Supplementary Information

Monitor Mitochondrial Viscosity with Anticancer Phosphorescent Ir(III) Complexes via Two-Photon Lifetime Imaging

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Supplementary experimental section

Materials

Iridium chloride hydrate (J&K Chemical, China), 2-phenylpyridine (ppy, Sigma Aldrich, USA), 4-(2-pyridyl)benzaldehyde (ppy-CHO, J&K Chemical, China), bis(diphenylphosphino)methane (L_1, J&K Chemical, China), 1,2-bis(diphenylphophino)ethane (L_2, J&K Chemical, China), cis-1,2-bis(diphenylphosphino)ethylene (L_3, J&K Chemical, China), ammonium hexafluorophosphate (Alfa Aesar, USA), dimethyl sulfoxide (DMSO, Sigma Aldrich, USA), glycerol (Gly, J&K Chemical, China), trypsin (Hyclone Laboratoreis Inc, USA), Roswell Park Memorial Institute Medium (RPMI1640 Medium, Hyclone Laboratoreis Inc, USA), Dulbecco's Modified Eagle Medium (DMEM, Hyclone Laboratoreis Inc, USA), fetal bovine serum (FBS, Hyclone Laboratoreis Inc), MitoTracker® Deep Red FM (MTDR, Life Technologies, USA), LysoTracker® Deep Red (LTDR, Life Technologies, USA), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT, J&K Chemical, China), cisplatin (J&K Chemical, China), [Ru(bpy)3]Cl2 (bpy = 2,2-Bispyridine, Sigma Aldrich, USA), phosphate buffered saline (PBS, Sigma Aldrich, USA), 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma Aldrich, USA), 2'-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-1H,3'H-2,5'-bibenzimidazole (Hoechst 33342, Sigma Aldrich, USA) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine (JC-1, Sigma Aldrich, 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). Other materials and chemicals were purchased from the commercial sources. Zebrafish larvae was purchased from National Zebrafish Resource Centre (China). All the tested compounds were dissolved in DMSO as mother liquor before diluted into the experimental concentration with 1% (v/v) DMSO in the solvents.

Instrumentation

^{1}H NMR and ^{1}H-^{1}H NOESY spectra at different temperatures were recorded on a Bruker Avance 400 spectrometer (Germany). Shifts were referenced relative to the internal solvent signals. ESI-MS were recorded on a Thermo Finnigan LCQ DECA XP spectrometer (USA). The quoted m/z values

represented the major peaks in the isotopic distribution. Microanalysis (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). Crystal structure of the complexes were measured by an Agilent X-ray single-crystal diffractometer (USA). Emission measurements were conducted on an FLS 920 combined fluorescence lifetime and steady state spectrometer (Japan). Quantum yields of luminescence at room temperature were calculated according to literature procedures using $[Ru(bpy)_3]Cl_2$ as the reference.[1-3] Viscosity data were recorded on a Kinexus Pro+ rotational rheometer (UK). Confocal and PLIM images were recorded on a Carl Zeiss LSM 710 laser scanning confocal microscope (Germany). The cofocal microscope was combined with a Becker & Hickl (BH) time-correlated single photon counting (TCSPC) system. The PLIM data were analyzed using the SPCImage software available on www.becker-hickl.com (Becker & Hickl GmbH, the bh TCSPC Handbook sixth Edition). Tecan Infinite M200 Pro microplate reader (Switzerland) was used in MTT assay, Caspase-3/7 activity assay and ATP quantification assay to read the absorbance or chemiluminescence. Flow cytometric analysis was done using a BD FACS Calibur™ flow cytometer (Becton Dickinson, USA).

Stability in aqueous solution.

Ir1–**Ir6** was dissolved in a mixed solvent of d_6-DMSO/D_2O (7:3, v/v) and measured with a Bruker Avance 500 spectrometer (Germany) every 24 h to obtain a time-resolved ^1H NMR spectra.

Crystallographic structure determination.

Crystals of **Ir1**, **Ir4** and **Ir6** qualified for X-ray analysis were obtained by diffusion of diethyl ether to the CH_3CN solution. X-ray diffraction measurements were performed on a Bruker Smart1000 CCD diffractometer with Mo and Cu Kα radiation (λ = 0.71073 and 1.54178 Å) at 298K. The crystal structures of **Ir1**, **Ir4** and **Ir6** were solved through direct methods with program SHELXS and refined using the full-matrix least-squaresprogram SHELXL.

Optical properties

The UV-Vis absorption and emission spectra (λ_{ex} = 405 nm) of **Ir1**–**Ir6** in CH_2Cl_2, CH_3CN and PBS (10 μM) at 298 K were obtained on a Varian Cary 300 spectrophotometer (USA) and a FLS 920 combined fluorescence lifetime and steady state spectrometer (Japan), respectively.

The two-photon excited fluorescence (TPEF) spetra (DMSO) were acquired with a nanosecond pulsed laser. The two-photon induced fluorescence intensity was measured at 710–970 nm by using rhodamine 6G as the reference.[4] The intensities of TPEF of the reference and samples emitted at the same excitation wavelength were determined. The two-photon absorption (TPA) cross-section was calculated according to equation:

$$\delta_s = \delta_r \frac{\Phi_r c_r I_s n_s}{\Phi_s c_s I_r n_r}$$

Where I is the integrated fluorescence intensity, c is the concentration, n is the refractive index, Φ is the quantum yield, subscript 'r' stands for reference samples, and 's' stands for the samples.[5]

Viscosity response.

The emission spectra and phosphorescence lifetime of **Ir6** in the media of CH_3OH and Gly with varies percentage of Gly (0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, v/v) at 298K were obtained on a FLS 920 combined fluorescence lifetime and steady spectrometer (Japan). The viscosity of the tested media were subsequently measured with a Kinexus Pro+ rotational rheometer (UK). The resulting data were processed with Origin Pro v8.0 to obtain a linear regression model between the logarithm of compound's phosphorescence and solution viscosity.

Temperature response.

The phosphorescence lifetime of **Ir6** in CH_3OH and Gly (percentage of Gly: 70%, v/v) at different temperatures (278 K, 283 K, 288 K, 293 K, 298 K, 303 K, 308 K, 313 K, 318 K, 323 K) were obtained on a FLS 920 combined fluorescence lifetime and steady spectrometer (Japan). The resulting data was

processed with Origin Pro v8.0 to obtain a linear regression model between the logarithm of compound's phosphorescence and temperature.

The ^{1}H NMR spectra and ^{1}H–^{1}H NOESY spectra of **Ir6** in $CDCl_3$ at different temperatures were obtained on a Bruker Avance 400 spectrometer (Germany).

Polarity response

The phosphorescence lifetime of **Ir6** in mixed solvents containing water and 1,4-dioxane representing different polarities (percentage of 1,4-dioxane: 0%, 20%, 40%, 60%, 80%, 100%, v/v) or different solvents at 298K were obtained on an FLS 920 combined fluorescence lifetime and steady spectrometer (Japan). The resulting data were processed with Origin Pro v8.0.

***In vivo* ions and biomolecules response**

The phosphorescence lifetime of **Ir6** in aqueous solution with varies ions and biomolecules (c>1 M) at 298K were obtained on a FLS 920 combined fluorescence lifetime and steady spectrometer (Japan) after incubated for 1 week. The resulting data were processed with Origin Pro v8.0.

Cell lines and culture conditions.

A549 (human lung adenocarcinoma), HeLa (human cervical carcinoma), LO2 (human hepatic) and HepG2 (human hepatoblastoma) were obtained from Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). Cells were maintained in RPMI 1640 medium or DMEM containing 10% FBS (Gibco BRL), 100 μg/mL streptomycin (Gibco BRL), and 100 U/mL penicillin (Gibco BRL). The cells were cultured in a humidified incubator with an atmosphere of 5% CO_2 and 95% air at a constant temperature of 37°C. In each experiment, the reference group cells were treated with vehicle control (1% DMSO).

Cell viability assay

The cytotoxicity of **Ir1**–**Ir6** towards A549, HeLa and LO2 cell lines was determined by MTT assay. The compounds were dissolved in DMSO and diluted into gradient concentration with a final DMSO proportion of 1% (v/v). Cells cultured in 96-well plates were grown to confluence before incubated with **Ir1**–**Ir6** for 44 h. The media was then removed and replaced with fresh media. 20 μL of MTT solution (5 mg/mL) was then added to each well. The plates were incubated for an additional 4 h before the media was carefully removed, and DMSO was added (150 μL per well). The plate was shaken for 3 min. The absorbance at 595 nm was measured using a microplate reader (Infinite M200 Pro, Tecan, Switzerland).

Cellular uptake levels

A549 or HLF cells were seeded in 100 mm culture dishes (Corning) for 72 h. After incubated with **Ir1**–**Ir6** (20 μM) at 37 ºC for 1 h, cells were washed with PBS for three times and digested by trypsin. The mitochondrial and cytoplasmic fractions were isolated by Mitochondria Isolation Kit according to the manufacturer's instructions. The extractions were treated with 68% nitric acid and the concentration of iridium was determined by inductively coupled plasma mass spectrometry (ICP-MS) according to our previous description.[6]

Cellular localization.

A549 cells were seeded in 35 mm culture dishes (Corning) for 24 h. After incubated with **Ir1**–**Ir6** (20 μM) at 37 °C for 1 h, cells were washed with PBS for three times and visualized by confocal microscopy. λ_{ex}= 405 nm (**Ir1**–**Ir6**); λ_{em} = 460 ± 20 nm (**Ir1**–**Ir3**), 550 ± 20 nm (**Ir3**–**Ir6**).

For the colocalization experiments, A549 cells were seeded in 35 mm culture dishes (Corning) for 24 h. After incubated with **Ir6** (20 μM) at 37 °C for 1 h, MTDR (100 nM)/LTDR (200 nM) was added. After 15 min, cells were washed with PBS for three times and visualized by confocal microscopy. λ_{ex}= 405 nm (**Ir6**, One-Photon Microscopy (OPM)), 750 nm (**Ir6**, Two-Photon Microscopy (TPM)) or 633 nm (MTDR/LTDR); λ_{em} = 550 ± 20 nm (**Ir6**) or 665 ± 20 nm (MTDR/LTDR).

Hoechst 33342 staining

A549 cells were seeded in 35 mm culture dishes (Corning) for 48 h. After incubated with **Ir3** or **Ir6** at 37°C for 24 h, cells were treated with paraformaldehyde for 10 min and washed with PBS for three times. Then the fixed cells were further incubated with Hoechst 33342 for 30 min before visualized by confocal microscopy. λ_{ex}= 405 nm ; λ_{em} = 460 ± 20 nm.

Transmission electron microscopy (TEM) experiments.

A549 cells were seeded in 100 mm culture dishes (Corning) for 48 h. After incubated with **Ir6** at 37°C for 24 h, cells were washed with PBS for three times and digested by trypsin. The cells were collected by centrifugation and further pretreated for TEM (JEM 100 CX, JEOL, Tokyo, Japan). Images were captured using the Eversmart Jazz program (Scitex).

Annexin V staining assay

The assay was performed according to the manufacturer's (Sigma Aldrich, USA) protocol.

For flow cytometry analysis, A549 cells were seeded in 6-well plates (Corning) for 48 h. After incubated with **Ir6** at 37 °C for 24 h, cells were washed with PBS for three times and digested by trypsin. The suspension cells were further incubated with 500 μL annexin-binding buffer supplemented with 5 μL Annexin V in the dark before analyzed by flow cytometry. λ_{ex}= 488 nm ; λ_{em} = 527 nm (Annexin V), 590 nm (propidium iodide, PI).

For confocal microscopy analysis, A549 cells were seeded in 35 mm culture dishes (Corning) for 48 h. After incubated with **Ir6** at 37 °C for 24 h, cells were washed with ice-cold PBS for three times and further incubated with 500 μL annexin-binding buffer supplemented with 5 μL Annexin V and 10 μL PI for 15 min in the dark before visualized immediately by confocal microscopy. λ_{ex}= 488 nm ; λ_{em} = 520 ± 20 nm (Annexin V), 610 ± 20 nm (PI).

Caspase-3/7 activity assay

The assay was performed using Caspase-Glo® Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

A549 cells were seeded in 96-well plates (Corning) for 24 h. After the cells were incubated with **Ir6** at different concentrations at 37 °C for 6 h, 100 μL of Caspase-Glo® 3/7 reagent was added to each well containing 100 μL medium. The cells were further incubated at room temperature for 1 h. The luminesence was measured using a microplate reader (Infinite M200 Pro, Tecan, Switzerland).

Mitochondrial membrane potential (MMP) assay.

The assay was performed according to the manufacturer's (Beyotime, China) protocol. A549 cells were seeded in 6-well plates (Corning) for 48 h. After incubated with **Ir6** at 37 °C for 6 h, cells were washed with PBS and digested with trypsin. Each well of the suspension cells were further incubated with 500 μL of JC-1 working solution (1X) at 37 °C for 20 min before washed twice and resuspended with ice-cold JC-1 buffer solution. The stained suspension cells were analyzed by flow cytometry. Red and green mean fluorescence intensities were analyzed using FlowJo Software (Tree Star, OR, USA). λ_{ex}= 488 nm ; λ_{em} = 527 nm (Green, JC-1 monomers) or 590 nm (Red, JC-1 Monomers).

ATP production assay

The assay was performed using CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

A549 cells were seeded in 96-well plates (Corning) for 24 h. The cells were incubated with **Ir6** at different concentrations at 37 °C for 6 h and washed with PBS before 100 μL of CellTiter-Glo reagent was added into each well. The cells were further incubated in room temperature for 10 min. The luminesence was measured using a microplate reader (Infinite M200 Pro, Tecan, Switzerland).

Mitochondrial bioenergetics analysis.

The key parameters of mitochondrial function were obtained with the XF Cell Mito Stress Test Kit on a Seahorse XFe24 analyzer (Seahorse Bioscience, Billerica, USA) by measuring the oxygen consumption rate (OCR) according to the manufacturer's instructions. A series of preliminary experiments were performed to determine the optimal A549 cell and the proper concentrations of oligomycin (Oligo) (0.75 μM), trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP) (1.0 μM) and a mixture of rotenone/antimycin A (R/A, 0.5 μM each).

A549 cells were seeded in Seahorse 24-well XF Cell Culture Microplates at a density of 3×10^4 cells per well (0.275 cm^2) and incubated for 18 h before treated with **Ir6** at the indicated concentrations for 3 h at 37 °C under a 5% CO2 atmosphere. The cell culture growth medium was removed and replaced with assay medium before the plates were placed into a 37 °C non-CO_2 incubator to equilibrate for 1 h. During the equilibration, the test compounds diluted to optimal concentration were loaded into the XF sensor cartridges. After baseline measurement, these compounds targeting components of the electron transport chain (ETC) were injected consecutively to reveal the key metabolic parameters of mitochondria in **Ir6**-treated A549 cells.

Reactive oxygen species (ROS) generation assay

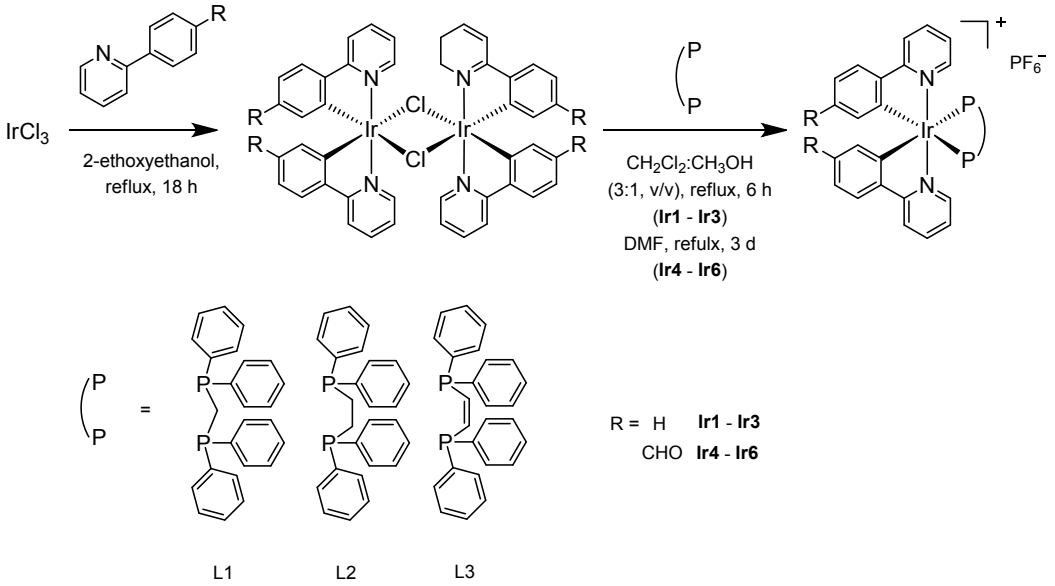
For flow cytometry analysis, A549 cells were seeded in 6-well plates (Corning) for 48 h. After incubated with **Ir6** at 37 °C for 6 h, cells were washed with PBS and digested by trypsin. The suspension cells were further incubated with DCFH-DA (10 μM) for 15 min in the dark and washed twice with PBS before analyzed by flow cytometry. The resulting histograms were analyzed by FlowJo Software. λ_{ex}= 488 nm ; λ_{em} = 527 nm.

For confocal microscopy analysis, A549 cells were seeded in 35 mm culture dishes (Corning) for 48 h. After incubated with **Ir6** at 37 °C for 6 h, cells were washed with PBS and further incubated with DCFH-DA (10 μM) for 15 min in the dark. After washed twice with PBS, cells were visualized immediately by confocal microscopy. λ_{ex}= 488 nm ; λ_{em} = 550 ± 20 nm.

***In vivo* imaging of zebrafish larvae.**

Zebrafish Larvae were bred in zebrafish embryo culture media for 48 h after hatch. The healthy larvae were selected and incubated with **Ir6** (20 μM) for 24 h before visualized by confocal microscopy. λ_{ex}= 405 nm (OPM), 750 nm (TPM); λ_{em} = 550 ± 20 nm.

Supporting Scheme, Figures and Tables



Scheme S1. Synthetic protocol and chemical structures of **Ir1**–**Ir6**.

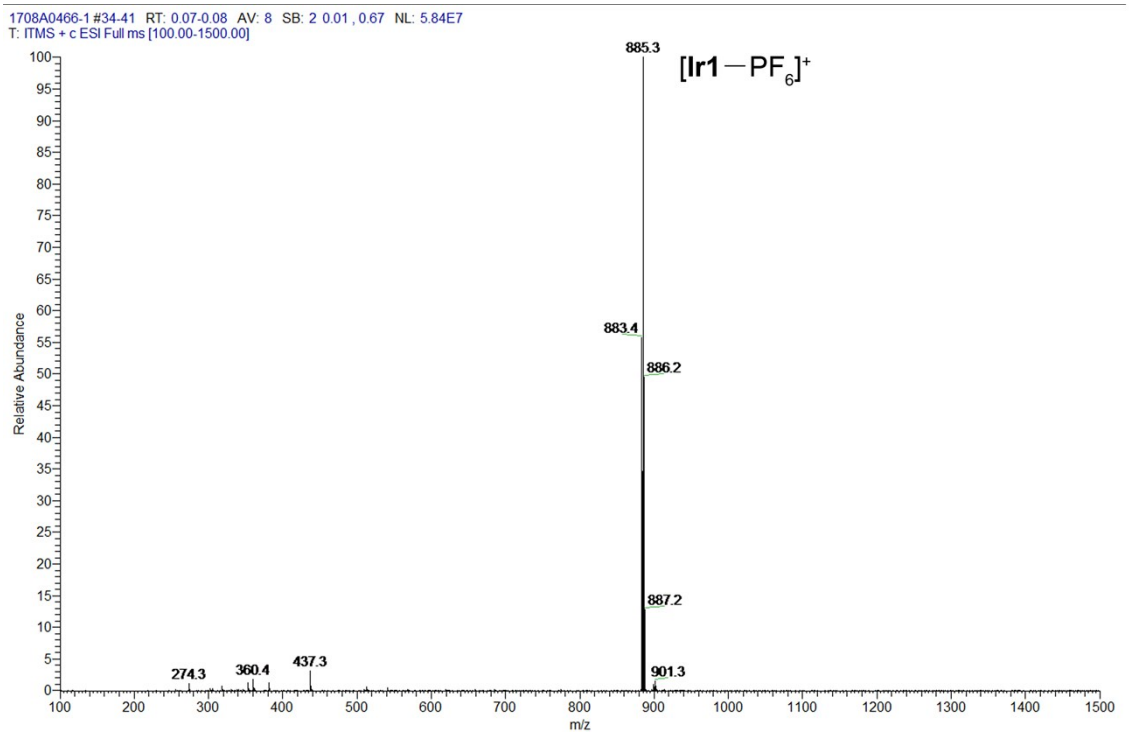


Fig. S1 ESI-MS spectrum of **Ir1** in CH_3OH.

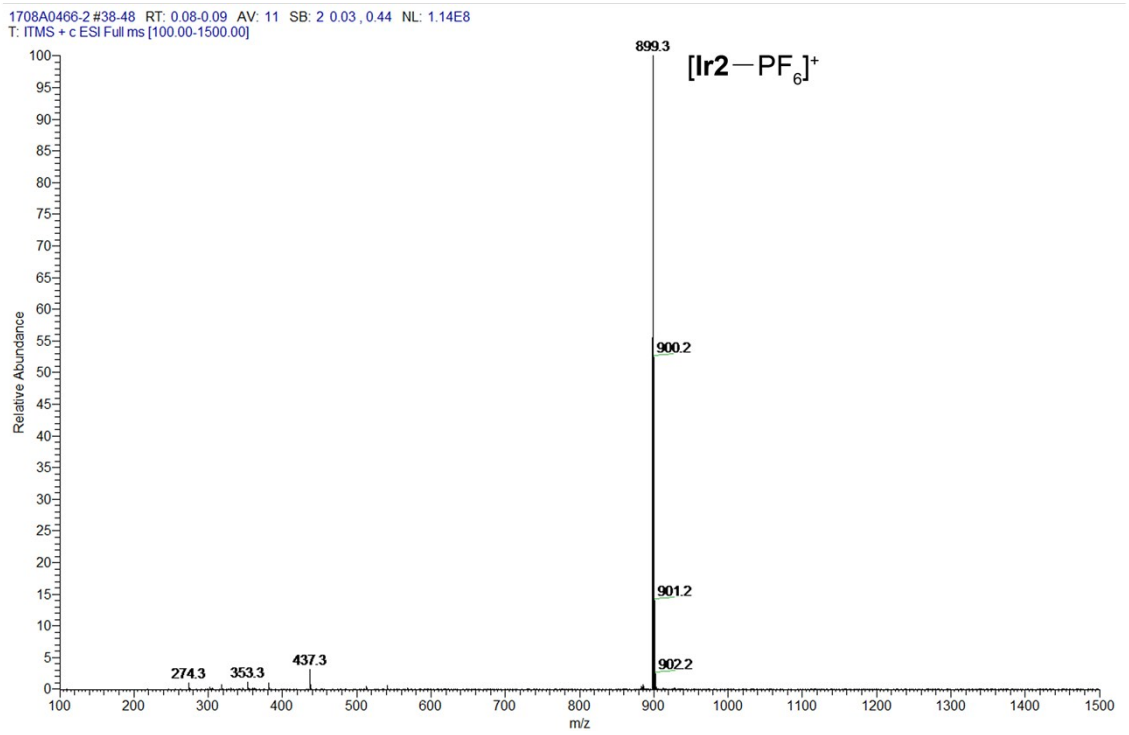


Fig. S2 ESI-MS spectrum of **Ir2** in CH_3OH.

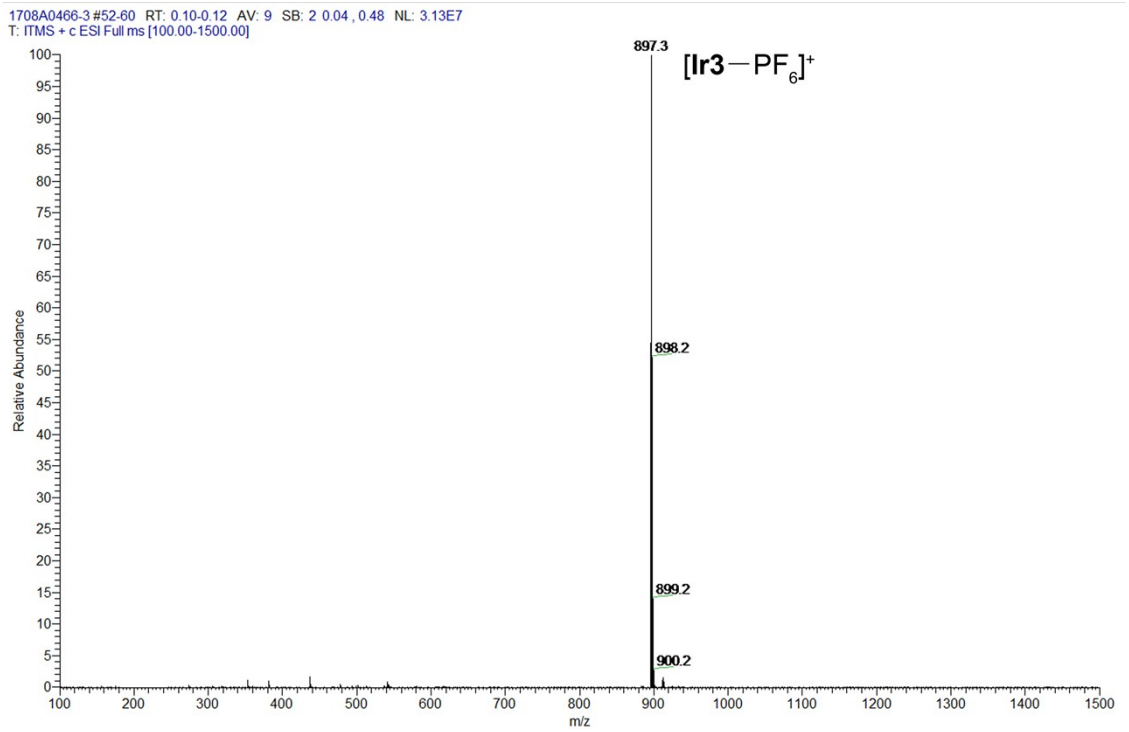


Fig. S3 ESI-MS spectrum of **Ir3** in CH_3OH.

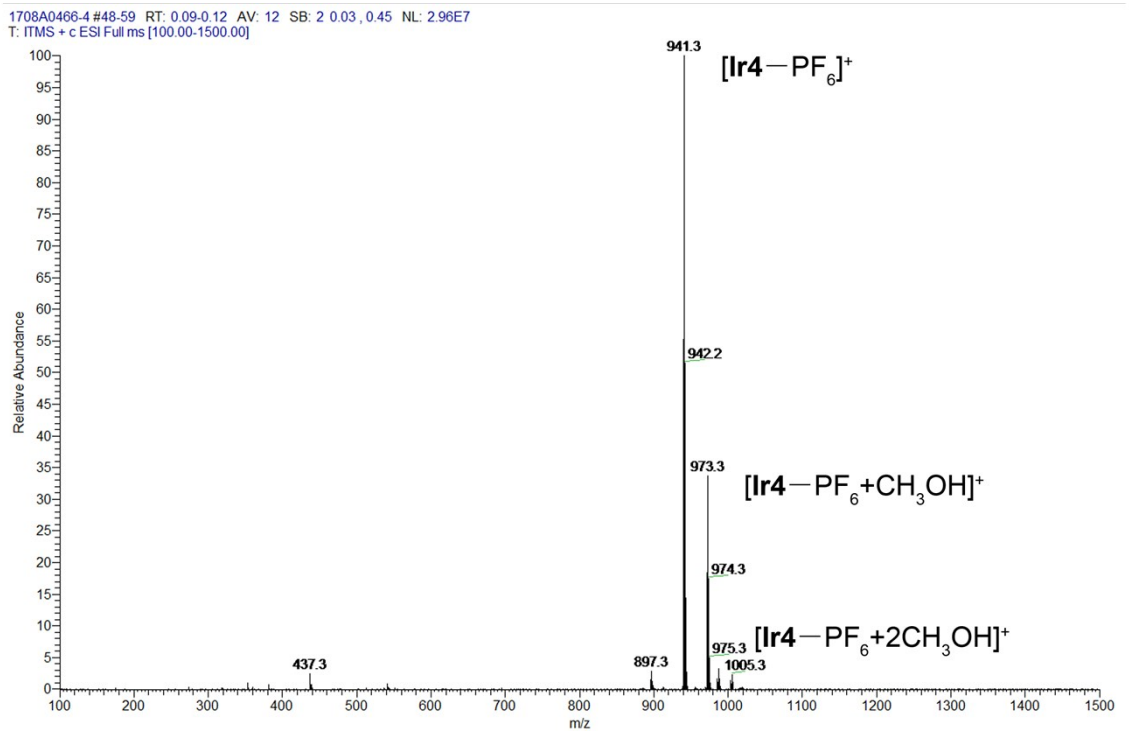


Fig. S4 ESI-MS spectrum of **Ir4** in CH_3OH.

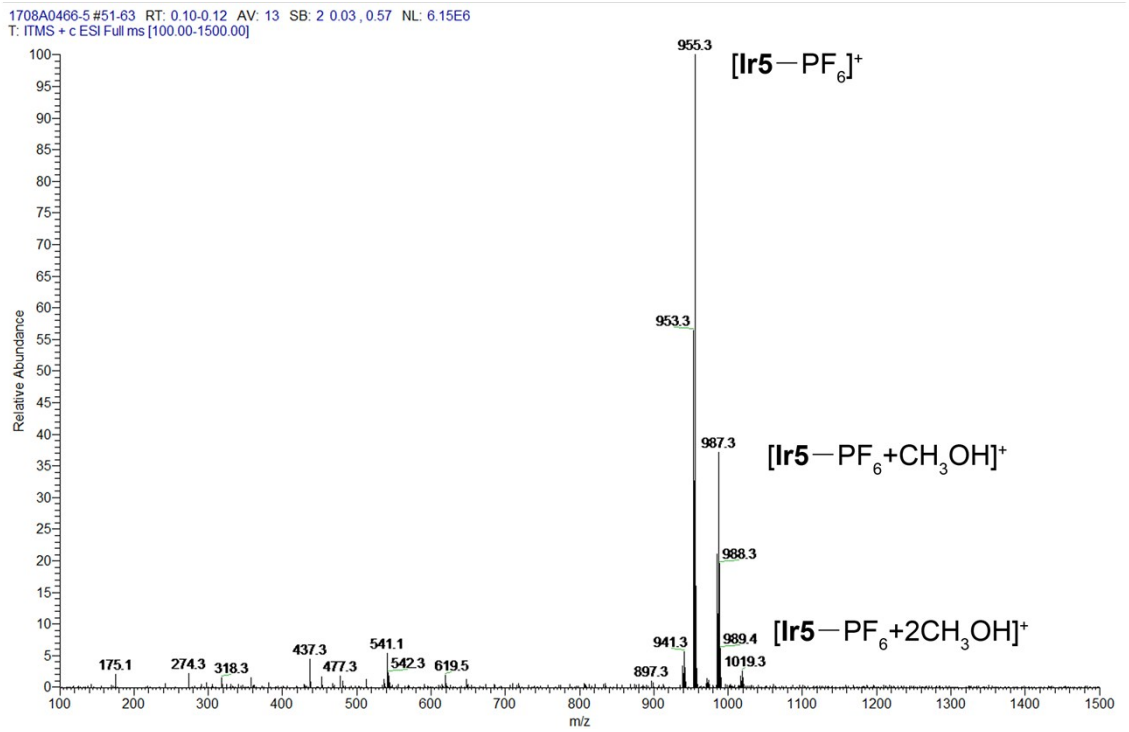


Fig. S5 ESI-MS spectrum of **Ir5** in CH_3OH.

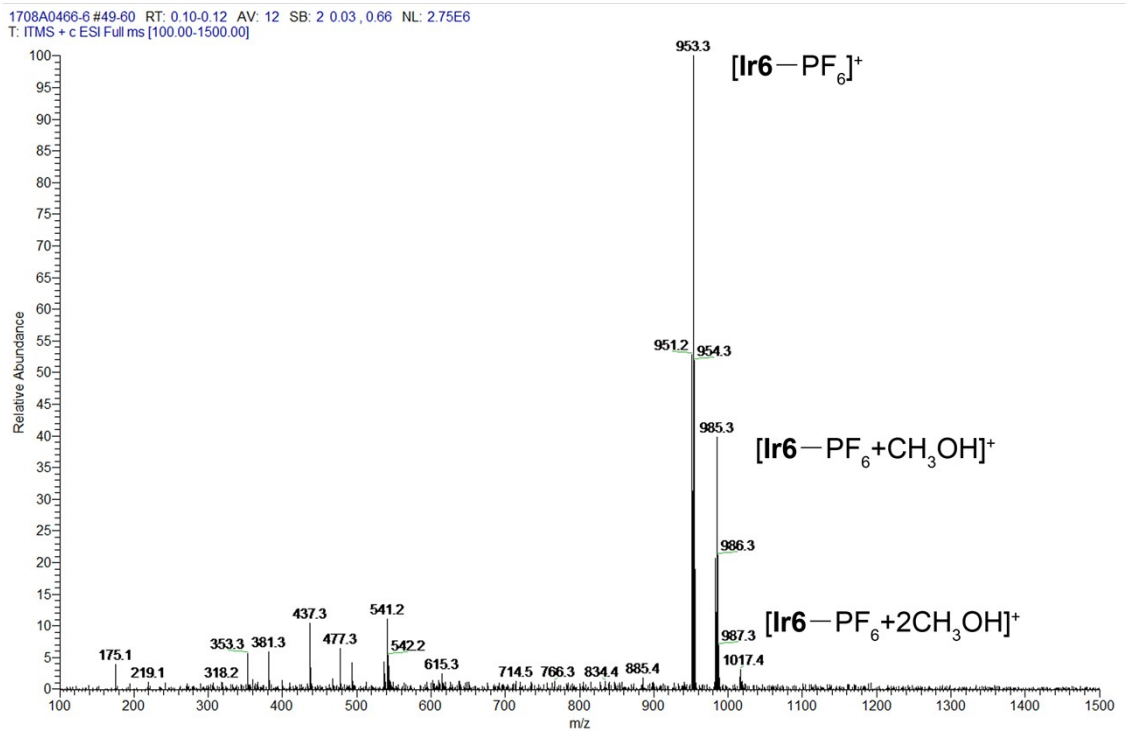


Fig. S6 ESI-MS spectrum of **Ir6** in CH_3OH.

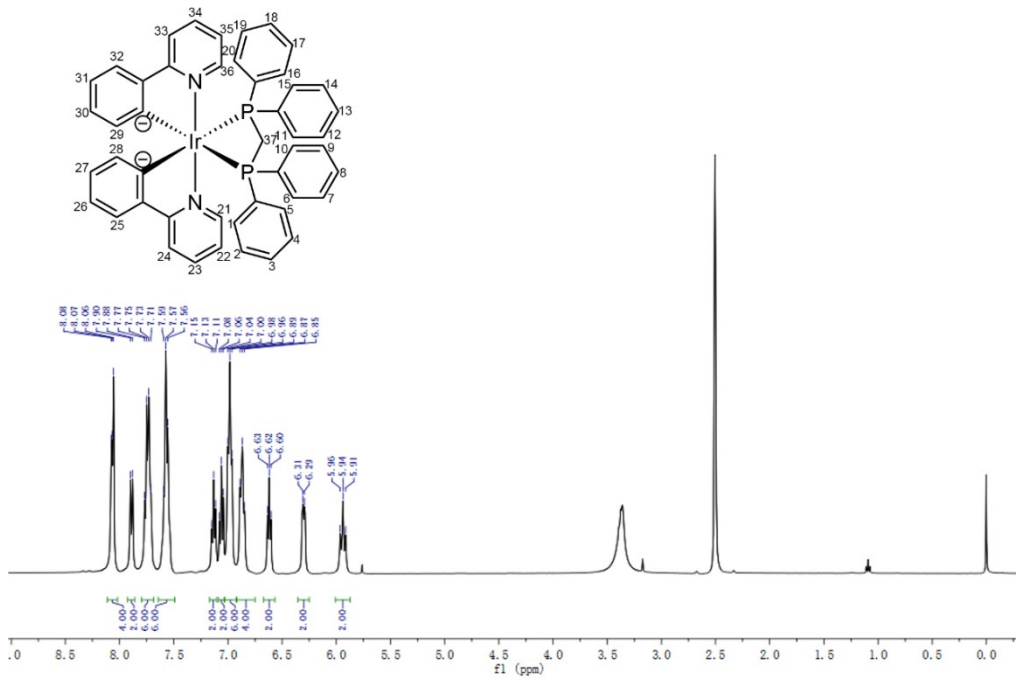


Fig. S7 ^1H NMR spectrum of **Ir1** in d_6-DMSO.

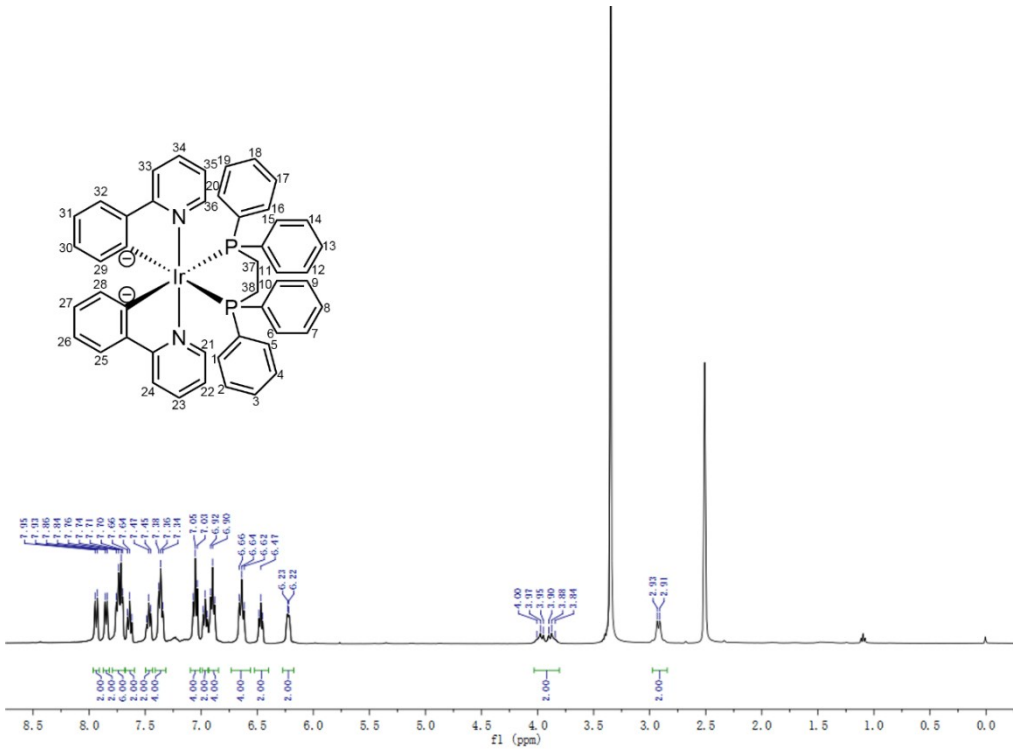


Fig. S8 ^{1}H NMR spectrum of **Ir2** in d_6-DMSO.

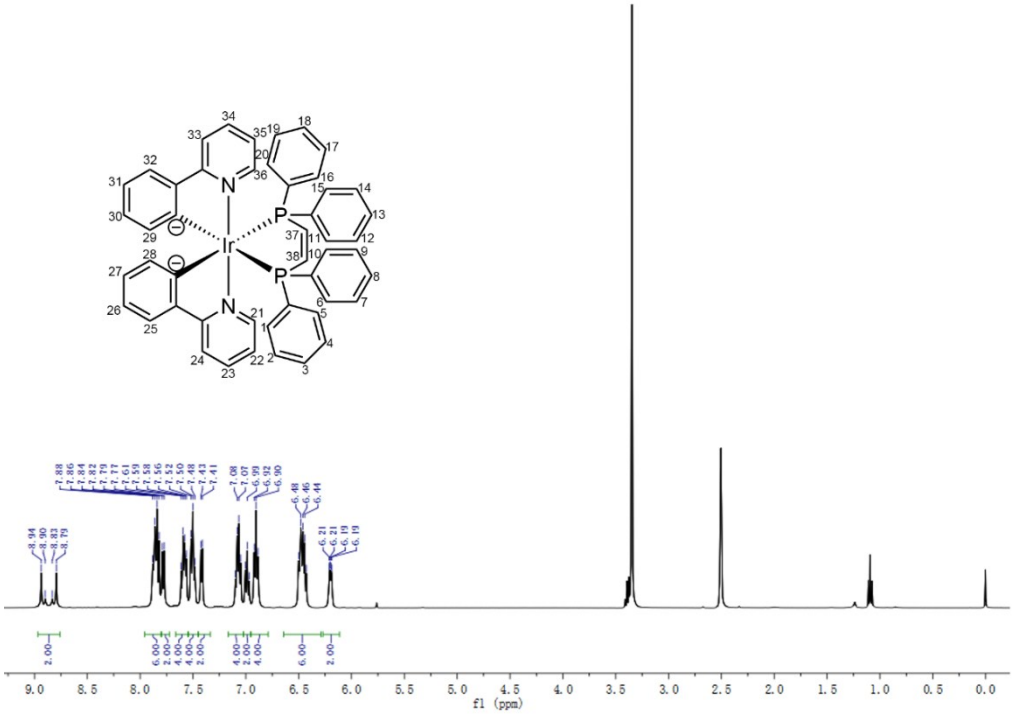


Fig. S9 ^{1}H NMR spectrum of **Ir3** in d_6-DMSO.

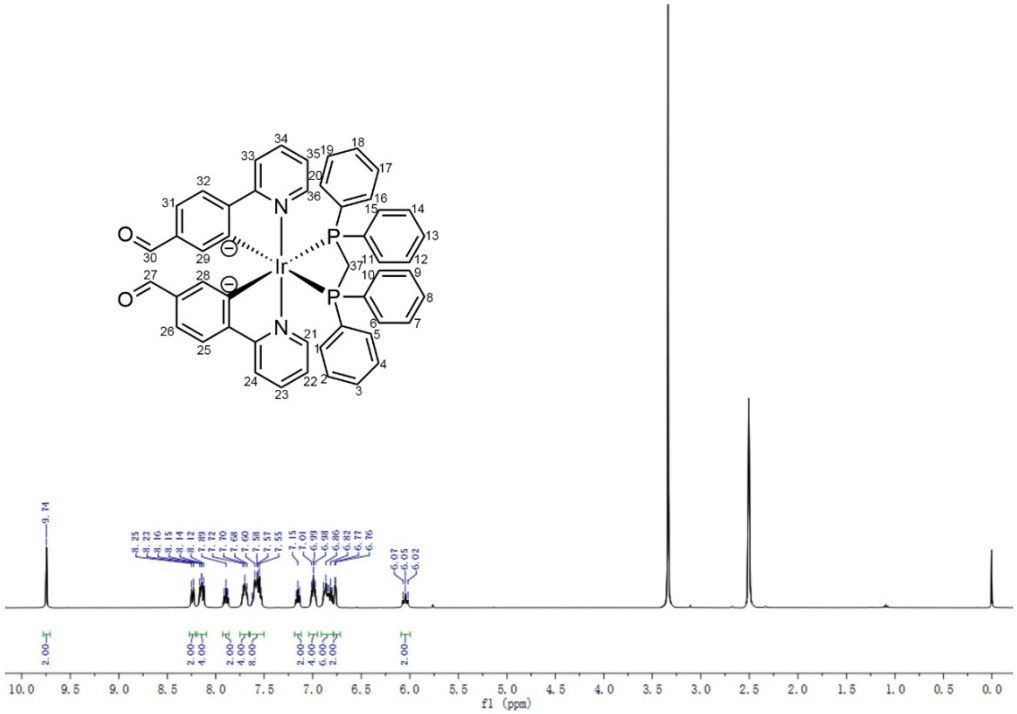


Fig. S10 ^{1}H NMR spectrum of **Ir4** in d_6-DMSO.

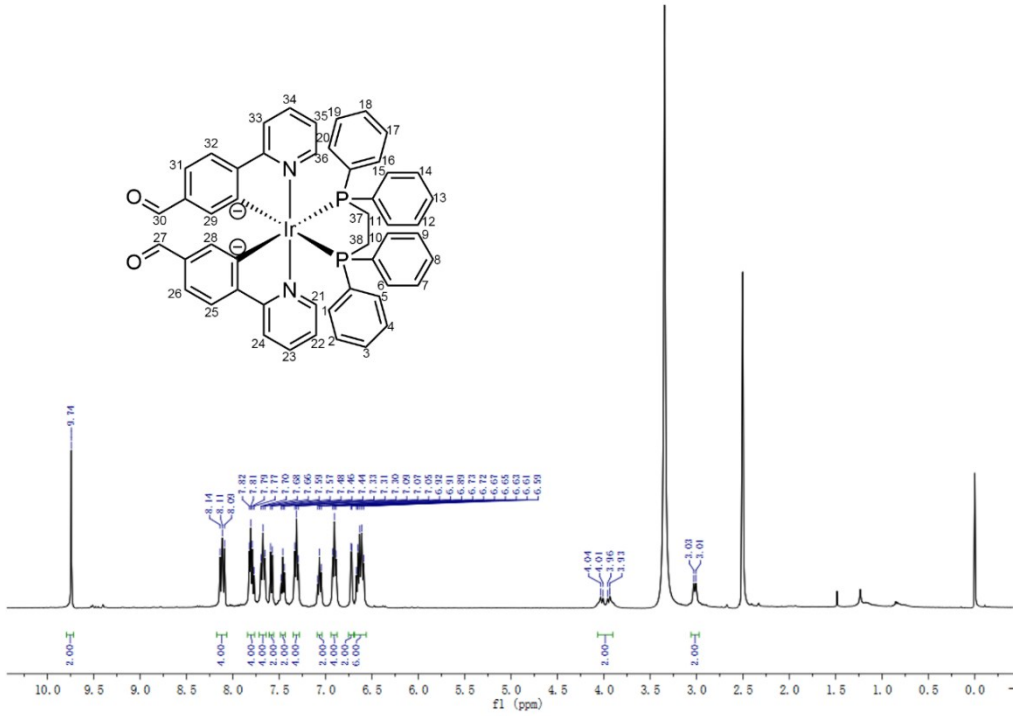


Fig. S11 ^{1}H NMR spectrum of **Ir5** in d_6-DMSO.

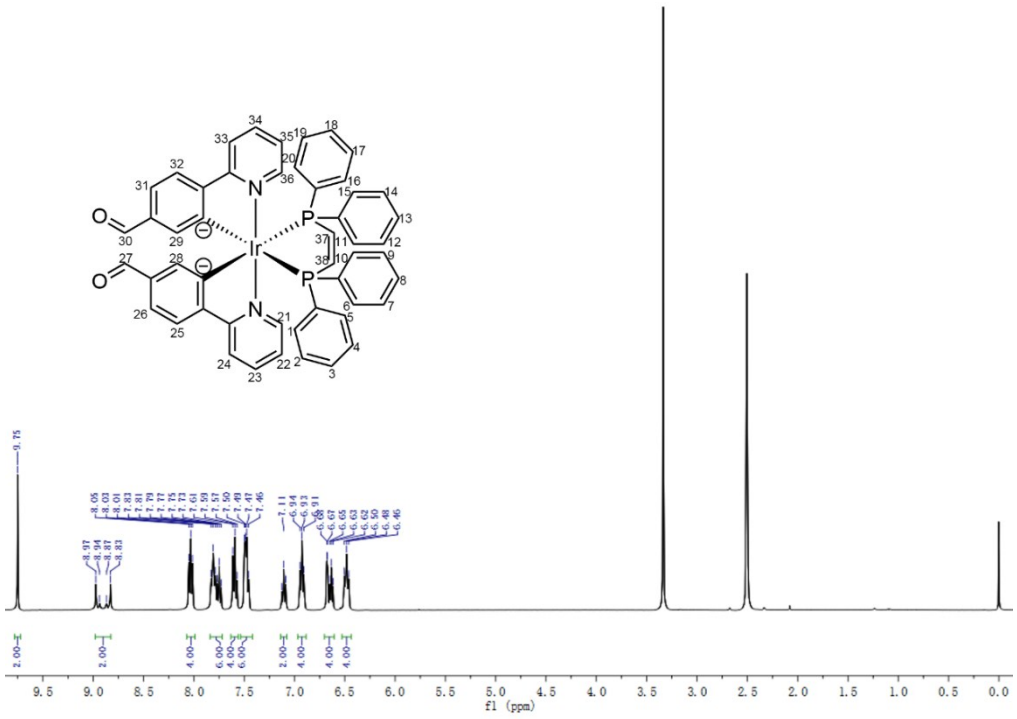


Fig. S12 ^{1}H NMR spectrum of **Ir6** in d_6-DMSO.

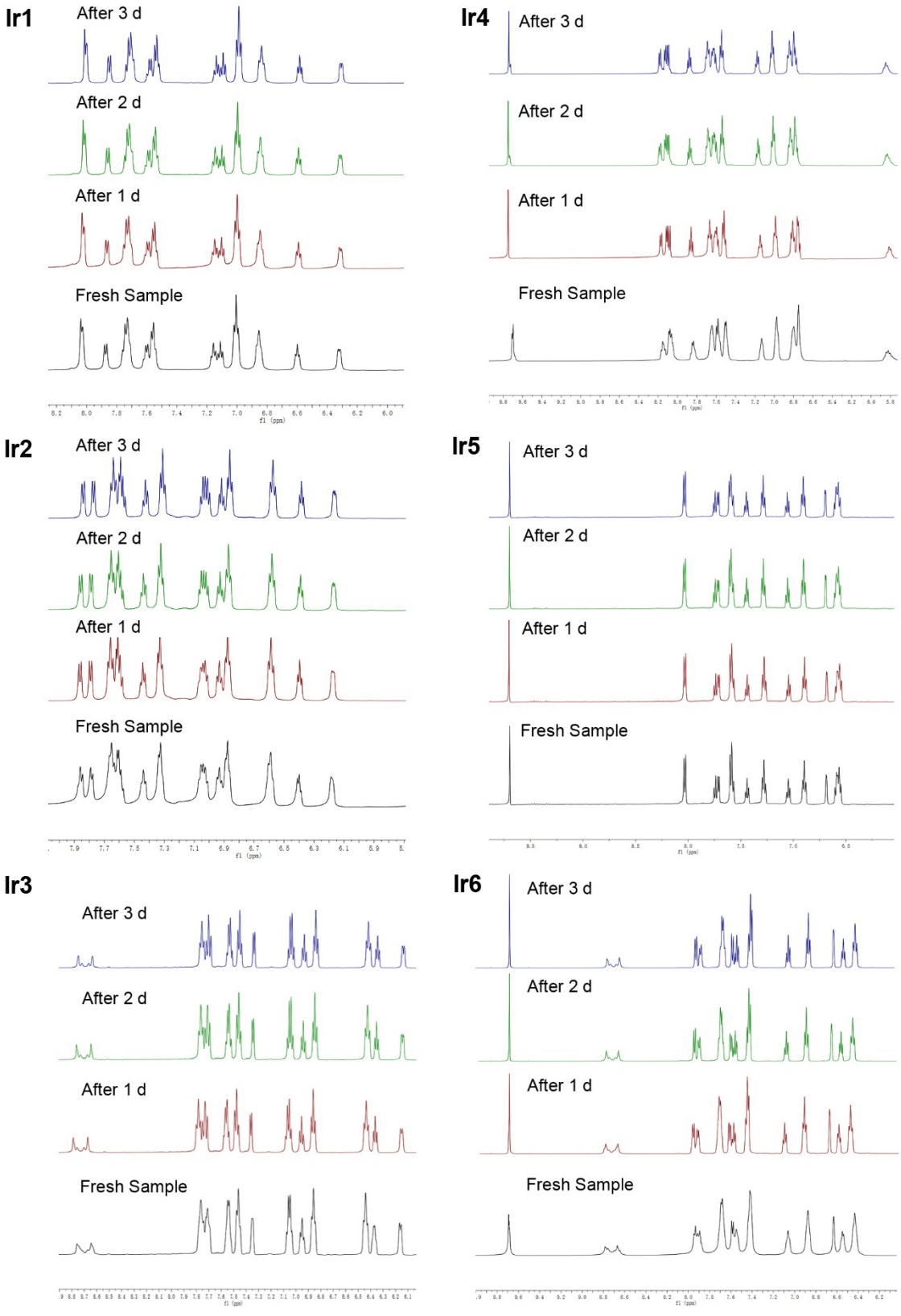


Fig. S13 Time-resolved ^{1}H NMR spectra of **Ir1**–**Ir6** in d_6-DMSO /D_2O (7:3, v/v).

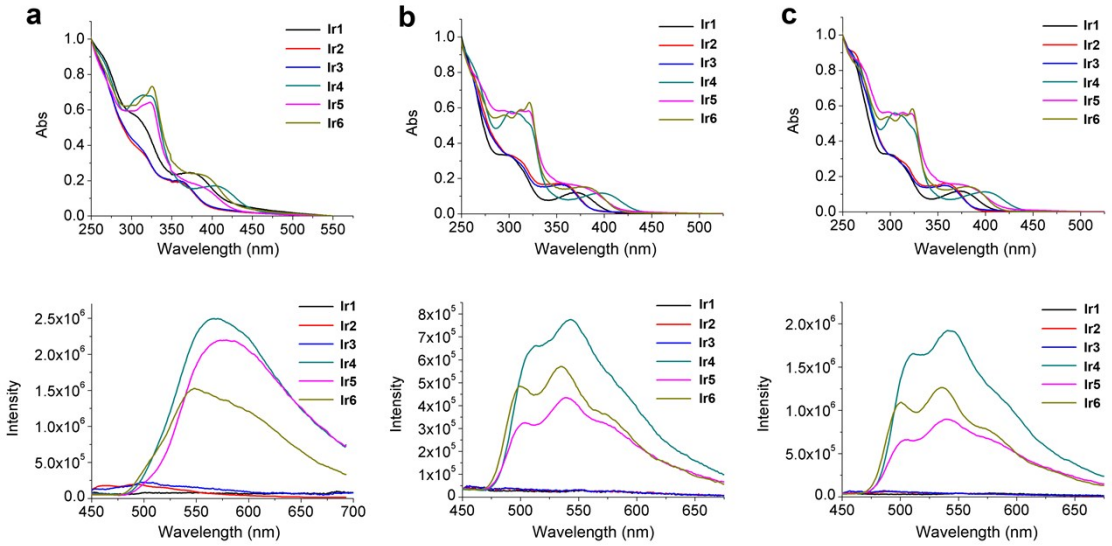


Fig. S14 UV-Vis and emission spectra of **Ir1**–**Ir6** (20 μM) in PBS (a), CH_3CN (b) and CH_2Cl_2 (c) at 298 K. **Ir1**–**Ir3** were excited at 365 nm, and **Ir4**–**Ir6** were excited at 405 nm.

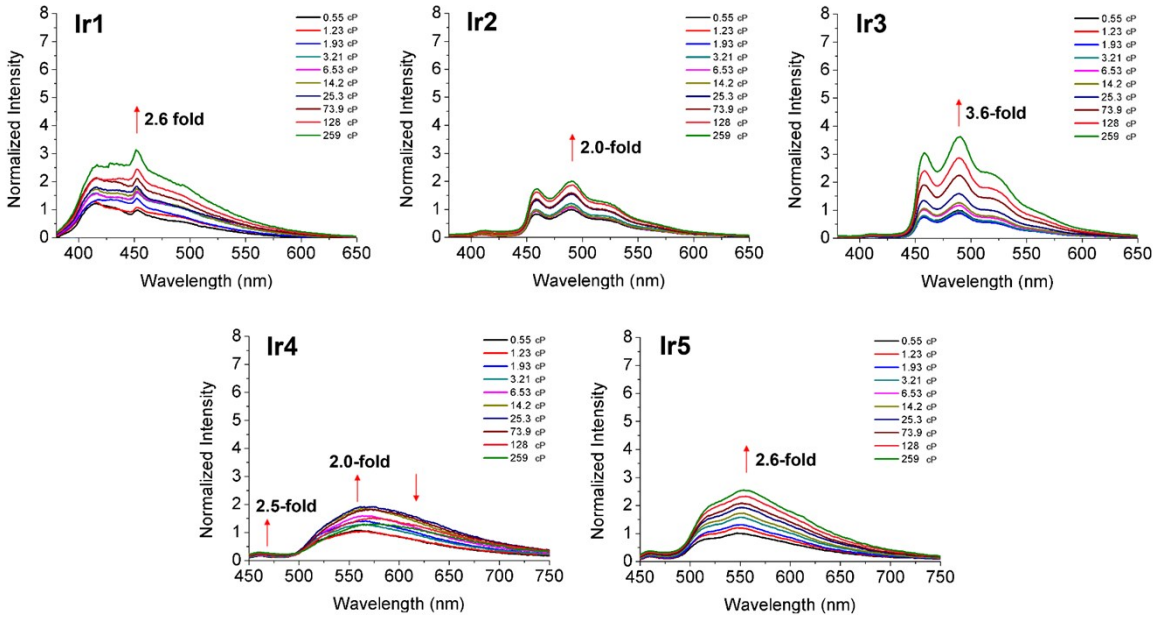


Fig. S15 Emission spectra of **Ir1**–**Ir5** (20 μM) in mixed solvents containing CH_3OH and Gly. Percentage of Gly = 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% and 90%. **Ir1**–**Ir3** were excited at 365 nm, and **Ir4** and **Ir5** were excited at 405 nm.

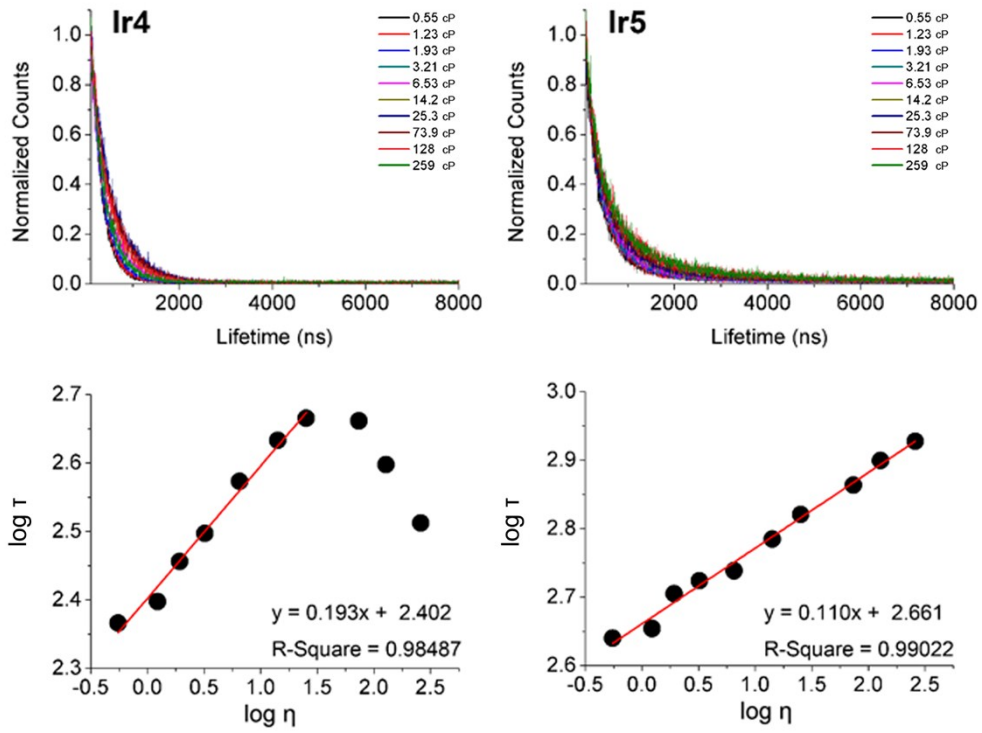


Fig. S16 Lifetime spectra and linear fit between lifetime and viscosity of **Ir4** and **Ir5** (20 μM) in mixed solvents containing CH_3OH and Gly. Percentage of Gly = 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% and 90%. **Ir4** and **Ir5** were excited at 405 nm.

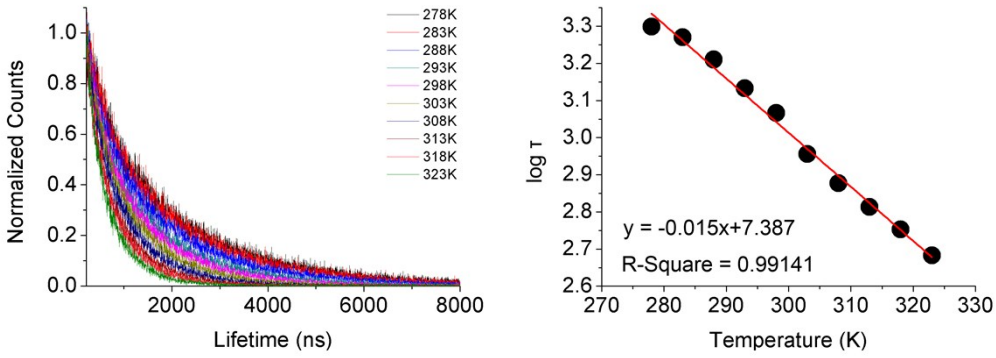


Fig. S17 Lifetime spectra and linear fit between lifetime and temperature measured in CH_3OH and Gly (percentage of Gly = 50%). **Ir6** (20 μM) was excited at 405 nm.

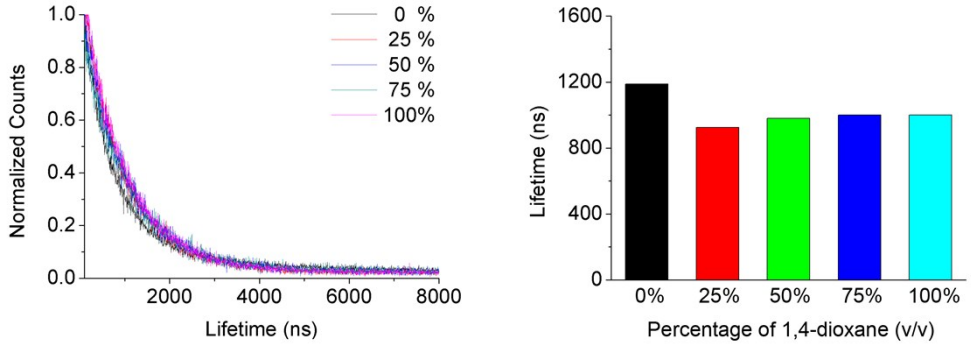


Fig. S18 Phosphorescence lifetime spectra of **Ir6** (20 μM) in mixed solvents containing water and 1,4-dioxane representing different polarities (Percentage of 1,4-dioxane: 0%, 20%, 40%, 60%, 80% and 100%). **Ir6** (20 μM) was excited at 405 nm.

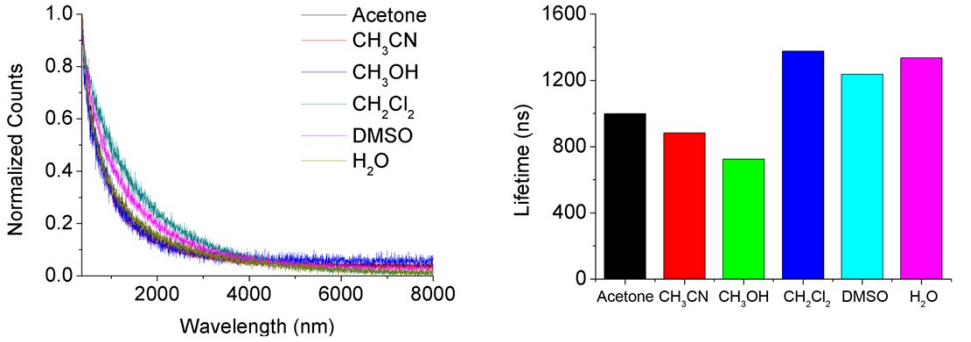


Fig. S19 Phosphorescence lifetime spectra of **Ir6** (20 μM) in different solvents. **Ir6** (20 μM) was excited at 405 nm.

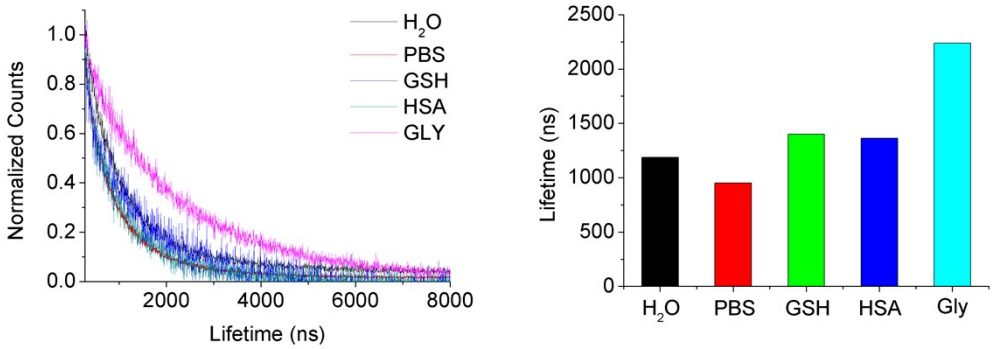


Fig. S20 Phosphorescence lifetime spectra of **Ir6** (20 μM) in different solutions: PBS (pH 7.4), GSH (200 μM) in H_2O, HSA (200 μM) in H_2O and Gly (90%, v/v) with CH_3OH. **Ir6** (20 μM) was excited at 405 nm.

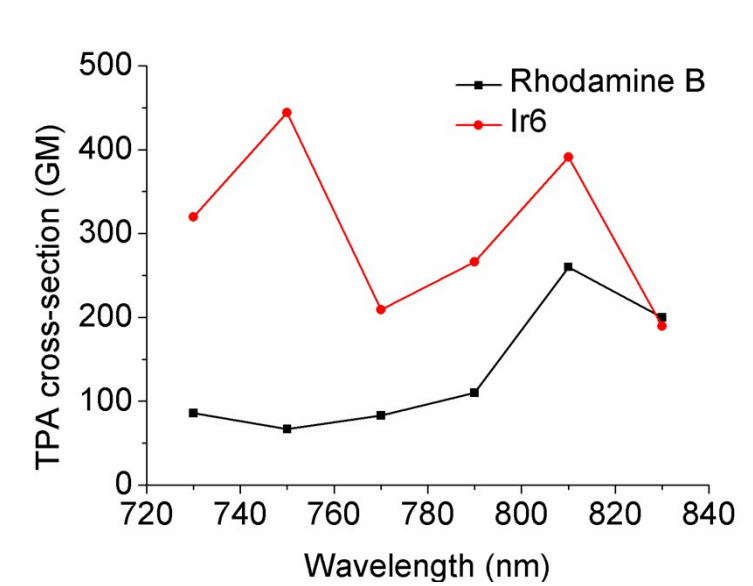


Fig. S21 Two-photon action (Φ_δ) spectra of **Ir6** (100 μM) and Rhodamine B (10 μM).

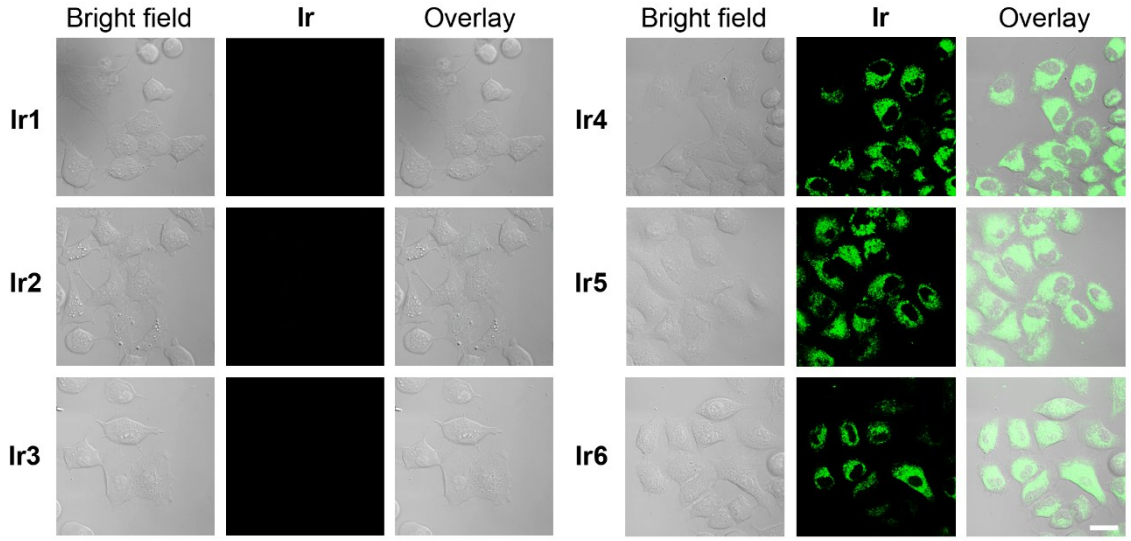


Fig. S22 Confocal microscopic images of A549 cells incubated with **Ir1**–**Ir6**. A549 cells were incubated with **Ir1**–**Ir6** (20 μM) for 1 h. λ_{ex}= 405 nm (**Ir1**–**Ir6**); λ_{em} = 460 ± 20 nm (**Ir1**–**Ir3)** or 550 ± 20 nm (**Ir3**–**Ir6**). **Overlay**: Cells shown were representative images from replicate experiments (n = 3). Scale bar: 20 μm.

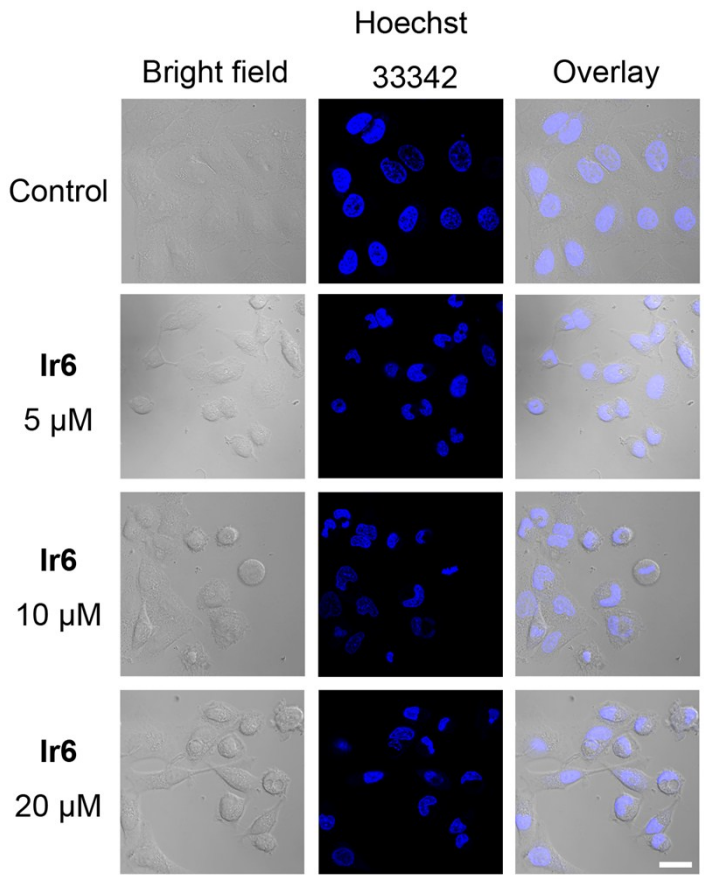


Fig. S23 Confocal microscopic images of A549 cells incubated with **Ir6** (5, 10, 20 μM, 24 h) and stained with Hoechst 33342 (5 μg·mL^{-1}, 30 min). λ_{ex} = 405 nm; λ_{em} = 460 ± 20 nm. Cells shown were representative images from replicate experiments (n = 3). Scale bar: 20 μm.

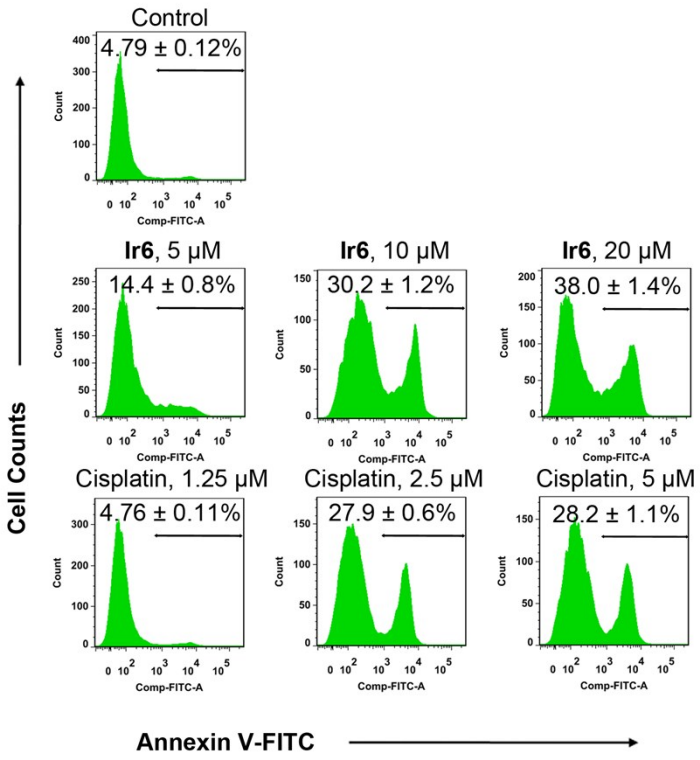


Fig. S24 Flow cytometric analysis of A549 cells incubated with **Ir6** at the indicated concentrations for 24 h. The cells were then stained with Annexin V for 30 min.

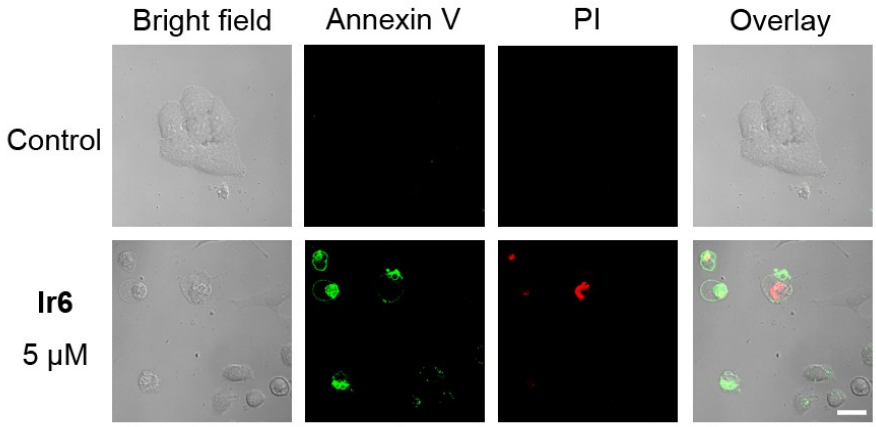


Fig. S25 Confocal microscopic images of A549 cells incubated with **Ir6** (5 μM, 24 h). The cells were stained with Annexin V (5 μL, 30 min) and PI (10 μL, 30 min). Annexin V: λ_{ex} = 488 nm; λ_{em} = 520 ± 20 nm. PI: λ_{ex} = 488 nm; λ_{em} =610 ± 20 nm. Cells shown were representative images from replicate experiments (n = 3). Scale bar: 20 μm.

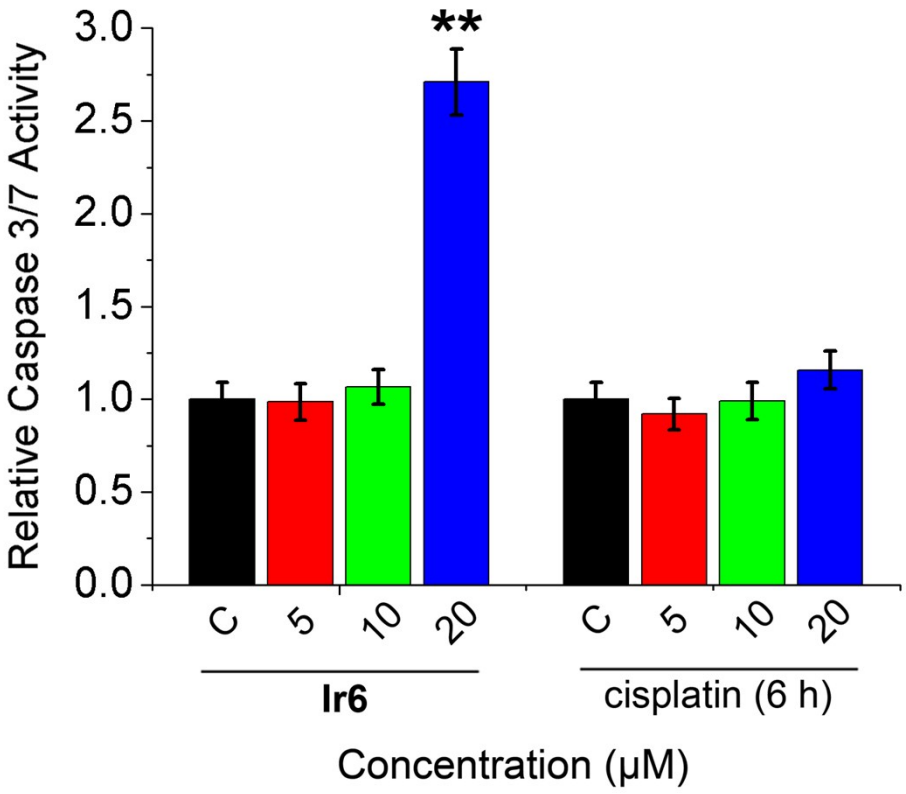


Fig. S26 Detection of caspase-3/7 activity in A549 cells treated with **Ir6** at the indicated concentrations for 6 h. Cisplatin was included as control in A549 cells. Data were represented as means ± SD of three independent experiments. *p< 0.05, **p< 0.01, as compared with control group.

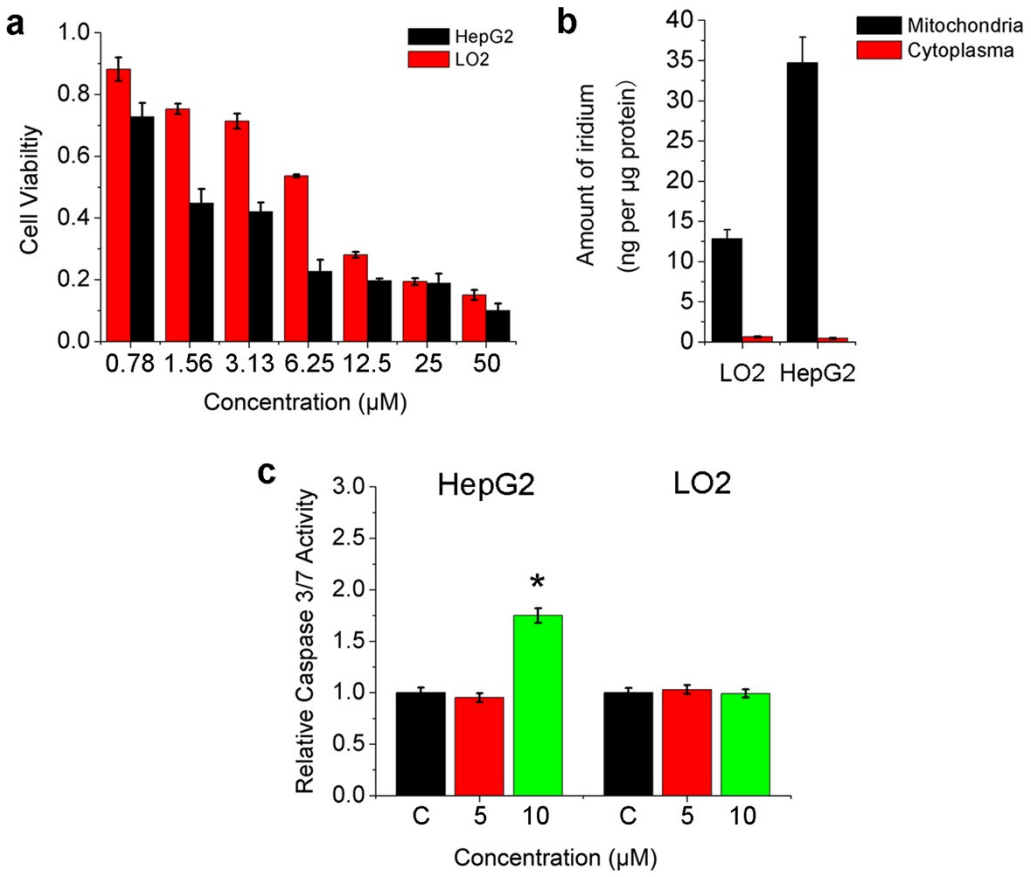


Fig. S27 (a) Cell viability of HepG2 and LO2 cells treated with gradient concentration of **Ir6**. (b) Detection of iridium in the extracts of HepG2 and LO2 cells treated with **Ir6** (20 μM, 1 h). (c) Detection of caspase-3/7 activity in HepG2 and LO2 cells treated with **Ir6** at the indicated concentrations for 6 h. Data were represented as means ± SD of three independent experiments. **p*< 0.05, ***p*< 0.01, as compared with control group.

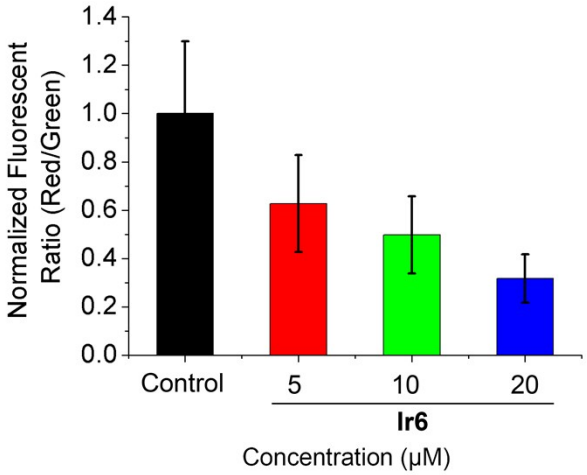


Fig. S28 Detection of MMP by flow cytometry. A549 cells were treated with **Ir6** at indicated concentrations for 4 h and stained with JC-1. λ_{ex}= 488 nm ; λ_{em} = 527 nm (Green), 590 nm (Red).

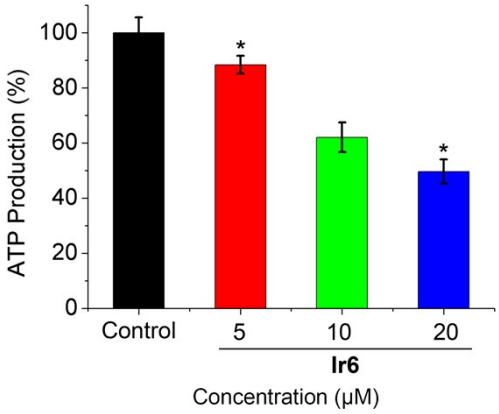


Fig. S29 Impact of **Ir6** on the ATP levels in A549 cells. The cells were treated with **Ir6** at the indicated concentrations for 4 h. *p< 0.05, **p< 0.01, as compared with control group.

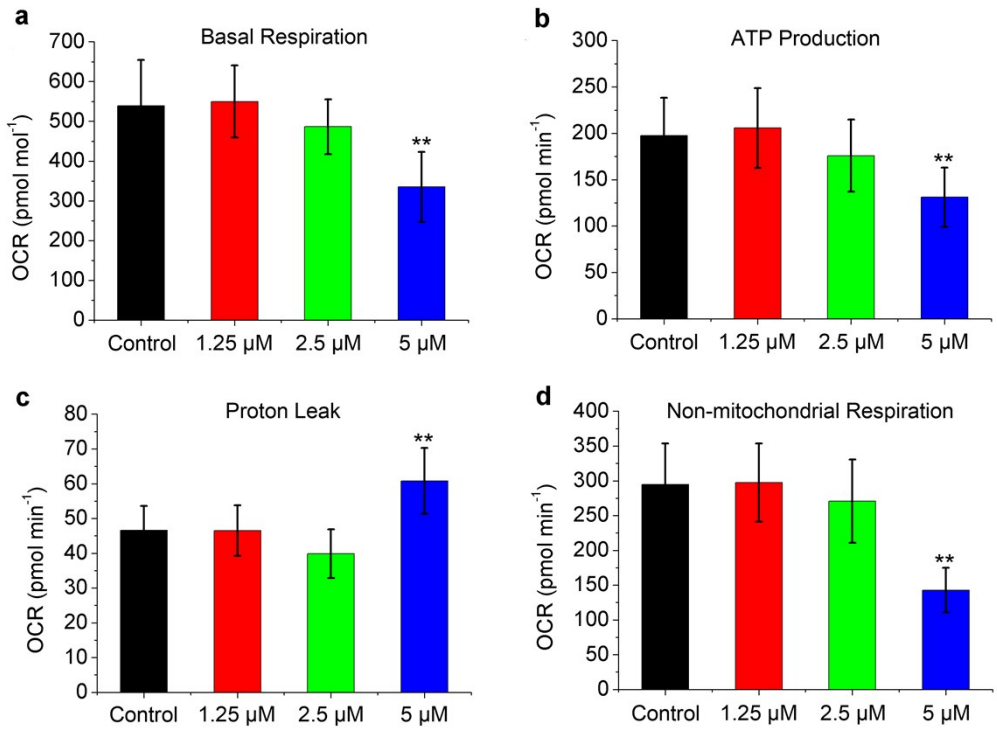


Fig. S30 (A) Basal respiration was calculated by subtracting OCR values after the addition of R/A from basal OCR. (B) ATP production was calculated by subtracting OCR values after the addition of Oligo from basal OCR. (C) Proton Leak was calculated by subtracting OCR values after the addition of R/A from OCR values obtained after adding FCCP. (D) Non-mitochondrial respiration was the OCR values after the addition of R/A. *p< 0.05, **p< 0.01, as compared with control group.

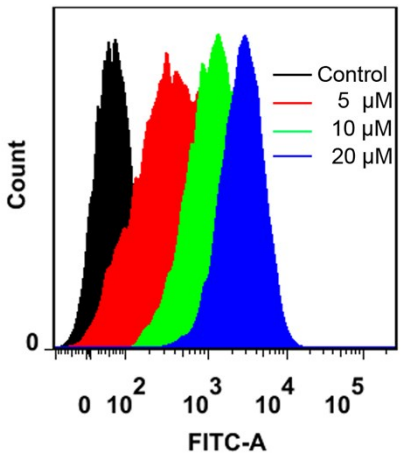


Fig. S31 Detection of ROS levels by flow cytometry after A549 cells were treated with **Ir6** at the indicated concentrations for 6 h and stained with DCFH-DA (10 μM, 30 min). λ_{ex}= 488 nm ; λ_{em} = 527 nm.

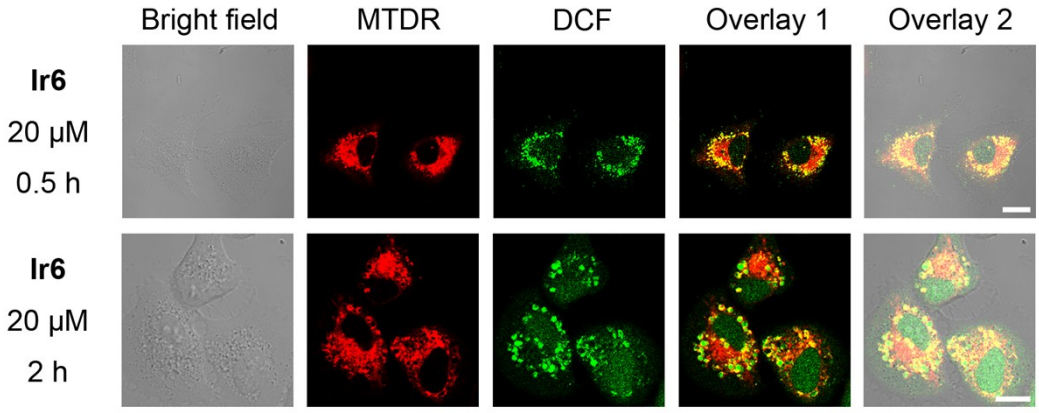


Fig. S32 Confocal microscopy image of ROS generation in A549 cells incubated with **Ir6** and co-stained DCFH-DA (10 μM, 30 min) and MTDR (100 nM, 30 min). 2',7'-dichlorofluorescein (DCF): λ_{ex}= 488 nm, λ_{em} = 550 ± 20 nm. MTDR: λ_{ex}= 633 nm; λ_{em} = 665 ± 20 nm. Overlay 1: Overlay of the 2nd and 3rd columns. Overlay 2: Overlay of the 1st, 2nd and 3rd columns. Scale bars: 10 μm.

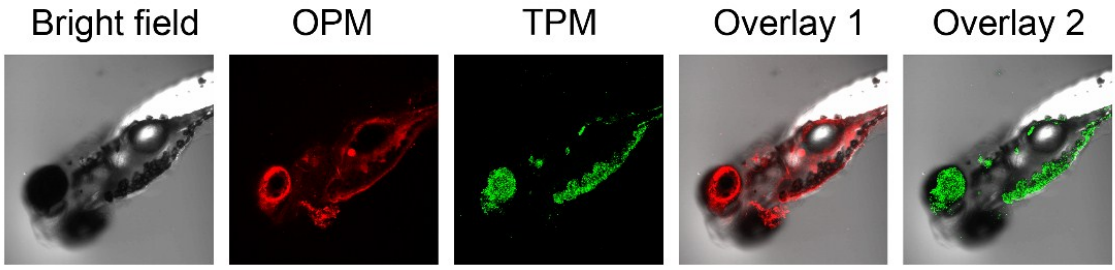


Fig. S33 *In vivo* confocal microscopy image of a zebrafish larva treated with **Ir6** (5 μM) for 24 h. λ_{ex} = 405 nm (OPM), 750 nm (TPM); λ_{em} = 550 ± 20 nm. **Overlay 1**: Overlay of the 1st and 2nd columns. **Overlay 2**: Overlay of the 1st and 3rd columns.

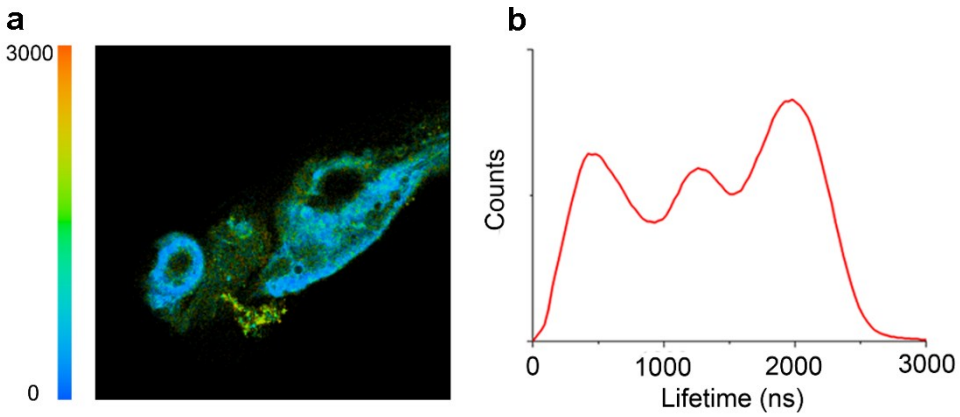


Fig. S34 (A) *In vivo* TPPLIM image of a zebrafish larva treated with **Ir6** (5 μM) for 24 h. (B) Histogram of phosphorescence lifetime of **Ir6** in the treated zebrafish larva. λ_{ex} = 405 nm (OPM) or 750 nm (TPM); λ_{em} = 550 ± 20 nm.

Table S1. Crystallographic data and structural refinements of **Ir1**, **Ir4** and **Ir6**.

Compound	**Ir1**	**Ir4**	**Ir6**
CCDC No.	1839168	1839169	1839170
Formula	$C_{47}H_{38}IrN_2P_3F_6$	$C_{49}H_{38}N_2O_2P_3F_6$	$C_{50}H_{38}N_2O_2P_3F_6$
Mr	1029.90	1085.92	1097.93
Temp. */ K*	279(2)	100	150.00(10)
Crystal system	Monoclinic	Monoclinic	Monoclinic
Space group	P21/c	P21	P21/c
a (Å)	16.9747(3)	10.5390(2)	17.4083(5)
b (Å)	12.0886(2)	19.2351(3)	13.0416(3)
c (Å)	21.1714(4)	10.9341(2)	20.5524(5)
α(°)	90	90	90
β (°)	103.859(2)	104.941(2)	108.427(3)
γ (°)	90	90	90
*V(*Å^3)	4217.90(13)	2141.61(7)	4426.79(18)
Z	4	2	4
Completeness to θ_{max}	0.998	0.997	0.998
ρcalcd. (g/cm^3)	1.622	1.684	1.647
μ (mm^{-1})	3.342	3.299	3.193
F (000)	2040	1076	2176
Reflns obsd [$I \geq 2\sigma(I)$]	28095	34605	30936
GOF on *F*2	1.048	1.024	1.112
*R*1 [$I \geq 2\sigma(I)$][a]	0.0286	0.0366	0.0351
*wR*2 (all data)[b]	0.0651	0.0821	0.0727

[a] $R_1 = \Sigma||F_0|-|F_c||/\Sigma|F_0|$. [b] $\omega R_2 = [\Sigma\omega(F_0^2 - F_c^2)^2/\Sigma\omega(F_0^2)^2]^{1/2}$

Table S2. Photophysical data of complexes **Ir4**–**Ir6**.

Compound	Solvent	λ_{em}[a]	Φ[b]	τ_0[c]
	PBS	567	0.032	1265
Ir4	CH_3CN	545	0.026	335
	CH_2Cl_2	548	0.028	710
	PBS	585	0.022	863
Ir5	CH_3CN	548	0.029	534
	CH_2Cl_2	546	0.036	823
	PBS	551	0.021	950
Ir6	CH_3CN	541	0.031	723
	CH_2Cl_2	542	0.036	1465
	PBS	626	0.042	362
$[Ru(bpy)_3]Cl_2$	CH_3CN	631	0.062	159
	CH_2Cl_2	618	0.059	280

[a] Maximum wavelengths of one-photon emission spectra (λ_{em}) in nm. [b] Quantum yields of phosphorescence at room temperature were determined using $[Ru(bpy)_3]Cl_2$ in PBS, CH_3CN and CH_2Cl_2[1-3]. [c] The lifetimes were measured at the emission maxima in ns.

Table S3. TPA cross-section data of **Ir6**.

Compound	Φ_σ[a]/GM					
	730	750	770	790	810	830
Ir6	319	444	209	266	391	189
Rhodamine B	86	67	83	110	260	200

[a] TPA cross-section in DMSO.

Table S4. Cytotoxicity of **Ir1**–**Ir6** and **L1**–**L3**[a]

Compound	IC_{50} (μM)		
	A549	HeLa	LO2
Ir1	1.00 ± 0.05	1.08 ± 0.01	1.00 ± 0.05
Ir2	1.95 ± 0.05	1.19 ± 0.07	1.72 ± 0.02
Ir3	0.72 ± 0.02	1.16 ± 0.04	1.92 ± 0.02
Ir4	6.92 ± 0.16	1.64 ± 0.05	4.96 ± 0.06
Ir5	10.35 ± 0.12	4.68 ± 0.10	25.77 ± 1.78
Ir6	4.12 ± 0.14	0.94 ± 0.01	4.17 ± 0.10
L1	> 50	> 50	> 50
L2	> 50	> 50	> 50
L3	> 50	> 50	> 50
cisplatin	17.38 ± 0.40	5.04 ± 0.26	5.78 ± 0.25

[a] Data are presented as the means ± standard deviations (SD), and cell viability was assessed after 48 h of incubation.

References

1 J. Van Houten and R. J. Watts, *J. Am. Chem. Soc.,* 1976, **98**, 4853-4858.

2 D. S. Tyson and F. N. Castellano, *J. Phys. Chem. A.,* 1999, **103**, 10955-10960.

3 D. Pucci, A. Bellusci, A. Crispini, M. Ghedini, N. Godbert, E. I. Szerb and A. M. Talarico, *J. Mater. Chem.,* 2009, **19**, 7643-7649.

4 N. S. Makarov, M. Drobizhev and A. Rebane, *Opt. Express,* 2008, **16**, 4029-4047.

5 C. Xu and W. W. Webb, *Journal of the Optical Society of America B,* 1996, **13**, 481-491.

6 J.-J. Cao, C.-P. Tan, M.-H. Chen, N. Wu, D.-Y. Yao, X.-G. Liu, L.-N. Ji and Z.-W. Mao, *Chem. Sci.,* 2017, **8**, 631-640.

Movies

Movie S1. One-photon excited real-time tracking by luminescence imaging (left) and two-photon excited real time tracking by phosphorescence lifetime imaging (right) of mitochondria. A549 cells were stained with **Ir6** (20 μM) for 20 min before visualized.