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Lysosome Targeted Drugs: Rhodamine B modified N^N-chelating Ligands for Half-sandwich Iridium(III) Anticancer Complexes

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Supporting Information

EXPERIMENTAL SECTION

Materials and Instrumentation

$IrCl_3 \cdot nH_2O$, 2,3,4,5-tetramethyl-2-cyclopentenone (95%), 1,2,3,4,5-pentamethylcyclopentadiene (95%), Butyllithium solution (1.6 M in hexane), rhodamine B, hydrazine hydrate, 4,4'-dimethyl-2,2'-bipyridine, selenium dioxide, 1,10-phenanthroline-5-carbaldehyde, purchased from Sigma-Aldrich. Dimer 1-2 and ligands L_1-L_2 were prepared as described. For biological experiments, BSA, DMEM medium, fetal bovine serum, penicillin / streptomycin mixture, trypsin / EDTA, cisplatin, MTT and phosphate buffered saline (PBS) were purchased from Sangon Biotech. Stock solutions of cisplatin (10 mM) and complexes **1** ~ **4** (10 mM) were prepared in DMSO. Test compounds were dissolved in DMSO, stock solutions stored at -20 °C, thawed prior to use and diluted with medium.

NMR Spectroscopy

^{1}H NMR spectra were acquired in 5 mm NMR tubes at 298 K on Bruker DPX 500 (1H= 500.13 MHz) spectrometers. 1H NMR chemical shifts were internally referenced to $(CHD_2)(CD_3)SO$ (2.50 ppm) for DMSO-d_6, $CHCl_3$ (7.26 ppm) for chloroform-d_1. All data processing was carried out using XWIN NMR version 3.6 (Bruker UK Ltd.).

UV-Vis Spectroscopy

The UV-Vis spectra of the compounds were recorded by TU-1901 UV spectrophotometer with 1 cm path-length quartz cuvettes (3 ml). Spectra were processed using UV Winlab software. Experiments were carried out at 298 K unless otherwise stated.

Reaction with NADH

The reaction of complexes **1** and **2** (ca. 1 μM) with NADH (ca. 100 μM) in 60% MeOH/40% H_2O (v/v) was monitored by UV-Vis at 298 K after various time intervals. TON was calculated from the difference in NADH concentration after 8 h divided by the concentration of iridium catalyst. The concentration of NADH was obtained using the extinction coefficient $\varepsilon_{339} = 6220\ M^{-1}cm^{-1}$.

BSA binding experiments

The titration experiments including UV-Vis absorption and fluorescence quenching were performed at constant concentration of BSA. A BSA stock solution was prepared in Tris buffer (5 mM Tris–HCl/10 mM NaCl at pH = 7.2) and stored at 4 ^{o} C. All spectra were recorded after each successive addition of the compounds and incubation at room temperature for 5 min to complete the interaction. In the UV–Vis absorption titration experiment, BSA solution (2.5 ml, 1×10^{-5} M) was titrated by successive additions of the stock solutions of Ir complex (1×10^{-6} M) and the changes in the BSA absorption were recorded after each addition. The fluorescence emission spectra of BSA in the absence and presence of Ir complex were also recorded with excitation at 285 nm. The concentrations of the Ir complex were 0–7.0 μM, and the concentration of BSA was fixed at 10 μM. Synchronous fluorescence spectra of BSA with various concentrations of complexes (0–7.0 μM) were obtained from 240 to 400 nm when $\Delta\lambda = 60$ nm and from 255 to 400 nm when $\Delta\lambda = 15$ nm.

Cell Culture

Hela human cervical cancer cells and A549 human lung cancer cells were obtained from Shanghai Institute of Biochemistry and Cell Biology (SIBCB) and were grown in Dubelco's Modified Eagle Medium (DMEM). All media were supplemented with 10% fetal bovine serum, and 1 % penicillin-streptomycin solution. All cells were grown at 310 K in a humidified incubator

undera 5 % CO_2 atmosphere.

Viability assay (MTT assay)

After plating 5000 A549 cells per well in 96-well plates, the cells were preincubated in drug-free media at 310 K for 24 h before adding different concentrations of the compounds to be tested. In order to prepare the stock solution of the drug, the solid complex was dissolved in DMSO. This stock was further diluted using cell culture medium until working concentrations were achieved. The drug exposure period was 24 h. Subsequently, 15 μl of 5 mg ml^{-1} MTT solution was added to form a purple formazan. Afterwards,100 μl of dimethyl sulfoxide (DMSO) was transferred into each well to dissolve the purple formazan, and results were measured using a microplate reader (DNM-9606, Perlong Medical, Beijing, China) at an absorbance of 570 nm. Each well was triplicated and each experiment repeated at least three times. IC_{50} values quoted are mean ± SEM. The complex was serially diluted in DMSO prior to bioassay. The maximum working concentration of serially diluted DMSO was 1% (v / v), so the 1% DMSO itself does not cause cell growth inhibition in all of the bioassays.

Cell Cycle Analysis

A549 cells at 1.5 × 10^6 per well were seeded in a six-well plate. Cells were preincubated in drug-free media at 310 K for 24 h, after which drugs were added at concentrations of 0.25 × IC_{50}, 0.5 × IC_{50}, 1 × IC_{50} and 2 × IC_{50}. After 24 h of drug exposure, supernatants were removed by suction and cells were washed with PBS. Finally, cells were harvested using trypsin-EDTA and fixed for 24 h using cold 70 % ethanol. DNA staining was achieved by resuspending the cell pellets in PBS containing propidium iodide (PI) and RNAse. Cell pellets were washed and resuspended in PBS before being analyzed in a flow cytometer (ACEA NovoCyte, Hangzhou, China) using excitation of DNA-bound PI at 488 nm, with emission at 585 nm. Data were processed using NovoExpress™ software. The cell cycle distribution is shown as the percentage of cells containing G_0/G_1, S and G_2/M DNA as identified by propidium iodide staining.

Induction of Apoptosis

Flow cytometry analysis of apoptotic populations of A549 cells caused by exposure to iridium complexes was carried out using the Annexin V-FITC Apoptosis Detection Kit (Beyotime Institute of Biotechnology, China) according to the supplier's instructions. Briefly, A549 cells (1.5 ×10^6 /2 ml per well) were seeded in a six-well plate. Cells were preincubated in drug-free media at 310 K for 24 h, after which drugs were added at concentrations of 0.25 × IC_{50}, 1 × IC_{50}, 2 × IC_{50} and 3 × IC_{50}. After 24 h of drug exposure, cells were collected, washed once with PBS, and resuspended in 195 μl of annexin V-FITC binding buffer which was then added to 5 μl of annexin V-FITC and 10 μl of PI, and then incubated at room temperature in the dark for 15 min. Subsequently, the buffer placed in an ice bath in the dark. The samples were analyzed by a flow cytometer (ACEA NovoCyte, Hangzhou, China).

ROS Determination

Flow cytometry analysis of ROS generation in A549 cells caused by exposure to iridium complex **3** was carried out using the Reactive Oxygen Species Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the supplier's instructions. Briefly, 1.5 × 10^6 A549 cells per well were seeded in a six-well plate. Cells were preincubated in drug-free media at 310 K for 24 h in a 5 % CO_2 humidified atmosphere, and then drugs were added at concentrations of 0.25 × IC_{50} and 0.5× IC_{50}. After 24 h of drug exposure, cells were washed twice with PBS and then incubated with the DCFH-DA probe (10 μM) at 37 °C for 30 min, and then washed triple

immediately with PBS. The fluorescence intensity was analyzed by flow cytometry (ACEA NovoCyte, Hangzhou, China). Data were processed using NovoExpress™ software. Samples were kept under dark conditions to avoid light-induced ROS production.

Mitochondrial Membrane Assay

Analysis of the changes of mitochondrial potential in cells after exposure to iridium complexes was carried out using the Mitochondrial membrane potential assay kit with JC-1(Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. Briefly, 1.5×10^6 A549 cancer cells were seeded in six-well plates left to incubate for 24 h in drug-free medium at 310K in a humidified atmosphere. Drug solutions, at concentrations of $0.25 \times IC_{50}$, $0.5 \times IC_{50}$, $1.0 \times IC_{50}$ and $2.0 \times IC_{50}$ of complex **3** against A549 cancer cells, were added in triplicate, and the cells were left to incubate for a further 24 h under similar conditions. Supernatants were removed by suction, and each well was washed with PBS before detaching the cells using trypsin-EDTA. Staining of the samples was done in flow cytometry tubes protected from light, incubating for 30 min at ambient temperature. The samples were immediately analyzed by a flow cytometer (ACEA NovoCyte, Hangzhou, China). For positive controls, the cells were exposed to carbonyl cyanide 3-chlorophenylhydrazone, CCCP (5 μM), for 20 min. Data were processed using NovoExpress™ software.

Cellular localization assay

Two Photon Laser Scanning Microscope(*/LSM/880NLO) is produced at Carl Zeiss AG, Germany. LTDR (Life Technologies, USA), MTDR (Life Technologies, USA), CCCP (Sigma Aldrich, USA), chloroquine(Sigma Aldrich, USA) were used as received. A549 cells were seeded into 35 mm dishes (Greiner, Germany) for confocal microscopy. After cultured overnight, the cells were incubated with complexes **1** ~ **4** at 10 μM for 1 h. The treated cells were observed immediately under a confocal microscope with excitation at 488 nm. For colocalization studies, the cells were incubated with complexes (10 μM) for 1 h. Subsequently, the medium was replaced with staining medium containing MTDR/LDTR (500 nM) and stained for another 30 min and 1h. The cells were washed twice with PBS, and then viewed immediately under a confocal microscope. Investigation of drug entry pattern: Cells were incubated with complexes (10 μM) for 1 hour, after which media was replaced with staining medium containing CCCP (50 μM, 1 h) chloroquine (50 μM, 1 h) and re-stained for 15 min. The cells were washed twice with PBS, and then viewed immediately under a confocal microscope.

Assay for the lysosomal membrane permeabilization (Acridine Orange assay)

Using two photon laser scanning microscope and AO (Sigma Aldrich, USA) A549 cells were seeded in 500 μL culture medium on a 35 mm imaging dish (μ-Dish 35 mm, high, ibidi GmbH, Germany) at a cell density of 80 cells/μL and stabilized for 24 h. Then the cells were washed with PBS (2 × 2 mL) and Acridine Orange (5 μM in PBS) was added to fresh medium (2 mL, RPMI 1640 medium supplemented with 5% FCS, 1% L-glutamine, and 1% penicillin/streptomycin) and incubated for 15 min at 37 °C and 5% CO_2. Then the cells were washed with PBS (3 × 2 mL) and fresh medium (2 mL) was added. Then the prodrugs (20 μL, 5 mM in DMSO) were added and incubated for 1 h at 37 °C and 5% CO_2. The fluorescence images were taken with two photon laser scanning microscope: excitation 510 ± 20 nm for green channel, excitation 625 ± 20 nm for yellow channel.

Experimental

1.1. Synthesis of rhodamine B hydrazid

Rhodamine B hydrazide was synthesized by a one-step reaction of rhodamine B with hydrazine hydrate in ethanol. To a 100 ml round bottom flask was added 4.80 g（10 mmol）rhodamine B, 30 ml absolute ethanol was added, stirred rapidly at room temperature, an excessive hydrazine hydrate（8 ml）was added, the mixture was stirred under reflux until pink color to orange color and yellow precipitate precipitated, then, The reaction was cooled to room temperature, 1 M HCl was added and the solution turned to blood red. The pH of the solution was adjusted to 9 - 10 with 1 M NaOH. The color of the solution changed to an orange-yellow color and a white precipitate was formed. The precipitate was filtered, washed with water (3 × 25 ml) and dried to give a white solid, the solid was dissolved in water and extracted with dichloromethane (3 × 25 ml) to remove excessive hydrazine hydrate, organic layer was dried to give a purple-white solid.

1.2. Synthesis of the ligands (L_1–L_2).

L_1：To a 0.912 g（2 mmol) of rhodamine B hydrazide dissolved in 30 ml of dichloromethane ,a 0.396 g (2 mmol) 4-methyl-4-carbonyl-2,2-bipyridine was added, a drop of formic acid was added dropwise and heated to reflux for 24h.The mixture was cooled to room temperature, the solvent was evaporated to dryness on a rotary evaporator to afford purple oil. Add a small amount of methanol dropwise to precipitate a white solid which was then filtered and washed with methanol.

L_2: The procedure was similar to that described for the synthesis of L_1. Reactant: rhodamine B hydrazide (2 mmol, 0.912 g) with 1,10-phenanthroline-5-carbaldehyde (2 mmol, 0.416 g).

*1.3. Synthesis of complexes **1~4**.*

Complex **1 ~ 4** was obtained by the reaction of L_1-L_2 (0.10 mmol) with **dimers 1–2** $[(\eta^5\text{-}Cp^x)IrCl_2]_2$ (0.05 mmol), in a solvent mixture (30 ml) of dichloromethane and methanol (1:1 v/v) under reflux condition for 12 h . The solution turned blood red, after a period of time, a red residue appears, spin the solution, dissolved in hot methanol, A saturated methanolic solution of NH_4PF_6 was added to precipitate the red solid the precipitate was orange red, the precipitate was completely precipitated, the solution and precipitate separately. The precipitate was dissolved in methylene chloride, filtered through celite, spin the liquid, dissolved with as little methylene chloride as possible, and recrystallized by adding ether. The solution was the same as the precipitate and the product was orange-red crystals. The ^{1}H NMR (500 MHz, DMSO-d_6) peak integrals of ligand complexes **1, 4** and the ^{1}H NMR (500 MHz, $CDCl_3$) peak integrals of ligand complexes **2,3** are shown in Fig. S13 – S16. The Mass spectrometry of complexes **1 ~ 4** can be seen in Fig. S17.

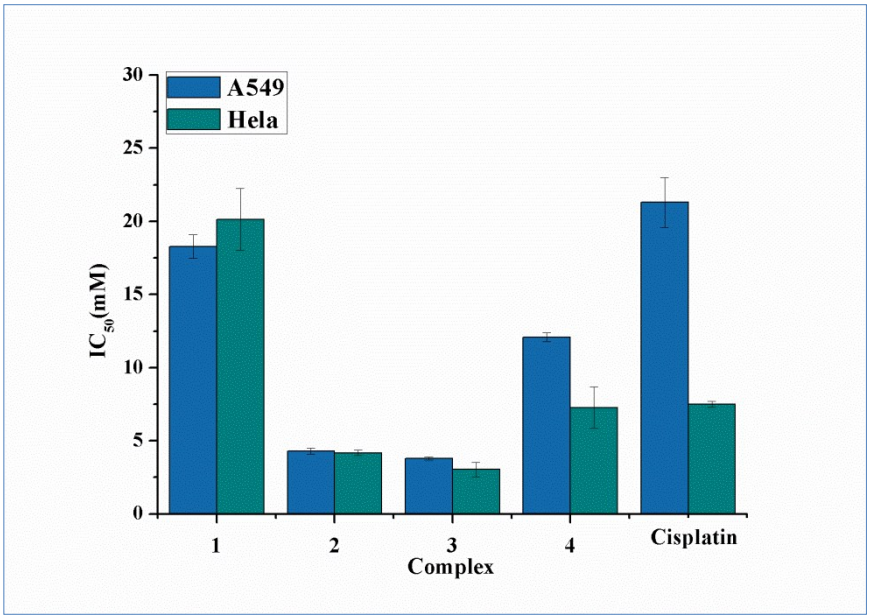


Fig. S1 Histogram showing the comparison of IC_{50} values of complexes **1** ~ **4** and cisplatin towards HeLa and A549 cancer cells after incubation for 24 h.

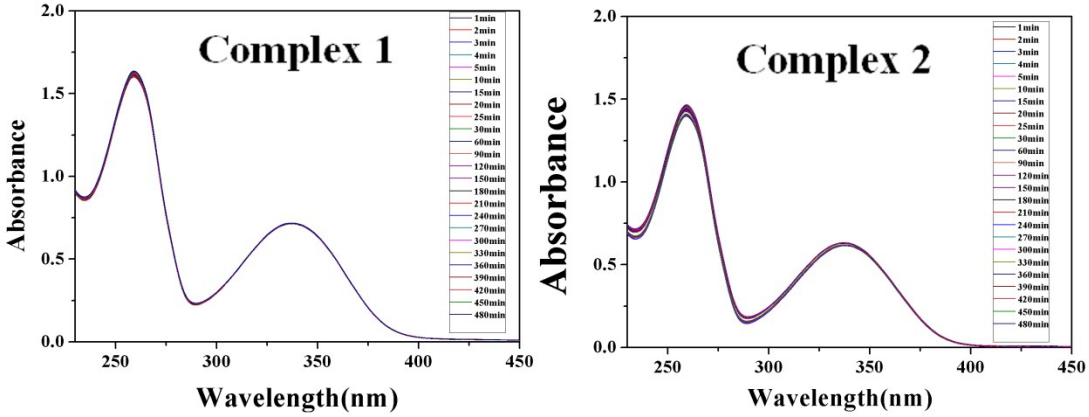


Fig. S2 UV-Vis spectra of the reaction of NADH (100 μM) with complex **1** (1 μM) and complex **2** (1 μM) in 60% MeOH/40% H_2O (v/v) at 298 K for 8 h.

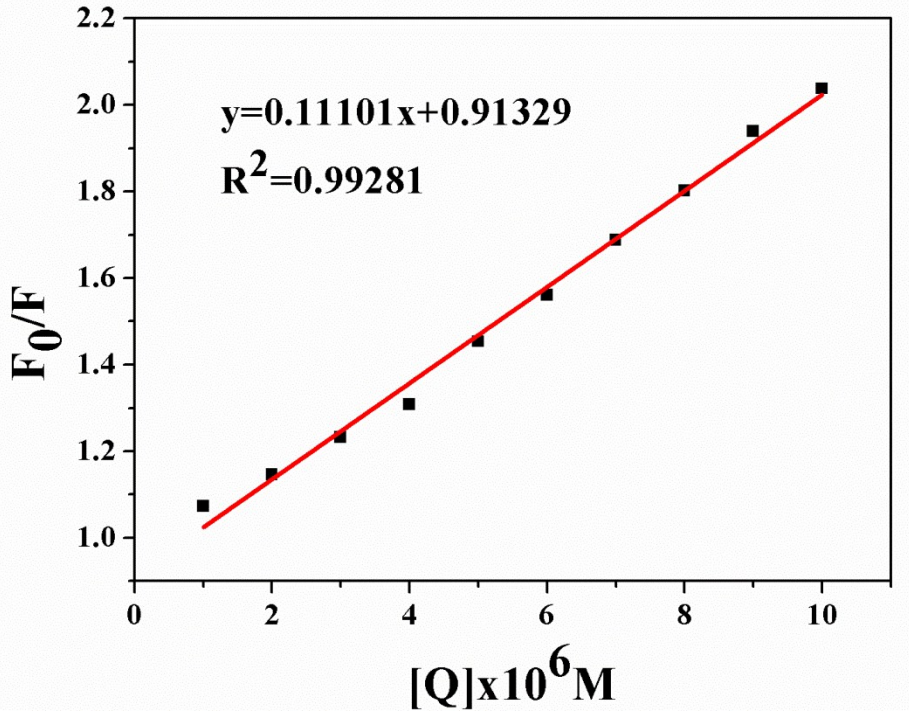


Fig. S3 Stern–Volmer plots of F_0/F against the concentration of complex **3.**

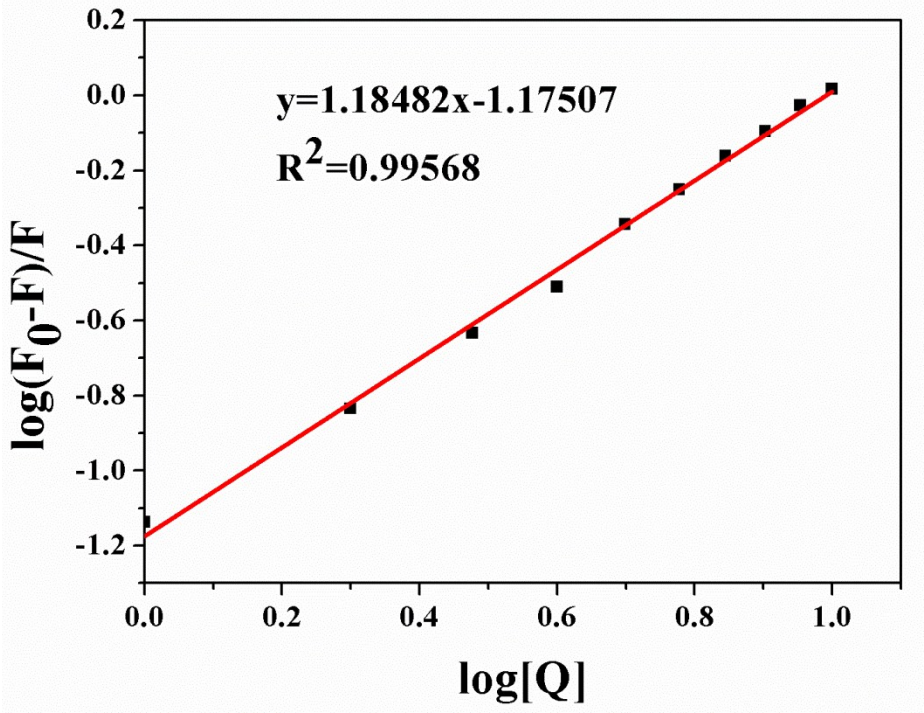


Fig. S4 Plots of $\log[(F_0 - F)/F]$ vs. log[Q] for the interaction of BSA with complex **3**

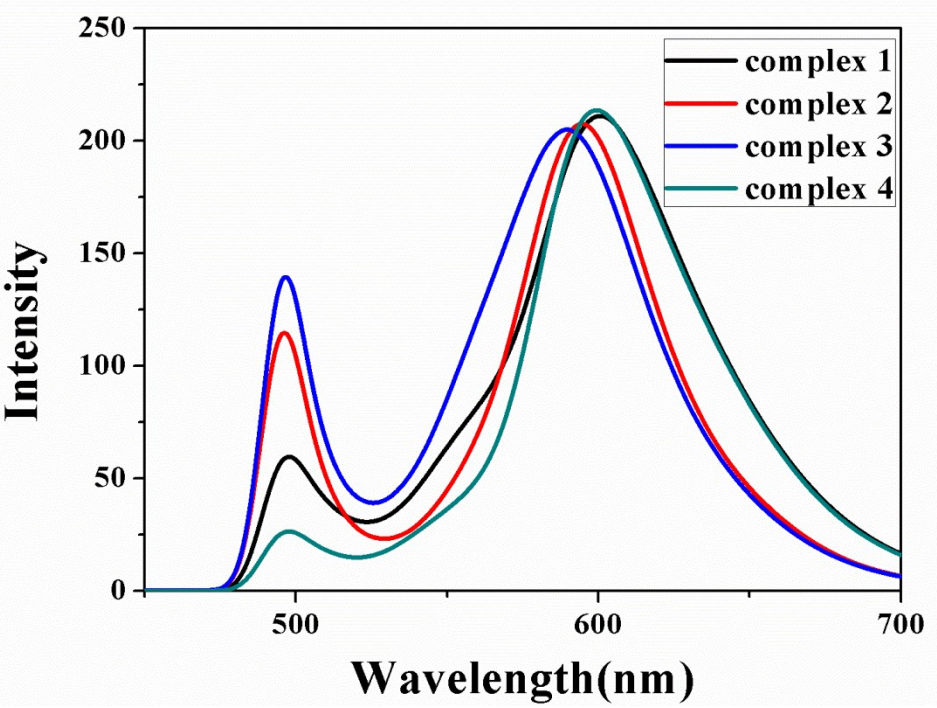


Fig. S5 Emissionspectra of complexes**1** ~ **4** (10 μM) measured in CH_3CN at 298 K (excitation at 488 nm).

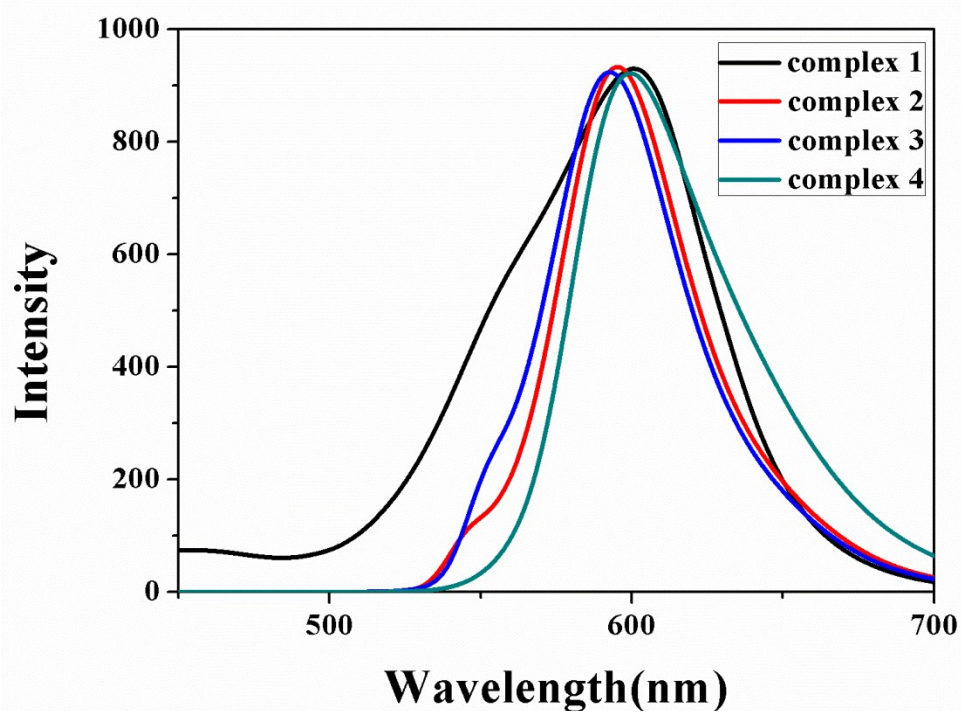


Fig. S6 Emissionspectra of complexes **1** ~ **4** (10 μM) measured in CH_3CN at 298 K (excitation at 550 nm).

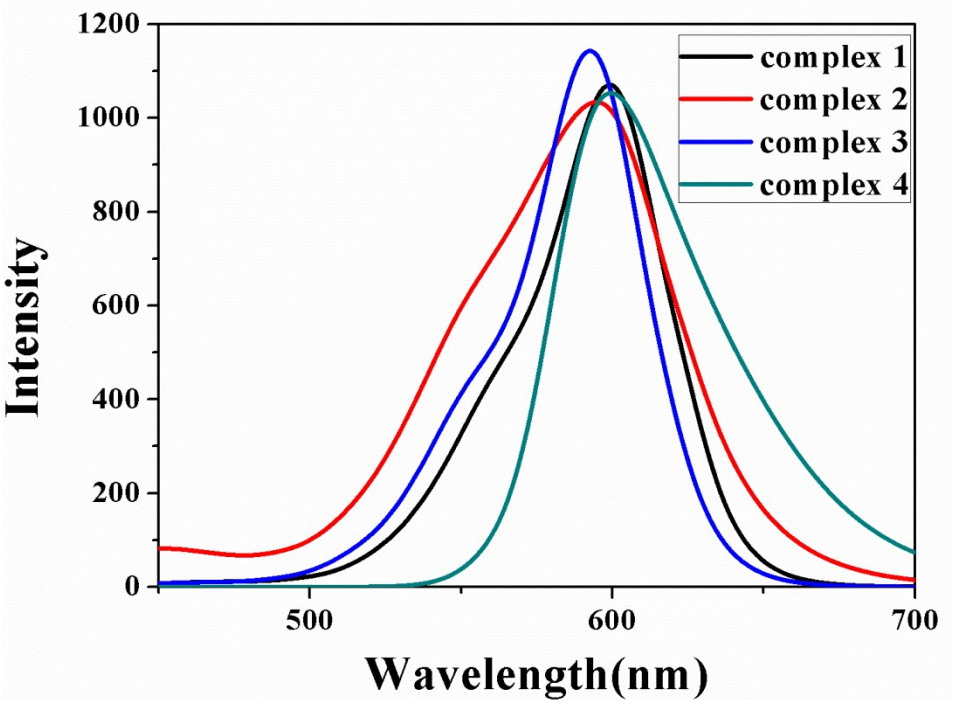


Fig. S7 Excitationspectra of complexes **1** ~ **4** (10 μM) measured in CH_3CN at 298 K (emission at 600 nm).

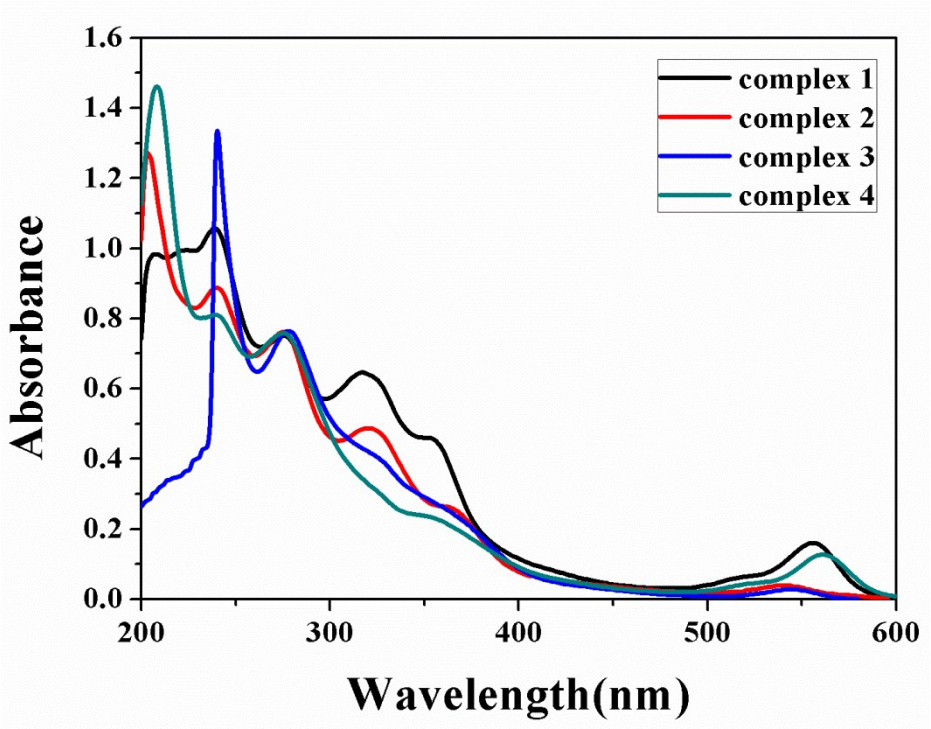


Fig. S8 UV -visible spectra of complexes **1** ~ **4** (20 μM) measured in CH_3CN at 298 K.

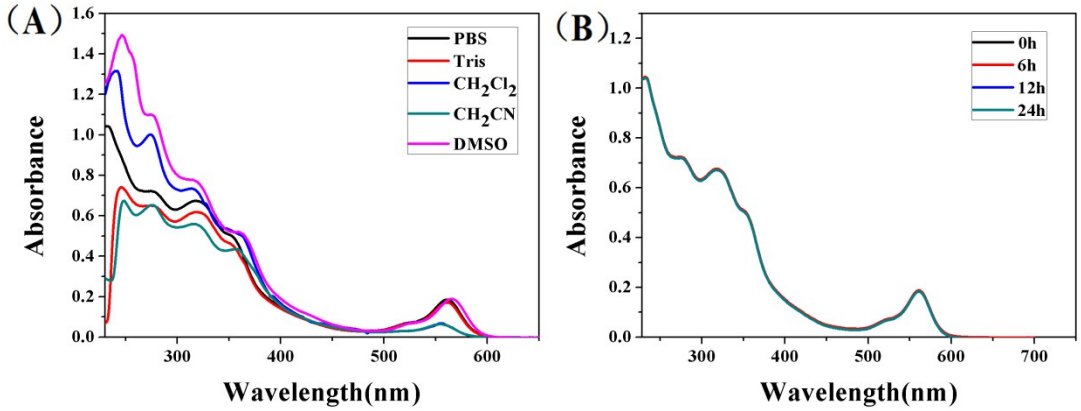
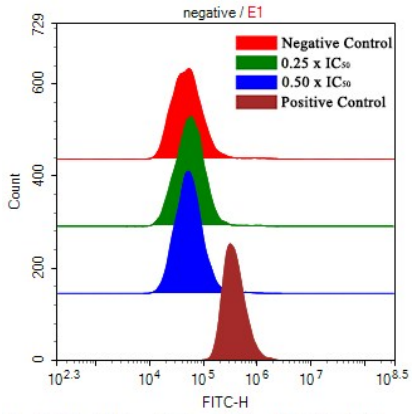


Fig. S9 (A)UV -visible spectra of complexes **1** (20 μM) measured in CH_2Cl_2, PBS, DMSO, Tris-HCl buffer solution and CH_3CN at 298 K.(B) UV-Vis absorption spectra of Ir complexes (20 μM) in PBS buffer solution during incubation at 25 °C.



#	Sample	Gate	Count	% E1	Mean X	CV X
1	negative	E1	13,456	100.00%	70,771	168.09%
2	1_4	E1*	15,267	100.00%	74,526	112.54%
3	1_2	E1*	15,886	100.00%	69,358	104.49%
4	positive	E1*	12,465	100.00%	431,149	58.54%

Fig. S10 ROS induction in A549 cancer cells treated with complex **3**.

Table S1 Cell cycle analysis carried out by flow cytometry using PI staining after exposing A549 cells to complex **3.**

		Population（%）		
Complex	**Ir Concentration**	**G_0/G_1 phase**	**S phase**	**G_2/M phase**
Control		53.0±2.4	32.33±1.9	11.88±0.1
Complex 3	0.25×IC_{50}	54.33±0.1	31.31±0.1	11.13±0.1
	0.5×IC_{50}	61.85±0.5	26.28±0.3	8.75±1.5
	1×IC_{50}	66.71±1.8	22.76±0.1	7.74±1.5
	2×IC_{50}	69.34±1.3	21.46±0.1	7.50±0.4

Table S2 Flow cytometry analysis to determine the percentages of apoptotic cells, using Annexin V-FITC vs S19 PI staining, after exposing A549 ells to complex **3.**

		Population (%)			
Complex	**Ir Concentration**	**Viable**	**Early apoptosis**	**Late apoptosis**	**Non-viable**
control		93.86±0.1	0.84±0.7	4.49±0.3	0.82±0.4
Complex 3	0.5× IC_{50}	92.24±0.7	1.15±0.1	5.71±0.2	0.91±0.6
	1 × IC_{50}	91.24±1.1	1.63±0.2	6.55±0.7	0.59±0.3
	2 × IC_{50}	86.54±0.7	3.56±1.1	9.16±0.1	0.74±0.3
	3 × IC_{50}	66.79±4.9	6.88±0.7	21.10±0.4	5.24±4.6

Table S3 The mitochondrial membrane polarization of A549 cells induced by complex **3**.

	Population (%)	
Complex	Ir concentration	Mean Fluorescence
Complex 3	0.25 × IC_{50}	72876.5±2332.7
	0.5 × IC_{50}	68929.5±605.9
Untreated cells (negative control)		70771±706
CCCP treated cells (positive control)		431149±1020

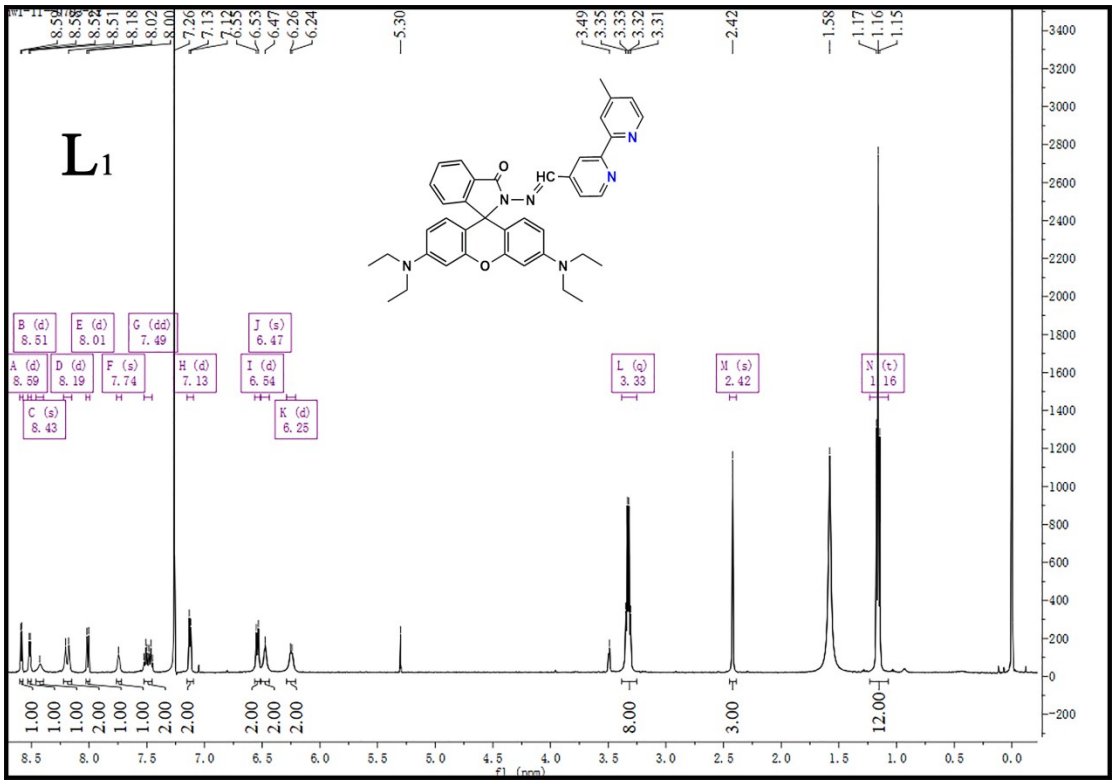


Fig. S11 The ^{1}H NMR (500.13 MHz, $CDCl_3$) peak integrals of L_1.

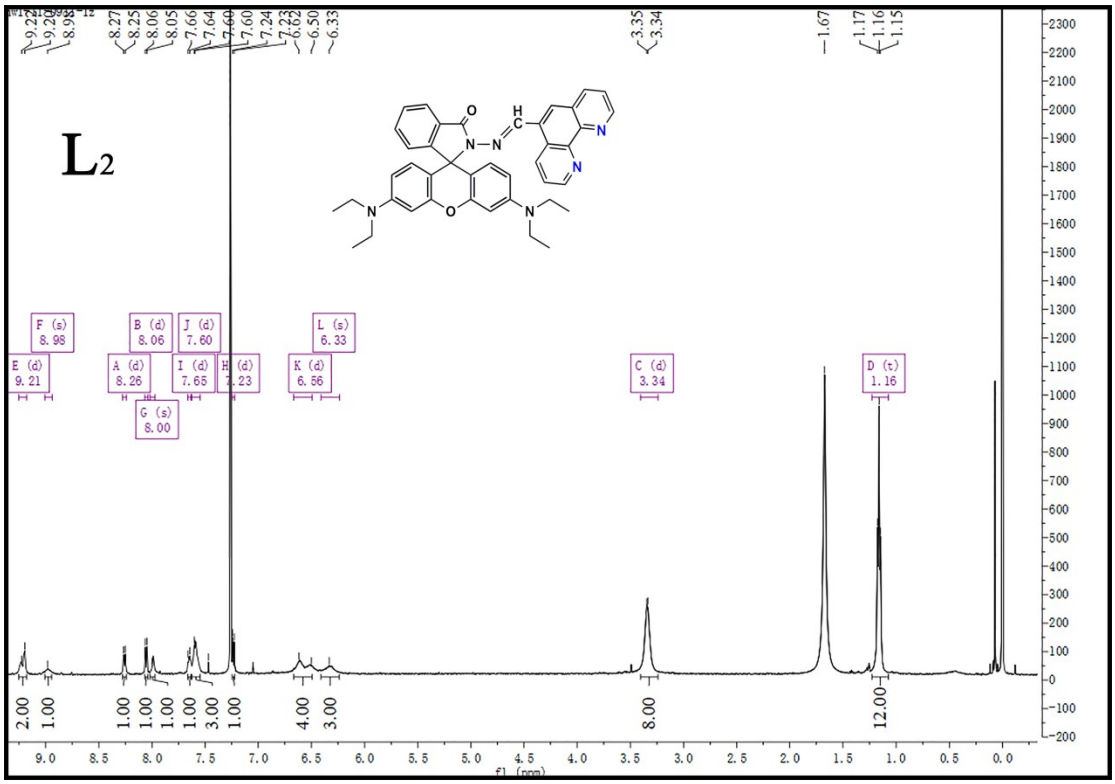


Fig. S12 The ^{1}H NMR (500.13 MHz,$CDCl_3$) peak integrals of L_2.

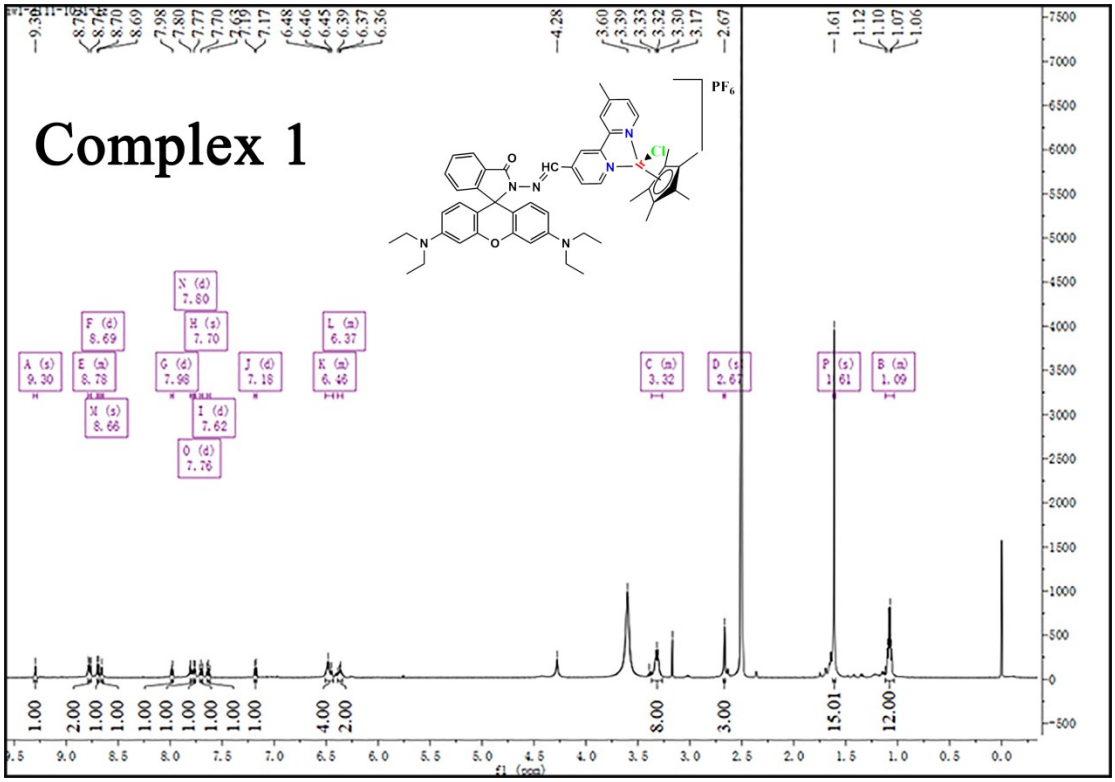


Fig. S13 The ^{1}H NMR (500.13 MHz, DMSO) peak integrals of $[(\eta^5\text{-}C_5Me_5)Ir(L_1)Cl]PF_6$ (complex **1**).

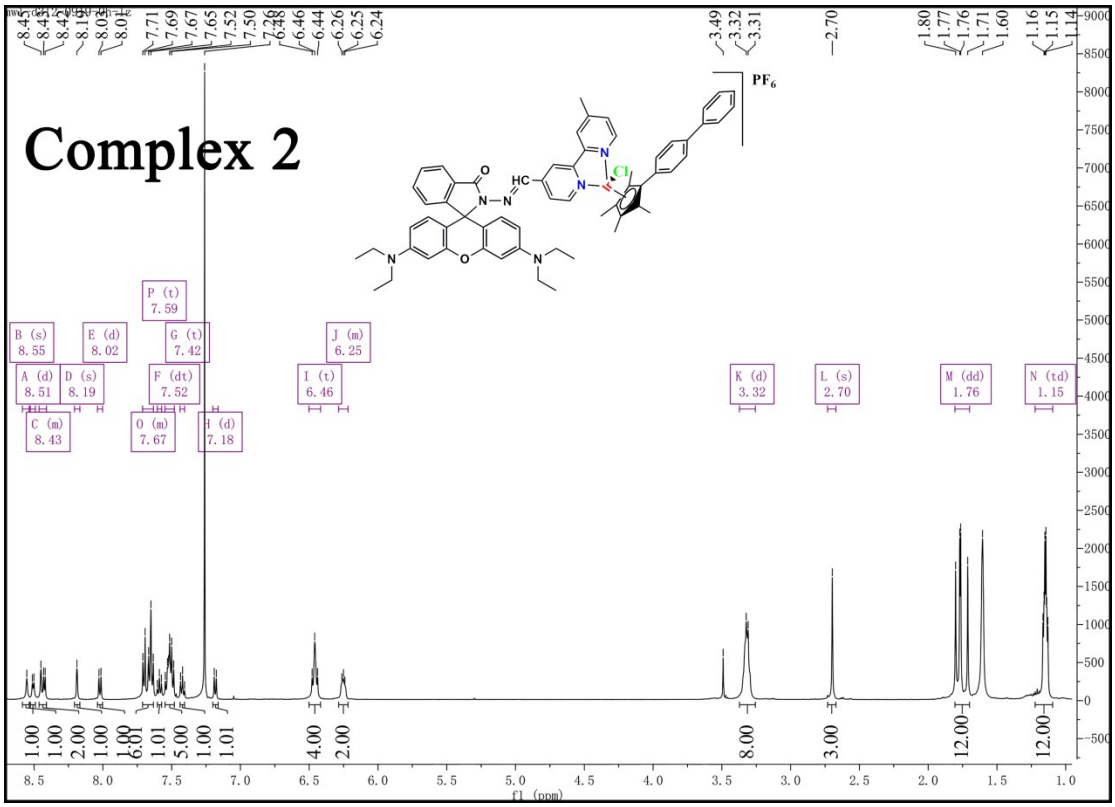


Fig. S14 The ^{1}H NMR (500.13 MHz, $CDCl_3$) peak integrals of $[(\eta^5\text{-}C_5Me_4C_6H_4C_6H_5)Ir(L_1)Cl]PF_6$ (Complex **2**) .

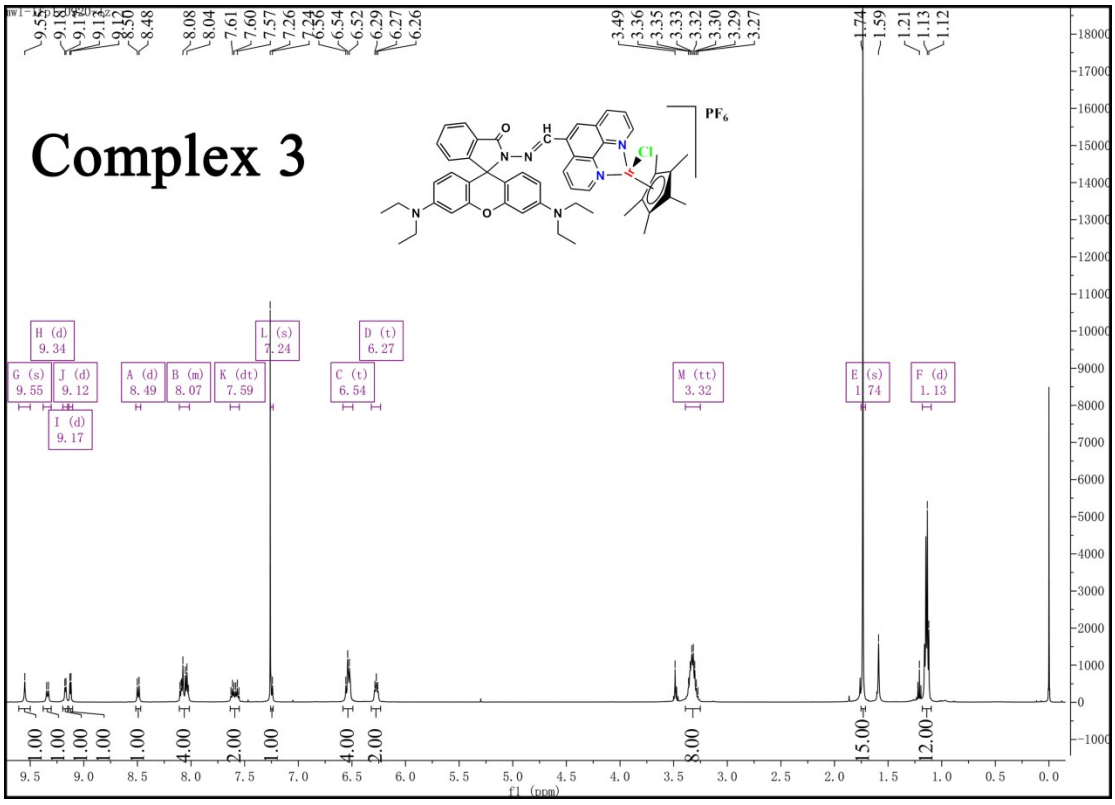


Fig. S15 The ^{1}H NMR (500.13 MHz, $CDCl_3$) peak integrals of $[(\eta^5\text{-}C_5Me_5)Ir(L_2)Cl]PF_6$ (Complex **3**).

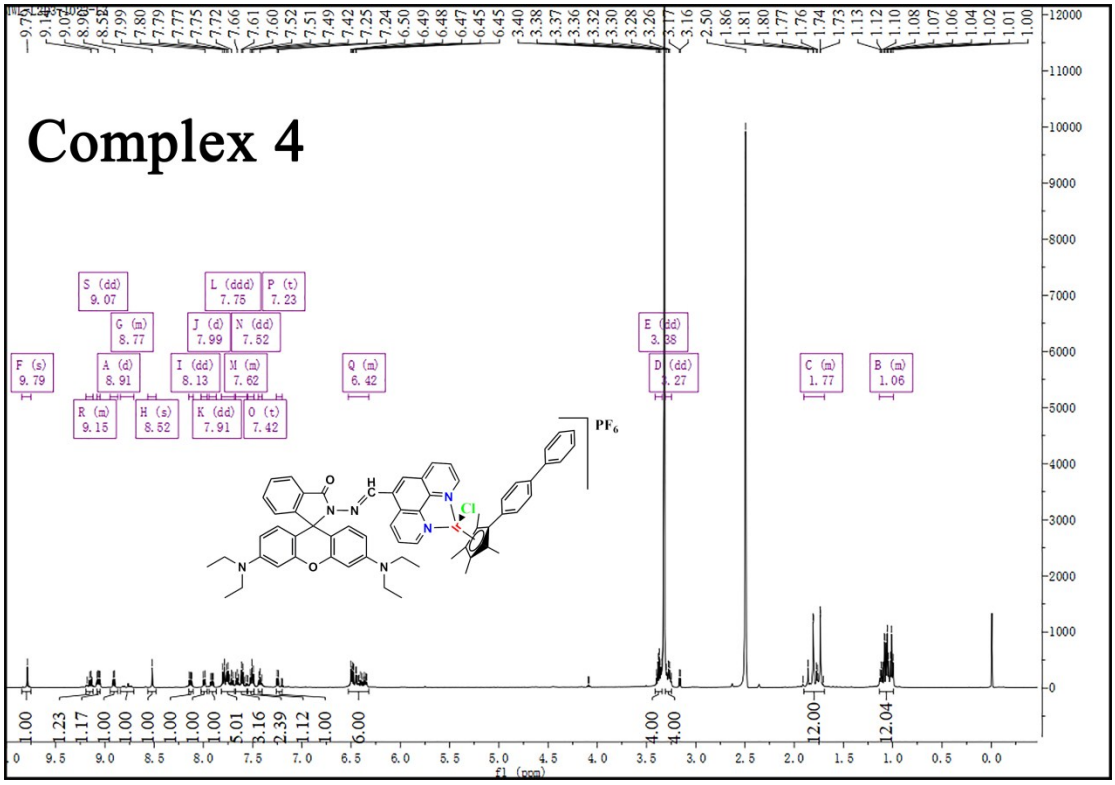
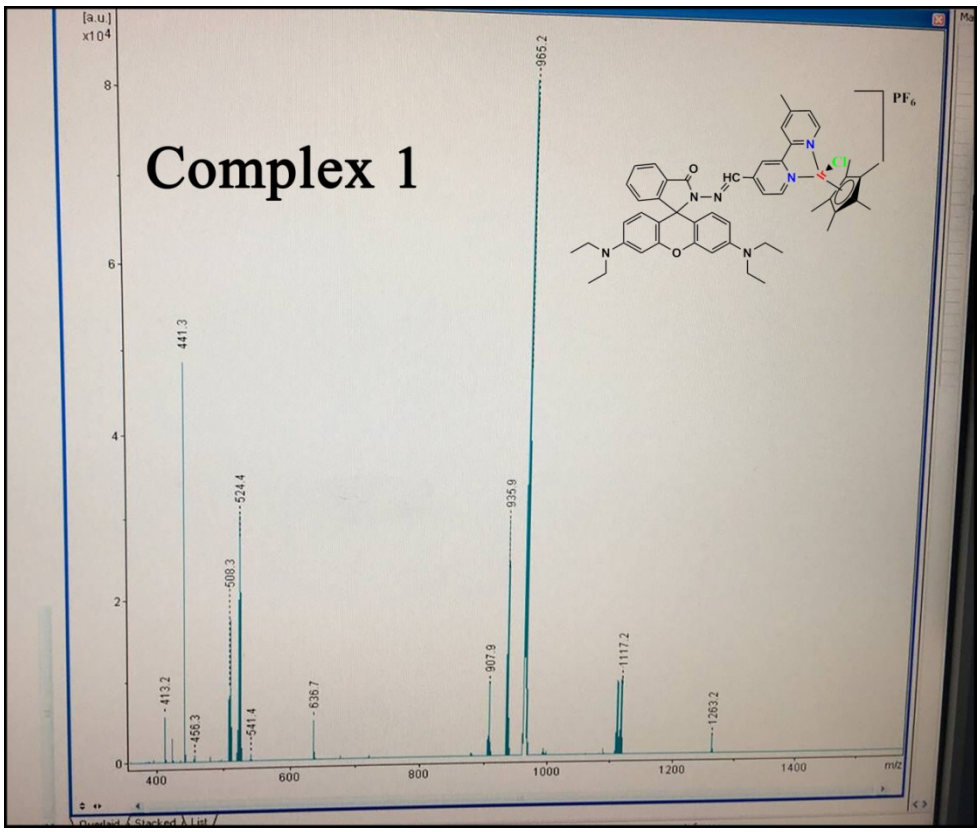
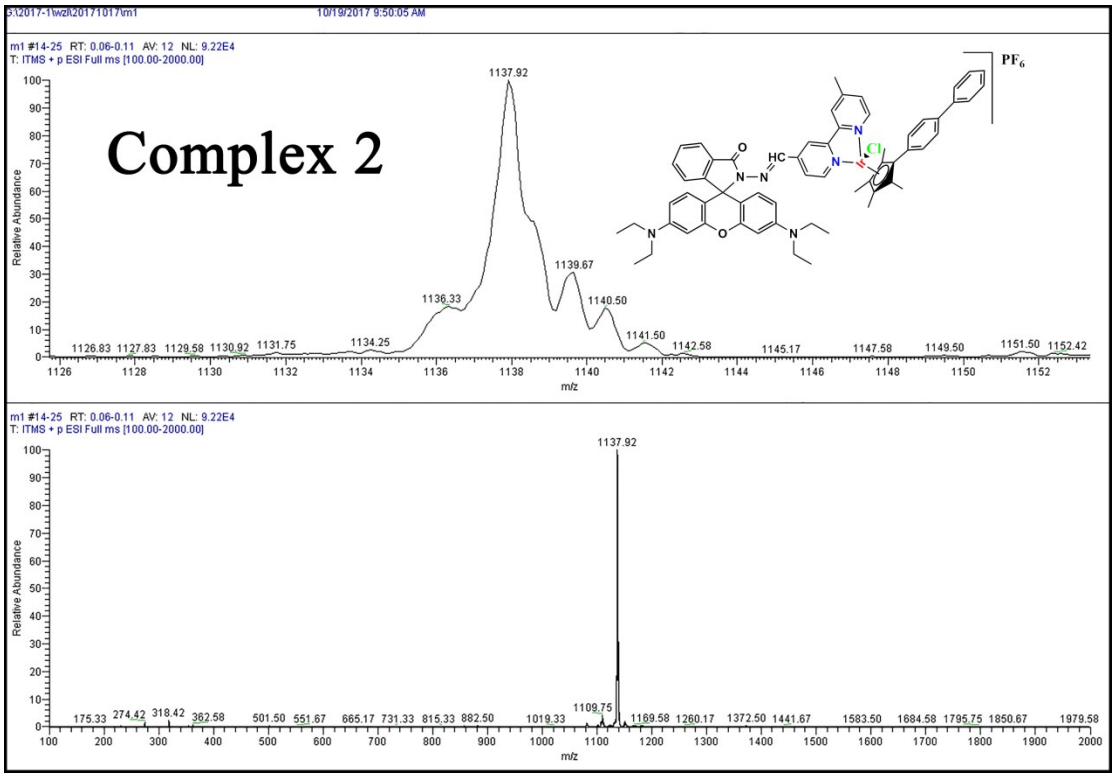


Fig. S16 The ^{1}H NMR (500.13 MHz, DMSO) peak integrals of $[(\eta^5\text{-}C_5Me_4C_6H_4C_6H_5)Ir(L_2)Cl]PF_6$ (Complex **4**) .

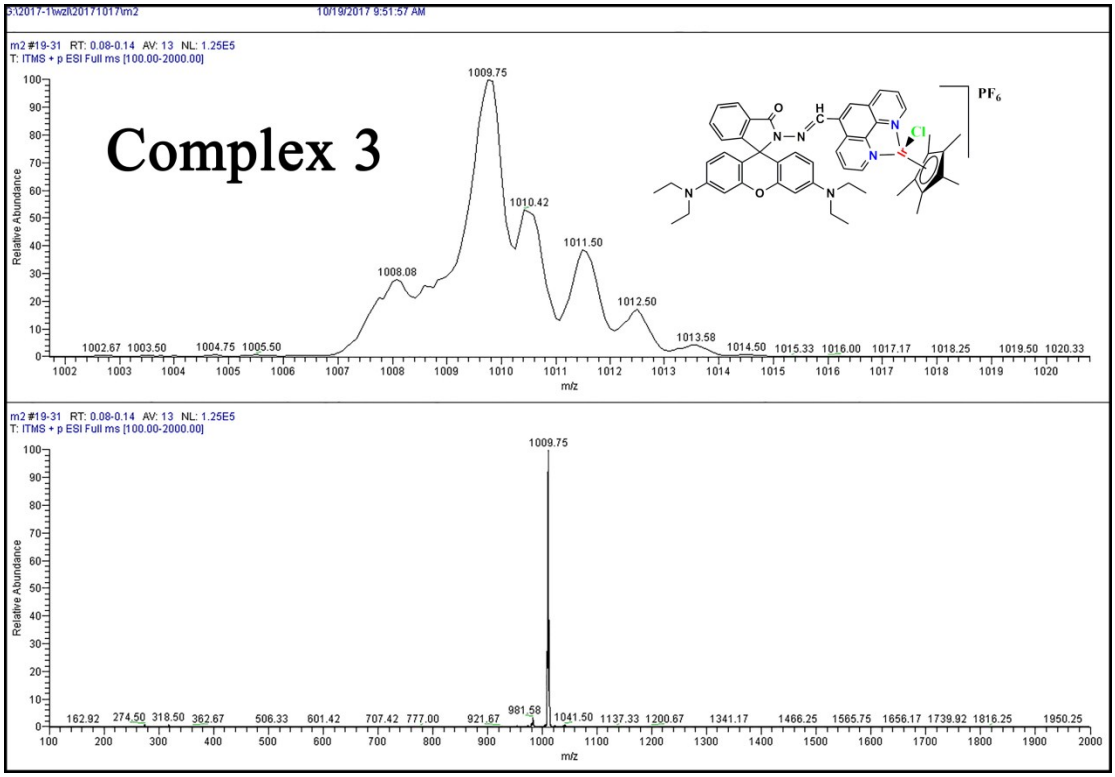
[(η^5-C_5Me_5)Ir(L_1)Cl]PF_6 (Complex **1**): MS: m/z 964.25(-PF_6, -Cl).



[(η^5-$C_5Me_4C_6H_4C_6H_5$)Ir(L_1)Cl]PF_6(Complex **2**): MS: m/z 1137.42 (-PF_6).



[(η^5-C_5Me_5)Ir(L_2)Cl]PF_6(Complex **3**): MS: m/z 1009.70 (-PF_6).



[(η^5-$C_5Me_4C_6H_4C_6H_5$)Ir(L_2)Cl]PF_6(Complex **4**): MS: m/z 1111.95 (-PF_6, -Cl).

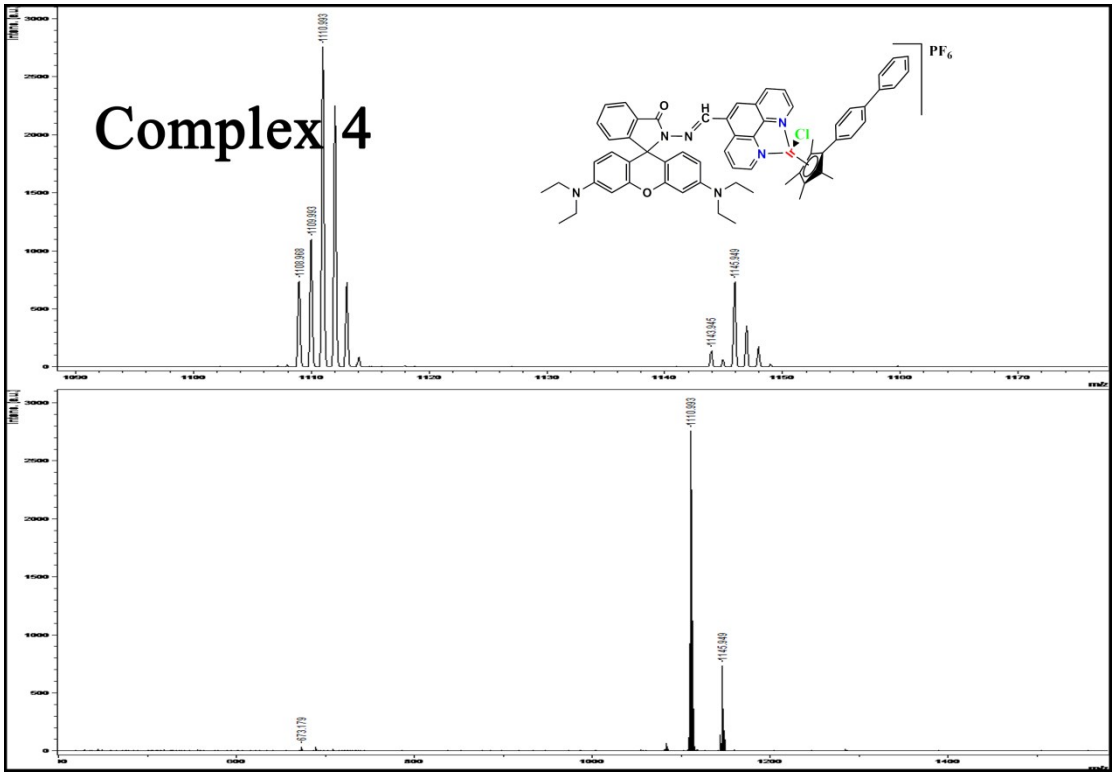


Fig. S17 The Mass spectrometry of complexes **1** ~ **4** [(η^5-Cp^x)Ir(N^N)Cl]PF_6.