Electronic Supplementary Information

Mitochondria-targeted cyclometalated rhodium(III) complexes：Synthesis, Characterization and Anticancer Research

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Table of Contents

Experimental section S3
Cell lines and culture conditions S3
Lipophilicity S3
Cytotoxicity assay S3
ICP-MS measurement S3
Analysis of MMP S4
Intracellular ATP level S4
Measurement of intracellular ROS S4
Colocalization assay of DCF and MTDR S4
Cell cycle analysis S4
Hoechst 33342 staining S4
Annexin V/PI staining assay S5
Western blotting analysis S5
Statistical analysis S5
Supporting Scheme and Figures S6
Scheme S1. Synthetic routes of ligands **L1–L3**. S6
Scheme S2. Synthetic routes of complexes **Rh1–Rh3.** S6
Fig. S1 ESI-MS spectrum of **Rh1** S7
Fig. S2 ^{1}H NMR spectrum of **Rh1**. S7

Fig. S3 ^{13}C NMR spectrum of **Rh1**. S8
Fig. S4 ESI-MS spectrum of **Rh2**.....S9
Fig. S5 ^{1}H NMR spectrum of **Rh2**. S9
Fig. S6 ^{13}C NMR spectrum of **Rh2**. S10
Fig. S7 ESI-MS spectrum of **Rh3**.....S10
Fig. S8 ^{1}H NMR spectrum of **Rh3**. S10
Fig. S9 ^{13}C NMR spectrum of **Rh3.** S11
Fig. S10 UV/Vis absorption spectroscopy of **Rh1-Rh3**……..…….. S11
Fig. S11 The solution stability of **Rh1-Rh3** S11
Supporting References.....S12

Experimental section

Cell lines and culture conditions

Human cervical carcinoma (HeLa), human lung adenocarcinoma (A549), and human breast carcinoma (MDA-MB-231) were obtained from Experimental Animal Center of Sun Yat-Sen University (Guangzhou, China). Cells were routinely maintained in DMEM medium (Dulbecco's modified Eagle's medium, Gibco BRL), RPMI 1640 (Roswell Park Memorial Institute 1640, Gibco BRL) medium containing 10% FBS (fetal bovine serum, Gibco BRL), 100 μg/mL streptomycin, and 100 U/mL penicillin (Gibco BRL). Cells were incubated in a humidified incubator (Atmosphere: 5% CO_2 and 95% air; Temperature: 37 °C). Cisplatin-resistant A549R cells were cultured in RPMI 1640 with cisplatin to maintain the resistance.

Lipophilicity

The lipophilicity of the Rhodium(III) complexes, determined as log $P_{o/w}$ values, was determined according to a reported procedure [1].

Cytotoxicity assay

The cells were cultured in 96-well culture plates at a density of 10^4 cells per well. After 12 h incubation, the cells were treated with a series of different concentrations of the compounds. After incubation for 68 h, 20 μL MTT (5 mg/mL) was added to each well. The plates were incubated for an additional 4 h. Then, the medium was carefully removed and DMSO (150 μL) was added to each well. For ROS or caspase inhibitor experiments, HeLa cells were pre-incubated with 10 mM NAC or 50 μM z-VAD-FMK for 1 h, and then treated with various concentrations of **Rh2** for 24 h, the cell viability was determined by measurement of the absorbance at 570 nm (Infinite F200, Tecan, Switzerland).

ICP-MS measurement

The cellular uptake capacity of the rhodium complexes was measured according to the method reported with slight modifications[2]. Briefly, HeLa cells were treated with the Rh (III) complexes (5 μM) for 8 h. After that, the cells were trypsinized and collected in PBS (3 mL). Mitochondria were isolated from Rh(III)-treated cells using the mitochondria isolation kit according to the manufacturer's instructions. Nuclear and cytosolic fractions were separated using a nucleoprotein extraction kit according to the manufacturer's instructions. The radio immunoprecipitation assay (RIPA) buffer was used for cell lyses. The concentrations of rhodium in mitochondria, nuclear and cytosolic fractions were measured using the ICP-MS. Cells treated without Rh complexes were detected as control.

Analysis of MMP

HeLa cells were cultured in 6 well plates and treated with **Rh2** at the indicated concentrations for 12 h. Then cells were stained with JC-1 and analyzed in a flow cytometer. Fluorescence was monitored by measuring both the monomer (530±20 nm emission; green) and the aggregate (590±20 nm emission; red) forms of JC-1 following excitation at 488 nm. Red and green MFI were analyzed using FlowJo 7.6 software (Tree Star, USA). 10, 000 events were acquired for each sample.

Intracellular ATP level

Cellular ATP levels were measured using the CellTiter-Glo® Luminescent Cell Viability Assay kit according to the manufacturer's instructions. HeLa cells were seeded in 96 well plates and treated with **Rh2** at the indicated concentrations for 12 h. These samples were equilibrated in PBS at room temperature for 30 min. The CellTiter-Glo® reagent was added and the plate was incubated for 10 min. The luminescence was acquired using a microplate reader.

Measurement of intracellular ROS

HeLa cells seeded into 6 well plates were treated with **Rh2** at the indicated concentrations for 12 h. Cells were stained with H_2DCFDA (10 μM) for 30 min at 37 °C in the dark. Then cells were collected and washed twice with serum-free DMEM. The fluorescence intensity of DCF in HeLa cells was measured by flow cytometry. λex = 488 nm; λem = 530 ± 20 nm.

Colocalization assay of DCF and MTDR

Hela cells were treated with complexes **Rh2** at the indicated concentrations for 6 h. The colocalization of the fluorescence of DCF and MTDR was obtained as previously described [3]. MTDR was excited at 633 nm, with emission was collected at 650 ± 20 nm. DCF was excited at 488 nm with emission collected at 530 ± 20 nm.

Cell cycle analysis

HeLa Cells were cultured in 6 well plates and treated with **Rh2** at the indicated concentrations incubated for 24 h. The cells were then collected and fixed in 1 mL of 70% aqueous ethanol (v/v). After overnight storage at -20 °C, cells were centrifuged (10 min at 1000 g) and washed twice with PBS, and then resuspended in 500 μL PBS containing PI (50 μg/mL) and DNase-free RNase (100 μg/mL). Data were acquired by a flow cytometer and analyzed by ModFit LT 2.0software. 10, 000 events were obtained for each sample. PI: λ_{ex} = 488 nm; λ_{em} = 590 ± 20 nm.

Hoechst 33342 staining

HeLa cells were cultured in 35 mm dishes for confocal microscopy to ensure about 70% confluence. Then, cells were treated with **Rh2** at the indicated concentrations incubated for 24 h. After fixed with 4% paraformaldehyde for 10 min at room temperature, the cells were washed with PBS twice and

labelled with Hoechst 33342 (5 μg/mL, 10 min) in the dark. The samples were washed with PBS again and surveyed by confocal microscopy. Hoechst 33342: λex = 405 nm, λem = 460 ± 20 nm.

Annexin V/PI staining assay

The assay was carried out according to the manufacturer's protocol. Cells were treated with **Rh2** for 24 h and labelled with annexin V and PI reagent at room temperature for 30 min in the dark. The samples were immediately analyzed by flow cytometry. FITC: λ_{ex} = 488 nm; λ_{em} = 530 ± 20 nm. PI: λ_{ex} = 488 nm; λ_{em} = 590 ± 20 nm.

Casepase-3/7 activity assay

Caspase-3/7 activity was detected using the Caspase-Glo® Assay kit according to the manufacturer's instructions. Cells cultured in 96 well plates were treated with **Rh2** for 6 h. Then, 100 μL of Caspase Glo® 3/7 reagent was added to each well containing 100 μL of culture medium. The mixture was incubated at room temperature for 1 h and then luminescence was measured using a micro-plate reader.

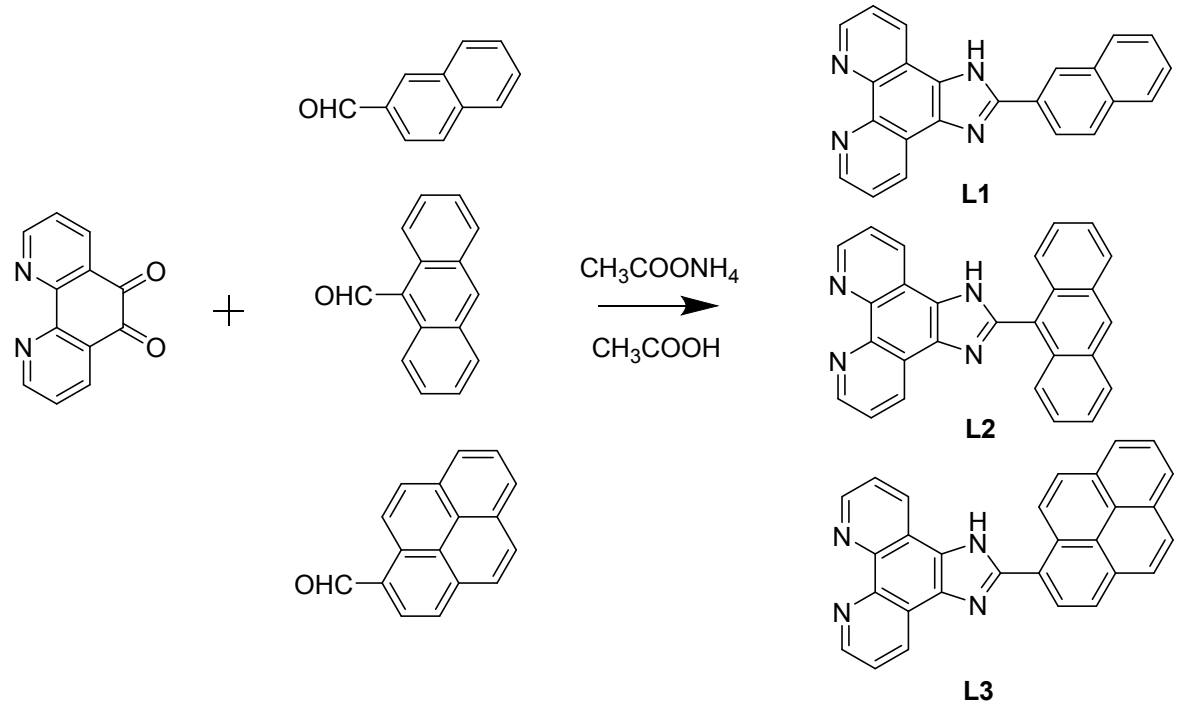
Western blotting analysis

HeLa cells were cultured in 6-well plates and incubated for 24 h, and then treated with **Rh2** complexes at indicated concentrations for 24 h. Cells were harvested and washed with ice-cold PBS twice, and then were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with inhibitors of proteases and inhibitor of phosphatases sodium orthovanadate. Equal amounts of cellular total proteins (20-80 μg) were separated on SDS-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes. Membranes were blocked in 10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20 (TBST, Sigma Aldrich, Missouri, USA) containing 5% nonfat dried milk, and then incubated with the primary antibodies specific to β-actin, P53, Bcl-2, Bax and cytochrome C. After a subsequent washing step, the membrane was incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The immunoreactivity was measured using the enhanced chemiluminescence detection kit. Images were acquired using a FluorChem Mi maging station and analyzed manually in AlphaView software (ProteinSimple, CA, USA)

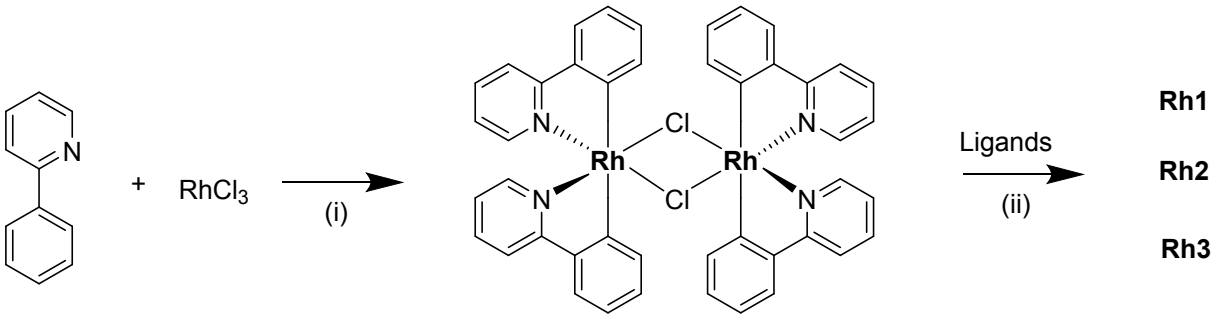
Statistical analysis

All biological experiments were performed at least twice with triplicates in each experiment. Representative results were depicted in this report and data were presented as means ± standard deviations.

Supporting Scheme and Figures



Scheme S1. Synthetic routes of ligands **L1–L3.**



Condition: (i) $CH_3CH_2OCH_2CH_2OH/H_2O$, N_2, 24 h, 135 ^oC

(ii) CH_3OH/CH_2Cl_2, N_2, 4 h, 50 ^oC

Scheme S2. Synthetic routes of complexes **Rh1–Rh3.**

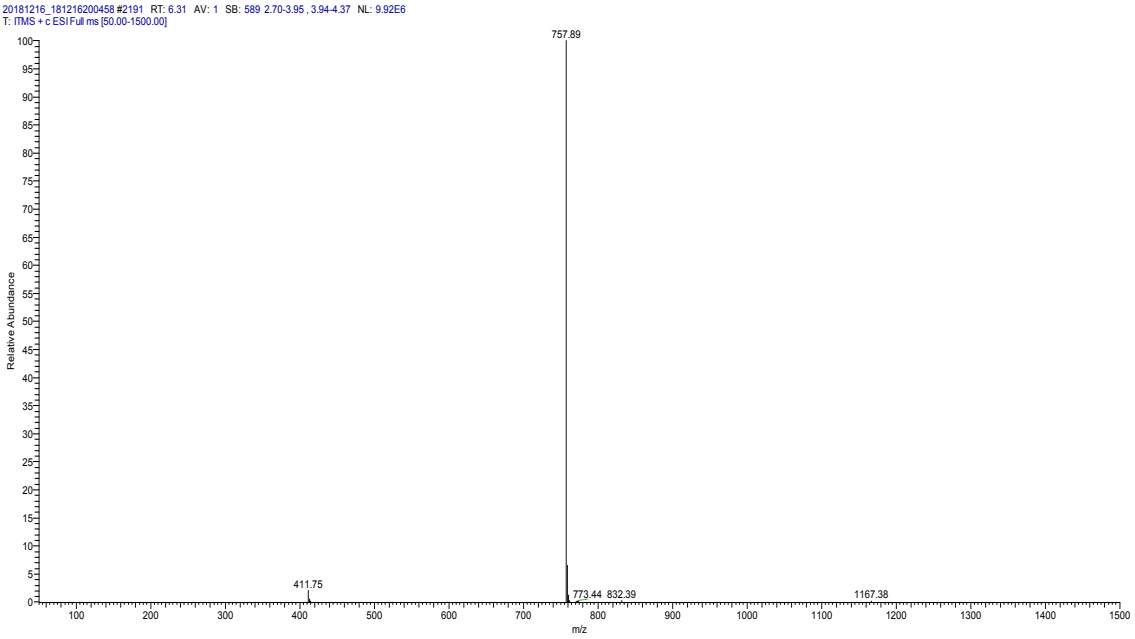


Fig. S1 ESI-MS spectrum of **Rh1**

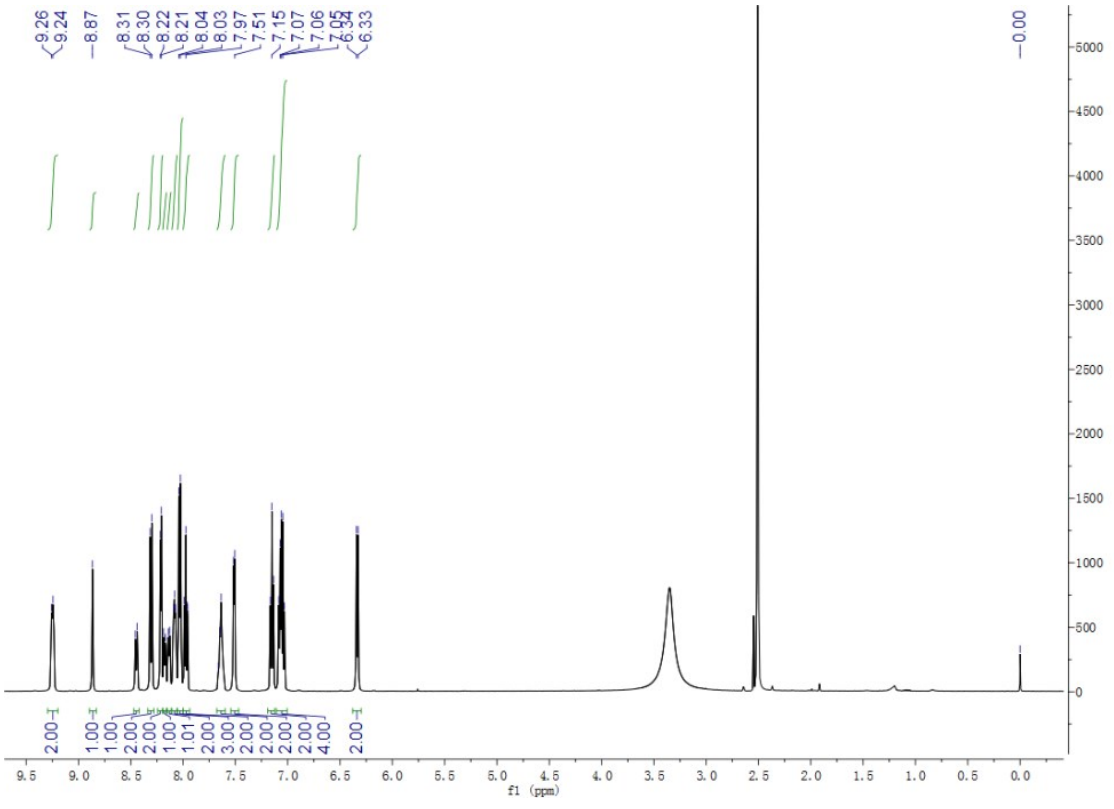


Fig. S2 ^{1}H NMR spectrum of **Rh1.**

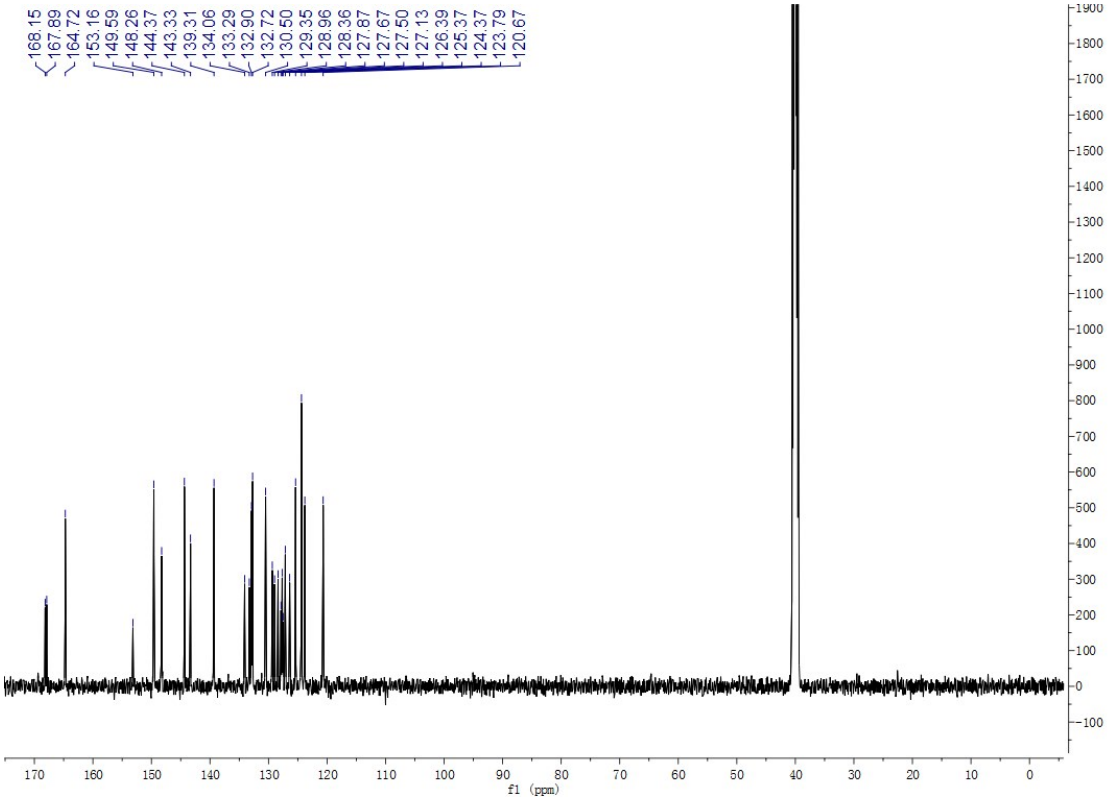


Fig. S3 ESI-MS spectrum of **Rh1.**

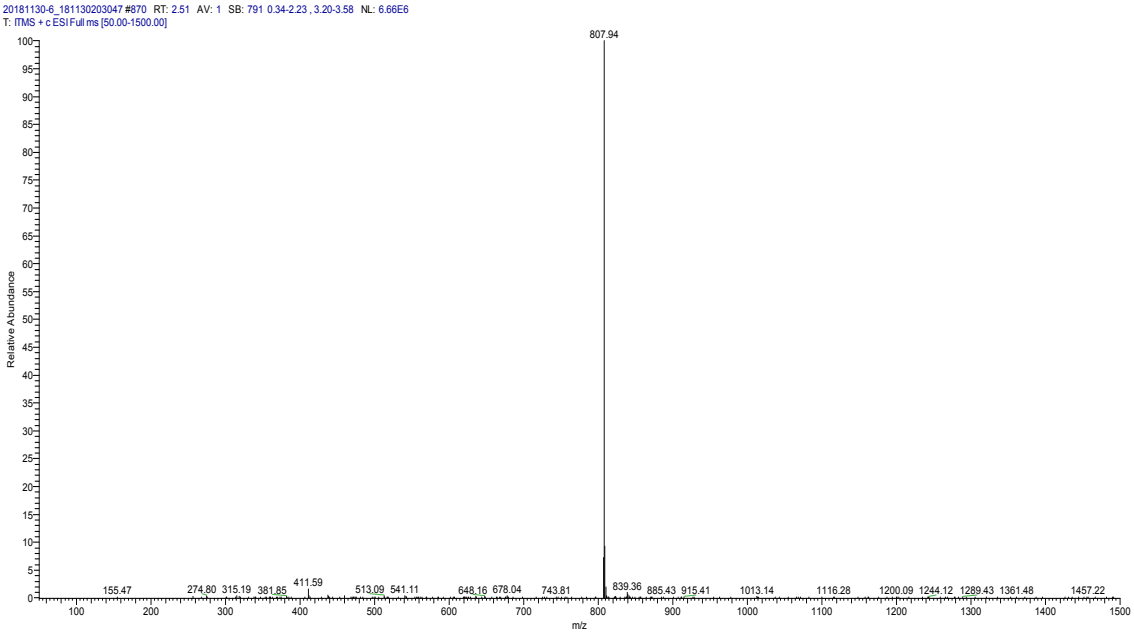


Fig. S4 ESI-MS spectrum of **Rh2.**

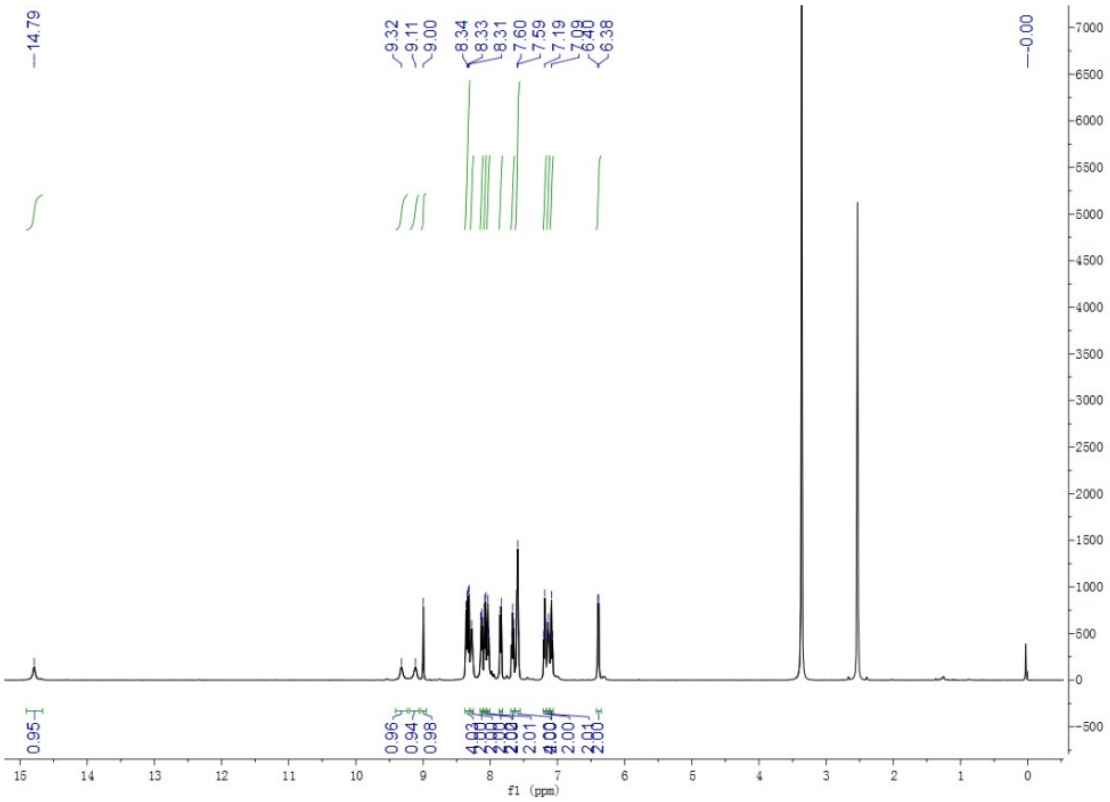


Fig. S5 ^{1}H NMR spectrum of **Rh2.**

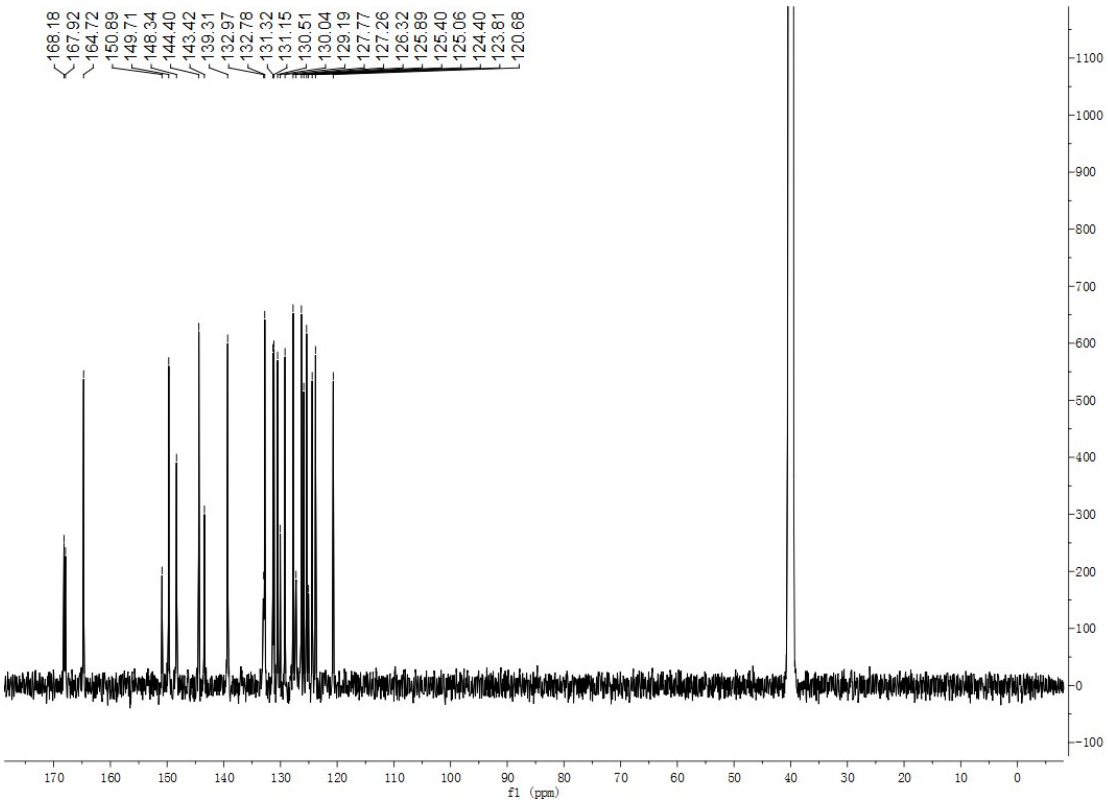


Fig. S6 ^{13}C NMR spectrum of **Rh2.**

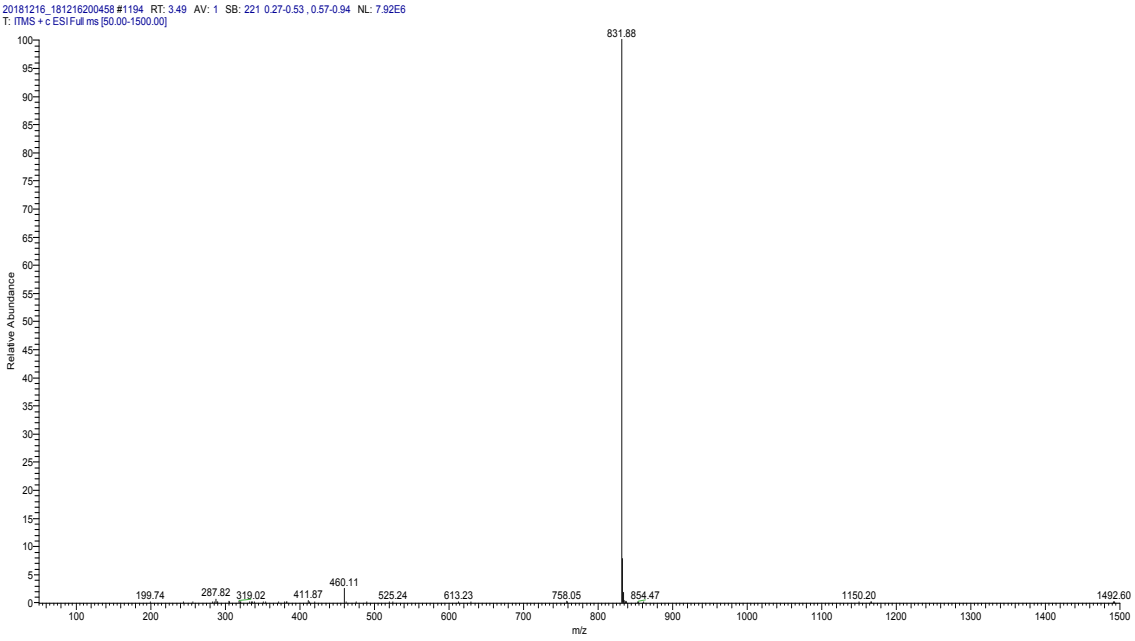


Fig. S7 ESI-MS spectrum of **Rh3.**

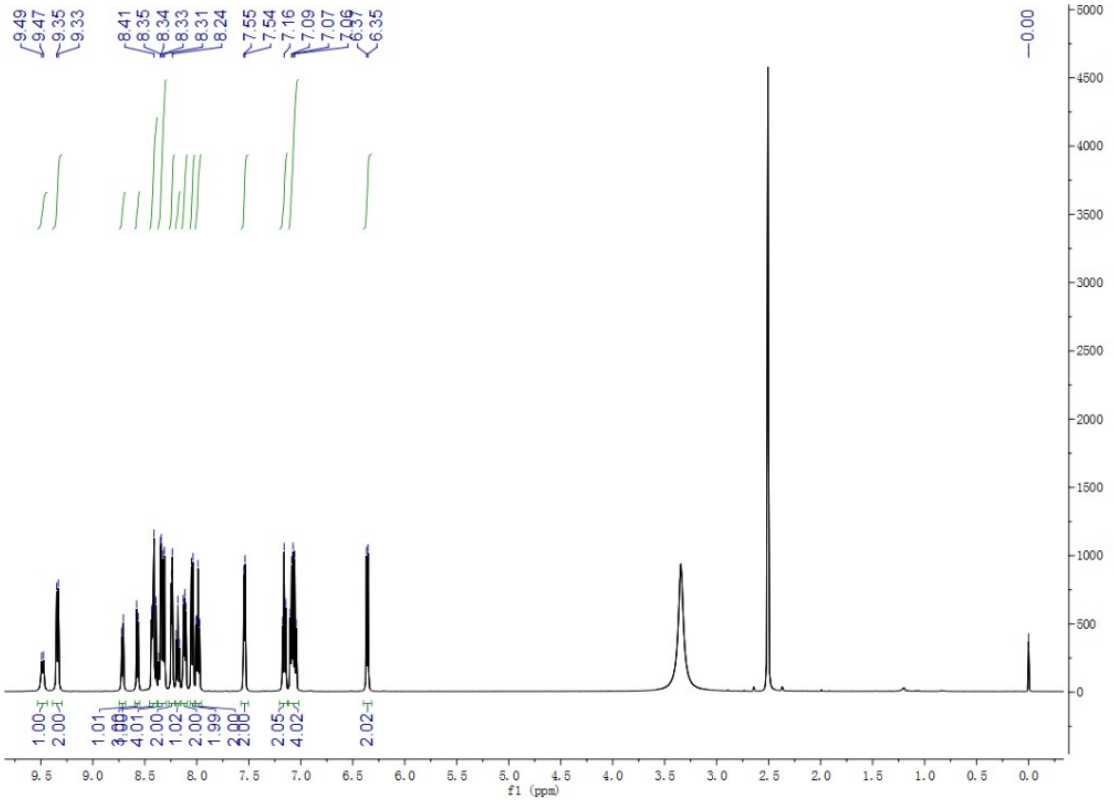


Fig. S8 ^{1}H NMR spectrum of **Rh3.**

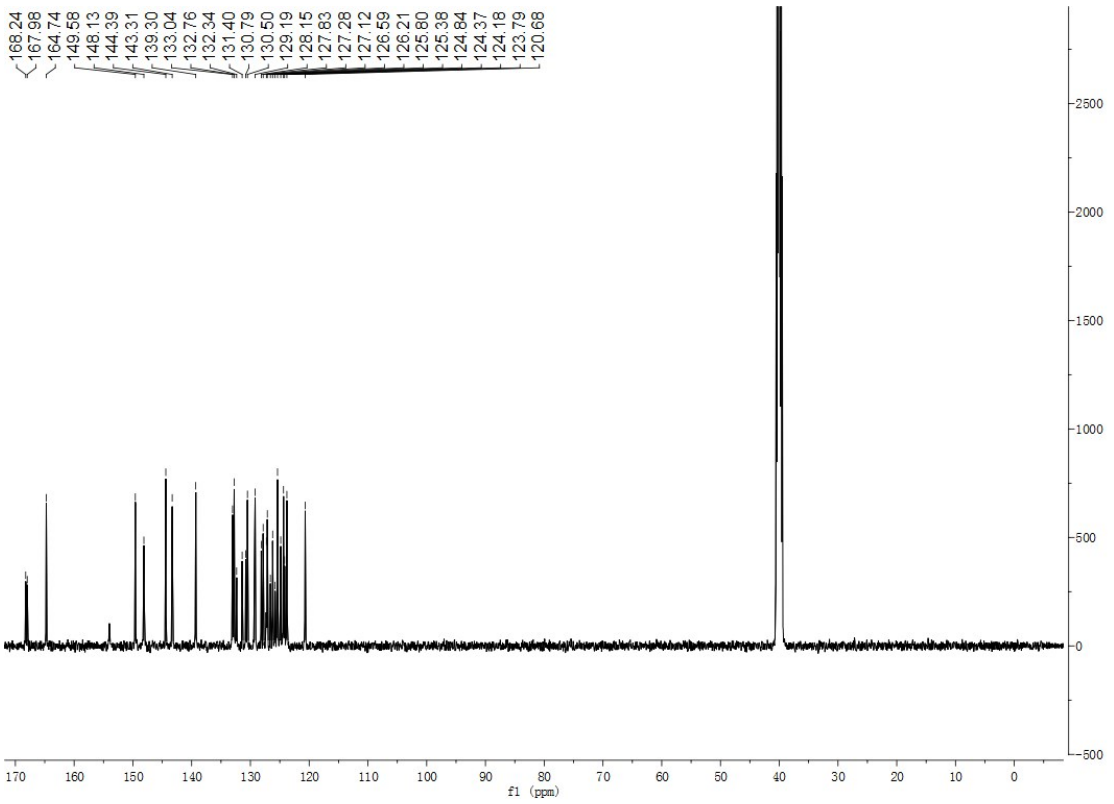


Fig. S9 ^{13}C NMR spectrum of **Rh3.**

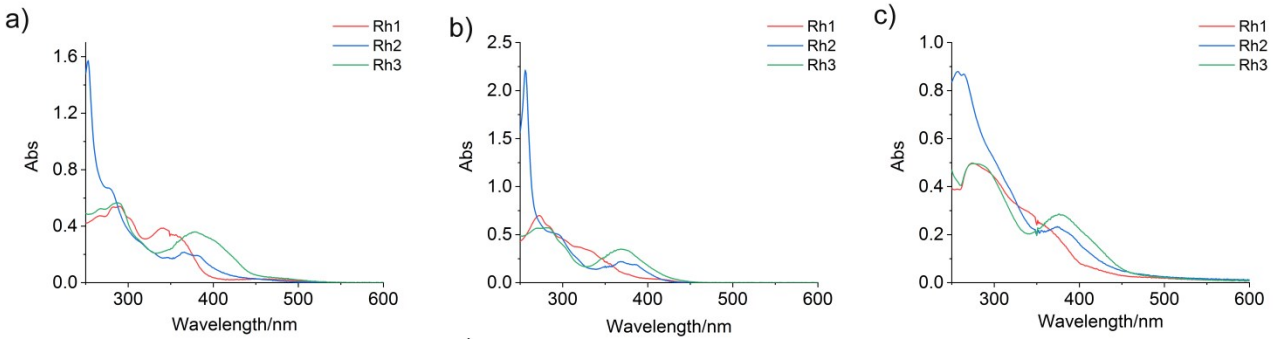


Fig. S10 UV-Vis spectra of **Rh1, Rh2** and **Rh3** in CH_3CN (a), CH_2Cl_2 (b) and PBS (c)**.**

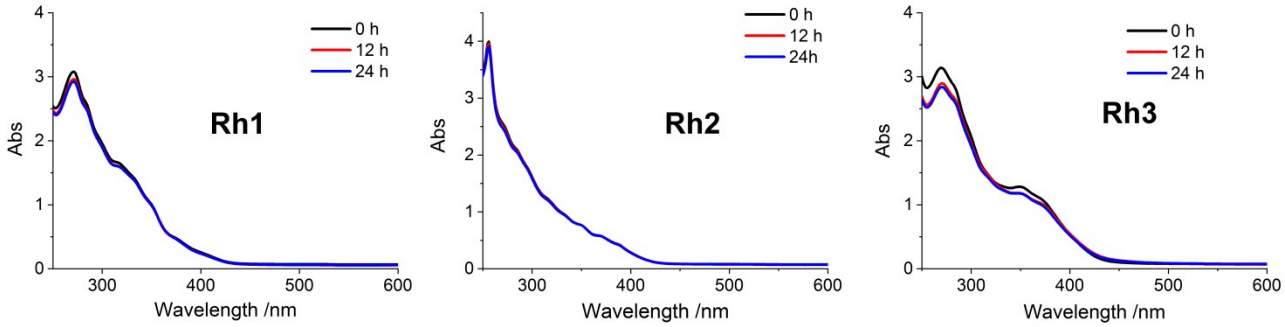


Fig. S11 Time-dependent UV/Vis absorption spectroscopy of Rh1-Rh3 aqueous solution measured in H_2O. **Rh1** (10 μM) , **Rh2** (10 μM) and **Rh3** (10 μM).

Supporting References

1. F. X. Wang, M. H. Chen, X. Y. Hu, R. R. Ye, C. P. Tan, L. N. Ji and Z. W. Mao, *Sci Rep*, 2016, **6**, 38954.
2. 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.
3. Y. Zheng, D. Y. Zhang, H. Zhang, J. J. Cao, C. P. Tan, L. N. Ji and Z. W. Mao, *Chem. - Eur. J.*, 2018, **24**, 18971-18980.