Electronic Supporting Information Materials

Evaluation on the iodine-substituted effect of 8-hydroxyquinoline on its platinum(II) complex: Cytotoxicity, cell apoptosis and telomerase inhibition

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Table S1. Crystal data and structure refinement details for **1**.

Empirical formula	$C_{11}H_{12}ClNO_2PtS$
Formula weight	452.82
Temperature/K	296.15
Crystal system	orthorhombic
Space group	$Pna2_1$
a/Å	7.4733(2)
b/Å	17.1260(6)
c/Å	9.9156(3)
α/°	90.00
β/°	90.00
γ/°	90.00
Volume/Å^3	1269.06(7)
Z	4
ρ_{calc}mg/mm^3	2.370
m/mm^{-1}	11.419
F(000)	848.0
Crystal size/mm^3	0.22 × 0.2 × 0.18
2θ range for data collection	5.94 to 52.74°
Index ranges	$-9 \le h \le 9$, $-18 \le k \le 21$, $-12 \le l \le 11$
Reflections collected	3726
Independent reflections	1888[R(int) = 0.0603]
Data/restraints/parameters	1888/31/156
Goodness-of-fit on F^2	1.076
Final R indexes [I>=2σ (I)]	R_1 = 0.0606, ωR_2 = 0.1408
Final R indexes [all data]	R_1 = 0.0681, ωR_2 = 0.1534
Largest diff. peak/hole / e Å^{-3}	1.88/-3.70
Flack parameter	-0.07(3)

[a] $R_1 = \Sigma \, ||F_o| - |F_c|| / \Sigma |F_o|$; [b] $wR_2 = [\Sigma w(F_o^2 - F_c^2)^2 / \Sigma w(F_o^2)^2]^{1/2}$.

Table S2. Selected bond lengths (Å) and bond angles (°) for **1**.

Pt1-Cl1	2.289(6)	N1-Pt1-Cl1	169.0(5)
Pt1-S1	2.193(5)	N1-Pt1-S1	100.9(5)
Pt1-O2	2.084(14)	N1-Pt1-O2	80.4(6)
Pt1-N1	1.988(19)	O1-S1-Pt1	121.6(7)
S1-Pt1-Cl1	89.9(2)	O2-Pt1-S1	178.5(4)
O2-Pt1-Cl1	88.8(4)		

Table S3. Crystal data and structure refinement details for **2**.

Empirical formula	$C_{22}H_{20}Cl_2I_4N_2O_4Pt_2S_2$
Formula weight	1409.23
Temperature/K	296.15
Crystal system	orthorhombic
Space group	Pbca
a/Å	12.2175(4)
b/Å	11.5867(4)
c/Å	45.0661(13)
α/°	90
β/°	90
γ/°	90
Volume/Å^3	6379.6(4)
Z	8
ρ_{calc}g/cm^3	2.9342
μ/mm^{-1}	12.961
F(000)	5025.1
Crystal size/mm^3	0.22 × 0.2 × 0.18
Radiation	Mo Kα (λ = 0.71073)
2θ range for data collection/°	6.04 to 52.74
Index ranges	$-15 \leq h \leq 9$, $-15 \leq k \leq 15$, $-57 \leq l \leq 60$
Reflections collected	17159
Independent reflections	6506 [R_{int} = 0.0369, R_{sigma} = 0.0522]
Data/restraints/parameters	6506/0/346
Goodness-of-fit on F^2	1.061
Final R indexes [I>=2σ (I)]	R_1 = 0.0470, ωR_2 = 0.0934
Final R indexes [all data]	R_1 = 0.0581, ωR_2 = 0.0998
Largest diff. peak/hole / e Å^{-3}	2.16/-1.95

[a] $R_1 = \Sigma ||F_o| - |F_c|| / \Sigma |F_o|$; [b] $wR_2 = [\Sigma w(F_o^2 - F_c^2)^2 / \Sigma w(F_o^2)^2]^{½}$.

Table S4. Selected bond lengths (Å) and bond angles (°) for **2.**

Pt1-S1	2.207(2)	Cl1-Pt1-S1	90.64(10)
Pt1-Cl1	2.311(3)	O0aa-Pt1-S1	91.7(2)
Pt1-O0aa	2.002(7)	O0aa-Pt1-Cl1	177.5(2)
Pt1-N1	2.036(8)	N1-Pt1-S1	170.8(2)
Pt2-Cl2	2.304(3)	N1-Pt1-Cl1	95.8(2)
Pt2-O6	2.016(7)	N1-Pt1-O0aa	82.0(3)
Pt2-N2	2.031(8)	O6-Pt2-Cl2	88.5(2)
Pt2-S2	2.207(3)	N2-Pt2-Cl2	168.8(3)
N2-Pt2-O6	81.5(3)	S2-Pt2-N2	100.7(2)
S2-Pt2-Cl2	89.60(11)	O1-S1-Pt1	114.8(3)
S2-Pt2-O6	175.9(2)	C10-S1-Pt1	108.7(4)

Table S5. The inhibitory ratios (%) of H-Q, H-IQ, **1**, **2**, cis-[$Pt(DMSO)_2Cl_2$] and cisplatin towards five tumor cell lines and the normal liver HL-7702 for 48 h.

Compounds	BEL-7404	Hep-G2	NCI-H460	T-24	A549	HL-7702
H-Q [a]	20.36±2.05	18.56±3.07	15.67±0.52	15.14±2.99	23.54±0.83	25.24±0.75
H-IQ [a]	27.34±0.36	25.01±0.29	15.03±3.11	18.25±0.93	34.13±1.25	21.93±1.37
1 [a]	57.12±0.91	65.04±0.84	51.17±1.56	48.02±1.24	40.27±0.36	38.01±3.09
2 [a]	80.05±1.54	85.37±2.14	67.05±2.31	78.73±0.76	76.31±2.12	32.07±0.55
cis-[$PtCl_2(DMSO)_2$] [b]	20.18±0.78	21.93±2.75	22.41±1.19	NA [c]	15.02±3.27	13.05±1.33
cisplatin [a]	68.71±0.56	61.01±3.29	50.87±0.65	49.09± 0.58	64.29± 2.06	55.66±3.46

Results represent mean ± SD of at least five independent experiments. SD represents the standard deviation.

[a] The concentration was 20 μM. [b] The concentration was 100 μM. [c] NA : No activity.

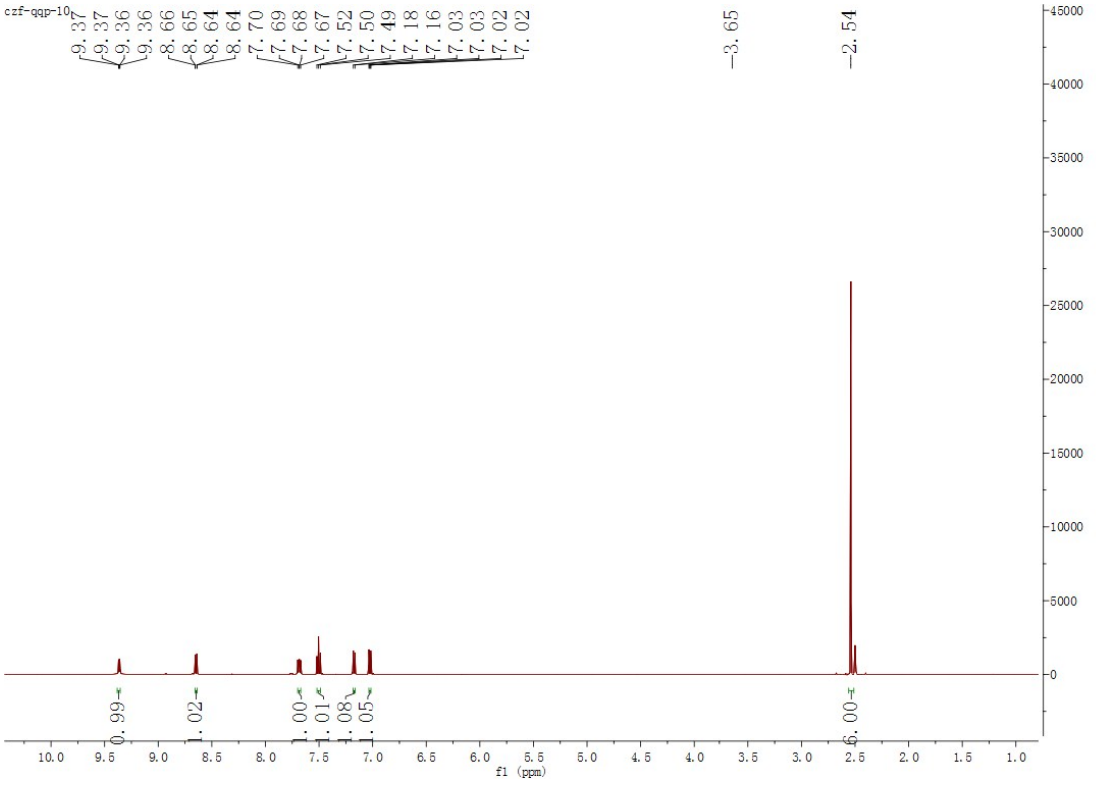


Figure S1. ^{1}H NMR spectrum (500MHz, DMSO-d_6) of complex **1**.

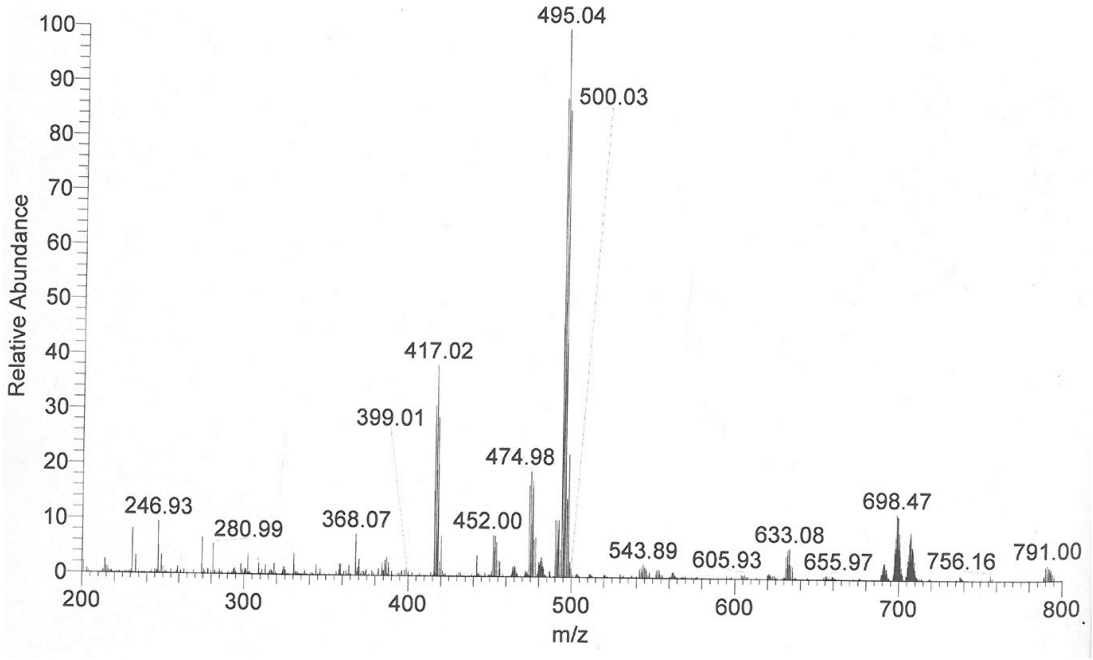


Figure S2. ESI-MS spectrum of complex **1**.

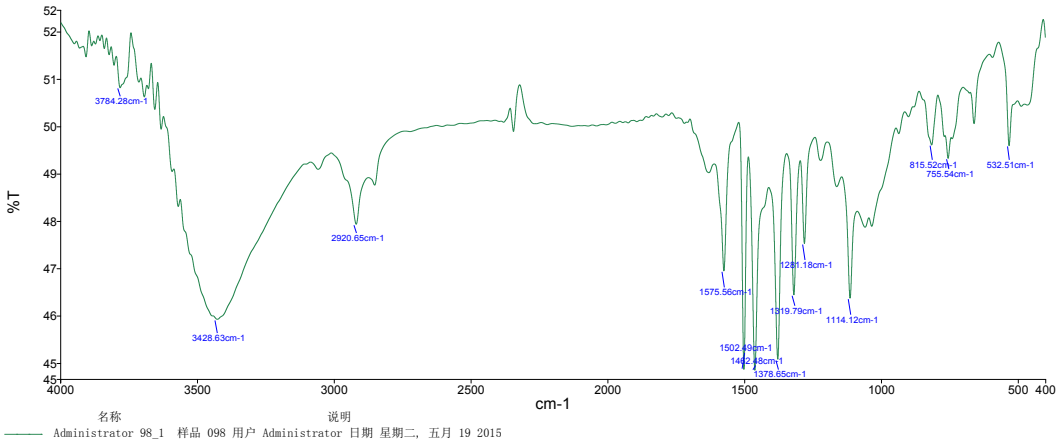


Figure S3. IR (KBr) spectrum of complex **1**.

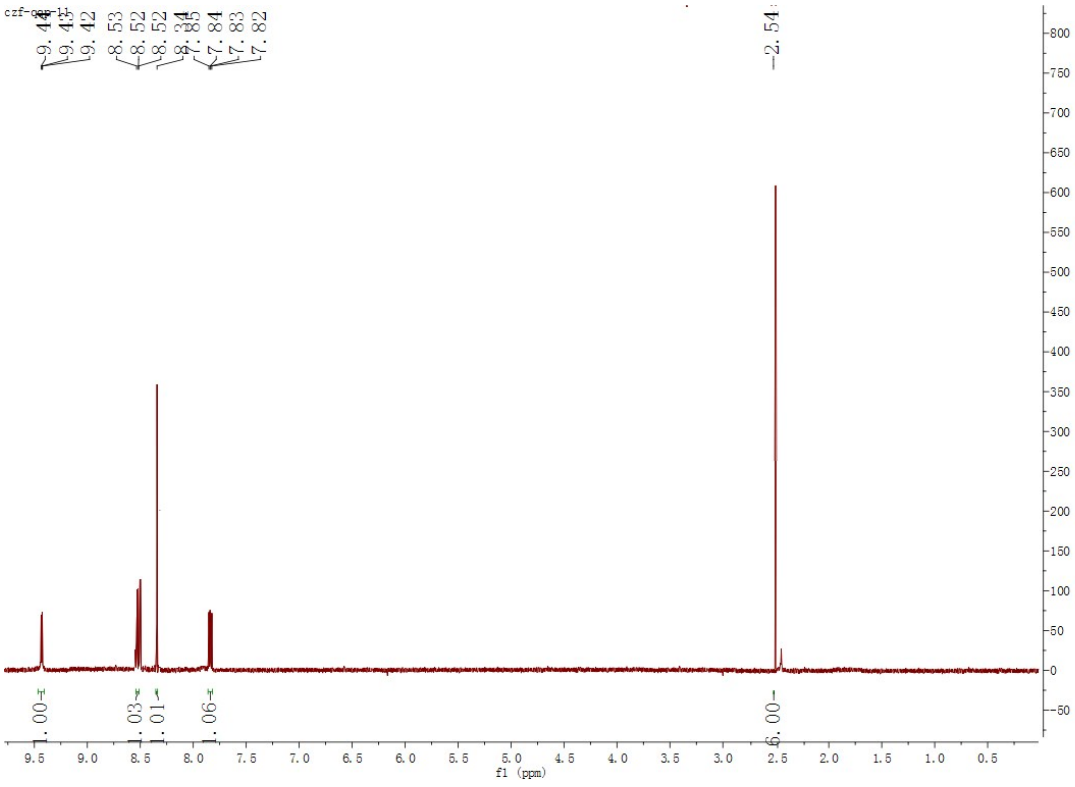


Figure S4. ^{1}H NMR spectrum (500MHz, DMSO-d_6) of complex **2**.

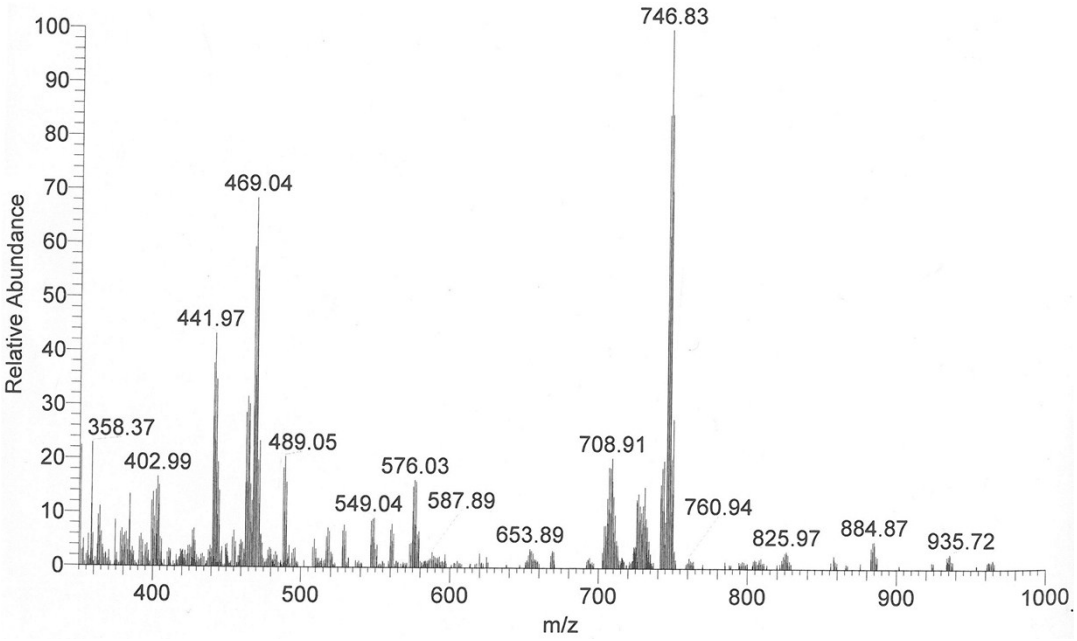


Figure S5. ESI-MS spectrum of complex **2**.

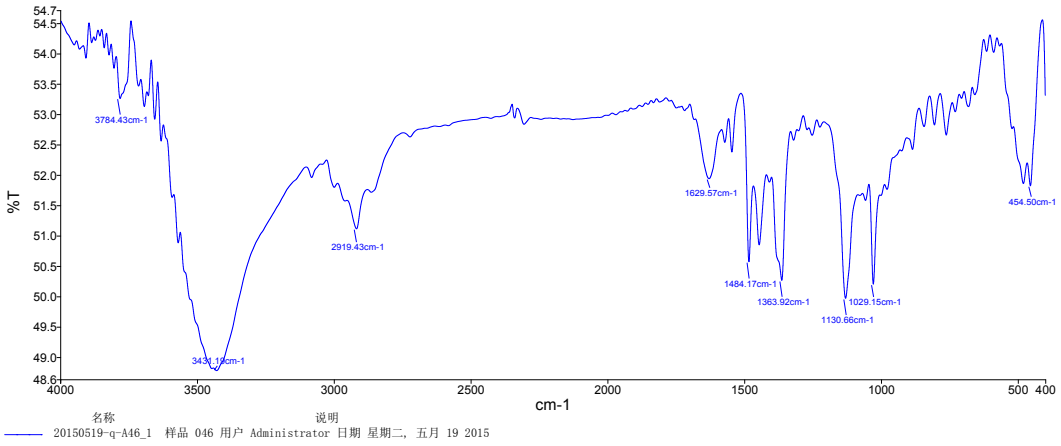


Figure S6. IR (KBr) spectrum of complex **2**.

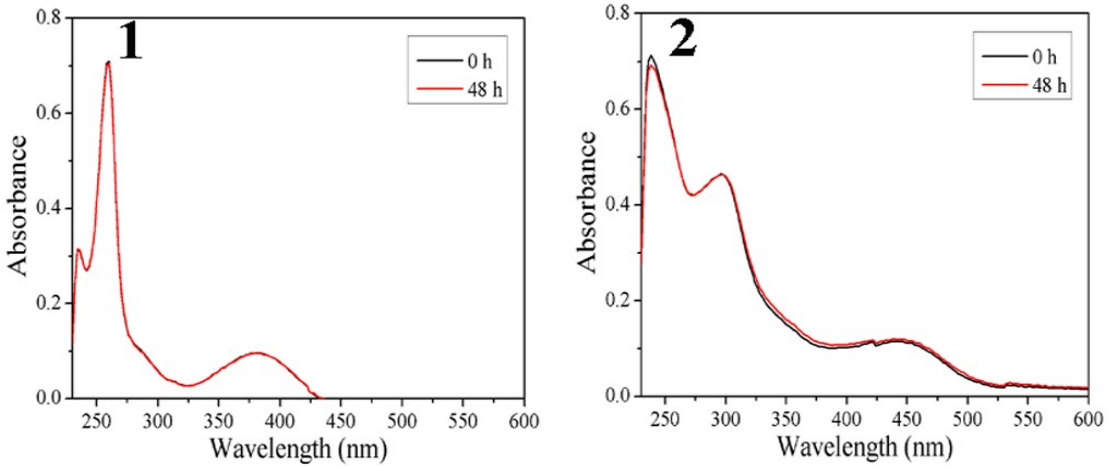
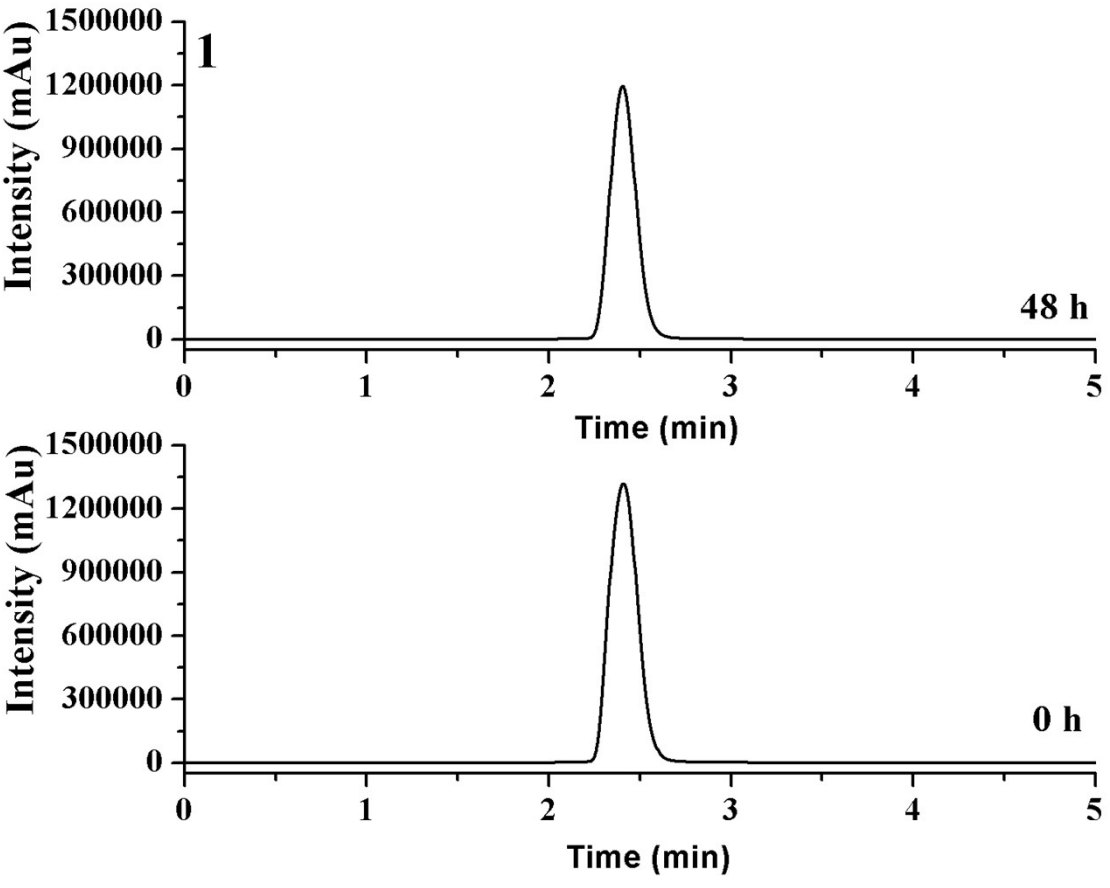


Figure S7. UV-Vis absorption spectra of **1** and **2** (3.0×10^{-5} M) in Tris-KCl solution in a 48 h time course, respectively.



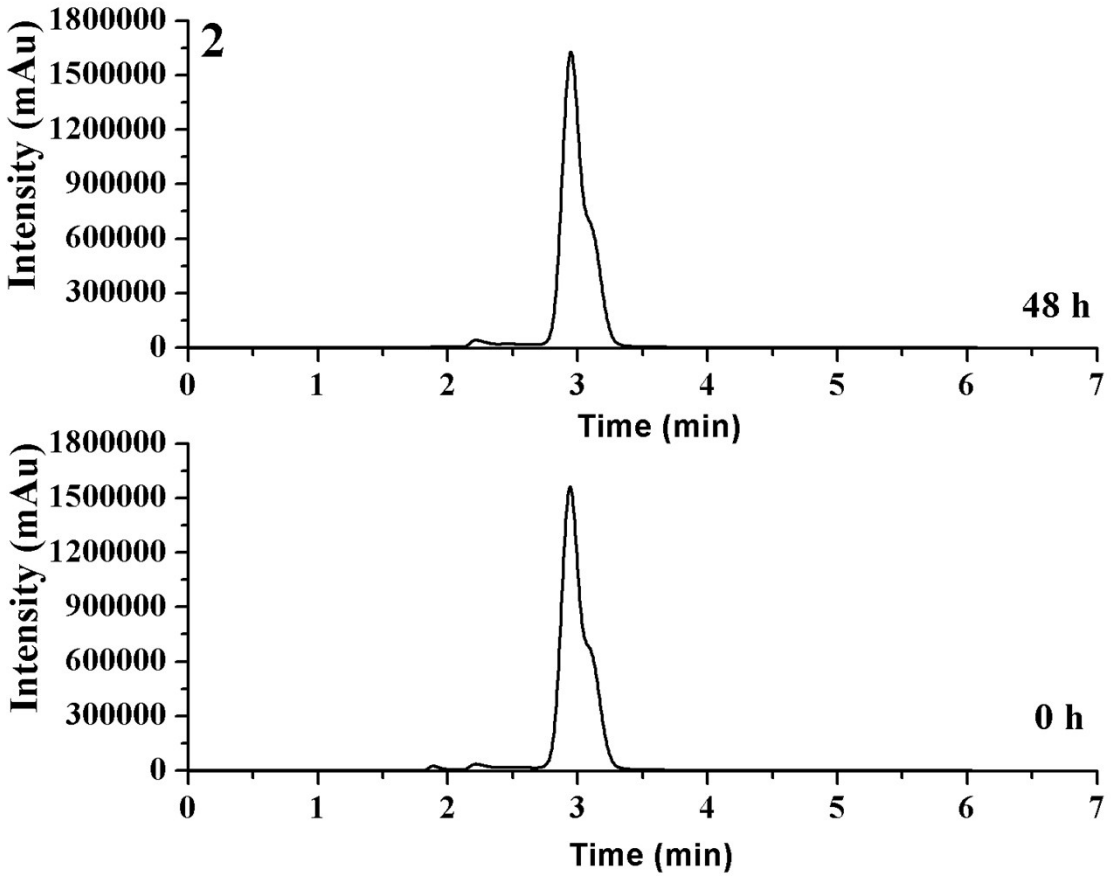


Figure S8. HPLC spectra for **1** and **2** in DMSO (6.0×10^{-3} M) for 0 h and 48 h, respectively. Column: Inertsustain C18 column (LC-20AT, SPD-20A HPLC COLUMN, 150mm×5.0μm I.D.). Column temperature: 40ºC. Mobile phase: methanol/H_2O containing 0.01% TFA (81:19 methanol/H_2O). Flow rate: 0.8 mL/min. Injection volume: 6.0×10^{-4} M.

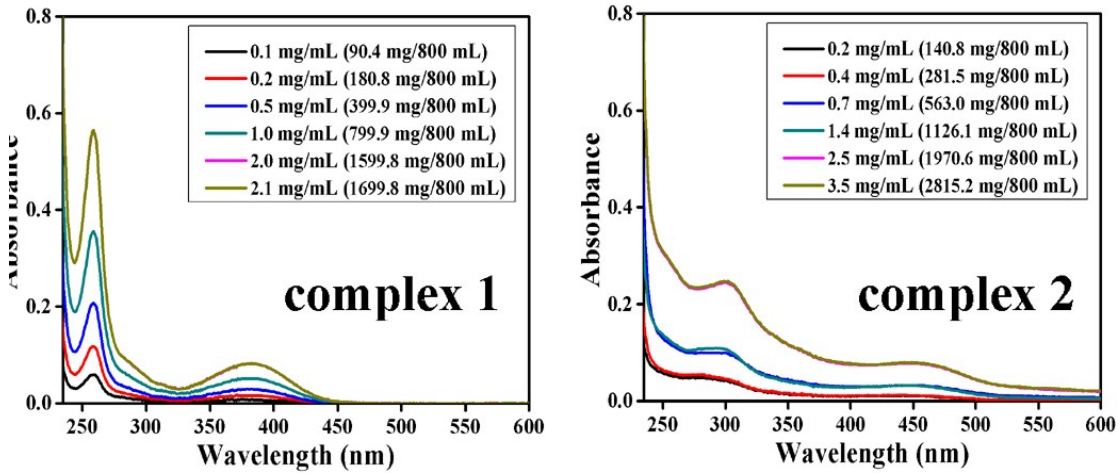


Figure S9. The water solubility of complexes **1** and **2** measured by UV-Vis absorption spectroscopy in distilled water at room temperature, respectively.

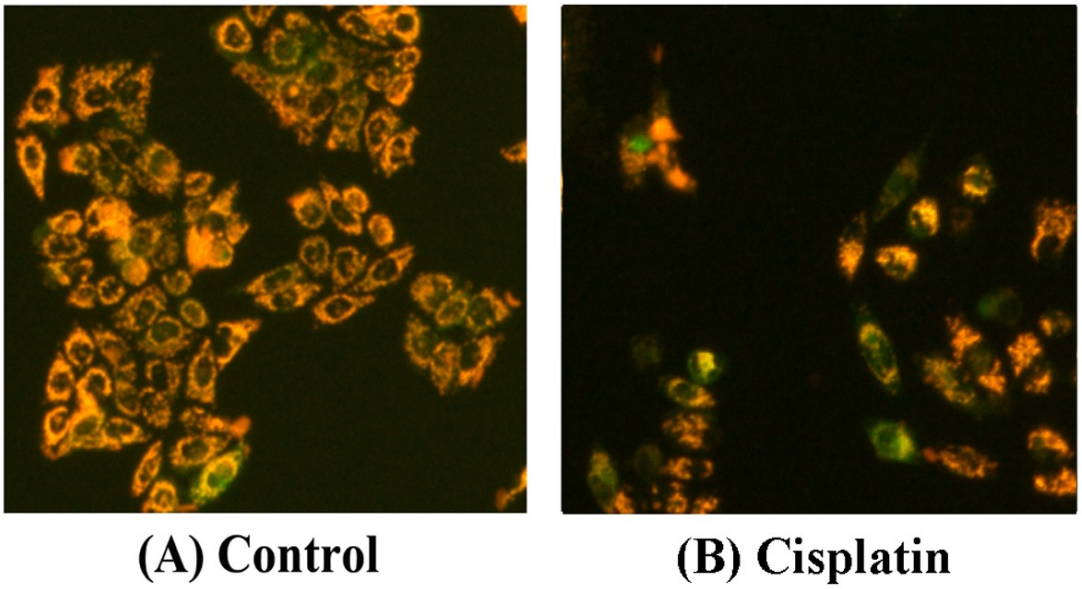


Figure S10. Collapse of mitochondrial membrane potential (Δψ) in the HepG2 cells treated with cisplatin (13 μM) for 24 h by JC-1 staining. Selected fields illustrated the corresponding living cells (orange-red) and apoptotic cells (green). Images were acquired using a Nikon Te2000 microscope (magnification 200×).

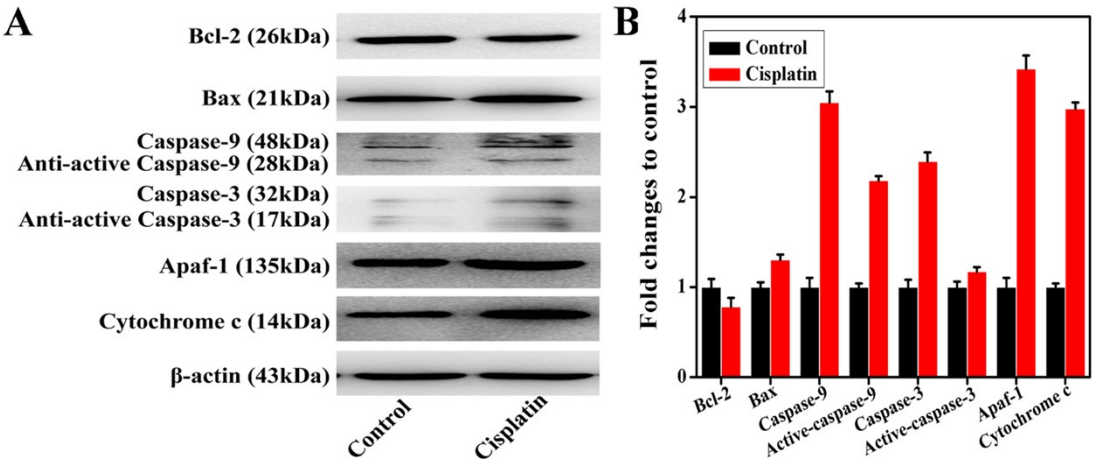


Figure S11. (A) Western blot assay to determine the expression of cytochrome c, apaf-1, bcl-2, bax and caspase-3/9 in the HepG2 cells treated with cisplatin (13 μM) for 24 h. (B) Densitometric analysis of cytochrome c, apaf-1, bcl-2, bax and caspase-3/9 normalized with β-actin. The relative expression of each protein was represented by the ratio of the protein band density to the β-actin band. The Mean SD was from three independent experiments.

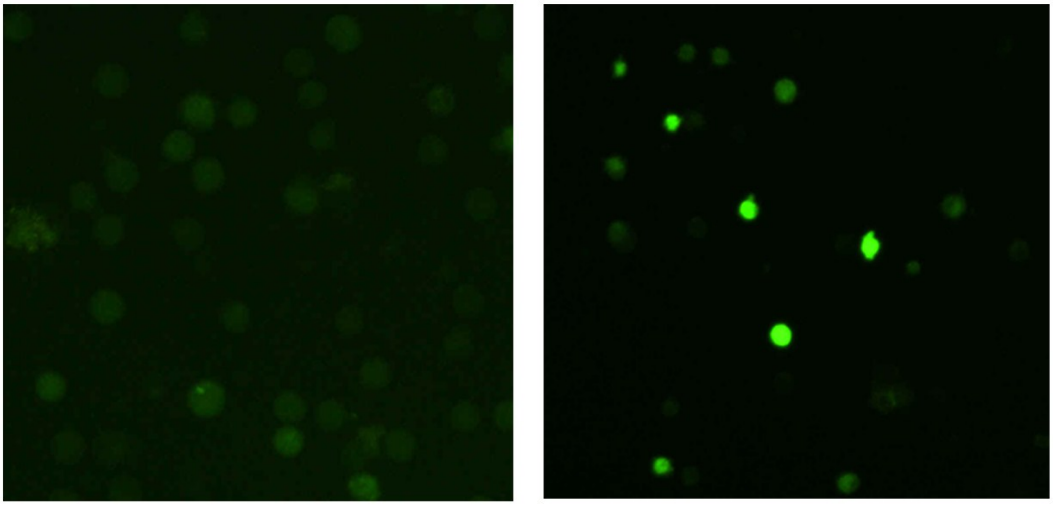


Figure S12. ROS generation detection in the HepG2 cells when treated by cisplatin (13 μM) for 24 h. Images were acquired using a Nikon Te2000 deconvolution microscope (magnification 200×).

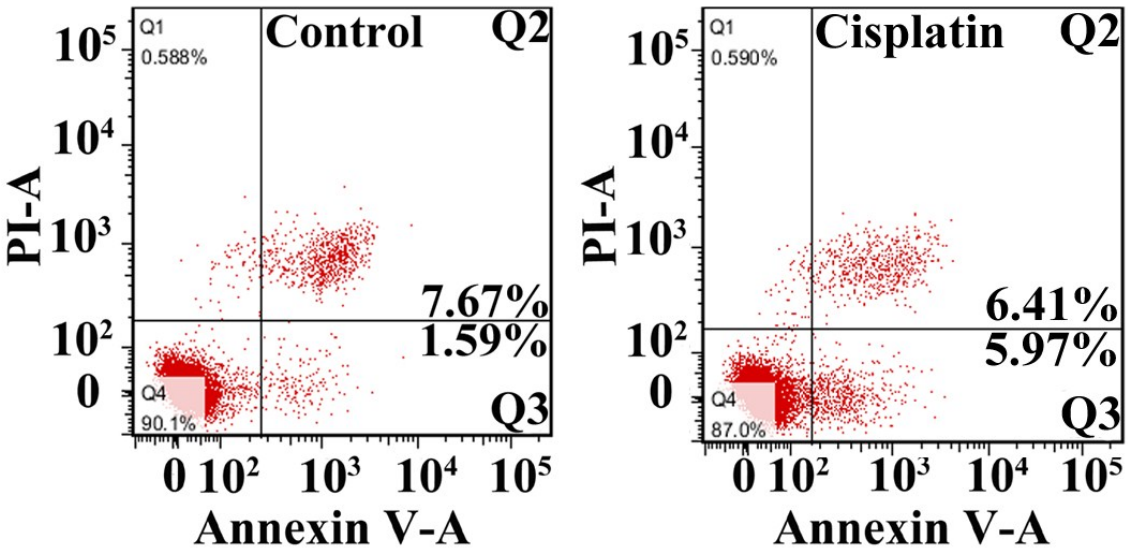


Figure S13. The cell apoptosis induction in the HepG2 cells treated with cisplatin (13 μM) for 24 h.

Experimental methods

Materials. Tris, RNase A, and propidium iodide (PI) were purchased from Sigma. The antibody of cdc25 A, cyclin A, cyclin B, CDK2, 53BP1, TRF1, TRF2, p21, p27, p53, cytochrome c, apaf-1, bax, caspase-3/9, and bcl-2 were purchased from Abcam. Unless otherwise stated, spectroscopic titration experiments were carried out in 10 mM Tris-HCl (pH 7.35) containing 100 mM KCl. The total RNA isolation kit and the two-step RT-PCR kit were purchased from TIANGEN. All tumor cell lines (BEL-7404, HepG2, NCI-H460, T-24, A549 and the normal liver cell line HL-7702) were obtained from the Shanghai Institute for Biological Science (China). Stock solutions of all the compounds (2 mM) were made in DMSO, and further dilutions to working concentrations were made with corresponding buffer.

Instrumentation. Infrared spectra were obtained on a PerkinElmer FT-IR Spectrometer. Elemental analyses (C, H, N) were carried out on a Perkin Elmer Series II CHNS/O 2400 elemental analyser. NMR spectra were recorded on a Bruker AV-500 NMR spectrometer. Fluorescence emission spectra were recorded on a Shimadzu RF-5301/PC Spectrofluorophotometer. ESI-MS spectra were obtained on Thermofisher Scientific Exactive LC-MS spectrometer (ThermalElctronic, USA). The circular dichroic spectra of DNA were obtained on a JASCO J-810 automatic recording spectropolarimeter operating at 25 °C. The region between 200 and 400 nm was scanned for each sample. MTT assay was performed on M1000 microplate reader (Tecan Trading Co. Ltd, Shanghai, China). Cell cycle and apoptosis analysis was recorded on FACS Aria II Flow Cytometer (BD Biocsiences, San Jose, USA). FRET assay was performed on 7500 fast Real-Time PCR (ABI Co. Ltd, USA).

Cytotoxicity assay. The cell culture was maintained on RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL

streptomycin in 25 cm^2 culture flasks at 37 °C humidified atmosphere with 5% CO_2. All cells to be tested in the following assays have a passage number of 3–6.

Cells 5.0×10^3 (BEL-7404, HepG2, NCI-H460, T-24, A549 and the normal liver cell line HL-7702) per well were seeded in triplicate in 96-well plates and incubated for 24 h at 37 °C and 5% CO_2/95% air. Then graded amounts of compound were added to the wells in 10 μL of FBS free culture medium and the plates were incubated in a 5% CO_2 humidified atmosphere for 48 h. Six replica wells were used as controls. Cells were grew for 12 h before treatment to reach 70% confluency and 20 μL of tested various concentrations of compounds were added to each well. The final concentration of the tested compounds were kept at 1.25, 2.5, 5, 10, 20 μM, respectively. After 48 h of culture, 0.1 mg of MTT (in 20 μL of PBS) was added to each well, and cells were incubated at 37 °C for 6 h. The formed formazan crystals were then dissolved in 100 μL of DMSO and the absorbance was read by enzyme labeling instrument with 490/630 nm double wavelength measurement. The final IC_{50} values were calculated by the Bliss method (n = 5). All tests were repeated in at least three independent trials.

Platinum uptake assay in Hep-G2 cells. Hep-G2 cells (~10 million cells) were treated with cisplatin (13 μM), complexes **1** (10 μM) and **2** (5.0 μM) for 24 h at 37 °C in a humidified 5% CO_2 incubator. The spent media was removed, and the cells were washed with 5 mL of PBS, scraped, and collected in 5 mL of PBS. The scrapped cells were spun down, by centrifuging at 2500 rpm for 10 min. The cell pellet obtained was dissolved in 1 M NaOH (1 mL) and diluted with 2% (v/v) HNO_3 (5 mL) for determining whole cell cobalt content. Another set was treated similarly, nuclear fraction, nuclear proteins, membrane proteins and cytoplasmic protein were isolated as described by Schreiber et al.[1] and the final solution was made up to 5 mL using 2%

(v/v) HNO_3. The amount of cobalt taken up by the cells was determined by ICP-MS. The instrument was calibrated for cobalt using standard solutions containing 10, 50, 100, 500 and 1000 ppb platinum.

SA-β-Gal assay. After the long-term incubation with complexes **1** (10 μM) and **2** (5.0 μM), the growth medium was aspirated and the cells were fixed in 2% formaldehyde/0.2% glutaraldehyde for 15 min at room temperature. The fixing solution was removed, and the cells were gently washed twice with PBS and then stained using the β-Gal staining solution containing 1 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactoside, finally followed with incubation overnight at 37 °C. The staining solution was removed, and the cells were washed three times with PBS. The cells were viewed under an optical microscope and photographed (Nikon Te2000, Japan).

Morphological observation of cell apoptosis by Hoechst33258 staining. To examine whether complexes **1** (10 μM) and **2** (5.0 μM) induced apoptosis in Hep-G2 cells, Hep-G2 cells were plated in six-well plates, and treated with complexes **1** (10 μM) and **2** (5.0 μM) for 24 h, respectively. After 24 h of treatment, the cells were washed with PBS and fixed for 10 min at room temperature. The cells were rinsed twice in PBS and stained with a Hoechst 33258 fluorescent dye (Beyotime, China), at room temperature at dark for 10 min. The cells were then washed twice with PBS, examined and immediately photographed under the fluorescence microscope (Nikon Te2000, Japan) with excitation wavelength of 330–380 nm. Apoptotic cells were defined on the basis of nuclear morphology changes, such as chromatin condensation and fragmentation.

Morphological observation of cell apoptosis by AO/EB staining. To Hep-G2 cells were grown on chamber slides to 70% confluence. Complexes **1** (10 μM) and **2**

(5.0 μM) were added to the culture medium (final DMSO concentration, 5% v/v) and incubated for 24 h at 37 °C, respectively. The cells were then washed with PBS, stained with medium containing AO/EB solution (100 mg/mL AO, 100 mg/mL EB) for 30 min, photographed with a Nikon Te2000 using a fluorescence deconvolution deconvolution microscopy (magnification 200×).

Cell cycle analysis. In cell cycle analysis, the Hep-G2 cells were maintained with 10% fetal calf serum in 5% CO_2 at 37 °C. Cells were harvested by trypsinization and rinsed with PBS. After centrifugation, the pellet (10^5–10^6 cells) was suspended in 1 mL PBS. The cells were washed in PBS and fixed with ice-cold 70% ethanol in PBS under violent shaking. Cells 1×10^6 were centrifuged and resuspended in a staining solution (0.5 mL of PBS containing 50 μg/mL PI and 75 kU/mL RNase A) for 30 min at room temperature in the dark. Finally, the cell cycle was analyzed by FACS Calibur flow cytometer (BD) and the cell cycle distribution and percentage of apoptotic cells were analyzed using Cell Quest (BDIS) and ModFit LT (Verity Software House, Topsham, ME).

Apoptosis analysis. Apoptosis was detected by flow cytometric analysis of annexin V staining. Annexin V-FITC vs PI assay was performed as previously described.[2,3] Briefly, adherent Hep-G2 cells were harvested and suspended in the annexin-binding buffer (5×10^5 cells/mL). Then, cells were incubated with annexin V-FITC and PI for 1 h at room temperature in the dark and immediately analyzed by flow cytometry. The data are presented as biparametric dot plots showing PI red fluorescence vs annexin V-FITC green fluorescence.

Measurement of ROS generation. DCFH-DA is a freely permeable tracer specific for ROS. At the same time, DCFH-DA can be deacetylated by intracellular esterase to the non-fluorescent DCFH which is oxidized by ROS to the fluorescent compound

2',7'-dichloroflorescein (DCF). Therefore, the fluorescence intensity of DCF is proportional to the amount of ROS produced by the cells.[4,6] Cells 1×10^6 were exposed to cisplatin (13 μM), complexes **1** (10 μM) and **2** (5.0 μM) for 24 h, respectively, and 1 mM H_2O_2 used as a positive control of ROS production. After the exposure, cells were harvested, washed once with ice-cold PBS and incubated with DCFH-DA (100 μM in a final concentration) at 37 ºC for 15 min in the dark.[5,6] Finally, the cells were washed again and maintained in 1 mL PBS. The ROS generation was assessed from 10,000 cells each sample by fluorescence microscope (Nikon Te2000, Japan) with excitation and emission wavelengths of 488 and 530 nm, respectively. All the experiments were performed at least three times.

Measurement of mitochondrial membrane potential. The loss of mitochondrial membrane potential (Δψ) was assessed using a lipophilic cationic fluorescent probe, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine; Beyotime). Cells treated with cisplatin (13 μM)**,** complexes **1** (10 μM) and **2** (5.0 μM) for 24 h were incubated with 5 μg/mL JC-1 for 20 min at 37 °C and examined under the fluorescence microscopy. The emission fluorescence for JC-1 was monitored at 530 and 590 nm, under the excitation wavelength at 488 nm. The orange-red emission of the dye is attributable to a potential-dependent aggregation in the mitochondria, which reflects the Δψ. Green fluorescence reflects the monomeric form of JC-1, appearing in the cytosol after mitochondrial membrane depolarization.

TRAP assay (TRAP-Silver Staining Assay). The telomerase extract was prepared from the Hep-G2 cells: a total of 5×10^6 Hep-G2 tumor cells untreated or treated with complexes **1** (10 μM) and **2** (5.0 μM) were pelleted, and the cells were washed with 5 mL of PBS, scraped and lysed for 30 min on ice. Finally, the lysate was centrifuged at 13 000 rpm for 30 min at 4 °C; the supernatant was collected and stored at −80 °C

before use.[6,7] The TRAP assay was performed by following previously published procedures.[7–9] Telomerase extract was prepared from Hep-G2 cells. A modified version of the TRAP assay was used.[8] PCR was performed in a final 50 mL reaction volume composed of reaction mix (45 mL) containing Tris-HCl (20 mM, pH 8.0), deoxynucleotide triphosphates (50 mM), $MgCl_2$ (1.5 mM), KCl (63 mM), EGTA (1 mM), Tween-20 (0.005%), BSA (20 mg/mL), primer H21T (3.5 pmol; 5'-$G_3[T_2AG_3]_3$-3'), primer TS (18 pmol; 5'-AATCCGTCGAGCAGAGTT-3'), primer Cxext (22.5 pmol; 5'-GTGCCCTTACCCTTACCCTTACCCTAA-3'), primer NT (7.5 pmol; 5'-ATCGCTTCTCGGCCTTTT-3'), TSNT internal control (0.01 amol; 5'-ATTCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3'), Taq DNA polymerase (2.5 U), and telomerase (100 ng). Compounds or distilled water was added (5 mL). PCR was performed in an Eppendorf Master cycler equipped with a hot lid and incubated for 30 min at 30 ℃, followed by 92 ℃ 30 s, 52 ℃ 30 s, and 72℃ 30 s for 30 cycles. After amplification, loading buffer (8 mL; 5×TBE buffer, 0.2% bromophenol blue, and 0.2% xylene cyanol) was added to the reaction. An aliquot (15 mL) was loaded onto a nondenaturing acrylamide gel (16%; 19:1) in 1×TBE buffer and resolved at 200 V for 1 h. Gels were fixed and then stained with $AgNO_3$.

RNA extraction. Cell pellets harvested from each well of the culture plates were lysed in RZ Lysis solution. RNA was extracted with RNAsimple Total RNA kit (TIANGEN) according to manufacturer's protocol and eluted in distilled, deionized water with 0.1% diethyl pyrocarbonate (DEPC) to a final volume of 50 μL. RNA was stored at −150 °C before use.

RT-PCR. Total RNA was used as a template for reverse transcription using the following protocol: each 20 μL reaction contained 2.0 μL 10×RT mix., 2.0 μL dNTP (2.5 mM), 2.0 μL Oligo-dT15 primer, 1.0 μL Quant Reverse Transcriptase, 10 μL

DEPC-H_2O, and 2 μg of total RNA. Briefly, RNA and oligo dT15 primer was incubated at 37 °C for 60 min and then immediately placed on ice. Finally, the reacted solution was stored at −80℃. Real-time PCR was performed on 7500fast Real-Time PCR (ABI Co. Ltd, USA) by using 2.5×RealMasterMix/20×SYBR solution (TIANGEN), according to the manufacturer's protocol. The total volume of 20 μL real-time RT-PCR reaction mixtures contained 9.0 μL of 2.5×RealMasterMix/20×SYBR solution, 0.25 μM each of forward and reverse primers, 1.0 μL of cDNA, and nuclease-free water. The program used for all genes consisted of a denaturing cycle of 3 min at 95 °C, 45 cycles of PCR (95 °C for 20 s, 58 °C for 30 s, and 68 °C for 30 s), a melting cycle consisting of 95 °C for 15 s, 65 °C for 15 s, and a step cycle starting at 65 °C with a 0.2 °C/s transition rate to 95 °C. The specificity of the real-time RT-PCR product was confirmed by melting curve analysis. The PCR product sizes were confirmed by agarose gel electrophoresis and ethidium bromide staining. Three replications were performed, and then hTERT mRNA or c-myc mRNA level was normalized with the GAPDH mRNA level of each sample. Results of real-time PCR were analyzed using the $2^{-\Delta\Delta CT}$ method in the program Origin 8.0 to compare the transcriptional levels of hTERT or c-myc genes in each sample relative to nondrug treated control.

Western blotting. Cells harvested from each well of the culture plates were lysed in 150 μL of extraction buffer consisting of 149 μL of RIPA Lysis Buffer and 1 μL PMSF (100 mM). The suspension was centrifuged at 10000 rpm at 4 °C for 10 min, and the supernatant (10 μL for each sample) was loaded onto 10% polyacrylamide gel and then transferred to a microporous polyvinylidene difluoride (PVDF) membrane. Western blotting was performed using anti-hTERT, c-myc, cdc25 A, cyclin A, cyclin B, CDK2, p53, p21, p27, cytochrome c, apaf-1, bcl-2, bax, caspase-3/9 and β-actin

antibody and horseradish peroxidase-conjugated antimouse or antirabbit secondary antibody. Protein bands were visualized using chemiluminescence substrate.

Transfection. After Hep-G2 cells (8.0×10^5) were grew in 3 cm Petri dishes for 24 h, DNA transfections were performed using the following procedure. Firstly, 2.0 μg EGFP plasmid[10] and 2.0 μg c-myc plasmid[11] were cotranfected into Hep-G2 cells using Lipo2000 (Invitrogen).Then, complexes **2** (10 μM) and cisplatin (10 μM) were added into medium, respectively, after 6 h of transfection. After another 24 h of drug treatment, the cells were imaged using Nikon TE2000 (Japan) scanning fluorescence microscope and studied by Luciferase Reporter Gene Assay Kit.

Statistical analysis. The experiments have been repeated from three to five times, and the results obtained were presented as means ±standard deviation (SD). Significant changes were assessed by using Student's *t* test for unpaired data, and p values of <0.05 were considered statistically significant.

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