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2-(3,5-Dibromo-4-hydroxyphenyl)imidazo [4,5-*f*][1,10]phenanthrolinoruthenium(II) complexes: synthesis, characterization, cytotoxicity, apoptosis, DNA-binding and antioxidant activity

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Abstract A new ligand DBHIP and its two ruthenium(II) complexes $[Ru(dmb)_2(DBHIP)](ClO_4)_2$ (**1**) and $[Ru(dmp)_2(DBHIP)](ClO_4)_2$ (**2**) have been synthesized and characterized. The cytotoxicity of DBHIP and complexes **1** and **2** has been assessed by MTT assay. The apoptosis studies were carried out with acridine orange/ethidium bromide (AO/EB) staining methods. The binding behaviors of these complexes to calf thymus DNA (CT-DNA) were studied by absorption titration, viscosity measurements, thermal denaturation and photoactivated cleavage. The DNA-binding constants of complexes **1** and **2** were determined to be $8.64 \pm 0.16 \times 10^4$ (s = 1.34) and $2.79 \pm 0.21 \times 10^4$ (s = 2.17) M^{-1}. The results suggest that these complexes interact with DNA through intercalative mode. The studies on the mechanism of photocleavage demonstrate that superoxide anion radical ($O_2^{\bullet-}$) and singlet oxygen ($^1O_2$) may play an important role in the DNA cleavage. The experiments on antioxidant activity show that these compounds also exhibit good antioxidant activity against hydroxyl radical ($OH^{\bullet}$).

Keywords Ruthenium(II) complexes · DNA · Cytotoxicity · Apoptosis · Antioxidant activity

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Abbreviations

DBHIP	2-(3,5-Dibromo-4-hydroxyphenyl)imidazo[4,5-*f*][1,10]phenanthroline
dmb	4,4′-Dimethyl-2,2′-bipyridine
dmp	2,9-Dimethyl-1,10-phenanthroline
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
AO/EB	Acridine orange/ethidium bromide
DMSO	Dimethyl sulfoxide
RPMI	Roswell Park Memorial Institute
DMF	*N*,*N*-Dimethylformamide
CT-DNA	Calf thymus DNA
MLCT	Metal to ligand charge transfer
Tris	Tris(hydroxymethyl)aminomethane
ES–MS	Electrospray mass spectroscopy
NAMI-A	[ImH][trans-$RuCl_4$(DMSO)(Im)]
KP1019	[IndH][trans-RuCl4$(Ind)_2$]

Introduction

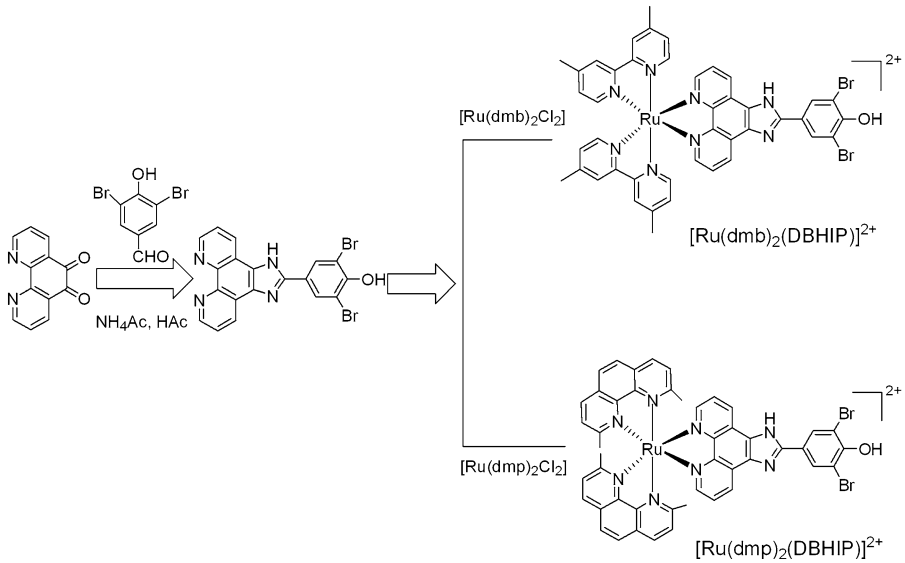
Due to rich photochemical and photophysical properties, polypyridyl ruthenium(II) complexes have received considerable attention as DNA-binding substrates. They are photochemical stable in their ground and excited state. The rich optical properties of these complexes facilitate assessments of their DNA binding capabilities as binding to DNA can be probed through changes in absorption and emission spectra. Many octahedral ruthenium(II) complexes have been proved to bind with DNA through intercalative mode (Erkkila et al. 1999; Metcalfe and Thomas 2003; Zeglis et al. 2007; Ghosh et al. 2009; Liu et al. 2009, 2010; Sun et al. 2009; Tan et al. 2009; Arockiasamy et al. 2009; Yu et al. 2009; Friedman et al. 1990). These ruthenium complexes show interesting properties. Complex $[Ru(bpy)_2(dppz)]^{2+}$ (dppz = dipyrido[3,2-*a*:2′,3′-*c*]phenazine) may act as "molecular light switch" (Friedman et al. 1990) and exhibits cytotoxic activity at low micromolar IC_{50} values (Schatzschneider et al. 2008). Organometallic arene ruthenium compounds of the general formula $[Ru(\eta^6\text{-arene})(L)(L')(L'')]X_n$, in which at least some of the ligands L are labile, have also received a lot of attention recently, with structure–activity studies against a variety of cancer cells lines (Yan et al. 2005; Melchart and Sadler 2006; Dyson 2007; Dougan and Sadler 2007; Schäfer et al. 2007). Ruthenium anticancer chemistry has already yielded many promising results. Several compounds have been described which display an activity comparable to that of cisplatin, and in some cases, activities are even better (Reedijk 2003; Hotze et al. 2004; Velders et al. 2000; Habtemariam et al. 2006; Vilaplana et al. 1994, 2006). Ruthenium complex ImH[trans-Ru(III)Cl_4(DMSO)Im] (Im = imidazole, NAMI-A) was the first to enter clinical trials against metastases (Cocchietto and Sava 2000; Zorzet et al. 2001), and KP 1019 has been introduced into phase I clinical trials against colon carcinomas and their metastases (Hartinger et al. 2006). More recent, studies demonstrate that ruthenium(II) complexes containing polypyridyl ligand also show high antitumor activity in vitro (Schatzschneider et al. 2008; Liu et al. 2008, 2009; Tan et al. 2008; Gao et al. 2007), these complexes can effectively inhibit the cell proliferation. In this article, we report the synthesis, characterization, DNA-binding, cytotoxicity, apoptosis in vitro and antioxidant activity of a new ligand DBHIP and its two ruthenium(II) complexes $[Ru(dmb)_2(DBHIP)](ClO_4)_2$ **1** and $[Ru(dmp)_2(DBHIP)](ClO_4)_2$ **2** (Scheme 1). Thermal denaturation and viscosity measurements show that the two complexes interact with DNA by intercalative mode. The studies on the mechanism of photocleavage demonstrate that superoxide anion radical ($O_2^{\bullet-}$) and singlet oxygen ($^1O_2$) may play an important role in the DNA cleavage. The cytotoxicity of these compounds has been evaluated by MTT assay. The studies on the cytotoxicity show these compounds exhibit high activity against BEL-7402 (hepatocellular), C-6 (Rat glioma), HepG-2 (hepatocellular) and MCF-7 (breast cancer) cells in a dose-dependent manner. The results of apoptosis assay suggest that these complexes **1** and **2** can induce the apoptosis of BEL-7402 cells. The experiments on antioxidant activity show that these compounds exhibit good antioxidant activity against hydroxyl radical ($OH^{\bullet}$).

Materials and methods

Calf thymus DNA (CT-DNA) was obtained from the Sino-American Biotechnology Company. pBR 322 DNA was obtained from Shanghai Sangon Biological Engineering & Services Co., Ltd. Dimethyl sulfoxide (DMSO) and RPMI 1640 were purchased from Sigma. Cell lines of BEL-7402 (hepatocellular), C-6 (Rat glioma), HepG-2 (hepatocellular) and MCF-7 (breast cancer) were purchased from American Type Culture Collection, agarose and ethidium bromide were obtained from Aldrich. $RuCl_3 \cdot xH_2O$ was purchased from Kunming Institution of Precious Metals. Doubly distilled water was used to prepare buffers (5 mM Tris(hydroxymethylaminomethane)–HCl, 50 mM NaCl, pH = 7.2). A solution of calf thymus DNA (CT-DNA) in the buffer gave a ratio of UV absorbance at 260 and 280 nm of ca. 1.8–1.9:1, indicating that the DNA was sufficiently free of protein (Reichmann et al. 1954). The DNA concentration per nucleotide was determined by absorption spectroscopy using the molar absorption coefficient (6,600 M^{-1} cm^{-1}) at 260 nm (Chaires et al. 1982).

Physical measurements

Microanalysis (C, H, and N) was carried out with a Perkin-Elmer 240Q elemental analyzer. Fast atom bombardment (FAB) mass spectra were recorded on a VG ZAB-HS spectrometer in a 3-nitrobenzyl alcohol



Scheme 1 The synthetic route for ligand and complexes

matrix. Electrospray mass spectra (ES–MS) were recorded on a LCQ system (Finnigan MAT, USA) using methanol as mobile phase. The spray voltage, tube lens offset, capillary voltage and capillary temperature were set at 4.50 kV, 30.00 V, 23.00 V and 200°C, respectively, and the quoted *m/z* values are for the major peaks in the isotope distribution. ^{1}H NMR spectra were recorded on a Varian-500 spectrometer. All chemical shifts were given relative to tetramethylsilane (TMS). UV/Vis spectra were recorded on a Shimadzu UV-3101PC spectrophotometer at room temperature.

Synthesis of the ligand and complexes

Synthesis of 2-(3,5-dibromo-4-hydroxyphenyl)imidazo[4,5-f][1,10]phenanthroline (DBHIP)

A mixture of 1,10-phenanthroline-5,6-dione (0.315 g, 1.5 mmol), 3,5-dibromo-4-hydroxyphenylaldehyde (0.419 g, 1.5 mmol), ammonium acetate (2.31 g, 30 mmol) and glacial acetic acid (30 cm^3) was refluxed with stirring for 2 h. The cooled solution was diluted with water and neutralized with concentrated aqueous ammonia. The precipitate was collected and purified by column chromatography on silica gel (60–100 mesh) with ethanol as eluent to give the compound as yellow powder. Yield: 81%. Anal. Calcd. for $C_{19}H_{10}N_4OBr_2$: C, 48.54; H, 2.14; N, 11.92; Found: C, 48.51; H, 2.18; N, 11.89%. FAB–MS: $m/z = 471$ $[M + 1]^+$. IR (KBr, cm^{-1}): 3437, 2927, 1604, 1589, 1476, 1449, 1357, 1173, 1109, 1073, 805, 736, 670. ^{1}H NMR (500 MHz, DMSO-d_6): 9.02 (d, 2H, $J = 8.5$ Hz), 8.33 (d, 2H, $J = 8.4$ Hz), 8.01 (d, 2H, $J = 8.6$ Hz), 7.22 (d, 2H, $J = 8.2$ Hz), 3.26 (s, 1H).

*Synthesis of [Ru(dmb)$_2$(DBHIP)](ClO$_4$)$_2$ (**1**)*

A mixture of *cis*-[Ru(dmb)$_2$Cl$_2$]·2H$_2$O (0.280 g, 0.5 mmol) (Sullivan et al. 1978) and DBHIP (0.185 g, 0.5 mmol) in ethanol (30 cm^3) was heated to reflux under argon for 8 h to give a clear red solution. Upon cooling, a red precipitate was obtained by dropwise addition of saturated aqueous NaClO$_4$ solution. The crude product was purified by column chromatography on a neutral alumina with a mixture of CH$_3$CN–toluene (3:1, v/v) as eluant. The mainly red band was collected. The solvent was removed under reduced pressure and a red powder was obtained. Yield: 71%. Anal. Calcd. for $C_{43}H_{34}N_8Cl_2Br_2O_9Ru$: C, 45.36; H, 3.01; N, 9.84; Found: C, 45.31; H, 3.05; N, 9.87%. ES–MS [CH$_3$CN, *m/z*]: 939.1 ([M–2ClO$_4$–H]$^+$), 470.3 ([M–2ClO$_4$]$^{2+}$). IR (KBr, cm^{-1}): 3434, 2925, 1619, 1445, 1366, 1121, 1090, 876, 729, 636. ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.01 (d, 2H, $J = 8.2$ Hz), 8.72 (d, 2H, $J = 8.8$ Hz), 8.23 (s, 4H),

7.99 (s, 2H), 7.85 (d, 4H, $J = 8.2$ Hz), 7.67(d, 2H, $J = 8.0$ Hz), 7.17 (d, 4H, $J = 8.0$ Hz), 3.38 (s, 1H,), 2.56 (s, 6H), 2.51 (s, 6H).

*Synthesis of [Ru(dmp)$_2$(DBHIP)](ClO$_4$)$_2$ (**2**)*

This complex was synthesized in an manner identical to that described for complex **1**, with *cis*-[Ru(dmp)$_2$-Cl$_2$]·2H$_2$O (0.312 g, 0.5 mmol) (Collin and Sauvage 1986) in place of *cis*-[Ru(dmb)$_2$ Cl$_2$]·2H$_2$O. Yield: 70%. Anal. Calcd. for $C_{47}H_{34}Cl_2Br_2N_8O_9Ru$: C, 47.57; H, 2.89; N, 9.44; Found: C, 47.54; H, 2.94; N, 9.48%. ES–MS [CH_3CN, *m/z*]: 987.0 ([M–2ClO$_4$–H]$^+$), 494.2 ([M–2ClO$_4$]$^{2+}$). IR (KBr, cm^{-1}): 3436, 2926, 1628, 1586, 1444, 1366, 1121, 1084, 857, 728, 636. ^{1}H NMR (500 MHz, DMSO-d$_6$): δ 8.91 (d, 2H, $J = 8.6$ Hz), 8.84 (d, 2H, $J = 8.5$ Hz), 8.42 (t, 4H, $J = 7.8$ Hz), 8.24 (d, 2H, $J = 8.2$ Hz), 8.14 (s, 4H), 7.97 (d, 2H, $J = 8.2$ Hz), 7.38 (d, 4H, $J = 8.4$ Hz), 3.35 (s, 1H), 2.51 (s, 6H), 2.50 (s, 6H).

Caution Perchlorate salts of metal compounds with organic ligands are potentially explosive, and only small amounts of the material should be prepared and handled with great care.

In vitro cytotoxicity assay

Standard 3-(4,5-dimethylthiazole)-2,5-diphenyltetrazolium bromide (MTT) assay procedures were used (Mosmann 1983). Cells were placed in 96-well microassay culture plates (1×10^4 cells per well) and grown overnight at 37°C in a 5% CO_2 incubator. Compounds tested were dissolved in DMSO and diluted with RPMI 1640 to the required concentrations prior to use. Control wells were prepared by addition of culture medium (100 μl). Wells containing culture medium without cells were used as blanks. The plates were incubated at 37°C in a 5% CO_2 incubator for 48 h. Upon completion of the incubation, stock MTT dye solution (20 μl, 5 mg ml^{-1}) was added to each well. After 4 h incubation, buffer (100 μl) containing *N*,*N*-dimethylformamide (50%) and sodium dodecyl sulfate (20%) was added to solubilize the MTT formazan. The optical density of each well was then measured on a microplate spectrophotometer at a wavelength of 490 nm. The IC_{50} values were determined by plotting the percentage viability versus concentration on a logarithmic graph and reading off the concentration at which 50% of cells remain viable relative to the control. Each experiment was repeated at least three times to get the mean values. Four different tumor cell lines were the subjects of this study: BEL-7402 (hepatocellular), C-6 (Rat glioma), HepG-2 (hepatocellular) and MCF-7 (breast cancer) (purchased from American Type Culture Collection).

Apoptosis studies

Apoptosis studies were performed with a staining method utilizing acridine orange AO) and ethidium bromide (EB) (Spector et al. 1998). According to the difference in membrane integrity between necrotic and apoptosis, AO can pass through cell membrane, but EB cannot. Under fluorescence microscope, live cells appear green, necrotic cells stain red but have a nuclear morphology resembling that of viable cells, apoptosis cells appear green. And morphological changes such as cell blebbing and formation of apoptotic bodies will be observed.

A monolayer of BEL-7402 cells was incubated in the absence or presence of complex **2** at concentration of 25 μM at 37°C and 5% CO_2 for 24 h. After 24 h, each cell culture was stained with AO/EB solution (100 μg ml^{-1} AO, 100 μg ml^{-1} EB). Samples were observed under a fluorescence microscope.

DNA binding and photoactivated cleavage

The DNA-binding and photoactivated cleavage experiments were performed at room temperature. Buffer A [5 mM tris(hydroxymethyl)aminomethane (Tris) hydrochloride, 50 mM NaCl, pH 7.0] was used for absorption titration, luminescence titration and viscosity measurements. Buffer B (50 mM Tris–HCl, 18 mM NaCl, pH 7.2) was used for DNA photocleavage experiments. Buffer C (1.5 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, 0.25 mM Na_2EDTA, pH 7.0) was used for thermal DNA denaturation experiments. Buffer D (0.9% of physiological saline) was used for retardation assay of pGL 3 plasmid DNA.

The absorption titrations of the complexes in buffer were performed using a fixed concentration (20 μM) for complexes to which increments of the DNA stock solution were added. Ru–DNA solutions were allowed to incubate for 5 min before the absorption spectra were recorded. The intrinsic binding constants *K*, based on the absorption titration, were measured by

monitoring the changes of absorption in the MLCT band with increasing concentration of DNA using the following equation (Carter et al. 1989).

$$\frac{\varepsilon_a - \varepsilon_f}{\varepsilon_b - \varepsilon_f} = \frac{\sqrt{b - (b^2 - 2K^2C_t[\mathrm{DNA}]/s)}}{2KC_t} \quad (1a)$$

$$b = 1 + KC_t + K[\mathrm{DNA}]/2s \quad (1b)$$

where [DNA] is the concentration of CT-DNA in base pairs, the apparent absorption coefficients ε_a, ε_f and ε_b correspond to A_{obsed}/[Ru], the absorbance for the free ruthenium complex, and the absorbance for the ruthenium complex in fully bound form, respectively. K is the equilibrium binding constant, C_t is the total metal complex concentration in nucleotides and s is the binding site size.

Thermal denaturation studies were carried out with a Perkin-Elmer Lambda 35 spectrophotometer equipped with a Peltier temperature-controlling programmer (±0.1°C). The melting temperature T_m, which is defined as the temperature where half of the total base pairs is unbonded, is usually introduced. The melting curves were obtained by measuring the absorbance at 260 nm for solutions of CT-DNA (80 μM) in the absence and presence of the Ru(II) complex (32 μM) as a function of the temperature. The temperature was scanned from 50 to 90°C at a speed of 1°C min^{-1}.

Viscosity measurements were carried out using an Ubbelodhe viscometer maintained at a constant temperature at 25.0 (±0.1)°C in a thermostatic bath. DNA samples approximately 200 base pairs in average length were prepared by sonicating in order to minimize complexities arising from DNA flexibility (Chaires et al. 1982). Flow time was measured with a digital stopwatch, and each sample was measured three times, and an average flow time was calculated. Data were presented as $(\eta/\eta_0)^{1/3}$ versus binding ratio (Cohen and Eisenberg 1969), where η is the viscosity of DNA in the presence of complexes and η_0 is the viscosity of DNA alone.

For the gel electrophoresis experiment, supercoiled pBR 322 DNA (0.1 μg) was treated with the Ru(II) complexes in buffer B, and the solution was then irradiated at room temperature with a UV lamp (365 nm, 10 W). The samples were analyzed by electrophoresis for 1.5 h at 80 V on a 0.8% agarose gel in TBE (89 mM Tris–borate acid, 2 mM EDTA, pH = 8.3). The gel was stained with 1 μg/ml ethidium bromide and photographed on an Alpha Innotech IS-5500 fluorescence chemiluminescence and visible imaging system. The integrated density values (IDV) were given by FluorChem 5500 software. The percentage of cleavage (C) was calculated according to Equation, where D_I, D_{II} and D_{III} are the IDVs of Form I (supercoil form), Form II (nicking form) and Form III (linear form), respectively.

$$C = \frac{D_{II} + 2D_{III}}{D_I + D_{II} + D_{III}} \quad (2)$$

Scavenger measurements of hydroxyl radical ($OH^{\bullet}$)

The hydroxyl radical ($OH^{\bullet}$) in aqueous media was generated by the Fenton system (Li et al. 2008). The solution of the tested complexes was prepared with DMF. The 4 ml of assay mixture contained following reagents: safranin (28.5 μM), EDTA–Fe(II) (100 μM), H_2O_2 (44.0 μM), the tested compounds (0.5–3.5 μM) and a phosphate buffer (67 mM, pH = 7.4). The assay mixtures were incubated at 37°C for 30 min in a water bath. After which, the absorbance was measured at 520 nm. All the tests were run in triplicate and expressed as the mean. A_i was the absorbance in the presence of the tested compound; A_0 was the absorbance in the absence of tested compounds; A_c was the absorbance in the absence of tested compound, EDTA–Fe(II), H_2O_2. The suppression ratio (η_a) was calculated on the basis of $(A_i - A_0)/(A_c - A_0) \times 100\%$.

Results and discussion

Synthesis and characterization

The ligand DBHIP was prepared through condensation of 1,10-phenanthroline-5,6-dione with 3,5-dibromo-4-hydroxyphenylaldehyde using a similar method to that described by Steck and Day (1943). The corresponding ruthenium(II) complexes were synthesized by direct reaction of DBHIP with the appropriate precursor complexes in ethanol and purified by column chromatography.

For each of the two complexes, two sets of ^1H NMR signals were observed. One set corresponds to the ancillary ligands, dmb or dmp, and the other set corresponds to the intercalative ligand, DBHIP. In

each of the two complexes, the chemical shifts of protons on the nitrogen atom of imidazole ring were not observed, probably because the protons are very active and easy to be exchanged quickly between the two nitrogens of the imidazole ring in solution.

The complexes were also characterized by electrospray mass spectrometry (ES–MS). In the ES–MS spectra for the complexes **1** and **2**, as expected, the intense signals for $[M–2ClO_4–H]^+$ and $[M–2ClO_4]^{2+}$ were observed, the obtained molecular weights are consistent with the expected values.

DNA-binding of ruthenium(II) complexes

Viscosity measurements

The viscosity of a DNA solution was sensitive to the addition of organic drugs and metal complexes bound by intercalation. And relative viscosity measurements have proved to be a reliable method for the assignment of the mode of binding compounds to DNA. In general, intercalation of a ligand into DNA is known to cause a significant increase in the viscosity of a DNA solution due to an increase in the separation of the base pairs at the intercalation site and, hence, an increase in the overall DNA molecular length; A partial and/or nonclassical intercalation of ligand could bend (or kink) the DNA helix, reduces its effective length and, concomitantly, its viscosity (Satyanarayana et al. 1993; Waring 1965). The relative change in viscosity was measured using CT-DNA with increasing concentrations of the complexes **1** and **2**. The effects of complexes **1** and **2**, together with ethidium bromide (EB) on the relative viscosity of rod-like DNA are shown in Fig. 1. It is well-known that the DNA intercalator EB increases the viscosity of DNA with increments of the concentration. On increasing the concentration of complexes **1** and **2**, the relative viscosity of the DNA increased steadily. These behaviors are similar to that of EB. The results suggest that complexes **1** and **2** intercalate between the base pairs of CT-DNA.

Electronic absorption spectra studies

Due to the intercalative mode involving a stacking interaction between the aromatic chromophore and the DNA base pairs, the large change in absorbance can be observed while a complex binds to DNA through

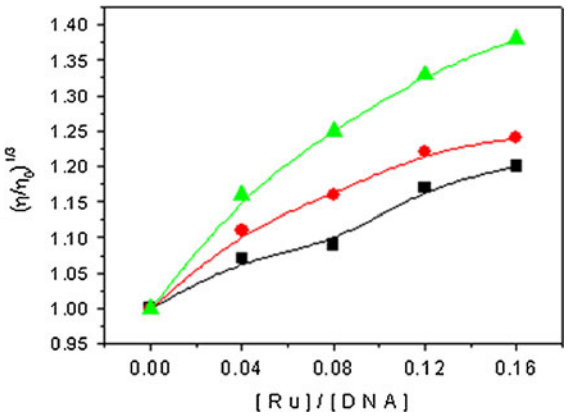


Fig. 1 Effect of increasing amounts of complexes EB (*filled triangle*), **1** (*filled circle*) and **2** (*filled square*) on the relative viscosity of calf thymus DNA at 25 (± 0.1)°C. [DNA] = 0.30 mM

intercalation. The absorption spectra of complexes **1** and **2** mainly consist of three resolved bands in the range 200–600 nm. The bands below 300 nm are attributed intraligand (IL) π–π^* transitions, the bands at the range of 300–400 nm are attributed to π–π^* transitions, and the lowest energy bands at 464 nm for **1** and 463 nm for **2** are assigned to the metal-to-ligand charge transfer (MLCT) transition.

Figure 2 shows the absorption spectra of complexes **1** and **2** in the presence of increasing concentration of DNA. As increasing the concentration of CT-DNA, the MLCT transition band of complexes **1** at 464 nm and **2** at 463 nm exhibit hypochromism of 17.5 and 16.8%, and bathochromism of 2 and 3 nm, respectively. The intrinsic constants K_b were determined by monitoring the changes of absorbance in the MLCT band with increasing concentration of CT-DNA. The values of K_b are determined to be $8.64 \pm 0.16 \times 10^4$ (s = 1.34) and $2.79 \pm 0.21 \times 10^4$ (s = 2.17) M^{-1} for **1** and **2**, respectively. These values are comparable to that of complexes $[Ru(phen)_2(PBIP)]^{2+}$ (5.91×10^4 M^{-1}) (Xu et al. 2004) and $[Ru(bpy)_2PIP]^{2+}$ ($4.7 \pm 0.2 \times 10^5$ M^{-1}) (Wu et al. 1997), but smaller than that of complexes $[Ru(bpy)_2(dppz)]^{2+}$ (4.90×10^6 M^{-1}) (Friedman et al. 1990).

Thermal denaturation studies

When the temperature in solution increases, the double-stranded DNA gradually dissociates to single strands, and generates a hyperchromic effect on the

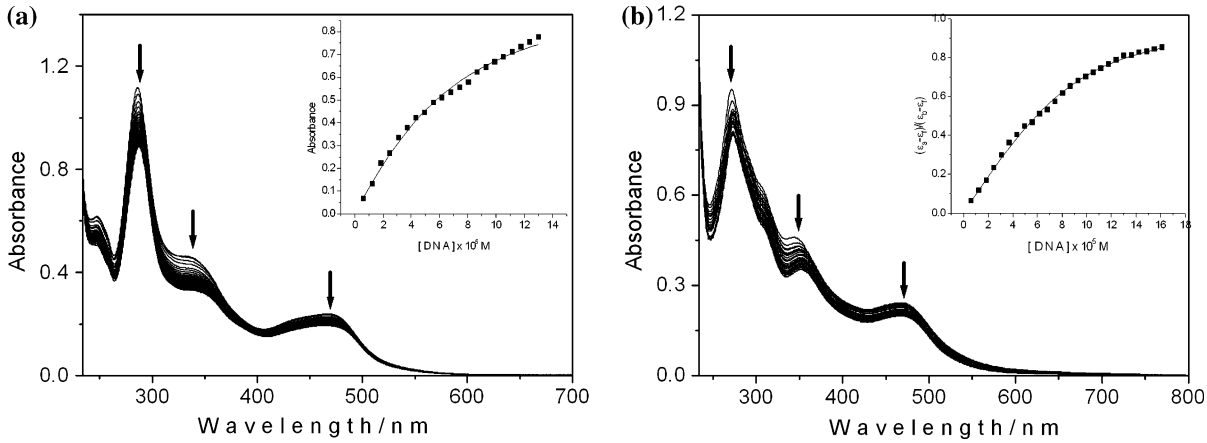


Fig. 2 Absorption spectra of complexes **1** (**a**) and **2** (**b**) in Tris–HCl buffer upon addition of CT-DNA. [Ru] = 20 μM. *Arrow* shows the absorbance change upon the increase of DNA concentration. Plots of $(\varepsilon_a - \varepsilon_f)/(\varepsilon_b - \varepsilon_f)$ versus [DNA] for the titration of DNA with Ru(II) complexes

absorption spectra of DNA base pairs ($\lambda = 260$ nm). A large change in the T_m of DNA is an indication of strong interaction between the complexes and DNA. The interactions of all ruthenium(II) complexes with CT-DNA are characterized by measuring the effects on melting temperature of DNA (Fig. 3). In the absence of any added complexes, the thermal denaturation determined for DNA gave a T_m of 61.30 ± 0.54°C under our experimental conditions. The observed melting temperatures in the presence of complexes **1** and **2** were 67.30 ± 0.50 and 66.99 ± 0.53°C, respectively. The increases in T_m of the two Ru(II) complexes (the ΔT_m is 6.00 and 5.69°C for **1** and **2**) are comparable to that observed for Ru(II) complexes (Chen et al. 2008; Tan et al. 2009; Liu et al. 2006) and lend strong support for intercalation into the helix of DNA. The experimental results also indicate that complex **1** exhibits larger DNA-binding affinity than complex **2** does.

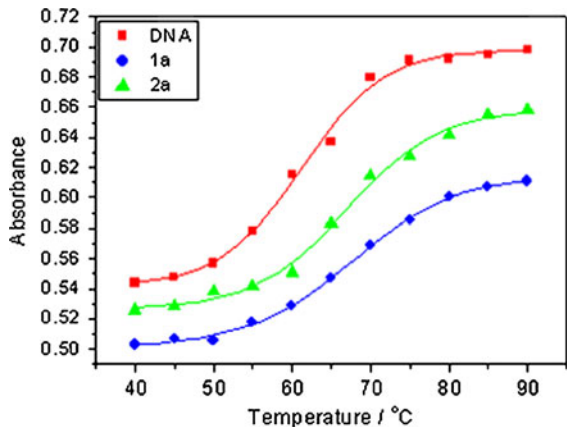


Fig. 3 Thermal denaturation of CT-DNA in the absence (*filled square*) and presence of complexes **1** (*filled circle*) and **2** (*filled triangle*). [Ru] = 32 μM, [DNA] = 80 μM

DNA photocleavage

The cleavage reaction on plasmid DNA induced by ruthenium(II) complexes can be monitored by agarose gel electrophoresis. When circular plasmid DNA is subject to electrophoresis, relatively fast migration will be observed for the intact supercoiled form (Form I); If scission occurs on one strand (nicked), the supercoiled will relax to generate a slower-moving open circular form (Form II). If two strands are cleaved, a linear form (Form III) that migrates between Form I and Form II will be generated (Barton and Raphael 1984). Figure 4a shows gel electrophoresis separation of pBR 322 DNA after incubation with different concentrations of Ru(II) complexes and irradiation at 365 nm for 30 min. No obvious DNA cleavage was observed for controls in which complex was absent, or incubation of the plasmid with the Ru(II) complex in dark (data not presented). With increasing concentration of the Ru(II) complexes, the amount of Form I of pBR 322 DNA diminishes gradually, whereas that of Form II increase. Under the same experimental conditions, complex **1** exhibits more effective DNA cleavage activity than complex **2**. The different cleaving

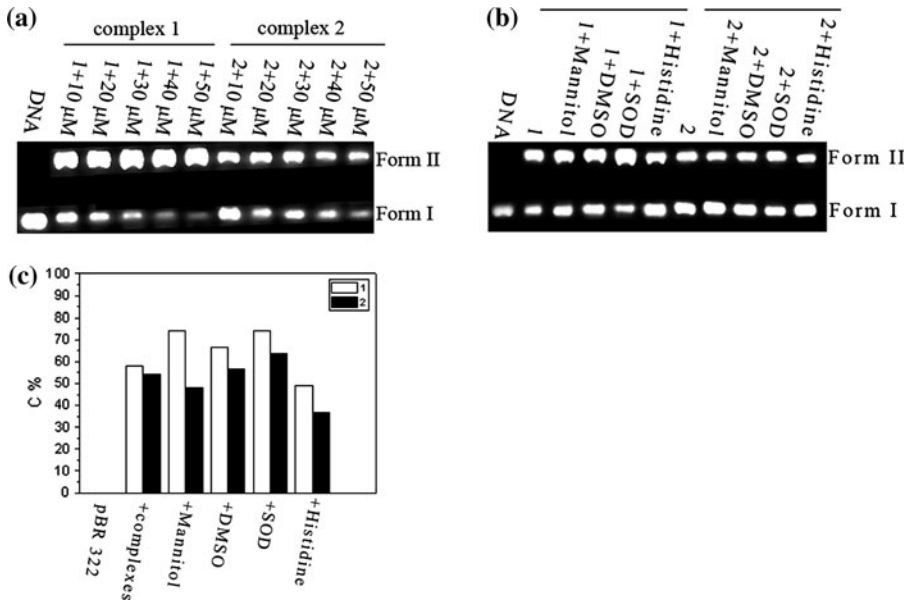


Fig. 4 **a** Photoactivated cleavage of pBR 322 DNA in the presence of different concentrations of Ru(II) complexes after irradiation at 365 nm for 30 min. **b** Photoactivated cleavage of supercoiled pBR 322 DNA by complexes **1** and **2** (20 μM) in the absence and presence of different inhibitors [100 mM mannitol, 200 mM dimethyl sulfoxide (DMSO), 1,000 U ml^{-1} superoxide dismutase (SOD), 1.2 mM distidine] after irradiation at 365 nm for 30 min. **c** Bar diagram representation of the effect of inhibitors on the photoactivated cleavage activity of complexes **1** and **2**

efficiency may be ascribed to the different DNA-binding affinity of two Ru(II) complexes.

To explore the DNA photocleavage mechanism, control experiments were carried out for complex **1** and **2** in the presence of the D-mannitol, dimethylsulfoxide (DMSO), superoxide dismutase (SOD) enzyme and histidine (Fig. 4b). The cleavage of the plasmid was not inhibited in the presence of hydroxyl radical ($OH^{\bullet}$) scavengers such as mannitol (Cheng et al. 1993) and dimethylsulfoxide (Lesko et al. 1980), which indicated that hydroxyl radicals were not likely to be the cleaving agent. In the presence of superoxide dismutase (SOD), a facile superoxide anion radical ($O_2^{\bullet-}$) quencher, the cleavage was obviously improved. In the presence of the singlet oxygen ($^1O_2$) scavenger histidine (Nilsson et al. 1972), the DNA cleavage of the plasmid by complexes **1** and **2** was inhibited, suggesting that $^1O_2$ is likely to be the reactive species responsible for the cleavage reaction. Figure 4c shows the bar diagram representation of the percentage of cleavage (C) for complexes **1** and **2**.

Retardation of pGL 3 plasmid DNA

DNA condensation into contact structures has been received considerable attention to understand the

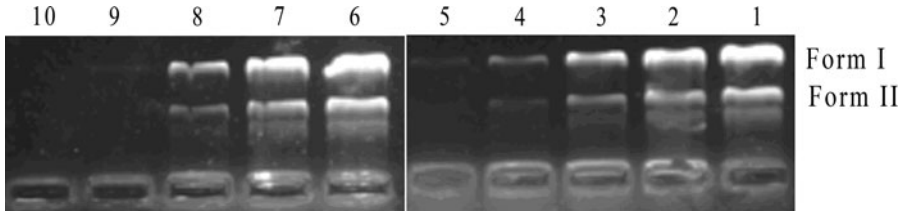


Fig. 5 Agarose gel electrophoresis retardation of pGL 3 plasmid DNA in the presence different concentrations of complexes **1** and **2**. **1**: *Lane (1)* DNA alone, *(2)* 0.33 mM; *(3)* 1 mM; *(4)* 1.67 mM; *(5)* 2.33 mM. **2**: *(6)* DNA alone, *(7)* 0.33 mM; *(8)* 1.0 mM; *(9)* 1.67 mM; *(10)* 2.33 mM. [DNA] = 0.5 μg

Table 1 The IC_{50} values for DBHIP, **1** and **2** against selected cell lines

Compounds	IC_{50} (μM)				
	BEL-7402 (24 h)	BEL-7402 (48 h)	C-6	HepG-2	MCF-7
DBHIP	15.26 ± 4.11	11.18 ± 3.87	15.05 ± 4.45	10.65 ± 3.62	18.45 ± 2.89
1	73.51 ± 3.54	62.19 ± 5.57	52.68 ± 3.43	172.38 ± 3.52	170.20 ± 4.37
2	21.72 ± 3.12	17.11 ± 3.18	19.63 ± 3.54	14.09 ± 4.81	22.58 ± 3.69
Cisplatin	30.25 ± 3.64	20.12 ± 2.35	10.26 ± 2.78	26.25 ± 3.12	11.34 ± 2.38

mechanism of uptake of gene vectors in living cells. It is well known that polyamine can effectively condense DNA (Huang et al. 2006; Vijayanathan et al. 2001; Sarkar et al. 2009). However, the studies of small molecules to condense DNA have been less paid attention. Ji and co-workers reported the Ru(II) complexes $[Ru(bpy)_2(PIPSH)]^{2+}$ and $[Ru(phen)_2(PIPSH)]^{2+}$ (Sun et al. 2009) can effectively condense DNA at a concentration of 80 μM. The abilities of complexes **1** and **2** to condense pGL 3 DNA were

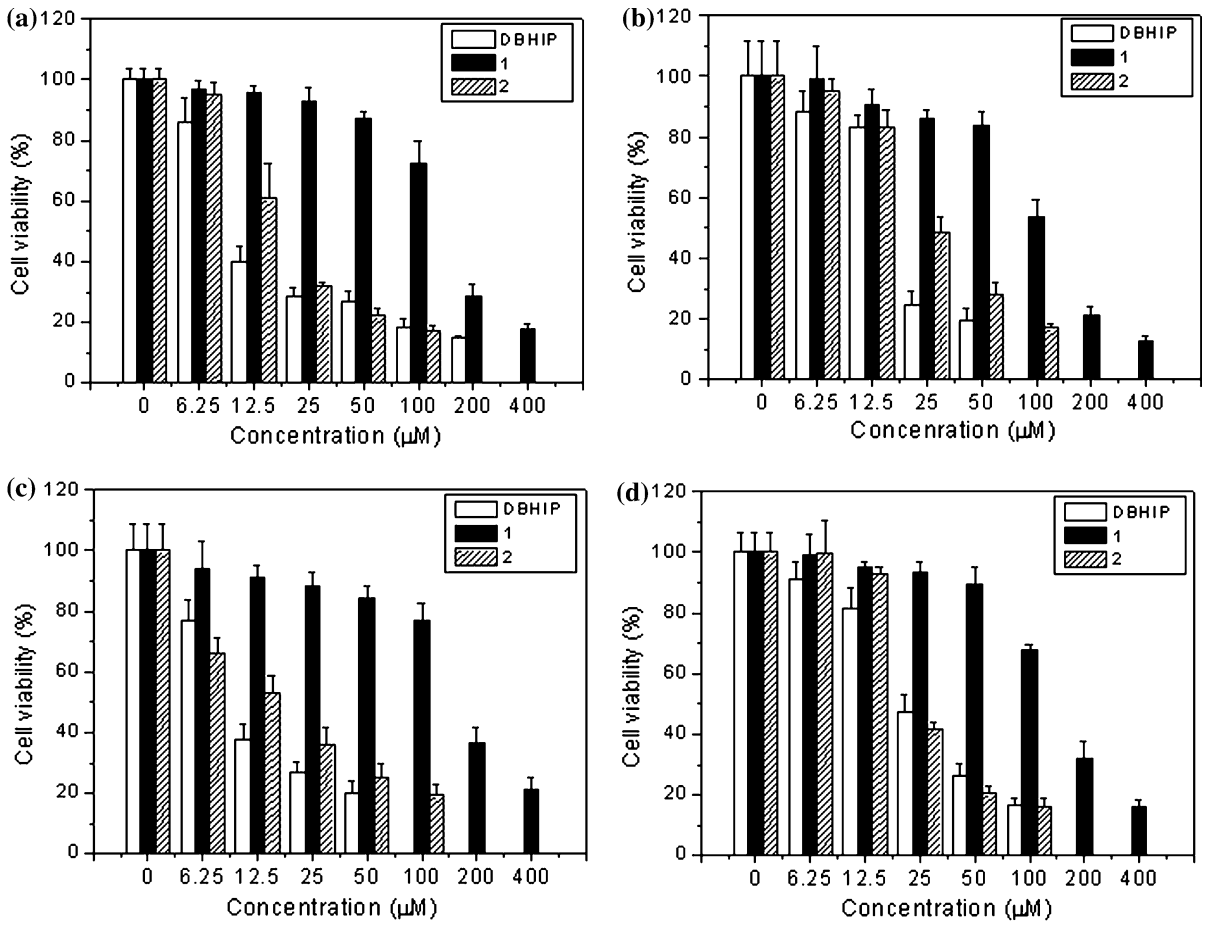
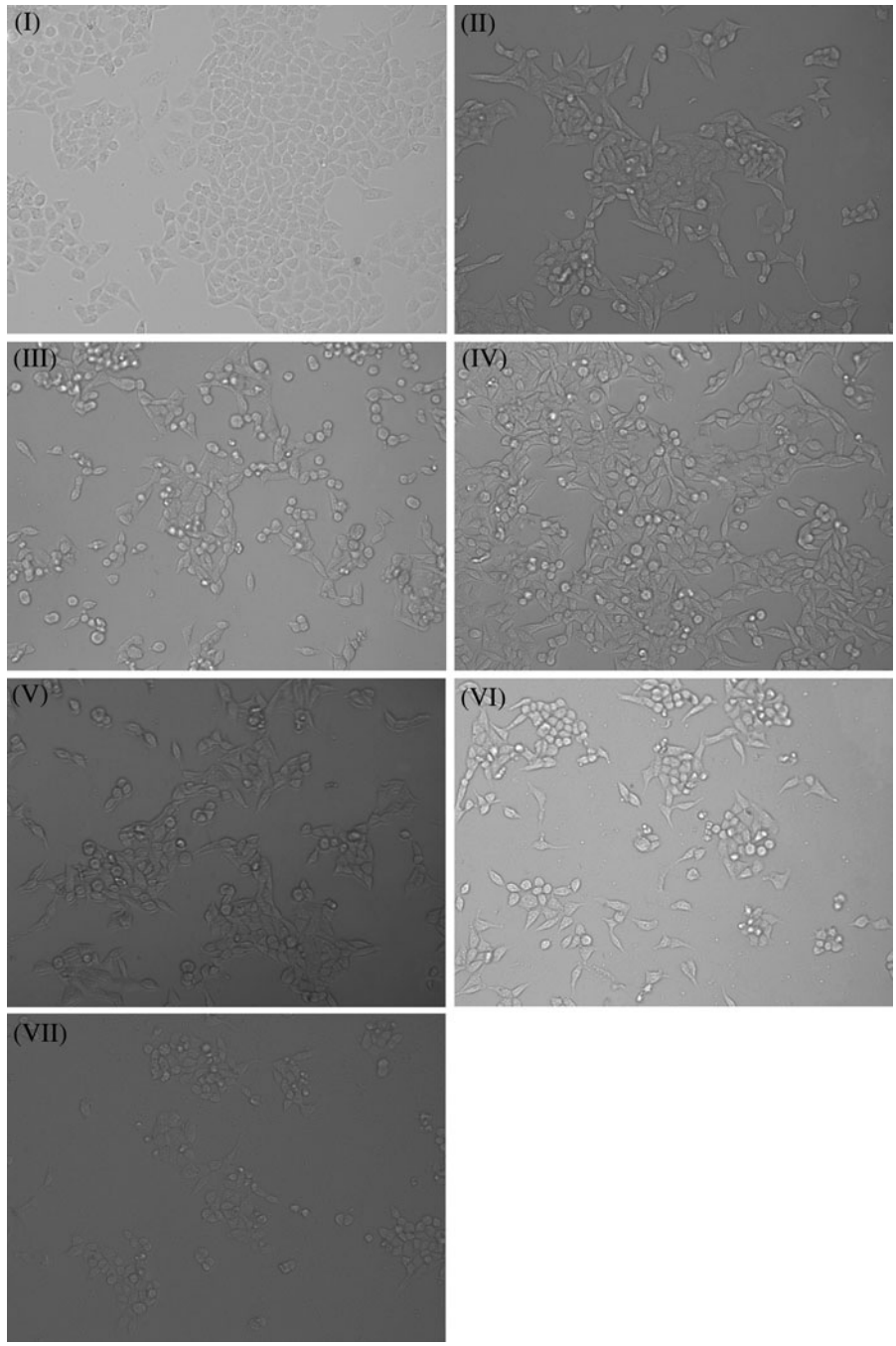


Fig. 6 **a** Cell viability of DBHIP, **1** and **2** on tumor BEL-7402 (*a*), C-6 (*b*), HepG-2 (*c*) and MCF-7 (*d*) cell proliferation in vitro. Each data point is the mean ± standard error obtained from at least three independent experiments. **b** Light microscopy of C-6 cell line after treated for 48 h in the absence I (control) and presence of different concentrations complex **1**: II: [Ru] = 6.25 μM; III: [Ru] = 12.5 μM; IV: [Ru] = 25 μM; V: [Ru] = 50 μM; VI: [Ru] = 100 μM; VII: [Ru] = 200 μM. Cell was observed using an inverted microscope and photographed by a digital camera

Fig. 6 continued



assessed by gel retardation assay. Figure 5 shows that the concentrations of complexes **1** and **2** are 0.33 and 1.0 mM, complexes **1** and **2** can not condense the DNA. However, at the high concentrations of 2.33 mM, the effects of condensation of DNA by complexes **1** and **2** were obviously observed.

In vitro cytotoxicity

The cytotoxicity of DBHIP and complexes **1** and **2** was assayed on BEL-7402 (hepatocellular), C-6 (Rat glioma), HepG-2 (hepatocellular) and MCF-7 (breast cancer) by cell survival after 48 h of exposure to the

desired concentration (6.25, 12.5, 25, 50, 100, 200 and 400 μM) using the MTT assay. The IC_{50} values were calculated after 48 h of incubation with ligand DBHIP, complexes **1**, **2** together with cisplatin in different concentrations and are listed in Table 1. Table 1 show that the IC_{50} values are time-dependent. Though none of the cytotoxicity of ligand and complexes is as high as that of cisplatin, it is clear that the complex **2** is more sensitive against selected four cell lines than complex **1**. The IC_{50} values for complex **2** (17.11 ± 3.18, 19.63 ± 3.54, 14.09 ± 4.81 and 22.58 ± 3.69 on BEL-7402, C-6, HepG-2 and MCF-7, respectively) are far lower than that of complex **1** (62.19 ± 5.57, 52.68 ± 3.43, 172.38 ± 3.52 and 170.20 ± 4.37), under the same condition, complex **2** show higher activity on four tumor cell lines than complex **1**. Comparing the cytotoxicity of ligand and complex **2**, we can conclude that the antitumor activities against selected four tumor cell lines have been slightly weakened when ligand bonds with the Ru(II) metal center to form complexes. Figure 6a show the results of cell viability assay for different compounds in BEL-7402, C-6, HepG-2 and MCF-7 cell lines. The cell viability was found to be concentration dependent, and increasing the concentrations of DBHIP, complexes **1** and **2**, an obvious decrease was observed for the cell viability. Figure 6b (I–VII) was the antiproliferative activity for complex **1** against tumor cells of C-6, with increasing the concentration of complex **1** (from II to VII), the proliferation of tumor cells of C-6 was effectively inhibited.

Apoptosis assay

Apoptosis induced by compounds is one of the considerations in drug development. Apoptosis assay were carried out with a staining method utilizing acridine orange AO) and ethidium bromide (EB). The AO/EB staining is sensitive to DNA and was used to assess changes in nuclear morphology. Apoptotic and necrotic cells can be distinguished from one another

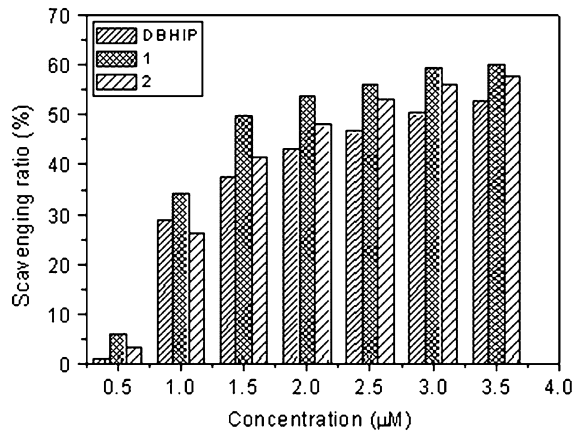


Fig. 8 Scavenging effect of the ligand DBHIP and complexes **1** and **2** on hydroxyl radicals. Experiments were performed in triplicate

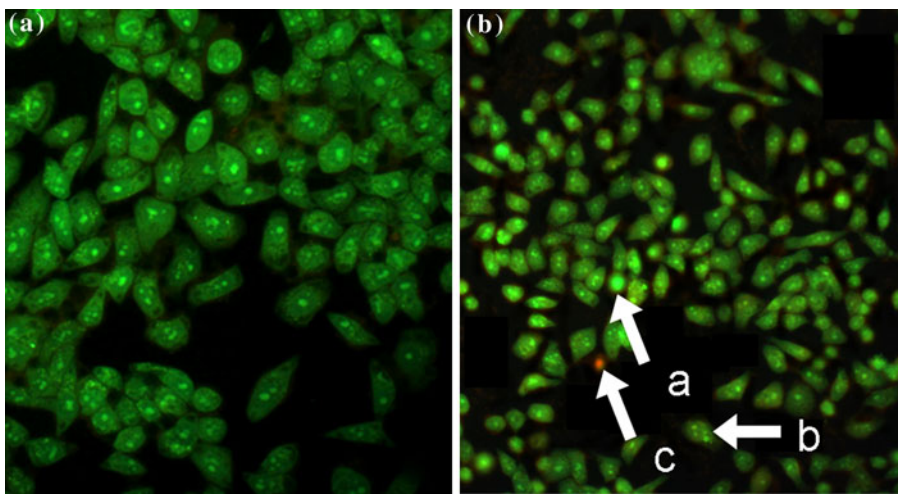


Fig. 7 BEL-7402 cell were stained by AO/EB and observed under fluorescence microscopy. BEL-7402 cell without treatment (**a**) and in the presence of complex **2** (**b**) incubated at 37°C and 5% CO_2 for 24 h. Cells in *a*, *b* and *C* are apoptotic, living and necrotic cells, respectively

Table 2 The influence of investigated compounds for $OH^{\bullet}$

Compounds	Average inhibition (%) for OH (μM)							Equation	IC_{50} (μM)	R^2
	0.5	1.0	1.5	2.0	2.5	3.0	3.5			
DBHIP	1.05	28.85	37.52	43.05	46.64	50.37	52.92	$Y = 47.23 + 58.97x$	1.11	0.9801
1	6.13	34.23	49.63	53.81	56.05	59.34	60.24	$Y = 56.43 + 63.49x$	0.79	0.9678
2	3.44	26.31	41.56	47.98	53.21	56.20	57.85	$Y = 52.11 + 65.74x$	0.93	0.9900

IC_{50} values were calculated from regression lines where: x was log of the tested compound concentration and Y was percent inhibition of the tested compounds. When the percent inhibition of the tested compounds was 50%, the tested compound concentration was IC_{50}. R^2 is the correlation coefficient

using fluorescence microscope after being stained with AO/EB solution, apoptotic cells showed apoptotic features such as nuclear shrinkage, chromatin condensation. In the absence of complexes **1**, the living cells were stained bright green in spots (Fig. 7a). However, after treatment with complex **1**, green apoptotic cells containing apoptotic bodies, as well as red necrotic cells, were also observed (Fig. 7b). Similar result for complex **2** was also observed.

Antioxidant activity studies

Since complexes **1** and **2** showed good DNA-binding affinity, it was considered worthwhile to study other potential aspects of these complexes such as antioxidant activity. The hydroxyl radical ($OH^{\bullet}$) was one of the most reactive products of reactive oxygen species (ROS), which could result in cell membrane disintegration, membrane protein damage, DNA mutation and further initiate or propagate the development of many diseases. The antioxidant activities on hydroxyl radical of complexes **1** and **2** together with ligand DBHIP were investigated. As shown in Fig. 8 and Table 2. The inhibitory effect of the ligands DBHIP and their Ru(II) complexes **1** and **2** on $OH^{\bullet}$ was concentration-dependent and the suppression ratio increased with increasing of sample concentration in the range of 0.5–3.5 μM. The suppression ratio against $OH^{\bullet}$ valued from 1.05 to 52.92% for DBHIP, 6.13 to 60.24% for complex **1** and 3.44 to 57.85% for complex **2**. The antioxidant activity against hydroxyl radical of complex **1** ($IC_{50} = 0.79$ μM) is higher than that of complex **2** ($IC_{50} = 0.93$ μM) under the same experimental condition. It is clear that the hydroxyl radical scavenging activity can be enhanced when ligand ($IC_{50} = 1.11$ μM) bonds Ru(II) metal center to form complexes. The information obtained from the present work would be helpful to develop new potential antioxidants and new therapeutic reagents for some diseases.

Conclusion

A new ligand DBHIP and its two ruthenium(II) complexes $[Ru(dmb)_2(DBHIP)]^{2+}$ and $[Ru(dmb)_2(DBHIP)]^{2+}$ were successfully prepared and characterized by elemental analysis, ES–MS, IR, ^1H NMR. These complexes have high DNA-binding affinity and excellent antioxidative abilities. The results show they interact with DNA by intercalative mode. Complex **2** shows high cytotoxicity against the selected four tumor cell lines and the two complexes can effectively induce the apoptosis of BET-7402 cells. Upon irradiation at 365 nm, complexes **1** and **2** can also cleave plasmid DNA. At high concentration, complexes **1** and **2** can effectively condense the pGL 3 DNA.

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