Rhodium(III) complexes with isoquinoline derivatives as potential anticancer agents: *in vitro* and *in vivo* activity studies

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Table S1: Crystal structure data and refinement details of Rh1 and Rh2

	Rh1	**Rh2**
Empirical formula	$C_{19}H_{24}Cl_3N_2O_3RhS$	$C_{18}H_{22}Cl_3N_2O_2RhS$
Formula weight (*M*)	569.72	539.70
Crystal system	Monoclinic	Monoclinic
Space group	$P2_1/c$	$P2_1/c$
a (Å)	7.1112 (7)	11.293 (3)
b (Å)	22.275 (2)	19.617 (3)
c (Å)	14.0569 (14)	10.5135 (17)
α (°)	90.00	90.00
β (°)	94.284(7)	117.668(2)
γ (°)	90.00	90.00
V/(Å^3)	2220.5 (4)	2062.7(7)
Z	4	4
D_c(Mg m^{-3})	1.704	1.738
F(000)	1152	1088
θ range for data collection (°)	3.9–25.4°	2.3–26.5°
Reflections collected / unique	4524/3063	22138/3773
Rint	0.049	0.041
Goodness-of-fit on F^2	1.068	1.104
Final *R* indices [$I > 2\sigma(I)$]	R_1= 0.0510	R_1= 0.0238
	ωR_2= 0.0958	ωR_2= 0.0638
R indices (all data)	R_1 = 0.0877	R_1 = 0.0296
	ωR_2= 0.1148	ωR_2= 0.0680

Table S2: Selected Bond Length and angle of Rh1 and Rh2

Rh1			
Rh1—Cl1	2.3327 (15)	S1—Rh1—Cl3	89.72 (6)
Rh1—Cl2	2.3585 (13)	N2—Rh1—Cl1	95.11 (12)
Rh1—Cl3	2.3482 (14)	N2—Rh1—Cl2	174.98 (12)
Rh1—S1	2.2799 (15)	N2—Rh1—Cl3	84.72 (12)
Rh1—N2	2.065 (3)	N2—Rh1—S1	91.87 (12)
Rh1—N1	2.104 (4)	N2—Rh1—N1	82.32 (15)
Cl1—Rh1—Cl2	89.89 (5)	N1—Rh1—Cl1	89.54 (12)
Cl1—Rh1—Cl3	179.71 (5)	N1—Rh1—Cl2	98.17 (11)
Cl3—Rh1—Cl2	90.27 (5)	N1—Rh1—Cl3	90.68 (12)
S1—Rh1—Cl1	90.04 (6)	N1—Rh1—S1	174.12 (10)
S1—Rh1—Cl2	87.70 (5)		
Rh2			
Rh1—Cl1	2.3699 (7)	S1—Rh1—Cl3	88.89 (3)
Rh1—Cl2	2.3545 (8)	N1—Rh1—Cl1	85.94 (6)
Rh1—Cl3	2.3418 (7)	N1—Rh1—Cl2	177.45 (6)
Rh1—S1	2.2653 (7)	N1—Rh1—Cl3	92.32 (6)
Rh1—N1	2.089 (2)	N1—Rh1—S1	92.79 (6)
Rh1—N2	2.085 (2)	N2—Rh1—Cl1	88.77 (6)
Cl2—Rh1—Cl1	91.69 (3)	N2—Rh1—Cl2	95.55 (6)
Cl3—Rh1—Cl1	177.77 (3)	N2—Rh1—Cl3	92.44 (6)
Cl3—Rh1—Cl2	90.07 (3)	N2—Rh1—S1	176.09 (6)
S1—Rh1—Cl1	89.79 (2)	N2—Rh1—N1	83.48 (8)
S1—Rh1—Cl2	88.13 (2)		

Table S3. Hydrogen bonds for Rh1 [Å and °].

D-H...A	d(D-H)	d(H...A)	d(D...A)	<(DHA)
N(1)-H(1A)...Cl(2)#1	0.89	2.62	3.409(4)	148.7
N(1)-H(1A)...O(3)	0.89	2.33	2.988(8)	131.3
N(1)-H(1B)...Cl(3)#2	0.89	2.58	3.421(4)	157.7
C(2)-H(2)...Cl(2)#1	0.93	2.77	3.570(6)	144.4
C(4)-H(4)...O(1)#5	0.93	2.49	3.288(8)	143.6
C(14)-H(14A)...Cl(3)#3	0.97	2.71	3.527(5)	141.7
C(15)-H(15A)...Cl(2)	0.97	2.67	3.416(6)	133.8
C(18)-H(18C)...Cl(1)#4	0.96	2.69	3.574(7)	153.1

Symmetry transformations used to generate equivalent atoms:

#1 x-1,y,z #2 -x+1,-y+2,-z+1 #3 -x+2,-y+2,-z+1

#4 x,-y+3/2,z+1/2 #5 1-x,-y,2-z

Table S4. Hydrogen bonds for Rh2 [Å and °].

D-H...A	d(D-H)	d(H...A)	d(D...A)	<(DHA)
N(1)-H(1A)...Cl(1)#1	0.89	2.50	3.342(2)	158.6
N(1)-H(1B)...O(2)	0.89	2.37	3.031(3)	131.5
C(10)-H(10)...O(2)#2	0.93	2.43	3.258(3)	147.6
C(15)-H(15B)...Cl(2)	0.97	2.62	3.352(3)	132.9
C(16)-H(16B)...Cl(2)#3	0.96	2.74	3.621(3)	153.1

Symmetry transformations used to generate equivalent atoms:

#1 -x,-y+1,-z+1 #2 -x,y+1/2,-z+3/2 #3 -x+1,y+1/2,-z+3/2

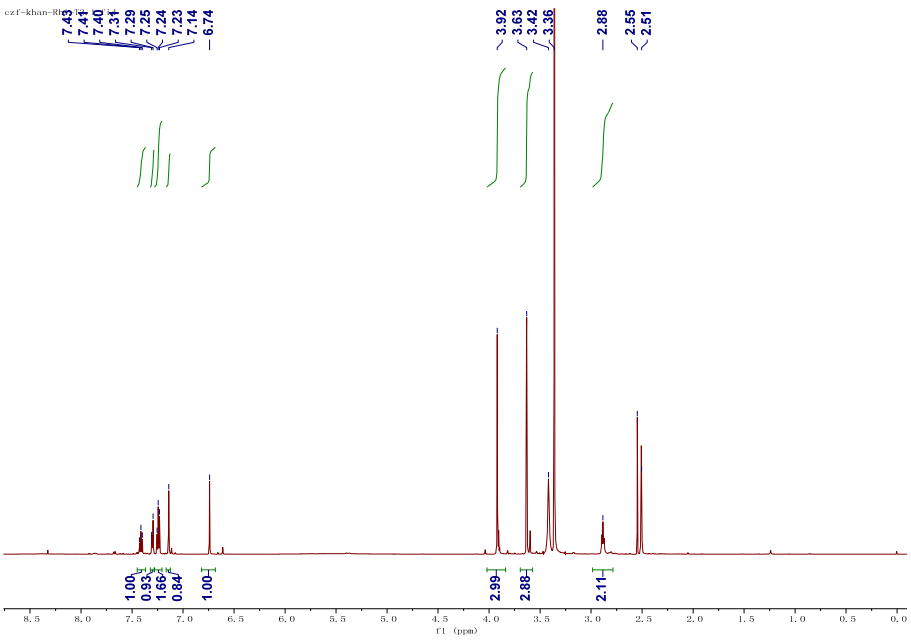

Figure S1: ^{1}H-NMR Spectrum of Rh1

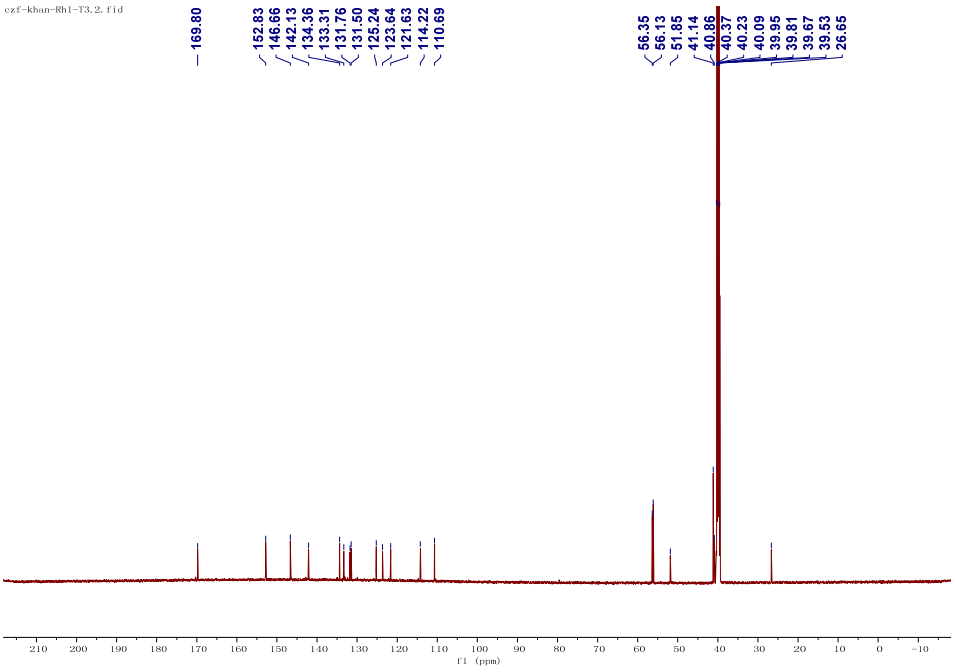

Figure S2: ^{13}C-NMR Spectrum of Rh1

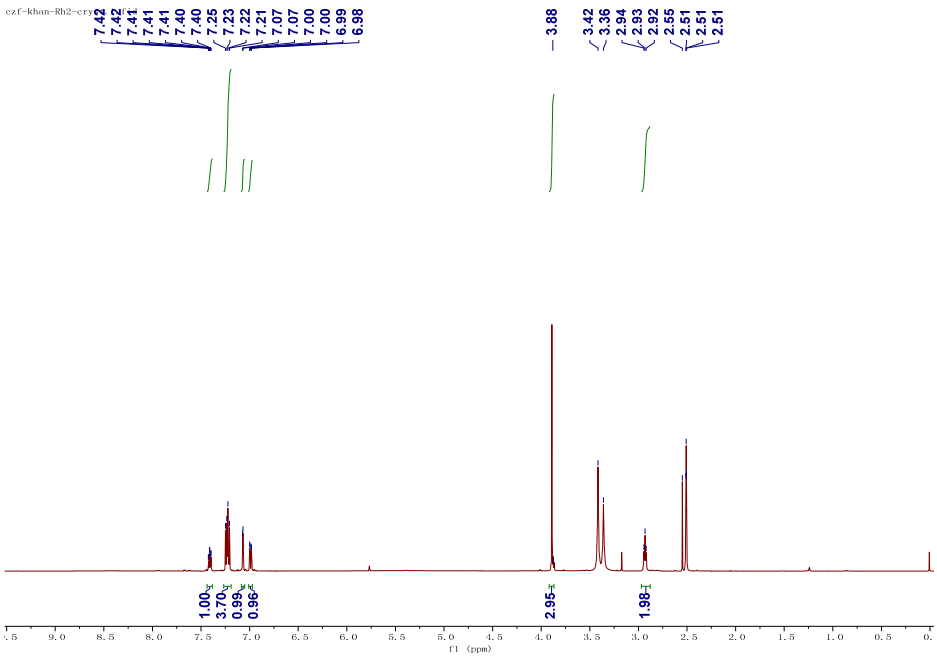

Figure S3: ^{1}H-NMR Spectrum of Rh2

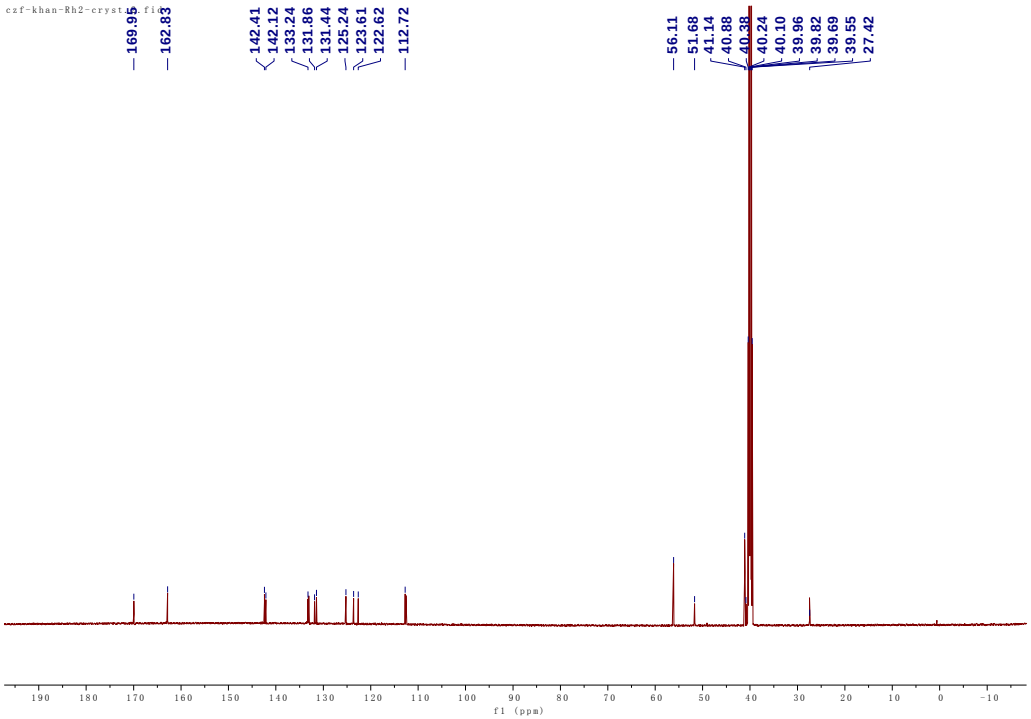

Figure S4: ^{13}C-NMR Spectrum of Rh2

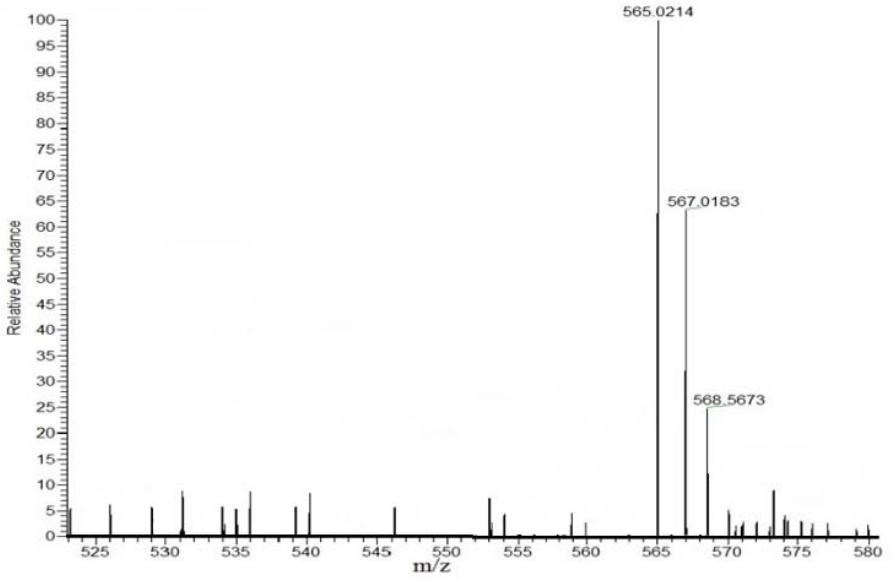

Figure S5: Mass Spectrum of Rh1

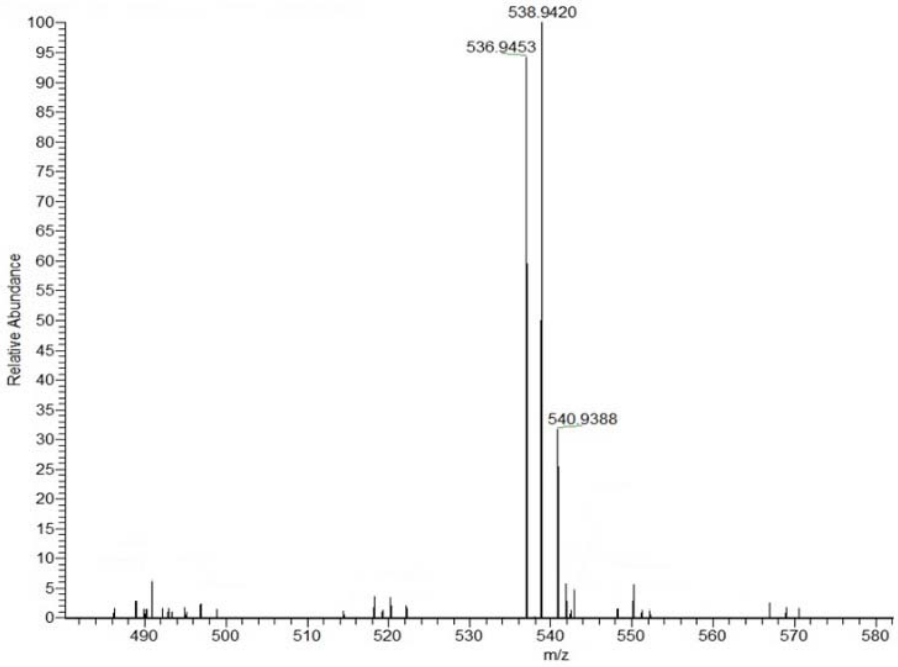

Figure S6: Mass Spectrum of Rh2

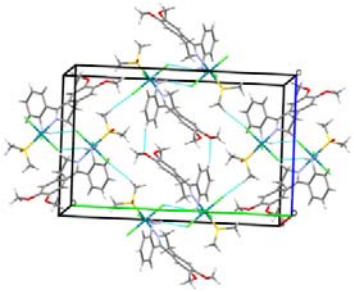

Figure S7: Unit cell packing, and ring set motif for Rh1

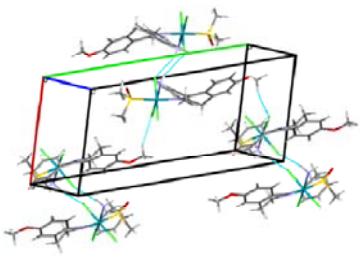

Figure S8: Unit cell packing, and ring set motif for Rh2

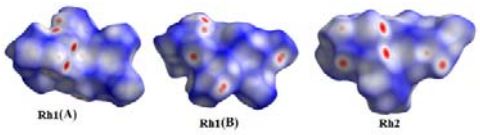

Figure S9: Three dimensional Hirshfeld surface generated for complexes Rh1 and Rh2

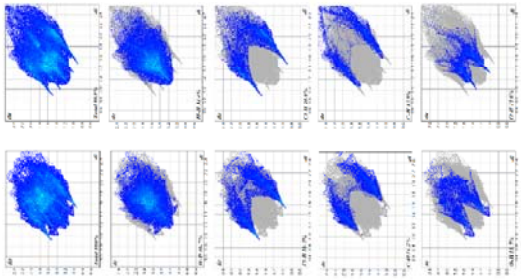

Figure S10: 2D fingerprint plot showing the percentage contribution of the contacts towards the overall crystal packing.

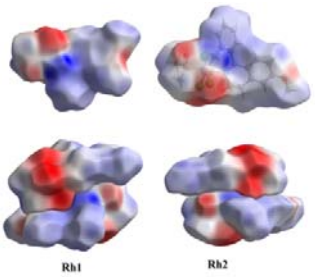

Figure S11**:** Electrostatic potential surfaces generated over the Hirshfeld surface.

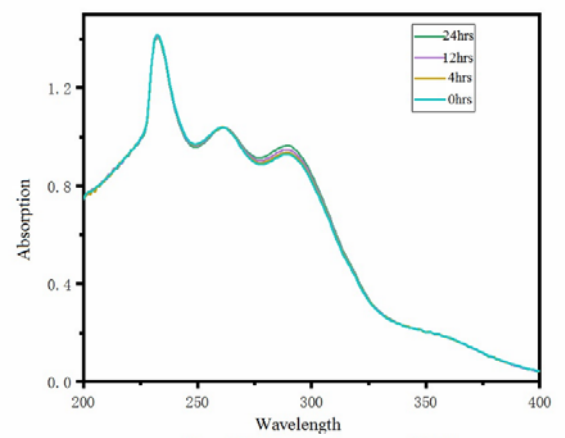

Figure S12: UV-Visible Spectrum of Rh1

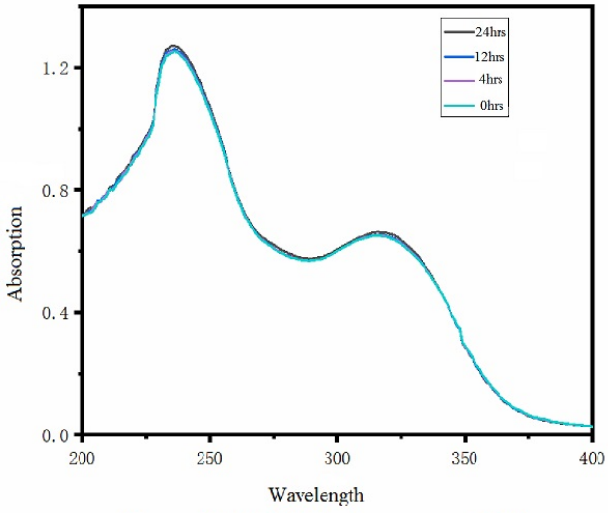

Figure S13: UV-Visible Spectrum of Rh2

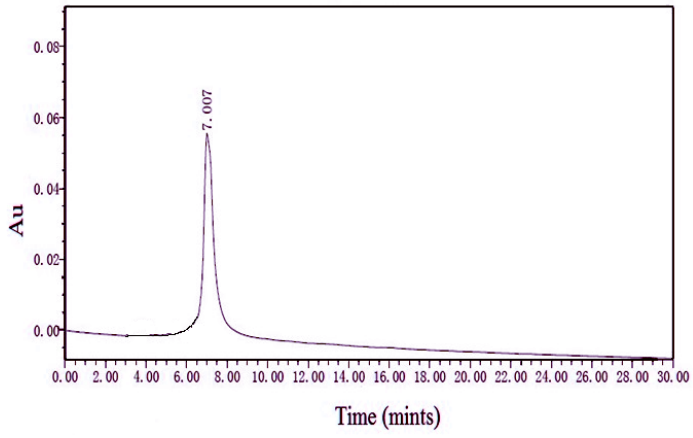

Figure S14: HPLC Spectrum of Rh1

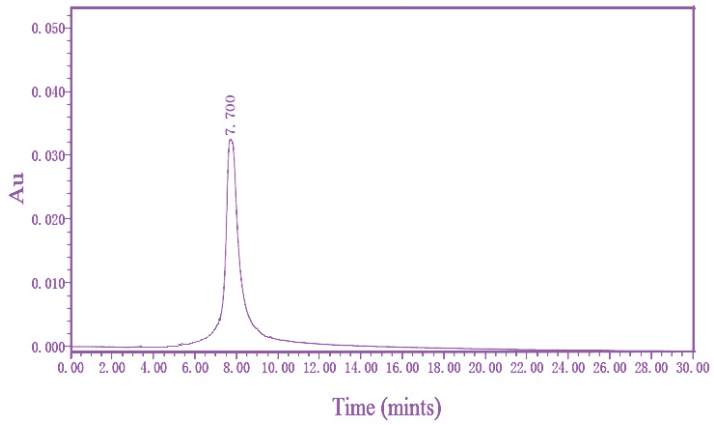

Figure S15: HPLC Spectrum of Rh2

Experimental detail

Cell Culture

All the tumor cell lines (T-24, BEL-7402, MGC80-3, HeLa, A549 and SKOV-3) and human normal liver cell line HL-7702 have been obtained from Shanghai Cell Bank of Chinese Academy of Sciences. Cells were cultured at 37 °C in DMEM (Dulbecco's Modified Eagle's Medium) supplied with 10% FBS (Fetal Bovine Serum) and 100µM streptomycin and penicillin, provided 5% humidified atmosphere of 5% CO_2 and 95% air. The compounds were dissolved in DMSO for making stock solution and PBS were used for making of working solutions ranging from 1.25µM to 200µM. The concentration of DMSO in stock solution was 100%, whereas the concentration of DMSO in working solution was 0.5%. Cisplatin was used as a positive control which was dissolved in 0.9% sodium chloride solution.

MTT Assay

Cells were seeded in 96 well plates with a population of 6×10^3/180 µL per well for 24 h. To each well various concentrations of compounds have been added to become the final concentration of 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0, 160.0 and 200.0 µM. The cells were incubated for 48 h with compounds. 0.5% DMSO was added to control wells. Cells were further incubated with 10µL MTT (5mg/mL in PBS) added to each well for more 6 h at 37 °C. All the media in wells have been discarded, and formazan crystals were dissolved by the addition of 100 µL of DMSO. The absorbance was measured on the microplate reader with double wavelength of 490nm/ 630nm. IC_{50} values were calculated by using the Bliss method (n=5). All the tests have been repeated three times independently [Table 1].

Cell Cycle Analysis.

Cells were cultured in a 75mm plate at a density of 5-6 $\times10^6$ per well for 24 h. They were treated with various concentration of Rh1, Rh2 and cisplatin. After 24 h cells were collected and fixed with 75% chilled ethanol and placed at -20 °C for one night. Before the assay, cells were washed, resuspended and incubated with PBS (100µg/mL RNAs and 50µg/mL PI) for 20 min in the dark. FACS Calibur Flow cytometer (BD) was used for determination of cell cycle. Cell cycle distribution was calculated by ModFIT LT software [Fig S16].

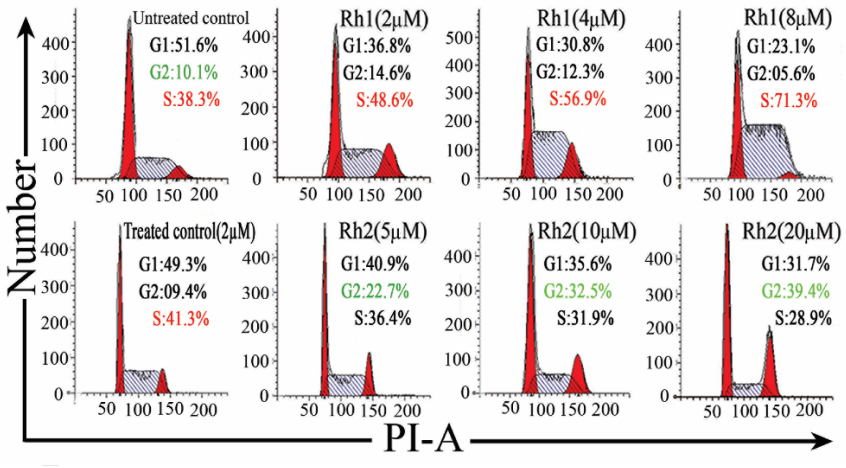

Figure S16: Flow cytometric analysis of cell cycle arrest by treatment of T-24 cells for 24 h at 37°C with Rh1, Rh2, and cisplatin (treated control). Determinations were conducted in triplicate, and one representative experiment is shown.

Apoptosis

Cells were cultured in 6 well-plate at the population of 2×10^5 for overnight at 37 °C. The cells were exposed to the complexes for 24 h. After inoculation with Rh1, Rh2 and cisplatin the cells were collected and washed with PBS. By using the "Apoptosis Detection Kit FITC Annexin V," the assay was carried out according to the manufacturer's instruction [Fig S17].

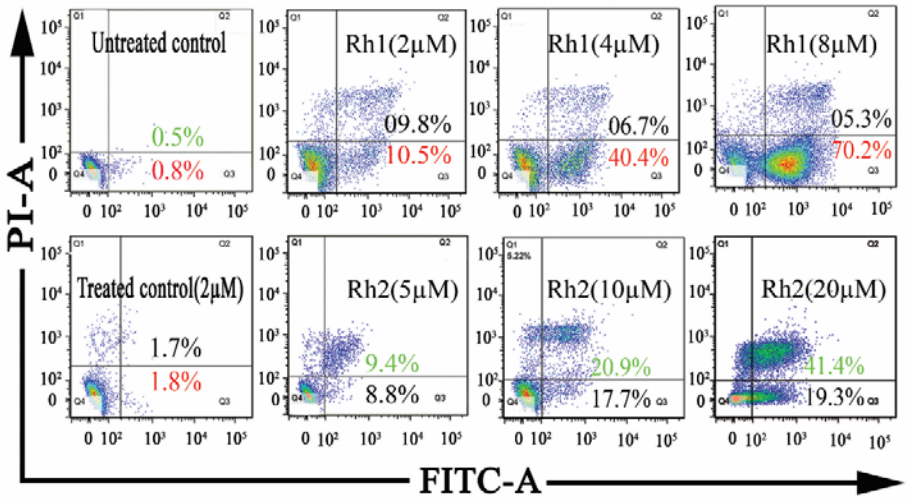

Figure S17: Flow cytometric analysis of apoptosis in T-24 cell line triggered by Rh1, Rh2, and cisplatin (treated control). Experiments have been done in triplicate, and one representative experiment is shown.

ROS generation

To evaluate the effect of complexes on ROS generation, DCFH-DA method was used. In the cells, DCFH-DA (stable, non-fluorescent) was hydrolyzed to DCFH (non-fluorescent) by the enzymatic action of esterase. After the formation of DCFH, it was rapidly converted to a highly fluorescent compound, DCF (dichlorofluorescein) by ROS. The intensity of fluorescence depends on the amount of ROS production in the cells. Therefore, T-24 cells (1×10^6) were seeded in a 6-well culture plate overnight. Cells were inoculated with tested compounds for 24 h at 37 °C. After treatment, the cells were collected, washed and incubated with DCFH-DA (100μM) for 20 min in the dark. Cells were washed with 1mL of PBS followed by the incubation with DCFH-DA. Generation of ROS was analyzed by using FACS Calibur flow cytometer (BD) and measured the fluorescence of DCF of the collected samples [Figure 7].

Intracellular Ca^{2+}

Level of Intracellular Ca^{2+}were investigated by a fluorescent dye Fluo-3 AM. It could cross the membrane and separated to Fluo-3 by the cellular esterase. Flou-3 was bind to Ca^{2+} and gave a strong fluorescence on 488 nm. T-24 cells were exposed to tested complexes for 24 h at 37 °C. After treatment, the cells were harvested, washed, resuspended in Fluo-3 AM (5μM) and incubated for 40 min in the dark. At the wavelength of 525 nm, the detection of Ca^{2+} has been carried out by flow cytometer [Figure 8].

MMP (mitochondrial membrane potential).

JC-1 assay was used for the evaluation of MMP. 1×10^6 T-24 cells were placed in a 6 well-plate overnight at 37 °C. Then the cells were treated with Rh1, Rh2 and cisplatin for 24 h. The content of the media was removed and washed thrice with PBS after 24 h treatment and incubated with JC-1 for 20 min in the dark. After incubation 0.5 mL of PBS was added and by FACS Calibur

flow cytometer, the membrane potential measurement was determined in percent values [Figure 9].

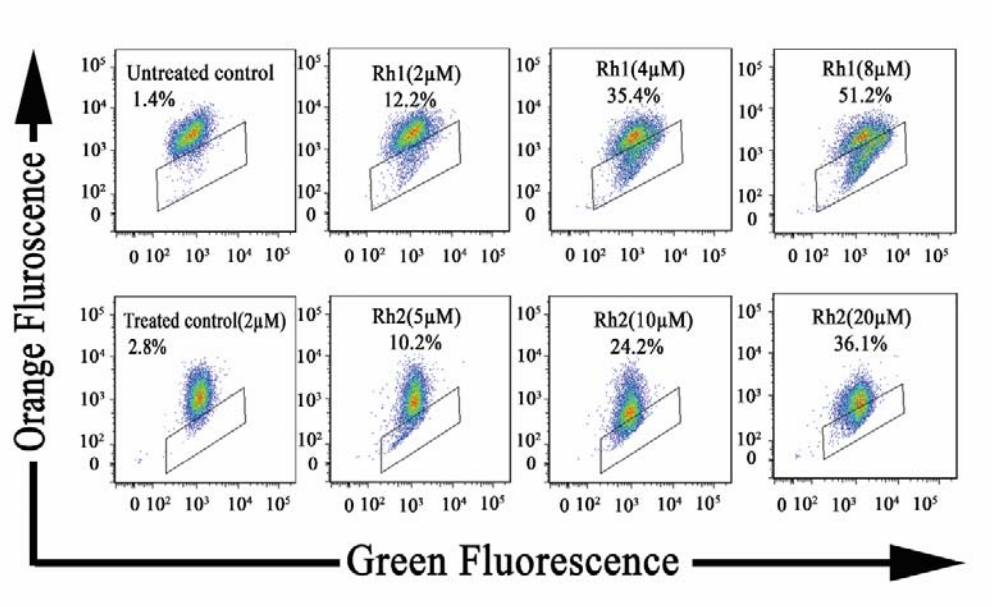

Figure S18: Measurement of mitochondrial membrane potential by flow cytometry in T-24 cells after 24 h treatment with different concentrations of Rh1, Rh2 and cisplatin (treated control) in comparison with untreated control. Experiments were performed in triplicate, and one representative experiment was shown.

Determination of Activated caspases 3, 8, 9.

Measurement of caspases (3/8/9) activities were carried out by using a staining kit of CaspGLOW fluorescein. The cells were exposed to tested complexes for 24 h at 37 °C at various concentrations. Before the assay, cells were collected, washed and suspended in 300 µL cold PBS. Each collected sample was incubated with 1µL of caspase-3, 8, 9 inhibitors (FITC-DEVD-FMK) for 60 min in the dark. Using FACS flow cytometer the percent activation of caspases was measured compared with negative and positive controls [Figure 10].

Western blotting analysis

T-24 cells were cultured in 100 mm culture plate for 24 h. Cells were treated with various concentrations of Rh1 and Rh2 at 37 °C for 24 h. After treatment, the cells were collected, washed and lysed by 150 µL of lysis buffer (149µL RIPA and 1 µL PMSF). The samples were put on ice for 30 min. Total proteins were extracted from cells by centrifugation at 1000 rpm for 15 min at 4 °C. A mixture of an equal volume of electrophoresis sample buffer and 50 µg of purified protein was prepared and boiled for 5 min of all samples including control. The prepared samples were loaded on 10% SDS-PAGE gels and then transferred to PVDF membrane. After the complete transfer of protein, the membrane was blocked by TBST buffer containing 5% BSA for 120 min. The layer was then washed with PBS and incubated with specific primary antibodies of suitable dilution overnight at 4 °C. Then incubated with secondary antibodies for one h at room temperature. Every time the membrane was washed with TBST buffer three times before and after the incubation with antibodies. For the detection of immunoreactive signals, chemo-luminance kit (Pierce ECL Western Blotting Substrate) were used according to the procedure described by the manufacturer [Figure 11(A, B, C) and 15(A, B)].

In vivo experiment.

Guangxi Medical University Laboratory Animal Centre (Guangxi, China) has provided the BALB/c nude male mice (aged 5-6 weeks and 17-20g). The animals were kept in an IVC (individual ventilated caging) environment with the constant condition of light (12 h) and dark (12 h) at 25-30 °C and 45-46 % humidity. T-24 cells (5×10^6) were injected into the right flank of nude mice to developed tumor. When the xenograft tumor reached the volume of 1000 mm^3, the mice were killed, and the tumor tissue was cut into small pieces of 1.5 mm^3 and translocated to the male nude mice. When the tumor reached about 90-150 mm^3, the mice were randomly

divided into solvent control and complex-treated groups. Control mice were supplemented with DMSO of 5% v/v per saline. Cisplatin was administrated 2mg/kg with an interval of two days and used as a positive control. Rh1 were given in lower dose (7.5mg/kg) and higher dose (15mg/kg). Every three days the body weight and tumor size were observed. Body weight was used as a parameter for drug systemic toxicity. After 15 days of treatment, the mice were killed for the sake of humanity, weighed and recorded the tumor. The tumor volume was determined by measuring the length and width and calculated with the formula $V = l \times w^2 / 2$. The rate of tumor growth was calculated by using the formula (1-TWt / TWc) × 100, where TWt stands for complex treated mice, TWc stands for vehicle-treated mice [Figure 13].

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