Electronic Supplementary Information

Sparfloxacin - Cu(II) - aromatic heterocycle complexes: Synthesis, characterization and in vitro anticancer evaluation

Qi-Yan Liu, Yong-Yu Qi, Dai-Hong Cai, Yun-Jun Liu, Liang He and Xue-Yi Le

a. Key Laboratory for Biobased Materials and Energy of Ministry of Education, College of Materials and Energy, South China Agricultural University, Guangzhou 510642, People's Republic of China. E-mail: heliang@scau.edu.cn (L. He), lexyfu@163.com (X.-Y. Le)
b. Department of Applied Chemistry, South China Agricultural University, Guangzhou, 510642, People's Republic of China.
c. School of Pharmacy, Guangdong Pharmaceutical University, Guangzhou, 510006, People's Republic of China. E-mail: lyjche@gdpu.edu.cn (Y.-J. Liu)
# Table of Contents

**Experimental section** ........................................................................................................... S4

- Materials and Apparatuses .................................................................................................. S4
- Stability test ....................................................................................................................... S4
- Measurement of the oil-water partition coefficients ($\log P$) ............................................. S4
- UV-Vis absorption spectra ................................................................................................. S5
- EB competition experiment .............................................................................................. S5
- Viscosity experiment ......................................................................................................... S6
- Circular dichroism ............................................................................................................. S6
- Molecular docking ............................................................................................................ S6
- pBR322 DNA cleavage experiment .................................................................................. S7
- *In vitro* cytotoxicity evaluation ........................................................................................ S7
- Single cell gel electrophoresis assay (SCGE) .................................................................. S7
- Hoechst 33342 staining ..................................................................................................... S8
- Annexin V-FITC/PI double staining .................................................................................. S9
- Western blot ..................................................................................................................... S9
- Mitochondrial membrane potential (MMP) detection ....................................................... S9
- ROS detection .................................................................................................................. S10
- Determination of cyt c ....................................................................................................... S10
- Determination of intracellular Ca^{2+} concentration ....................................................... S10
- Cell cycle arrest ................................................................................................................. S10

**Supporting figures and tables** ............................................................................................ S12

- Fig. S1 FT-IR spectra of complexes 1 (a) and 2 (b) ........................................................... S12
- Fig. S2 UV-Vis spectra of complexes 1 (a) and 2 (b) ........................................................ S13
- Fig. S3 X-band EPR spectra of complexes 1 (a) and 2 (b) ............................................... S13
Fig. S4 UV absorption spectra of complexes 1 (a) and 2 (b)……………………………………S14
Fig. S5 The UV-vis absorption standard curves of complexes 1 (a) and 2 (b)………………S14
Fig. S6 Stacking distance diagram of complexes 1 (a) and 2 (b)……………………………S15
Fig. S7 Cleavage of pBR322 DNA (250 ng) by complexes 1 and 2 in the presence of various typical ROS scavengers…………………………………………………………………………………S15
Fig. S8 UV-Vis spectra of MB degradation in different solutions………………………….S16
Fig. S9 Cell viability after treatment with the complexes in the absence and presence of z-VAD-fmk……………………………………………………………………………………………S16
Fig. S10 Cell viability of Eca-109 cells treated with the complexes in the absence and presence of various ROS inhibitors……………………………………………………………………………………………S17
Table S1 UV-vis values of the complexes in oil and water phases, respectively…………..S17
Table S2 the nucleus tail length after 24 h with the complexes (average ± SD)………………S17

Supporting references…………………………………………………………………………………S17
Experimental section

Materials and Apparatuses

Materials. All reagents and solvents were of commercial reagent grade and were used without further purification, and deionized water was used throughout the experiments. Calf-thymus DNA (CT-DNA), ethidium bromide (EB = 3,8-diamino-5-ethyl-6-phenyl-phenanthridinium bromide), dimethylsulfoxide (DMSO), RPMI-1640 and DMEM were purchased from sigma, and pBR322 DNA was provided by MBI Fermentas (Lithuania). MTT were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Eca-109 (human esophageal cancer cell line), Bel-7402 (human liver cancer cell line), A549 (human lung cancer cell line) and LO2 (human normal liver cell) were purchased from the Laboratory Animal Center of Sun Yat-Sen University (Guangzhou, China).

Apparatuses. Elemental analyses (C, H, and N) were obtained on a Vario EL elemental analyzer (Elementar, Germany). FT-IR spectra were determined with KBr particles (4000-400 cm⁻¹) on a VERTEX 70 FT-TR spectrometer (Brook, Germany). Molar conductivities were measured on a DDS-11A digital conductance (LeiCi, China) in 10⁻³ M and methanol and DMSO solutions at room temperature. ESR spectra were measured on a Bruker A300 (Bruker, Germany). Electrospray ionization mass spectra (ESI-MS), UV-vis, fluorescence spectra and CD spectra were recorded on API3200 liquid chromatography tandem mass spectrometer (AB Sciex, Singapore), Cary 60 spectrophotometer (Agilent, USA), Hitachi F-7000 fluorescence spectrophotometer (Hitachi, Japan) and Chriascan (Applied Photophysics Ltd, UK), respectively.

Stability test

The UV absorption spectra of the complexes (20 μmol/L) in a mixture of Tris-HCl buffer (pH=7.2) and DMSO (v/v, 2:1) were measured at 0 h, 12 h, and 24 h.

Measurement of the oil-water partition coefficients (log P)

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 (2-10 μg/mL) were prepared. The UV-vis spectra of the above concentrations were scanned and the absorption standard curves of the complexes at the wavelengths of the maximum absorption peaks (304 and 306 nm for complexes 1 and 2, respectively) were constructed.

An equal volume of n-octanol saturated aqueous solution was mixed with 5 ml of water-saturated n-octanol solution of Cu(II) complex (300 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 complexes 1 and 2 were diluted 30 and 44 times, respectively, while the water phases were undiluted, and log P values were calculated according to Eq. S1.

\[
\log P = \log \left( \frac{C_O}{C_W} \right) \quad \text{Eq. S1}
\]

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

**UV-Vis absorption spectra**

The complexes (40 μM) were incubated with various concentrations of CT-DNA in the Tris-HCl buffer (5 mM Tris-HCl/50 mM NaCl buffer = pH 7.2) at room temperature, and recorded in the scanning wavelength range of 200-500 nm. From the spectral titration data, the binding constant \(K_b\) were calculated using the following Eq. S2:

\[
\frac{[\text{DNA}]}{(\varepsilon_a - \varepsilon_f)} = \frac{[\text{DNA}]}{(\varepsilon_b - \varepsilon_f)} + \frac{1}{K_b(\varepsilon_b - \varepsilon_f)} \quad \text{Eq. S2}
\]

where, [DNA] represents the concentration of DNA base pairs, \(\varepsilon_a\), \(\varepsilon_b\), and \(\varepsilon_f\) represent \(A_{\text{obs}}/C_{\text{Cu}}\), the extinction coefficients of the complexes in the bound and free forms, respectively. In the graph of \([\text{DNA}]/(\varepsilon_a - \varepsilon_f) \text{ versus } [\text{DNA}]\), \(K_b\) is given by the ratio of the slope \(1/(\varepsilon_b - \varepsilon_f)\) to the intercept \(1/K_b(\varepsilon_b - \varepsilon_f)\).\(^1\)

**EB competition experiment**

The mixture solutions (3 ml, EB: 8 μM; DNA: 10 μM) of EB-CT-DNA were titrated by solutions of Cu(II) complexes ranging from 10 to 80 μM, and the fluorescence spectra ranging from 550 to 700 nm (525 nm excitation) were recorded. The fluorescence quenching constants \(K_{sv}\) were calculated using the following Eq. S3:\(^2\)
\[ F_0 F = 1 + K_{sv}[C] \]  
\text{Eq. S3}

where, \( F_0 \) and \( F \) are the fluorescence intensities of EB-CT-DNA system in the absence and presence of Cu(II) complex, respectively, \([C]\) represents the molar concentration of the complex and \( K_{sv} \) represents the quenching constant of the Stern-Volmer equation.

**Viscosity experiment**

Viscosity experiments were carried out using Ubbelohde viscometer at room temperature. The viscosity determination was performed for a fixed amount of CT-DNA solution (200 mM) in the absence and presence of various concentrations of the complexes. The flow times were measured and each sample was measured three times. The relative viscosities \( \eta \) of DNA solutions were calculated according to the following Eq. S4:

\[ \eta = (t-t_0)/t_0 \]  
\text{Eq. S4}

Where, \( t_0 \) is the flow time of the buffer solution alone, \( t \) is the flow time of the DNA-bound complexes. In the graph of \( (\eta/\eta_0)^{1/3} \) (where \( \eta \) and \( \eta_0 \) are the specific viscosities of DNA in the presence and absence of the complex, respectively) \text{versus} \ r ([\text{Complex}]/[\text{DNA}]), while EB is used as a positive control, the trend of the effect of the complex on the viscosity of the DNA solution is obtained.

**Circular dichroism**

The solutions of Cu(II) complexes ranging from 10 to 60 μM were added to the solutions of CT-DNA (100 μM), and the spectra ranging from 220 to 320 nm were recorded.

**Molecular docking**

The molecular structures of the complexes were drawn using YASARA1 software, and the dispersion-corrected density functional theory (DFT-D3) in GAMESS (US) software was used to geometrically optimize the structure of the complexes. The continuous polarization model (CPCM) was used to simulate water solvation effect, followed by docking after optimization. The crystal structure of DNA (PDB ID: 454D) was obtained from RCSB protein database, and the file was dehydrated and hydrogenated before docking. The molecular docking process was performed on AutoDock 4.2 software using a grid spacing of 0.375 Å. The center of the complexes was located at
coordinates \( x = 29.986, y = 9.578, z = 20.079 \), and the grid size was \( 60 \times 60 \times 60 \). According to the Lamarckian Genetic Algorithm (LGA), the number of calculation rounds have been set to 100. Other parameters such as distance range, 3D level, grid dimension were set to default. The results were analyzed using Autodock 1.5.6 software and visualized using PyMol software.

**pBR322 DNA cleavage experiment**
In the presence or absence of the reducing agent ascorbic acid (500 \( \mu \text{M} \)), pBR322 DNA (250 \( \text{ng} \)) was mixed with the complexes and stabilized to 27 \( \mu \text{L} \) with Tris-HCl buffer (pH = 7.2). The mixed solutions were incubated in the dark for 1 h. Subsequently, 3 \( \mu \text{L} \) 6\( \times \) loading buffer solution was added to each mixed solution, and the samples were loaded into an agarose gel containing GelRed, and electrophoretic at 100V in standard Tris-Boric Acid-EDTA (TBE) buffer (pH 8.3) for 40 min. The treated gels were visualized and photographed using BIO-RAD Laboratories-Segrate. In addition, hydroxyl radical scavengers (DMSO, ethanol and tert-butyl), superoxide anion radical scavenger (SOD) and singlet oxygen quencher (NaN\(_3\)) were added according to the above experimental procedures in the exploration of DNA cracking mechanism.

**In vitro cytotoxicity evaluation**
The MTT method was used to determine the cytotoxicity of the complexes. RPMI-1640 and DMEM media were used to culture the tested cells. Cells were seeded in 96-well plates and incubated at constant temperature incubator (37 \( ^\circ \text{C}, 5\% \text{ CO}_2 \)) for the screening experiments. The cells were incubated with various doses of the drug from 3.125 to 200 \( \mu \text{M} \) when the cell growth reached 70-80\% for 48 h. The MTT reagent was added into each well at a final concentration of 5 mg·mL\(^{-1}\) for the formation of formazans, and then the incubation was continued for 4 h. After that, 100 \( \mu \text{L} \) of DMSO solution was added to solubilize the precipitate. After shaking for 10 min, the absorbance was measured at 490 nm using a microplate reader. The experiments were performed in triplicate, and then the semi-inhibitory concentrations (IC\(_{50}\)) were calculated.

**Single cell gel electrophoresis assay (SCGE)**
Eca-109 cells were plated and cultured in CO\(_2\) incubator (37 \( ^\circ \text{C}, 5\% \text{ CO}_2 \)) in a 12-well plate (2\( \times \)10\(^5\) cells·well\(^{-1}\)) overnight. The cells were exposed to different concentrations of copper complexes for
24h. 60 mL of 0.5 % normal agarose (NMA) PBS solution was gently poured on the glass slide, covered with a cover glass, and dried at 37 °C overnight. The cover glass was removed to obtain the first layer of gel. In a centrifuge tube, 60 μL of cell suspension (2×10^5 cells·mL⁻¹) and 60 μL of 1 % low melting point agarose gel (LMA) were pipetted uniformly, and 100 μL of the mixed solution was added dropwise to the first layer of gel. Thereafter, the cover glass was quickly covered and solidified at 4 °C for 10 min to obtain a second layer of glue. The coverslip was removed, and 100 μL of 0.5% LMA was dropped on the second layer of gel and allowed to solidify at 4 °C for 10 min. After the agarose had solidified, the coverslip was removed and the slide was immersed in an ice-cold alkaline cell lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 90 mM sodium sarcosine, NaOH, pH 10, 1 % Triton X-100 and 10 % DMSO), lysed at 4 °C for 60 min. All of the above operations were performed under low light conditions to avoid additional DNA damage. The slide was washed with distilled water and electrophoresis for 20 min (25 V, 300 mA) in electrophoresis buffer (300 mM NaOH, 1.2 mM EDTA). After that, it was washed three times with neutralization buffer (400 mM Tris, HCl, pH 7.5) for 10 min each time, and then stained with 200 μL EtBr (10 μg·mL⁻¹) in the dark for 20 min and washed with neutralization buffer for 15 min. Finally, the cells were detected by fluorescence microscopy. The obtained data were analyzed with ZEN lite 2012 software. The images were analyzed by an ImageXpress Micro XLS confocal fluorescence microscope.

**Hoechst 33342 staining**

Eca-109 cells were seeded in a 12-well plate (2×10^5 cells·well⁻¹) and incubated overnight in a CO₂ incubator (37 °C, 5% CO₂). Various doses of drugs were added to each well, and a control group (non-medicated group) was set up. After 24 h of incubation, the old culture media were discarded and washed with PBS. 0.5 mL cell fixation solution (methanol: glacial acetic acid = 3:1) were added. After being fixed at 4 °C for 10 minutes, the fixative was aspirated and washed with PBS. 200 μL Hoechst 33342 staining solution were added to each well for 10 min, and the staining solutions were discarded and washed with PBS. The images were acquired by a fluorescence microscope and avoid light as much as possible throughout the processes.

**Annexin V-FITC/PI double staining**
Eca-109 cells were seeded in 6-well plates (6×10^5 cells per well) and incubated overnight. The cells were incubated with various doses of the drug for 24 h when the cell growth reached 80 %. The old culture solutions were aspirated and washed with PBS. The cells were collected and centrifuged (1000 rpm, 6 min), washed with PBS. 195 μL of 1× Annexin V-FITC binding solution, 5 μL of Annexin V-FITC and 10 μL of PI working solution were added to per well, the cell mixture was incubated at 37 °C for 15 min in the dark, tested by the BD FACSCalibur flow cytometry system and analyzed with CytExpert software. Avoid light as much as possible throughout the processes.

**Western blot**

After Eca-109 cells were seeded in a 6-well plate and the cell growth reached 90 %, the complexes were added and incubated for 24 h. The cells were lysed for 30 min by the cell lysate (PMSF:RIPA = 1:100), and centrifuged at 12000 r/min and 4 °C for 15 min to extract protein. The BCA kit was used to determine the total protein concentration, followed by gel preparation and sample loading. SDS-PAGE electrophoresis was carried out in 1× electrophoresis solution, and the separation gel of the target protein was cut and transferred to the polyvinylidene fluoride membrane (PVDF) after the end of electrophoresis. The membrane was blocked in 5 % skimmed milk powder at room temperature for 4 h. After being washed with TBST, the membrane was further incubated with the corresponding primary antibody at 4 °C overnight, and then incubated with the secondary antibody at room temperature for 1 h. Finally, the membrane was washed with TBST and added with chemiluminescent solution, which was used for exposure in the imaging system, and Image J software was used to quantitatively calculate protein expression.

**Mitochondrial membrane potential (MMP) detection.**

Eca-109 cells were seeded in 12-well plates (2×10^5 cells per well) and incubated overnight. Then, the cells were incubated with 15, 30 μM Cu(II) complexes at 37 °C for 24 h and washed with PBS. 200 μL JC-1 staining solution (1 mg·mL⁻¹) was added to each well, and the incubation was carried out for 20 min at 37 °C in the dark, followed by being washed with PBS and cell images were acquired by high-content cell imaging system.
**ROS detection**

Eca-109 cells were seeded in confocal dish (2×10^5 cells) and the cell growth reached 60 %. The cells were washed with serum-free medium for three times after the complexes were added and incubated at 37 °C for 24 hours. The cells were then incubated with 10 μM DCFH-DA for 30 minutes and were washed with serum-free medium for three times. The cells were detected by confocal laser microscopy (TCS SP8, LEICA, GER), with excitation at 488 nm and emission at 525 nm.

**Determination of cyt c**

Eca-109 cells were seeded in 12-well plates (2×10^5 cells per well) and incubated overnight. The cells were incubated with the specified concentration of the complexes at 37 °C for 24 h, and then fixative was added at 4 °C. The next day, each well was washed with TBST, and blocking solution was added. After 60 min, it was washed with TBST, and the Cytochrome c antibody was added and incubated for 24 h at 4 °C, followed by being washed with TBST. The corresponding secondary antibody was added and incubated for 60 min at room temperature and then washed with TBST. DAPI staining solution was added to each well and incubated in the dark at 37 °C for 20 min. After dyeing, cells were washed with PBS. High-content cell imaging system was used to take pictures and quantify. Avoid light as much as possible during the whole process.

**Determination of intracellular Ca^{2+} concentration**

The tested cells were seeded in 12-well plates (2×10^5 cells per well) and incubated at 37 °C overnight. After the cells were treated with the complexes at the indicated concentrations for 24 h, each well was washed with PBS twice and incubated with 200 μL Fluo-3 Am staining solution for 30 min at 37 °C in the dark. Finally, the cells were washed with PBS and collected, followed by being detected by a high-content cell imaging system.

**Cell cycle arrest**

Eca-109 cells were seeded in 6-well plates (4×10^5 cells per well) and incubated for 24 h. The culture media were replaced by the fresh media containing various concentrations of the complexes. After 24 h, the cells were washed with PBS and digested with trypsin and immobilized with 70 % ice-cold ethanol, and eventually preserved at 4 °C. The experimental data were acquired by the BD FACSCaliburflow cytometry system and analyzed with FlowJo X software.
Supporting figures and tables

**Fig. S1** FT-IR spectra of complexes 1 (a) and 2 (b).
**Fig. S2** UV-Vis spectra of complexes 1 (a) and 2 (b) in DMSO \((1.0 \times 10^{-5} \text{ M})\). Inset: \(1.0 \times 10^{-2} \text{ M}\).

**Fig. S3** X-band EPR spectra of complexes 1 (a) and 2 (b) at 100 K in DMF \((1.0 \times 10^{-3} \text{ M})\).

*EPR conditions:* Microwave frequency, 9.85 GHz; Microwave power, 19.07 mW; mod. amplitude, 1.0 G.
Fig. S4 UV absorption spectra of complexes 1 (a) and 2 (b) at room temperature for 0, 12, and 24 h.

Fig. S5 The UV-vis absorption standard curves of complexes 1 (a) and 2 (b).
Fig. S6 Stacking distance diagram of complexes 1 (a) and 2 (b).

Fig. S7 Cleavage of pBR322 DNA (250 ng) by complexes 1 and 2 in the presence of various typical ROS scavengers. (a) Lane 1, DNA control; lane 2, DNA + VC (500 μM) + SOD (15 units); lane 3, DNA + VC (500 μM) + 1 (10 μM); lanes 4-8, DNA + VC (500 μM) + 1 (10 μM) + [DMSO(0.2M), tert-butyl alcohol (0.2 M), EtOH (0.2 M), NaN₃ (0.2 M) and SOD (15 units), respectively]; (b) Lane 1, DNA control; lane 2, DNA + VC (500 μM) + SOD (15 units); lane 3, DNA + VC (500 μM) + 2 (35 μM); lanes 4-8, DNA + VC (500 μM) + 1 (35 μM) + [DMSO(0.2M), tert-butyl alcohol (0.2 M), EtOH (0.2 M), NaN₃ (0.2 M) and SOD (15 units),
Fig. S8 UV-Vis spectra of MB degradation in different solutions.

Fig. S9 Cell viability after treatment with the complexes in the absence and presence of z-VAD-fmk (average ± SD, **p < 0.01).
**Fig. S10** Cell viability of Eca-109 cells treated with the complexes in the absence and presence of various ROS inhibitors (average ± SD, **p < 0.01**).

### Table S1 UV-vis values of the complexes in oil and water phases, respectively.

<table>
<thead>
<tr>
<th>Complex</th>
<th>( \bar{A}_O )</th>
<th>( \bar{C}_O ) (μg/ml)</th>
<th>( \bar{A}_W )</th>
<th>( \bar{C}_W ) (μg/ml)</th>
<th>( \log P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.545</td>
<td>0.549</td>
<td>0.551</td>
<td>284</td>
<td>0.554</td>
</tr>
<tr>
<td>2</td>
<td>0.472</td>
<td>0.456</td>
<td>0.454</td>
<td>292</td>
<td>0.405</td>
</tr>
</tbody>
</table>

### Table S2 The nucleus tail length after 24 h with the complexes (average ± SD).

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Control</th>
<th>Complex 1</th>
<th>Complex 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>24.6 ± 0.2</td>
<td>99.5 ± 0.8</td>
<td>64.6 ± 0.5</td>
</tr>
<tr>
<td>30</td>
<td>137.1 ± 0.2</td>
<td>84.7 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>
Supporting references

