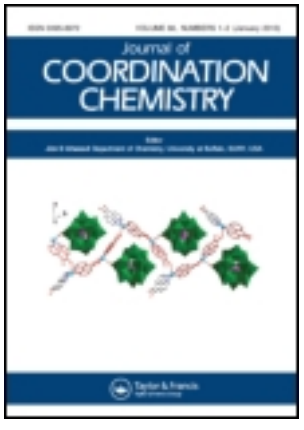

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Syntheses, characterizations and bioactivities on HeLa cells and KB cells of two dinuclear manganese complexes with carboxylate bridges

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Syntheses, characterizations and bioactivities on HeLa cells and KB cells of two dinuclear manganese complexes with carboxylate bridges

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To explore anti-tumor activities of manganese complexes, two complexes have been synthesized and characterized. Complex **1** is bridged by 1,10-phenanthroline and 2,4-biphenyl dicarboxylate. The two complexes have strong fluorescent emission and interact with DNA in an intercalative mode. The complexes also exhibit significant cytotoxic specificity and cancer cell inhibition.

Keywords: HeLa cells; KB cells; Manganese complexes; Gel electrophoresis; Cytotoxicity; Apoptosis

1. Introduction

Development of new pharmaceuticals has undergone substantial change and continues to change rapidly [1–3]. Especially in anticancer treatment, since the discovery of cisplatin, many other transition metal complexes and anti-tumor agents have been developed and used in clinical trials [4–6]. To overcome clinical side effect problems of cisplatin, other transition metal complexes have been examined [5, 7]. Our laboratory has reported palladium(II), platinum(II) and copper(II) complexes with aromatic ligands partially embedded in adjacent DNA base pairs, changing the conformation of DNA and showing anticancer activities [8–11]. As an important transition metal, manganese complexes play critical roles in dioxygen metabolism of many biological systems [12, 13]. Manganese is an essential trace element, involved in numerous biological redox reactions [14, 15].

Ligands can significantly alter the biological activities of complexes [16]. Carboxylic acids are widely used as ligands as they can easily coordinate with metal by deprotonation [17]. 1,10-Phenanthroline and substituted derivatives can also coordinate with transition metals and disturb functions of biological systems. Phenanthroline has long been used as a component of hemoglobin models, with strong coordination ability and biological activity [18–20].

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Therefore, $[Mn_2(phen)_4(L_1)_2]\cdot(phen)\cdot 11H_2O$ (where phen = 1,10-phenanthroline and L_1 = 2,4-biphenyl dicarboxylate) has been characterized by X-ray single-crystal diffraction, elemental analysis, IR, fluorescence, gel electrophoresis, and crystal structure determination [21–23]. The complex was bridged by dicarboxylate lipophilic fragments and hydrophilic fragments. The anti-tumor activity may stem from the lipophilic, hydrophilic and cationic properties [24].

2. Experimental

2.1. *Materials*

All chemicals and reagents were of reagent grade and used without purification, except that the solvents used for physical measurements were purified by classical methods. HeLa and KB cells DNA applied here were extracted in our lab.

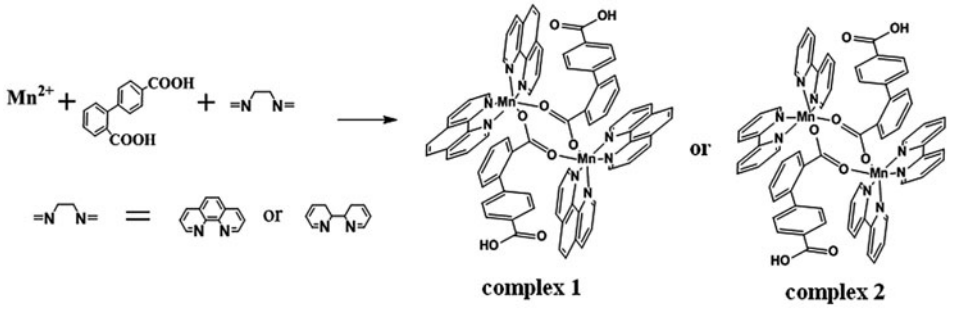
2.2. *Complex synthesis*

2.2.1. Synthesis of $C_{88}H_{80}O_{20}N_{10}Mn_2$. Complex **1** was prepared from Mn $(CH3COO)_2$ aqueous solution (10 mmol, 10 mL) and 2,4-biphenyl dicarboxylic acid solution (10 mmol, 10 mL) (Scheme 1). With potassium hydroxide solution (0.1 mol L^{-1}) the pH of the solution was adjusted to 6.5. After 4 h of stirring, 10 mL of 1,10-phenanthroline (0.1 mol L^{-1}) was added; after another 4 h of stirring, the solution was filtered and the filtrate was evaporated slowly in air. After 30 days, slightly yellow transparent crystals were obtained. Anal. Calcd for $C_{88}H_{80}O_{20}N_{10}Mn_2$ (%): C, 61.90; H, 4.72; N, 8.20. Found (%): C, 61.86; H, 4.75; N, 8.18. IR: 3385 (s), 158 (s), 1400 (s), 1241 (m), 1021 (m), 839 (s), 783 (s) cm^{-1}.

2.2.2. Synthesis of $C_{80}H_{76}O_{20}N_{10}Mn_2$. Complex **2** was synthesized in the same way, but using 2,2′-bipyridine instead of 1,10-phenanthroline solution (0.1 mol L^{-1}, 10 mL) (Scheme 1). The product was obtained as yellow powder. Anal. Calcd for $C_{80}H_{52}O_{20}N_{10}Mn_2$ (%): C, 59.78; H, 4.77; N, 8.71. Found (%): C, 59.74; H, 4.72; N, 8.76. IR: 3380 (s), 1576 (s), 1426 (s), 1231 (m), 1028 (m), 827 (s), 776 (s) cm^{-1}.

2.3. *Physical measurements*

Elemental analysis (C, H and N) was performed on a Finnigan EA 1112. IR spectra were obtained as KBr pellets on a Nicolet FT-IR 470 spectrometer. Fluorescence measurements were carried out on a Perkin Elmer LS55 fluorescence spectrofluorometer.



Scheme 1. The synthesis of **1** and **2**.

2.4. *X-ray crystal structure determination for 1*

The crystal structure of **1** was determined by single-crystal X-ray diffraction. Data were collected on a Bruker Smart 100 CCD X-ray single-crystal diffractometer with MoKα radiation ($\lambda = 0.71073$ Å) at 293(2) *K* from $1.32° < \theta < 25.03°$. The structure was solved by direct methods using SHELXS-97 [25, 26]. All non-hydrogen atoms were determined with successive difference Fourier syntheses and refined by full-matrix least-squares on F^2 [27]. All hydrogens were located at the calculated positions.

2.5. *Fluorescence spectroscopic studies*

The buffer used in the binding studies was 50 mM Tris-HCl, pH 7.4, containing 10 mM NaCl. For all fluorescence measurements, the entrance and exit slits were maintained at 10 nm. The sample was excited at 526 nm and the emission range was set between 540 and 750 nm. Under these conditions, the fluorescence intensity of the respective complexes, extracted DNA and EtBr were very small and could be ignored [28].

2.6. *Determination of electronic absorption spectra*

UV-visible spectra were recorded on a SHIMADZU-UV-2550. Tris-HC buffer (pH 7.0) and (10 mM) solutions of **1** and **2** were titrated with HeLa and KB cells DNA solutions [29].

2.7. *Cleavage of extracted cells DNA*

In this experiment, DNA (kept at −20 °C, 0.3 mg mL^{-1}) was treated with the complex (dissolved in DMF) in Tris buffer (50 mM Tris–acetate, 18 μM NaCl buffer, pH 7.2) and the contents were incubated for 1 h at room temperature. The samples were electrophoresed for 1.5 h at 120 V on 0.85% agarose gel in Tris-acetate buffer. After electrophoresis, the gel was stained with 1 mg mL^{-1} EtBr and photographed under UV light [30].

2.8. In vitro *apoptosis assay*

Hematoxylin–eosin (H–E) staining was carried out as described previously with some modifications. Briefly, HeLa cells were first grown to 80% confluence on cover slips followed by incubation with concentrations (50 μM) of the complex for 24 h. Control (untreated), cisplatin, and the complex treated cells were gently washed twice with cold phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) and then fixed with 4% paraformaldehyde in PBS for 15 min. Cells were washed three times with PBS, permeabilized with 0.2% Triton X-100 in PBS for 5 min, and washed three times with cold PBS followed by incubation with H–E at 37 °C for 1 h. H–E stained cells were washed three times with cold PBS, mounted on a microscope slide, and visualized under a light microscope fitted with a photometric camera [31].

2.9. *Cytotoxicity assay*

The cytotoxicity of the complex and cisplatin against HeLa cells and KB cells were evaluated by tetrazollium (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT) assay as described. Briefly, cells were seeded into a 96-well culture plate at 2×10^5 cells/

well in a 100 µL culture medium. After incubation at 37 °C in a 5% CO_2 incubator for 24 h, cells were exposed to the tested complex of serial concentrations. The cells were incubated for 24 h and 72 h, followed by the addition of 20 µL MTT solution (5 mg mL^{-1}) to each well and further cultivation for 4 h. The absorbance of each cell at 550 nm was determined by analysis with a microplate spectrophotometer. The IC_{50} values were obtained from the results of quadruplicate determinations of at least three independent experiments [32].

In another trial the effects on cell growth for the two complexes were studied by culturing the cells in medium alone for 24 h, followed by 48 h treatment with 3 µg mL^{-1} concentrations. The viable cells remaining at the end of treatment period were determined by MTT assay and calculated as the percent of control, treated with DMSO alone under similar conditions.

3. Results and discussion

3.1. *Crystal structure of 1*

The crystal structure of **1** was determined by X-ray crystallography (figure 1). Both Mn(II) are six coordinate by four nitrogens from two 1,10-phenanthrolines and two oxygens from two 2,4-biphenyl dicarboxylates, exhibiting a slightly distorted octahedral structure. As shown in figure 1, the complex is coordinated by two oxygens from two 2,4-biphenyl dicarboxylates, binding with Mn(1) and Mn(2) fragments; the Mn(1) and Mn(2) fragments are coordinated by two 1,10-phenanthrolines.

The bridging around Mn(II) is planar, composed of four oxygens (Mn1-O1 2.1275 Å, Mn1-O5 2.1118 Å, Mn2-O2 2.1596 Å, Mn2-O6 2.1243 Å) with two manganese. Crystal data and structure refinement details are summarized in table 1 and selected bond lengths and angles are given in table 2. CCDC reference number is 829203 (figure 2).

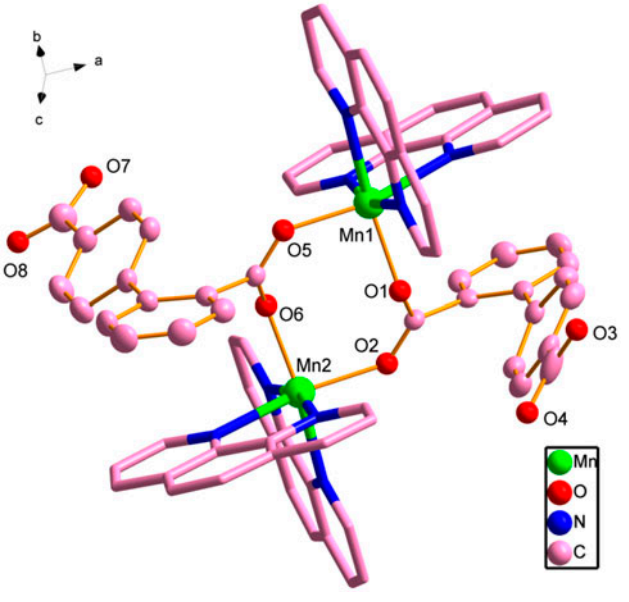


Figure 1. The molecular structure of **1**.

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

Parameter	Value
Formula weight	1707.5 g/mol
Crystal system	Monoclinic
Space group	P 1 21/n 1 (14)
Unit cell dimensions	
a, Å	22.2451(14)
b, Å	12.5037(8)
c, Å	32.613(2)
β, deg	107.86(0)
V, Å^3	8633.98(294)
Z	4
Calculated density, g/cm^{-3}	1.31351
Crystal size	$0.26 \times 0.17 \times 0.10$
Reflections collected/unique	50,126/15,235 [R(int) = 0.0327]
$I > 2\sigma(I)$	
Completeness, %	99.9
Goodness of fit on F^2	1.065
Final R indices ($I > 2s(I)$)	R1 = 0.0463, wR2 = 0.1203
R indices (all data)	R1 = 0.0623, wR2 = 0.1275
Residual electronic density (max/min), e Å^{-3}	0.473 and −0.415

Table 2. Selected bond lengths and angles for **1**.

Bond	d, Å	Bond	d, Å
Mn1–O5	2.1118(15)	Mn2–O6	2.1243(15)
Mn1–O1	2.1275(14)	Mn2–O2	2.1596(15)
Mn1–N2	2.261(2)	Mn2–N5	2.240(2)
Mn1–N3	2.2679(18)	Mn2–N8	2.2575(18)
Mn1–N1	2.2915(19)	Mn2–N7	2.2848(19)
Mn1–N4	2.2916(18)	Mn2–N6	2.2879(19)
Angle	ω, deg	Angle	ω, deg
O5–Mn1–O1	93.02(6)	N5–Mn2–N8	164.80(7)
O5–Mn1–N2	96.25(7)	O6–Mn2–N7	163.35(7)
O1–Mn1–N2	100.78(6)	O2–Mn2–N7	87.01(6)
O5–Mn1–N3	92.93(6)	N5–Mn2–N7	96.92(7)
O1–Mn1–N3	89.19(6)	N8–Mn2–N7	73.36(7)
N2–Mn1–N3	166.03(7)	O6–Mn2–N6	92.86(6)
O5–Mn1–N1	165.95(7)	O2–Mn2–N6	170.24(7)
O1–Mn1–N1	97.72(6)	N5–Mn2–N6	73.32(7)
N2–Mn1–N1	72.92(7)	N8–Mn2–N6	94.24(7)
N3–Mn1–N1	96.23(7)	N7–Mn2–N6	87.47(7)
O5–Mn1–N4	87.47(6)	C25–O1–Mn1	131.18(15)
O1–Mn1–N4	162.20(6)	C25–O2–Mn2	127.11(13)
N2–Mn1–N4	96.84(7)	C1–N1–Mn1	126.71(18)
N3–Mn1–N4	73.02(7)	C10–N2–Mn1	125.85(17)
N1–Mn1–N4	85.06(6)	C6–N2–Mn1	116.56(15)
O6–Mn2–O2	94.74(6)	C63–O5–Mn1	129.36(14)
O6–Mn2–N5	99.12(7)	C63–O6–Mn2	132.76(15)
O2–Mn2–N5	99.37(7)	C5–N1–Mn1	115.44(14)
O6–Mn2–N8	90.02(6)	C1–N1–C5	117.8(2)
O2–Mn2–N8	91.89(6)	C10–N2–C6	117.6(2)

Weak interactions such as π–π stacking [33] are significant in supermolecular complexes. Adjacent molecules are connected into a 1-D supramolecular structure through π–π interactions at 3.829 Å; the stacking framework of **1** is shown in figure 3.

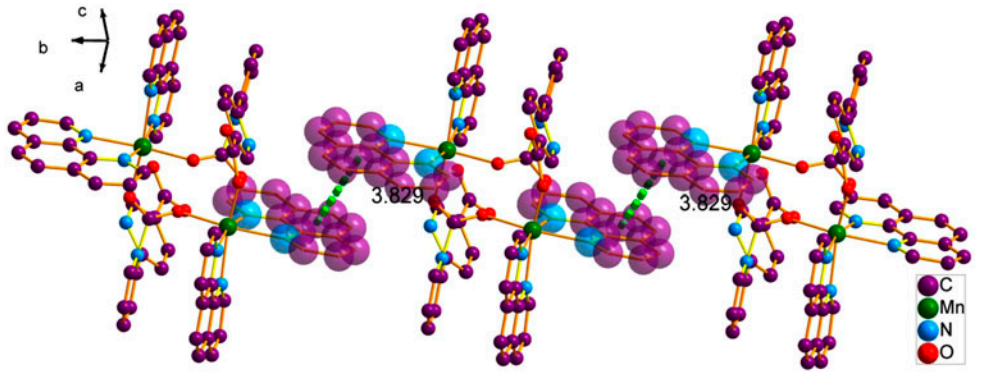


Figure 2. The 1-D metal complex framework formed by intermolecular π-π interactions.

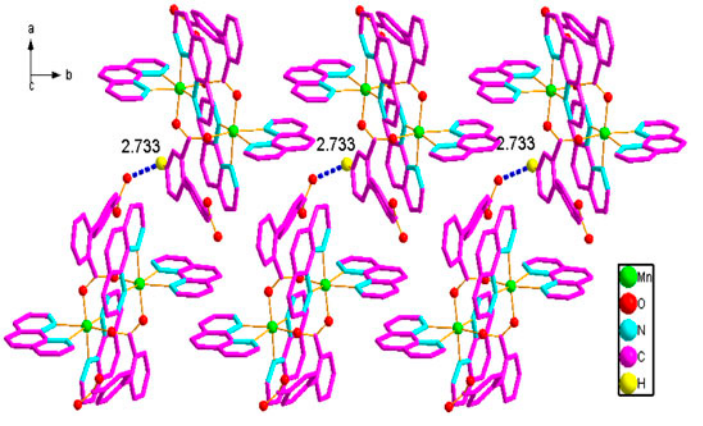


Figure 3. The 2-D network of **1** constructed through hydrogen bonds.

The 2-D layers are further connected through hydrogen bonds [34] (the distance of O1-H-O2 is 2.733 Å) forming a 2-D framework, shown in figure 3.

3.2. *Fluorescence spectroscopic studies*

Ethidium bromide (EB) is a conjugated planar molecule and its fluorescence intensity is very weak [35]. EB emits intense fluorescence in the presence of DNA due to its strong intercalation between adjacent DNA base pairs. The extent of fluorescence quenching for EB bound to DNA is used to determine the extent of binding between another molecule and DNA [36, 37]. Figure 4 shows the EB/DNA complexes ($C_{EtBr} = 5 \times 10^{-5}$ M, $C_{DNA} = 5 \times 10^{-5}$ M) with addition of complex solution decreases the fluorescence. According to the classical Stern–Volmer equation: $I_0/I = 1 + K_{sq}r$ [38], 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 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} values for the two complexes are $K_{sq1} = 0.819$, $K_{sq2} = 0.6749$, $K_{sq3} = 0.1711$,

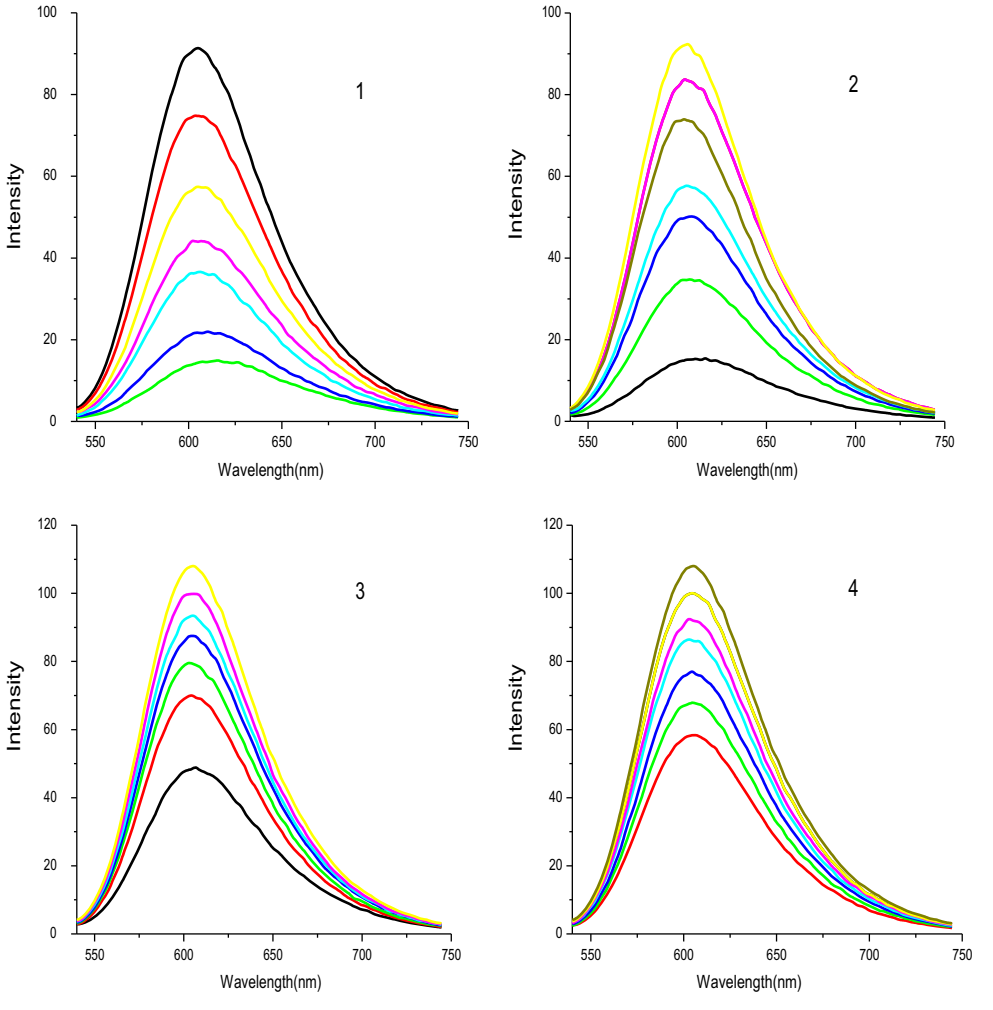


Figure 4. The emission spectrum of EB bound to HeLa and KB cells DNA in the presence of **1** and **2** (λ_{ex} = 526 nm, [EtBr] = 1 μM, [DNA] = 2.5 M̀m, 1: complex **1** with HeLa cells DNA, 2: complex **2** with HeLa cells DNA, 3: complex **1** with KB cells DNA, 4: complex **2** with KB cells DNA).

K_{sq4} = 0.1361. Such values of quenching constant suggest that the interaction of the complex with DNA is moderate intercalation. This led us to the following conclusions: (1) Two complexes have the ability to interact with DNA in an intercalative mode. (2) The quenching plots of EB–DNA by successive addition of **1** and **2** gave linear Stern–Volmer equations with K_{sq} values, indicating stronger interaction of **1** with two types of DNA than **2**.

3.3. *UV absorption spectroscopy*

Electronic absorption spectroscopy is useful for DNA-binding studies of metal complexes [39]. A complex binding to DNA through intercalation usually results in hypochromism and bathchromism, due to intercalation involving a strong π–π stacking interaction between an aromatic chromophore and the base pairs of DNA. The extent of hypochromism in the

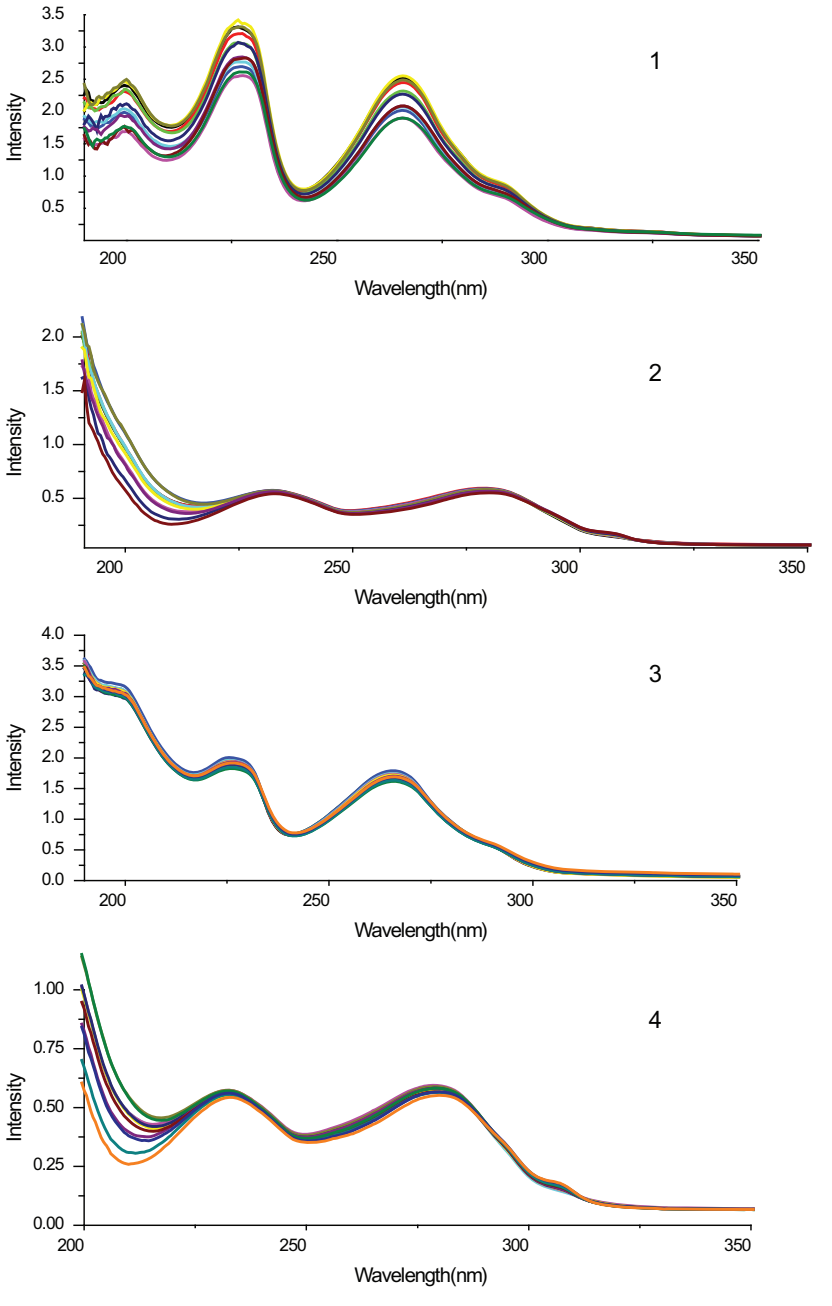


Figure 5. Effect of two different cell's DNA on the absorption spectrum of **1** and **2** (1: complex **1** with HeLa cells DNA, 2: complex **2** with HeLa cells DNA, 3: complex **1** with KB cells DNA, 4: complex **2** with KB cells DNA).

UV band is consistent with the strength of intercalation [40–42]. The spectral changes are characterized (figure 5) by one or more isosbestic points, maintained till the end of the titrations of the complexes with DNA so one can rule out the presence of species other

than the free and the intercalated complex [43]. The intense absorptions at 220 and 260 nm reveal the intraligand π–π transition of phen (figure 5.1 and 5.3), attributed to the strong stacking interaction between aromatic group and the base pairs of DNA when the complexes intercalate to DNA. Other absorptions from 230 to 280 nm reveal the intraligand π-π transition of 2,2′-bipyridine (figure 5.2 and 5.4). DNA binding of **1** is stronger than that of **2** due to the larger hydrophobicity of 1,10-phenanthroline than 2,2′-bipyridine. This result suggested that complex binding to DNA involves a strong stacking interaction between an aromatic group and the base pairs of DNA.

3.4. *Cleavage of extracted cells DNA*

In past research, pBR322 plasmid DNA or pUC19 DNA were used but do not originate from cell [44, 45]. However, we analyzed DNA strand breaks in HeLa and KB cells treated with the two complexes, so the mechanism coincides with the activity. Because the DNA intercalator EB is known to cause DNA elongation, the cleavage efficiency of extracted DNA can be characterized by agarose gel electrophoresis. As shown in figure 6, two different cells DNA fragmentations with a characteristic laddering pattern were observed for the complexes. In the two cells DNA displayed, lanes 1 and 7 were untreated DNA ladders. In contrast, the DNA samples treated with lanes 2–6 and lanes 8–12 show

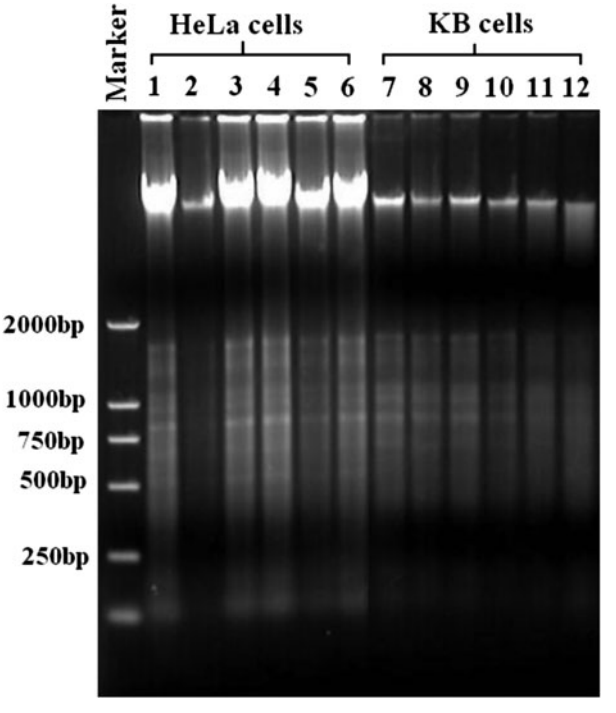


Figure 6. DNA strand break in two different cells treated with two complexes. (Lane 1 is HeLa cells DNA alone; Lanes 2–4 are HeLa cells DNA with different concentration of complex **1**; Lanes 4 and 5 are HeLa cells DNA with different concentration of complex **2**; Lane 7 is KB cells DNA alone; Lanes 8–10 are HeLa cells DNA with different concentration of complex **1**; Lanes 11 and 12 are HeLa cells DNA with different concentration of complex **2**).

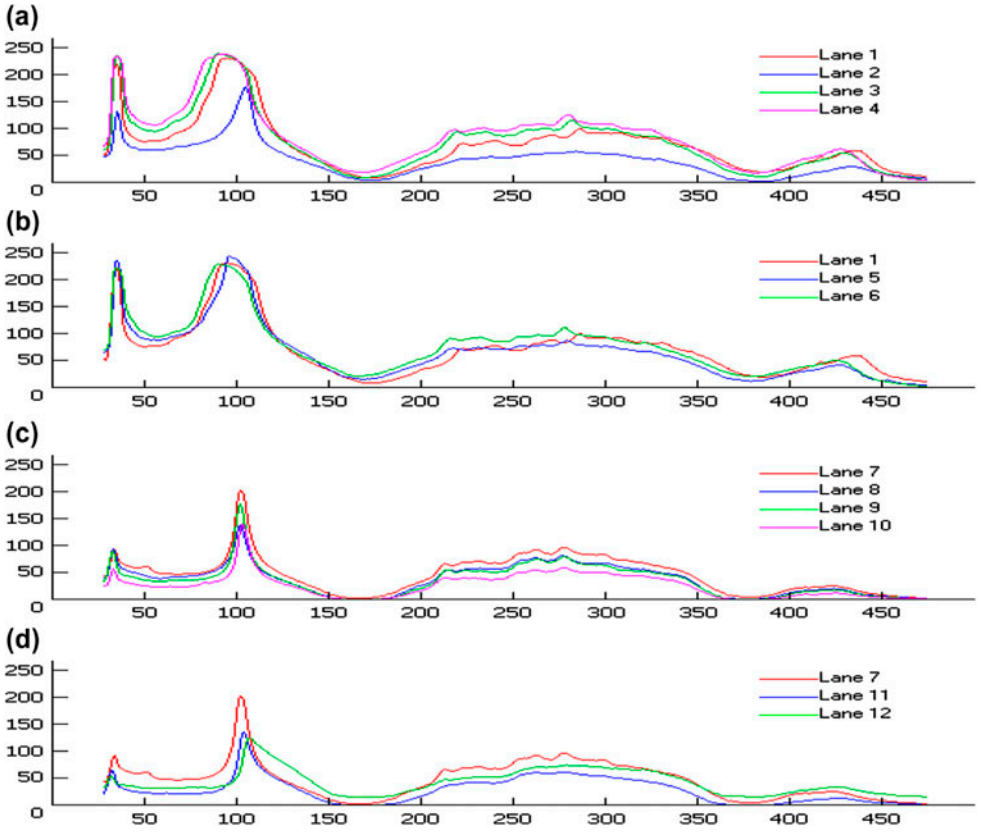


Figure 7. The DNA-cleavage activity of **1** and **2** by the optical density scanning experiment (Lanes 1 and 7: DNA alone, Lanes 2–6: complex **1**, Lanes 8–12: complex **2**).

retarded mobility (tailing effect), which may be attributed to intercalation into the DNA base pairs. These results further suggest that **1** and **2** can bind to DNA through intercalation. We investigated the DNA-cleavage activity of **1** and **2** by the optical density scanning experiment (figure 7, analysis software of electrophoresis system). The two complexes at different concentrations exhibit more effective DNA-cleavage activity [46].

3.5. *Apoptotic study*

Apoptosis is a highly orchestrated cell suicidal program required to maintain a balance between cell proliferation and death. Apoptosis is usually induced by physiological and external stimuli [47, 48]. In figure 8, we observed the morphological changes of the two cells by light microscopy. The cytoplasm and nucleolus had been stained; normal cells exhibited dense state and intercellular tight junction, the nuclei of the normal cells were intact and had no pyknosis. After addition of cisplatin (figure 8(b)) and the complexes (figure 8(c–h)) for 24 h, the cells appeared small, different from the apoptotic cells, with cell shrinkage in size, nuclear condensation, fragmentation and ultimately death. Consequently, the two complexes have apoptotic effect on the HeLa and KB cells.

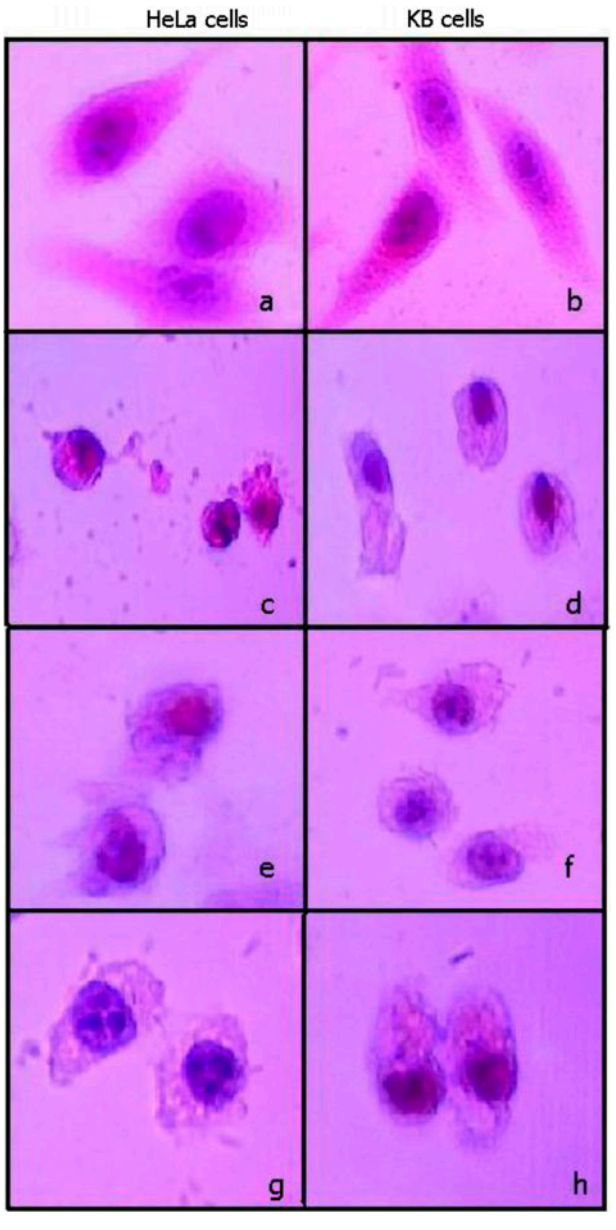


Figure 8. Morphological changes of two cells. (a, b: normal HeLa and KB cells for 24 h; c, d: two cells treated with cisplatin for 24 h; e, f: two cells treated with complex **1**; e, f: two cells treated with complex **2**).

3.6. *Cytotoxicity* in vitro *study*

As several manganese complexes show efficient cell killing activities (with IC_{50} comparable to cisplatin), we investigated their potential in anti-tumor drug application by analyzing their IC_{50} values towards different cancer and normal cell lines. The *in vitro* growth inhibitory effect of **1**, **2** and cisplatin were evaluated in figure 9. The IC_{50} values of Mn

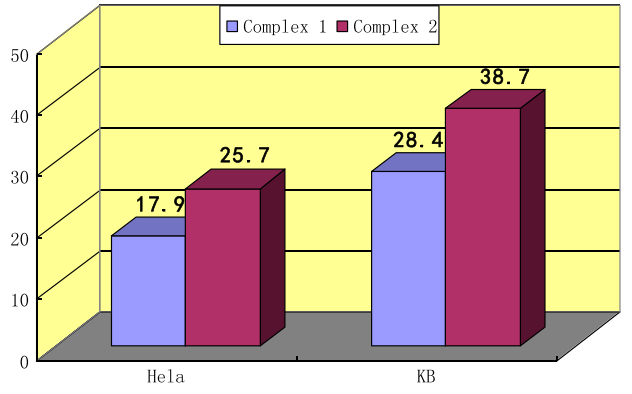


Figure 9. Effect of 3.0 mg mL^{-1} of the complexes on breast cancer cells viability after 48 h of incubation.

Table 3. Cytotoxicity of the complexes and cisplatin against selected human tumor cells after 24 h and 72 h of incubation (data are expressed as mean ± SD (n = 4)).

		In vitro activity (IC_{50} ± SD, μM)		
Time of incubation	Tumor cells	Complex **1**	Complex **2**	Cisplatin
24 h	HeLa	18 ± 3	26 ± 3	17 ± 3
	KB	39 ± 6	44 ± 8	9 ± 2
72 h	HeLa	5 ± 2	8 ± 2	1 ± 0
	KB	11 ± 2	16 ± 2	2 ± 0

complexes (along with cisplatin) for HeLa cells and KB cells are shown in table 3. A viability rate by day 3 to less than 50% of the control values was observed for the complexes [49]. Based on our analysis, cisplatin also showed about 3-fold preference for HeLa cells over KB cells. The level of cytotoxicity of the manganese complexes towards HeLa cells and KB cells are similar to cisplatin, indicating that manganese complexes are potential anti-tumor agents.

3.7. *Extraction cells-DNA*

HeLa and KB cells were adhering 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. 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 and transferred into an Eppendorff tube. The HeLa specimens were centrifuged under 10,000 rpm at room temperature. The sediments were suspended in 0.4 mL TE, digested in 40 μL of a buffer containing 20 mg mL^{-1} proteinase K and 10% SDS and incubated overnight at 37 °C. A 150 μL volume of saturated sodium chloride was added to the specimen and incubated at 40 °C for 10 min. The mixture was centrifuged at 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. An equal volume of absolute ethyl alcohol 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 the DNA is sufficiently free from protein.

4. Conclusions

Two manganese complexes, **1** and **2**, were strong DNA intercalators. The experimental results suggest that the two complexes intercalated between adjacent base pairs of DNA through classical intercalation with **1** bound to the DNA more tightly than **2**. The different interaction between the two complexes with DNA arises from additional nitrogen substitution on the benzene ring, inhibiting DNA binding. Recent advances in understanding biochemical roles of metal complexes and their medicinal applications demonstrated significance of metal complexes as drugs. Herein, we explored the biochemical and potential anti-tumor activities of two manganese complexes with different DNA. The results obtained from electrophoresis showed stronger cleavage. The complexes affect cell viability and efficient apoptosis. The toxicities of **1** and **2** toward cancer cells suggest potential in anti-tumor applications.

Acknowledgements

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