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Copper(II) complexes based on quinoline-derived Schiff-base ligands: synthesis, characterization, HSA/DNA binding ability, and anticancer activity†

Kun Hu, *^a Chensi Liu,^a Jingui Li^a and Fupei Liang *^{ab}

Three copper(II) complexes, [Cu(L1)(NO₃)₂] (C1), [Cu(L2)Cl₂] (C2) and [Cu(L2)SO₄]₂·H₂O (C3), were designed and synthesized by the reaction of Cu(NO₃)₂·3H₂O, CuCl₂·2H₂O and CuSO₄·5H₂O with a quinoline-derived Schiff base ligand, L1 or L2, prepared by the condensation of quinoline-8-carbaldehyde with 4-aminobenzoic acid methyl ester or 4-aminobenzoic acid ethyl ester (benzocaine). The efficient bindings of the C1–C3 complexes with human serum albumin (HSA) and calf thymus DNA (CT-DNA) were analyzed by spectroscopy and molecular docking. These complexes could significantly quench the fluorescence of HSA through the static quenching process, and hydrophobic interactions with HSA through the sub-domain IIA and IIIA cavities. The complexes bind to DNA via the intercalative mode and they fit well into the curved contour of the DNA target in the minor groove region. Furthermore, the interaction abilities of the Cu(II) complexes with HSA/DNA were greater as compared to their corresponding ligands. Interestingly, C1–C3, particularly C3, exhibited more cytotoxicity toward HeLa cells compared to normal HL-7702 cells and three other tumor cell lines (Hep-G2, NCI-H460, and MGC80-3). Their cytotoxicity toward the HeLa cell lines was 1.9–3.5-fold more potent than cisplatin. Further studies indicated that these complexes arrested the cell cycle in the G0/G1 phase and promoted tumor cell apoptosis via a reactive oxygen species (ROS)-mediated mitochondrial pathway.

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1. Introduction

With the development of medicinal inorganic chemistry, especially the anticancer activity of cisplatin being found, metal-based drugs have been placed in the frontline in the fight against cancer.¹ Among these bio-essential metals, the homeostasis and metabolism of copper are important to all kinds of human cancers, and copper levels in tumor tissues of cancer patients are significantly elevated compared to normal tissue.² As such, cancer cells may represent a suitable and selective target for copper-based agents.³ Copper also alters the metabolism cancer cells and exhibits a differential response to tumor cells as compared with normal cells, which endows copper complexes with essential anti-neoplastic char-

acteristics. Copper complexes have, therefore, shown encouraging prospects and are considered as promising alternatives to platinum drugs in this field.^{1,4,5}

Recent literature has revealed that the activity of metal-based anticancer agents is as a result of the ligand framework because the organic ligand can regulate metal absorption, distribution, and metabolism in biological systems and thus reduce toxic side effects, improve efficiency, enhance delivery, and provide better activities in drug resistance cells.⁶ As such, the ligand introduced is crucial for the metal-based anticancer agents. As an important class of compounds, Schiff bases have drawn considerable attention due to their bioactivities, pharmacological activities and good coordination ability with metal ions.^{7–9} Some Schiff bases and their copper complexes have been designed and employed as very effective metal-based multiple drug resistance modulators.^{10–12} Moreover, Schiff base copper complexes can also bind DNA or cause DNA damage, promote intracellular reactive oxygen species (ROS) levels, as well as the activation of the mitochondrial pathway and induce caspase-dependent apoptosis.^{13,14}

Quinoline and its derivatives, an important class of biologically active heterocyclic compounds, exhibit various pharmaceutical activities.^{15–18} In particular, quinoline scaffolds play a key role in the development of anticancer drugs because they exhibit excellent characteristics by different mechanisms of action;

^a State Key Laboratory Cultivation Base for Chemistry and Molecular Engineering of Medicinal Resources, School of Chemistry and Pharmacy, Guangxi Normal University, 15 Yucai Road, Guilin 541004, P. R. China.

E-mail: hukun216@163.com, fliangoffice@yahoo.com; Fax: +86 773 2120958

^b Guangxi Key Laboratory of Electrochemical and Magnetochemical Functional Materials, College of Chemistry and Bioengineering, Guilin University of Technology, Guilin 541004, China

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e.g., growth inhibitors by apoptosis induction, cell cycle arrest, and the disruption of cell migration and inhibition of angiogenesis.¹⁶ Copper complexes based on quinoline Schiff bases have been found to show significant perspectives for anticancer therapies. For example, several quinoline-2-carboxaldehyde Schiff base copper complexes have been reported, which show antiproliferative activity toward prostate cancer cells and cervical cancer cells.^{19–22} Following these reports, aminoquinoline Schiff base copper complexes were also synthesized and their anticancer activity evaluated.^{22,23} A series of 2-oxo-1,2-dihydroquinoline-3-carbaldehyde (2'-hydroxybenzoyl,²⁴ benzoyl,²⁵ 2'-methylbenzoyl,²⁶ semicarbazone²⁷) hydrazone copper complexes have been found to demonstrate low cytotoxic activity toward the normal mouse embryonic fibroblast cell line (NIH 3T3) ($IC_{50} > 200 \mu M$). However, they showed significantly less activity against HeLa cell lines than cisplatin. Moreover, transition metal complexes with quinoline Schiff base ligands have kindled the interest of many researchers in many studies, especially as anticancer chemotherapeutic agents.^{21,28} In previous studies, we found that the Cu(II) quinoline hydrazone Schiff base complex showed high anticancer activity against HeLa and other cancer cell lines.^{29,30} Although these quinoline Schiff base copper complexes exhibit good biological activities, their cytotoxicity to normal cell lines is still unsatisfactory, which may restrict their application. The design and synthesis of novel quinoline Schiff base copper complexes with high efficacy and less toxicity are therefore of great interest.

Herein, we report the design and synthesis of three copper(II) complexes $[Cu(L1)(NO_3)_2]$ (C1), $[Cu(L2)Cl_2]$ (C2) and $[Cu(L2)SO_4] \cdot 2H_2O$ (C3) with quinoline-derived Schiff base ligands (L1 = (*E*)-methyl 4-((quinolin-8-ylmethylene)amino)benzoate, L2 = (*E*)-ethyl 4-((quinolin-8-ylmethylene)amino)benzoate). The interactions of these complexes with HSA/DNA were evaluated through spectroscopy and molecular docking methods. The antiproliferative activities of ligands and C1–C3 in the cell lines were investigated, and the anticancer mechanisms were further surveyed.

2. Experimental section

Antibodies, CT-DNA, HSA, quinoline-8-carboxaldehyde, benzocaine and 4-aminobenzoic acid methyl ester were purchased from Sigma Chemical Co. (USA). Hep-G2, NCI-H460, MGC80-3, HeLa cell lines and normal liver cells (HL-7702) were obtained from ATCC.

Flow cytometric analysis was carried out using a FACS Aria II flow cytometer. Fluorescence spectra were obtained on PE LS55 spectrometer. UV-vis spectra were detected using an Agilent Carry 100. ¹H NMR spectra were obtained on a Bruker Avance 500 spectrometer. High-resolution mass spectra were recorded on a Thermofisher Scientific Exactive LC-MS mass spectrometer.

2.1. Synthesis and structures of Cu(II) complexes

2.1.1 Synthesis

Synthesis of L1 and L2. L1 and L2 ligands were synthesized according to the reported methods.^{31,32} In brief, a mixture of

quinoline-8-carbaldehyde (1.57 g, 10 mmol) and 4-aminobenzoic acid methyl ester (1.51 g, 10 mmol) or benzocaine (1.65 g, 10 mmol) in 50 mL methanol was stirred overnight (60 °C). The resulting yellow solid products suitable for structural analysis were obtained.

L1. Yield: 93%. Anal. calcd for $C_{18}H_{14}N_2O_2$: C, H and N of 74.47, 4.86 and 9.65, respectively. Found: C, H and N of 74.35, 4.90 and 9.51, respectively. FT-IR (KBr, cm^{-1}): 1704, 1599, 1578, 1274, 1105, 779, 706 and 652. HRMS (ESI): $m/z = 291.1127 [M + H]^+$, and calcd for: 291.1134 $[M + H]^+$. ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.84 (s, 1H, $-CH=N$), 9.05 (d, $J = 3.8$ Hz, 1H, ArH), 8.55 (d, $J = 7.2$ Hz, 1H, ArH), 8.51 (d, $J = 8.2$ Hz, 1H, ArH), 8.24 (d, $J = 8.0$ Hz, 1H, ArH), 8.04 (d, $J = 8.2$ Hz, 2H, ArH), 7.79 (t, $J = 7.7$ Hz, 1H, ArH), 7.67 (dd, $J = 8.3, 4.1$ Hz, 1H, ArH), 7.41 (d, $J = 8.3$ Hz, 2H, ArH), 3.87 (s, 3H, CH_3O).

L2. Yield: 94%. Anal. calcd for $C_{19}H_{16}N_2O_2$: C, H and N of 74.73, 5.61 and 9.17, respectively. Found: C, H and N of 74.80, 5.72 and 9.01, respectively. FT-IR (KBr, cm^{-1}): 1704, 1599, 1578, 1274, 1105, 779, 706 and 652. HRMS (ESI): $m/z = 305.1280 [M + H]^+$, and calcd for: 305.1290 $[M + H]^+$. ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.85 (s, 1H, $-CH=N-$), 9.05 (dd, $J = 4.1, 1.8$ Hz, 1H, ArH), 8.57 (dd, $J = 7.3, 1.2$ Hz, 1H, ArH), 8.52 (dd, $J = 8.4, 1.7$ Hz, 1H, ArH), 8.26 (dd, $J = 8.1, 1.2$ Hz, 1H, ArH), 8.07–8.03 (m, 2H, ArH), 7.81 (t, $J = 7.7$ Hz, 1H, ArH), 7.68 (dd, $J = 8.3, 4.2$ Hz, 1H, ArH), 7.44–7.39 (m, 2H, ArH), 4.34 (q, $J = 7.1$ Hz, 2H, $-CH_2CH_3$), 1.35 (t, $J = 7.1$ Hz, 3H, $-CH_2CH_3$).

Synthesis of C1, C2 and C3. Copper salt (0.15 mmol, $Cu(NO_3)_2 \cdot 3H_2O$ (36.3 mg) for C1, $CuCl_2 \cdot 2H_2O$ (25.5 mg) for C2 and $CuSO_4 \cdot 5H_2O$ (37.5 mg) for C3) and ligand (0.15 mmol, L1 for C1, L2 for C2 and C3) were put in a thick Pyrex tube, which was quenched in liquid N_2 and sealed, then heated for four days at 80 °C, and cooled to room temperature (programmed at 5 °C h^{-1}). Crystals suitable for X-ray structure analysis were obtained.

C1. Green block crystals. Yield: 82%. Anal. calcd for $C_{18}H_{14}CuN_4O_8$ (477.87): C, H and N of 45.24, 2.95 and 11.72, respectively. Found: C, H and N of 45.05, 3.11 and 11.42, respectively. FT-IR (KBr, cm^{-1}): 3440, 1704, 1637, 1589, 1409, 1378, 1285, 1184, 1116, 1015, 775, 705, 610, 521, 479 and 430. HRMS (ESI): $C_{18}H_{14}CuN_3O_5 [M - NO_3]^+$, calcd for: 415.0230, found 415.0227.

C2. Green block crystals. Yield: 80%. Anal. calcd for $C_{19}H_{16}N_2O_2CuCl_2$ (403.34): C, H and N of 52.01, 3.68 and 6.38, respectively. Found: C, H and N of 51.87, 3.55 and 6.59, respectively. FT-IR (KBr, cm^{-1}): 3444, 1715, 1595, 1511, 1279, 1184, 1100, 1022, 843, 775, 695, 606, 521, 479 and 433. HRMS (ESI): $C_{19}H_{16}N_2O_2CuCl [M - Cl]^+$, calcd for: 402.0196, found 402.0183.

C3. Bright green block crystals. Yield: 78%. Anal. calcd for $C_{38}H_{32}N_4O_{14}Cu_2S_2$ (959.88): C, H and N of 47.55, 3.36 and 5.84, respectively. Found: C, H and N of 47.15, 3.81 and N 5.65, respectively. FT-IR (KBr, cm^{-1}): 3440, 1715, 1637, 1384, 1274, 1142, 838, 780, 659, 574, 490 and 427. HRMS (ESI): $C_{19}H_{17}N_2O_6CuS [M - H_2O + H]^+$, calcd for: 464.0103, found 464.0048.

2.1.2 X-Ray crystallography. The crystal structure data of complexes C1–C3 were collected using a SuperNova CCD area detector (Mo-K α , λ = 0.71073 Å). Data collection and refinement were made using the Apex2 software.³³ The SADABS program was used for absorption corrections.³⁴ The structures of complexes C1–C3 were solved through SHELX-97 programs. The complexes C1–C3 crystallographic information is given in Tables S1 and S2.†

2.2. HSA binding studies

Fluorescence method. A 2.0 mL HSA aqueous solution (1 μ M) was titrated by the C1–C3 and L1, L2. The changes in fluorescence of HSA were recorded at the excitation wavelength of 275 nm. By using the Scatchard equation (eqn (1)),³⁵ $\log[(F_0 - F)/F] = \log K_{\text{bin}} + n \times \log(Q)$, the binding constant (K_{bin}) of the test compounds to HSA were analyzed. Furthermore, the quenching rate constant (K_q) and quenching constant (K_{sv}) were calculated (eqn (2)): $F_0/F = 1 + K_q\tau_0 [Q] = 1 + K_{\text{sv}} [Q]$, where F_0 is the fluorescence of HSA; F is the fluorescence of HSA after adding the test compound; $[Q]$ is the test compound concentration; τ_0 = 4.43 ns.³⁶ The synchronous fluorescence spectra of HSA were also scanned when $\Delta\lambda$ = 60, 15 nm, respectively.

UV-visible absorption method. The concentrations of the compound and HSA were kept at 2.5 μ M. The absorption spectra were scanned from 250 to 350 nm at room temperature.

2.3. DNA binding studies

The CT-DNA/EB system (10 μ M) was titrated by the test compounds ranging from 0 to 30 μ M and their fluorescence spectra were measured. The quenching constant (K_q) and the apparent DNA binding constant (K_{app}) were determined using eqn (3), $I_0/I = 1 + K_q [Q]$, and eqn (4), $K_{\text{EB}} [\text{EB}] = K_{\text{app}} [\text{complex}]$,^{37,38} respectively, where I_0 is the fluorescence of the CT-DNA/EB system, and I is the fluorescence of CT-DNA/EB after adding test compounds. $[Q]$ is the compound concentration; $K_{\text{EB}} = 1.0 \times 10^7 \text{ M}^{-1}$; $[\text{complex}]$ is the compound concentration at 50% fluorescence of the CT-DNA/EB system.

2.4. Molecular docking analysis

Docking studies were performed in order to analyze the interactions of the test complex with HSA/DNA. The AutoDock Tools with the 1.5.6 docking programme and Autodock Vina were employed to analyze the molecular docking.^{39,40} The HSA and B-DNA crystal structures were obtained from the Protein Data Bank (HSA: PDB ID: 1E7H; DNA: PDB ID: 1BNA; (CGCGAATTC GCG)₂). The complex structure with HSA/DNA was prepared according to the reference method.^{41,42} Briefly, the Cu(II) complexes structures were converted into PDB format from CIF format using Mercury software. The structures of DNA and protein were modified to include polar hydrogen atoms and water molecules and the ligands were removed, and Gasteiger charges were added to the complex by Autodock Tools (ADT) before performing docking calculations.

2.5. MTT assays

The cytotoxicity of all the compounds against cell lines was screened through the MTT standard method. In brief, in a 96-well plate, the cells were seeded (5×10^3 cells per well) and cultured for 24 h in 5% CO₂. The test compounds (0–100 μ M) were added and further incubated for 48 h. MTT solution (10 μ L, 5 mg mL⁻¹) was put into each well and cultured for 5 h, and then DMSO (100 μ L) was injected after removal of the supernatant. Finally, we used the ELISA microplate reader to measure the absorbance of each well and calculate the IC₅₀ values.

2.6. Cell cycle distribution analysis and cellular uptake

After HeLa cells were cultured, the C3 was added at the indicated concentration. The mixture was kept in 5% CO₂ at 37 °C for 24 h and then the cells were collected and fixed with ice-cold ethanol (70%, -20 °C) overnight. The 100 μ g mL⁻¹ RNase A was put into the cells and further cultured (30 min, 37 °C). In the dark, the cells were stained using PI for 15 min. Finally, the above solution was analyzed immediately.

HeLa cells were treated with C3 (10 μ M) and cultured for 24 h. The cells were washed five times with ice-cold buffer and treated with concentrated HNO₃. The amount of Cu(II) compound in cells was monitored by ICP-MS.

2.7. Cell apoptosis

HeLa cells were cultured with C3 at different concentrations for 48 h and then harvested and suspended in annexin-binding buffer (200 μ L 1 $\times$). We used the annexin V (5 μ L) and PI (5 μ L) to stain the cell suspension in the dark (15 min), then a binding buffer (300 μ L 1 $\times$) was added while gently mixing. Finally, the mixtures were analyzed immediately using a FACS Calibur flow cytometer (BD).

2.8. ROS level

The HeLa cells were treated with C3 (0–20 μ M) and incubated in a CO₂ incubator (24 h, 37 °C) after attachment. HeLa cells were then collected and incubated for 30 min at 37 °C with H₂DCFDA (10 μ M) in serum-free medium. Afterward, the HeLa cells were washed and the fluorescence intensity was recorded.

2.9. Mitochondrial membrane potential

After the HeLa cells were seeded and incubated with complete DMEM for 24 h, C3 was added and further cultured (24 h). The cells were collected and re-suspended in PBS buffer containing pre-warmed JC-1 (5 μ g mL⁻¹) and incubated at 37 °C (30 min). Subsequently, we used PBS buffer to wash the cells three times. Finally, the mixture was analyzed immediately by FACS Calibur flow cytometer (BD).

2.10. Western blot assay

After the HeLa cells were cultured overnight, C3 was added and further incubated (24 h). The total proteins were

extracted after cell-lysis and the extract was loaded onto SDS polyacrylamide gel then transferred to PVDF membranes. BSA (5%) in TBST buffer was used to block the membrane. The primary antibodies were added and incubated overnight (4 °C). After washing, the secondary antibody was also added and cultured (120 min). Finally, the immunoreactive signal was detected.

3. Results and discussion

3.1. Characterization of L1, L2, C1–C3

L1, L2, C1–C3 were synthesized and characterized (Fig. S1–S12†). The results indicated that the structures of these compounds are consistent with their compositions.

The crystal structures of C1 and C2 are shown in Fig. 1. C1 and C2 belong to the triclinic ($P\bar{1}$) and monoclinic ($P2_1/n$) crystal systems, respectively. In complex C1, the copper atom is four-coordinate, bonded to two N (N1 and N2) donors from the L1 ligand and two terminal NO_3^- ions. All Cu–X bond lengths are consistent with those of Cu(II) complexes in literature.⁴³ In complex C2, copper atoms are four coordinate, with two nitrogen atoms (N1 and N2) of the L2 ligand and two terminal chlorines (Cl1 and Cl2).

Compared to C1 and C2, C3 is a neutral dinuclear complex, $[\text{Cu}(\text{L2})\text{SO}_4]_2$, with SO_4^{2-} anion bridges (Fig. 1). Every central copper(II) in the dimer is five-coordinate in the form of a slightly distorted square-pyramidal geometry with $\tau = 0.0141$. Interestingly, the Cu–N (quinoline) lengths (Cu1–N1 = 1.985 (4) Å in C1, Cu1–N1 = 1.987 (2) Å in C2 and Cu1–N2 = 1.979 (5) Å in C3) of complexes C1–C3 are longer than the Cu–N (imine) lengths (Cu1–N2 = 1.972 (4) Å in C1, Cu1–N2 = 1.9855 (19) in C2 and Cu1–N1 = 1.958 (5) Å in C3) in these complexes, which is possibly due to weaker π -back-bonding in the Cu–N (quinoline) bond than in the Cu–N (imine) bond.

3.2. Stability of C1–C3

The stability of the metal complex has an effect on the anti-tumor activity; therefore, the hydrolytic stabilities of the

Cu(II) complexes were investigated. The UV-vis absorption spectra of C1–C3 in aqueous solution (TBS, pH 7.4, 1% DMSO) at different times (0, 24 and 48 h) are shown in Fig. S13†. The UV-vis spectra do not differ significantly from those observed even after 48 h at laboratory temperature, which indicated that the C1–C3 complexes were stable in TBS buffer within 48 h.

3.3. HSA binding ability

The binding ability of anticancer agents with HSA affects their distribution, uptake, transfer, metabolism and mechanism of action toward cancer cells.^{44–46} First, we studied the nature of the C1–C3 binding with HSA using fluorescence and the UV-vis absorption method.

The fluorescence spectral changes were investigated before and after titration using the Cu(II) complexes (Fig. 2A–C) and ligands (Fig. S14†). The addition of these compounds to HSA resulted in the essential fluorescence quenching of HSA with a small blue shift (59%, 1 nm, C1; 60%, 3 nm, C2; 62%, 7 nm, C3; 43%, 1 nm, L1; 47%, 2 nm, L2). The quenching efficiency of these complexes for HSA was greater than with the free ligands. These facts indicate that the binding of the test compounds to HSA did occur and the binding sites of HSA are buried in a hydrophobic environment.

To further study the quenching process, K_q , K_{sv} and K_{bin} were assessed using eqn (1) and (2) (Fig. S15†) (Table 1). As can be seen from Table 1, the higher K_{sv} and K_{bin} values of C3 compared to C1 and C2 may be due to its higher solubility in aqueous medium. The K_q values of these complexes are 1000-fold higher than the maximum collisional quenching constant ($2.0 \times 10^{10} \text{ mol L}^{-1} \text{ s}^{-1}$),³⁴ so the fluorescence of HSA is quenched by these Cu(II) complexes through static quenching processes. These larger values of K_{sv} and K_{bin} demonstrate the strong interaction of C1–C3 with HSA.

UV-vis absorption spectroscopy is a simple method to explore the type of quenching mechanism of HSA. We chose C3 as an example for the further study of the quenching mechanism. The absorption intensity of HSA at 280 nm was

Fig. 1 The ORTEP drawing of the C1–C3 complexes with the atom numbering scheme. Color scheme: Cu, aqua; O, red; N, blue; C, gray; Cl, green.

Fig. 2 The emission spectrum of HSA (1 μ M; λ_{ex} = 280 nm) in the absence and presence of C1 (A), C2 (B) and C3 (C) with increasing concentrations (0, 1.0, 2.0, 3.0, 4.0 and 5.0 μ M). Fluorescence quenching spectra of DNA-EB (10 μ M) in the presence of C1 (D), C2 (E) and C3 (F) (0, 5.0, 10, 15, 20, 25 and 30 μ M).

enhanced and a slight blue shift of about 2 nm was observed with the addition of C3 (Fig. S16[†]), which demonstrated that the quenching of the fluorescence of HSA by the test compounds is mainly *via* static processes.

Furthermore, synchronous fluorescence spectra were used for the analysis of changes in the microenvironment around the fluorophore functional groups. The information about the microenvironment for tryptophan and tyrosine was reflected when $\Delta\lambda$ = 60 nm and 15 nm, respectively.^{47,48} Synchronous fluorescence spectra of HSA were obtained with different concentrations of the C1–C3 complexes (Fig. S17[†]). The addition of test compounds gradually led to a hypochromic effect in HSA. Moreover, when $\Delta\lambda$ = 60 nm, the maximum emission wavelength of HSA not only gradually decreased but was also accompanied by a slight blue shift (1 nm for C1 and C2, 2 nm for C3). However, the maximum emission wavelength had no shift at $\Delta\lambda$ = 15 nm. The results demonstrated that the C1–C3 bound effectively with HSA, and thus quenched the fluorescence of the tyrosine and tryptophan residues of HSA. The fluorescence changes also indicated that the interactions may occur in domain II because the Tyr and Trp residues are located in domain II of HSA.^{42,49} These

observed changes in fluorescence are different from the results obtained for cisplatin, which induced the red-shifted emission wavelength and was bound to HSA mainly *via* covalent bonds.^{49–51} Therefore, the results suggest that the interaction of C1–C3 with HSA should be noncovalent and reversible.⁴⁹

High-performance liquid chromatography (HPLC)⁵² was often used to study the interaction of guest molecules with HSA. The chromatographic results for the mixture of C3 or L2 and HSA were similar to the pure HSA, indicating that the binding of C3 and L2 induces slight structural changes in the protein (Fig. S18[†]). These experimental results imply that the nature of the interaction of C1–C3 and their respective ligands with HSA is noncovalent.^{42,52}

The above results confirm that the C1–C3 complexes exhibit prominent HSA binding ability *via* noncovalent interactions and the binding ability of these complexes toward HSA was greater compared to the free ligands. The complexes simultaneously weakened the fluorescence of tyrosine and tryptophan, but their interaction affected the micro-region of tryptophan more than the tyrosine. The strong binding ability of the complexes to HSA suggested that the C1–C3 can easily be stored in protein, then carried to the objective area and released from the protein to the desired targets.²⁹

Table 1 Stern–Volmer quenching constants and binding parameters of the HSA–complex drug systems at 295 K

	K_q ($M^{-1} s^{-1}$)	K_{sv} (M^{-1})	K_{bin} (M^{-1})	n
C1	$6.36 \pm 0.31 \times 10^{13}$	$2.83 \pm 0.14 \times 10^5$	$0.94 \pm 0.09 \times 10^8$	1.37
C2	$6.49 \pm 0.25 \times 10^{13}$	$2.89 \pm 0.11 \times 10^5$	$1.15 \pm 0.04 \times 10^8$	1.49
C3	$7.42 \pm 0.27 \times 10^{13}$	$3.30 \pm 0.12 \times 10^5$	$1.60 \pm 0.07 \times 10^8$	1.51
L1	$3.12 \pm 0.18 \times 10^{13}$	$1.38 \pm 0.08 \times 10^5$	$0.47 \pm 0.05 \times 10^6$	1.29
L2	$4.02 \pm 0.20 \times 10^{13}$	$1.78 \pm 0.09 \times 10^5$	$1.20 \pm 0.09 \times 10^6$	1.38

3.4. DNA binding

DNA is one of the main molecular targets for cancer therapeutics;^{53,54} hence, the fluorescence method based on the EB competitive experiment was used to investigate the nature of the interactions of C1–C3 (Fig. 2–D–F) and ligands (Fig. S19[†]) with

CT-DNA. The emission band of CT-DNA/EB was gradually decreased when these compounds were titrated. The effect of the hypochromism of the complex on the CT-DNA/EB system was greater than that of the corresponding ligand. Furthermore, the K_q and K_{app} values were analyzed (Table 2) according to eqn (3) and (4) (Fig. S20†) for evaluating the binding ability of the complexes and free ligands. As can be seen from Table 2, the binding of C1–C3 with CT-DNA is stronger than that of the free ligand. The higher DNA binding affinity of C3 may be due to its solubility in aqueous medium.⁵⁵

The complexes have no UV-vis absorption (Fig. S13†) and fluorescence excitation spectra in the 550–700 nm range (Fig. S21†), which indicate that these compounds cannot quench the fluorescence of EB by using the inner effect or energy transfer mechanism. It may be inferred that the EB is being efficiently replaced by these test compounds from the binding sites of CT-DNA. The above results demonstrate that the C1–C3 can interact with CT-DNA *via* an intercalative mode and their binding ability is greater compared to the free ligands.⁵⁶

3.5. Molecular docking studies

Docking studies were conducted in order to complement the spectroscopy results and provide more insight into the complex–HSA interactions. The resulting docking patterns of C3 with the lowest energy and the entire protein structure are shown in Fig. 3 and S22,† respectively. The results reveal that complex C3 was bound at the hydrophobic cavities of HSA subdomains IIIA and IIA, but mainly in IIA, and it was surrounded by Lys199, Ser202, Phe211, Ala210, Trp214, Val344, Leu453, Leu457, Leu481, Arg485, Ser454, Glu450 and Ser342. Molecular models showed that complexes C2 (Fig. S23-B†) and C3 were almost in the same position. The docking results showed potential for Trp214. C1 (Fig. S23-A†) was located in the hydrophobic cavity of subdomain IIA, and it was surrounded by Leu260, Leu219, Leu238, Lys199, Arg218, Arg257, His242, Val241; there were π – π stacking interactions of C2 and C3 with the residues Ala291, Glu153, Gln196, Ser192, His288 and Ser287. The methyl 4-aminobenzoate fragment of the C1 interacts with Gln196 and Glu153 amino acid residues through hydrogen bonds. The lipophilic quinoline fragment of the C1 is coiled into a hydrophobic channel lined by Leu260, Leu219, Leu238, Ala291 and Val241. We found that their binding sites with HSA are different from cisplatin.⁵⁷ The lowest binding energies of C1, C2, and C3 respectively are –9.3, –10.8, and –11.8

Fig. 3 Molecular docked structure of the C3 interacting with HSA (PDB ID: 1E7H).

kcal mol^{–1}, which suggest that their binding affinities for HSA are greater compared to the other copper(II) complexes.⁵⁸

The interaction of C1–C3 with B-DNA (PDB ID: 1BNA) was also investigated by docking studies. Energetically favourable docked poses obtained from the molecular docking of the C3 to the B-DNA are shown in Fig. 4, and C1 and C2 are demonstrated in Fig. S24.† Their lowest binding energies are –8.1 (C1), –8.0 (C2), and –8.4 (C3) kcal mol^{–1}, respectively, and these are lower compared to the other copper(II) Schiff base complexes.⁵⁹ The C1–C3 fitted well into the curved contour of the B-DNA target in the minor groove region, which brings about hydrogen bonds, hydrophobic interactions and van der Waals forces with DNA functional groups that define the groove. Their region of interaction with DNA is different from that of cisplatin, which binds to the DNA major groove facing inward.⁶⁰ Furthermore, the energy minimized structures suggest that the C1–C3 is stabilized by π – π stacking (edge to face interactions). Therefore, the data suggest that the docking studies results corroborate the spectroscopic results.

Fig. 4 Molecular docked model of complex C3 with DNA (PDB ID: 1BNA).

Table 2 Stern–Volmer constant (K_q) and the apparent binding constant (K_{app}) for the Cu(II) complexes

	K_q (M ^{–1} s ^{–1})	K_{app} (M ^{–1})
C1	$0.93 \pm 0.04 \times 10^4$	$0.95 \pm 0.05 \times 10^4$
C2	$0.92 \pm 0.05 \times 10^4$	$0.94 \pm 0.06 \times 10^4$
C3	$1.10 \pm 0.06 \times 10^4$	$1.11 \pm 0.06 \times 10^4$
L1	$0.22 \pm 0.02 \times 10^4$	$0.23 \pm 0.02 \times 10^4$
L2	$0.44 \pm 0.03 \times 10^4$	$0.45 \pm 0.02 \times 10^4$

3.6. IC₅₀ level

The ligand, metal salt, C1–C3 and cisplatin cytotoxicities of toward the tested cells were examined *in vitro* via the MTT method; the IC₅₀ values obtained are summarized in Table 3.

The C1–C3 showed potent inhibition activity toward HeLa, with IC₅₀ in the micromolar range and obviously lower than cisplatin. In contrast, the free ligand, and metal salt displayed relatively low activity toward HeLa cells. The enhanced antiproliferative activity of C1–C3 may result from the synergistic effect of Cu(II) ions and ligands. Despite the potency, the Cu(II) complexes were much less toxic toward human normal cells (HL-7702), with IC₅₀ values ranging from 60.39 to 92.81 μ M, which are significantly higher than that of cisplatin (23.02 μ M). The cytotoxicities of C1–C3 toward the normal HL-7702 cells were, respectively, 4.7, 5.9 and 6.1 times lower than the activity on HeLa cells, which suggest that the C1–C3 complexes might demonstrate selective inhibition activity against HeLa cells.

In comparison, the cytotoxic activity toward HeLa cells follows the order C3 > C2 > C1, which is consistent with the DNA/HSA binding results. These results imply that the introduction of the drug molecule of benzocaine was more effective for enhancing the anticancer activity compared to the 4-aminobenzoic acid methyl ester.

Moreover, the IC₅₀ values of these complexes, especially C3, to HeLa cell are comparable or lower than most of the previously reported quinoline-derived Schiff base complexes.^{20,21,23–30} The cytotoxic activity toward HL-7702 was clearly decreased compared with our previously reported quinoline-8-carboxaldehyde hydrazone copper complexes,^{29,30} which may be because the introduction of benzocaine into quinoline scaffolds induces a synergistic cytotoxicity. The results suggest that the Cu(II) complexes show good selectivity between normal and cancer cells, and thus have the potential for application in human cervical cancer chemotherapy.

Based on the above results, it can be inferred that C3 possesses higher cytotoxicity toward HeLa cell lines compared to the free ligand as well as C1 and C2. Thus, C3 was selected to investigate the anticancer mechanism.

3.7. Possible anticancer mechanism of C3

Because complex C3 exhibits the highest cytotoxicity to HeLa, it was used for further cellular studies to understand the

mechanism of action. Most of the previous reports on cell apoptosis induction and cell cycle arrest were closely correlated with cancer cell death,^{61,62} so we monitored features related to these pathways.

The cellular uptake properties affect the metal-based anticancer drug antiproliferative performance, so we first assessed the uptake of C3 in HeLa cells using ICP-MS assay. The accumulated amount of C3 was 9.6 ± 2.5 nM/10⁶ cells in the HeLa cells after 24 h of treatment. Subsequently, the cell cycle in HeLa cells induced by C3 (5, 10 and 20 μ M) was examined by flow cytometry (Fig. 5). With the increase in complex C3, the S-phase and G2/M-phase cell population was gradually reduced. However, a dramatic increase in G0/G1-phase was obtained from 53.79% to 83.66%. The results indicate that C3 stalled the cell cycle at the G0/G1-phase in a concentration-dependent manner. It is known that cyclin-dependent kinases (CDKs) and cyclin complexes affect the cell cycle progression at each phase.⁶³ Therefore, the expression of cell cycle regulatory proteins of G0/G1-phase was determined using the western blotting assay to study the molecular mechanisms of the C3 complex-induced G0/G1-phase block (Fig. 6). As can be seen from Fig. 6, the progression of CDK2, cyclin D1 and E was reduced in a dose-dependent manner, and the expression of CDK4 had almost no changes. The observed phenomenon revealed that the inhibition of the activity of the G0/G1-phase-promoting cyclin-CDK complexes by limiting the supply of CDK2, cyclins D1 and E1 may be a mechanism by which the C3 mediated growth G0/G1-phase arrest.

It has been reported that most metal anticancer agents generally kill tumor cells through the induction of apoptosis;^{64,65} we, therefore, monitored the ability of complex C3 to promote apoptosis in HeLa cells (Fig. 7). It was found that the populations of the apoptotic HeLa cells (both at the early stage and the late stage of apoptosis, Q2 + Q3) distinctly increased in a dose-dependent manner when incubated with the C3. The apoptosis populations of HeLa cells in early-and late-stages were 16.7% and 9.28%, respectively, which suggested that cell apoptosis in HeLa cells can be effectively induced by the complexes.

Mitochondrial dysfunction plays an important role in triggering various apoptotic pathways.⁶⁶ To confirm whether mitochondrial dysfunction induces cell apoptosis, we used flow

Table 3 IC₅₀^a (μ M) values of L1, L2 and C1–C3 on the selected cells for 48 h

Compound	Hep-G2	NCI-H460	MGC80-3	HeLa	HL-7702
4-Amino-benzoic Acid methyl ester	>100	>100	>100	>100	>100
Benzocaine	>100	>100	>100	>100	>100
L1	>100	>100	>100	>100	>100
L2	>100	>100	>100	>100	>100
C1	>100	>100	>100	18.72 $\pm$ 1.03	87.70 $\pm$ 4.01
C2	>100	35.45 $\pm$ 2.35	16.87 $\pm$ 2.01	15.76 $\pm$ 1.19	92.81 $\pm$ 6.03
C3	>100	>100	17.52 $\pm$ 1.81	9.98 $\pm$ 0.87	60.39 $\pm$ 5.86
Cisplatin	27.31 $\pm$ 2.01	47.68 $\pm$ 1.98	21.28 $\pm$ 2.81	35.25 $\pm$ 2.80	23.02 $\pm$ 5.68
Copper(II) salt	>100	>100	>100	>100	>100

^a IC₅₀ values are presented as the mean. SD (standard error of the mean) from five independent experiments.

Fig. 5 Cell cycle analysis by flow cytometry for HeLa cells treated with C3 (5, 10 and 20 μ M).

Fig. 8 Effects of C3 (5, 10 and 20 μ M) on MMP as analyzed by JC-1 staining and flow cytometry.

Fig. 6 Western blot was used to determine the expression of CDK2, CDK4, cyclin E, cyclin D1 in HeLa cells treated with C3 (5 and 10 μ M).

cytometry to measure the mitochondrial membrane potential (MMP) (Fig. 8). C3 led to a reduction in MMP compared with the control cells and exhibited a dose-dependence, which implies that the mitochondrially-mediated apoptosis was activated.

Fig. 7 Annexin-V/propidium iodide assay of HeLa cells treated by C3 (5, 10 and 20 μ M) measured by flow cytometry.

In the apoptosis pathway, the elevation of reactive oxygen species (ROS) is closely associated with mitochondrial dysfunction.⁶⁷ Thus, the C3 complex catalytic ability of the intracellular ROS elevation in HeLa cells was monitored by flow cytometry and the fluorescent 2',7'-dichlorofluorescein (DCF) probe. As shown in Fig. 9A, compared with the control groups, the incubation of HeLa cells with the C3 complex can increase the levels of DCF fluorescence and lead to the red shift in the fluorescence peak, which implies that C3 increased the oxidative damage to the HeLa cells. The ROS levels show dose-dependent behaviour (Fig. 9B).

The Bcl-2 family of proteins has been verified as key regulators of apoptosis.⁶⁸ To further study the mechanism of cell apoptosis, we tested the Bcl-2 family of proteins expression through C3 treated cells (Fig. 10).

The western blotting data revealed that the complex induced significant down-regulation of Bcl-2 proteins and slight up-regulation of Bax proteins in a concentration-dependent manner. These results demonstrate that C3 could activate the Bcl-2 and Bax proteins and decrease the ratio of

Fig. 9 A) Analysis of the ROS levels by flow cytometry after the HeLa cells were treated with C3 at indicated concentrations for 12 h and stained with H2DCFDA. B) Quantification of the flow cytometric results in (A) showing the percentage of cells with increased intracellular DCF oxidation compared to control cells. Results are the mean $\pm$ SD ($n = 5$); (**) $p < 0.01$.

Fig. 10 Western blotting was used to determine the expression of Bcl-2 and Bax in HeLa cells treated with C3 (5 and 10 μ M).

Bcl-2 protein to Bax protein, which leads to the mitochondrial release of apoptogenic factors in HeLa cells and ultimately induces cell apoptosis.

4. Conclusions

In conclusion, three Cu(II) complexes of quinoline-derived Schiff bases were designed, synthesized and characterized. The C1–C3 complexes display high quenching ability of HSA fluorescence and strong binding activity by non-covalent interactions, and hydrophobic interactions with HSA through sub-domain IIA and IIIA cavities. Moreover, the complexes effectively bind to DNA by an intercalative mode, and they fit well into the curved contour of the DNA target in the minor groove region. The cytotoxicity assay revealed that these complexes significantly enhanced the cytotoxic activity toward HeLa cell lines with their IC₅₀ values being markedly lower than those of cisplatin, the ligands and metal salt. The *in vitro* antiproliferative activities of C2 and C3 against the HeLa cells are greater than that of C1, suggesting that the introduction of the drug molecule of benzocaine was more effective for enhancing the anticancer activity than that of 4-aminobenzoic acid methyl ester. The complexes show low cytotoxicity against normal HL-7702 cell lines, which demonstrates that these complexes are more selective toward HeLa cells than normal HL-7702 cells. The mechanism studies indicate that C3 can regulate the expression of CDKs along with cyclins, and arrest the cell cycle at the G0/G1 phase. At the same time, C3 can effectively induce HeLa cell line apoptosis *via* the ROS-mediated mitochondrial pathways, and activate the Bcl-2 family of proteins. In general, the introduction of active medicament into ligands could improve the biological effects of the copper complexes with quinoline-derived Schiff bases with respect to DNA/HSA affinity and cell cytotoxicity. The present work implies that it may be helpful for the design and development of potential anticancer complexes to use modified clinical drugs as ligands.

Conflicts of interest

The authors declare no competing interests.

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