European Journal of Medicinal Chemistry 46 (2011) 160–167

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and crystal structure of two new dinuclear cobalt(II) complexes interaction with HeLa cells

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ARTICLE INFO

Article history:
Received 21 July 2010
Received in revised form
20 October 2010
Accepted 29 October 2010
Available online 3 November 2010

Keywords:
Co(II) coordinate complexes
Extracted DNA binding
Cleavage
Cytotoxicity in vitro
Apoptotic

ABSTRACT

Two novel dinuclear complexes $[Co_2(L)_2(phen)_2{\cdot}2H_2O]_n$ (**1**), $[Co_2(L)_2(bipy)_2]_n$ (**2**) (where phen = 1,10-phenanthroline, bipy = 2,2′-bipyridine, and L = biphenyl-2,4′-dicarboxylic acid) have been synthesized and characterized using IR, ^1H NMR, element analysis and single-crystal X-ray diffractometry. The binding of the complexes with HC-DNA (HeLa cells DNA, which was extracted by ourselves) was investigated by fluorescence spectrum. The experimental results show that the two complexes have the ability to bind with extracted HC-DNA. Gel electrophoresis assay demonstrates the ability of the complexes to cleave the extracted HC-DNA. The complexes exhibit a higher cytotoxicity against tumor cells as against normal cells in vitro. Further more, the apoptotic tests indicate that the complexes have an apoptotic effect on HeLa cells.

1. Introduction

During the past decades, tremendous interest has been aroused to explore the potential applications of metal complexes as possible DNA cleaving agents [1–8]. In these complexes, the metal or ligands may be varied in an easily controlled way to facilitate the individual application. There have been a number of reports highlighting the use of transition metal complexes as anticancer agents [9–12], which can interact non-covalently with DNA by intercalation, groove binding, or external electrostatic binding [6,13,14]. Among transition metal compounds, cisplatin is one of the most effective anticancer drugs in the treatment of various human tumor [15,16]. Nevertheless, cisplatin and other second-generation platinum drugs have such major drawbacks as severe tissue toxicity, and relative inactivity against gastrointestinal tumors [17]. Thus, the design and testing of new complexes is practically important. Cobalt, as transition metal and an essential trace element in the human body or in the living body, exists in the form of complexes. Cobalt complexes plays an important role in the research of pharmacology, coordination chemistry and biological inorganic chemistry [18], some of these complexes also exhibit interesting properties upon binding to DNA [19–21], among these cobalt complexes that have been studied, which containing 1,10-phenanthoroline/2,2′-bipyridine is of interest because of its biological activities and ability to bind to DNA [22,23], but that containing dicarboxylic acids ligands are seldom reported.

Many different bridging ligands such as pyridine (di)carboxylic acids and imidazole (di)carboxylic acids [24,25] have been successfully used to construct complexic coordination compounds structures. As a bridging ligand, biphenyl-2,4′-dicarboxylic acid with hydrophilic carbonyl oxygen have good coordination with the metal to form coordinate complexes.

In this paper, we choose to concentrate our work on the Co(II) complexes, based on the structural analogy between phen and bipy complexes. Herein, we describe the synthesis and characterization of two dinuclear complexes dibridged by L (L = biphenyl-2,4′-dicarboxylic acid) ligand (Scheme 1). In addition, the binding behaviors of the complexes with extracted HC-DNA were investigated by fluorescence spectroscopy. Their cleavage behavior toward extracted HC-DNA, cytotoxicity in vitro and apoptosis were also investigated.

2. Results and discussion

2.1. Synthesis of complexes *1* and *2*

2.1.1. Synthesis of the $C_{52}H_{32}N_4O_8Co_2{\cdot}2H_2O$ (*1*)

Complex **1** was prepared as follows: a mixture of Co(II) (0.029 g, 10 mmol), L (0.024 g, 10 mmol), phen(1,10-phenanthroline)

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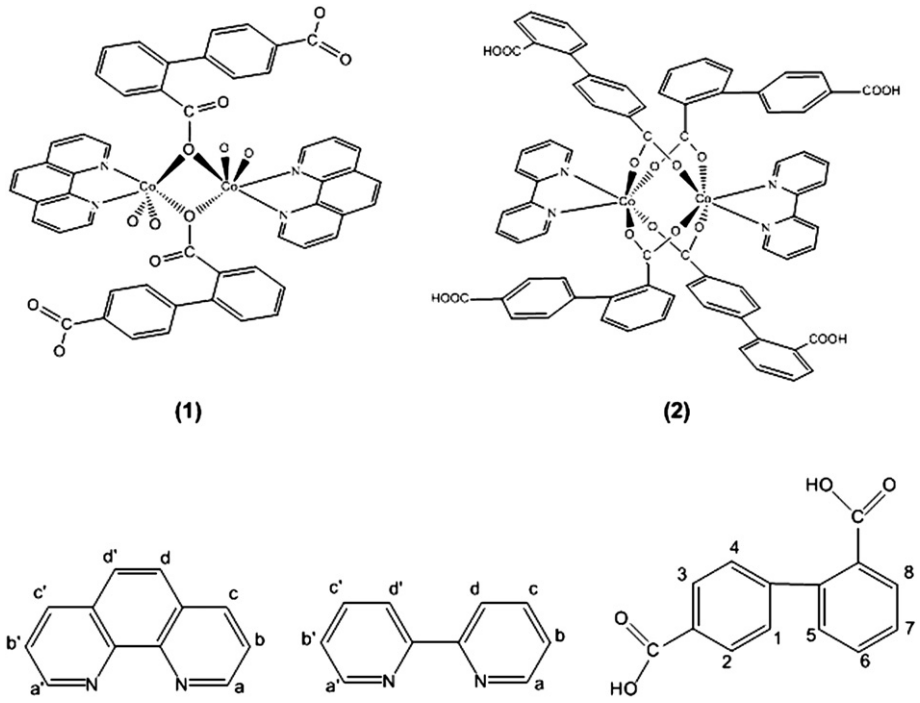
doi:10.1016/j.ejmech.2010.10.031



Scheme 1. Schematic structure of the two complexes and the numbering scheme for ^{1}H NMR spectroscopy.

(0.020 g, 10 mmol) and H_2O (10 mL) was heated at 160 °C for 3 days in a 25 mL Teflon-lined stainless steel vessel under autogenous pressure. After the reaction mixture was slowly cooled down to room temperature, pink crystals were produced in a yield of 60%. Anal. calcd. (%) for complex $C_{52}H_{32}N_4O_8Co_2{\cdot}2H_2O$(**1**): C, 62.79; H, 3.65; N, 5.63; Found: C, 62.87; H, 3.62; N, 5.60. IR (cm^{-1}, s, strong; m, medium; w, weak): γ(O—H) 3391(m); γ(COO^-) 1575(s), 1384(s); γ (C=C) 1637(s), 1429(m); γ(C—N) 1147(s); δ(C—H) 719(m). ^{1}H NMR (DMSO-d_6, 300 MHz); t t, two triplet; t, triplet; d, doublet: δ9.38 (d, J = 7.2 Hz, 2H, Ha, Ha′), 9.14 (d, J = 7.5 Hz, 2H, Hc, Hc′), 8.89 (t, J = 8.1 Hz, 2H, Hd, Hd′), 8.63 (d, J = 5.4 Hz, 2H, Hb, Hb′), 8.26 (d, J = 5.4 Hz, 1H, H5), 8.07 (t, J = 5.7 Hz, 2H, H2, H3), 7.83 (d, J = 7.2 Hz, 1H, H8), 7.54 (t, J = 7.8 Hz, 1H, H6), 7.49 (t, J = 5.7 Hz, 2H, H1, H4), 7.38 (t, J = 6.6 Hz, 1H, H7).

2.1.2. *Synthesis of the $C_{48}H_{32}N_4O_8Co_2$ (**2**)*

The complex was synthesized in an identical manner as that described for (**1**) with bipy (0.016 g, 10 mmol) in place of phen. Grey crystals were produced in a yield of 53%. Anal. calcd. (%) for $C_{48}H_{32}N_4O_8Co_2$(**2**): C, 63.31; H, 3.54; N, 6.15. Found (%): C, 63.39; H, 3.51; N, 6.12. IR (cm^{-1}, s, strong; m, medium; w, weak): γ(COO^-) 1575(s), 1387(s); γ(C=C) 1606(s), 1448(m); γ(C—N) 1154(s); δ(C—H) 714(m). ^{1}H NMR (DMSO-d_6, 300 MHz); t t, two triplet; t, triplet; d, doublet: δ8.56 (d, J = 4.5 Hz, 2H, Hd, Hd′), 8.48 (t, J = 7.5 Hz, 2H, Ha,

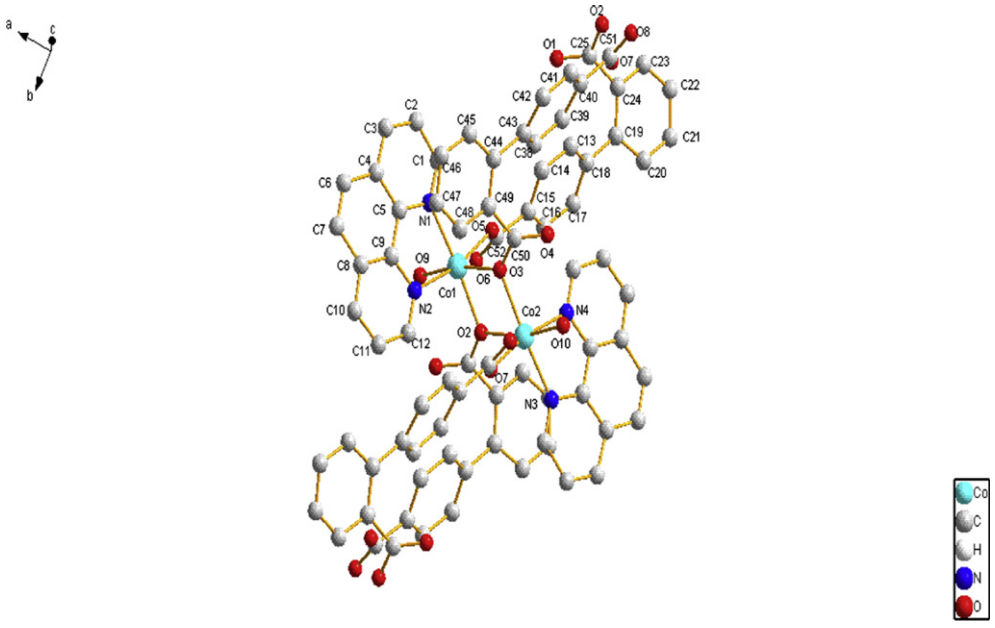


Fig. 1. Complex **1** with numbering of atoms (H atoms were omitted for clarity) at 30% probability thermal ellipsoids.

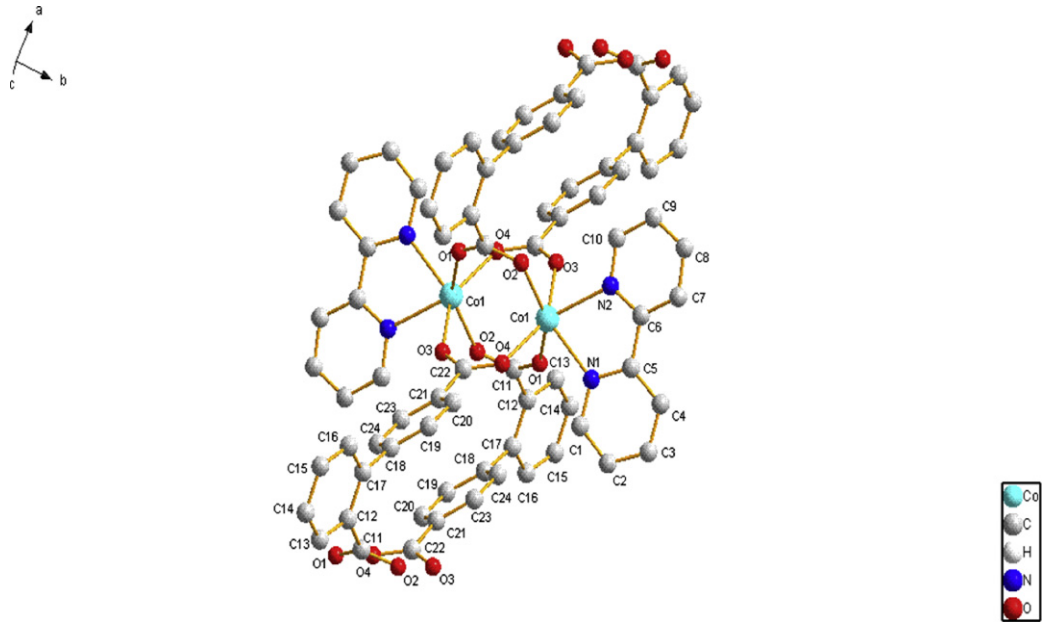


Fig. 2. Complex **2** with numbering of atoms (H atoms were omitted for clarity) at 30% probability thermal ellipsoids.

Ha′), 8.23 (t, $J = 7.2$ Hz, 2H, Hc, Hc′), 8.11 (d, $J = 5.1$ Hz, 1H, H5), 7.95 (t, $J = 5.4$ Hz, 2H, H2, H3), 7.83 (d, $J = 6.0$ Hz, 2H, Hb, Hb′), 7.70 (d, $J = 7.2$ Hz, 1H, H8), 7.61 (t, $J = 7.8$ Hz, 1H, H6), 7.52 (t, $J = 5.7$ Hz, 2H, H1, H4), 7.36 (t, $J = 6.6$ Hz, 1H, H7).

2.2. Crystal structure of **1** and **2**

The crystal structures of complex **1** and **2** were determined by X-ray crystallography, which respectively shown in Figs. 1 and 2, the structure of complex **1** belongs to the orthorhombic system with space group Pna2(1), and the structure of complex **2** belongs to the monoclinic system with space group P2(1)/c, a summary of data collection and refinement for the two compounds are presented in Table 1, selected bond distances and angles are given in Tables 2 and 3.

As can be seen from Fig. 1, both Co(II) atoms are six coordinated by two nitrogen atoms from the phen, three oxygen atoms from three different biphenyl-2,4′-dicarboxylic acid ligands and one oxygen atom from H_2O, exhibiting a irregular octahedral structure. Oxygen atoms from different biphenyl-2,4′-dicarboxylic acid ligands dibridge Co(1) and Co(2), and form an approximate parallelogram structure, the main bond lengths and bond angles are as follows: Co(1)–O(2) 2.108(4) Å, Co(1)–O(3) 2.142(5) Å, Co(2)–O(2) 2.145(4) Å, Co(2)–O(3) 2.141(5) Å, O(3)–Co(1)–O(2): 76.54(16)°, O(3)–Co(2)–O(2): 75.79(16)°, Co(1)–O(2)–Co(2): 104.35(3)°, Co(1)–O(3)–Co(2): 103.3(3)°.

As can be seen from Fig. 2, both Co(II) atoms adopt the same coordinate pattern, and each Co(II) atom is coordinated by two nitrogen atoms from the bipy, four oxygen atoms from four different biphenyl-2,4′-dicarboxylic acid ligands. Two Co(II) centers are connected by two identical biphenyl-2,4′-dicarboxylic acid ligands, resulting in a $[Co_2L_2]$ metallocycle which represents a small closed cyclic structure with a 1:1 metal-to-ligand ratio. The Co–O distances indicate that these bridges are close to symmetric, Co(1)–O(1) 2.1756(20) Å, Co(1)–O(4) 2.1787(2) Å, Co(1)–O(2) 2.1432(20) Å, Co(1)–O(3) 2.1522(20) Å.

Weak interactions such as hydrogen bonding [26,27] and π–π stacking [28], are the significant factors in supermolecular complex. In the two complexes, one-dimensional chain structures consist of the complexes through bridging ligands, and the three-dimensional structures are constructed by one-dimensional chain structures through π…π stacking, the connection of π…π stacking and hydrogen bonding may be propitious for intercalative into DNA [29].

2.3. Fluorescence spectroscopic studies

Fluorescence quenching measurements can be used to monitor metal binding [30]. EtBr is a conjugate planar molecule. Its

Table 1
Crystal data and refinement for **1** and **2**.

	1	**2**
Empirical formula	$C_{52}H_{36}N_4O_{10}Co_2$	$C_{48}H_{32}N_4O_8Co_2$
Formula weight	994.74	910.67
Temperature (K)	273	273
Wavelength (Å)	0.71073	0.71073
Crystal system	Orthorhombic	Monoclinic
Space group	P n a 2(1)	P 2(1)/c
a (Å)	20.0467(13)	9.5066(14)
b (Å)	10.4142(7)	10.5311(16)
c (Å)	20.7496(14)	21.035(3)
α (deg)	90	90
β (deg)	90	93.707(2)
γ (deg)	90	90
Volume (Å^3)	4331.9(5)	2101.5(6)
Z	55	4
D_{calc} (mg/m^3)	1.519	1.439
Absorption coefficient (mm^{-1})	0.835	0.850
F(000)	2695.0	932.0
Crystal size	0.25 × 0.15 × 0.12	0.10 × 0.08 × 0.06
θ Range for data collection (deg)	1.96–25.08	1.94–26.06
Index ranges	$-23 \le h \le 23$, $-12 \le k \le 11$, $-22 \le l \le 24$	$-11 \le h \le 11$, $-12 \le k \le 12$, $-25 \le l \le 25$
Reflections collected	22,751	12,600
Independent reflections (R_{int})	6603 ($R_{int} = 0.0570$)	4089 ($R_{int} = 0.0148$)
Data/restraints/parameters	6603/1/603	4089/0/280
S	1.053	1.097
Final R indices [$I > 2\sigma(I)$]	$R_1 = 0.0567$, $wR_2 = 0.1351$	$R_1 = 0.0440$, $wR_2 = 0.1390$
R indices (all data)	$R_1 = 0.0724$, $wR_2 = 0.1460$	$R_1 = 0.0468$, $wR_2 = 0.1423$
Largest diffraction peak and hole (Å e^3)	1.095 and −0.275	0.495 and −0.479

Table 2
Selected bond lengths (Å) and angels (deg) for **1**.

Co(1)–O(2)	2.108(4)	Co(2)–O(2)	2.145(4)
Co(1)–O(3)	2.142(5)	Co(2)–O(3)	2.141(5)
Co(1)–O(5)	2.055(5)	Co(2)–O(7)	2.063(5)
Co(1)–O(9)	2.162(5)	Co(2)–O(10)	2.183(5)
Co(1)–N(1)	2.158(6)	Co(2)–N(3)	2.119(5)
Co(1)–N(2)	2.142(6)	Co(2)–N(4)	2.124(6)
O(2)–Co(1)–O(3)	76.54(16)	O(3)–Co(2)–O(2)	75.79(16)
Co(1)–O(2)–Co(2)	104.35(3)	Co(1)–O(3)–Co(2)	103.3(3)
N(1)–Co(1)–O(9)	89.8(2)	N(3)–Co(2)–O(10)	88.15(2)

fluorescence intensity is very weak, but it is greatly increased when EtBr is specifically intercalated into the base pairs of double-stranded [31,32]. EtBr emits intense fluorescent light in the presence of extracted HC-DNA due to its strong intercalation between the adjacent DNA base pairs. It was previously reported that the enhanced fluorescence could be quenched by the addition of another molecule [33,34]. Competitive binding studies with EtBr were carried out to gain support for the mode of binding of the complexes with DNA. The study involves addition of the complexes to DNA pretreated with EtBr ([DNA]/[EtBr] = 2.5) and then measures the intensity of emission. The emission spectra of EtBr bound to extracted HC-DNA in the absence and presence of the two complexes are shown in Fig. 3. The addition of the complex to extracted HC-DNA pretreated with EtBr causes an appreciable reduction in fluorescence intensity, showing that the Co(II) complexes compete with EtBr to bind with extracted HC-DNA. According to the classical Stern–Volmer equation [34]: $I_0/I = 1 + K_{sq}r$, where I_0 and I represent the fluorescence intensities in the absence and presence of the complex, respectively, and r is the concentration ratio of the complex to DNA, K_{sq} is a linear Stern–Volmer quenching constant dependent on the ratio of the bound concentration of EtBr to the concentration of DNA. The K_{sq} value is obtained as the slope of I_0/I versus r linear plot. The fluorescence quenching curves of DNA-bound EtBr by the two complexes are given in Fig. 4. The quenching curves illustrate that the complexes bind to DNA in the order of K_{sq} **1** > K_{sq} **2**. The data also shows that the intercalary ability of the coordinated ligands varies as phen > bipy in the two complexes [19]. Obviously, the result follows the order of complex **1** > complex **2**. Thus, it can be confirmed that the reactions of the two intercalary complexes between the adjacent DNA base pairs have taken place [35].

2.4. Cleavage of extracted HC-DNA

The cleavage reaction on extracted HC-DNA can be monitored by agarose gel electrophoresis. The efficiency of cleavage of these molecules was probed using agarose gel electrophoresis [36,37]. When circular plasmid DNA is subject to electrophoresis, relatively fast migration will be observed for the intact supercoil form (Form I). If scission occurs on one strand (nicking), the supercoil will relax to generate a slower-moving open circular form (Form II). If both strands are cleaved, a linear form (Form III) that migrates between Form I and Form II will be generated [38]. Complexes **1**

Table 3
Selected bond lengths (Å) and angels (deg) for **2**.

Co(1)–O(1)	2.1756(20)	Co(1)–Co(1)	3.4614(6)
Co(1)–O(2)	2.1432(20)	Co(1)–N(1)	2.310(2)
Co(1)–O(3)	2.1512(20)	Co(1)–N(2)	2.319(2)
Co(1)–O(4)	2.1787(2)	O(1)–Co(1)–O(3)	81.85(9)
N(1)–Co(1)–O(4)	79.80(8)	N(2)–Co(1)–O(2)	82.49(8)
O(1)–Co(1)–O(4)	91.12(8)	O(2)–Co(1)–O(3)	85.82(9)

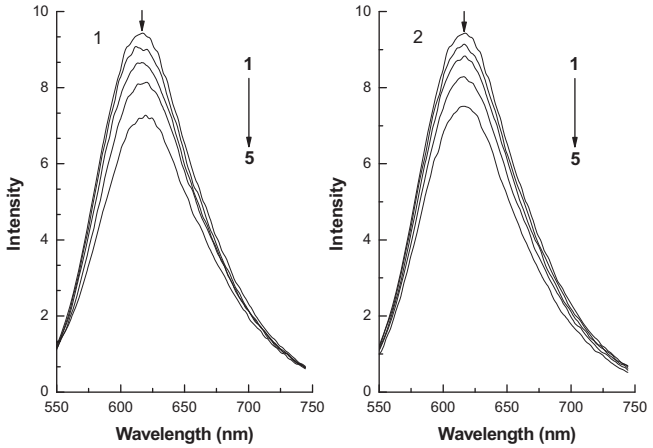


Fig. 3. Fluorescence spectra of the binding of EtBr to extracted HC-DNA in the absence (line 1) and presence (line 2–5) of increasing amounts of the complexes λ_{ex} = 526 nm, [EtBr] = 1 μM, [DNA] = 2.5 μM, [complex (**1**,**2**)] (line 2–5): 2.5, 5, 7.5, 10, 12.5 (μM).

and **2** were found to promote the cleavage of extracted HC-DNA from supercoiled Form I to the nicked Form II (Fig. 5). As shown in Fig. 5, with the decreasing of the complex concentration, the intensity of the circular supercoiled DNA (Form I) band diminished gradually, while that of nicked (Form II) band apparently increased [complex **1** (lanes 1–3), complex **2** (lanes 4–6)]. The gel electrophoretic separations showing the cleavage of extracted HC-DNA induced by complex at different concentrations, and the complex can induce the obvious cleavage of the extracted HC-DNA at the concentration of 13.2 μM. Each lanes have obviously tow the marks, compare lanes 1–6 with lane 0, we can see the two complexes have biological activity that cuts the extracted HC-DNA into series size basic group to the fragment.

2.5. Cytotoxicity in vitro study

The human tumor cell lines HeLa used in vitro cytotoxicity tests for two complexes was evaluated. The IC_{50} values are listed in Table 4. The effect on cell growth after a treatment period of 48 h and treatment with 3 μM concentration were depicted in Fig. 6.

By studying two compounds here, complex **1** demonstrates more active than complex **2**. A viability rate by day 3 to less than 50% of the control values was observed for the complexes. On the

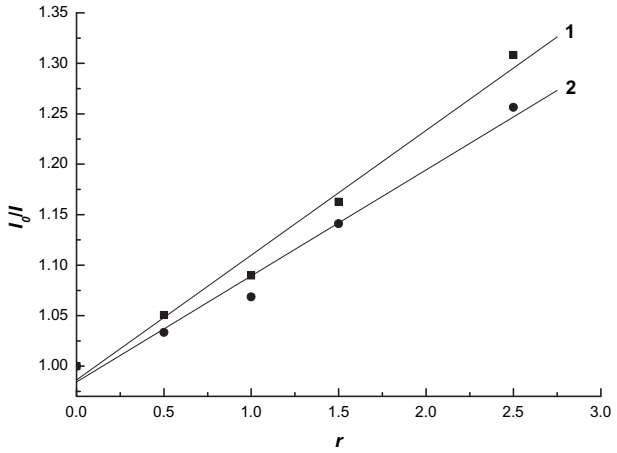


Fig. 4. Stern–Volmer quenching plots of the complex **1** and **2** with the value of slope 0.1235 (complex **1**), 0.1050 (complex **2**).

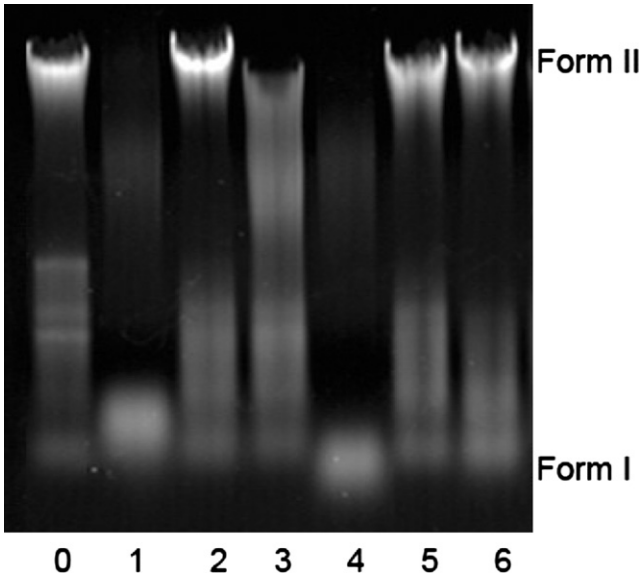


Fig. 5. Photoactivated cleavage of extracted HC-DNA (0.2 μg/μL) in the absence (lane 0) and presence (lane 1–6) in the different concentrations of the complex: (1) 13.2; (2) 6.6; (3) 3.3; (4) 13.2; (5) 6.6; (6) 3.3 μM (lane 1–3: complex **1**, lane 4–6: complex **2**).

whole, the two Co(II) complexes are effective in restraining the growth of HeLa and show a similar activity to cisplatin against the human tumor cell line. The results coincide with IC_{50} values reveals.

2.6. Apoptotic study

The most common and well defined form of programmed cell death is apoptosis, which is a physiological cell suicide program that is essential for the maintenance of tissue homeostasis in multicellular organisms. In contrast to the self-contained nature of apoptotic cell death, necrosis is a messy, unregulated process of traumatic cell destruction, which is followed by the release of intracellular components [39]. Under the light microscope, the morphological changes of the HeLa cells have been observed. In Figs. 7a and b, the normal cells exhibited dense state and intercellular tight junction, single cell is polygonal in shape or irregular cell morphology and cytoplasm is a clear appearance. After addition of the complexes, the cells appeared small size and became round in morphology comparing with normal cell and the state of being separated between them. As shown in Fig. 7, the cells stained by hematoxylin–eosin were observed and the morphology of the cell nucleus have changed obviously. In Fig. 7a and b, the nuclei of the normal cells were intact and had no pyknosis, which differed from the apoptotic cells, where the cell nucleus have become pyknotic (shrunken and dark) and condensed chromatin located on the nuclear membrane in Fig. 7c–h. As stated previously, the two complexes have apoptosis on the HeLa cells apparently, but lower than cisplatin.

Table 4
Cytotoxicity of the complexes against selected human tumor cells after 48 h of incubation.

Tumor cells	In vitro activity (IC_{50}±SD, μM)		
	Complex **1**	Complex **2**	Cisplatin
HeLa	8.83 ± 2.31	17.61 ± 3.58	0.76 ± 0.11

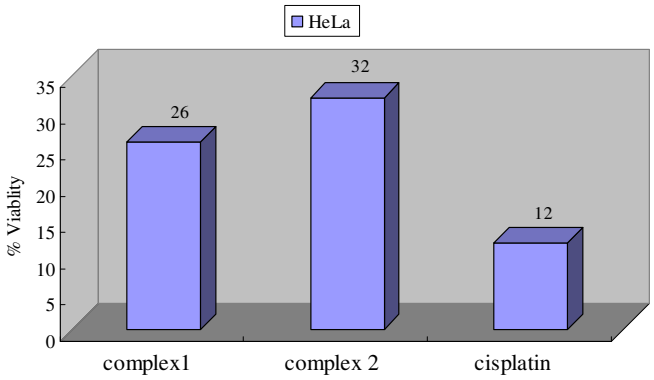


Fig. 6. Effect of 3 μg/mL of the complexes on HeLa cells viability after 48 h of incubation. All determinations are expressed as percentage of the control (untreated cells).

3. Conclusions

Two novel Co(II) complexes dibridged by biphenyl-2,4′-dicarboxylic acid ligand were synthesized and characterized. The crystal structure of the complexes was determined by single-crystal X-ray diffraction. From X-ray single-crystal diffractometer we can see both of the Co(II) complexes are six coordination, which have hydrophilic carboxyl group, coordinate carboxyl group and plan aromatic ring. The extracted HC-DNA binding properties of the complexes were examined by fluorescence spectra. The results support the fact that the complexes bind to extracted DNA in an intercalative mode. What's more, the capability of cleavage of extracted HC-DNA by the complexes was investigated using agarose gel electrophoresis and the results also indicated that the two complexes exhibit an DNA-cleavage activity. Cytotoxic and antiproliferative studies show that the two complexes exhibit cytotoxic activity against extracted HC-DNA. Additionally, the apoptotic tests indicated that the complexes had an apoptotic effect on HeLa cells.

4. Experimental

4.1. Materials

Total chemicals and agents purchased were of reagent-grade and used without further purification unless otherwise noted. All the spectroscopic titration was carried out in aerated buffer (5 mM Tris–HCl, 50 mM NaCl, pH 7.2) at room temperature. HC-DNA applied here was extracted by ourselves. The HeLa cells (HeLa human cervix epitheloid carcinoma cells) were obtained from American Type Culture Collection.

4.2. Physical measurements

Elemental analysis (C, H and N) was performed on a model Finnigan EA 1112. The IR spectra were obtained as KBr pellets on Nicolet FT-IR 470 spectrometer. ^{1}H NMR spectra were measured with a Bruker Avance 300 MHz spectrometer. Fluorescence measurements were carried out on a PerkinElmer LS55 fluorescence spectrofluorometer.

4.3. X-ray crystal structure determination for the complex *1* and *2*

Data of complex **1** and **2** were collected on a Brucker Smart 1000 CCD X-ray single-crystal diffractometer with Mo Kα radiation ($k = 0.71073$ Å) at 273 K in the range of $1.96° < h < 25.08°$ and $1.94° < h < 26.06°$.

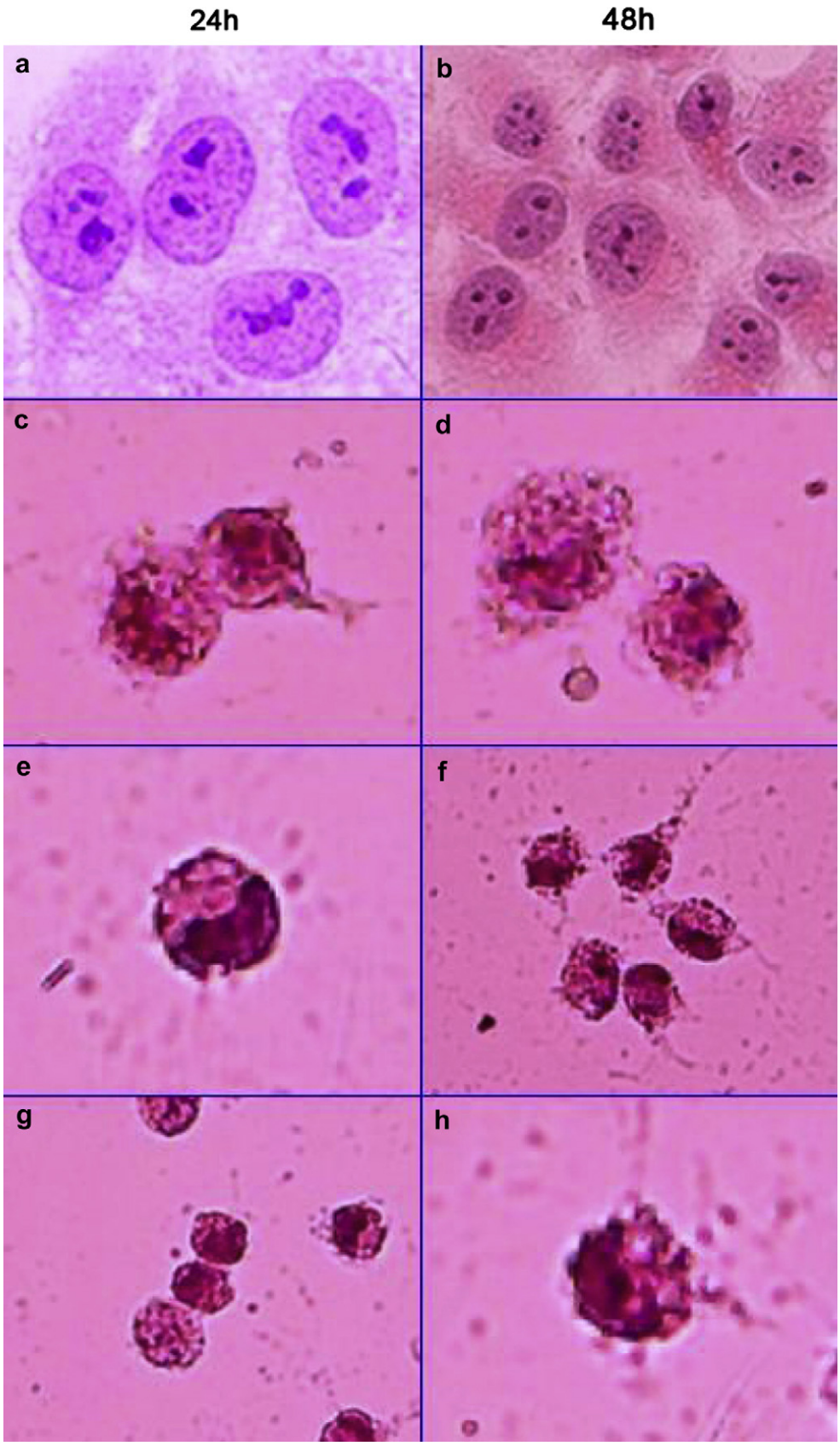


Fig. 7. Morphological changes of HeLa cells. a, b: nomal HeLa cells for 24 h and 48 h; c, d: HeLa cells treated with cisplatin for 24 h and 48 h; e, f: HeLa cells treated with complex **1** for 24 h and 48 h; g, h: HeLa cells treated with complex **2** for 24 h and 48 h.

The structures were solved by direct method and Fourier difference synthesis with SHELXS-97 [40], and refined by full-matrix leasts-quares techniques on F^2 with SHELXL-97 [41,42]. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were located from different Fourier maps.

4.4. Fluorescence spectroscopic studies

For all fluorescence measurements, the entrance and exit slits were maintained at 15 and 15 nm, respectively. The sample was excited at 526 nm and its emission observed at 617 nm. The buffer used in the binding studies was 50 mM Tris–HCl, pH 7.2, containing 10 mM NaCl. The sample was incubated for 4 h at room temperature (20 °C) before spectral measurements. Under these conditions, the fluorescence intensity of the respective complexes, extracted HC-DNA and EtBr was very small and could be ignored. The interaction of the respective complexes with DNA in vitro was studied as described in the literature [43,44].

4.5. Cleavage of extracted HC-DNA

For the gel electrophoresis experiments, extracted HC-DNA (2 μL) was treated with the complexes in Tris(Tris(Hydroxymethyl) aminomethane) buffer (50 mM Tris–acetate, 18 mM NaCl buffer, pH 7.2), and the contents were incubated for 1 h at room temperature. The samples were eletrophoresed for 3 h at 90 V on 0.8% agarose gel in Tris–acetate buffer. After eletrophoresis, the gel was stained with 5 μg/mL ethidium bromide and photographed under UV light.

4.6. Cell line and culture

The cell lines used in this experiment were routinely maintained in a RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mmol/L of glutamine, 100 u/mL of penicillin, and 100 μg/mL of streptomycin in a highly humidified atmosphere of 95% air with 5% CO_2 at 37 °C.

4.7. Cell sensitivity assay

Cell proliferation was evaluated by using a system based on the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT] which is reduced by living cells to yield a soluble formazan product that can be assayed colorimetrically [45,46]. The cytotoxicity of complexes was investigated on HeLa cells. The cell lines were grown in 25 cm^2 tissue culture flasks in an incubator at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air. The cells were maintained in logarithmic growth phase in complete medium consisting of RPMI 1640, 10% (v/v) heat-inactivated fetal calf serum, 20 mM Hepes, 0.112% bicarbonate, and 2 mM glutamine. Cell viability was assessed by the microculture tetrazollium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphe nyltetrazolium bromide, MTT] assay [47]. In brief, cells were seeded into a 96-well culture plate at 2×10^5 cells/well in a 100 μL culture medium. After incubation for 24 h, cells were exposed to the tested complexes of serial concentrations. The complexes were dissolved in DMF and diluted with RPMI 1640 or DMEM to the required concentrations prior to use (0.1% DMF final concentration). The cells were incubated for 24 h, followed by the addition of 20 μL MTT solution (5 mg/mL) to each well and further cultivation for 4 h. The media with MTT were removed, and 100 μL of DMSO was added to dissolve formazan crystal at room temperature for 30 min. The absorbance of each cell at 450 nm was determined by analysis with a micro plate spectrophotometer. The IC_{50} values were obtained from the results of quadruplicate determinations of at least three independent experiments.

In another trial the effect on cell growth for the two complexes was studied by culturing the cells in medium alone for 24 h, followed by 48 h treatment with 3 μg/mL concentrations. The viable cells remaining at the end of treatment period were determined by MTT assay and calculated as % of control, treated with vehicle alone (DMSO) under similar conditions.

4.8. In vitro apoptosis assay

The HeLa cells in usable condition were seeded in a 24-well culture plate at 2×10^5 cells/well in a 1 mL culture medium and 24 h later the medium including the cisplatin was given. The concentration of the two complexes was 1 μM/mL. The cells were further incubated for 48 h, then the level of apoptosis was evaluated at least three independent experiments. The hematoxylin–eosin stain was the means of the apoptotic morphology observed by light microscopy [48]. Under the light microscope, the cytoplasm had been stained pink and with blue-black in the nucleolus.

4.9. Extraction HC-DNA

HeLa cells were grown adherently on the culture flask at 37 °C under sterile conditions after 2–3 days of growth for extraction. Saturated sodium chloride extraction method was taken to extract DNA [49].

I. The cells were washed with 1 mL PBS (0.1 M, NaCl, $Na_2HPO_4 \cdot 12H_2O$, $NaH_2PO_4 \cdot 2H_2O$, pH 7.4) buffer system twice, then 0.8 mL TE (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) was added and the cells were removed by a cell scraper, which were transferred into a eppendorff tube.
II. The HeLa specimens were centrifuged under 10,000 rpm at room temperature.
III. The sediments were suspended in 0.4 mL TE, digested in 40 μL of a buffer containing 20 mg/mL proteinase K and 10% SDs and incubated overnight at 37 °C.
IV. A 150 μL volume of saturated sodium chloride was added to the speciment and incubated at 40 °C for 10 min.
V. The mixture was centrifuged under 10,000 rpm for 10 min to get the supernatant to another tube, in which 715 μL chloroform was added and blended with supernatant, then centrifuged under 10,000 rpm for 10 min again and the supernatant carefully decanted.
VI. An equal volume of absolute ethanol and 1/10 [v/v] of 3 M NaAc pH 5.2 was added, the solution was mixed for 5 min and then allowed to stand at room temperature for 5 min. The mixture was centrifuged at 10,000 rpm for 1 min at room temperature, and the recovered pellet washed with 0.5 mL of 70% ethanol. After removing all traces of ethanol, the pellet was dissolved in 100 μL of TE.

A solution of DNA in the buffer gave a ratio of UV absorbance at 260 and 280 nm of about 1.9:1, indicating that the DNA is sufficiently free from protein.

Acknowledgements

We gratefully acknowledge the Natural Science Foundation of China (No.20971090), the science and technology projects fund of Shenyang City (No.F10-215-1-00), Foundation of Educational Department of Liaoning Province (No.L2010430), the foundation of Liaoning Bai Qian Wan Talents Program (No.2008921054) and the

Phenom Foundation of Liaoning colleges and universities (No. 2007R30).

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