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Electronic Supporting Information Materials

Structure and Anticancer Activities of Four Cu(II) Complexes Bearing Tropolone

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

	1	**2**
Formula	$C_{14}H_{10}CuO_4$	$C_{38}H_{28}C_{l2}Cu_2N_4O_5$
f w	305.76	818.62
T / K	296(2)	296(2)
Crystal system	Monoclinic,	Triclinic
Space group	$P2_1/c$	$P\bar{1}$
a / Å	3.772(3)	9.1448(4)
b / Å	13.655(12)	13.4418(7)
c / Å	11.517(11)	14.6873(7)
α / °	90	110.896(5)
β / °	92.726(14)	92.600(4)
γ / °	90	99.526(4)
V / Å^3	592.5(9)	1652.69(15)
Z	2	2
μ / mm^{-1}	1.714	1.645
D_c / g cm^{-3}	1.849	1.502
F(000)	310	832
θ / °	2.315 -26.663	3.540 - 25.010
Reflns collected	5037	18072
Reflns unique	1239	5788
R_{int}	0.0745	0.0559
GOF on F^2	1.060	1.061
R_1 [I> 2σ(I)]	0.0506	0.0453
wR_2 [I>2σ(I))]	0.1230	0.1091
R_1 (all data)	0.0735	0.0652
wR_2 (all data)	0.1380	0.1252
Largest diff. peak/hole / e Å^{-3}	0.944 and -0.583	0.493 and -0.434

[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 S2. Crystal data and structure refinement details for **3** and **4**.

	3	**4**
Formula	C_{34} H_{26} Cu_2 N_2 O_6	C_{17} H_{15} Cl Cu N_2 O_3
f w	685.65	394.30
T / K	296(2)	296(2)
Crystal system	Triclinic	Monoclinic
Space group	$P\bar{1}$	$P2_1/n$
a / Å	8.0152(11)	9.547(3)
b / Å	12.4307(17)	12.575(4)
c / Å	14.712(2)	14.020(4)
α / º	90.432(2)	90
β / º	95.729(2)	94.564(4)
γ / º	102.287(2)	90
V / Å^3	1424.4(3)	1677.8(9)
Z	2	4
μ / mm^{-1}	1.599	1.561
D_c / g cm^{-3}	1.545	1.478
F(000)	700	804
θ / °	1.392 - 25.015	2.179 - 26.648
Reflns collected	15435	19702
Reflns unique	5028	3507
R_{int}	0.0243	0.0260
GOF on F^2	1.054	1.024
R_1 [I> 2σ(I)]	0.0285	0.0241
wR_2 [I>2σ(I))]	0.0805	0.0668
R_1 (all data)	0.0343	0.0284
wR_2 (all data)	0.0852	0.0700
Largest diff. peak/hole / e Å^{-3}	0.381 and -0.263	0.291 and -0.279

[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 S3. Selected bond lengths (Å) and angles [deg] for complexes1-4.

Complex **1**			
Cu(1)-O(1)	1.908(3)	Cu(1)-O(2)	1.903(3)
O(2)#1-Cu(1)-O(2)	180.0	O(2)#1-Cu(1)-O(1)	96.27(12)
O(2)-Cu(1)-O(1)	83.73(12)	O(2)#1-Cu(1)-O(1)#1	83.73(12)
O(2)-Cu(1)-O(1)#1	96.27(12)	O(1)-Cu(1)-O(1)#1	180.0
Complex **2**			
Cu(1)-O(1)	1.923(2)	Cu(1)-O(2)	1.941(2)
Cu(1)-N(2)	2.016(3)	Cu(1)-N(1)	2.021(3)
Cu(1)-Cl(1)	2.5335(11)	Cu(2)-O(3)	1.930(2)
Cu(2)-O(4)	1.942(3)	Cu(2)-N(4)	2.003(3)
Cu(2)-N(3)	2.007(3)	Cu(2)-Cl(2)	2.5561(11)
O(1)-Cu(1)-O(2)	83.50(10)	O(1)-Cu(1)-N(2)	166.20(12)
O(2)-Cu(1)-N(2	96.39(11)	O(1)-Cu(1)-N(1)	93.91(11)
O(2)-Cu(1)-N(1)	159.82(11)	N(2)-Cu(1)-N(1)	81.41(12)
O(1)-Cu(1)-Cl(1)	101.92(9)	O(2)-Cu(1)-Cl(1)	98.57(8)
N(2)-Cu(1)-Cl(1)	91.75(9)	N(1)-Cu(1)-Cl(1)	101.54(8)
O(3)-Cu(2)-O(4)	83.28(10)	O(3)-Cu(2)-N(4)	167.10(11)
O(4)-Cu(2)-N(4)	97.00(11)	O(3)-Cu(2)-N(3)	95.01(11)
O(4)-Cu(2)-N(3)	167.20(12)	N(4)-Cu(2)-N(3)	81.85(12)
O(3)-Cu(2)-Cl(2)	97.99(8)	O(4)-Cu(2)-Cl(2)	96.54(9)
N(4)-Cu(2)-Cl(2)	94.80(9)	N(3)-Cu(2)-Cl(2)	96.27(9)
Complex **3**			
Cu(1)-O(2)	1.9286(16)	Cu(1)-O(3)	1.9299(15)
Cu(1)-O(1)	1.9620(16)	Cu(1)-N(1)	2.0268(18)
Cu(1)-O(3)#1	2.4123(17)	Cu(2)-O(6)	1.9237(15)
Cu(2)-O(5)	1.9354(16)	Cu(2)-O(4)	1.9479(15)
Cu(2)-N(2)	2.0283(19)	O(2)-Cu(1)-O(3)	174.07(6)
O(2)-Cu(1)-O(1)	81.63(7)	O(3)-Cu(1)-O(1)	92.58(6)
O(2)-Cu(1)-N(1)	100.38(7)	O(3)-Cu(1)-N(1)	84.70(7)
O(1)-Cu(1)-N(1)	161.01(8)	O(2)-Cu(1)-O(3)#1	97.21(7)
O(3)-Cu(1)-O(3)#1	84.26(6)	O(1)-Cu(1)-O(3)#1	92.92(7)
N(1)-Cu(1)-O(3)#1	105.46(6)	O(6)-Cu(2)-O(5)	173.00(7)
O(6)-Cu(2)-O(4)	91.19(6)	O(5)-Cu(2)-O(4)	81.81(7)
O(6)-Cu(2)-N(2)	84.36(7)	O(5)-Cu(2)-N(2)	102.41(7)
O(4)-Cu(2)-N(2)	163.00(7)		
Complex **4**			
Cu(1)-O(2)	1.9349(12)	Cu(1)-O(1)	1.9478(13)
Cu(1)-N(1)	1.9858(15)	Cu(1)-N(2)	1.9999(14)
Cu(1)-Cl(1)	2.5622(9)	O(2)-Cu(1)-O(1)	83.07(5)
O(2)-Cu(1)-N(1)	168.29(6)	O(1)-Cu(1)-N(1)	96.09(6)
O(2)-Cu(1)-N(2)	95.95(5)	O(1)-Cu(1)-N(2)	162.34(6)
N(1)-Cu(1)-N(2)	81.29(6)	O(2)-Cu(1)-Cl(1)	95.50(4)
O(1)-Cu(1)-Cl(1)	95.48(4)	N(1)-Cu(1)-Cl(1)	96.20(4)
N(2)-Cu(1)-Cl(1)	102.16(4)		

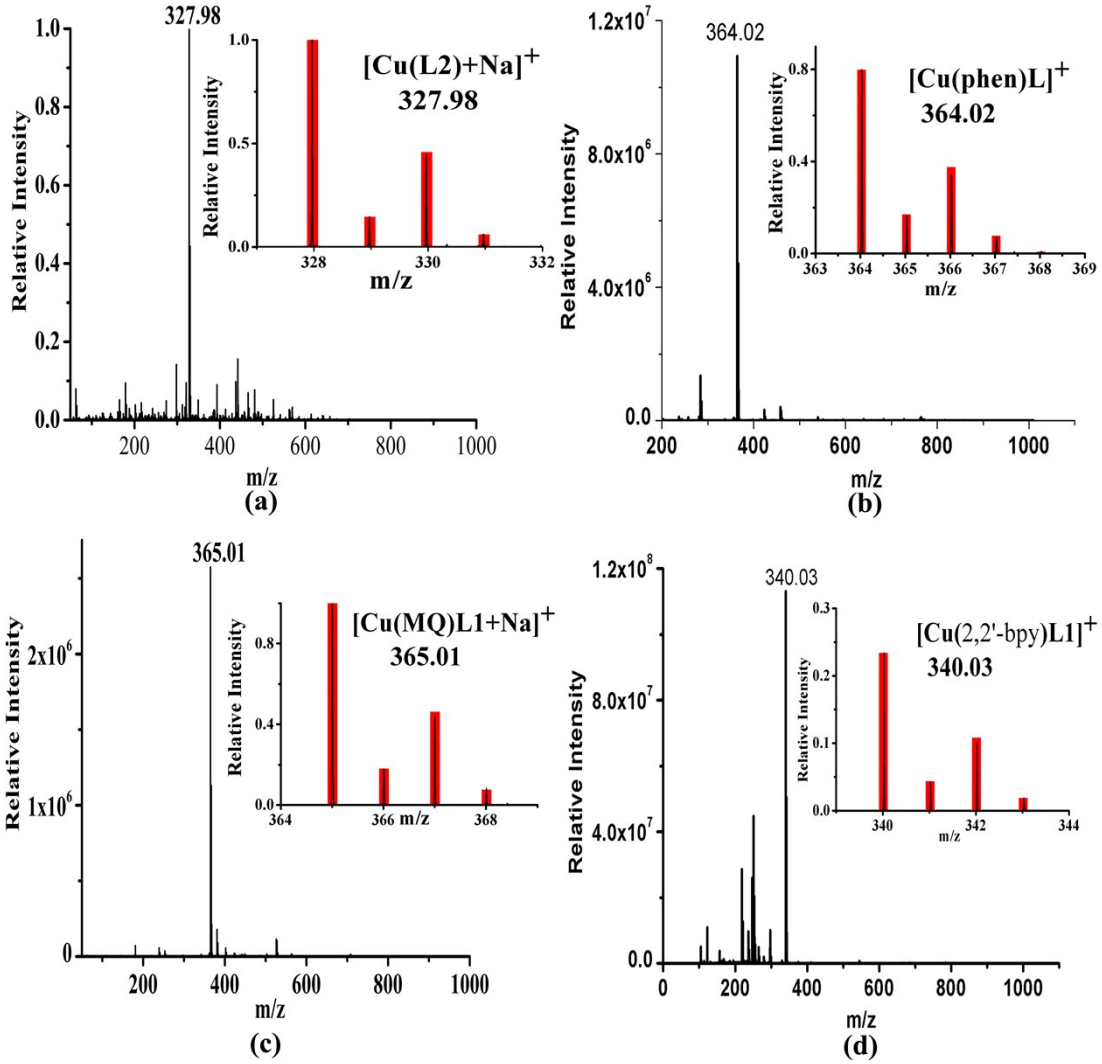

Fig. S1 ESI-MS spectra of complexes **1-4** (a-d, respectively) in MeOH containing DMSO (10% v/v) with the in-source energy being set at 0 eV. The red lines represent simulated m/z envelope with black lines for observed spectra.

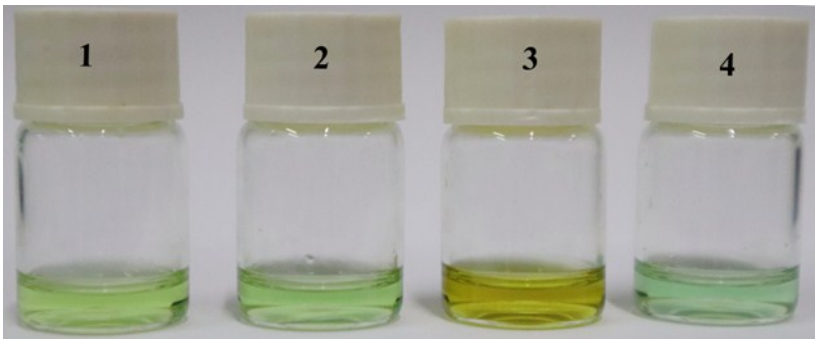

Fig. S2 The stock solutions of **1**-**4** in DMSO.

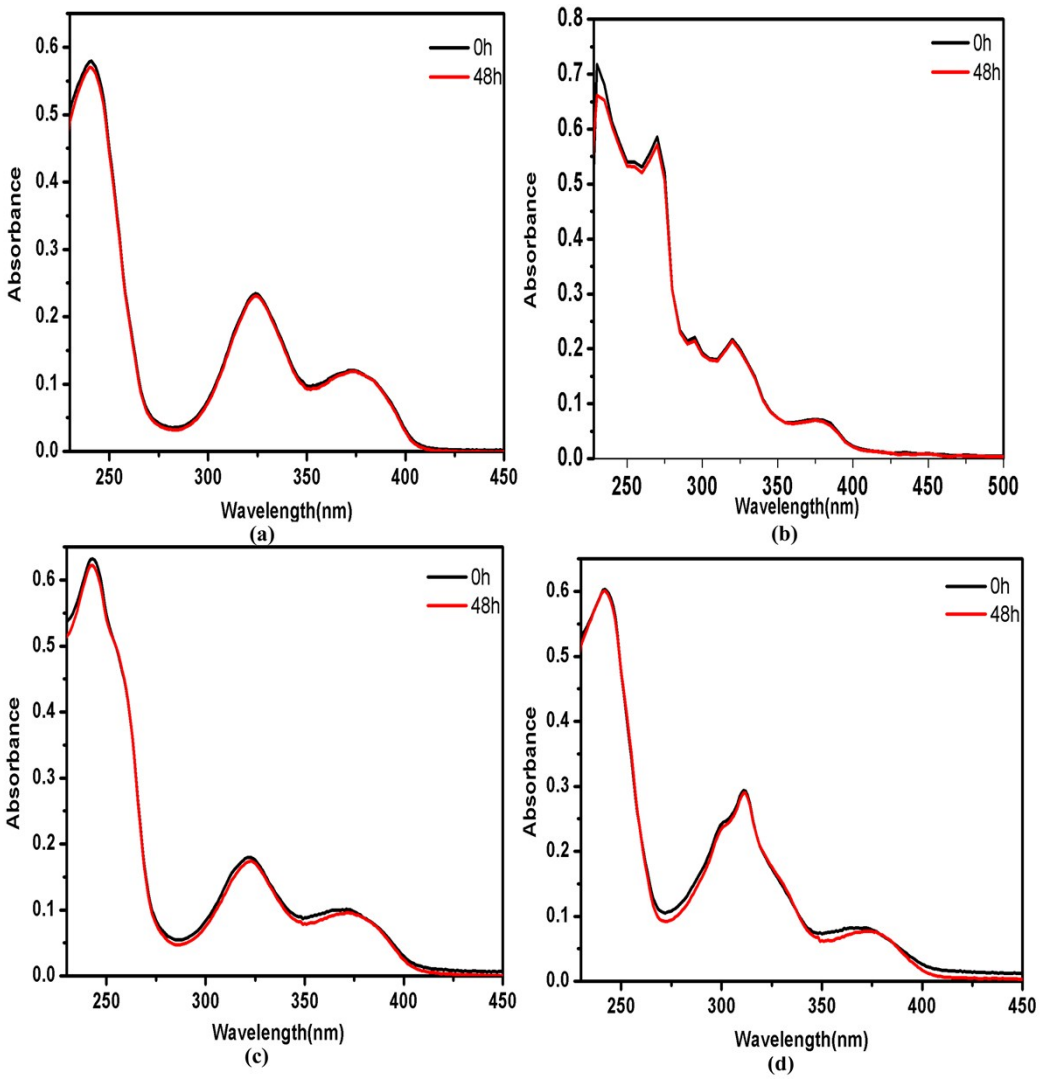

Fig. S3 (a-d) UV-Vis spectra of complexes **1** - **4** in PBS with 1% DMSO for 0 and 48 h. Black, red traces represent the relative viscosity curves for 0 and 48 h, respectively.

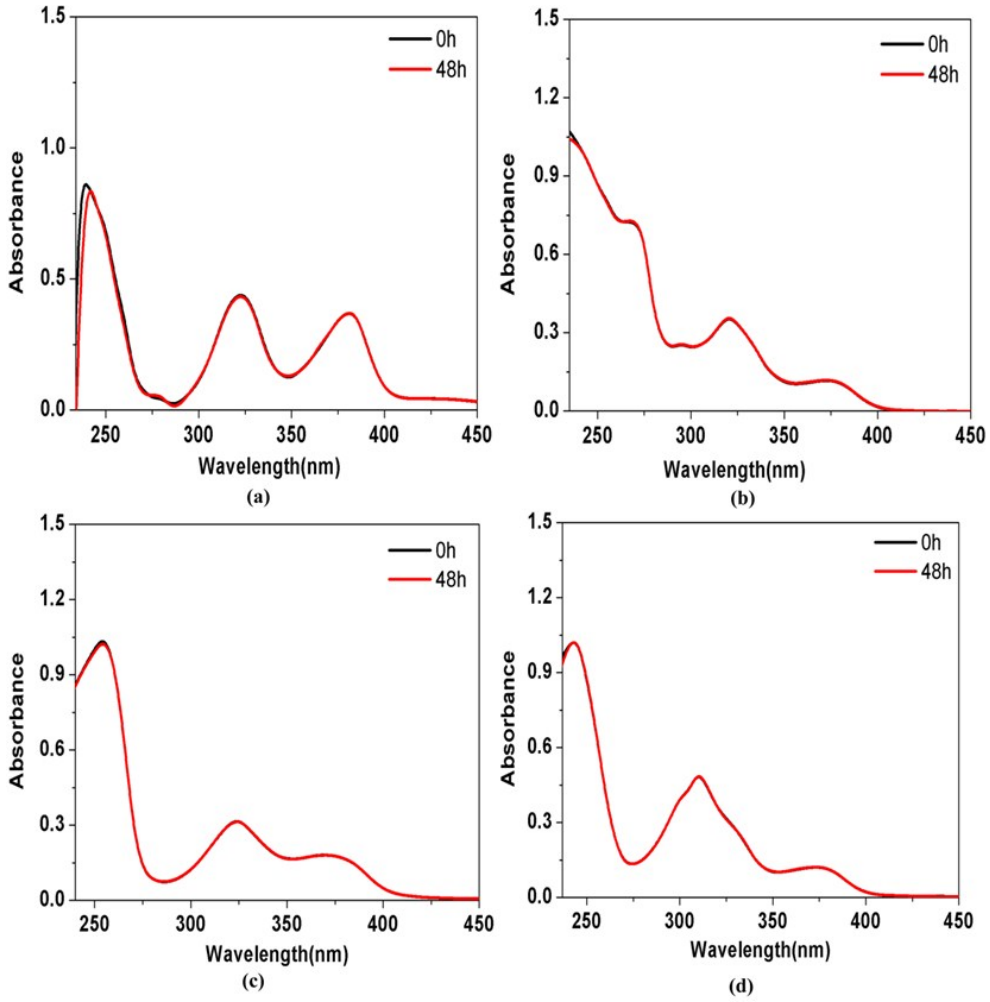

Fig. S4 (a-d) UV-Vis spectra of complexes **1** - **4** in DMEM with 5% fetal bovine serum (FBS) for 0 and 48 h. Black, red traces represent the relative viscosity curves for 0 and 48 h, respectively.

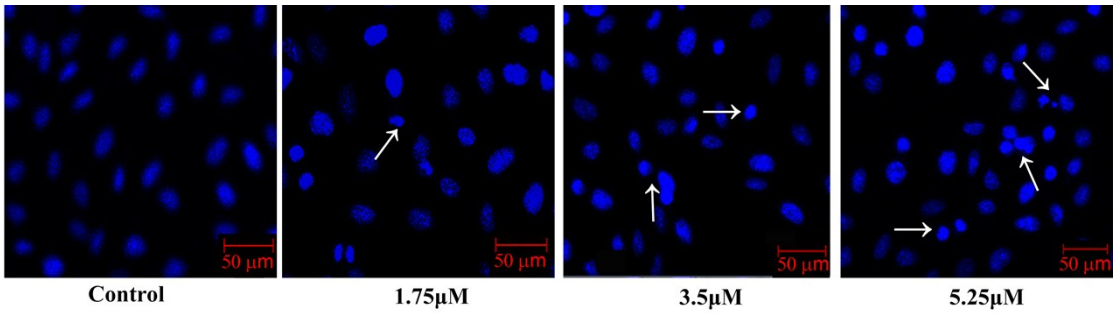

Fig. S5 Nuclear morphological change within MGC80-3 cells detected by Hoechst 33258 after incubation for 24 hours with different concentrations of complex **2**.

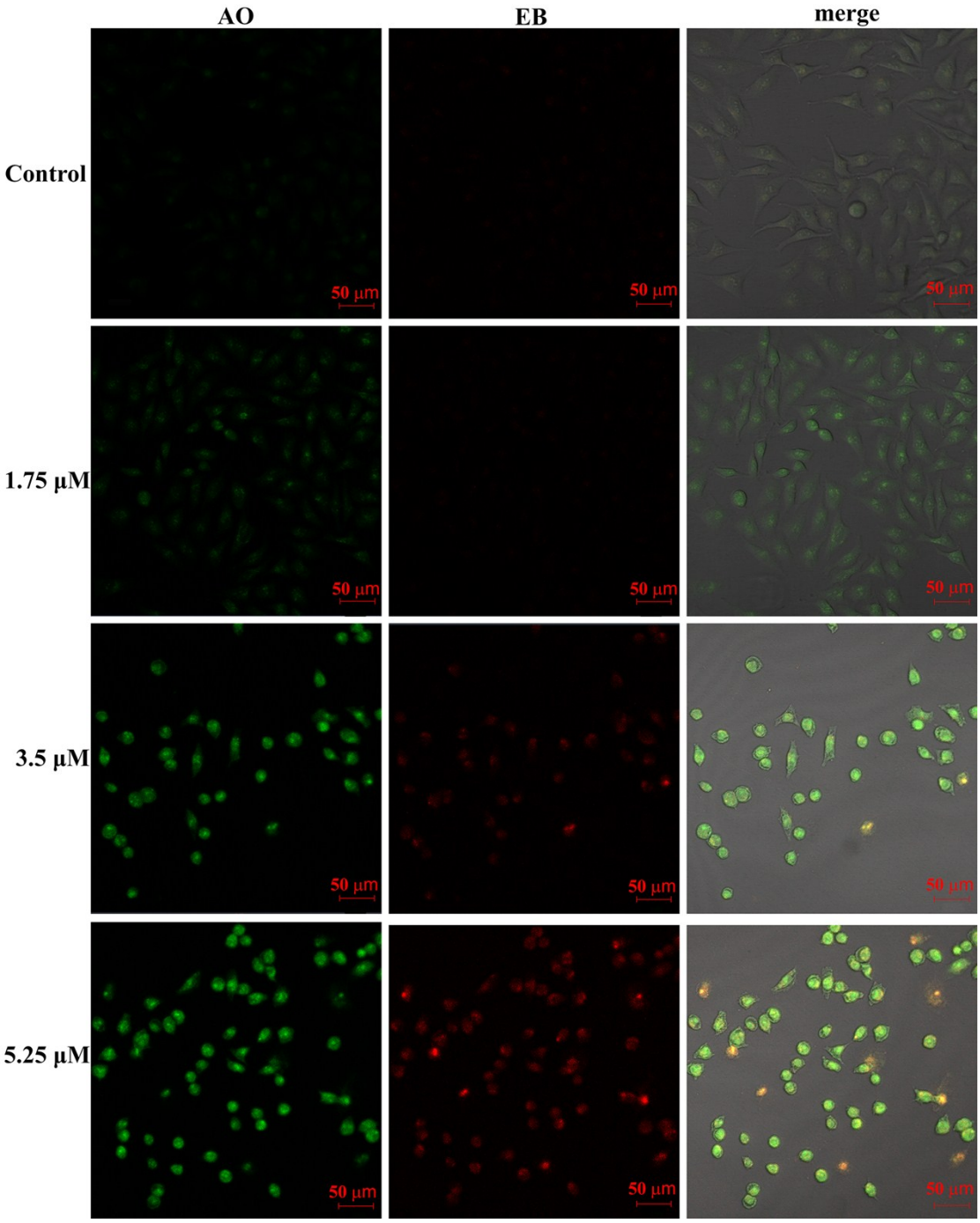

Fig. S6 MGC80-3 cells were stained by AO/EB and observed under a fluorescence microscope after 24 h of exposure to complex **2** (1.75, 3.5, 5.25μM). Living and necrotic cells are stained green and orange/red, respectively. Early and late apoptotic cells are densely stained green/yellow and orange, respectively.

Experimental methods

Pretreatment and preparation of stock solution: The solubilities of the titled complexes were tested. It revealed nice solubility in water, DMSO, and biological milieu for all titled complexes. The stock solutions of the titled complexes were prepared by dissolving the titled complexes in DMSO into 2 mol / L solutions. These stock solutions have the pH value of about 6 with their color being light green for **1**, **2** and **4** and greenish yellow for **3**, as shown in Fig. S2.

Absorption spectral titrations: The absorption titration experiments were performed using UV-vis spectrophotometer in the range of 190-700 nm were recorded by increasing the amounts of original concentration 2 mmol/L calf-thymus DNA (CT-DNA) introduced to the test complexes in pH 7.4 buffer (5 mmol TrisHCl / 50 mmol NaCl) at room temperature. In each experiment, a fixed concentration of metal complex was titrated with increasing concentrations of calf thymus DNA. For each addition, the samples were allowed to equilibrate for 10 min, and then to record the changes in the absorption spectra.

Fluorescence spectroscopic studies: The voltage is 600 and the nip width is 10 nm, emission spectra in the range of 530-750 nm were determined by setting the excitation wavelength at 510 nm. Complexes were added to a mixture containing 10 mmol CT-DNA and 8 mmol GelRed (GR) in pH 7.4 Tris-HCl buffer. The changes for each experimental was recorded in the absorption spectra.

Cell culture and maintenance: All human tumor cell lines and human normal liver cell line HL-7702 in this study were purchased from China Life Science Collage

(Shanghai, PRC). Culture medium Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS, pH=7.2), and Antibiotice-Antimycotic were purchased from KeyGen Biotech Company (China). Cell lines were grown in the supplemented with 10% FBS, 100 units/mL of penicillin and 100 g/mL of streptomycin in a humidified atmosphere of 5% CO_2 at 37 ºC.

Cell lines: The human lung carcinoma A549 cell lines, human hepatocellular Bel-7402 cell lines, human gastric MGC80-3 cell lines, human bladder T24 cell lines, human ovarian SK-0V-3 cell lines，human lung NCI-H460 cell lines and normal liver HL-7702 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). These cells were cultured in DMEM culture media supplemented with 1% antibioticantimycotic solution and 10% fetal bovine serum (FBS) at 37 ºC and 5% CO_2.

Cytotoxicity assay: The anticancer activity of all compounds were investigated in six human cancer cell lines (A549, Bel-7402, MGC80-3, T24, SK-0V-3, NCI-H460 cells) and human normal liver cell line (HL-7702). About 1×10^5 cells/mL cells, which were in the logarithmic phase, were grown in each well of 96-well plates and incubated for 12 h of 5% CO_2 at 37 °C. All compounds at five different concentrations were then added to the test well and the cells were incubated at 37 °C in a 5% CO_2 atmosphere for 48 h, respectively. A total of 10 μL MTT (5 mg/mL) saline solution was added into each well and then incubated for 5 h at 37 ºC under 5% CO_2 . The medium was then discarded, and 100 mL of DMSO (dimethyl sulfoxide) was added into each well. An enzyme labeling instrument was used to read absorbance with 570/630 nm double wavelength measurement. Cytotoxicity was evaluated on the percentage of cell

survival compared with control group. The final IC50 values were calculated by the Bliss method (n = 5). All of the tests were repeated in triplicate.

Cell morphology analysis: The assay was performed with MGC80-3 cells. The MGC80-3 cells (1×10^5 cells/mL) were seeded in six-well plates for 24 h at 37 ºC under 5% CO_2 and then incubated with Cu(II) complex **2** at 37 ºC for another 24 h. Thereafter, the cells were visualized under microscope.

Cellular uptake study: The intracellular uptake of complex **2** was performed with MGC80-3 cells. Briefly, 2×10^6 cells were seeded in 10 cm culture dishes at 37 ºC under 5% CO_2. After 24 h, the medium was replaced, and the cells were incubated with complex **2** (10 μM) for 12 h. The cells were then carefully washed with phosphate buffered saline (PBS) several times and harvested by trypsinization. Cell digestion was performed using 3 mL of HNO_3 for 2 h at 60 ºC. The copper content in the cells was analyzed by inductively coupled plasma mass spectrometry (ICP-MS)

ROS Assay: The production of ROS was examined by flow cytometry using DCFH-DA (Molecular Probe, Beyotime, Haimen, China), as previously described. MGC80-3 cells were grown into six-well plates and subjected to various treatments. On the following treatment, cells were collected at 1300 rpm and washed twice with ice-cold PBS, and then resuspend cells in 10mM DCFH-DA dissolved in cell free medium at 37 °C for 20 minutes in dark, and then washed twice with ice-cold PBS. Cellular fluorescence was analyzed by flow cytometry at an excitation of 485 nm and an emission of 538 nm.

Mitochondrial membrane potential assay: The mitochondrial membrane potential was measured by flow cytometry using the JC-1 fluorescent probe (Beyotime, Haimen, China, Molecular Probe), as previously described. MGC80-3 cells were treated with different concentrations of complex **2** for 24 h. After 24 h, the JC-1 fluorescent probe was added 20 minutes after replacing with fresh medium. Cells were harvested at 1300 rpm and washed twice with ice-cold PBS and the losses of mitochondrial membrane potential were investigated by flow cytometry. The emission fluorescence for JC-1 was monitored at 530 and 590 nm, under the excitation wavelength at 488 nm, respectively.

Ca^{2+} levels Assay: MGC80-3 cells were grown into six-well plates and treated with different concentrations of complex **2** for 24 h, the cells were incubated with Fuo-3AM for 20 min at 37 ºC in the dark, and washed three times with ice-cold PBS, then incubated an additional 20 min with PBS at 37 ºC to ensure that Fluo-3AM has been completely transformed into Fluo-3, which can specifically bind to Ca^{2+} and cellular fluorescence was analyzed by flow cytometry at an excitation wavelength of 488 nm.

Determination of Caspase-3 and Caspase-9 Activity by Flow Cytometric Analysis: The measurement of caspase-3 and caspase-9 activity was performed by CaspGLOW fluorescein active caspase-3 and caspase-9 staining kit. After treatment with complexes **2** 0, 1.75 μM, 3.5 μM and 5.25 μM for 24 h, respectively, the cells at a density of 1×10^6 cells/mL were harvested, washed with PBS three times, and then resuspended in 300 μL volume; 1 μL of FITC-DEVDFMK or FITC-LEHD-FMK was consequently added and incubated for 1.0 h at 37 °C in 5% CO_2 incubator. The cells was analyzed by flow cytometry. The results were represented as the percent

change on the activity comparing with the control

Flow cytometry analysis of cell cycle distribution: The MGC80-3 cells were grown on 6-well plates and treated with complex **2** (1.75, 3.5,5.25 μM), and maintained In the proper culture medium full of 5% CO_2 at 37 °C for 24 h. After completion of incubation, cells were harvested and washed twice with ice-cold PBS, fixed with ice-cold 70% ethanol at -20 °C for overnight. The cells were treated with 100 μg /mL RNaseA for 30 minutes at 37 °C after being washed twice with ice-cold PBS, and finally stained with 1 mg/mL propidium iodide (PI) in the dark at 4 °C for 1 h. Analysis was performed with flow cytometry.

AO/EB double staining for cell morphology: The Select cells with good growth and logarithmic growth phase were washed twice with phosphate buffer solution (PBS), dealt with trypsinize. After the addition of culture medium containing 10% new bovine serum and 1% streptomycin, the mixture was mixed well into a suspension of cells. Confocal dishes with a density of 1 $\times 10^5$ cells were seeded. After the growth of cells to a plate area of 1/3 to 2/3, the increasing concentrations of the complexes were added to the cells. After 24 hours, it was washed once with PBS. Then PBS: AO/EB was added to the confocal dish at a ratio of 1:1, and incubated at room temperature for 5-10 minutes in the dark. The cells were then washed 2 to 3 times with PBS and examined using confocal microscopy.

Nuclear staining (Hoechst 33258) for detection of apoptotic morphology: the select cells with good growth and logarithmic growth phase were washed twice with phosphate buffer solution (PBS) and dealt with trypsinize. After the addition of

culture medium containing 10% new bovine serum and 1% streptomycin, it was mixed well to form a suspension of cells. Confocal dishes with a density of 1 $\times 10^5$ cells were seeded. Whn the cells grow to a plate area of 1/3 to 2/3 , the increasing concentrations of the complexes were added to the cells. After 24 hours, it was washed once with PBS. then 0.5 mL of Hoechst 33258 staining solution (Sigma, final concentration: 10 μg mL^{-1}) was added. It was then incubated for 5-10 minutes at room temperature in the dark. The cells were then washed 2 to 3 times with PBS and examined using confocal microscopy.

Apoptosis analysis: Apoptosis was examined by flow cytometry analysis of annexin V/PI staining. MGC80-3 cells were seeded in each well of 6-well plates at the density of 1×10^5 cells/mL of the DMEM medium with 10% FBS to the final volume of 2 mL. The plates were incubated for overnight and treated with different concentrations of Complex **2** for 24 h. Briefly, cells were harvested and washed three times using ice-cold PBS, and then suspended in the annexin-binding buffer at a concentration of 5×10^5 cells / mL. Subsequently, the cells were then incubated with 5 μL of annexin V-FITC and 5 μL of PI for 30 minutes at room temperature in the dark. The cells were analyzed by system software (Cell Quest; BD Biosciences).

Western Blot Analysis: Western blot analysis was performed as described previously. MGC80-3 cells were treated with different concentrations of complex **2** for 24 h. After for 24 h, cells were harvested, centrifuged, and washed twice with ice-cold PBS. The pellet was then resuspended in lysis buffer. After the cells were lysed on ice for 30 min, lysates were centrifuged at 13000 rmp at 4 °C for 15 min. The protein concentration in the supernatant was analyzed using the BCA protein assay reagents (Imgenex, USA). Equal amounts of protein per line were separated on 12% SDS

polyacrylamide gel electrophoresis and transferred to PVDF Hybond-P membrane (GE Healthcare). Membranes were incubated with 5% skim milk in Tris-buffered saline with Tween 20 (TBST) buffer for 1 h and then the membranes being gently rotated overnight at 4 °C. Membranes were then incubated with primary antibodies against Cytochrome c, Bak, Bax, Bcl-2, Apaf-1, LC3 II and Beclin-1 or β-actin for overnight at 4°C. Membranes were next incubated with peroxidase labeled secondary antibodies for 2 h. Then all membranes were washed with TBST three times for 15 minutes and the protein blots were investigated with chemiluminescence reagent (Thermo Fischer Scientifics Ltd.).

Statistical analysis. The experiments have been repeated from three times, and the results obtained were presented as means ± standard deviation (SD), and considered to be significant when $p < 0.05$.