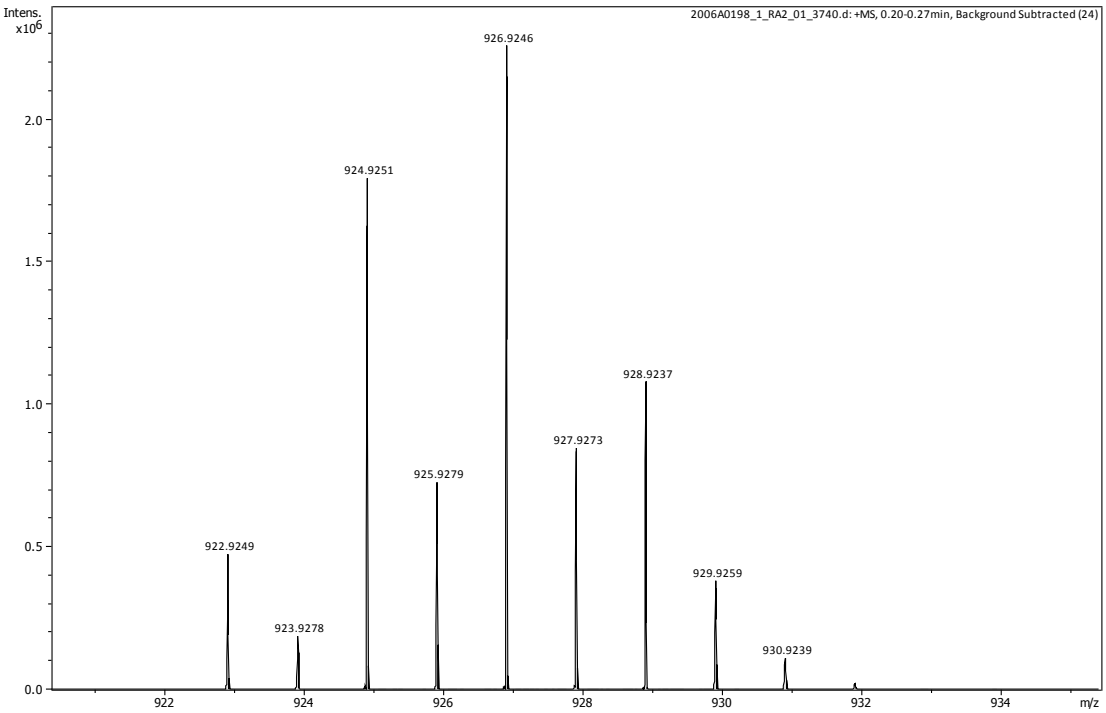


Fig. S10 Ion isotopes spectrum of complex **Ir3**.

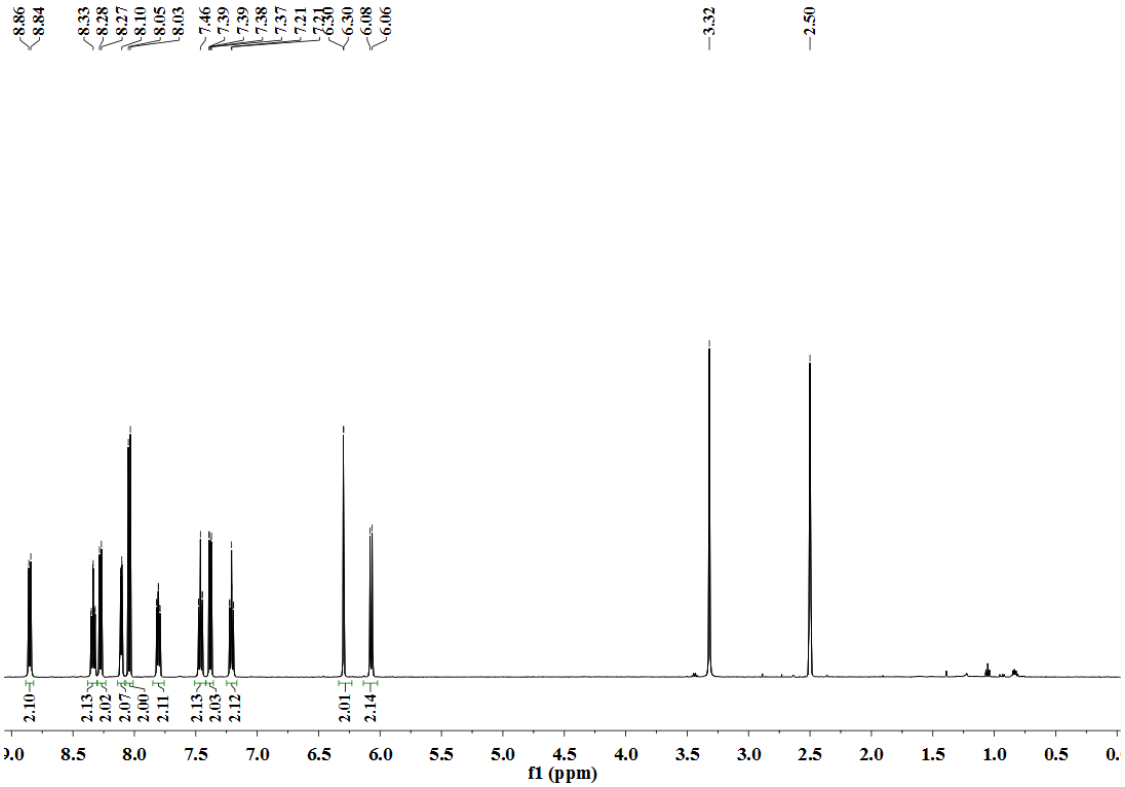


Fig. S11 ^{1}H NMR spectrum of complex **Ir3**.

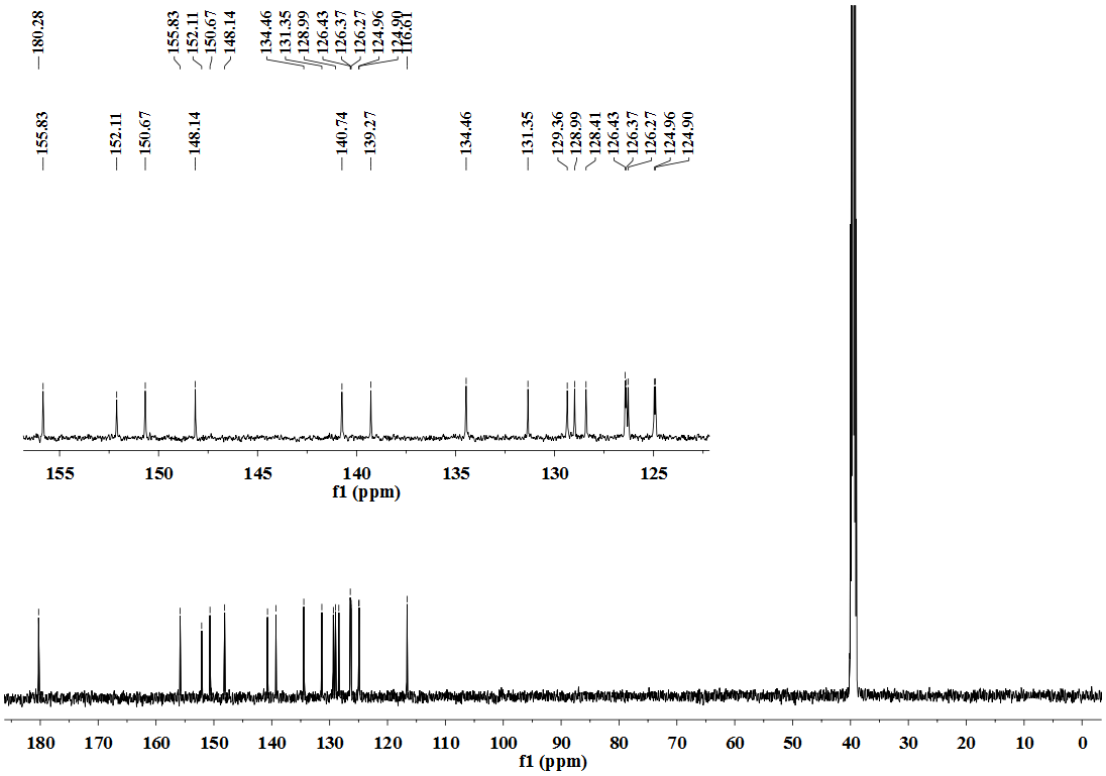


Fig. S12 ^{13}C NMR spectrum of complex **Ir3**.

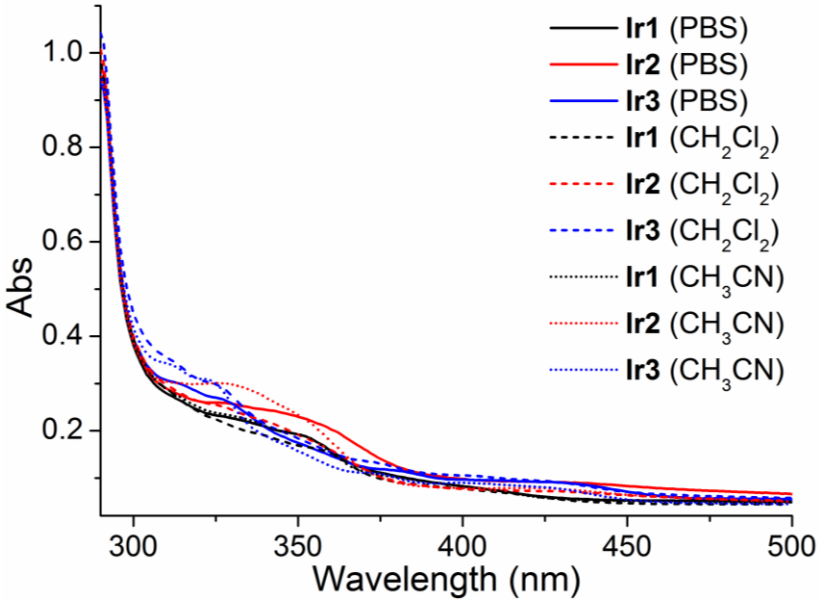


Fig. S13 UV-vis absorption spectra of **Ir1**-**Ir3** (20 μM) in PBS, CH_2Cl_2 and CH_3CN, respectively.

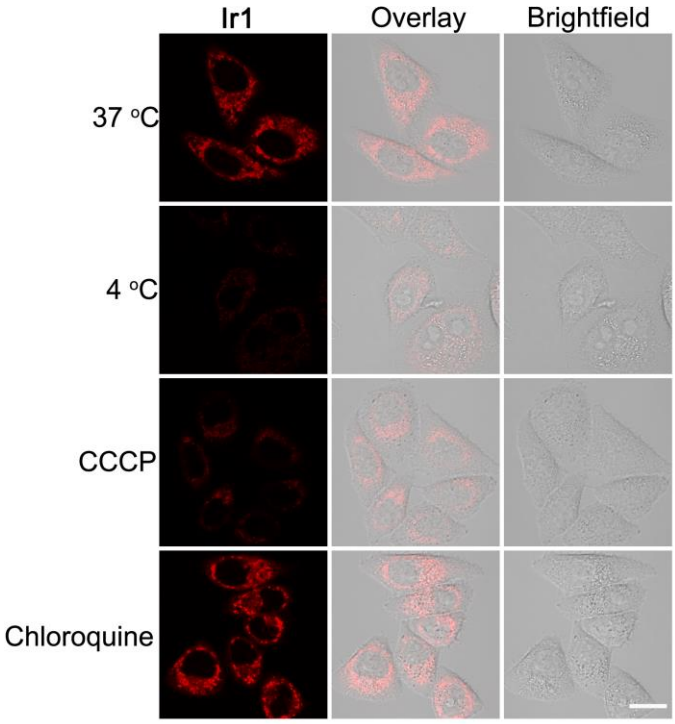


Fig. S14 Confocal images of HeLa cells after incubation with **Ir1** (10 μM) under different conditions. (A) Cells were incubated with **Ir1** (10 μM) at 37 °C for 10 min. (B) Cells were incubated with **Ir1** (10 μM) at 4 °C for 10 min. (C) Cells were pre-incubated with CCCP (10 uM) for 1 h at 37 ℃ and then incubated with **Ir1** (10 μM) at 37 °C for 10 min. (D) Cells were pre-incubated with chloroquine (50 μM) for 1 h at 37 ℃ and then incubated with **Ir1** (10 μM) at 37 °C for 10 min. Complex **Ir1** was excited at 405 nm and emission was collected at 650 ± 20 nm. Scale bar: 10 μm.

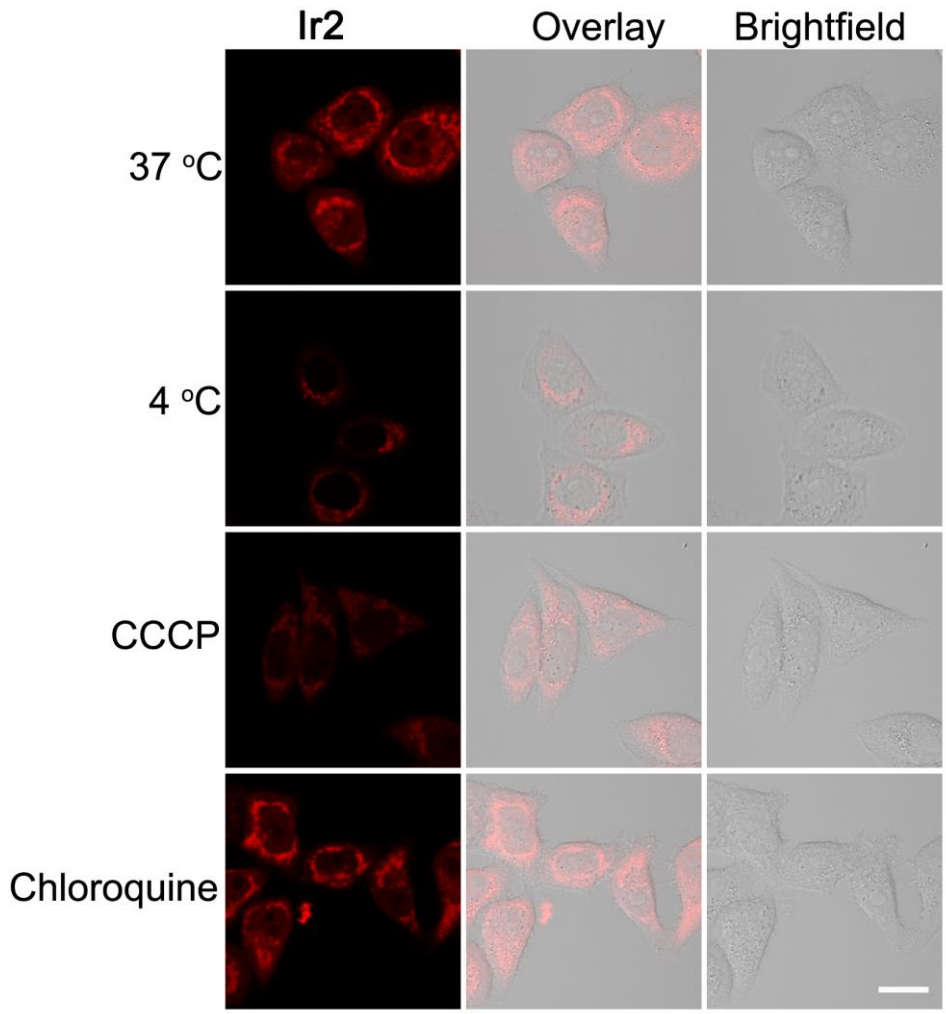


Fig. S15 Confocal images of HeLa cells after incubation with **Ir2** (10 μM) under different conditions. (A) Cells were incubated with **Ir2** (10 μM) at 37 °C for 10 min. (B) Cells were incubated with **Ir2** (10 μM) at 4 ℃ for 10 min. (C) Cells were pre-incubated with CCCP (10 uM) for 1 h at 37 ℃ and then incubated with **Ir2** (10 μM) at 37 °C for 10 min. (D) Cells were pre-incubated with chloroquine (50 μM) for 1 h at 37 ℃ and then incubated with **Ir2** (10 μM) at 37 °C for 10 min. Complex **Ir2** was excited at 405 nm and emission was collected at 640 ± 20 nm. Scale bar: 10 μm.

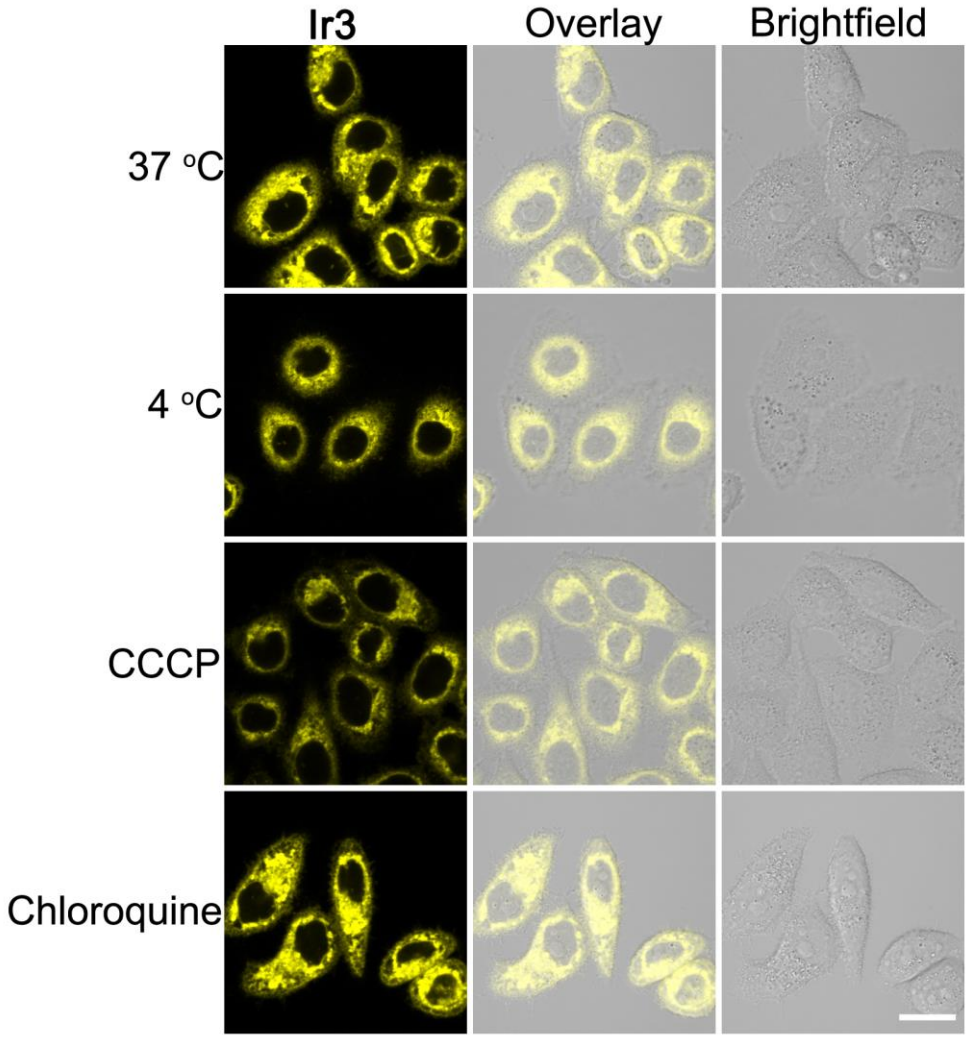


Fig. S16 Confocal images of HeLa cells after incubation with **Ir3** (10 μM) under different conditions. (A) Cells were incubated with **Ir3** (10 μM) at 37 ℃ for 10 min. (B) Cells were incubated with **Ir3** (10 μM) at 4 °C for 10 min. (C) Cells were pre-incubated with CCCP (10 uM) for 1 h at 37 ℃ and then incubated with **Ir3** (10 μM) at 37 °C for 10 min. (D) Cells were pre-incubated with chloroquine (50 μM) for 1 h at 37 ℃ and then incubated with **Ir3** (10 μM) at 37 °C for 10 min. Complex **Ir3** was excited at 405 nm and emission was collected at 560 ± 20 nm. Scale bar: 10 μm.

Table S1 The different conditions and cellular uptake efficiency of complexes **Ir1-Ir3**.

Compounds	conditions	Amount of iridium (ng per 10^6 cells)[a]
Ir1	37 ºC	306.29 ± 12.4
	4 ºC	56.38 ± 6.2
	CCCP	118.43 ± 9.7
	chloroquine	235.23 ± 10.6
Ir2	37 ºC	455.30 ± 16.5
	4 ºC	114.07 ± 7.8
	CCCP	146.40 ± 11.2
	chloroquine	375.14 ±
Ir3	37 ºC	183.24 ± 13.5
	4 ºC	27.36 ± 3.1
	CCCP	60.13 ± 5.6
	chloroquine	155.38 ± 13.3

[a]Data are presented as means ± standard deviation obtained in at least three independent experiments.

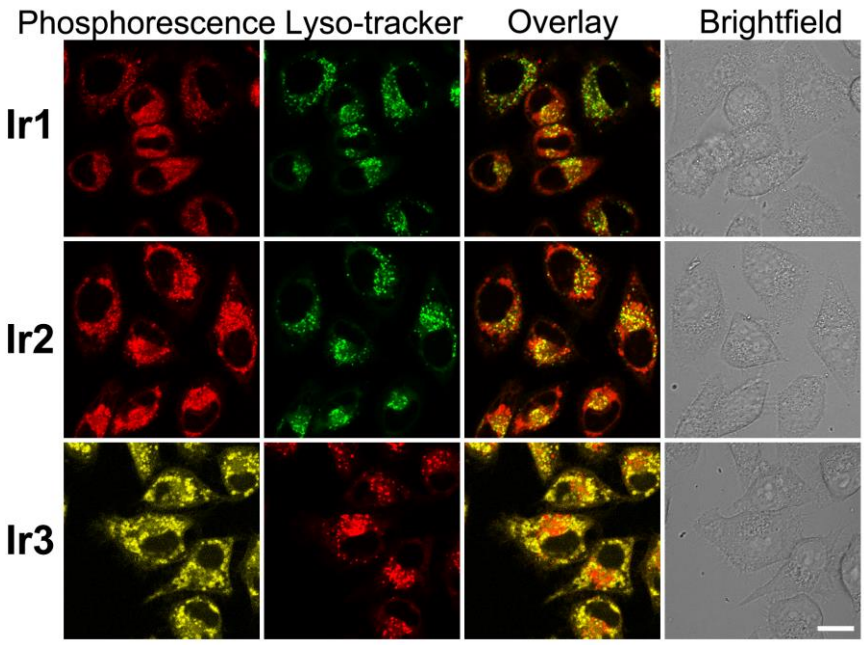


Fig. **S17** Determination of intercellular localization of complexes **Ir1**–**Ir3** by confocal microscopy. A549 cells were incubated with LTDR (75 nM) for 20 min and then co-incubated with **Ir1** (20 μM), **Ir2** (20 μM) and **Ir3** (20 μM) for 10 min at 37 ºC. The complexes **Ir1**–**Ir3** were excited at 405 nm, and the emission was collected at 650 ± 20 nm, 640 ± 20 nm, 560 ± 20 nm, respectively. LTDR was excited at 630 nm and the emission was collected at 660 ± 20 nm. Scale bar: 10 μm.

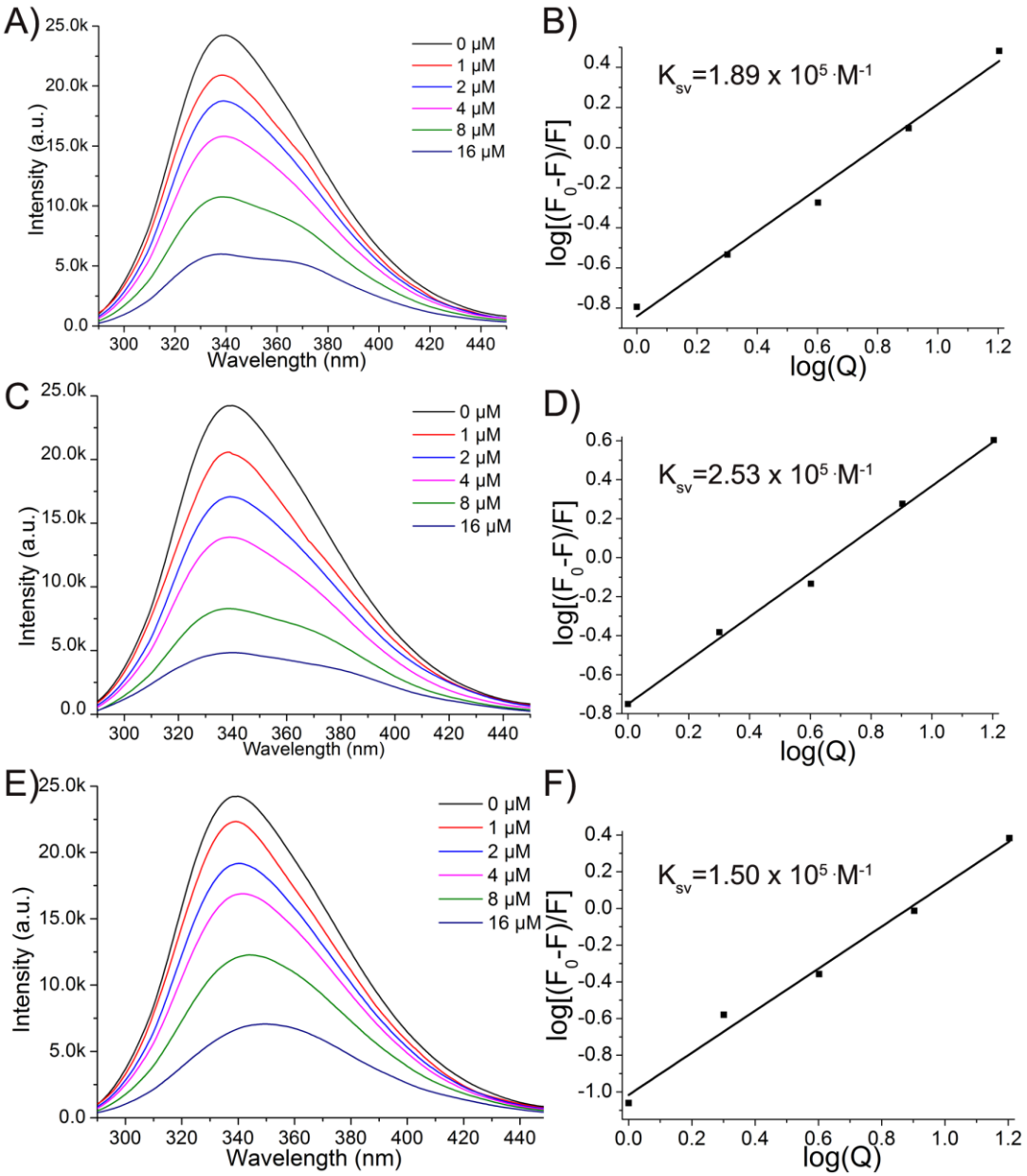


Fig. S18 Emission spectra of BSA quenched by complexes **Ir1**-**Ir3**. BSA (10 μM) was incubated with the Ir(III) complexes at indicated concentrations for 30 min. λ_{ex} = 280 nm.

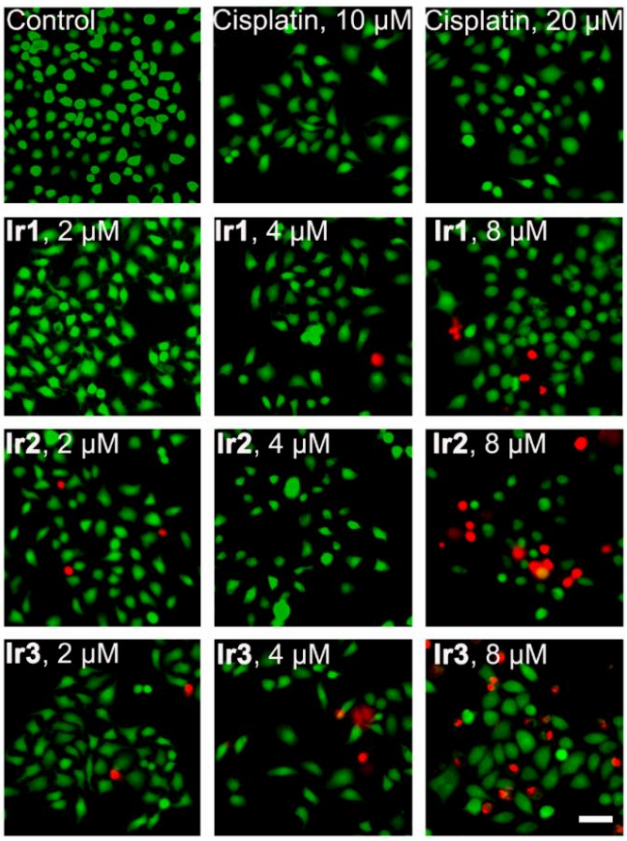


Fig. S19 Fluorescence images of HeLa cells costained with calcein AM (staining live cells, green color) and propidium iodide (staining dead cells, red color) after treated with different complexes at indicated concentrations. Scale bar: 50 μm.

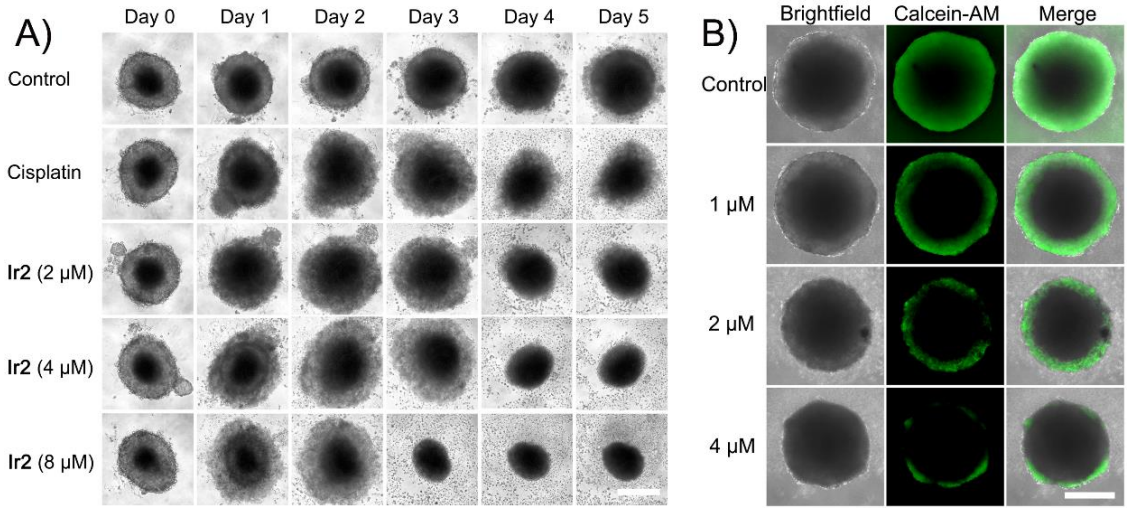


Fig. S20 A) Growth inhibition of drug-treated HeLa MCTSs by using cisplatin (10 μM) and complex **Ir2** at indicated concentrations. B) HeLa MCTSs were treated with complex **Ir2** at indicated concentrations for 60 h and then stained with Calcein AM dyes (λ_{ex}=488, λ_{em}=550 nm). Scale bar: 300 μm.

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