Supplementary materials

DNA interactions and *in vitro* anticancer evaluations of pyridin-benzimidazole-based Cu complexes

Jiyong Hu[1], Chunli Liao[2], Ruina Mao[3], Junshuai Zhang[1], Jin'an Zhao[1,*]Zhenzhen Gu

1. Experimental section

1.1 Materials and general methods.

All chemicals used were of analytical grade and utilized without further purification. 4,4'–bis((2-(pyridin-2-yl)-1H-benzo[d]imidazol-1-yl)methyl)biphenyl (bpbb) was synthesized according to previously published methods with some modifications [1]. Ethidium bromide (EB), 3-(4,5-dimathylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and Calf thymus DNA (CT-DNA), were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Tris-HCl buffer solution was prepared using triple-distilled water. Fetal bovine serum (FBS) was purchased from Hyclone. Cisplatin was obtained from Shanghai Energy Chemical Co., Ltd. Protein concentrations were measured using the bicinchoninic acid (BCA) assay, applying a Varioskan multimode microplate spectrophotometer. Additionally, apoptosis and ROS for the samples were analyzed using a Guava easyCyte 6-2I flow cytometer (Millipore, USA), whereas cell cycle and mitochondrial membrane potential assays were performed on a BD LSRFortessa™ Cell Analyzer (USA). The visualization of comet assay cells and ROS production were performed using Zeiss Axio Vert. A1 inverted fluorescence microscope. The Cu contents were obtained on an inductively coupled plasma mass spectrometry (ICP-MS) with a Nex ION 300X instrument (PerkinElmer, USA). The circular dichroism (CD) spectrum was recorded on a Bio-logic MOS-500 spectropolarimeter. Elemental analysis was performed using a NETZSCH STA 409 PC/PG instrument. The Fourier transform infrared spectroscopy (FT-IR) spectra were recorded on a PerkinElmer Frontier spectrophotometer using KBr pellets in the region of 400–4000 cm^{-1}; the far-infrared spectroscopy (far-IR) spectra were performed on a PerkinElmer spectrum400 spectrophotometer with ATR attachment. Elemental analyses (C, H, and N) were measured using a Flash EA 1112 elemental analyzer.

1.2 The synthesis of the complexes

*1.2.1 Synthesis of [Cu(bpbb)$_{0.5}$·Cl·SCN]·(CH$_3$OH) (**1**).*

A solution of CuSCN (0.0037 g, 0.03 mmol) in methanol (1 mL) was added to a stirred solution of bpbb (0.0065 g, 0.02 mmol) in chloroform (1 mL) and N,N-dimethylacetamide (0.5 mL) at room temperature. The reaction mixture was then sealed in a glass reactor and heated at 85 °C for two days. Subsequently, the reaction system was cooled to room temperature. The solution produced blue rod crystals suitable for X-ray analysis. Yield: 64% (based on Cu). Calcd. for ($C_{21}H_{18}ClCuN_4OS$): C 53.27, H 3.83, N 11.83; found: C 53.01, H 3.54, N 12.01. IR(KBr/pellet, cm^{-1}): 3466w, 2113s, 2018s, 1601m, 1572m, 1518w, 1482s, 1455s, 1438s, 1335m, 1286m, 1021m, 745s; Far-IR(ATR, cm^{-1}): 430m, 409m, 395w, 321m, 286s, 228w. The stability of **1** in solution was investigated using electrospray ionization mass spectrometry (ESI-MS). ESI-MS: (Found, m/z: 846.52), could be assigned to [Cu$_2$(bpbb)·Cl·(SCN)$_2$]$^+$, (calcd, m/z: 847.38); (Found, m/z: 823.92), could be assigned to [Cu$_2$(bpbb)·Cl$_2$·SCN]$^+$, (calcd, m/z: 824.75).

*1.2.2 Synthesis of [Cu$_2$(bpbb)·Br$_3$·(OH)]$_n$ (**2**).*

A solution of CuBr$_2$ (0.0067 g, 0.03 mmol) in methanol (4 mL) was added to a solution of bpbb (0.0098 g, 0.03 mmol) in chloroform (1 mL) and N,N-dimethylformamide (1 mL). The reaction solution was kept at room temperature for 5 weeks, which produced blue block crystals. Yield: 50% (based on Cu). IR(KBr/pellet, cm^{-1}): 3390m, 3054w, 2938w, 1659m, 1600s, 1481s, 1456s, 1437s, 1403m, 1338m, 1290m, 1177w, 1055w, 1005m, 748s, 696w; Far-IR(ATR, cm^{-1}): 433w, 412s, 309m, 250w, 174s. The peaks appearing at m/z 789.6 could be assigned to the {[Cu$_2$(bpbb)·Br·(OH)] – H}$^+$ (calcd, m/z: 790.67).

1.3 Crystal structure determination

Data for both complexes were collected at 100 K on a SuperNova single-crystal X-ray diffractometer using Cu-Kα radiation (λ = 1.54184 Å). The structures were solved by direct methods and expanded with Fourier techniques. The non-hydrogen atoms were first refined isotropically and afterward anisotropically. Hydrogen atoms were placed in calculated geometrically positions. The final cycle of full-matrix least-squares refinement was based on the observed reflections and variable parameters. All refinements were performed using the

SHELXL and olex2 programs [2–5]. The residual electron density in complex **2** that could not be modeled as the solvent was removed using the SQUEEZE function of PLATON [6–7]. Table S1 lists cell parameters and refinement conditions for complexes **1** and **2**, whereas Table S2 shows the main bond lengths and angles.

1.4 Stability determination

Stability of the complexes is vital for biological studies. Complexes were soluble at 4×10^{-5} M in Tris-HCl/NaCl buffer (5 mM Tris-HCl/50 mM NaCl, pH 7.40) with 0.4% dimethylsulfoxide (DMSO) as the cosolvent. The ultra violet-visible (UV-vis) absorption spectra of the complexes were carried out using a Specord 200 UV-visible spectrophotometer. The ESI-MS spectra of **1** and **2** were performed using an Agilent 1100 LC/MSD Trap SL Electrospray Ionization Mass Spectrometer.

1.5 Cell Culture Experiments

Four different human tumor cell lines and one normal liver cell line were used. Colon cancer cell line (HCT116), gastric cancer cell line (BGC823) and normal liver cell line (LO2 cells) were cultured in DMEM media, while colon cancer cell line (HT29) and liver cancer cell line (SMMC7721) were grown in RMPI-1640 media containing 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C and CO_2 (5%). HCT116, BGC823, HT29, SMMC7721 and LO2 cell lines were purchased from Jiangsu KeyGEN BioTECH Corp., Ltp (Nanjing, China). The cells, after appropriate incubation, were seeded into different plates for the *in vitro* assays.

1.6 Cell Viability Assay

Cell viability was determined using standard MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] assays after 24, 48, and 72 h of incubation. Briefly, a cell suspension (200 μL) was seeded into 96-well plates (4.5×10^3 to 8×10^3 cells per well) and incubated overnight. The tested complexes were dissolved in DMSO and subsequently diluted

with 10% FBS-supplemented DMEM (HCT116, BGC823) or RMPI-1640 (SMMCF7721, Ht29) media to the indicated concentrations (ranging from 5 to 60 μM). Five replica wells containing culture media (200 μL) without cells were used as blanks. Cisplatin was used as a positive control and was dissolved with phosphate buffered saline (PBS) before dilution. After the required treatment time, the cells were incubated with MTT solution (20 μL of 5 mg/mL; Sigma) for 4 h at 37 °C. The medium was then replaced by 150 μL of DMSO to dissolve the formed formazan crystals. The plate was then shaken well to form a homogeneous mixture, and the absorbance was measured at 492 nm using a microplate reader (TECAN). The cytotoxicity of each sample was expressed as the IC_{50} value, which was determined by plotting the percentage viability versus concentration on a logarithmic graph and reading off the control. All samples were assayed in triplicate, and the final IC_{50} values were calculated by the average of the triplicate experimental results.

1.7 Cellular uptake determination

The uptake and DNA accumulation of copper in HCT116 cells after exposure to complex **1** for 12 h was determined using ICP-MS measurement. The experiment was performed according to a previously published method [8]. The isolated cell nuclei, mitochondria, and DNA samples were digested with concentrated HNO_3 at 95 °C for 2 h, 30% H_2O_2 at 95 °C for 1.5 h, and concentrated HCl at 37 °C for 1 h, sequentially. The protein concentration was assayed using the BCA method, according to the manufacturer's instructions (micro-well plate protocol). The Cu content in HCT116 cell nuclei and mitochondria was determined after the cells were exposed to complex **1** (15 μM). The genomic DNA was extracted using a TIANamp Genomic DNA Kit.

1.8 DNA binding, cleavage and comet assays

All experiments involving DNA were determined in a Tris-HCl buffer solution (5 mM Tris-HCl/50 mM NaCl, pH 7.40). The concentration of DNA was determined by its UV absorbance at 260 nm, taking 6600 $M^{-1}cm^{-1}$ as the molar absorption coefficient. The UV absorbance ratio at 260 and 280 nm (A260/A280) was 1.89, suggesting that the DNA solution was sufficiently free of protein [9]. The stock solution of DNA was stored at 4 °C.

1.8.1 UV-vis absorption spectral titration experiments

Absorption spectra titrations were carried out on a Specord 200 UV-Vis spectrophotometer using varying concentrations of DNA to the complex **1** (5.0×10^{-5} M) with the $R_{[DNA/complex\ 1]}$ = 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.2 and 1.6. Before the absorption spectra were measured, the solutions were incubated at 37 °C for 60 min.

1.8.2 Competitive DNA-binding studies with EB

The competitive binding of the tested complexes to DNA was determined using an EB displacement assay (PH = 7.4, 5 mM Tris-HCl/50 mM NaCl buffer). The experiments were carried out by adding an increasing amount of the complexes ([complex] = 1–50 μM) into the EB-DNA solution (10 μM), and incubating the mixture at 37 °C for 4 h. The influence of addition of complex **1** to the EB-DNA solution was determined by measuring the variation of fluorescence emission spectra (λ_{ex} = 490 nm, λ_{em} = 510.0–800.0 nm).

1.8.3 CD measurement

The CD spectra of DNA (100 μM) were determined in 5 mM Tris-HCl/50 mM NaCl buffer solution (pH 7.2) at room temperature with increasing [Complex]/[DNA] ratios (r = 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0). The results were determined as the average of three replications from 230 to 320 nm and deducting the buffer background.

1.8.4 DNA cleavage studies

DNA cleavage by complex **1** was assayed using agarose (1%) gel electrophoresis of a 10 μL total sample volume in 0.2 mL transparent microcentrifuge tubes containing pBR322 DNA (50 ng/μL) in a 50 mM Tris-HCl/50 mM NaCl buffer (pH = 7.3). For the gel-electrophoresis experiments, supercoiled pBR322 DNA was incubated with the complex (60–160 μM), and the mixtures were maintained in the dark for 4 h at 37 °C.

1.8.5 Comet assay

HCT116 cells (3.0×10^5) were seeded in six-well plate. After 24 h, the cells were incubated

with different concentrations of complex **1** (0, 5, 10, and 20 μM) for another 12 h. A comet assay was then performed as previously described [10]. Briefly, 50 μL of 0.5% normal agarose in PBS was heated to 45 °C and dropped lightly onto a fully frosted microslide, covered by a coverslip immediately, and maintained at 4 °C for 10 min. The coverslip was removed after the gel set, and 25 μL cell suspension was mixed with 75 μL of 1% low-melting agarose at 37 °C. A total volume of 100 μL of the mixture was used rapidly on top of the gel, coated over the microslide, covered promptly with a coverslip, and then kept at 4 °C for 10 min. After agarose solidification, the coverslips were removed, and immersed in an ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, and 10% DMSO; pH 10). The samples were then retained at 4 °C for 4 h in the dark. Thereafter, the slides were incubated with the electrophoresis buffer (300 mM NaOH, 1.2 mM EDTA) for 20 min to allow unwinding of the DNA, and electrophoresis was performed at 25 V for 40 min. Subsequently, the slides were washed three times with PBS, and the cells were stained with 50 μL EB in the dark for 15 min. DNA fragmentation was visualized and analyzed under an inverted fluorescence microscopy.

1.9 Apoptosis evaluation

1.9.1 Cell Cycle Distribution Analysis

A cell cycle assay was performed using a Cell cycle detection Kit (KeyGen BioTECH). HCT116 cells were plated in 6-well culture plate, grown for 24 h, and then treated with various concentrations of complex **1** (0.4% DMSO as a cosolvent) for another 12 h. At the indicated time points, cells were trypsinized and collected into centrifuge tubes. The cells washed once with PBS and fixed by adding 500 μL of 70% (V/V) chilled ethanol overnight at 4 °C. The fixed cells were then washed with PBS before staining. RNase A (100 μL) was added and incubated for 30 min at 37 °C. Lastly, PI (400 μL) was added to each tube, and incubated for 30 min at 4 °C.

1.9.2 Measurement of mitochondrial transmembrane potential ($\Delta\psi_m$)

The mitochondrial membrane potential $\Delta\psi_m$ in HCT116 cells was measured using the JC-1 reagent (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide). First, HCT116 cells (3.0×10^5) were plated in 6-well culture plate and exposed to three different

concentrations (0, 5, 10, and 20 μM) of complex **1** for 12 h. After incubation, the medium was removed and the cells were resuspended with PBS twice. The cells were collected and resuspended in medium (0.5 mL) and JC-1 dye (0.5 mL). After 20 min of incubation at 37 °C in the dark, the cells were collected and washed with JC-1 dye buffer (1 mL) twice. The supernatants were gently discarded after centrifugation. Subsequently, JC-1 dye buffer (0.5 mL) was added for detection.

1.9.3 Intracellular ROS measurement

The production of intracellular ROS was investigated using the fluorescent probe 2',7'-dichlorofluorescein diacetate (H_2DCF-DA, Sigma-Aldrich). The cultured cancer cells were incubated with diverse concentrations of **1** (5, 10, and 20 μM) for 12 h, and the untreated cells were used as the control. The cells were then incubated with 10 μM of H_2DCF-DA at 37 °C for 20 min. The DCF fluorescence intensity is positively related to the amount of intracellular ROS generated. The level of intracellular ROS was examined using flow cytometry and inverted fluorescence microscopy.

1.9.4 Flow Cytometric Analysis of Apoptosis

HCT116 cells in the logarithmic growth phase (3.0×10^5) were seeded in a 6-well culture plate. After incubation for 24 h, these cells were treated with various concentrations of complex **1** (0, 5, 10, and 20 μM) for 20 h; an untreated sample was used as the control. Following digestion with trypsin, the cells were collected, washed with PBS twice, resuspended in binding buffer, and then 5 μL annexin V-FITC and 5 μL propidium iodide (PI) were added. The cells were kept in the dark for 15 min to allow cell apoptosis to be determined. Subsequently, the samples were analyzed by flow cytometery.

References

[1] A. W. Addison, P. J. Burke, Synthesis of some imidazole- and pyrazole- derived chelating agents. *J. Heterocyclic Chem.*, 1981, **18**, 803–805.

[2] G. M. Sheldrick, SHELXTL-97, Program for Refining Crystal Structure Refinement,

University of Göttingen (Germany). 1997

[3] G. M. Sheldrick, SHELXS-97, Program for Crystal Structure Solution, University of Gcttingen (Germany), 1997

[4] G. M. Sheldrick, A short history of *SHELX*. *Acta Crystallographica*, 2008, **A64**, 112–122.

[5] O. V. Dolomanov, L. J. Bourhis, R. J. Gildea, J. A. K. Howard, H. Puschmann, OLEX2: a complete structure solution, refinement and analysis program. *J. Appl. Crystallogr.*, 2009. **42**, 339–342.

[6] Spek, A.L. (2003). Single-crystal structure validation with the program *PLATON*. *Journal of Applied Crystallography*, 36, 7–13.

[7] Spek, A. L. (2009). Structure validation in chemical crystallography. *Acta Crystallographica*, D65, 148–155.

[8] Zhu, Z., Wang, X., Li, T., Aime, S., Sadler, P. J., Guo, Z. (2014). Platinum(II)–Gadolinium(III) Complexes as Potential Single-Molecular Theranostic Agents for Cancer Treatment. *Angewandte Chemie International Edition*, *53*, 13225–13228.

[9] Alagesan, M., Sathyadevi, P., Krishnamoorthy, P., Bhuvanesh, N. S. P., Dharmaraj, N. (2014). DMSO containing ruthenium(II) hydrazone complexes: *in vitro* evaluation of biomolecular interaction and anticancer activity. *Dalton Transactions*, *43*, 15829–15840.

[10] Maclaren, J. K., Sanchiz, J., Gili, P., Janiak, C. (2012). Hydrophobic-exterior layer structures and magnetic properties of trinuclear copper complexes with chiral amino alcoholate ligands. *New Journal of Chemistry*, *36*, 1596–1609.

Table S1. Crystal data and structure refinement for complexes **1** and **2**

Complex	**1**	**2**
Formula	$C_{21}H_{18}ClCuN_4OS$	$C_{38}H_{28}Br_3Cu_2N_6O$
fw	473.44	951.47
Temp(K)	100.01(10)	100.00(10)
λ (Cu, Mo Kα), Å	1.54184	1.54184
Crystal system	triclinic	monoclinic
Space group	P-1	$P2_1/m$
a (Å)	9.4142(8)	9.7938(3)
b (Å)	10.2389(8)	19.0133(15)
c (Å)	11.9095(10)	14.1848(8)
α(deg)	87.064(7)	90
β(deg)	67.901(8)	103.517(4)
γ(deg)	71.422(7)	90
V ($Å^3$)	1005.13(16)	2568.2(3)
Z	2	2
F(000)	484.0	938.0
θ range for data (deg) collection (deg)	4.019–76.432	3.204–76.319
Final R1,[a]wR2[b]	0.0376, 0.0893	0.0794, 0.2185
Goodness-of-fit on F^2	1.042	0.981

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

Table S2. Selected bond lengths (Å) and angles (°) for complexes **1** and **2**

Complex **1**			
Cu1–Cl1	2.2615(7)	Cu1–N2	1.983(2)
Cu1–N4	1.949(2)	Cu1–N1	2.045(2)
N2–Cu1–Cl1	96.29(7)	N2–Cu1–N1	80.52(9)
N4–Cu1–Cl1	90.99(7)	N4–Cu1–N2	172.69(10)
N4–Cu1–N1	92.36(9)	N1–Cu1–Cl1	160.98(6)
Complex **2**			
Br1–Cu1	2.4816(13)	Cu1–Br2	2.593(2)
Cu1–N1	2.114(5)	Cu1–N2	1.950(9)
Cu1–O1	1.914(5)	Br1–Cu1–Br2	120.67(8)
N1–Cu1–Br1	123.0(2)	N1–Cu1–Br2	116.2(2)
N2–Cu1–N1	78.3(3)	O1–Cu1–Br1	94.2(2)

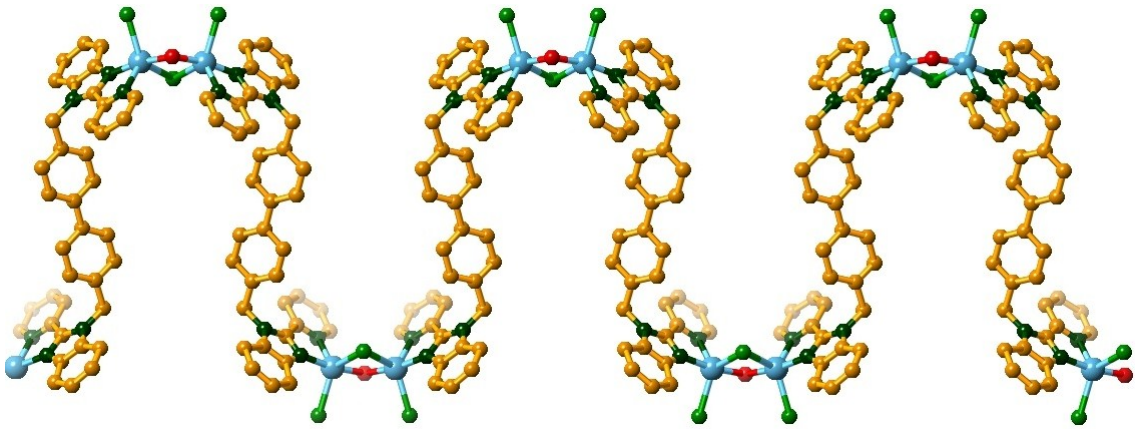

Fig. S1 The 1D chain of complex **2**.

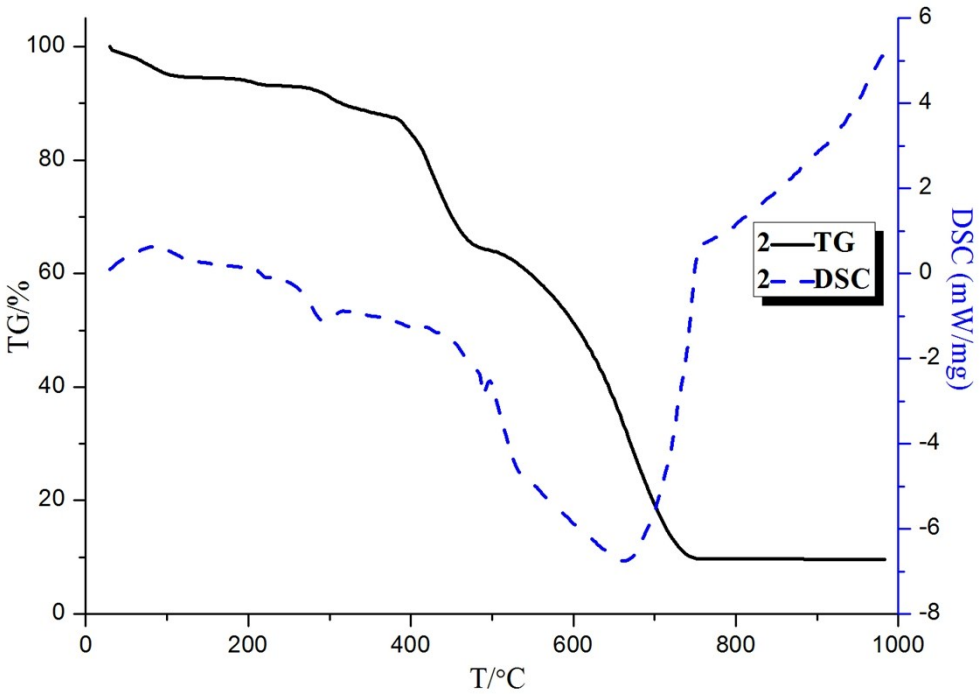

Fig. S2 The plots of TG and DSC analyses of complex **2**.

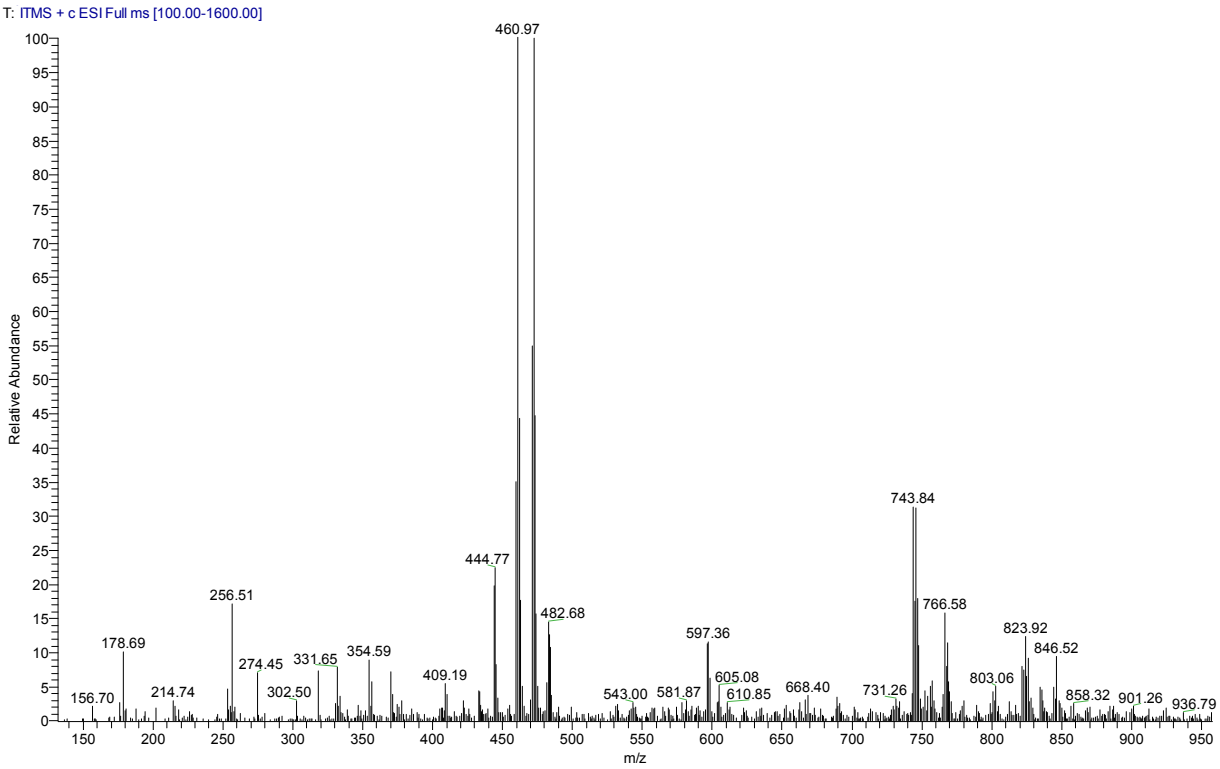

Fig. S3 ESI-MS spectrum of complex **1**.

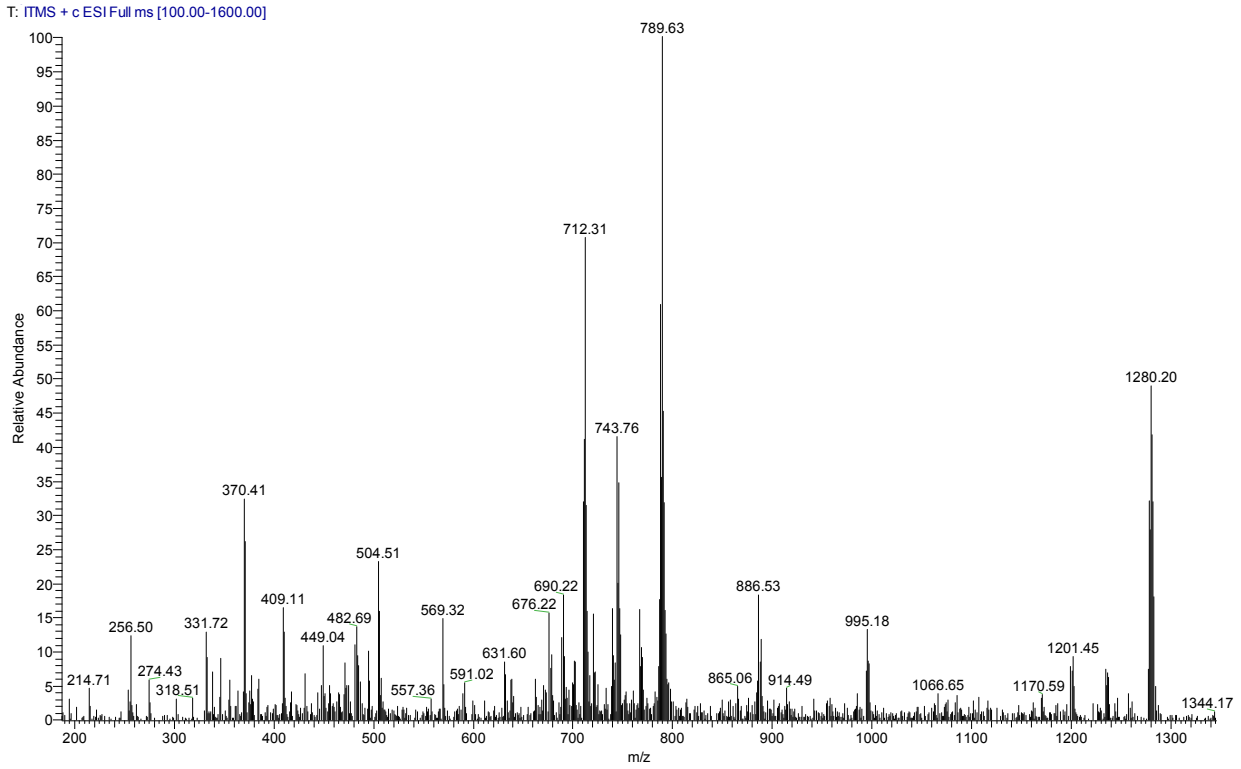

Fig. S4 ESI-MS spectrum of complex **2**.

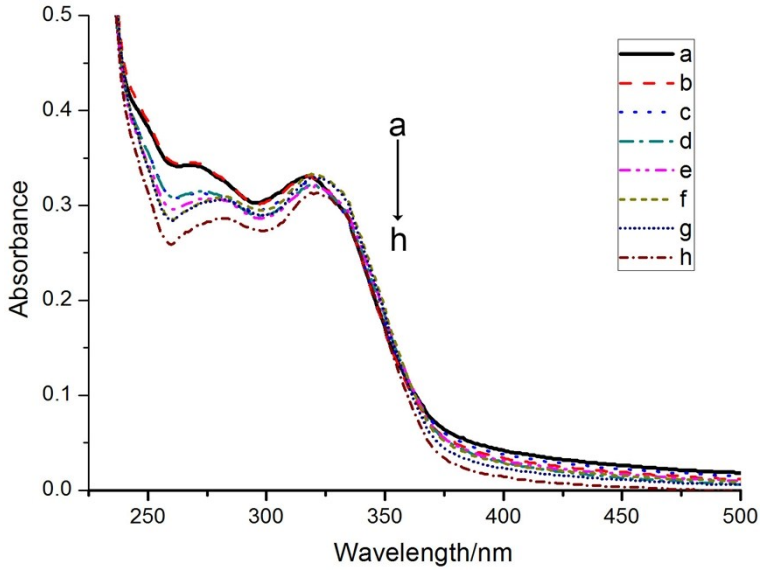

Fig. S5 Absorption spectra of complex **1** [complex **1** = 5×10^{-5} M] in the absence (solid line) and presence (dashed line) of increasing amounts of CT-DNA at room temperature in Tris-HCl/NaCl buffer (pH=7.4). The arrow (↓) shows the absorbance changes upon increasing the DNA concentration (a: $C_{\text{complex 1}} = 5\times10^{-5}$ M; b–j: $R_{\text{[DNA]/[complex 1]}} = 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.2, 1.6$, respectively).

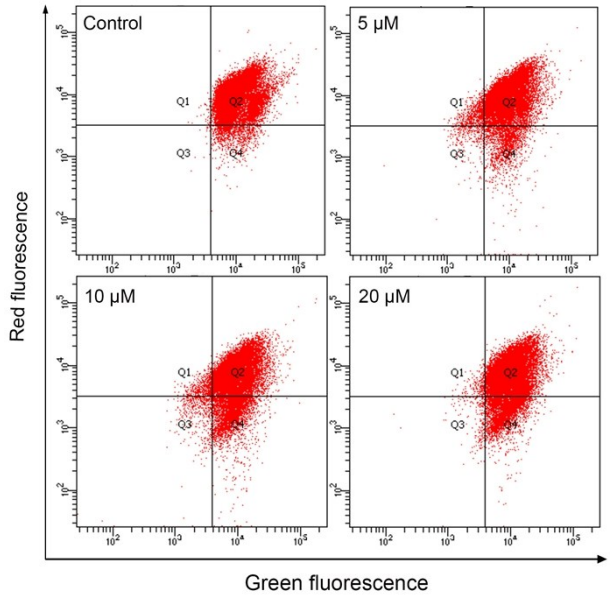

Fig. S6 Induction of mitochondrial dysfunction by complex **1** for 12 h (0, 5, 10, 20 μM).

Table S3 Comparison of the cytotoxic effects of two complexes, bpbb and cisplatin (IC_{50} values were tested for 48 h).

	BGC823	HT29	HCT116	SMMC7721
bpbb	40 ± 5	20 ± 2	34 ± 5	55 ± 9
cisplatin	5 ± 1	32 ± 4	23 ± 1	10 ± 0.4

Table S4 Comparison of the cytotoxic effects of two complexes, bpbb and cisplatin (IC_{50} values were tested for 48 h).

	LO2
bpbb	13 ± 2
cisplatin	5 ± 1
Complex **1**	6 ± 1
Complex **2**	5 ± 1