In vitro and in vivo antitumor activity of novel half-sandwich ruthenium complexes containing quinoline derivative ligands

Xiangdong He,[a] Jun Chen,[b] Martha Kandawa-Shultz,[c] Guoqiang Shao,[b*] Yihong Wang[a*]

a School of Chemistry and Chemical Engineering, Southeast University, Nanjing, 211189, China

b Department of Nuclear Medicine, Nanjing First Hospital, Nanjing Medical University, Nanjing 211166, China.

C Department of Chemistry and Biochemistry, University of Namibia, Windhoek 13301, Namibia

Materials and instruments

The raw materials involved in the experiment were obtained through commercial procurement, and no further purification was required during use. Unless otherwise stated, all raw materials were obtained through commercial procurement without further purification. 2-methyl-8-hydroxyquinoline, p-fluorobenzaldehyde, p-chlorobenzaldehyde, p-bromobenzaldehyde, p-iodob -enzaldehyde, dimer$[(\eta^6\text{-p-cymene})RuCl_2]_2$, sodium acetate, purchased from Aladdin, Shanghai. Phosphate buffer saline PBS, Dulbecco's modified Eagle medium （DMEM）, Roswell Park Memorial Institute 1640 (RPMI-1640), fetal bovine serum, trypsin, penicillin/streptomycin were purchased from Jiangsu Keygen Biotech Corp., Ltd. Thiazolyl blue tetrazolium Bromide (MTT), MitoMaker Red CMXRos (MTDR), lysosomal red probe Lyso-Tracker Red (LTDR), Annexin V-FITC apoptosis detection kit, JC-1 assay kit, ROS assay kit, acridine orange, Methylrosanilnium chloride solution crystal violet was purchased from Beyotime Biotechnology, Shanghai. Anti-Bcl-2 and Bax antibodies are products of Proteintech. Cells were cultured in a Thermo 3543 carbon dioxide cell incubator. In the MTT experiment, the absorbance at 570 nm was read on the Thermo Fisher Multiskan FC microplate reader. Carl Zeiss AG* / LSM / 880NLO two-photon laser scanning microscope was used for laser confocal experiments. Cell apoptosis and mitochondrial membrane potential experiments were analyzed by BD Calibur cytometer. Immunoblot experiments were visualized with the Omega Lum C imaging system (Aplegen, USA). ^1H NMR spectra were measured with Burker 600 MHZ. An electrospray ionization mass spectrometer (ESI-MS) was performed on an Agilent 6520Q-TOF LC / MS system.

Cell line and cell culture

The cells were cryopreserved in liquid nitrogen by previous laboratory personnel and were stored until they were cultured. All cell types were maintained at 37°C in a humidified 5% CO_2 environment. MDA-MB-231, MCF-7, Hela, and HepG2 cells were grown in DMEM (Gibco) containing 10% fetal bovine serum and A549 cells in RPMI 1640 (Gibco) with 10% fetal bovine serum.

MTT

The cells in the logarithmic growth phase were planted in 96 well plates at a

density of 5000 per well and incubated in an incubator until they grew to the appropriate concentration. The 10 mM complex solution dissolved in DMSO was diluted according to the concentration and added to the well plate, and the content of DMSO in the well plate was controlled to be less than 2%. The final concentration of the complex in the pore was 0.78125, 1.5625, 3.125, 6.25, 12.5, 25 50, 100 μm/L. Cisplatin (cis-diammineplatinum II dichloride; Sigma-Aldrich) was dissolved in 0.9% of PBS at a concentration of 2 mm/L. Cells were exposed to the drug environment for 24 h. A 20 μL (5 mg/mL) MTT solution was added to each well and incubated for 4 h. The liquid in the well plate was replaced with 100 uL DMSO and the absorbance was read at 570 nm with a microplate reader after shaking for 30 min.

Apoptosis analysis

The logarithmic growth phase of MCF-7 cells was collected and planted on the well plate. After 24 h of exposure to the drug, the cells were collected and stained according to the manufacturer's instructions for use. Furthermore, the cells were washed with PBS twice to remove the excess medium, and 0.5 mL trypsin was added to each well for digestion for 5 min. The digestion was terminated by adding the medium, the cells were blown, and the cell suspension was transferred to a centrifuge tube for centrifugation at 1000 rpm for 5 min. Next, 1 mL PBS was added to re-suspend the cells and centrifuged again. After the last centrifugation, the supernatant was removed and 500 uL staining binding solution was added. Thereafter, 5 μL Annexin V-PE and 10 uL 7-AAD were added into the centrifuge tube successively. The tubes were incubated for 0.5 h in the dark and the samples were analyzed on the flow cytometer to obtain the experimental results.

Colocalization experiment

After the MCF-7 cells were planted in a six-well plate, they were exposed to the 4-2 for 4 h with 1-fold IC_{50} concentration after growing to a suitable concentration. The mitochondria and lysosomes were stained for 30 min according to the manufacturer's instructions for use, and the colocalization effect was analyzed on a confocal microscope.

Lysosomal damage detection

MCF-7 cells were planted in a confocal culture dish with appropriate concentration, and complex 4-2 with the concentration of 1,2,3-fold IC_{50} was added. After incubation for 12 h, acridine orange (5 μM) stained the cells for 30 min, and the experimental results were analyzed with a confocal microscope.

Cathepsin B release

MCF-7 cells were exposed to the drug for 12 h and then stained with 100 nM Magic Red MR-$(RR)_2$ for 15 min. Confocal imaging was performed in the shortest time to observe the release of cathepsin.

Mitochondrial membrane potential analysis

MCF-7 cells were planted in 6-well plates at the rate of 2.5×10^5 /mL per well, exposed to different concentrations of 4-2 for 24 h, and then collected. According to the manufacturer's instructions for use, the cells were stained with

a JC-1 probe for 30 min, and the experimental results were analyzed using flow cytometry.

ROS analysis

After the MCF-7 cells were exposed to the drug for 24 h, the cells were washed twice with PBS and digested with pancreatic enzymes for 5 min. A complete medium was added to terminate digestion and the cells were blown gently and transferred to a 15 mL centrifuge tube for 5 min at 1000 rpm. The cells were suspended and centrifuged with PBS and repeated twice. Next, DCFH-DA was used to dye the cells for 30 min according to the manufacturer's instructions for use. After this, the experimental results were analyzed by flow cytometry.

Western blot

Bax (20 kDa) and Bcl-2 (26 kDa) were detected. GAPDH (42 kDa) was used as the loading control. Antibodies were diluted to 1:4000 for GAPDH, 1:2000 for Bax, and Bcl-2.

MCF-7 cells were cultured with different concentrations of the complexes for 24 h, and the 6-well plates were placed on ice. The cells were washed three times with pre-cooled PBS, and 100 μL lysate was added to each well. After 5 min, the cells were scraped with cell scrapers and transferred to 1.5 mL centrifuge tubes with the tip of a gun. The above-obtained samples were processed by ultrasonic treatment with a cell crusher, the ultrasonic time was 3 s, the intermittent time was 10 s, and the samples were repeated three times. The supernatant was centrifuged at 12000 rpm at 4 ℃ for 15 min. The BCA protein kit determines the protein concentration. Equal amounts of sample protein were mixed with 5× SDS-PAGE loading buffer and heated at 100 °C for 10 min, the proteins were loaded onto SDS-PAGE spacer gel and separated at 120 V for 1 h. After that, the proteins were electrotransferred to PVDF membranes at 100 V for 1 h. The membranes were blocked by blocking buffer (5% nonfat-dried milk) at room temperature for 7 h. Subsequently, the membranes were incubated with primary antibodies which were diluted by TBST containing 2% nonfat-dried milk at 4 °C overnight. Then, the membranes were washed with TBST for 15 min and repeated three times, followed by the treatment with the corresponding HRP-conjugated secondary antibodies which were also diluted by TBST containing 2% nonfat-dried milk at room temperature for 4 h. Afterwards, the membranes were washed with TBST for 15 min and repeated three times and visualized with an Omega Lum C imaging system (Aplegen, USA).

Wound healing experiment

MCF-7 cells were planted in a 6-well plate at an appropriate concentration. After incubation for 24 h, the monolayer cells were scraped off with the tip of a pipette gun and the cells attached to the edge were cleaned with PBS. The expected concentration of complex 4-2 was added to the 6-well plate. The cells were imaged at 0 h and 24 h, respectively.

Clonogenic assay

In the clonogenic assay, the cells in the logarithmic growth period were first

taken, followed by the addition of a culture solution to prepare 1000 cells/mL after digestion and collection. Next, 1 mL of the cell suspension was added to each well of 12-well plates. Thereafter, different concentration of complex 4-2 was added after incubation for 24 h. The culture medium was changed every three days and fixed with paraformaldehyde after 10 days. Furthermore, 0.1% crystal violet staining working solution was added for 30 min and washed three times to remove the background color, and the cloning ability was observed under a microscope.

Antitumor effect of complex 4-2 in xenograft tumor mouse model

Mouse MCF-7 animal experiment: BALB/c-Nu female mice with a body weight of 20-22 g were used for 6-8 weeks. The animals were cared for, tested, and killed according to the principles in the guide for the care and use of laboratory animals. All efforts were made to reduce the pain of mice in the experiment. Before the start of tumor xenotransplantation, mice were domesticated for 7 days after arriving at the animal room, and MCF-7 was collected and suspended in PBS buffer solution to establish a tumor model. All tumors were inoculated subcutaneously in the right armpit. When the tumor volume reached 50-80 mm^3, the mice were randomly divided into two groups with 4 mice in each group. Complex 4-2 (5 mg/kg) was injected into the tumor and normal saline was injected into the control group. Tumor volume and mouse body weight were recorded every two days. The experiment ended on the 24th day, tumors were excised and weighed. The tumor volume was calculated according to the formula $v = ab^2 \times 0.52$, where a is the longest diameter of the tumor and b is the shortest diameter. The results of tumor mean weight was expressed as mean ± standard deviation.

H&E and KI67 staining

1. Paraffin sections were dewaxed to water: the sections were washed in environmentally friendly dewaxing solution I, 10 min-environmentally friendly solution II, 10 min-environmentally friendly dewaxing solution III, 10 min-anhydrous ethanol I, 5 min-anhydrous ethanol II, 5 min-anhydrous ethanol III, 5 min-distilled water successively;
2. Paraffin sections were dewaxed and soaked in citric acid antigen repair buffer to repair the antigen. After natural cooling, the slides were placed in PBS (pH 7.4) and washed by shaking on a decolorizing shaker 3 times, 5 min each time.

3. Blocking endogenous peroxidase: The slices were put into 3% hydrogen peroxide solution, incubated at room temperature and away from light for 25 min, and the slides were placed in PBS (pH 7.4) and washed by shaking on a decolorizing shaking bed for 3 times, 5 min each time.
4. Serum blocking: The tissue was uniformly covered with 3% BSA in the tissue ring and closed at room temperature for 30 min. (Primary antibody of goat origin is blocked with rabbit serum, other sources are blocked with BSA)
5. Add primary antibody: gently shake off the sealing liquid, drop PBS primary antibody in a certain proportion on the section, and place the section flat in a

wet box at 4°C for overnight incubation.
6. Add secondary antibody: The slide was placed in PBS (pH 7.4) and washed by shaking on the decolorizing shaker 3 times, 5 min each time. After the slices were slightly dried, the secondary antibody of the corresponding species of the primary antibody (HRP labeling) was added to cover the tissue in the ring and incubated at room temperature for 50 min.
7. DAB color development: The slide was placed in PBS (pH 7.4) and washed by shaking on the decolorizing shaker 3 times, 5 min each time. After the sections were slightly dried, the DAB color-developing solution freshly prepared was added to the circle. The color developing time was controlled under the microscope. The positive color was brown and yellow, and the sections were washed with tap water to terminate the color development.
8. Restaining of cell nuclei: hematoxylin was restained for about 3 min, washed with tap water, hematoxylin differentiation solution differentiated for several seconds, rinsed with tap water, hematoxylin reverting blue solution reverting blue, and rinsed with running water.
9. Dehydration sealing: the slices were successively dehydrated and transparent in 75% alcohol, 5 min--85% alcohol, 5 min-- anhydrous ethanol ⅰ, 5 min -- anhydrous ethanol ⅱ, 5 min -- n-butanol, 5 min-- xylene Ⅰ, 5 min, and the slices were taken out of xylene to dry slightly and sealed with glue.
10. Microscopy: interpretation of results under a white light microscope.
Tunel
1. Deparaffinize and rehydrate: incubate sections in 2 changes of xylene, 15-20 min each. Dehydrate in 2 changes of pure ethanol for 10 min each, followed by dehydrate in gradient ethanol of 95%, 90%, 80%, and 70% ethanol, respectively, 5 min each (extend deparaffinized time slightly in winter). Wash in distilled water.
2. Antigen retrieval: eliminate obvious liquid, and mark the objective tissue with a liquid blocker pen. Add proteinase K working solution to cover objectives and incubate at 37 ℃ for 25 min. Then wash three times with PBS (pH 7.4) in a Rocker device, 5 min each. (Method for configuring working solution of proteinase K, stock solution: PBS=1:9)
3. Permeabilization: eliminate excess liquid, add permeabilize working solution to cover objective tissue, then incubate at room temperature for 20 min. wash three times with PBS (pH 7.4) in a Rocker device, 5 min each. (The membrane-breaking fluid is 0.1% triton. Configuration method, triron stock solution: PBS=1:1000)
4. Equilibrium at room temperature: After the slices are slightly dried, a buffer is added to the tissues in the circle, and the buffer is incubated at room temperature for 10 min.
5. Tunel reaction: Take the appropriate amount of TDT enzyme, dUTP, and

buffer in the tunel kit according to the number of slices and tissue size and mix at a 1:5:50 ratio. Prepare this reaction solution according to demand before use. Add this mixture to objective tissue placed in a flat wet box, and incubate at 37 ℃ for 2 h. Be sure to keep the wet box moist by adding water.

6. DAPI counterstain in the nucleus: wash three times with PBS (pH 7.4) in a Rocker device, 5 min each. Then incubate with DAPI solution at room temperature for 10 min, and kept in a dark place.
7. Mount: wash three times with PBS (pH 7.4) in a Rocker device, 5 min each. Throw away the liquid slightly, then cover the slip with an anti-fade mounting medium.
8. Microscopic examination and collecting images through a fluorescence microscope. DAPI emits blue light at an ultraviolet excitation wavelength of 330-380 nm and an emission wavelength of 420 nm; FITC has an excitation wavelength of 465-495 nm and an emission wavelength of 515-555 nm and emits green.

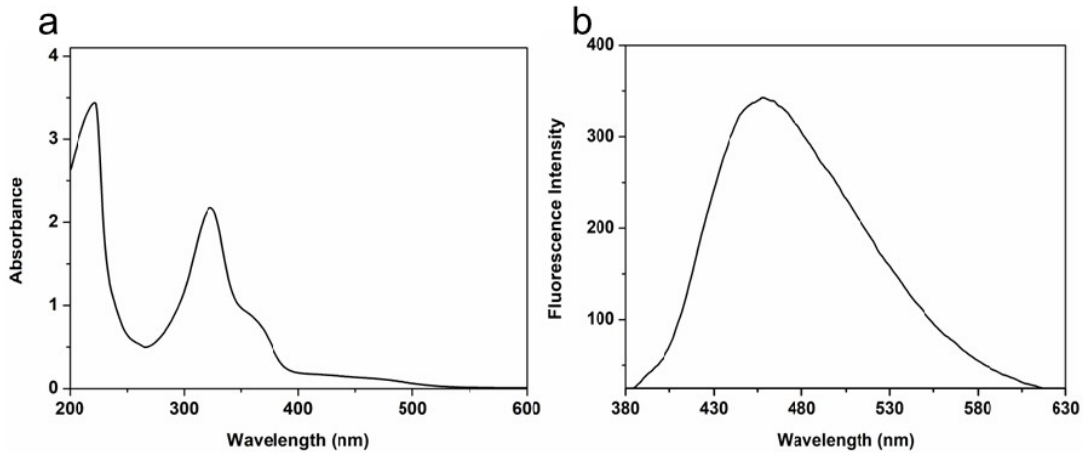

Figure S1. UV-Vis and fluorescence spectra of complex 4-2.

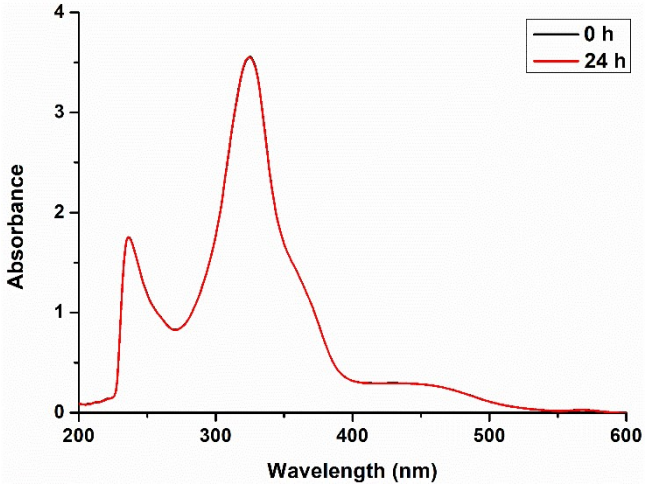

Figure S2. Stability of complex 4-2 at 0 and 24 h in DMEM media.

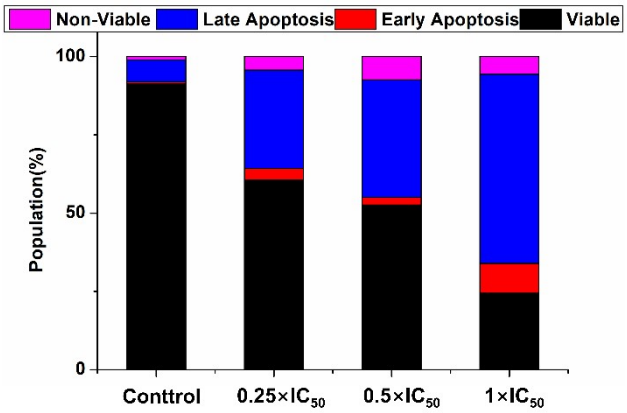

Figure S3. Apoptosis histogram for MCF-7 cells treated with different concentrations of complex 4-2 for 24 h.

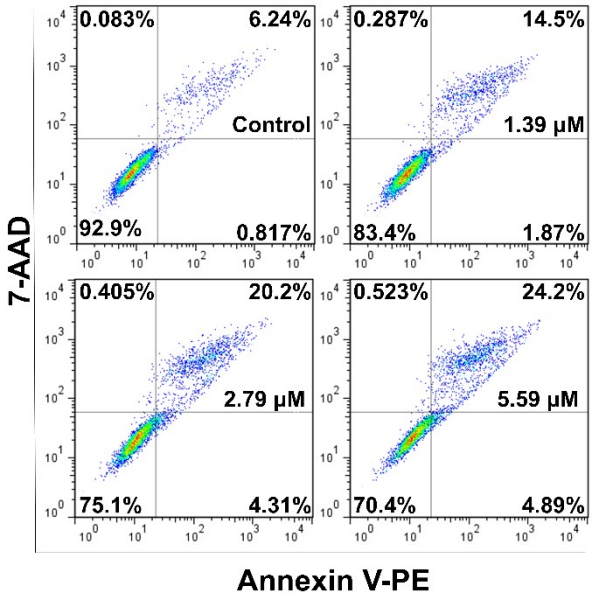

Figure S4. Effects of Cisplatin on apoptosis of MCF-7 cells treated indicated concentrations for 24 h at 310 K. Cells were resuspended in a 500 μL 1×buffer and stained with Annexin V-PE (5 μL) and 7-AAD (10 μL) each for 30 min. The cells were detected by flow cytometry.

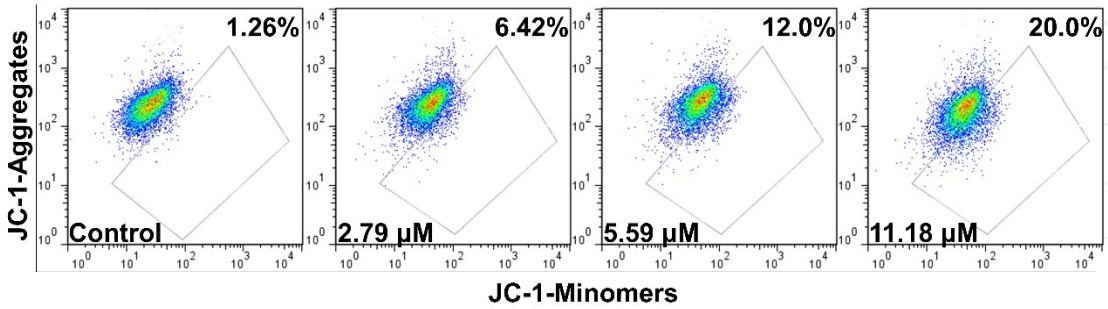

Figure S5. Effects of Cisplatin on the ΔΨm of MCF-7 cells treated indicated concentrations for 24 h at 310 K. Cells were resuspended in

1×buffer and stained by adding a JC-1 probe. The decreased degree of mitochondrial membrane potential was measured by flow cytometry.

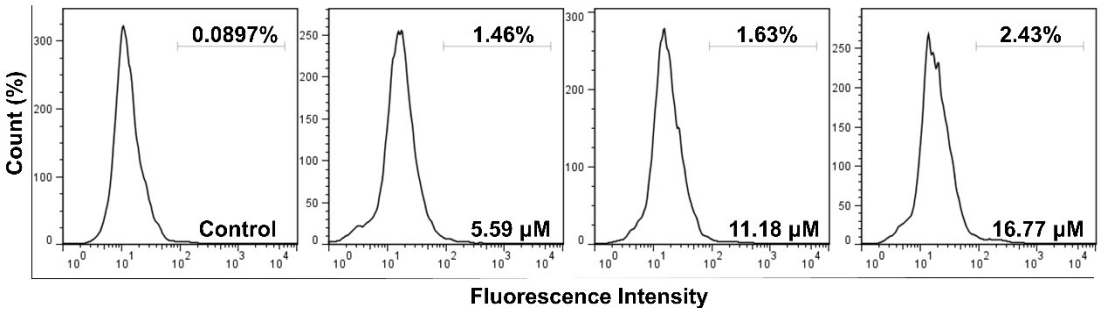

Figure S6. Effects of Cisplatin on ROS of MCF-7 cells treated indicated concentrations for 24 h at 310 K. Cells were resuspended in buffer and stained with DCFH-DA. The level of ROS production was detected by flow cytometry.

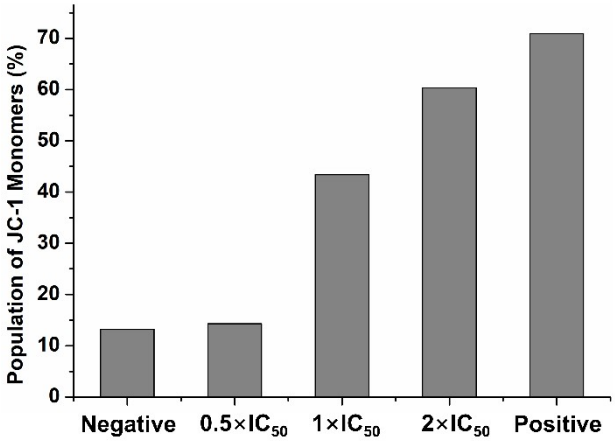

Figure S7. JC-1 histogram for MCF-7 cells treated with different concentrations of complex 4-2 for 24 h.

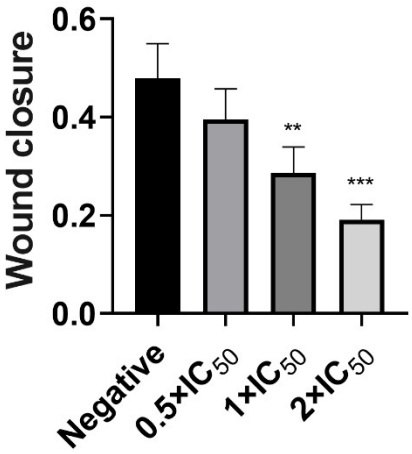

Figure S8. Wound closure histogram for MCF-7 cells treated with different concentrations of complex 4-2 for 24 h.

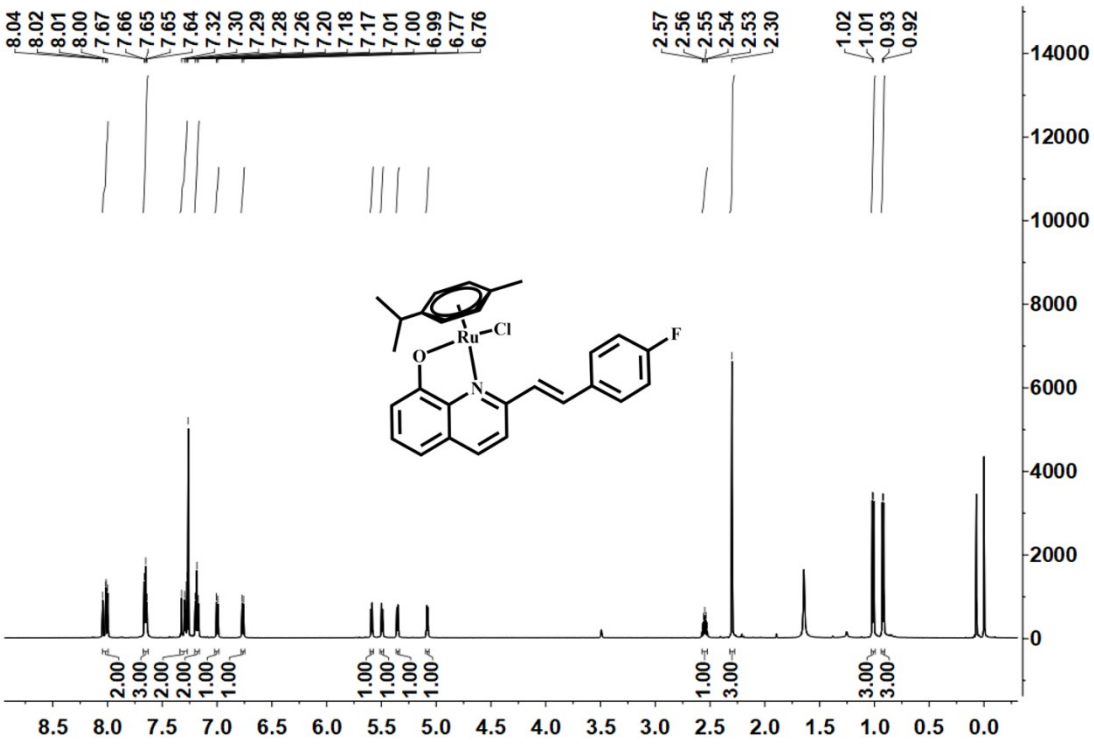

Figure S9. ^{1}H NMR (600 MHz) of complex 4-1

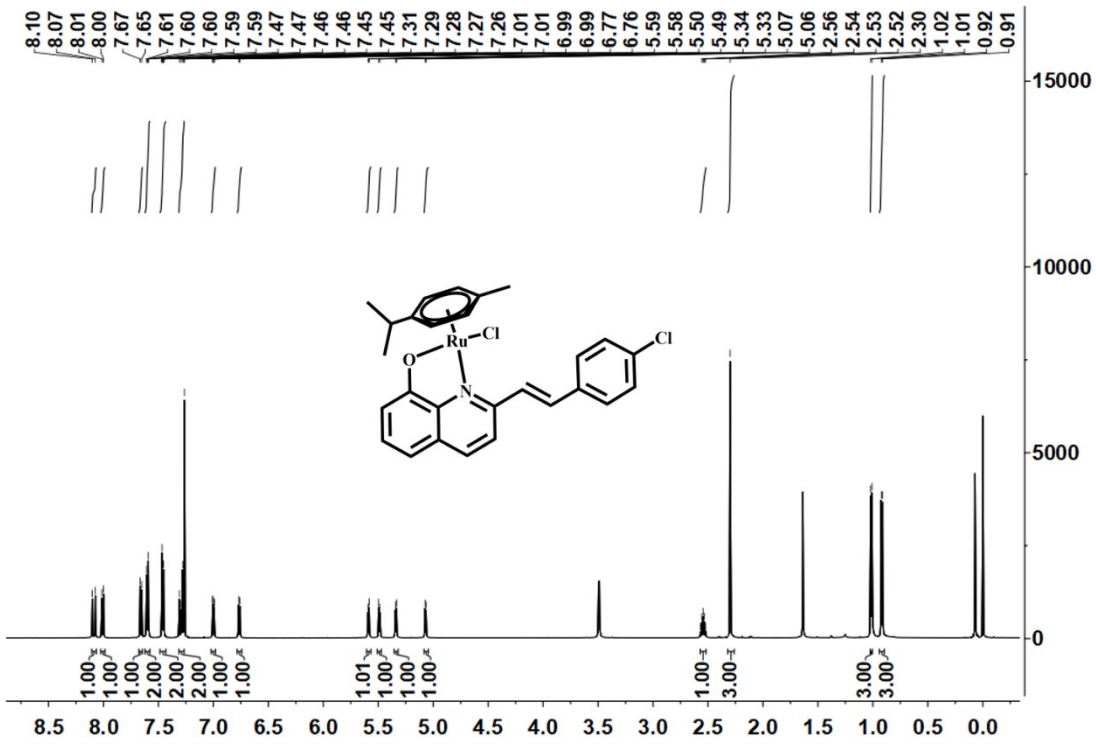

Figure S10. ^{1}H NMR (600 MHz) of complex 4-2

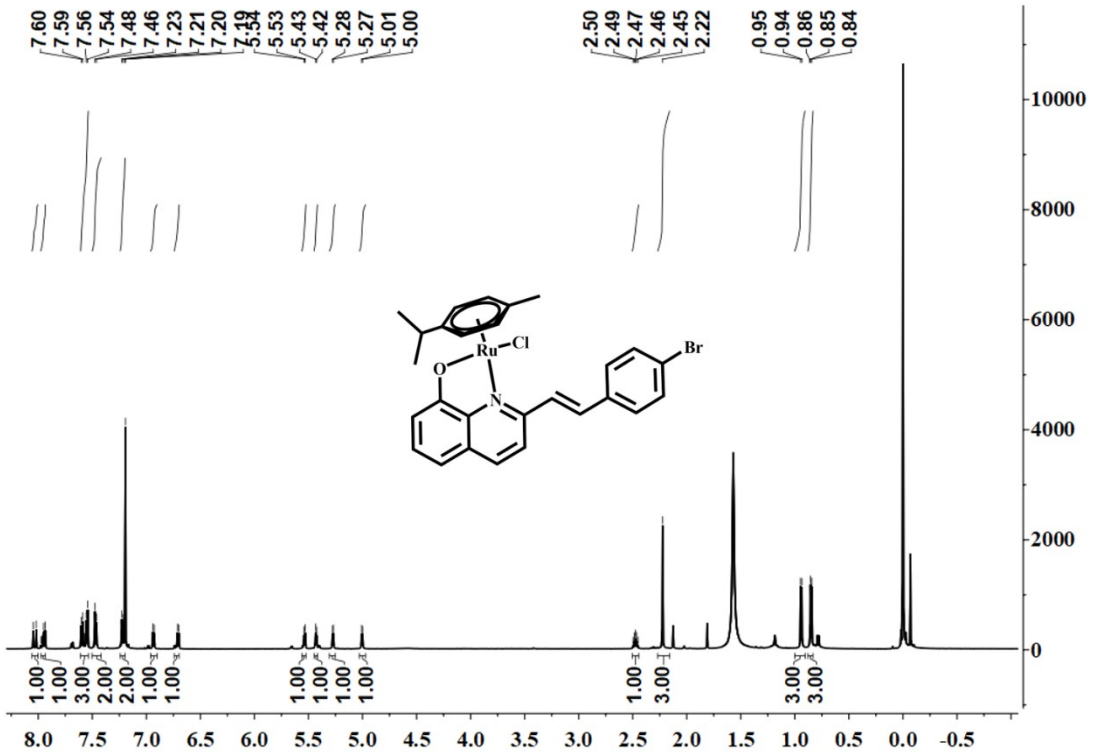

Figure S11. ^{1}H NMR (600 MHz) of complex 4-3

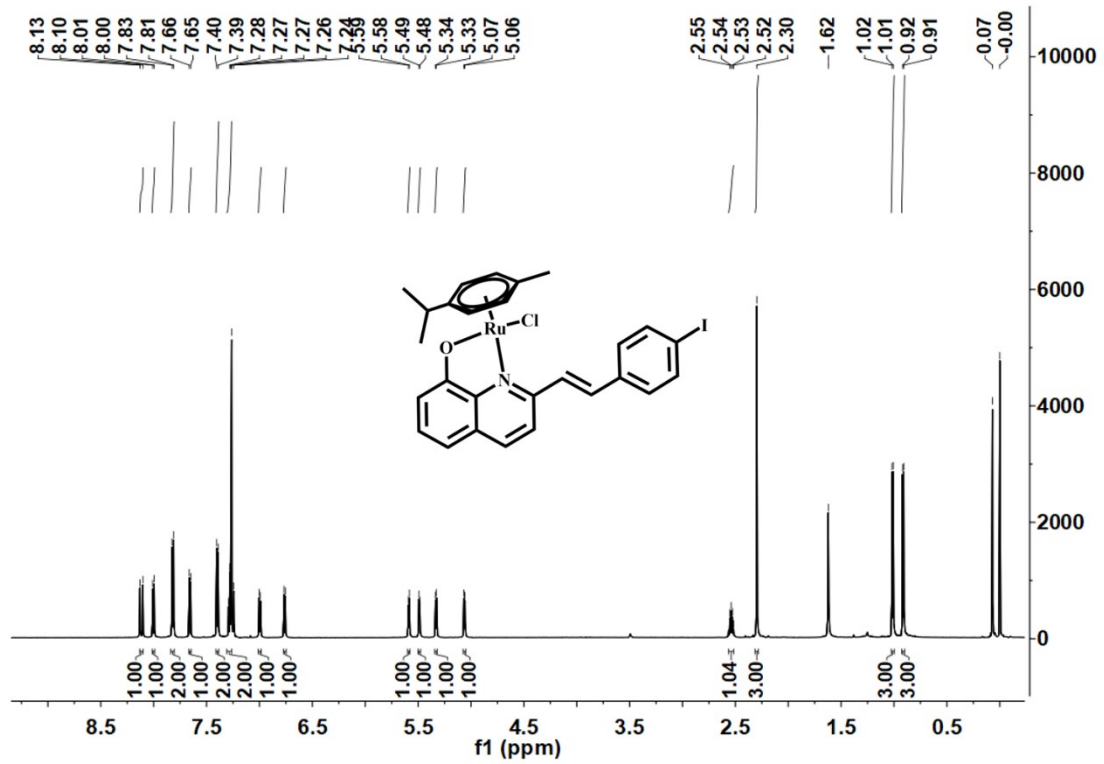

Figure S12. ^{1}H NMR (600 MHz) of complex 4-4

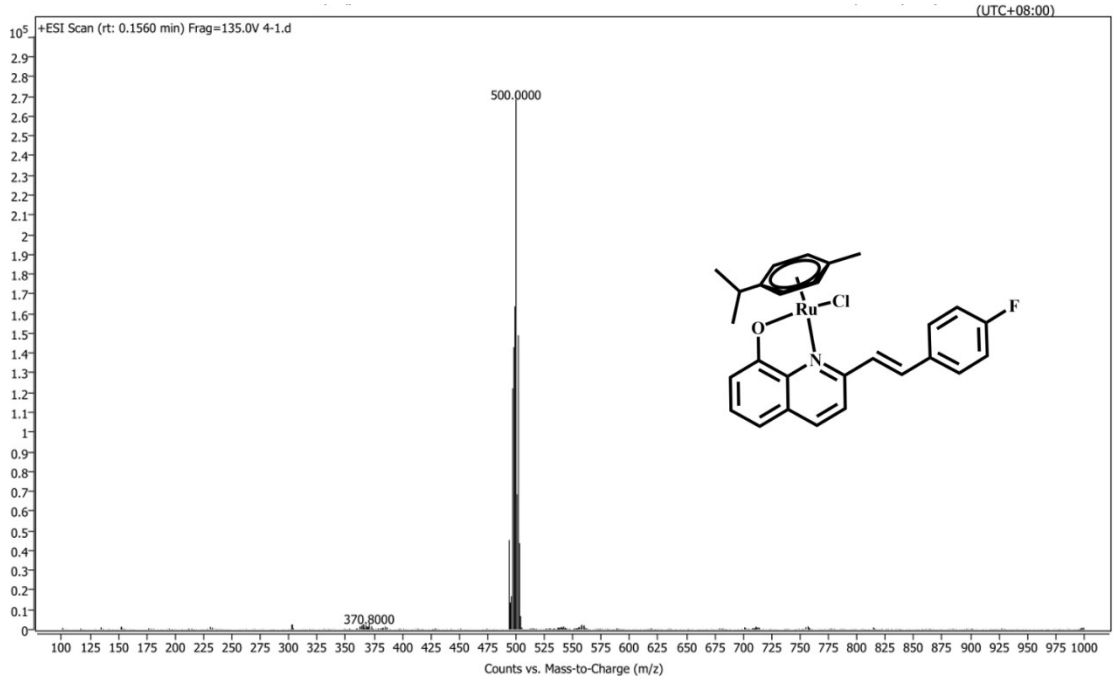

Figure S13. EIS-MS of complex 4-1.

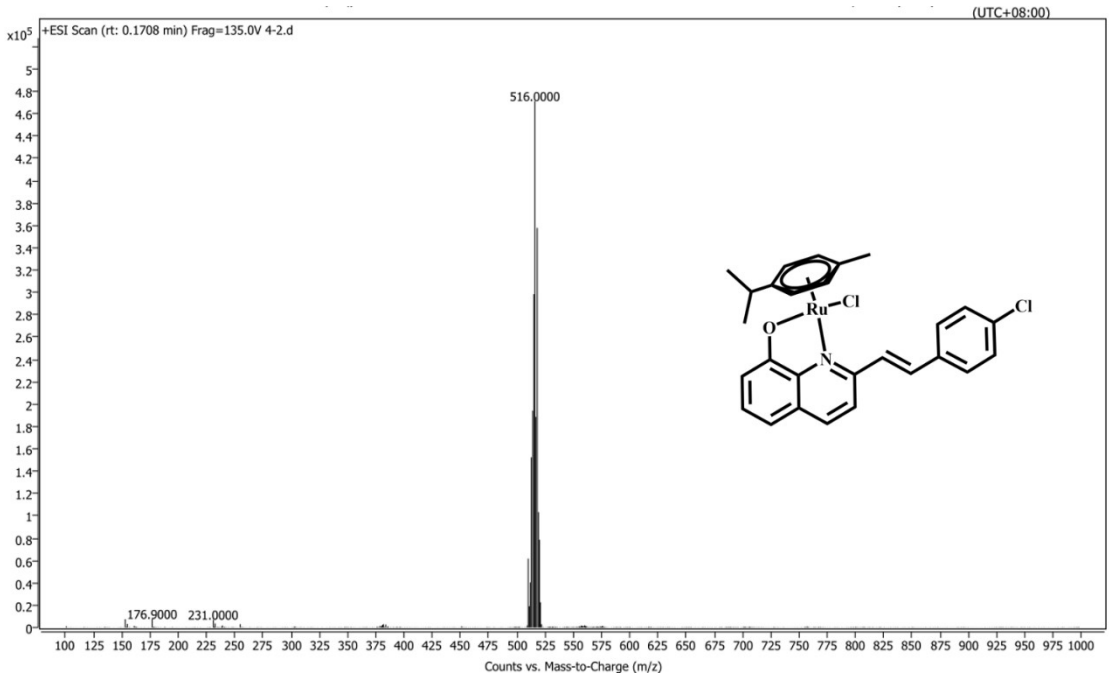

Figure S14. EIS-MS of complex 4-2.

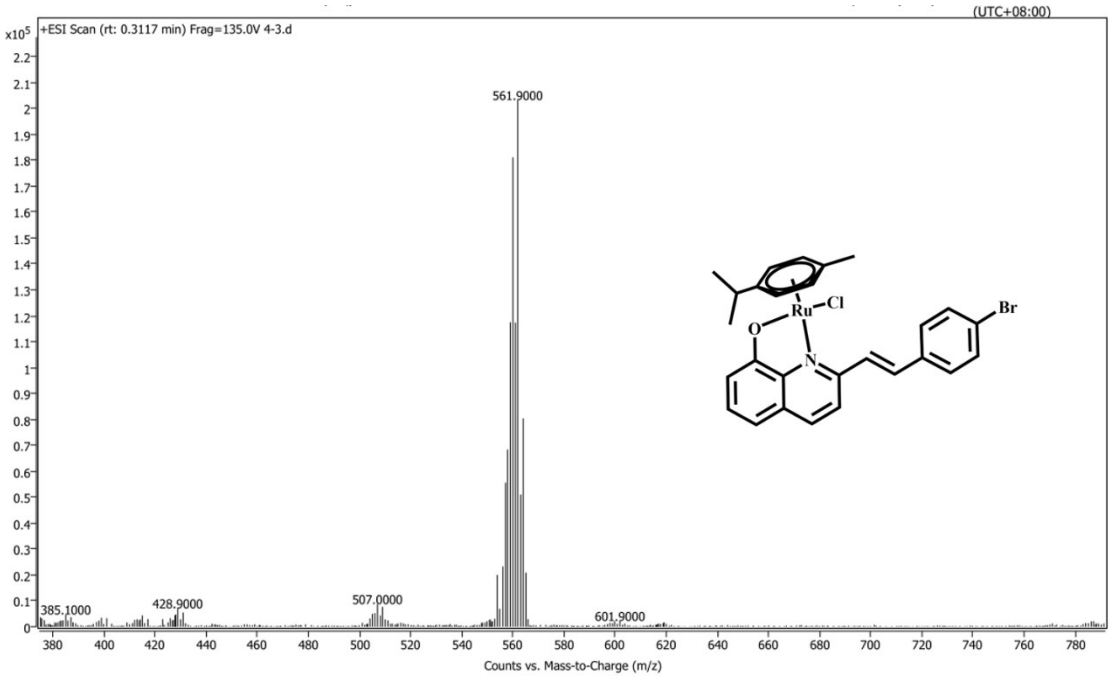

Figure S15. EIS-MS of complex 4-3.

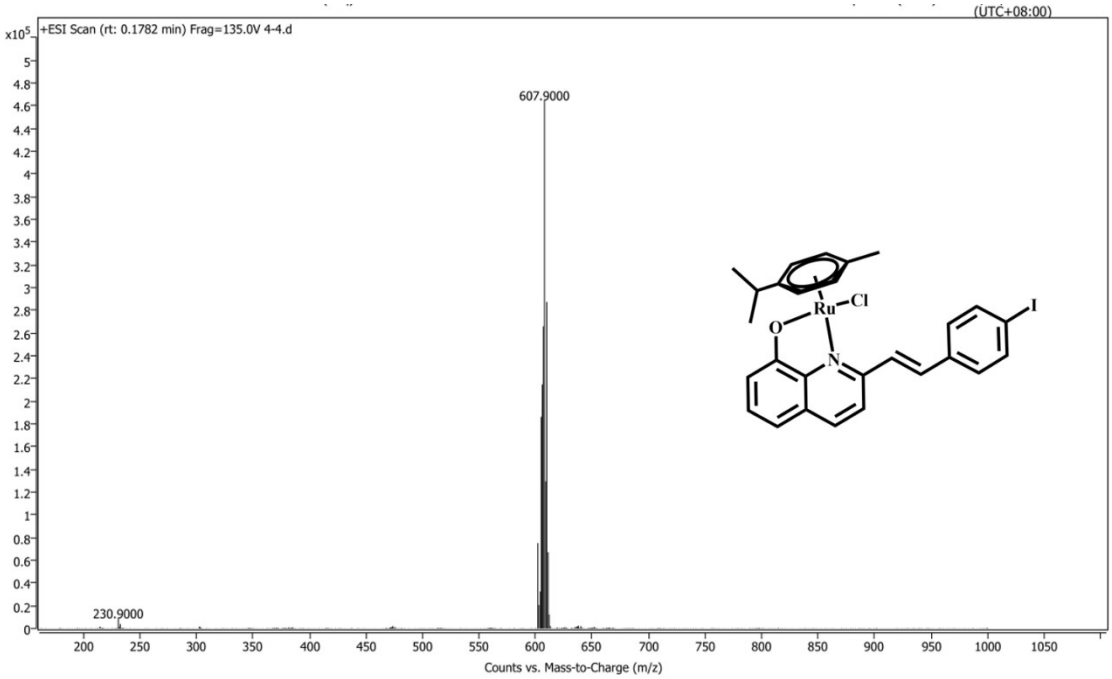

Figure S16. EIS-MS of complex 4-4.