Electronic Supplementary Information

Synthesis, structural studies, interaction with DNA/HSA and antitumor evaluation of new Cu(II) complexes containing 2-(1H-imidazol-2-yl)pyridine and amino acids

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1 Supplementary experimental section

1.1 Materials and methods

All reagents and solvents were of commercial sources and could be used without further purification, and the deionized water was used in all the experiments. Calf thymus DNA (CT-DNA), ethidium bromide (EB), and human serum albumin (HSA) were purchased from Sigma (USA). All the cells used in the anticancer experiments were purchased from the Laboratory Animal Center of Sun Yat-Sen University (Guangzhou, China). The Caspase-Glo® 3/7 Assay kit was obtained from Promega (USA), while other biological kits were purchased from Beyotime Biotechnology (China).

IR spectra were recorded on a Nicolet ACATAR 360 FTIR spectrometer (Nicolet, USA) using KBr particles (4000-400 cm^{-1}). Electronic absorption spectra were measured using a Pharmacia 2550 UV–visible spectrophotometer (Shimadzu, Japan). Elemental analyses of C, H and N were obtained on a Vario EL elemental analyzer (Elementar, Germany). The molar conductivities were determined in aqueous solution at room temperature using a DDS-11A digital conductivity meter (Leici, China). The fluorescence spectra were recorded using a Hitachi RF-4500 fluorescence spectrometer (Japan). Cell pictures were taken by confocal laser scanning microscopy TCS SP8 (Lecia, Germany).

1.2 DNA-binding experiments

The electron absorption titration experiments were carried out in the range of 200-400 nm by keeping the concentration of Cu(II) complex constant (50 μM) and gradually increasing the concentration of CT-DNA (0~53.3 μM). Besides, fluorescence emission spectra were measured by gradually increasing the concentration of the Cu(II) complexes (0~59.3 μM) in the EB (8 μM)−CT DNA (10 μM) system, and the spectra were recorded in the range of 550 to 700 nm with excitation wavelength at 525 nm. The viscosity of the CT-DNA solution (200 μM) was measured by an Ubbelodhe viscometer in the absence and presence of EB (0~60 μM) or the complexes (0~60 μM), and the mixture solution was incubated for 5 min at 29.0 °C before each measurement.

1.3 HSA-binding experiments

The UV absorption spectra of HSA (5 μM) in the absence and presence of the complexes (10 μM) were measured in the range of 240-360 nm at room temperature. To study the interaction

between HSA and the complexes, the fluorescence quenching spectra and synchronous fluorescence spectra were measured by keeping the HSA concentration constant (5 μM) at room temperature and continuously increasing the complex concentration (0~23.3 μM). The fluorescence quenching spectra were scanned from 290 to 450 nm with excitation wavelength at 280 nm. The synchronous fluorescence spectra of Tyr and Trp residues were measured from 270 to 345 nm at $\Delta\lambda$ = 15 nm and from 240 to 350 nm at $\Delta\lambda$ = 60 nm, respectively. Each sample needed to be incubated at room temperature for 5 min and then measured so that the complex can fully interact with HSA.

1.4 Molecular docking experiment

The Autodock 4.2 program was used to simulate and study the interaction sites and binding modes of the complexes with DNA/HSA. The X-ray crystal structures of the complexes were conversed to PDB format by Mercury software for docking, and the water molecules and free anions were needed to be removed prior to the experiments. The crystal structures of DNA (PDB ID: 454d) and HSA (PDB ID: 1H9Z) were derived from the Protein Data Bank, which was subjected to delete the water molecules and add polar hydrogen atoms before docking calculations were performed. The complexes were located at coordinates x = 29.581, y = 19.337, z = 70.656 of DNA for further docking, whereas for HSA, the coordinates were fixed at x = 24.986, y = 9.578, z = 20.079. The classic Lamarck genetic algorithm (LGA) was used for docking simulation with 100 runs for each binding site, and the semi-empirical free energy calculation method was used to analyze the docking results. Finally, the PyMol software was used to display the docking sites and interaction modes.

1.5 DNA cleavage experiment

The cleavage experiments of the pBR322 DNA (250 ng) were performed in the presence of the complexes at different concentrations (5, 10, 15, 20 μM) and the reducing agent ascorbic acid (50 μM). The samples were incubated at 37 °C for 1 h in the dark, and subsequently 6× loading buffer (3 μL each sample) was added to stop the reaction. Finally, the samples were loaded on 0.8% agarose gel (0.1 μL/mL GelRed) and gel electrophoresis in TBE solution (pH = 8.4) was carried out at 100 V for 45 min. The results were observed and analyzed by the gel imaging system (BIO-RAD Laboratories-Segrate).

In order to explore the possible oxidative cleavage mechanisms of DNA by the complexes, the

ROS scavengers such as DMSO (200 mM), tert-butanol (200 mM), SOD (15 units), NaN_3 (200 mM) and KI (200 mM) were added to pretreat pBR322 DNA (250 ng) and the complexes (15 μM) for 20 min. After that, each sample was mixed with ascorbic acid and the gel electrophoresis was performed to study the DNA cleavage mechanisms.

1.6 Hydroxyl radical detection by methylene blue method

First, the Cu(II) complexes (50 μM) were pretreated by ascorbic acid (2.5 mM) at room temperature for 5 min, after that H_2O_2 (8 mM) and MB (30 μM) were added into the mixture solutions to incubate in the dark for 1 h, and the control groups were carried out under the same conditions. The UV-Vis spectra of the samples were measured to observe the changes of the absorption peak intensity of MB at 665 nm, which could directly determine whether H_2O_2 was catalyzed to form ·OH.

1.7 Determination of cytotoxicity *in vitro*

The cytotoxicity of the ligand IPY and the complexes toward HeLa, HepG2, BEL-7402, SGC-7901 cancer cells and normal liver LO2 cells was evaluated *via* MTT assay. Cells were plated into 96-well plate (1.2×10^5 cells/well) and treated with different compounds concentrations (0, 1.56, 3.13, 6.25, 12.5, 25, 50, 100 μM) for 44 h when the cells were incubated to 70% confluence (37 °C, 5% CO_2). Subsequently, fresh serum-free media (90 μL) and MTT solution (10 μL, 5 mg/mL) were added to each well to be continuously incubated for 4 h. Afterward, the mixed solution was carefully removed and DMSO (100 μL/well) was added to fully dissolve formazan crystals. Finally, the optical density values were recorded at 490 nm by a microplate spectrophotometer (Varioskan Flash, Thermo Scientific, USA) and the IC_{50} values were obtained to evaluate the cytotoxicity of the compounds.

1.8 Antitumor mechanism studies

1.8.1 Apoptosis detection

Hoechst staining method: HepG2 cells were seeded in a confocal dish (2×10^5 cells/well) and incubated overnight at 37 °C in 5% CO_2. Subsequently, the cells were treated with different complexes (18.8 μM for **CuI1**, 15.6 μM for **CuI2** and 23.6 μM for **CuI3**, respectively) and incubated

for another 24 h. The cells were then fixed with 4% paraformaldehyde at room temperature for 15 min prior to stain with Hoechst 33342 in the dark for 20 min. At last, the samples were observed and photographed with the confocal microscope (λ_{ex} = 405 nm).

Caspase 3/7 activity study: After the cells were seeded into 96-well plates (1.5 × 10^4 cells/well) and incubated for adhering to 70% confluence, they were incubated with the various complexes (50 μM) or cisplatin (50 μM) for 18 h. Afterward, the cells were treated with Caspase-Glo® 3/7 Assay kit according to the manufacturer's instructions, and the chemiluminescence of the samples was measured by a multifunctional microplate reader. Besides, the cells were exposed to the complexes (50 μM) for 24 h with or without pretreatment of z-VAD-fmk (50 or 100 μM) for 1 h, and then the cell viability was measured by MTT assay.

1.8.2 Cell cycle arrest studies

HepG2 cells were seeded in 6-well plates (5 × 10^5 cells/well) to be cultivated for 24 h at 37 °C under 5% CO_2, after which the cells were treated with **CuI1** (18.8 μM), **CuI2** (15.6 μM) and **CuI3** (23.6 μM) for 24 h, respectively. Subsequently, the cells were digested by trypsinization and collected after washing by cold PBS, and then the cells were resuspended with 70% ethanol and incubated overnight at 4 °C. The cells were stained by RNase A (0.2 mg/mL) and PI (0.02 mg/mL) for 20 min at room temperature. Finally, the samples were measured immediately by a FACS Calibur flow cytometry (BD, USA) to explore the cell cycle changes.

1.8.3 Mitochondrial membrane potential (MMP) detection

The confocal dish was used to inoculated cells with a density of 2 × 10^5 cells per well and cultured in a 5% CO_2 incubator at 37 °C overnight, after which the cells were treated with **CuI1** (18.8 μM), **CuI2** (15.6 μM) and **CuI3** (23.6 μM) for 24 h, respectively. Subsequently, the samples were stained with JC-1 staining solution (1 mg/mL) for 20 min at 37 °C in the dark. Finally, the cold PBS was used to wash the cells and the confocal microscope was used to visualize and analyze the changes in fluorescence intensity of the cells.

1.8.4 Cellular ROS levels determination

After seeding in confocal dishes at a density of 2 × 10^5 cells/well and growing overnight, the cells were treated with **CuI1** (18.8 μM), **CuI2** (15.6 μM) and **CuI3** (23.6 μM) for 24 h, respectively. Subsequently, the cells were washed and exposed to 2′,7′-dichlorodihydro-fluorescein diacetate (DCHF-DA, 10 μM) at 37 °C for 30 min. The intracellular ROS levels were immediately analyzed

by confocal microscope (λ_{ex} = 488 nm).

HepG2 cells were seeded into 6-well plates (5 × 10^5 cells/well) to be incubated for 24 h, and then the cells were exposed to different complexes (18.8 μM for **CuI1**, 15.6 μM for **CuI2** and 23.6 μM for **CuI3**, respectively) for 24 h. The cells were harvested and resuspended in PBS containing DCHF-DA (10 μM) at 37 °C for 30 min, and then the treated samples were washed with PBS and promptly measured by flow cytometry to quantify the ROS levels.

The potential toxic ROS were identified by using common radical scavengers such as SOD (500 U/mL), catalase (1000 U/mL), NAC (10 mM), NaN_3 (5 mM) and mannitol (10 mM). In short, the cells were treated with the complexes (50 μM for **CuI1**, 60 μM for **CuI2**/**CuI3**) for 24 h with or without a pretreatment of different radical scavengers for 1 h, and the cell viability was evaluated *via* MTT assay to analyze the toxic ROS.

2 Supplementary figures and tables

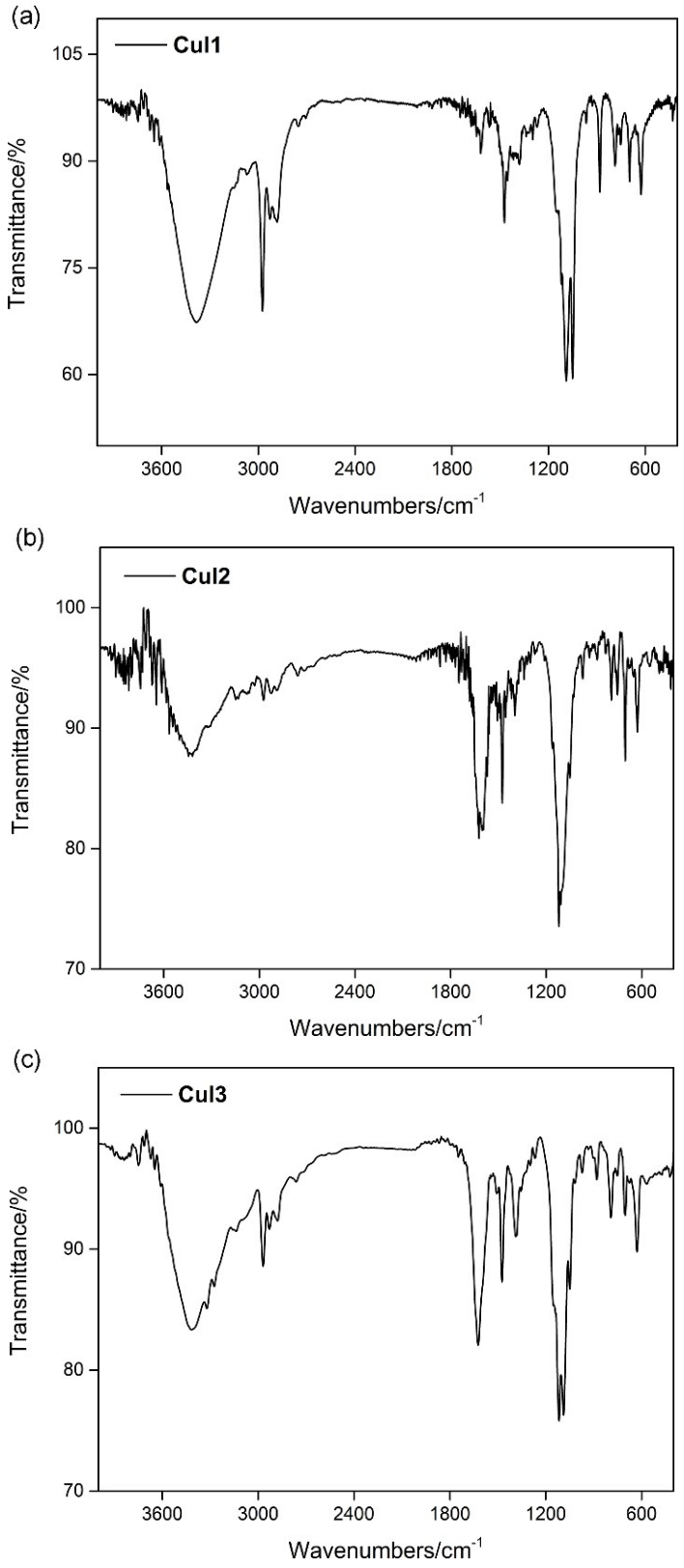

Fig. S1 Infrared spectra of **CuI1** (a), **CuI2** (b) and **CuI3** (c) with KBr tablets at room temperature.

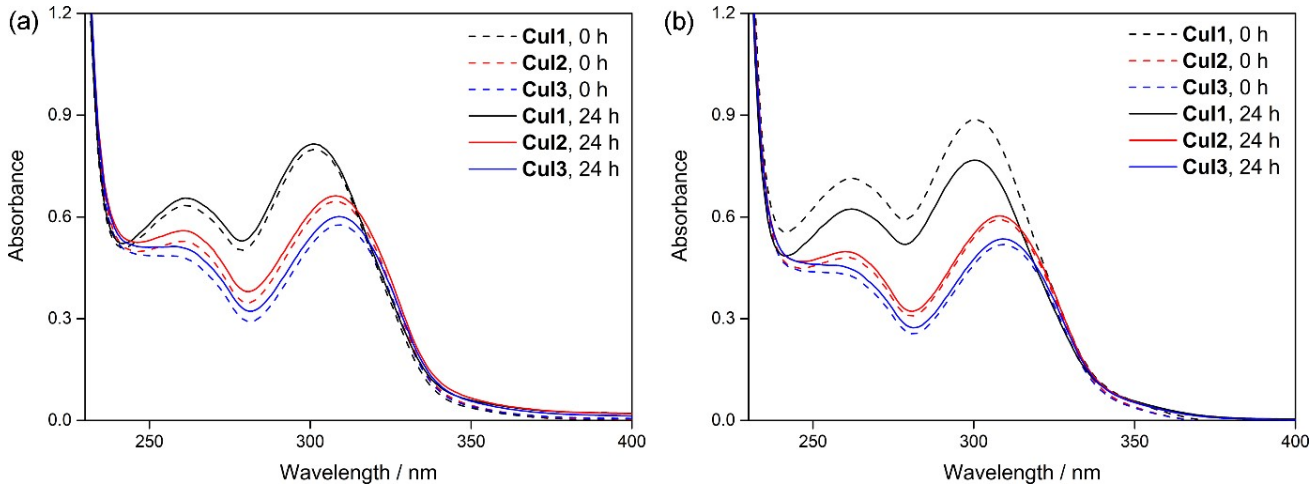

Fig. S2 UV-Vis spectra of the complexes (50 μM) in (a) Tris-HCl/NaCl buffer (pH = 7.2) and (b) PBS (pH = 7.4). Time intervals: 0 and 24 h.

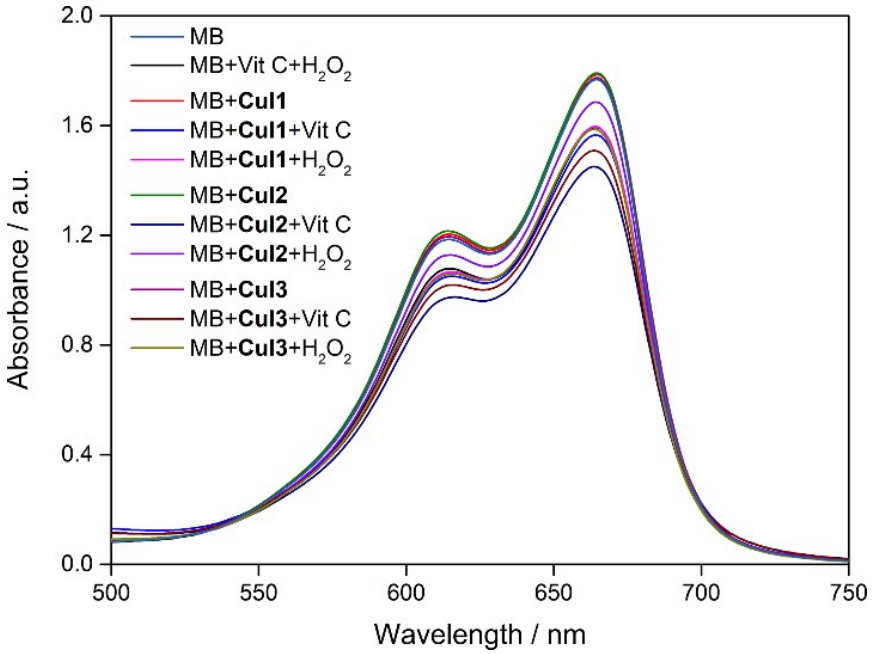

Fig. S3 Detection of ·OH mediated MB degradation in different solutions by UV-Vis absorption spectra ([Cu] = 0.05 mM, [H_2O_2] = 8 mM, [Vit C] = 2.5 mM).

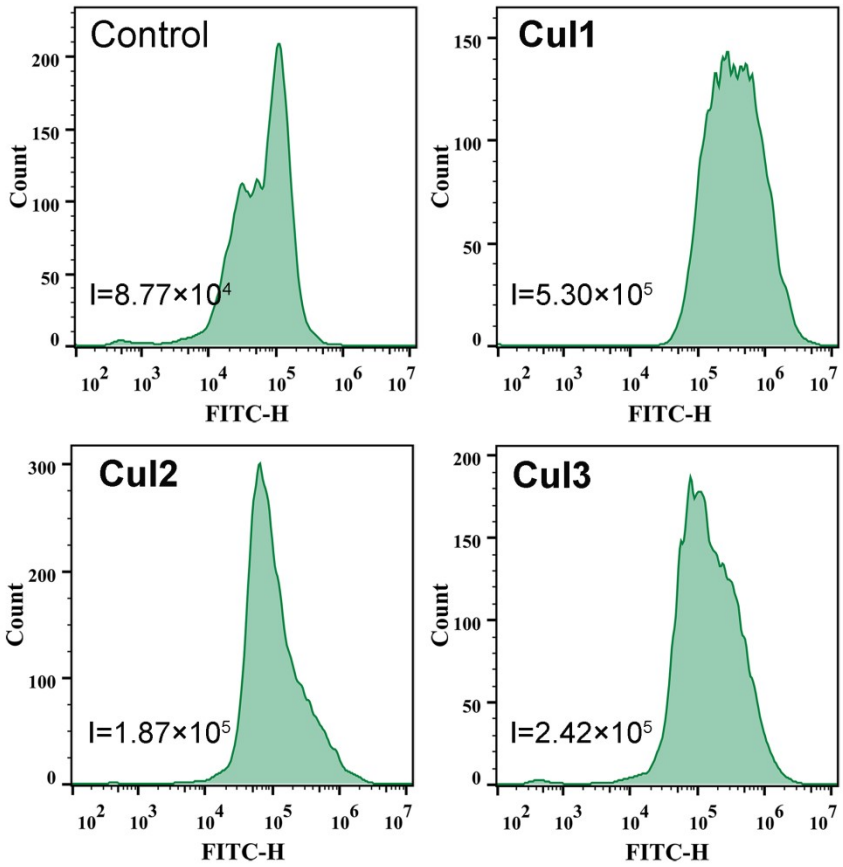

Fig. S4 ROS levels in HepG2 cells detected with DCHF-DA using flow cytometry.

Table S1 Crystallographic data and details of refinements for **CuI1** and **CuI2**

Compound	$C_{16}H_{14}Cl_2CuN_6O_8$ (**CuI1**)	$C_{17}H_{19}ClCuN_4O_{7.5}$ (**CuI2**)
CCDC	2041880	2193796
Formula weight	552.77	498.35
Crystal size (mm^3)	0.10×0.15×0.20	0.2×0.2×0.1
Temperature (K)	293	150
Wavelength (Å)	1.5418	1.5418
Crystal system	Triclinic	Orthorhombic
Space group	P-1	$P2_12_12_1$
a (Å)	8.4596(5)	12.3660(2)
b (Å)	9.9394(8)	15.6213(3)
c (Å)	13.2717(9)	21.2384(4)
B (°)	78.34(1)	90.00
Volume ($Å^3$)	1015.54(14)	4102.69(13)
Z	2	4
Density (calculated, $g·cm^{-3}$)	1.808	1.614
Absorption coefficient (mm^{-1})	4.504	3.163
F(000)	558	2040.0
θ range (°)	4.79 to 64.58	3.51 to 73.56
Index ranges	$-9\le h\le 8$; $-11\le k\le 11$; $-14\le l\le 15$	$-15\le h\le 14$; $-17\le k\le 19$; $-26\le l\le 26$
Reflection collected	5709	15289
Independent reflection	3373	8049
R_{int}	0.0240	0.0285
Refinement method	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2
Data, restraints, parameters	3373, 60, 372	8049, 0, 559
Goodness-of-fit on F^2	1.037	1.053
R_1, wR_2 ($I > 2\sigma(I)$)	$R_1 = 0.0497$, $wR_2 = 0.1357$	$R_1 = 0.0573$, $wR_2 = 0.1596$
R_1, wR_2 (all data)	$R_1 = 0.0596$, $wR_2 = 0.1487$	$R_1 = 0.0592$, $wR_2 = 0.1623$
Largest diff. peak and hole ($e·Å^{-3}$)	0.720 and 1.924	1.090 and 0.902

Table S2 Crystallographic data and details of refinements for **CuI3**

Compound	$C_{13}H_{19}ClCuN_4O_7$ (**CuI3**)
CCDC	2193797
Formula weight	442.31
Crystal size (mm^3)	0.15×0.12×0.1
Temperature (K)	150
Wavelength (Å)	1.54184
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
a (Å)	7.8142(1)
b (Å)	11.0887(1)
c (Å)	20.4823(1)
B (°)	90.00
Volume ($Å^3$)	1774.76(2)
Z	4
Density (calculated, $g·cm^{-3}$)	1.655
Absorption coefficient (mm^{-1})	3.541
F(000)	908.0
θ range (°)	4.32 to 73.45
Index ranges	$-9\leq h\leq 9$; $-13\leq k\leq 13$; $-25\leq l\leq 25$
Reflection collected	47774
Independent reflection	3572
R_{int}	0.0429
Refinement method	Full-matrix least-squares on F^2
Data, restraints, parameters	3572, 3, 312
Goodness-of-fit on F^2	1.152
R_1, wR_2 ($I > 2\sigma(I)$)	$R_1 = 0.0196$, $wR_2 = 0.0534$
R_1, wR_2 (all data)	$R_1 = 0.0197$, $wR_2 = 0.0535$
Largest diff. peak and hole ($e·Å^{-3}$)	1.344 and 0.829

Table S3 Selected bond lengths and bond angles for the complexes

CuI1		**CuI2**		**CuI3**	
Bond length (Å)					
Cu1–N1	2.051(3)	Cu1–O1	1.952(5)	Cu1–O1	1.919(2)
Cu1–N2	1.990(3)	Cu1–O3	2.227(6)	Cu1–O3	2.633(2)
Cu1–N3	2.042(3)	Cu1–N1	2.028(6)	Cu1–N1	1.963(2)
Cu1–N4	1.990(3)	Cu1–N2	1.984(5)	Cu1–N4	1.991(2)
		Cu1–N4	2.011(6)	Cu1–N2	2.015(2)
Bond angle (º)					
N1–Cu1–N2	81.39(12)	O1–Cu1–O3	93.86(23)	O1–Cu1–N1	174.16(8)
N1–Cu1–N3	169.28(11)	O1–Cu1–N1	90.29(21)	O1–Cu1–N4	85.08(7)
N1–Cu1–N4	100.60(12)	O1–Cu1–N2	166.34(19)	O1–Cu1–N2	91.54(8)
N2–Cu1–N3	98.96(12)	O1–Cu1–N4	83.70(22)	O1–Cu1–O3	96.60(6)
N2–Cu1–N4	166.34(12)	O3–Cu1–N1	97.90(21)	N1–Cu1–N4	100.57(8)
N3–Cu1–N4	81.61(11)	O3–Cu1–N2	98.30(24)	N1–Cu1–N2	83.07(9)
		O3–Cu1–N4	99.87(22)	N4–Cu1–N2	171.12(9)
		N1–Cu1–N2	82.00(22)	O3–Cu1–N1	80.78(6)
		N1–Cu1–N4	161.57(23)	O3–Cu1–N2	85.75(7)
		N2–Cu1–N4	100.15(23)	O3–Cu1–N4	102.79(8)

Table S4 Quenching constants and thermodynamic parameters of the interaction between the complexes and HSA

Complex	$K_{SV}(M^{-1})$	$K_q(M^{-1}\cdot S^{-1})$	R	$K_b(M^{-1})$	*n*	R
CuI1	4.02×10^4	4.02×10^{12}	0.999	2.70×10^4	0.962	0.999
CuI2	3.17×10^4	3.17×10^{12}	0.996	0.92×10^4	0.884	0.988
CuI3	3.73×10^4	3.73×10^{12}	0.998	2.31×10^4	0.957	0.995