Cobalt(II) 8-hydroxyquinoline complexes: crystal structures, cytotoxicity, and action mechanism

Hai-Rong Zhang,[a,b,c] Ke-Bin Huang,[b]* Zhen-Feng Chen,[b] Yan-Cheng Liu,[b] You-Nian Liu,[a] Ting Meng,[b] Qi-Pin Qin,[b] Bi-Qun Zou[b] and Hong Liang[a,b]*

Experimental

1. Materials

All commercially available reagents were used directly without further purification unless otherwise noted. 5-nitro-8-hydroxyquinoline (NOQ), 5-bromo-8-hydroxylquinoline (BrQ), 2-amino-8-hydroxylquinoline (NHQ) were purchased from Alfa-aesar. RPMI 1640 and fetal bovine serum (FBS) were obtained from Hyclone (USA). 3-(4,5-dimathylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Propidium iodide (PI), Rnase A, Hoechst 33258, AO/EB (Acridine orange/Ethidium bromide), 2',7'-dichlorofluorescein-diacetate (DCFH-DA) and Fluo-3 AM were purchased from Sigma Chemicals Co. (USA). The CasPGLOW™ Fluorescein Activite Caspase-9/3 Staining kit were purchased from BioVision. Calf thymus DNA (ct-DNA) was purchased from SIGMA. The oligonucleotide d(TCGGCGCCGA) was purchased from Invitrogen. Tris-HCl-NaCl buffer (5 mM Tris, 50 mM NaCl, pH 7.40) was prepared using double distilled water.

2. Instrumentation

Infrared spectra were recorded on a PerkineElmer FT-IR Spectrometer. Electrospray ionization mass spectrum (ESI-MS) was carried out on a Bruker HCT Electrospray Ionization Mass Spectrometer. Elemental analyses (C, H, N) were collected on Perkin Elmer Series II CHNS/O 2400 elemental analyzer. Flow Cytometry (FCM) was collected on FACS AriaII Flow Cytometer (BD Biosciences, San Jose, USA). UV-visible (UV-Vis) absorption spectra were recorded on a Varian Cary100 UV-Visible spectrophotometer. Fluorescence measurements were obtained on a Shimadzu RF-5301/PC spectrofluorophotometer. The circular dichroic (CD) spectra were performed on a JASCOJ-810 automatic recording spectropolarimeter.

3. Synthesis

The present three metal complexes of $[Co(NOQ)_2(C_5H_5N)_2]C_2H_5OH$ (**1**), $[Co(BrQ)_2(C_5H_5N)_2]$ (**2**), and $[Co(NQ)_2(C_5H_5N)_2]$ (**3**) were prepared by treating ligands 5-nitro-8-hydroxyquinoline, 5-bromo-8-hydroxylquinoline, 2-amino-8-hydroxylquinoline with $Co(NO_3)_2.6H_2O$ in different ratios of ethanol, water and pyridine under the solvothermal conditions using procedures previously reported.[25] The structures of these compounds were determined by single crystal X-ray diffraction analysis, elemental analysis, IR and ESI-MS.

4. Synthesis of complex 1

$Co(NO_3)_2 \cdot 6H_2O$ (0.1 mmol, 0.029 g), NOQ (0.1 mmol, 0.019 g), ethanol (1.0 mL) and pyridine (0.1 mL) were placed in a 25 cm long Pyrex tube, then the mixture was frozen by liquid N_2, evacuated under vacuum, sealed and reacted at 80 °C for three days. Red brown block crystals harvested and suitable crystals were selected for X-ray diffraction analysis. Yield (0.032 g, 93%). ESI-MS (in DMSO): *+m/z* 595

$[Co(NOQ)_2(C_5H_5N)_2]^+$, 662.9 $[Co(NOQ)_2(C_5H_5N)_2 + C_2H_5OH + Na]^+$. Selected IR (KBr, cm^{-1}): 3423 (m, *v*(OH)), 1568 (s, *v*(C=N)), 1464 (s, *v*(C=C)), 1283 (s, *v*(C-N)), 1111 (s, *v*(C-O)), 575 (s, *v*(Co-N)), 497 (s, *v*(Co-O)). Anal. Calc. for $C_{30}H_{25}CoN_6O_7$: C 56.26; H 3.93; N 13.12 %. Found: C 55.9; H 4.20; N 12.5%. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.35 (dd, *J* = 8.9, 0.8 Hz, 1H), 9.21 (dd, *J* = 8.8, 0.8 Hz, 1H), 8.82 (d, *J* = 4.4 Hz, 1H), 8.74 (d, *J* = 5.5 Hz, 2H), 8.66 (d, *J* = 6.1 Hz, 2H), 8.65 (d, *J* = 9.2 Hz, 1H), 8.53 (d, *J* = 9.1 Hz, 1H), 8.30 (dd, *J* = 8.9, 5.1 Hz, 1H), 8.04 (t, *J* = 7.5 Hz, 1H), 7.90 (dd, *J* = 8.9, 5.2 Hz, 1H), 7.83 (t, *J* = 7.5 Hz, 1H), 7.62 (d, *J* = 4.5 Hz, 1H), 7.60 (d, *J* = 7.1 Hz, 1H), 7.57 (d, *J* = 9.1 Hz, 1H), 7.45 – 7.40 (m, 4H).

5. Synthesis of complex 2

$Co(NO_3)_2{\cdot}6H_2O$ (0.1 mmol, 0.029 g), BrQ (0.1 mmol, 0.022 g), ethanol (0.6 mL), pyridine (0.05 mL) and H_2O (0.05 mL) were placed in a 25 cm long Pyrex tube, then use the same processing step for complex **1**. Red brown crystals of complex **2** were generated after three days, suitable crystals were selected for X-ray diffraction analysis. Yield (0.032 g, 92%). ESI-MS (in DMSO): +*m/z* 662.9 $[Co(BrQ)_2(C_5H_5N)_2]^+$, 583.8 $[Co(BrQ)_2(C_5H_5N)]^+$. Selected IR (KBr, cm^{-1}): 3417 (m, *v*(OH)), 3054 (w, (C-H), 1575 (s, *v*(C=N)), 1455 (s, *v*(C=C)), 1263 (s, *v*(C-N)), 1082 (s, *v*(C-O)), 553 (s, *v*(Co-N)), 486 (s, *v*(Co-O)). Anal. Calc. for $C_{28}H_{20}Br_2CoN_4O_2$: C 50.71; H 3.04; N 8.45 %, Found: C 50.49; H 3.17; N 8.01%. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.69 (d, *J* = 4.9 Hz, 2H), 8.77 (d, *J* = 5.6 Hz, 4H), 8.47 (d, *J* = 8.5 Hz, 2H), 8.09 (dd, *J* = 8.5, 5.1 Hz, 2H), 7.79 (t, *J* = 7.4 Hz, 2H), 7.69 (d, *J* = 8.5 Hz, 2H), 7.40 (t, *J* = 6.8 Hz, 4H), 7.31 (d, *J* = 8.5 Hz, 2H).

6. Synthesis of complex 3

$Co(NO_3)_2{\cdot}6H_2O$ (0.1 mmol, 0.029 g), NHQ (0.1 mmol, 0.016 g), ethanol (1.65 mL) and pyridine (0.05 mL) were placed in a 25 cm long Pyrex tube, the procedure is similar to that of complex **1**. Red brown crystals of complex **3** were collected after three days, suitable crystals for X-ray analysis were selected. Yield (0.025 g, 71%). ESI-MS (in DMSO): +*m/z* 535 $[Co(NHQ)_2(C_5H_5N)_2]^+$, 455.9 $[Co(NHQ)_2(C_5H_5N)]^+$. Selected IR (KBr, cm^{-1}): 3301 (m, *v*(OH)), 3053 (w, (C□H), 1568 (s, *v*(C=N)), 1445 (s, *v*(C=C)), 1291 (s, *v*(C-N)), 1093 (s, *v*(C-O)), 538 (s, *v*(Co-N)), 416 (s, *v*(Co-O)). Anal. Calc. for $C_{28}H_{24}CoN_6O_2$: C 62.81; H 4.52; N 15.69 %, Found: C 62.80; H 4.67; N 15.62%. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.40 (s, 2H), 8.74 (d, *J* = 5.5 Hz, 4H), 8.19 (d, *J* = 17.0 Hz, 2H), 7.84 (t, *J* = 7.5 Hz, 4H), 7.49 (t, *J* = 7.0 Hz, 4H), 7.32 (d, *J* = 7.4 Hz, 2H), 6.99 (t, *J* = 7.8 Hz, 2H), 6.82 (d, *J* = 9.0 Hz, 2H), 6.75 (d, *J* = 7.6 Hz, 2H).

7. X-ray crystallography

The data for complexes **1-3** were collected on a Bruker Smart Apex II CCD diffractometer equipped with graphite monochromated Mo *Kα* radiation (λ = 0.71073 Å). Structure solution, refinement and data output were solved by OLEX2 and SHELX-97 programs.[49] The non-hydrogen atoms were located in successive difference Fourier synthesis. The final refinement was completed by full-matrix least squares methods with anisotropic thermal parameters for non-hydrogen atoms on F_2. All hydrogen atoms were added theoretically, riding on the concerned atoms. The images of the complexes **1-3** were created with the DIAMOND program.

Table S1: Crystal data and structure refinement for complexes **1**-**3**.

Compounds	**1**	**2**	**3**
Formula	$C_{30}H_{26}N_6O_7Co$	$C_{28}H_{20}N_4O_2Br_2Co$	$C_{28}H_{24}N_6O_2Co$
Fw	641.48	663.23	535.46
T / K	293(2)	293(2)	293(2)
crystal system	monoclinic	orthorhombic	tetragonal
space group	C_2/c	$Pnna$	P_4/nnc
a, Å	17.880(4)	14.6162(11)	17.9171(8)
b, Å	13.110(3)	16.3774(8)	17.9171(8)
c, Å	14.440(3)	12.5927(11)	17.1784(8)
α, °	90.00	90.00	90.00
b, °	112.47(3)	90.00	90.00
γ, °	90.00	90.00	90.00
V, Å^3	3127.9(11)	3014.4(4)	5514.6(4)
Z	4	4	8
D_c, g cm^{-3}	1.460	1.532	1.290
μ, mm^{-1}	0.611	3.258	0.657
GOF on F^2	1.159	1.048	1.114
Reflns(collected/unique)	16985/3110	8717/ 3084	9818/2778
R_{int}	0.0455	0.0367	0.0538
R_1[a] ($I > 2\sigma(I)$)	0.0937	0.0934	0.0663
wR_2[b] (all data)	0.2412	0.2749	0.1998

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

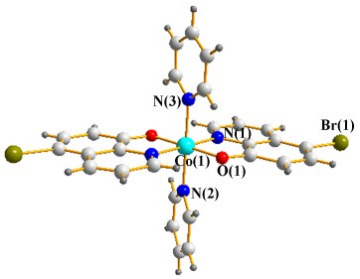

Fig. S1 ORTEP drawing of complex **2** at 30% ellipsoid probability. Selected bond lengths (Å) and angles (°), Co(1)–N(1) 1.910(7), Co(1)–N(2) 1.926(10), Co(1)–O(1) 1.878(6); N(1)–Co(1)–N(2) 90.33(18), O(1)–Co(1)–N(1) 86.4(3), O(1)-Co(1)-N(2) 89.68(17), O(1)–Co(1)–N(3) 90.32(17).

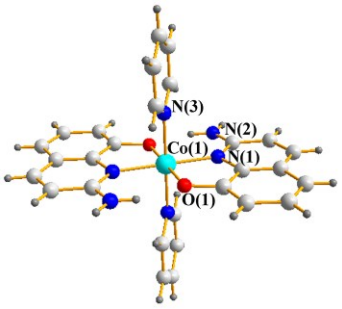

Fig. S2 ORTEP drawing of complex **3** at 30% ellipsoid probability. Selected bond lengths (Å) and angles (°), Co(1)–O(1) 1.884(3), Co(1)–N(1) 1.968(3), Co(1)–N(3) 1.964(4); O(1)–Co(1)–N(1) 85.04(14), O(1)–Co(1)–N(3) 89.23(15), N(3)–Co(1)–N(1) 90.27(14).

8. In vitro Cytotoxicity assay

T-24, BEL7404, HepG2, HeLa, MGC-803, SKOV-3, HL-7702, WI-38 cell lines were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. Cell lines were maintained in RPMI-1640 medium supplemented (including 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin) at 37 ºC in a humidified atmosphere of 5% CO_2/95% air. Cisplatin was selected as a positive metallodrug for investigating the potency of complexes **1**-**3**.

Tests of cytotoxicity were carried out in 96-well, flat bottomed microtitre plates by MTT assay. 1×10^4 cells $well^{-1}$ were seeded in 180 μL of supplemented culture medium in the plates, which were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h for allowing cell adhesion. Then different concentrations (2.5, 5, 10, 20, 40 μM, respectively) of NOQ, BrQ, NHQ, complexes **1**-**3** and cisplatin were added in the plates and cells were incubated for 48 h. After that, 10 μL of MTT (5mg/mL) in phosphate buffered saline (PBS, pH 7.40) was added to each well at the end of each incubation period, and cells were continued to incubate for 4 h. After removal of the supernatant, 100 μL of DMSO was added to dissolve formed formazan crystals and the absorbance was examined by an Enzyme-linked Immunosorbent Assay (ELISA) reader (using enzyme labelling instrument with 570/630 nm double wavelength measurement). The cytotoxicity was evaluated according to IC_{50} values which were calculated by the Bliss method (n = 5). All tests were independently repeated at least three times.

9. Cell cycle and cell apoptosis analysis by flow cytometry

For cell cycle assay, the T-24 cells were incubated in 10% FBS supplemented culture medium with complex **1** at 5.0, 10.0 μM (or 5-fluorouracil at10.0 μM) for 24 h. After treatment, cells were collected and fixed with ice-cold 75% ethanol at -20 °C overnight. Fixed cells were stained by 50 μg/mL PI in 500 μL PBS containing 100 μg/mL RNase A at 37 °C. After staining for 45 min, the cell cycle distribution was counted with FACS AriaII flow cytometer (BD) and calculated using MFLT32 LT software.

Cell apoptosis was tested by flow cytometric analysis using annexin V-FITC/PI staining. Briefly, T-24 cells were harvested after stimulation with different concentrations (5.0, 10.0, 20.0 μM) of complex **1** or cisplatin (20.0 μM) for 8 h. Then, cells were added 100 μL 1× binding buffer, 5 μL of annexin V-FITC and 5 μL of PI, stained for 20 min at room temperature in the dark. Finally, 400 μL 1× binding buffer was added, the stained cells were kept shielded from light before being analyzed by flow cytometry.

10. The examination of morphological changes on cells

The morphological changes on the tumor cells for cellular apoptosis were examined by fluorescence microscope using Hoechst 33258 and AO/EB double staining. For Hoechst 33258 assay, T-24 cells were treated with 5.0, 10.0, 20.0 μM of complex **1** or cisplatin (20.0 μM) for 8 h, respectively. Then cells were added 0.5 mL of stationary liquid and fixed for 10 min at room temperature, washed twice with PBS, added 0.5 mL Hoechst 33258 fluorescent dye in dark for staining 5 minutes, rinsed twice with PBS again, dropped a blob of anti-fluorescence quenching liquid on a slide and covered by a coverslip. The cells were visualized by a CarlZeiss LSM710 confocal microscope.

For AO/EB double staining assay, briefly, T-24 cells were incubated with 5.0, 10.0, 20.0 μM of complex **1** or cisplatin (20.0 μM) for 8 h. Then cells were harvested, suspended in PBS and added 4 μL of AO-EB working solution (AO: 100 μg/mL; EB: 100 μg/mL) for staining 5 min at room temperature. Cells were then visualized and photographed immediately by fluorescence microscope (Nikon TE2000, Japan).

11. Measurement of mitochondrial membrane potential

The loss of mitochondria membrane potential ($\Delta\Psi$) was evaluated by using a fluorescent dye Rh 123. T-24 cells were seeded in 6-well plates and stimulated with 5.0, 10.0, 20.0 μM of complex **1** or cisplatin (20.0 μM) for 8 h. Then cells were harvested, washed twice with PBS, stained by 10 μg/ml Rh 123 for 30 min at 37 °C in the dark, centrifuged, washed twice with culture medium, and analyzed immediately by flow cytometry.

12. Measurement of reactive oxygen species (ROS) generation and the intracellular Ca^{2+} concentration

ROS generation of T-24 cells induced by complex **1** was measured by flow cytometry using the stain of DCFH-DA. T-24 cells were exposed to 10.0 μM of complex **1** or cisplatin for 8 h at 37 °C, harvested, stained with DCFH-DA (100 μM in a final concentration) at 37 °C for 30 min in the dark. Then cells were washed with serum-free cell culture medium, kept in 500 μL serum-free culture medium and analyzed by flow cytometry.

Intracellular Ca^{2+} concentration was analyzed by Fluo-3 acetoxymethyl ester (Fluo-3 AM) staining. Briefly, T-24 cells were treated to 10.0 μM of complex **1** or cisplatin, stained with 5 μM Fluo-3 AM in dark for 30 min at 37 °C, rinsed with PBS and analyzed immediately by flow cytometry.

13. Determination of caspase-9 and caspase-3 activity by flow cytometric analysis

The levels of caspase-9 and caspase-3 activity were analyzed by flow cytometric using FITC-LEHD-FMK (for caspase-9), FITC-DEVD-FMK (for caspase-3) staining, respectively. T-24 cells exposed to 10.0 μM of complex **1** or cisplatin for 8 h and the controlled cells were harvested, rinsed twice with PBS and centrifuged, then added 300 μL buffer, 1 μL of FITC-LEHD-FMK (for caspase-9) or FITC-DEVD-FMK (for caspase-3), and then incubated for 1.0 h at 37 °C consequently. Finally, cells were examined by a FACS AriaII flow cytometer equipped with a 488 nm argon laser. The analysis results were depicted as the percent change on the activity comparing with the untreated control.

14. Spectroscopic studies on DNA interaction

Ct-DNA stock solution (2.0 mM) was stored at 4 °C for no more than 5 days before using. The synthesized complex **1** were prepared as 2.0 mM DMSO stock solution for next studies and diluted to required concentration by Tris-HCl buffer before using. For UV-Vis absorption assay, a 3.0 mL working solutions containing 2.0×10^{-5} M of complex **1** or EB was constantly added ct-DNA stock solution and then measured after incubating for 5 min. For electrostatic interaction experiments, similarly, 3.0 mL working solutions containing 2.0×10^{-5} M of complex **1** or EB was titrated by successive additions of 2.0×10^{-3} M SDS solution. After each addition, the mixed solution was allowed to incubate for 5 min, and measured by Varian Cary100 UV-Visible spectrophotometer. For competitive binding assay between EB and complex **1**, the fluorescence emission intensities were performed by fluorescence titration experiments. A 3.0 mL working solution including 2.0×10^{-5} M ct-DNA and 2.0×10^{-6} M EB was titrated by successive additions of complex **1** stock solution, then recorded the fluorescence emission intensity with a slit width of 15 nm/15 nm for E_x/E_m. CD spectra were measured in 0.1 M Tris-HCl buffer (pH 7.43). The 1.0×10^{-4} M ct-DNA solution was added to 2.5×10^{-4} M solution of complex **1** or EB. The mixed solutions were pre-incubated for 5 min. Finally, each sample solution was scanned in the range of 200-400 nm. The background CD signal of Tris-buffer was subtracted in advance. FT-IR spectra were measured using the oligonucleotide d(TCGGCGCCGA), prepared the sample solutions of (d(TCGGCGCCGA) (100 μM) in the absence and presence of the complex **1** (d(TCGGCGCCGA) : complex = 1:3), after the mixed solutions were reacted for 5 min at 37 °C, dried enough samples with freeze drying machine, and the samples were scanned in the range of 1300-1800 cm^{-1}.

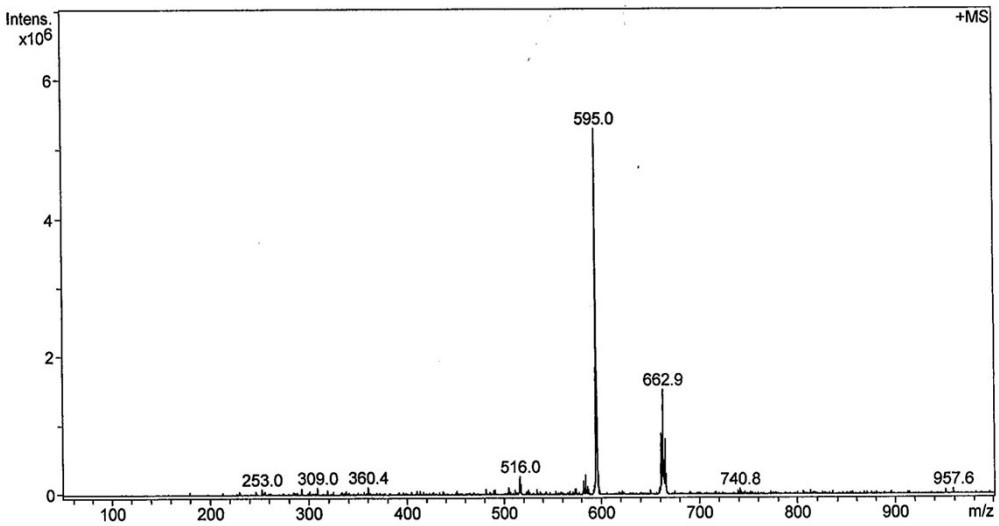

Figure S3. ESI-MS spectrum of complex **1**.

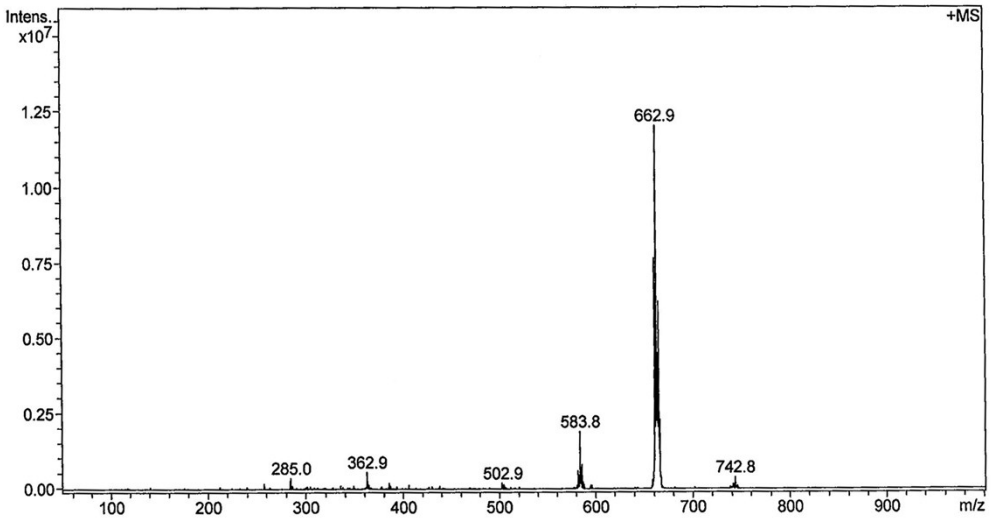

Figure S4. ESI-MS spectrum of complex **2**.

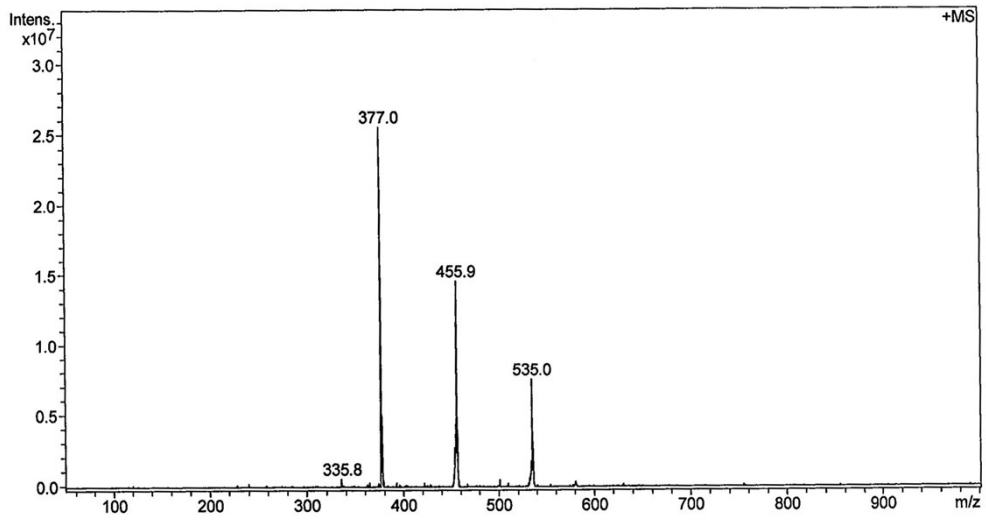

Figure S5. ESI-MS spectrum of complex **3**.

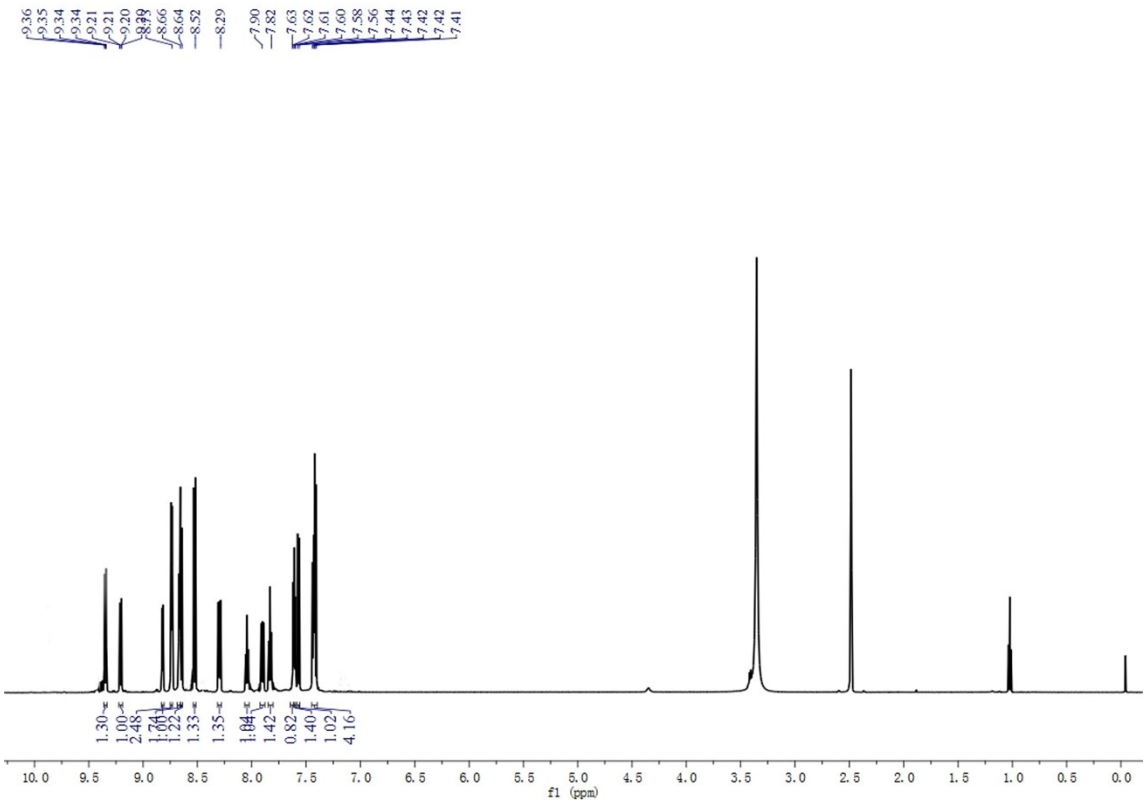

Figure S6. ^{1}H NMR spectrum of complex **1**.

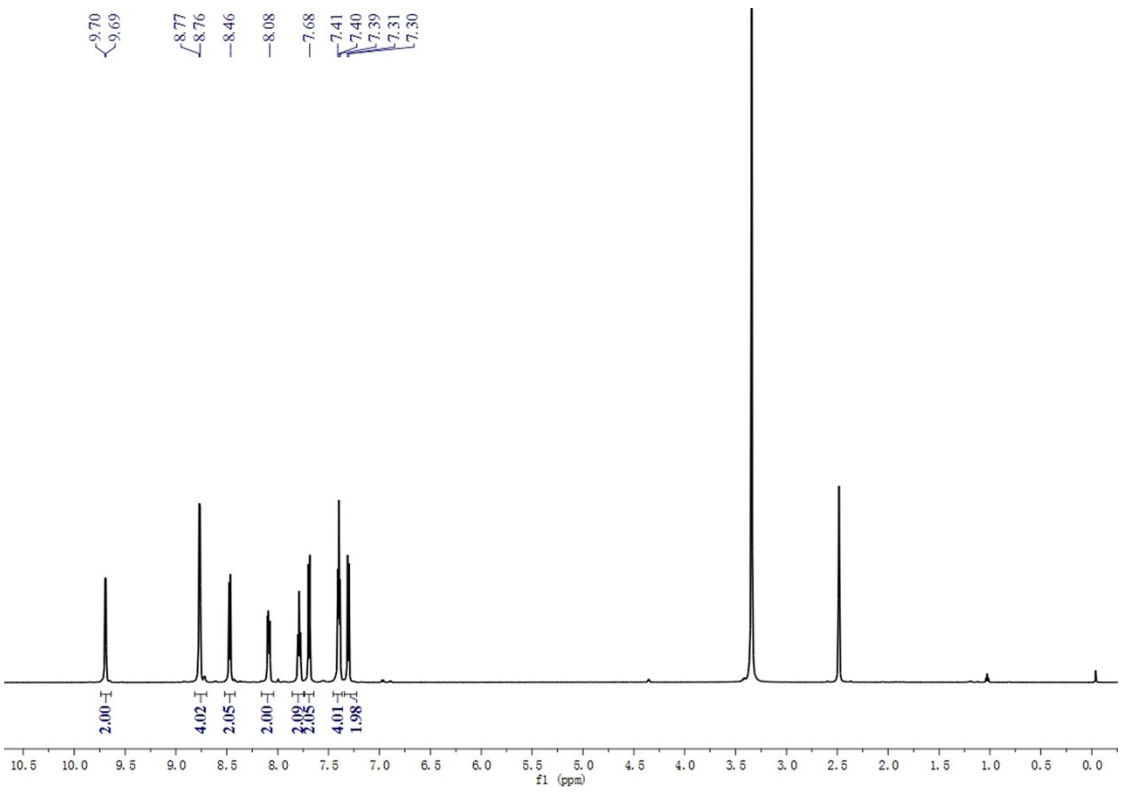

Figure S7. ^{1}H NMR spectrum of complex **2**.

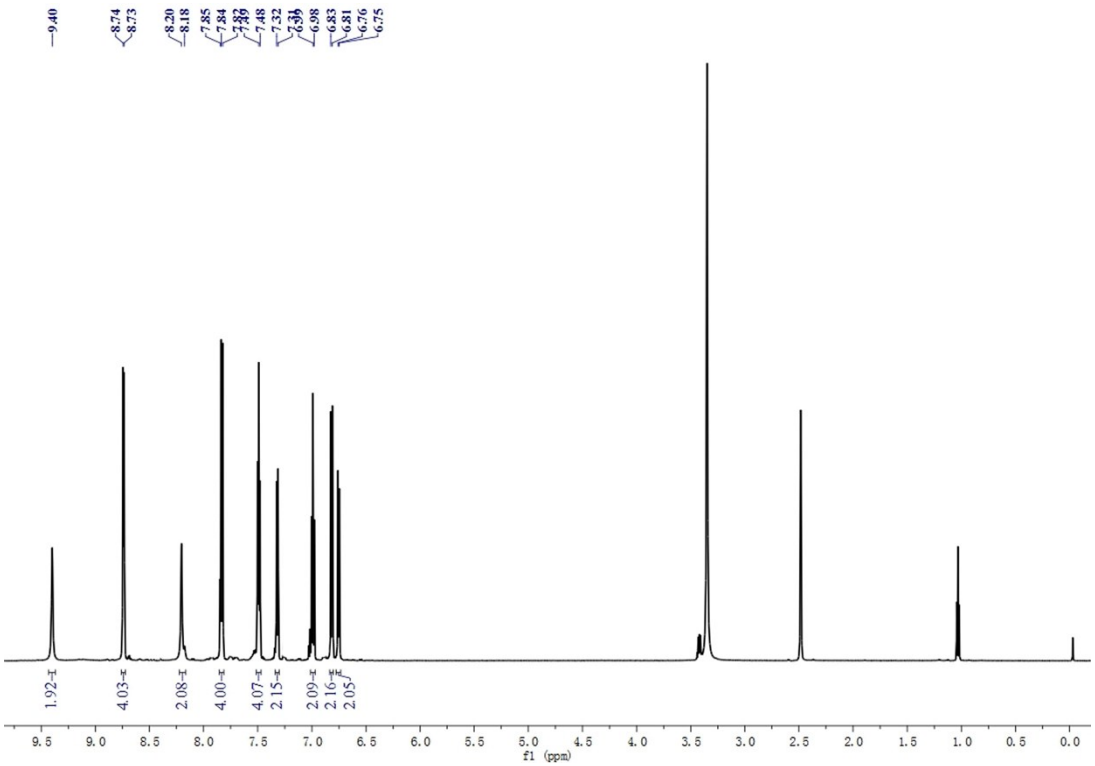

Figure S8. ^{1}H NMR spectrum of complex **3**.

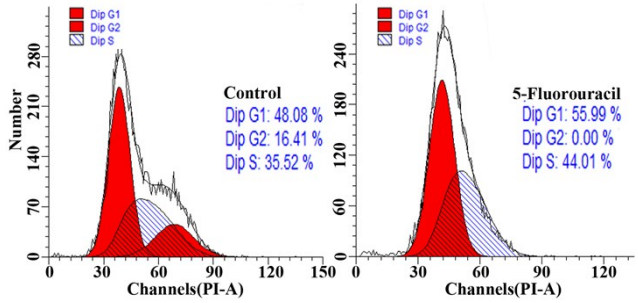

Figure S9. Cell cycle analysis by flow cytometry for the T-24 cells treated with 5-fluorouracil (10.0 μM).

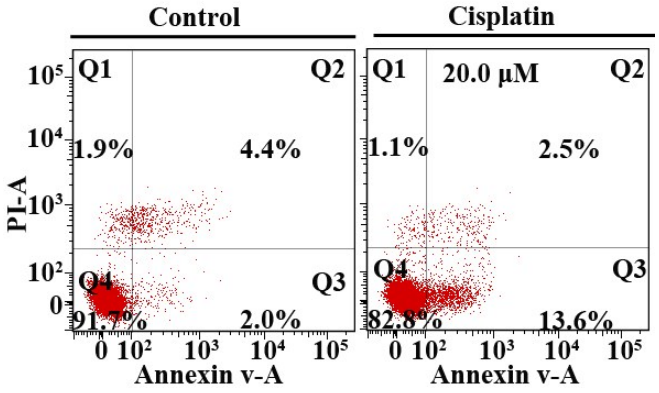

Figure S10. Annexin-V/propidium iodide assay of the T-24 cells treated with cisplatin (20.0 μM).

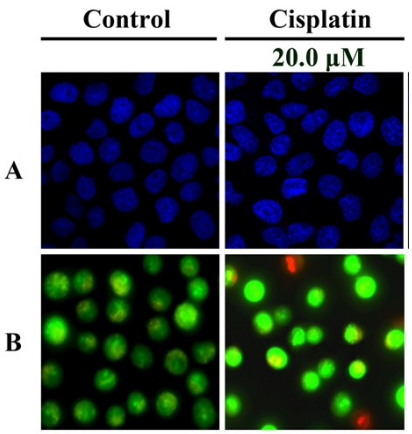

Figure S11. Morphological changes of the T-24 cells treated with cisplatin (0, 20 μM). The results of Hoechst 33258 staining (A) and AO/EB double staining (B) are shown.

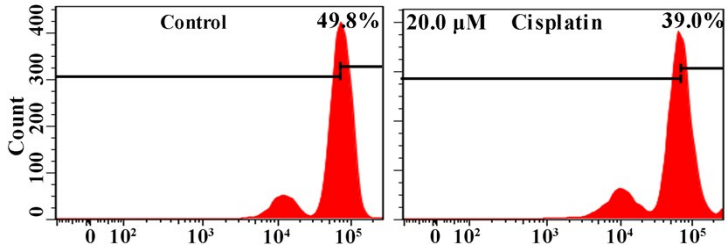

Figure S12. Rh-123 staining indicated the loss of the mitochondrial membrane potential in the T-24 cells treated with cisplatin (0, 20 μM).

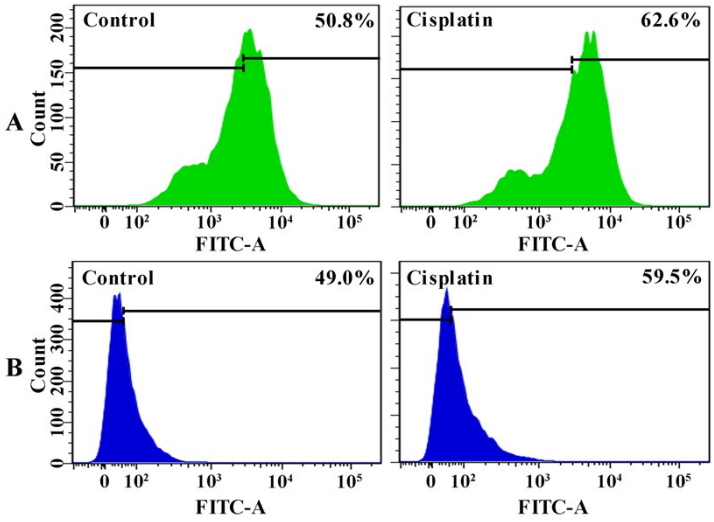

Figure S13. (A) Detection of ROS in the T-24 cells treated with cisplatin (10.0 μM). (B) Measurement of the intracellular level of Ca^{2+} in the T-24 cells treated with cisplatin (10 μM) using Fluo-3/AM as a fluorescent probe.

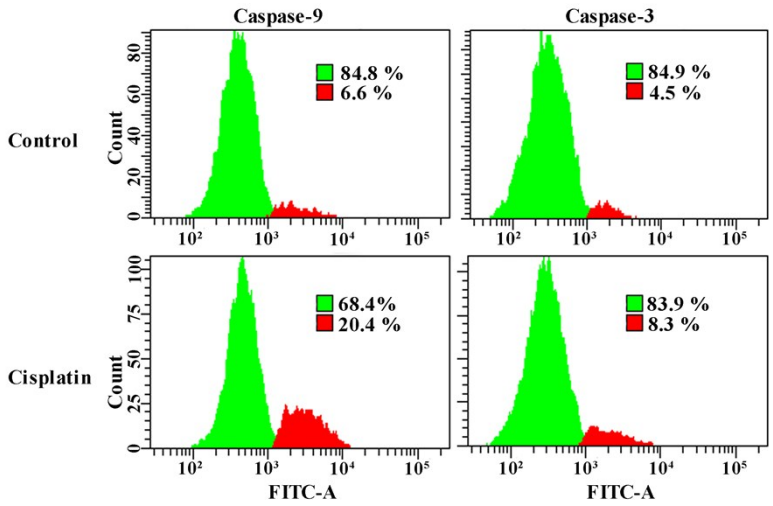

Figure S14. Activation state of caspase-9 and caspase-3 in the T-24 cells treated with cisplatin (10.0 μM).

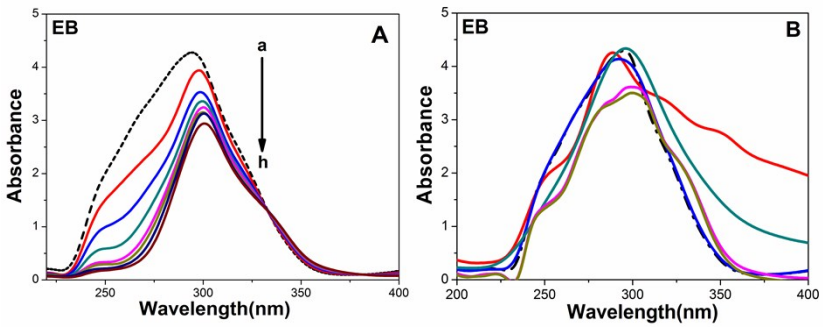

Figure S15. (A) UV-Vis spectra of EB (2.0×10^{-5} M) in the absence (dashed line) and presence (solid line) of increasing amounts of ct-DNA (from 1:1 to 7:1). (B) UV-Vis spectra of EB (2.0×10^{-5} M) in the absence (dashed line) and presence (solid line) of increasing amounts of SDS (from 1:1 to 5:1).

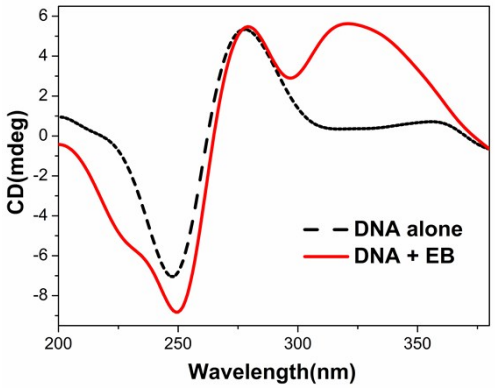

Figure S16. CD spectra of ct-DNA in the absence (black line) and presence (colour line) of EB ([DNA] = 1.0×10^{-4} M, [EB] = 2.5×10^{-4} M).