Electronic Supporting Information Materials

Synthesis and antitumor mechanism of a new iron(III) complex with 5,7-dichloro-2-methyl-8-quinolinol as ligands

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

Empirical formula	$C_{20}H_{12}Cl_5FeN_2O_2$
Formula weight	545.42
Temperature/K	293(2)
Crystal system	monoclinic
Space group	$P2_1/c$
a/Å	10.5841(2)
b/Å	29.3409(6)
c/Å	22.5449(6)
α/°	90.00
β/°	114.077(2)
γ/°	90.00
Volume/Å^3	6392.1(2)
Z	12
ρ_{calc}mg/mm^3	1.700
m/mm^{-1}	1.356
F(000)	3276.0
Crystal size/mm^3	0.41 × 0.23 × 0.12
2Θ range for data collection	6.76 to 52.74°
Index ranges	$-13 \le h \le 12$, $-36 \le k \le 34$, $-17 \le l \le 28$
Reflections collected	27953
Independent reflections	13041[R(int) = 0.0220]
Data/restraints/parameters	13041/0/817
Goodness-of-fit on F^2	1.034
Final R indexes [I>=2σ (I)]	R_1 = 0.0411, wR_2 = 0.0841
Final R indexes [all data]	R_1 = 0.0676, wR_2 = 0.0949
Largest diff. peak/hole / e Å^{-3}	0.44/-0.35

[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 complex **1**.

Bond lengths (Å) for 1							
Fe1-Cl10	2.2106(11)	Fe1-O1	1.898(2)	Fe1-O2	1.903(2)	Fe1-N3	2.146(2)
Fe1-N4	2.145(2)	Fe2-O5	1.903(2)				
Bond angles (°) for 1							
O1-Fe1-Cl10	117.59(8)	O1-Fe1-O2	123.71(10)	O1-Fe1-N3	96.13(9)	O1-Fe1-N4	80.54(9)
N3-Fe1-Cl10	96.00(7)	N4-Fe1-Cl10	90.40(7)	N4-Fe1-N3	173.59(10)	O2-Fe1-N4	96.78(9)
O2-Fe1-N3	80.46(9)	O2-Fe1-Cl10	118.65(8)				

Table S3. Inhibition rates of H-ClMQ, $FeCl_3$, Fe(III) complex **1** and cisplatin towards five selected tumor cell lines and one normal liver cell HL-7702 for 48 h.

Compounds	BEL-7404	NCI-H460	T-24	Hep-G2	A549	HL-7702
H-ClMQ [a]	30.14±0.99	29.89±1.99	25.46±0.54	20.11±0.66	19.59±1.74	24.52±0.44
1 [a]	62.87±1.03	69.54±2.49	65.02±0.93	75.64±0.75	70.45±1.88	40.15±0.82
$FeCl_3$ [b]	18.54±2.14	25.88±1.74	15.03±0.33	19.65±2.14	28.66±1.56	17.54±2.48
Cisplatin[c]	60.02±1.20	55.26±2.01	49.68±1.71	65.22±1.19	59.68±1.51	58.02±1.25

Results represent mean ± SD of at least five independent experiments. SD represents the standard deviation. [a] The concentration is 2 ×10^{-5} mol/L. [b] The concentration is 1× 10^{-4} mol/L. [c] Cisplatin was dissolved at a concentration of 1 mM in 0.154 M NaCl.

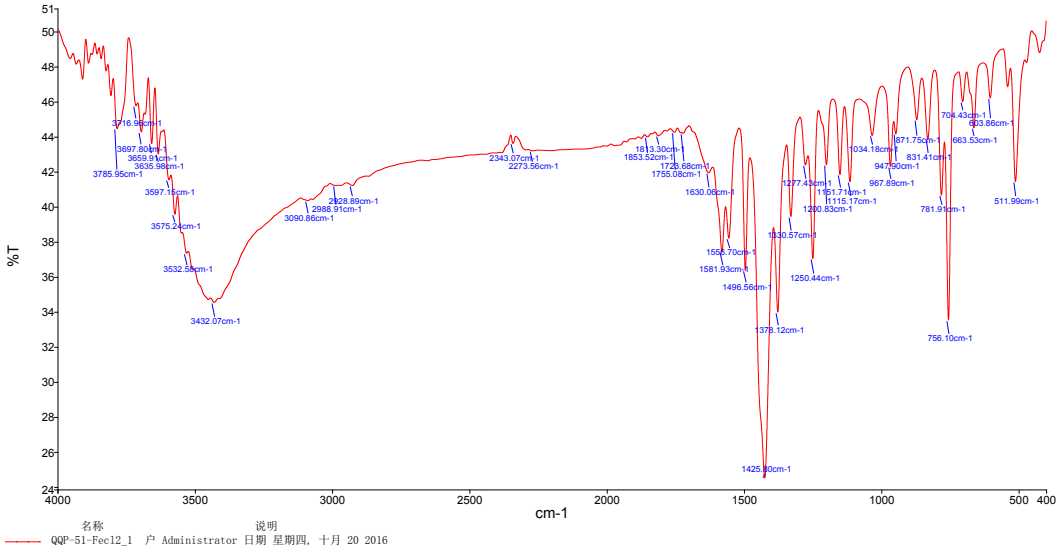

Figure S1. IR (KBr) spectra of complex **1**.

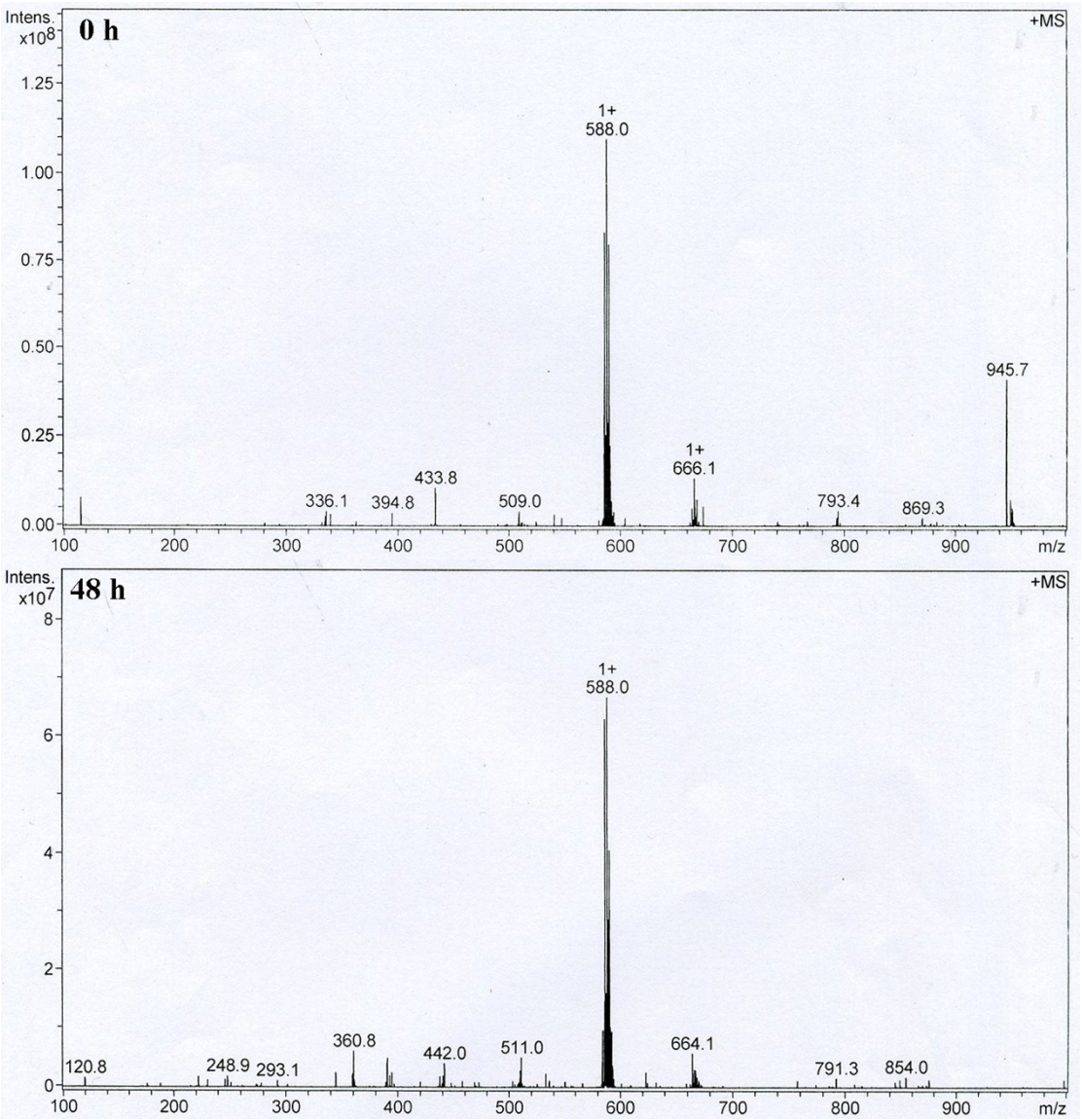

Figure S2. The mass spectra of complex **1** in Tris-HCl buffer solution (containing 5% DMSO) for 0 h (top) and 48 h (down), respectively.

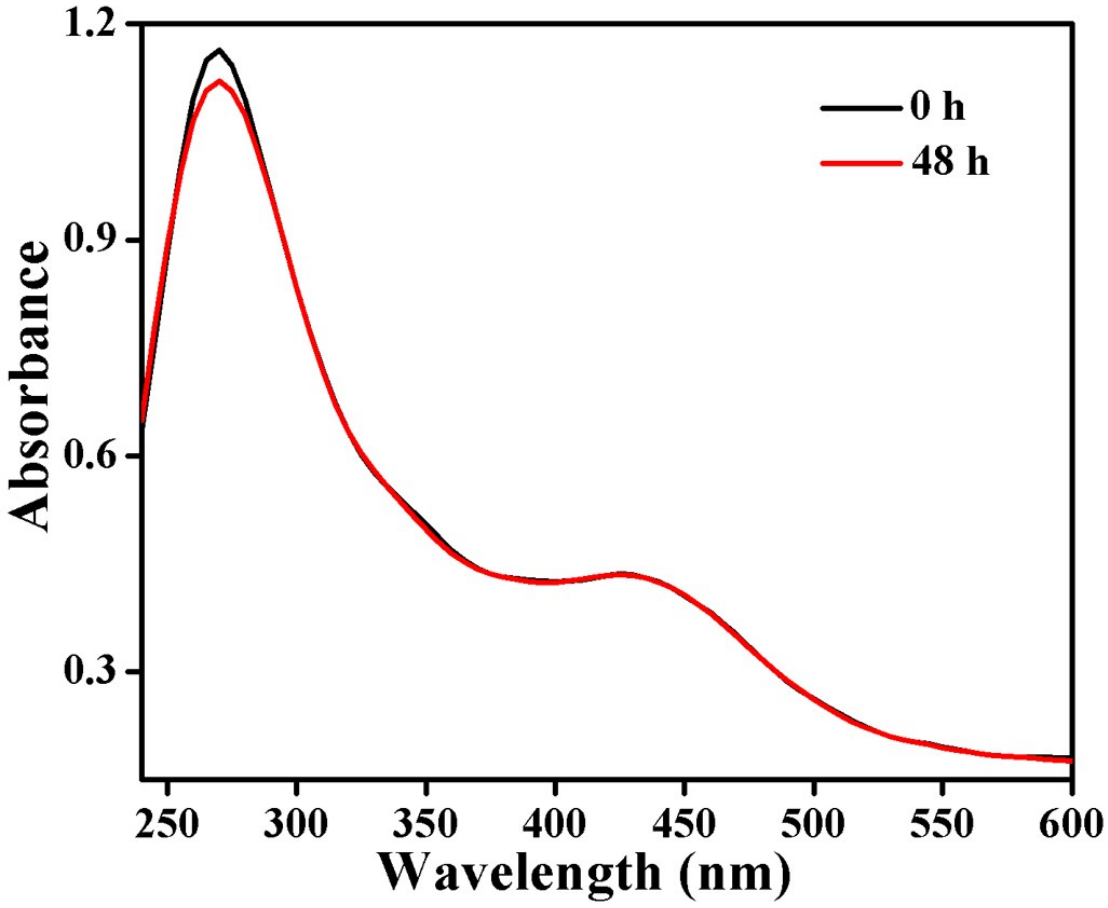

Figure S3. UV-Vis absorption spectra of complex **1** (1.0×10^{-5} M) in Tris-HCl solution (TBS) in the time course 0 and 48 h, respectively.

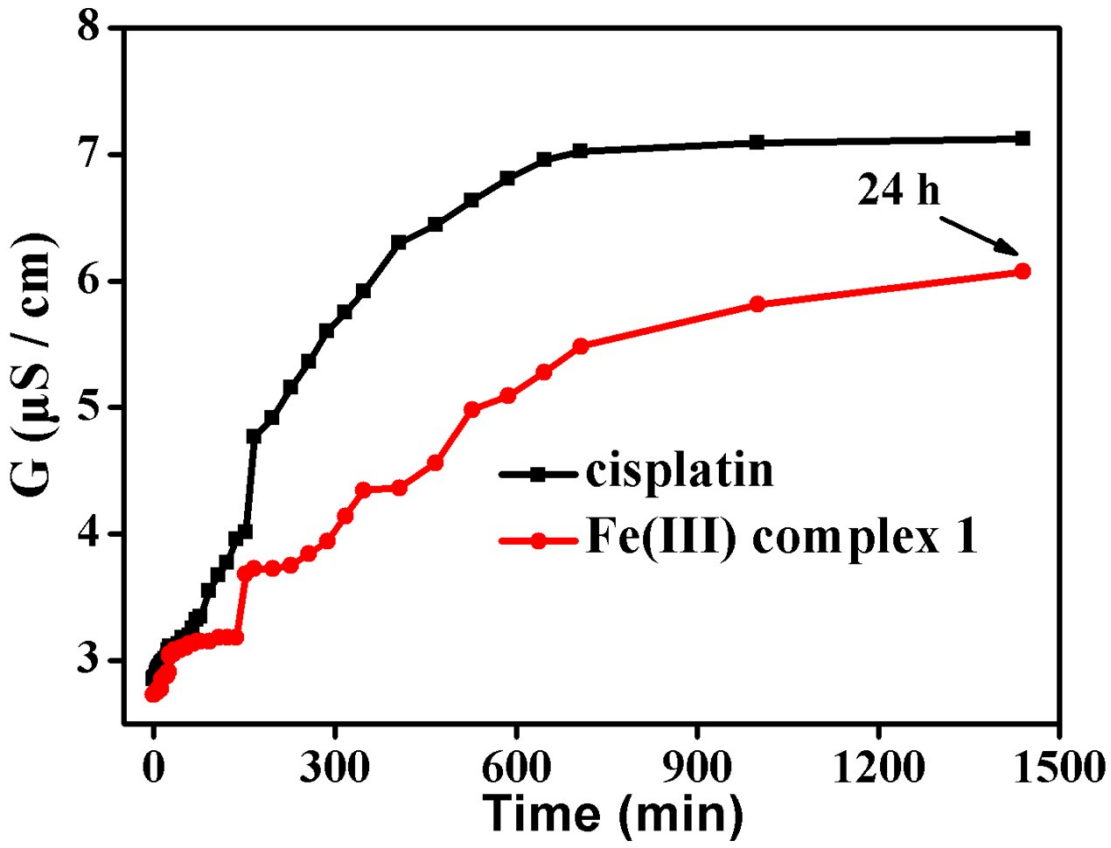

Figure S4. The variations on the conductivity of Fe(III) complex **1** (5 μM) and cisplatin (10 μM) in cellular lysate (Hep-G2 cells) at 37 ºC, shown as the average value in a triplicate experiment, which indicated that it would dissociated in TBS to give the species of e(III) complex **1** (5 μM) and cisplatin (10 μM), as suggested by the stability results of UV-Vis and ESI-MS spectroscopy.

Figure S5. The influence of Fe(III) complex **1** (5 μM) on the telomerase activity of the Hep-G2 cells for 24 h.

Table S4. DNA damage induced by Fe(III) complex **1** on Hep-G2 cells detected by Comet assay.

	Comet length	Tail length	Tail moment	Olive tail moment
Control	56.00±6.78	2.21±1.02	0.17±0.09	0.23±0.14
1	110.63±7.57*	54.98±6.81*	39.64±7.27*	20.94±5.11*

Compared with control group, *P<0.01.

Experimental methods

Materials. Tris, RNase A, and propidium iodide (PI) were purchased from Sigma. The antibody of c-myc and hTERT 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 cell lines (tumor cell lines BEL-7404, NCI-H460, T-24, Hep-G2, A549 and normal cell liver line HL-7702) were obtained from the Shanghai Institute for Biological Science (China). Stock solutions of H-ClMQ and Fe(III) complex **1** (2 mM) were made in DMSO, and further dilutions to working concentrations were made with corresponding buffer.

Instrumentation. Infrared spectra were obtained on a Perkine Elmer 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 measurements were performed on a Shimadzu RF-5301/PC spectro fluorophotometer. 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 7500fast 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, NCI-H460, T-24, Hep-G2, A549 tumor cell lines 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.

Uptake of Metal in Hep-G2 cells. Hep-G2 cells (~10 million cells) were treated with Fe(III) complex **1** (5.0 μM) and cisplatin (10.0 μM) for 24.0 h at 37.0 °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 5.0 mL of PBS. The scrapped cells were spun down, by centrifuging at 2500 rpm for 10 min. The cell pellet obtained was dissolved in 1.0 M NaOH (1.0 mL) and diluted with 2.0% (v/v) HNO_3 (5.0 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 5.0 mL using 2.0% (v/v) HNO_3. The amount of cobalt taken up by these Hep-G2 cells was determined by ICP-MS. The instrument was calibrated for platinum or iron using standard solutions containing 10, 50, 100, 500 and 1000 ppb Pt or/and Fe.

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 30-60 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.[2-5] 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.

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 Fe(III) complex **1** (5 μM) was 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.[10-14]

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.[10-14]

Western blotting. The Hep-G2 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, anti-c-myc and β-actin antibody and horseradish peroxidase-conjugated antimouse or antirabbit secondary antibody. Protein bands were visualized using chemiluminescence substrate.[12-14]

Transfection. After Hep-G2 cells (8.0×10^5 cells) were grew in 3 cm Petri dishes for 24.0 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.[12-14]

Comet Assay. Single-cell gel electrophoresis for detection of DNA damage was performed using the Comet assay reagent kit purchased from Trevigen according to the manufacturer's instructions. Briefly, cells after treatment were harvested by centrifugation at 1500 rpm (20 °C, 5 min) and resuspended at 1×10^6 Hep-G2 cells/mL in PBS. The cell suspension was mixed with melted LM agarose at a ratio of 1:10 (v/v). An aliquot (75 μL) of the mixture was immediately pipetted onto the slide (CometSlideTM). After refrigeration for 30 min, the slide was immersed in prechilled lysis solution and left on ice for 60 min, followed by immersing in freshly prepared alkaline solution (300 mM NaOH, 1 mM EDTA, pH>13) for 60 min on ice in darkness. After DNA unwinding, the slide was subjected to alkaline solution for electrophoresis in a Savant ps 250 system set at 300 mA and 1 volt/cm for 30 min. After electrophoresis, the slide was rinsed with distilled H_2O, fixed in 70% ethanol for 5 min and air-dried overnight. DNA was stained with SYBR Green I (Trevigen) and visualized under a fluorescence microscope (Nikon, Eclipse E600).[12-14]

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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