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Insight into the cytotoxicity, DNA binding and cell apoptosis induction of a zinc(II) complex of 5-bromo-8-hydroxylquinoline (HBrQ)

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1. Materials and methods

1.1 Materials and Methods

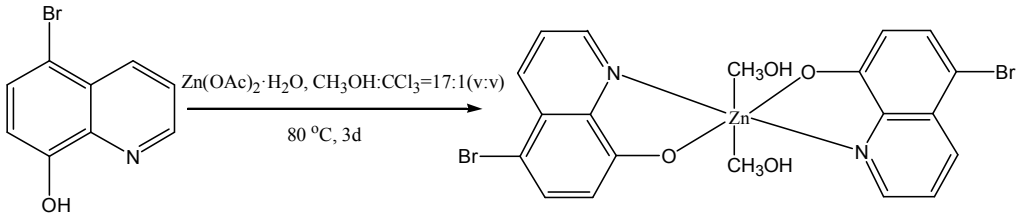
All the chemical reagents were commercially available and were used directly without further purification unless noted specifically. 5-bromo-8-hydroxylquinoline was purchased from Alfa-aesar. RPMI 1640 and fetal bovine serum (FBS) were purchased from Hyclone (USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium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

Electrospray ionization mass spectrum (ESI-MS) was recorded on a Bruker HCT Electrospray Ionization Mass Spectrometer. Infrared spectra were recorded on a PerkinElmer FT-IR Spectrometer. Elemental analyses (C, H, N) were collected on PerkinElmer Series II CHNS/O 2400 elemental analyzer. Flow Cytometry (FCM) was obtained using FACS AriaII Flow Cytometer (BD Biosciences, San Jose, USA). UV-visible (UV-Vis) absorption spectra were obtained on a Perkin-Elmer Lambda45 UV-Visible spectrophotometer. The circular dichroic (CD) spectra were recorded on a JASCO J-810 automatic recording spectropolarimeter. Fluorescence measurements were obtained on a Shimadzu RF-5301/PC spectrofluorophotometer.

1.2 Synthesis of complex 1

$Zn(OAc)_2 \cdot H_2O$ (0.1 mmol, 0.017 g), HBrQ (0.1 mmol, 0.022 g), 0.85 mL methanol and 0.05 mL chloroform were placed in a 25 cm long thick Pyrex tube. The mixture was frozen by liquid

N_2 and was vacuumized. Then the tube was sealed and kept in an oven at 80 °C for 3 days. Yellow block crystals of complex **1** were harvested and suitable single crystals were chosen for X-ray diffraction analysis. Yield (0.024 g, 83%). ESI-MS (in DMSO): *m/z* 546.7 $[Zn(BrQ)_2+H_2O+OH]^-$, 357.7 $[Zn(BrQ)+2H_2O+2OH]^-$, 699.1 $[\mu_2\text{-}Zn_2(BrQ)_2+4H_2O+3OH]^-$. ^{1}H-NMR (500 MHz, d_6-DMSO) δ 8.70 (2H, d, J = 5 Hz, 2-*H*), 8.47 (2H, d, J = 5 Hz, 4-*H*), 7.73 (2H, t, J = 5 Hz, 3-*H*), 7.64 (2H, d, J = 10 Hz, 6-*H*), 6.75 (2H, d, J = 5 Hz, 7-*H*), 3.18 / 3.17 (3H / 3H, s, $-CH_3$ in MeOH), 1.83 (1H, s, –OH in MeOH); ^{13}C-NMR (126 MHz, d_6-DMSO) δ 146.15, 141.19, 137.85, 133.82, 128.37, 123.55, 113.21, 98.79, 49.07.[23] FT-IR (KBr, cm^{-1}): 3426 (m, ν(OH)), 1580 (s, ν(C=N)), 1456 (s, ν(C=C)), 1253 (s, ν(C–N)), 1127 (s, ν(C–O)), 1045 (s, ν(C–Br)), 534 (s, ν(Zn–N)), 502 (s, ν(Zn–O)). Anal. Calc. for $C_{20}H_{18}Br_2N_2O_4Zn$: C 41.74; H 3.15; N 4.87 %. Found: C 41.30; H 3.26; N 4.82% (For the synthetic route, see Scheme S1).



Scheme S1 Synthesis route for complex **1**

1.3 X-ray crystallography

The single crystal X-ray diffraction data for complex **1** were collected on a Bruker Smart Apex II CCD diffractometer equipped with graphite monochromated Mo *K*α radiation (λ = 0.71073 Å) at 296.15 K. The structure was solved with direct methods and refined by full-matrix least-squares method using the OLEX2 and SHELXL-97 programs [1]. The non-hydrogen atoms were refined anisotropically by full-matrix least squares methods. All hydrogen atoms were added theoretically, riding on the concerned atoms. The image of the complex was created by the DIAMOND program

[2].

1.4 DNA binding studies

The UV-Vis spectral analysis for DNA binding studies were carried out in 0.1 M of Tris buffer saline (TBS, pH = 7.34), which gave a ratio of UV-Vis absorbance at 260 and 280 nm (A260/A280) of 1.8-1.9, indicating that the ct-DNA was sufficiently free of protein. The stock solution of ct-DNA (2.0 mM) was stored at 4 ºC no more than 5 days before use. The concentration of the stock solution of complex **1** dissolved in DMSO was also 2.0 mM. The experiments were carried out with fixed concentrations (3.0 mL) of complex **1** (2.0×10^{-5} M) by gradient addition of the stock solution of DNA. After the mixture was reacted for 5 min, the absorption spectra were recorded. The electrostatic interaction experiment for **1** was also performed by UV-Vis absorption spectrum. A solution (3.0 mL) containing 2.0×10^{-5} M of complex **1** was titrated by successive additions of sodium dodecyl sulfonate (SDS) solution (to give a final concentration of 1.0×10^{-4} M) and the absorption intensity was recorded. For the DNA competitive binding study between EB and complex **1** by the fluorescence emission spectral analysis, each 3.0 mL of working solution (including 2.0×10^{-5} M ct-DNA and 2.0×10^{-6} M EB) was titrated by successive addition of the stock solution of complex **1**, and was then recorded with a slit width of 15 nm/15 nm for λ_{Ex} / λ_{Em}. The CD (circular dichroism) spectra were also recorded in 0.1 M TBS (pH 7.34) working solution. A 3.0 mL solution of ct-DNA (1.0×10^{-4} M) was mixed by complex **1** under the gradient addition (1, 2, 3, 4×10^{-4} M, respectively), and was incubated for 5 min. The CD spectrum of each sample solution was then scanned in the range of 200–400 nm. The TBS solution was set as the blank sample.

1.5 Cell culture and MTT assay

The cells lines purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences including BEL-7404, HeLa, Hep-G2, MGC-803, T-24 tumor cell lines and HL-7702 human normal liver cell were maintained in RPMI-1640 medium supplemented with 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. Complex **1** was dissolved in DMSO at a concentration of 2.0 mM as a stock solution and diluted by PBS to the required concentration immediately before use. Cisplatin was selected as a reference metallodrug for investigating the potency of complex **1** [3].

For the *in vitro* MTT assay, 1×10^4 cells $well^{-1}$ were seeded in 180 μL of supplemented culture medium in 96-well, flat bottomed microtitre plates. The plates were incubated at 37 ºC in a humidified atmosphere of 5% CO_2 for 24 h to allow cell adhesion, prior to drug testing. Then different concentrations (2.5, 5, 10, 20, 40 μM, respectively) of HBrQ, complex **1** and cisplatin were added in the culture medium. Cells were exposed to HBrQ, complex **1** and cisplatin for another 48 h. At the end of each incubation period, 10 μL of MTT (5mg/mL) in phosphate buffered saline (PBS, pH 7.40) was added to each well, and cells were continued to incubate for 4 h. 100 μL of DMSO was added to dissolve formed formazan crystals after removal of the supernatant, and the absorbance was monitored using enzyme labelling instrument with 570/630 nm double wavelength measurement by an Enzyme-Linked Immunosorbent Assay (ELISA) reader. The cytotoxicity was evaluated by the IC_{50} values (drug concentration at which 50% of the cells are viable relative to the control) which were calculated by the Bliss method (n = 5).

1.6 Cell cycle assay

The BEL-7404 cells were incubated in 10% FBS supplemented culture medium with complex **1** at 4.5, 9.0, 18.0 μM for 24 h. After treatment, cells were harvested and fixed with ice-cold 75%

ethanol at –20 °C overnight. Fixed cells were resuspended in 500 μL of PBS containing 50 μg/mL PI and 100 μg/mL RNase A for staining 45 min at 37 ºC. The cell cycle distribution was analyzed by FACS AriaII flow cytometer (BD) and calculated using MFLT32 LT software.

1.7 Cell apoptosis assay

The BEL-7404 cells were plated in 6-well plates at the concentration of 1×10^5 cells/mL, incubated with complete medium only (control) and medium containing different concentrations (4.5, 9.0, 18.0 μM) of complex **1**, respectively. After chemical treatment for 8 h, cells were trypsinized and collected, added 100 μL 1× binding buffer, stained by 5 μL of annexin V-FITC and 5 μL of PI at 4 °C for 20 min in the dark, and finally resuspended in 400 μL 1× binding buffer. Cell apoptosis was assayed by flow cytometry within an hour.

1.8 Fluorescence morphological examination

The morphological changes on the tumor cells for cellular apoptosis were visualized by Hoechst 33258 and AO/EB double staining. For Hoechst 33258 assay, BEL-7404 cells were incubated in six-well plates, and were treated with 4.5, 9.0, 18.0 μM of complex **1** for 8 h, respectively. After that, the cells were washed with PBS, added 0.5 mL of stationary liquid and fixed for 10 min at room temperature, rinsed twice with PBS, stained by 0.5 mL Hoechst 33258 fluorescent dye in dark for 5 minutes, washed twice with PBS again, dropped a blob of anti-fluorescence quenching liquid on a slide and covered by a coverslip. The cells were visualized and photographed by a CarlZeiss LSM710 confocal microscope. For AO/EB double staining assay, briefly, the BEL-7404 cells were incubated with 4.5, 9.0, 18.0 μM of complex **1** for 8 h. Then the cells were trypsinized and harvested, suspended in PBS and stained by 4 μL of AO-EB working solution (AO: 100 μg/mL; EB: 100 μg/mL) for 5 min at room temperature. The cells were then

visualized immediately by fluorescence microscope (Nikon TE2000, Japan).

1.9 Measurement of mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\Psi$) was measured by 3,6-diamino-9-[2-(methoxycarbonyl)phenyl] xanthylium chloride (Rh 123) staining. BEL-7404 cells were seeded in 6-well plates and exposed to 9.0 μM of complex **1** for 8 h. The cells were then harvested, washed twice with PBS, stained by 10 μg/ml Rh 123 for 30 min at 37 ºC in the dark, centrifuged, washed twice with culture medium, and monitored immediately by flow cytometry.

1.10 Measurement of reactive oxygen species (ROS) production

The level of ROS production was measured using the stain of DCFH-DA by flow cytometry. BEL-7404 cells were incubated in 6-well culture plates at a cell density of 2×10^5 cells/well and exposed to 9.0 μM of complex **1** for 8 h at 37 ºC. The cells were then loaded with 100 μM DCFH-DA and incubated at 37 ºC for 30 min in the dark. After that, the cells were washed twice with serum-free cell culture medium, maintained in 500 μL serum-free culture medium. The ROS generation was measured immediately by flow cytometry.

1.11 Measurement of intracellular calcium concentration ($[Ca^{2+}]_c$)

Intracellular Ca^{2+} concentration was analyzed by flow cytometry using Fluo-3 acetoxymethyl ester (Fluo-3 AM) staining. Briefly, BEL-7404 cells were exposed to 9.0 μM of complex **1**, stained with 5 μM Fluo-3 AM in dark for 30 min at 37 ºC, washed with PBS and measured immediately by flow cytometry.

1.12 Determination of caspase-3/9 activity by flow cytometric analysis

The assessments of caspase-3 and caspase-9 activity were performed by flow cytometric using FITC-DEVD-FMK (for caspase-3) or FITC-LEHD-FMK (for caspase-9) staining. The BEL-7404

cells exposed to 9.0 μM of complex **1** for 8 h and the controlled cells were harvested, washed twice with PBS and centrifuged, then mixed with 300 μL buffer, added 1 μL of FITC-DEVD-FMK (for caspase-3) or FITC-LEHD-FMK (for caspase-9) consequently and incubated for 1.0 h at 37 ºC. Then, the cells were examined by a FACS AriaII flow cytometer equipped with a 488 nm argon laser. And the analysis results were represented as the percent change on the activity comparing with the untreated control.

Reference

[1] G.M. Sheldrick, SHELXS-97, Program for the Solution of Crystal Structures, University of Göttingen, Germany, 1997.

[2] J. Lu, Q. Sun, J.L. Li, L. Jiang, W. Gu, X. Liu, J.L. Tian, S.P. Yan, J. Inorg. Biochem. 137 (2014) 46-56.

[3] Q.P. Qin, Z.F. Chen, W.Y. Shen, Y.H. Jiang, D. Cao, Y.L. Li, Q.M. Xu, Y.C. Liu, K.B. Huang, H. Liang, Eur. J. Med. Chem. 89 (2015) 77-87.

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

Empirical formula	$C_{20}H_{18}Br_2N_2O_4Zn$
Formula weight	575.55
Temperature / *K*	293(2)
Crystal system	monoclinic
Space group	$P2_1/c$
a / *Å*, *b* / *Å*, *c* / *Å*	5.06412(19), 18.1609(12), 11.4569(4)
α / °, β / °, γ / °	90.00, 97.646(4), 90.00
Volume / $Å^3$	1044.31(9)
Z	2
ρ_{calc} / mg mm^{-3}	1.830
μ / mm^{-1}	5.031
F(000)	568
Crystal size / mm^3	0.44 × 0.20 × 0.15
2θ range for data collection	7.18 to 52.74°
Index ranges	$-4 \le h \le 6$, $-22 \le k \le 22$, $-14 \le l \le 13$
Reflections collected	5059
Independent reflections	2139 [*R*(int) = 0.0388]
Data/restraints/parameters	2139 / 2 / 137
Goodness-of-fit on F^2	1.084
Final *R* indexes [$I>2\sigma(I)$]	$R_1 = 0.0585$, $\omega R_2 = 0.1323$
Final *R* indexes [all data]	$R_1 = 0.0887$, $\omega R_2 = 0.1466$
Largest diff. peak/hole / *e* $Å^{-3}$	0.695 / –0.488

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

Zn(1)-O(1)	2.048(3)	O(1)-Zn(1)-O(2)	88.99(14)
Zn(1)-O(2)	2.193(3)	O(1)-Zn(1)-N(1)	80.02(14)
Zn(1)-N(1)	2.126(4)	N(1)-Zn(1)-O(2)	90.44(15)
Zn(1)-O(1^1)	2.048(3)	O(1^1)-Zn(1)-O(2^1)	88.99(14)
Zn(1)-O(2^1)	2.193(3)	O(1)-Zn(1)-O(2^1)	91.01(14)
Zn(1)-N(1^1)	2.126(4)	O(1^1)-Zn(1)-O(2)	91.01(14)
O(1^1)-Zn(1)-N(1)	99.98(14)	O(1^1)-Zn(1)-O(1)	180.00(1)
O(1^1)-Zn(1)-N(1^1)	80.02(14)	N(1)-Zn(1)-O(2^1)	89.56(15)
O(1)-Zn(1)-N(1^1)	99.98(14)	N(1^1)-Zn(1)-O(2^1)	90.44(15)
O(2^1)-Zn(1)-O(2)	180.00(1)	N(1^1)-Zn(1)-O(2)	89.56(15)

Table S3. IC_{50} (μM) values of complex **1**, comparing with HBrQ, $Zn(OAc)_2 \cdot H_2O$ and cisplatin, against five selected tumor cell lines and the normal human liver cell HL-7702 after incubation for 48 h.

Compounds	BEL-7404	Hep-G2	HeLa	T-24	MGC-803	HL-7702
$Zn(OAc)_2 \cdot H_2O$	>100	51.61±0.05	>100	99.52±0.06	41.20±0.04	>100
HBrQ	>100	39.53±0.05	>100	>100	23.77±0.04	43.77±0.05
1	8.69±0.04	22.2±0.04	25.41±0.05	11.09±0.05	21.22±0.05	28.40±0.05
Cisplatin	25.08±0.12	9.73±0.07	2.07±0.09	14.05±0.03	4.08±0.06	5.04±0.06

Noted: IC_{50} values are presented as the mean SD (standard error of the mean) from five separate experiments.

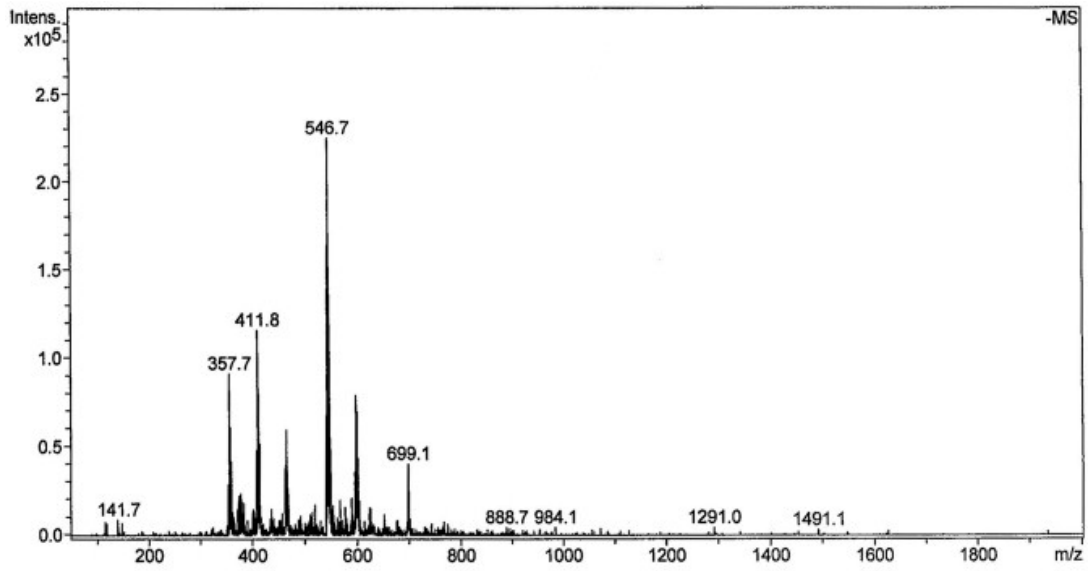


Figure S1. ESI-MS spectrum of the complex **1**.

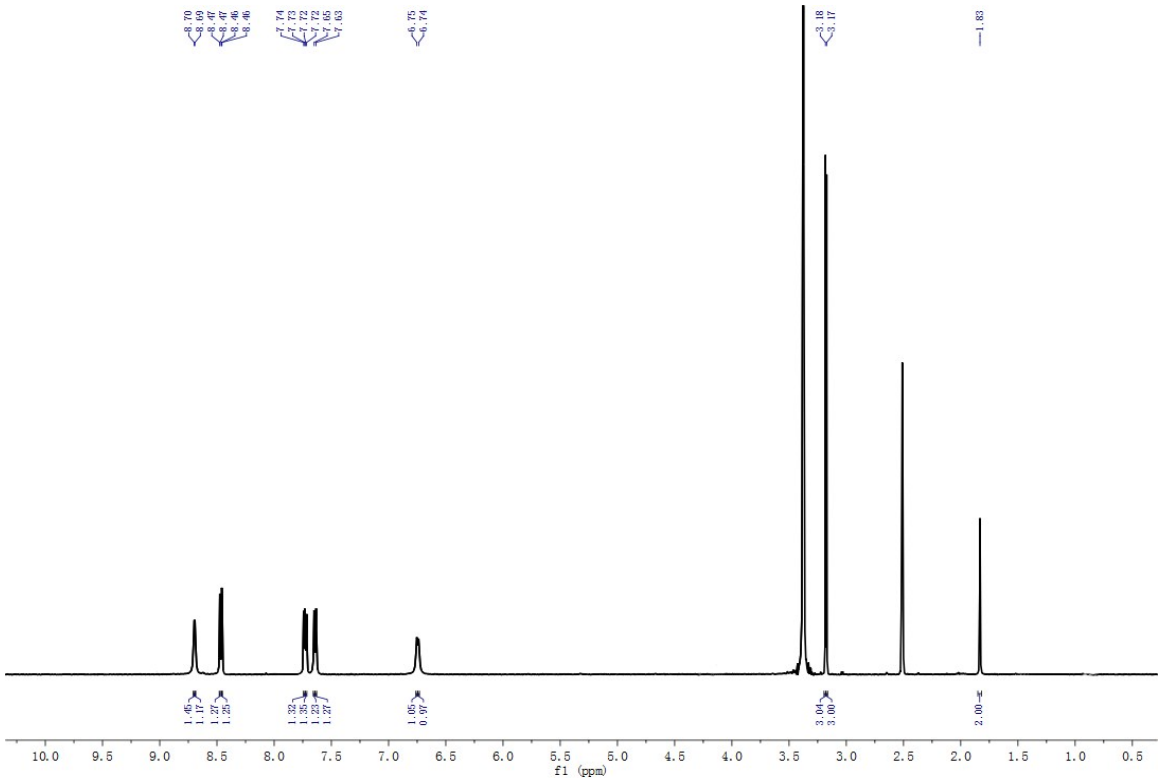


Figure S2. ^{1}H-NMR spectrum of the complex **1**.

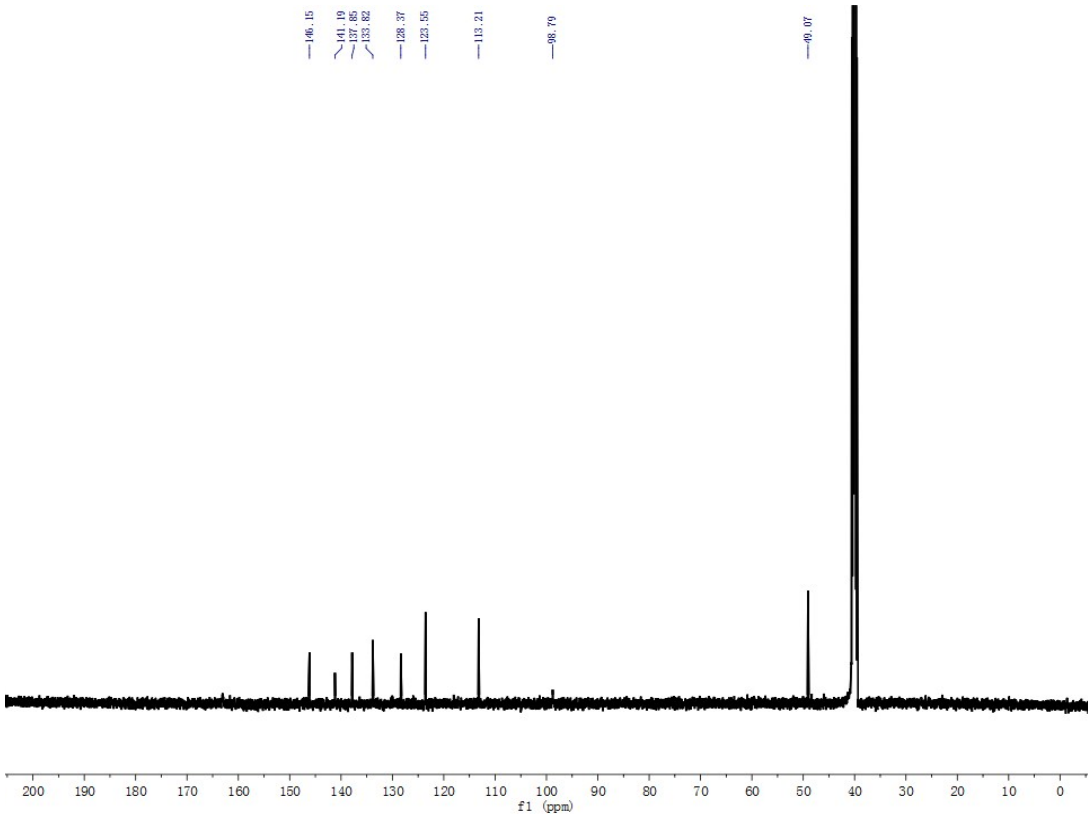


Figure S3. ^{13}C-NMR spectrum of the complex **1**.

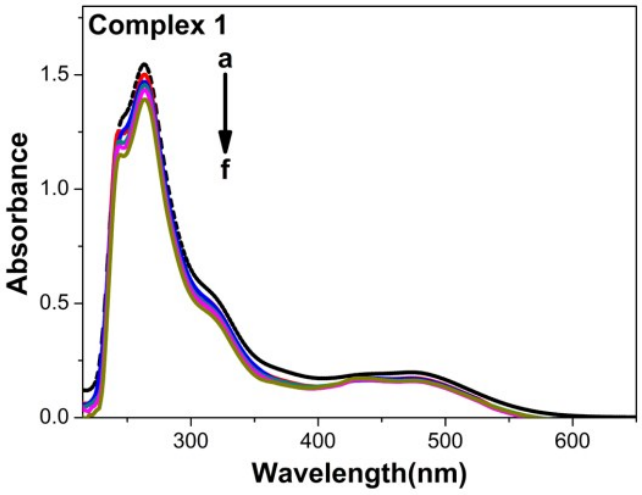


Figure S4. The absorption spectra of complex **1** (2.0×10^{-5} M) in the absence (dashed line) and presence (solid lines) of increasing amounts of SDS from 1 : 1 to 5 : 1.

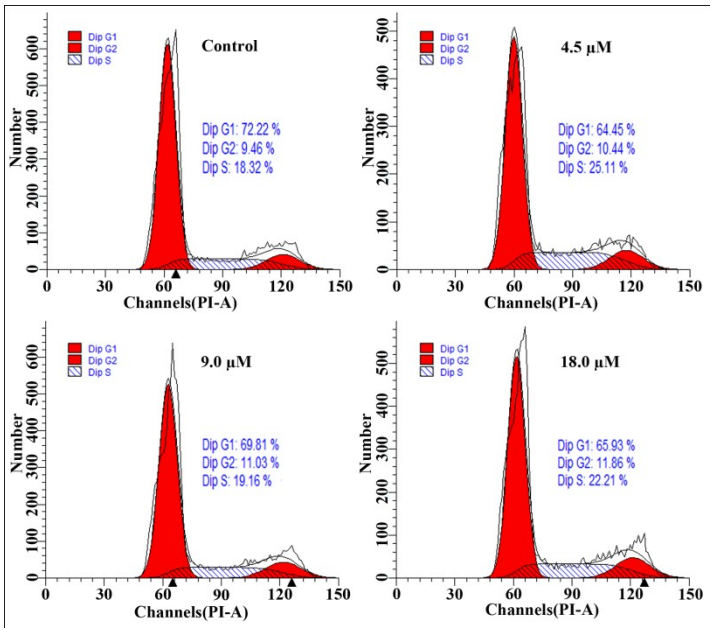


Figure S5. The percentage of cell populations in various phases (G1, S and G2 phase) for the cell cycle distribution in the BEL-7404 cells treated by 4.5, 9.0, 18.0 μM of $Zn(OAc)_2 \cdot H_2O$ for 48 h.

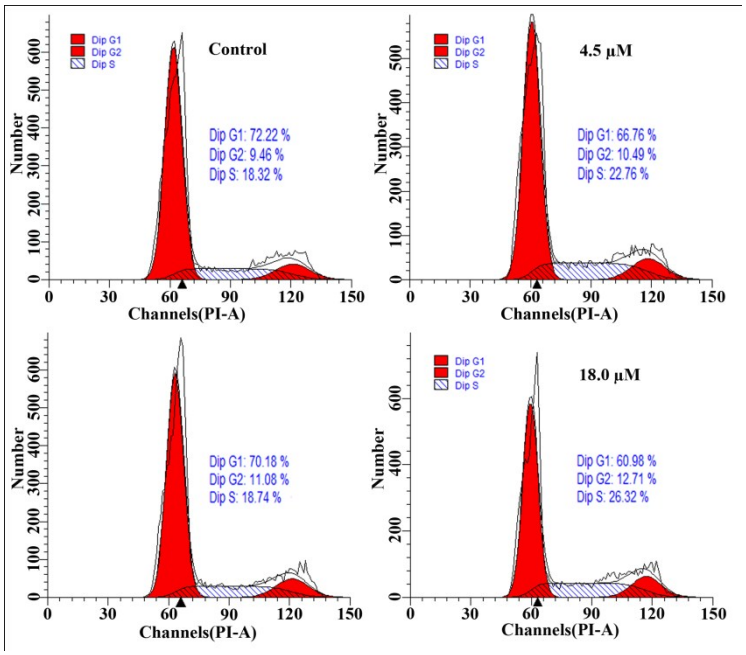


Figure S6. The percentage of cell populations in various phases (G1, S and G2 phase) for the cell cycle distribution in the BEL-7404 cells treated by 4.5, 9.0, 18.0 μM of HBrQ for 48 h.

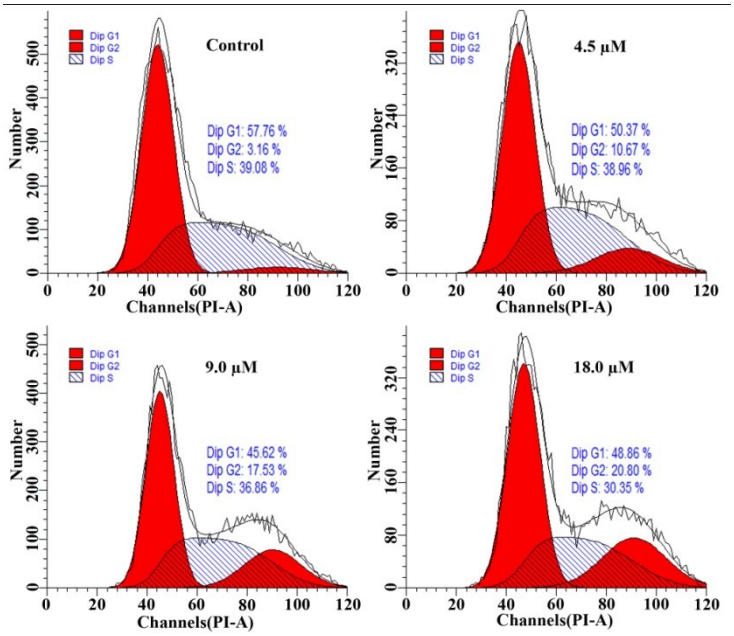


Figure S7. The percentage of cell populations in various phases (G1, S and G2 phase) for the cell cycle distribution in the BEL-7404 cells treated by 4.5, 9.0, 18.0 μM of complex **1** for 48 h.