Electronic Supplementary Information

Synthesis, characterization and biological activity of novel copper complexes containing a β-carboline derivative and amino acids

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1. Supplementary experimental section

1.1 Materials and methods

All reagents and solvents were from commercial sources and could be used without further purification, and 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 and CellTiter-Glo® kit were 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 an 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 Synthesis

Ligand 1-Im-βc was synthesized according to the published method in the literature[1].

Copper(II) complexes were synthesized by the following method: 0.25 mmol *L*-Val/Phe and 0.25 mmol NaOH were dissolved in 5 mL of deionized water and mixed thoroughly. Subsequently, 5 mL of 0.25 mmol $Cu(ClO_4)_2{\cdot}6H_2O$ methanol solution (water: methanol = 1:9, v/v) was slowly added dropwise to the above solution at 50 °C and stirred for 30 min. ***Please note: Cu(II)***

perchlorate is combustible and tends to burn and explode when mixed with reductants and combustibles. Finally, the methanolic solution of 1-Im-βc (0.25 mmol, 15 mL) was added, and the resulting solution was continued to be stirred at 50 °C for 1 h, cooled and filtered, and then slowly evaporated at room temperature to obtain grass green powder (**Cu1**) and dark green powder (**Cu2**).

1.3 HPLC analysis of purity

Copper complexes **Cu1** and **Cu2** were firstly dissolved in MeOH at a concentration of 10 μM, and 5 μL was used for each HPLC analysis with a C18 reverse phase column (100 mm × 2.1 mm, Thermo). Mobile phase A: H_2O + 0.01% trifluoroacetic acid (TFA). Mobile phase B: methanol. 0–20 min: From 10% B to 100% B; 20–30 min: 100% B. The absorption wavelength was set to 254 nm. Purity was obtained by calculating the proportion of peak areas.

1.4 Stability of the complexes

The solutions of the complexes (50 μM) were prepared with PBS to measure the UV absorption spectra at room temperature for 0 h, 24 h, and 48 h, respectively.

1.5 Lipophilicity of the complexes

The shake flask method was used to determine the log *P* values of the complexes. Saturated *n*-octanol aqueous solution and water-saturated *n*-octanol solution were first prepared, and then water-saturated n-octanol solutions of the complexes with a series of concentrations (4-36 μg/mL) were prepared. The UV-vis spectra of the above concentrations of the complexes were scanned and the absorption standard curves of the complexes at the wavelength of the maximum absorption peak (290 nm) were constructed.

An equal volume of the *n*-octanol-saturated aqueous solution was mixed with 5 mL of water-saturated *n*-octanol solution of Cu(II) complex (200 μg/mL).

After the mixed solution was shaken for 24 h (at 37 °C, 160 r/min), the absorbance values at the maximum absorption wavelength in the two phases were measured. The *n*-octanol phases of **Cu1** and **Cu2** were diluted 5 and 10 times, respectively, while the water phases were undiluted, and log *P* values were calculated according to Eq. S1.[2]

$$\log P = \log(C_O/C_W) \quad \text{(S1)}$$

Where, C_O and C_W represent the concentration of the complexes in the *n*-octanol phase and water phase (μg/mL), respectively.

1.6 Binding ability and interaction mode of the complexes with DNA

1.6.1 UV-Vis Spectroscopy

Add an equal amount of CT-DNA solution (20 μL, 1 mM) dropwise to the complex solution (3 mL, 50 μM) until there is no change in the spectrum. Each dropwise addition of CT-DNA solution should be blown well and incubated at room temperature for 6 min. Subsequently, the UV-visible spectra were measured at 245-800 nm.

1.6.2 EB competition experiment

The CT-DNA and EB solutions were mixed at constant concentrations (10 μM and 8 μM, respectively) and stood for 12 h to mix thoroughly. The fluorescence emission spectra were measured in the wavelength range of 535~700 nm under the excitation wavelength of 525 nm. Subsequently, an equal volume of the complex solution (2 μL, 10 mM) was added dropwise to the mixed EB-CT-DNA system until there was no change in the spectrum. Each drop of the complex solution should be blown well and incubated at room temperature for 6 min.

1.6.3 CD spectra

Preparing Tris solutions with a concentration of complex to CT-DNA (100 μM) in the ratio of 0, 0.2, 0.4, and 0.6, respectively. The CD spectra were scanned in the range of 235-295 nm under nitrogen protection. Each time the complex

is added, blowing is required for a uniform incubation for 6 min. The experimental data were processed using Chirascan and CD pro software to obtain the spectra.

1.6.4 Viscosity experiment

Viscosity experiments were conducted at 29 °C using an Uhr's viscometer. Different concentrations (0-0.06 mM) of the complexes were added dropwise to 0.2 mM of CT-DNA solution, each group reacted for 6 min, recorded the flow time of the samples, each group was parallel three times, and the relative viscosity (η) of the solution was calculated from the average value with Eq. S2 [3].

$$\eta = (t-t_0)/t_0 \qquad (2)$$

Where t_0 is the flow time of the blank buffer and t is the flow time of the experimental group. Then $(\eta/\eta_0)^{1/3}$ is plotted against r. η and η_0 are the CT-DNA viscosity in the presence and absence of the complexes, respectively.

1.7 Binding ability and interaction mode of the complexes with HSA

1.7.1 UV-Visible Spectroscopy

HSA (3 mL, 5 μM) solution was prepared and an equal amount of complex solution (2 μL, 10 mM) was added to the solution until there was no spectral change. The UV-visible spectra at 240-360 nm were measured after incubation for 6 min at room temperature. The intensity of its maximum absorption peak and its displacement were used to determine whether the complexes interacted with HSA.

1.7.2 Fluorescence spectra

The emission spectra of 5 μM HSA solution with a scanning range from 280 nm to 440 nm were measured at an excitation wavelength of 280 nm. An equal amount of the complex solution (2 μL, 10 mM)was added to the HSA solution by successive drops until there was no spectral change. Each drop addition of the complex solution needed to be blown evenly and incubated at room temperature for 6 minutes before measuring the fluorescence spectrum of

HSA.

1.7.3 Synchronous fluorescence spectra

Synchronous fluorescence spectra of tryptophan and tyrosine residues in HSA were determined separately. The synchronous fluorescence spectra with $\Delta\lambda$ = 60 nm in the wavelength range of 240-345 nm and $\Delta\lambda$ = 15 nm in the wavelength range of 270-345 nm were measured at the excitation wavelength of 240 nm and 270 nm, respectively.

1.8 Molecular docking

After the complex structure was optimized using Gaussview 6.0 software, the PDB file of the complex structure was exported. Autodock 4.0 software was used to simulate and study the interaction sites and modes between the complex and DNA/HSA. Water molecules and free anions need to be removed before docking. Crystal structures of DNA (PDB ID: 454d) and HSA (PDB ID: 1H9Z) were obtained from the Protein Data Bank, water molecules were deleted and polar hydrogen atoms were added before docking calculations, followed by docking calculations. The complex is located in the DNA coordinates x=29.466, y=19.382, and z=70.63. The coordinates of HSA were x= 24.986, y=9.578, and z=20.079. The classical Lamarck genetic algorithm (LGA) was used for docking simulation, with 100 runs for each binding site, and the docking results were analyzed by semi-empirical free energy calculation. Finally, PyMol software was used to display docking sites and interaction patterns.

1.9 *In vitro* cytotoxicity

The cytotoxicity of the ligand 1-Im-βc and the complexes toward HeLa, A549, MBA-MD-231 cancer cells, and normal HLF cells was evaluated *via* MTT assay. Cells cultured in 96-well plates (1.2×10^5 cells/well) were incubated to 70% confluence, and then treated with different concentrations of the compounds (0, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100 μM) for 48 h.

Subsequently, fresh serum-free media (90 μL) and MTT solution (10 μL, 5 mg/mL) were added to each well to continuously incubate 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.

1.10 Hoechst 33342 staining

About 2×10^5 cells were plated in a 35 mm laser confocal dish, and when the cells adhered to the wall and grew to 70%, the complex solution with 1, 2, and 4 times IC_{50} was added, respectively, and incubated at 37 °C for 24 h. At the end of the incubation period, the adherent cells were washed with PBS solution and subsequently fixed with paraformaldehyde. Finally, Hoechst 33342 (5 μg/mL) in PBS was added and incubated at 25 °C for 20 min for nuclear staining. Confocal laser scanning microscopy was used to observe and photograph the images.

1.11 Annexin V-FITC/PI double staining

About 5×10^5 cells were plated in 6-well plates, and when the cells adhered to the wall and grew to 70%, the complex solution with 1, 2, and 4 times IC_{50} was added, respectively, and incubated at 37 °C for 24 h. After that, the cells on the plates were digested with trypsin and collected by centrifugation at 4 °C (2500 r/min). After the supernatant was removed, 195 μL Annexin-binding buffer, 5 μL Annexin V-FITC and 10 μL PI were added successively, mixed and incubated at 25 °C in the dark for 20 min. The cell suspension was then transferred to a special flow tube and immediately detected by flow cytometry.

1.12 Activation of Caspase protein family

The cells were plated in 96-well plates (about 1.2×10^5 cells/well), and when the

cells adhered to the wall and grew to 70%, the complex solution with 1, 2, and 4 times IC_{50} was added, respectively, and incubated for 12 h at 37 °C. After the end of incubation, cells were subsequently detected for Caspase family activity using the Caspase-Glo® 3/7 kit from Promega, as described in the instructions. In addition, the cells were plated in 96-well plates (about 1.2×10^5 cells/well), and the cells were incubated with different concentrations of pan-caspase inhibitor (0, 25, 50, and 100 μM) for 1 h, and then a solution of the complexes (**Cu1**: 2 μM; **Cu2**: 1.5 μM) was added for 48 h at 37 °C. At the end of incubation, the medium was discarded, and subsequently 90 μL of DMEM medium and 10 μL of MTT solution were added to each well, thoroughly mixed, and incubated for 4 h at 37 °C. After 4 h, 100 μL DMSO was added to each well, and the 96-well plate was placed in a microplate reader and the absorbance measured at 490 nm. Finally, the cell survival rate was calculated.

1.13 Decrease of mitochondrial membrane potential (MMP)

About 2×10^5 cells were plated in a 35 mm laser confocal dish, and when the cells adhered to the wall and grew to 70%, the complex solution with 1, 2, and 4 times IC_{50} was added, respectively, and incubated at 37 °C for 12 h. At the end of the incubation, the cells were rinsed using a DMEM medium and incubated at 37 °C for 20 min with rhodamine 123 (5 μg/mL). Then the dye was removed, and the laser confocal microscope was used to observe and shoot in time.

In addition, the cells were plated in 96-well plates (about 1.2×10^5 cells/well), and when the cells adhered to the wall and grew to 70%, the complex solution of 1, 2, and 4 times IC_{50} was added, respectively, and incubated at 37 °C for 12 h. After the end of incubation, the ATP level of the cells was subsequently measured using the CellTiter-Glo® kit from Promega, as described in the instructions.

1.14 ROS assay

About 2×10^5 cells were plated in a 35 mm laser confocal dish, and when the cells adhered to the wall and grew to 70%, the complex solution with 1, 2, and 4 times IC_{50} was added, respectively, and incubated at 37 °C for 6 h. At the end of the incubation, the cells were rinsed using DMEM medium and subsequently incubated with DCFH-DA (10 μM) for 20 min in the dark. Then, the dye was removed, and the laser confocal microscope was used to observe and photograph in time to detect whether ROS was generated.

In addition, cells were plated in 96-well plates (about 1.2×10^5 cells/well), and when the cells adhered to the wall and grew to 70%, ROS inhibitors of different mechanisms were added for 1 h and then 1.5-fold IC_{50} complex solution was added for 48 h at 37 °C. At the end of incubation, the medium was discarded, and subsequently 90 μL of DMEM medium and 10 μL of MTT solution were added to each well, thoroughly mixed, and incubated for 4 h at 37 °C. After 4 h, 100 μL DMSO solution was added to each well, and the 96-well plate was placed in a microplate reader and the absorbance measured at 490 nm. Finally, the cell survival rate was calculated, and the type of reactive oxygen species leading to cell apoptosis was judged by the increase of cell survival rate.

2. Supplementary figures and tables

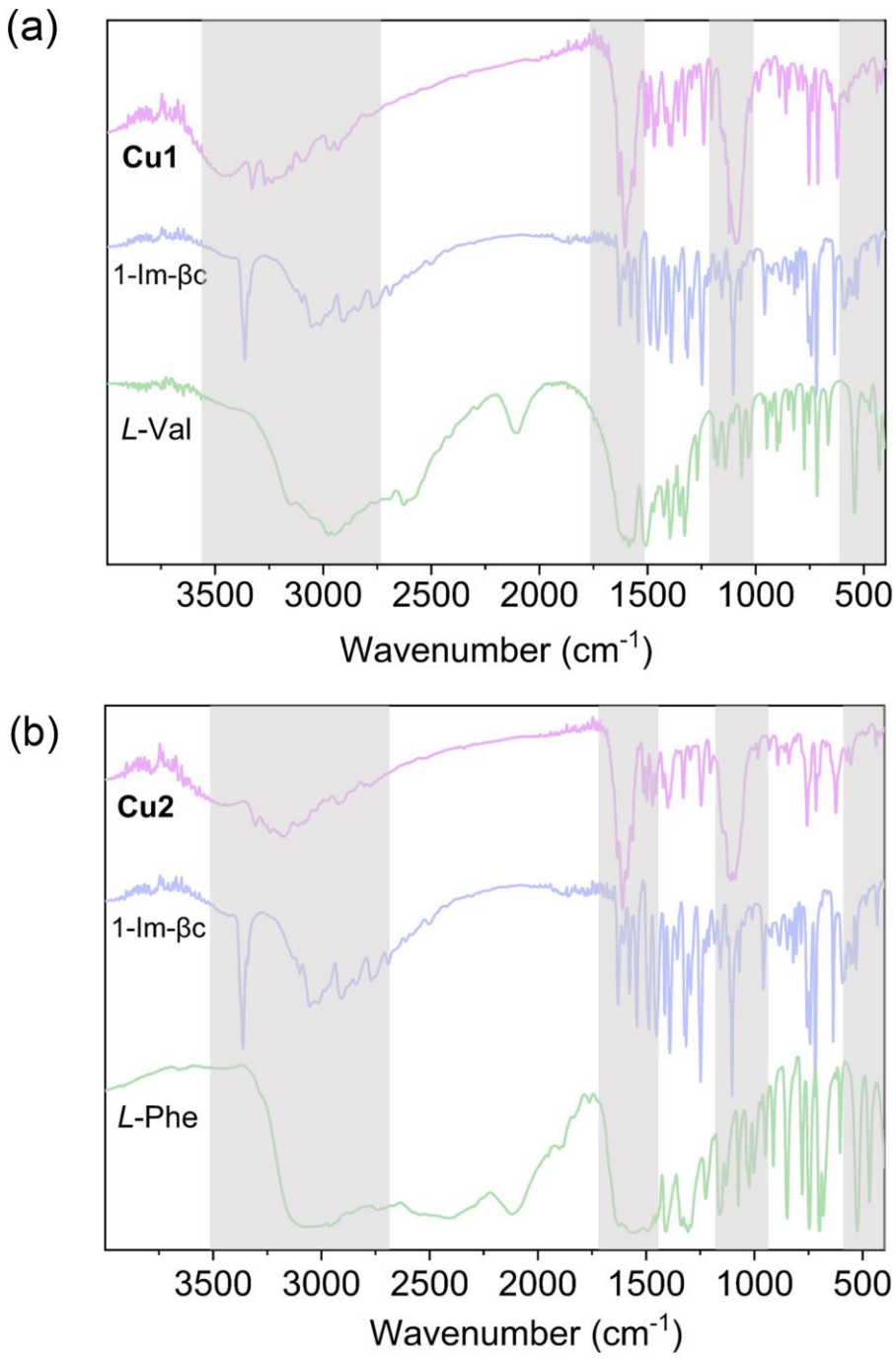


Fig. S1 FT-IR spectra of **Cu1** (a) and **Cu2** (b), and their respective ligands.

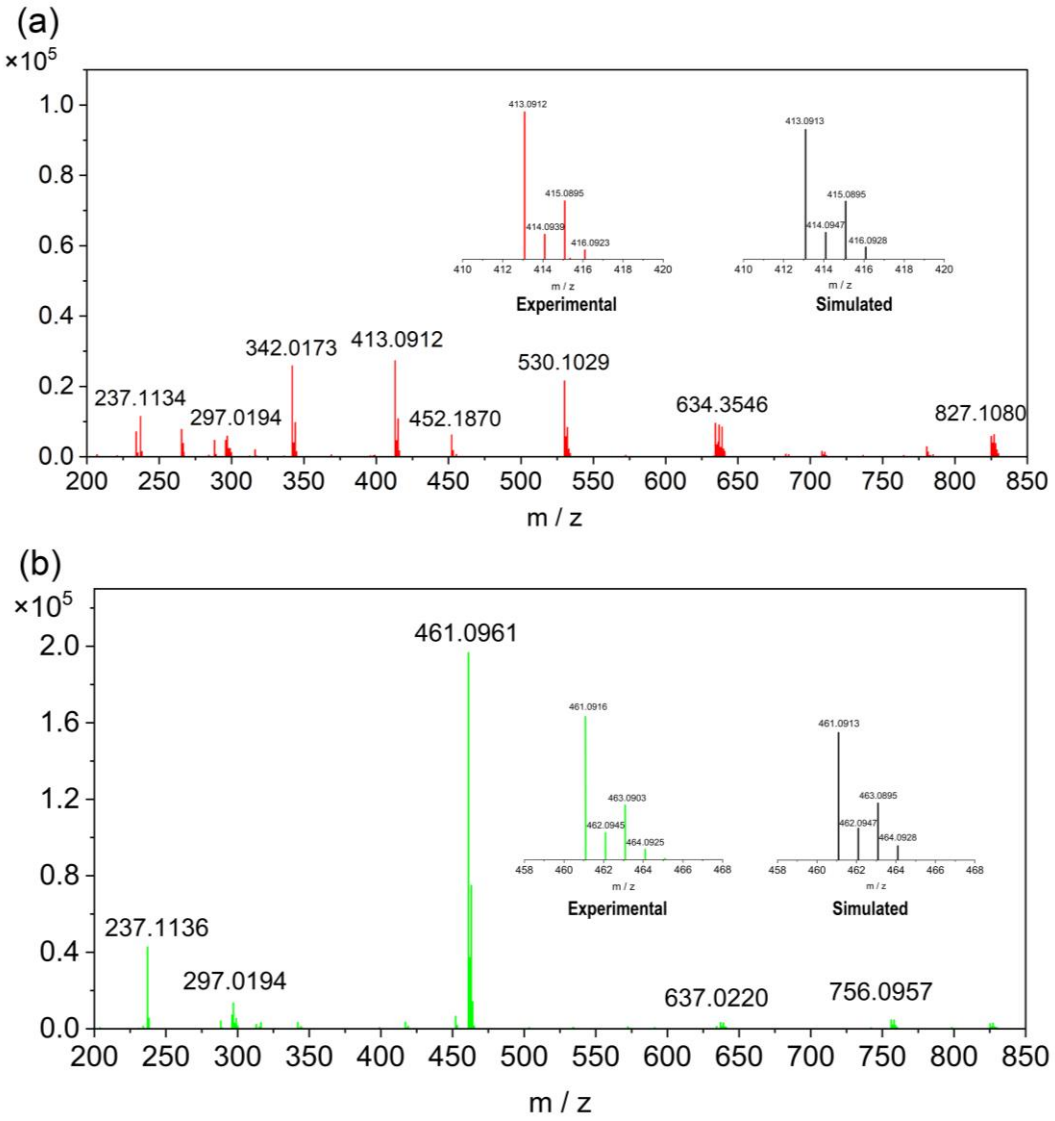
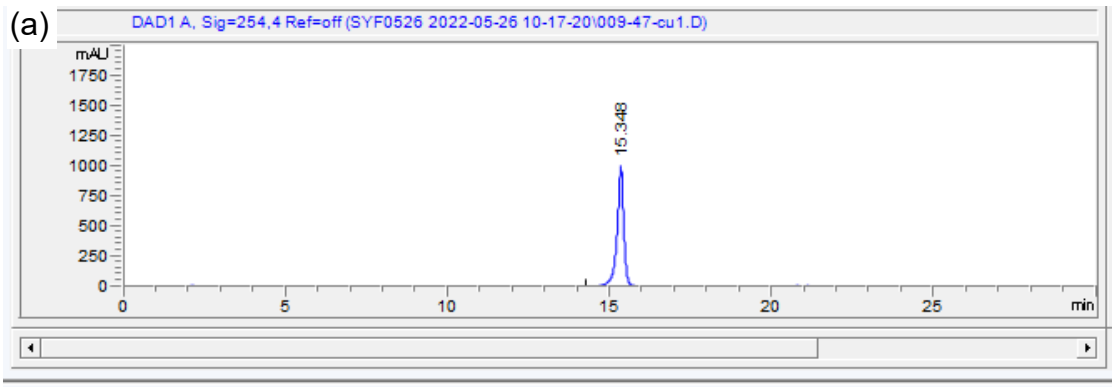


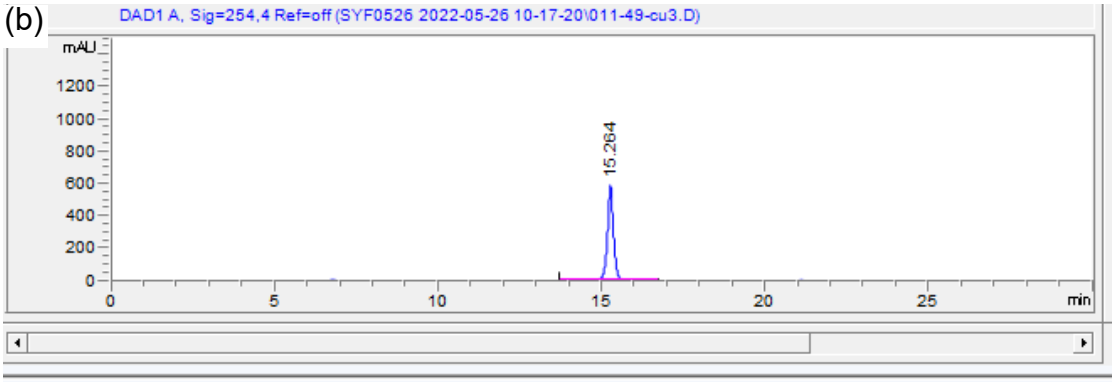
Fig. S2 Mass spectra of **Cu1** (a) and **Cu2** (b).

(a)



#	Time	Type	Area	Height	Width	Area%	Symmetry
1	15.348	BV R	14185.9	1000.9	0.1927	100.000	1.326

(b)



#	Time	Type	Area	Height	Width	Area%	Symmetry
1	15.264	BB	7675.6	592.8	0.1791	100.000	0.94

Fig. S3 HPLC spectra of **Cu1** (a) and **Cu2** (b). Mobile phase A: H_2O + 0.01% trifluoroacetic acid (TFA). Mobile phase B: methanol. 0–20 min: From 10% B to 100% B; 20–30 min: 100% B. The absorption wavelength was set to 254 nm. Purity was obtained by calculating the proportion of peak areas.

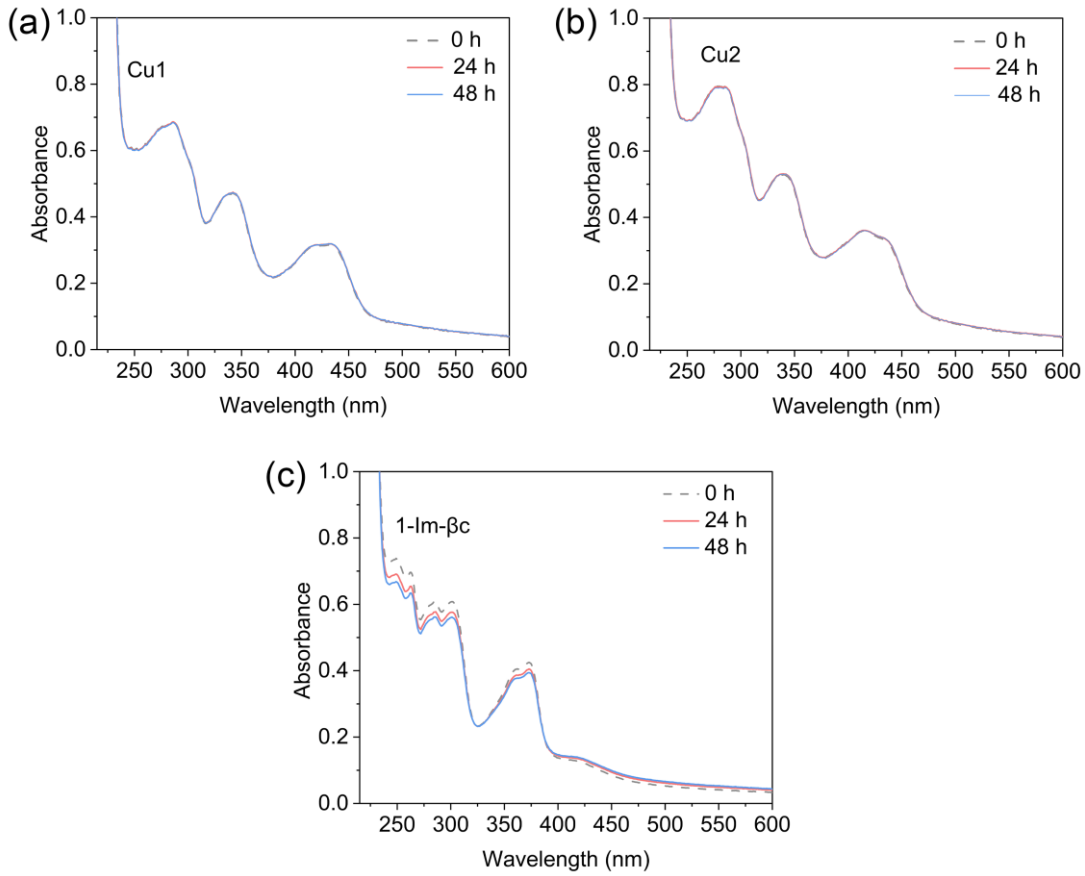


Fig. S4 Time-dependent changes in UV absorption of **Cu1** (a), **Cu2** (b) and 1-Im-βc (c) in PBS solution at room temperature.

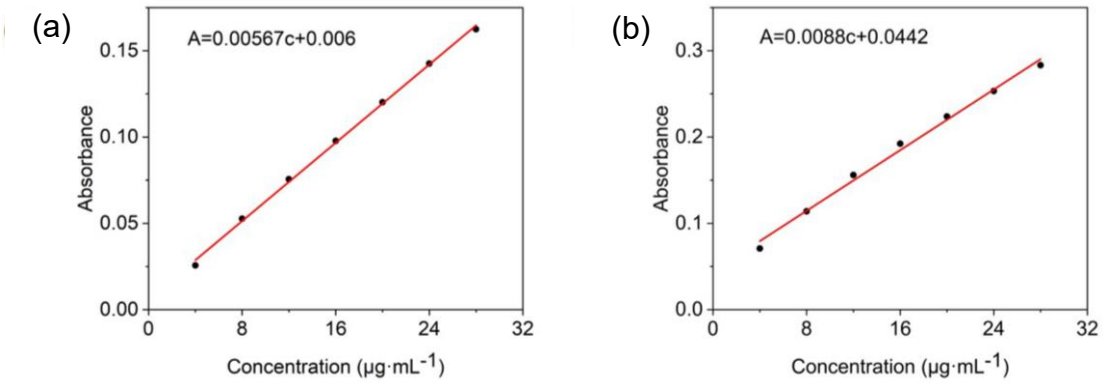


Fig. S5 The absorption standard curves for **Cu1** (a) and **Cu2** (b) at 290 nm.

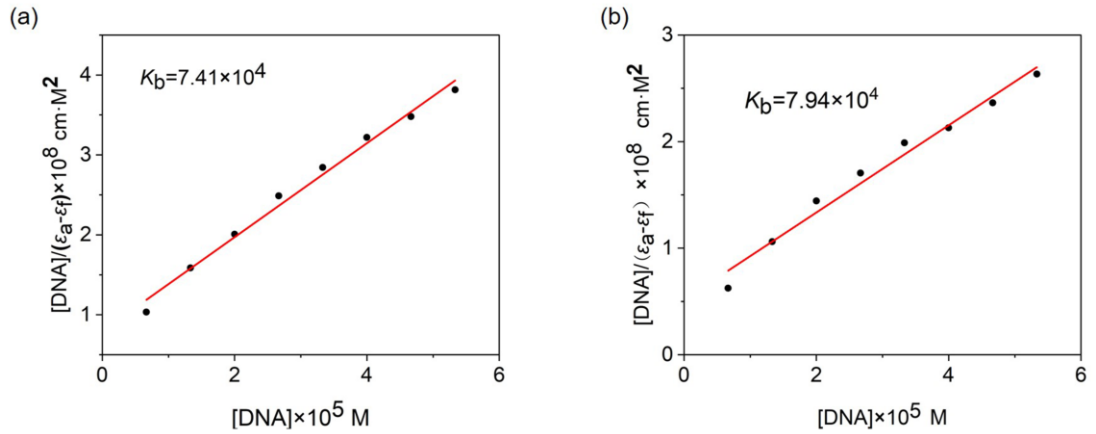


Fig. S6 Linear relationship graphs of [DNA] and [DNA]/(ε_a - ε_f) in DNA binding experiments, (a) and (b) for **Cu1** and **Cu2**, respectively.

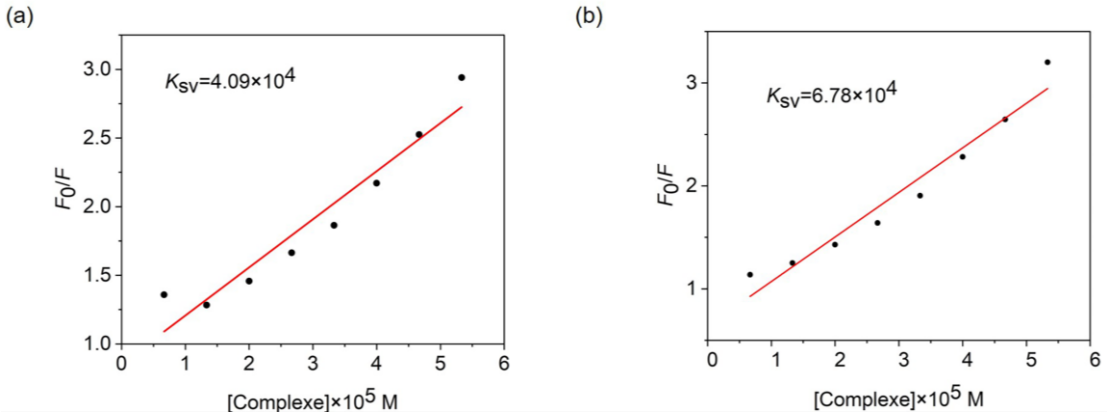


Fig. S7 Linear relationship plots for [DNA] and F_0/F in DNA binding experiments, (a) and (b) for **Cu1** and **Cu2**, respectively.

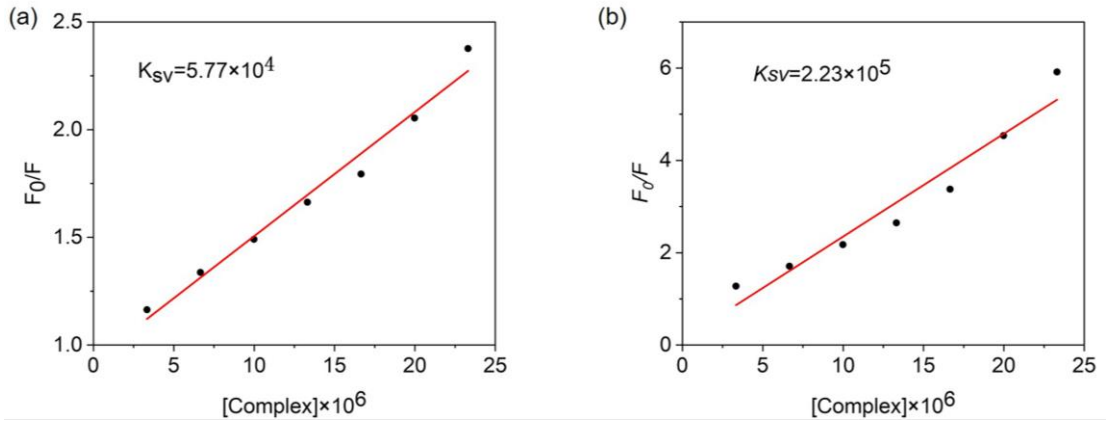


Fig. S8 Linear relationship plots for [Complex] and F_0/F in HSA binding experiments, (a) and (b) for **Cu1** and **Cu2**, respectively.

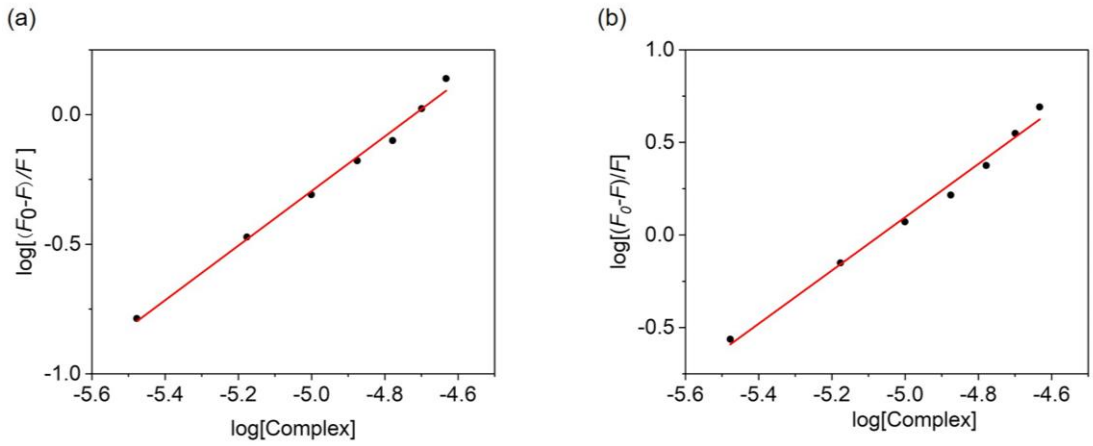


Fig. S9 Linear relationship plots for log[(F_0-F)/F] and log[Complex] in HSA binding experiments, (a) and (b) for **Cu1** and **Cu2**, respectively.

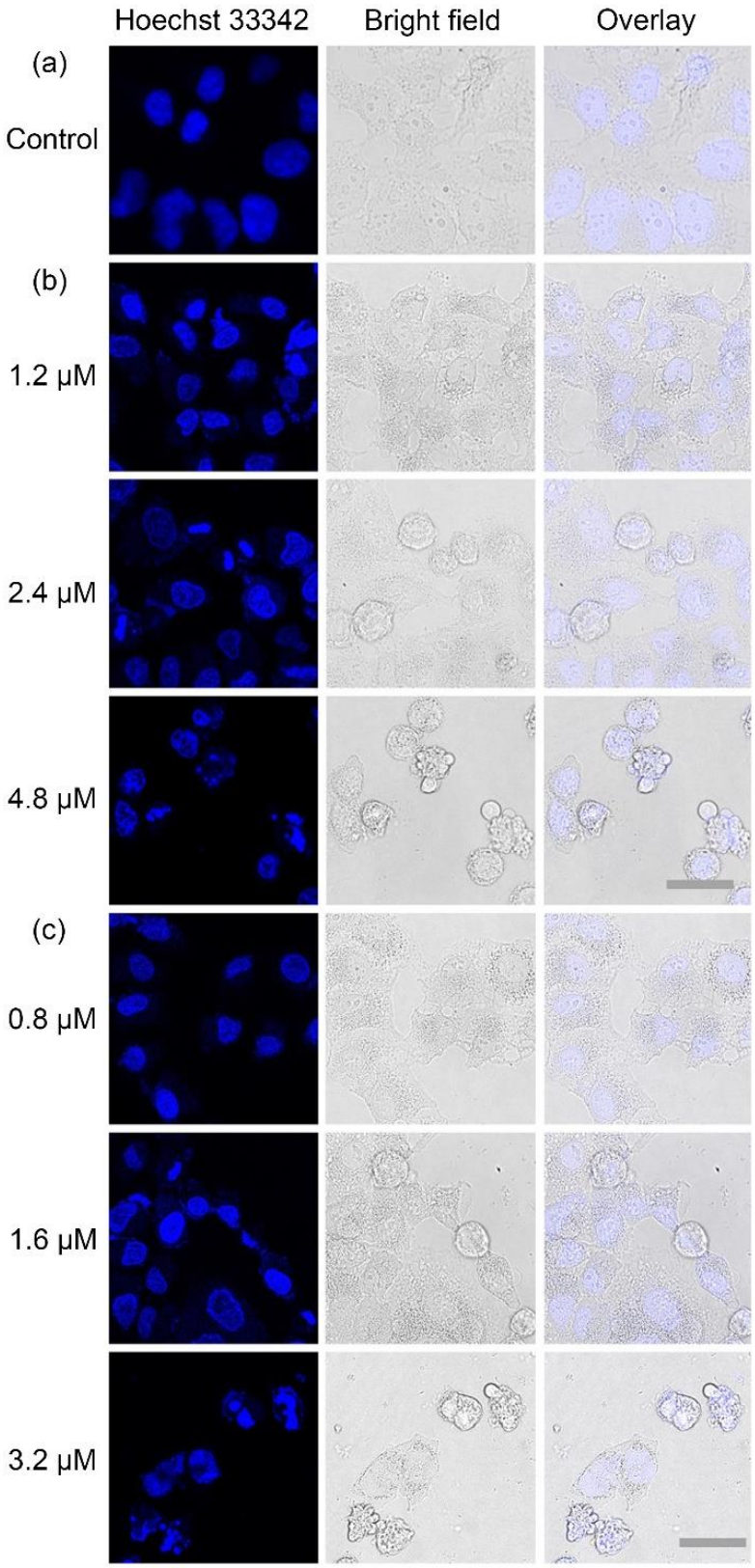


Fig. S10 HeLa cells were treated with the complexes for 24 h at 37 °C and then stained with Hoechst 33342 (5 μg/mL in PBS) for 20 min at 37 °C in the dark. Ex = 405 nm and Em = 470 ± 20 nm for Hoechst. (a) Control. (b) **Cu1**. (C) **Cu2**. Scale bar: 20 μm.

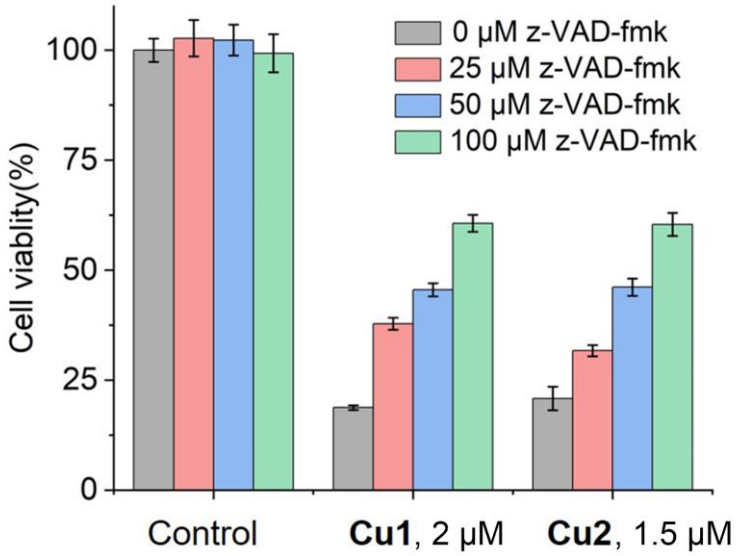


Fig. S11 Effect of z-VAD-fmk on the cytotoxicity of the complexes and cisplatin. HeLa cells were pretreated with z-VAD-fmk for 1 h at 37 °C, and then treated with **Cu1** (2 μM) and **Cu2** (1.5 μM) for 48 h at 37 °C. Cell viability was determined by the MTT method.

Table S1 Elemental analysis results for **Cu1** and **Cu2**

Complex	Molecular formula	Measured value (theoretical value)		
		C%	H%	N%
Cu1	$C_{19}H_{21}ClCuN_5O_{6.5}$	44.03(43.68)	3.98(4.05)	13.18(13.41)
Cu2	$C_{23}H_{21}ClCuN_5O_{6.5}$	47.03(48.43)	3.70(3.71)	12.04(12.28)

Table S2 The UV absorbance at 290 nm and log *P* values of the complexes

Complex	A_O			C_O ($\mu g \cdot mL^{-1}$)	A_W			C_W ($\mu g \cdot mL^{-1}$)	log *P*
Cu1	0.091	0.092	0.091	74.66	0.059	0.058	0.059	9.38	0.90
Cu2	0.186	0.186	0.185	163.86	0.104	0.105	0.103	6.76	1.38

Table S3 Quenching constants and thermodynamic parameters of the interaction of the complexes with HSA

Complex	K_{SV} (M^{-1})	K_q ($M^{-1} \cdot S^{-1}$)	R	K_a (M^{-1})	*n*	R
Cu1	5.77×10^4	5.77×10^{12}	0.988	9.36×10^4	1.05	0.996
Cu2	2.33×10^5	2.33×10^{13}	0.969	1.94×10^7	1.44	0.998

References

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