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# Evaluation of DNA binding, DNA cleavage, protein binding and *in vitro* cytotoxic activities of bivalent transition metal hydrazone complexes

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#### ABSTRACT

Divalent Co, Ni and Cu hydrazone complexes containing [N'-(phenyl(pyridine-2-yl)methylidene) benzohydrazide] ligand were synthesised and characterised. Interactions of these complexes with DNA revealed an intercalative mode of binding between them. Further, all the hydrazone chelates showed moderate ability to cleave pUC19 DNA. Synchronous fluorescence spectra proved that the interaction of metal complexes with bovine serum albumin (BSA) resulted in a conformational change of the latter. Assay on the cytotoxicity of the above complexes against HeLa tumor cells and NIH 3T3 normal cells revealed that the complexes are toxic only against tumor cells but not to normal cells. In all the biological assays, the complex with copper ion as the metal center showed enhanced activities than the other two. © 2011 Elsevier Masson SAS. All rights reserved.

#### 1. Introduction

Transition metal complexes containing Schiff base ligands are of interest to model the active sites of biologically important molecules. In particular, Schiff base derivatives of hydrazones exhibit various bioactivities and play vital role in certain physiological functions as well. Hydrazones and their metal complexes exhibit wide range of biological and pharmaceutical activities that includes antimicrobial, antituberculostatic, anticancer and antioxidant behavior. In addition, hydrazones also serve as an excellent polydentate chelating agent capable of forming coordination complexes with variety of both transition and inner transition metal cations. Metal complexes derived from hydrazides of carboxylic acids have been extensively investigated due to the high physiological activities of the free ligands and the presence of a chelatophore group of donor atoms in the coordination sphere [1–4].

Survey of literature demonstrates that interest on the design of novel transition metal complexes capable of binding and cleaving duplex DNA with high sequence and structure selectivity [5–7] increases continuously. Since DNA is particularly sensitive to oxidative cleavage, vast majority of the studies on metallonucleases have focused mainly on the molecules that cleave DNA oxidatively. The worldwide real success of *cisplatin* as an anticancer drug has stimulated interest in the synthesis of a wide range of transition metal complexes with potential anticancer activity [8]. Such complexes have been found to be useful for the design and development of compounds that can restrict certain enzymes. They also serve as DNA footprinting agents due to their potential to bind to DNA by a variety of interactions and thereby cleave the duplex by virtue of their intrinsic chemical, electrochemical and photochemical properties [9-12]. Hence, it is clear that the nature of the ligand as well as the identity and oxidation state of the metal play pivotal roles in their interaction with DNA molecule. Therefore, clear understanding on such interactions will facilitate the design and development of new drugs for DNA recognition, cleavage, DNA secondary structure probes and photocleavage reagents with high selectivity and efficiency [13,14].

Serum albumins are the most abundant proteins in plasma that have many physiological functions [15–17]. Particularly, they contribute to control osmotic blood pressure and maintenance of blood pH [18]. The most outstanding property of albumins is their ability to bind reversibly a large variety of ligands [19–21]. Reagents that react with protein chains are extremely useful in biochemistry and biology [22,23].

Based on the above facts, we herein report on the synthesis of  $[Co(L)_2]$  (1),  $[Ni(L)_2]$  (2), and  $[Cu(L)_2]$  (3) complexes containing



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N'-(phenyl(pyridine-2-yl)methylidene)benzohydrazide (HL) ligand. Transition metal ions such as Ni(II), Co(II) and Cu(II) were selected in this study due to their potential to enhance the biological activities of the free hydrazone ligands [24-26]. Single crystal X-ray structures of the complexes  $[Ni(L)_2](2)$  and  $[Cu(L)_2](3)$ , were determined in order to discern the mode of coordination of the hydrazone ligand (HL) to the transition metal ions. The observed tridentate coordination mode of the hydrazone ligands rendered them ideal candidates for bis-chelation to metal ions that prefer an octahedral coordination geometry. DNA binding abilities and DNA nuclease activities of the complexes 1-3 carried out with calfthymus (CT-DNA) and pUC19 DNA proved their ability to bind and cleave the DNA. The results of protein binding experiments carried out with bovine serum albumin (BSA) were also presented for all the newly synthesised complexes. The *in vitro* cytotoxic activities of complexes 1, 2 and 3 were tested against HeLa tumor cells and NIH 3T3 normal cells in order to assess whether these compounds are able to inhibit the proliferation of tumor cells without adverse effects on healthy cells.

#### 2. Chemistry

The hydrazone ligand (HL) was prepared by refluxing equimolar quantities of 2-benzoylpyridine and benzhydrazide in ethanol for 5 h. The resulting precipitate was filtered off, washed with cold ethanol and diethyl ether. The metal hydrazone complexes **1-3** were prepared by refluxing equimolar solutions of the respective starting complexes [CoCl<sub>2</sub>(PPh<sub>3</sub>)<sub>2</sub>], [NiCl<sub>2</sub>(PPh<sub>3</sub>)<sub>2</sub>] and [CuCl<sub>2</sub>(PPh<sub>3</sub>)<sub>2</sub>] [27,28] and ligand (HL) in methanol as outlined in Schemes 1 and 2. The resulting products were subsequently washed with methanol and dried under vacuum.

#### 3. Pharmacology

The hydrazone complexes **1**, **2** and **3** were evaluated for their DNA binding, DNA cleavage, protein binding, and cytotoxic activities. Binding abilities of all hydrazone complexes with CT-DNA and BSA, were determined using UV–visible, fluorescence and synchronous fluorescence spectroscopic methods. The cleavage efficiencies were measured by determining the ability of each complex to convert the supercoiled (SC) form of DNA to the nicked circular (NC) form. The proportion of DNA in each fraction was estimated quantitatively on the basis of the intensities of the bands using the BIORAD Gel Documentation System [29]. Cytotoxicity of the complexes were assessed against HeLa and NIH 3T3 cells by MTT assay [30].



Scheme 1. Synthesis of hydrazone ligand (HL).

#### 4. Results and discussion

#### 4.1. X-ray crystallographic studies

#### 4.1.1. Crystallographic study of [Ni(L)<sub>2</sub>] (2)

An ORTEP representation of the structure of  $[Ni(L)_2](2)$  inclusive of the atom-numbering scheme is shown in Fig. 1 and a selection of bond lengths and bond angles is presented in Table 1. The Ni(II) center of complex 1 is coordinated to two tridentate hydrazone ligands in a distorted octahedral fashion. The dimensions of the two coordinated ligands are very similar and both of the ligands adopt the enolate form (Scheme 2). The crystals of 2 are triclinic (space group *P*-1) with Z = 2 and unit cell dimensions a = 10.7892(6) Å, b = 12.3564(6) Å, c = 12.6713(7) Å; V = 1534.21(14) Å<sup>3</sup>; and  $D_c = 1.427 \text{ Mg/m}^3$ . The observed torsion angles of  $-179.2(2)^\circ$  for C5-C6-N2-N3 and -178.0(2)° for C22-C23-N5-N6 indicate that the ligand adopts the E conformation upon coordination [31]. The coordination environment around the nickel center is distorted octahedral and the two oxygen and four nitrogen atoms exhibit ligand-metal-ligand bite angles of 78.78(7)° [N1-Ni1-N2], 77.15(6)° [N2-Ni1-O1], 76.76(6)° [N5-Ni1-O2] and 78.47(7)° [N4-Ni1-N5]. The two central bonds Ni1-N1 [2.092(2) Å] and Ni1–O1 [2.061(2) Å] are comparable in length to those of the basal planar bonds Ni1-O2 [2.086(1) Å], Ni1-N5 [1.998(2) Å], Ni1-N4 [2.111(2) Å], Ni1–N2 [2.000(2) Å]. The trans angle of N2–Ni1–N5 [172.80(7)°] is close to the ideal value of 180°. However, the other trans angles, namely N4-Ni1-O2 [154.69(6)°] and N1-Ni1-O1 [155.70(6)°], are constrained by the meridional ligands. The Ni(II) center is coordinated to four fused five-membered chelate rings and the bicyclic chelate system {Ni102C32N6N5C25C24N4} and its counterpart {Ni1O1C13N3N2C6C5N1} such that the overall geometry at this center is distorted octahedral.

#### 4.1.2. Crystallographic study of $[Cu(L_2)]$ (3)

The crystal structure of complex **3** is displayed in Fig. 2 and a selection of pertinent crystallographic data is presented in Table 1. Selected bond distances and angles are listed in Table 2. Akin to complex **2**, complex **3** features the coordination of two (HL) ligands to the metal ion thus forming four fused five-membered chelate rings. The crystals of **3** are triclinic (space group *P*-1) with *Z* = 2 and unit cell dimensions a = 10.660(3) Å, b = 12.262(3) Å, c = 12.871(3) Å; V = 1540.9(7) Å<sup>3</sup>;  $D_c = 1.432$  Mg/m<sup>3</sup>.

The Cu(II) coordination geometry of **3** is distorted octahedral. The basal plane comprises an imine nitrogen (N2), an enolate oxygen (O2), an imine nitrogen (N5) and a pyridyl nitrogen (N4), and the pyridyl nitrogen (N1) and enolate oxygen (O1) atoms occupy the apical positions thus forming an overall distorted octahedral coordination geometry. The two central bonds Cu1-N1 [2.054(2) Å] and Cu1–O1 [2.023(2) Å] are comparable in length to those of the basal planar bonds Cu1–N2 [1.948(2) Å], Cu1–O2 [2.215(1) Å], Cu1–N5 [2.011(2) Å] and Cu1–N4 [2.320(2) Å]. Overall, the Cu(II) complex adopts a tetragonally elongated structure such that the trans pair of bonds Cu1-N1 and Cu1-O1 are longer than those of the remaining four bonds Cu1-N2, Cu1-O2, Cu1-N5 and Cu1-N4. This distortion is a consequence of the Jahn-Teller effect that is operative in the case of d<sup>9</sup> transition metal complexes. Thus, one *trans* pair of coordinate bonds is elongated while the remaining four are shortened. Collectively, the bond angles N1-Cu1-N2 [80.24(7)°], N2-Cu1-O1 [78.62(6)°], O2-Cu1-N5 [74.89(6)°] and N4-Cu1-N5 [75.45(7)°] confirm that the coordination geometry is distorted from that of a perfect octahedron. The [N2-Cu1-N5] trans bond angle is 172.48(7)°. However, the remaining *trans* bond angles of 158.68(6)° and 149.42(6)° for [N1-Cu1-O1] and [N4-Cu1-O2], respectively imply constraints within the meridional ligands. Overall, complex 3 adopts a distorted octahedral geometry.



Scheme 2. Synthesis of complexes 1, 2, 3 and 3a.

Complex **3a** was isolated as a minor product in the reaction of  $[CuCl_2(PPh_3)_2]$  with the hydrazone ligand (HL) and characterised using XRD. The crystal structure (Fig. 3), unit cell parameters and bond distances for 3a were found to be in good agreement with the literature values [32].

#### 4.2. Infrared spectra

The IR spectra of the metal hydrazone complexes were compared with that of the free hydrazone ligand in the region  $4000-200 \text{ cm}^{-1}$ .





The spectrum of the free hydrazone ligand displayed the characteristic absorption bands at 3143, 1678, 1581 and 1075 cm<sup>-1</sup> due to  $v_{(N-H)}$ ,  $v_{(C=O)}$ ,  $v_{(C=N)}$  and  $v_{(N-N)}$  vibrations, respectively. The bands due to the  $v_{(N-H)}$  and  $v_{(C=O)}$  vibrations of the free ligand were absent for complexes **1-3**, thus indicating that enolization and deprotonation had taken place prior to coordination. This view was confirmed by the detection of two new bands in the ranges

Table 1			
Crystal data	and	ctructuro	rofinor

	Crysta	l data	and	structure	refinement	data
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Empirical formula	C38H28 N6 Ni O2	C38H28 Cu N6 O2
Name	C38H28 N6 Ni O2	C38H28 Cu N6 O2
Formula weight	659.37	664.20
Crystal system	Triclinic	Triclinic
Space group	P-1	P-1
Temperature	100(2) K	100(2) K
Wavelength	0.71075 Å	0.71075 Å
Unit cell dimensions		
a (Å)	10.7892(6)	10.660(3)
b (Å)	12.3564(6)	12.262(3)
c (Å)	12.6713(7)	12.871(3
α (°)	66.617(1)	67.454(8)
β(°)	83.588(2)	84.086(11)
γ(°)	82.593(2)	83.656(10)
Color	Violet	Violet
D calc (Mg/m3)	1.427	1.432
F(000)	684	686
Crystal size (mm3)	$0.18\times0.15\times0.09$	$0.17 \times 0.14 \times 0.11$
hkl limits	$14 \leq h \leq 14$ ,	$13 \leq h \leq 13$
	$-16 \leq k \leq 16$	$-15 \leq k \leq 15$
	$-16 \leq l \leq 16$	$-14 \leq l \leq 16$
θ range for data collection	3.27-27.49°	1.93–27.50°
Reflections collected	36341	26163
R indices (all data)	R1 = 0.0487,	R1 = 0.0511,
	wR2 = 0.1268	wR2 = 0.1174
Data/restraints/	6985/0/42	7050/0/424
parameters		
Independent reflections	6985 [R(int) = 0.0706]	7050 [R(int) = 0.0496]
Goodness-of-fit on F2	1.036	1.053



Fig. 2. Molecular structure of complex 3 showing the atom-numbering scheme with ellipsoid of 25% probability.

1593–1488 cm<sup>-1</sup> and 1357–1373 cm<sup>-1</sup> that are assigned to  $v_{(C=N-N=C)}$  and  $v_{(C-O)}$  stretching vibrations, respectively [33–35]. The band attributed to  $v_{(C=N)}$  stretching was shifted to lower frequencies and accompanied by a positive shift in the  $v_{(N-N)}$  stretching vibration in the range of 3–11 cm<sup>-1</sup> in comparison with that of the free ligand, thus implying that coordination involves the nitrogen atom of the azomethine group [36]. Taken collectively, the foregoing spectral data indicate that the hydrazone behaves as a monobasic tridentate (NNO) chelating ligand in each of the complexes **1–3**.

#### 4.3. Electronic spectra

The electronic spectra of complexes **1–3** were recorded in methanol solution and are consistent with an octahedral metal(II) environment. The bands observed in the range 230–289 nm are probably due to the intra-ligand  $\pi \rightarrow \pi^*$  transitions of the hydrazone ligand [37]. The band representing the imino group (343 nm)

Ta	abl	le	2	
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Selected bond lengths (Å	Å) and bond angles (°	)
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was not detected in the spectra of the three complexes due to ketolenol tautomerism of the hydrazone ligand. The bands observed at approximately 400 nm are attributable to the ligand-to-metal charge transfer (LMCT) transitions [38].

#### 4.4. Luminescent properties

The emissive properties of complexes **1–3** were recorded in methanol solution at room temperature. The emission maxima fell in the range 330–465 nm. The emission maxima of the complexes evidenced a positive shift of the order of 50–150 nm in comparison with those of the excitation maxima. The observed charge transfer luminescence for these complexes may be due to the presence of the imine functional group. The luminescence in the cases of **1–3** is attributable to the  $\pi \rightarrow \pi^*$  transitions of the N-arylmethylene-benzhydrazones [39]. The observation of a more intense blue luminescence may due to the coordination of the ligand to the metal center which increases the rigidity of the ligand and thereby reduces the loss of energy via a non-radiative pathway, thus enhancing the probability of a  $\pi \rightarrow \pi^*$  transition on the part of the ligand.

#### 4.5. Cyclic voltammetry

The electrochemical properties of the complexes 1, 2 and 3 were studied in dichloromethane solution. Tetrabutylammonium perchlorate was employed as the supporting electrolyte. Since the ligand used in this work does not undergo reversible oxidation or reduction in the potential range employed, the observed redox processes are assigned exclusively to the metals. The cyclic voltammogram for complex **2** evidenced an anodic response at +0.975 V which is believed to be due to  $Ni(II) \rightarrow Ni(III)$  oxidation. The corresponding cathodic response at +0.836 V is attributable to Ni(III)  $\rightarrow$  Ni(II) reduction. The peak-to-peak separation ( $\Delta E_P$ ) of 139 mV revealed that this redox process is quasi-reversible. This quasi-reversibility is probably due to slow adsorption and electron transfer of the complexes on to the electrode surface [40]. The cyclic voltammograms for complexes 1 and 3 evidenced irreversible peaks attributable to Co(II)  $\rightarrow$  Co(III) and Cu(II)  $\rightarrow$  Cu(III) oxidations at +0.644 V and +1.304 V, respectively. The corresponding irreversible reductions were observed at -0.331 V and -0.310 V and are assigned to  $Co(II) \rightarrow Co(I)$  and  $Cu(II) \rightarrow Cu(I)$  reductions, respectively.

The  $i_c/i_a$  ratios for cathodic and anodic sweeps were found to deviate from unity in the scan range 25–200 mVs<sup>-1</sup>. The potential difference ( $\Delta E_p = E_{pa} - E_{pc}$ ) for the first electrode couple increased with increasing scan rate. Similar observations have been reported

C38H28 N6 Ni O2		C38H28 Cu N6 O2					
Bond lengths		Bond angles		Bond lengths		Bond angles	
Ni1-N1	2.092(2)	N2-Ni1-01	77.15(6)	Cu1–N1	2.054(2)	02-Cu1-N5	74.89(6)
Ni1-N2	2.000(2)	N2-Ni1-N1	78.78(7)	Cu1-N2	1.948(2)	N5-Cu1-N1	94.42(7)
Ni1-02	2.086(1)	N5-Ni1-N1	94.84(7)	Cu1-02	2.215(1)	N1-Cu1-N2	80.24(7)
Ni1-N5	1.998(2)	N5-Ni1-O1	109.38(7)	Cu1-N5	2.011(2)	N2-Cu1-O2	110.33(6)
Ni1-N4	2.111(2)	01-Ni1-N1	155.70(6)	Cu1-N4	2.320(2)	N1-Cu1-O2	91.51(6)
Ni1-01	2.083(2)	N2-Ni1-N5	172.48(7)	Cu1-01	2.023(2)	N2-Cu1-N5	172.48(7)
		N4-Ni-O2	154.69(6)			01-Cu1-N4	88.30(6)
		02-Ni1-N5	76.76(6)			01-Cu1-N2	78.62(6)
		O2-Ni1-N1	91.12(6)			01-Cu1-N1	158.68(6)
		O2-Ni1-N2	106.47(6)			01-Cu1-02	93.23(6)
		02-Ni1-01	92.52(6)			01-Cu1-N5	106.89(6)
		N4-Ni1-N2	98.73(7)			N4-Cu1-N1	98.05(7)
		N4-Ni1-01	90.79(6)			N4-Cu1-N2	99.92(7)
		N4-Ni1-N1	96.10(7)			N4-Cu1-O2	149.42(6)
		N4-Ni1-N5	78.47(7)			N4-Cu1-N5	75.45(7)



**Fig. 3.** Molecular structure of complex **3a** showing the atom-numbering scheme with ellipsoid of 25% probability.

earlier for related complexes and attributed to the occurrence of slow chemical reactions subsequent to the electrode processes [41].

#### 4.6. DNA binding studies

#### 4.6.1. Absorption spectroscopic studies

The electronic absorption spectra of complexes **1**, **2** and **3** consist of two or three well-resolved bands in the range of 200–450 nm. The high energy absorption band appeared in the spectra of respective complexes below 300 nm are assigned to  $\pi \rightarrow \pi^*$  intraligand charge transfer transitions and the band that were found



**Fig. 4.** Electronic absorption spectra of complex **1** (25  $\mu$ M) in the absence and presence of increasing amounts of CT DNA (2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0, 22.5 and 25  $\mu$ M). Arrows show the changes in absorbance with respect to an increase in the DNA concentration (Inset: Plot of [DNA] vs [DNA]/( $\varepsilon_a - \varepsilon_f$ )).



**Fig. 5.** Electronic absorption spectra of complex **2** (25  $\mu$ M) in the absence and presence of increasing amounts of CT DNA (2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0, 22.5 and 25  $\mu$ M). Arrows show the changes in absorbance with respect to an increase in the DNA concentration (Inset: Plot of [DNA] vs [DNA]/( $\varepsilon_a - \varepsilon_f$ )).

around 400 nm are assigned to ligand-to-metal charge transfer (LMCT). Upon the addition of DNA, the above bands corresponding to complexes **1** and **2** showed significant hypochromism accompanied with a red shift. However, in the case of complex **3**, we observed the same phenomenon of hypochromism but with blue shift. These results are similar to those reported earlier for various metallointercalators [42] suggesting that the complexes used in this study showed strong binding to DNA in an intercalative mode. The electronic absorption spectra of all the complexes with or without CT-DNA are shown in Figs. 4, 5 and 6.

In order to compare quantitatively the binding strength of the complexes, the intrinsic binding constants of them with CT-DNA



**Fig. 6.** Electronic absorption spectra of complex **3** (25  $\mu$ M) in the absence and presence of increasing amounts of CT DNA (2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0, 22.5 and 25  $\mu$ M). Arrows show the changes in absorbance with respect to an increase in the DNA concentration (Inset: Plot of [DNA] vs [DNA]/( $\varepsilon_a - \varepsilon_f$ )).



**Fig. 7.** Emission spectra of DNA-EB (10  $\mu$ M), in the presence of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130 and 140  $\mu$ M of complex **1.** Arrow indicates the changes in the emission intensity as a function of complex concentration. Inset: Stern–Volmer plot of the fluorescence titration data corresponding to the complex **1**.

were obtained by monitoring the changes in lower energy bands of complexes **1**, **2** and **3** respectively, with increasing concentration of DNA using the equation:  $[DNA]/[\varepsilon_a - \varepsilon_f] = [DNA]/[\varepsilon_b - \varepsilon_f] + 1/K_b[\varepsilon_b - \varepsilon_f]$ ; where [DNA] is the concentration of DNA in base pairs, the apparent absorption coefficients  $\varepsilon_a$ ,  $\varepsilon_f$ , and  $\varepsilon_b$  correspond to  $A_{obsd}/[Complex]$ , the extinction coefficient for the complex in free solution, and the extinction coefficient for the complex in the fully bound form, respectively.  $[DNA]/[\varepsilon_b - \varepsilon_f]$  vs [DNA] gave a slope and the intercept which are equal to  $1/[\varepsilon_a - \varepsilon_f]$  and  $(1/K_b)[\varepsilon_b - \varepsilon_f]$ , respectively;  $K_b$  is the ratio of the slope to the intercept. The magnitudes of intrinsic binding constants ( $K_b$ ) were calculated to be



**Fig. 8.** Emission spectra of DNA-EB (10  $\mu$ M), in the presence of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130 and 140  $\mu$ M of complex **2.** Arrow indicates the changes in the emission intensity as a function of complex concentration. Inset: Stern–Volmer plot of the fluorescence titration data corresponding to the complex **2**.



**Fig. 9.** Emission spectra of DNA-EB (10  $\mu$ M), in the presence of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130 and 140  $\mu$ M of complex **3**. Arrow indicates the changes in the emission intensity as a function of complex concentration. Inset: Stern–Volmer plot of the fluorescence titration data corresponding to the complex **3**.

 $2.296 \times 10^5$  M<sup>-1</sup>,  $8.616 \times 10^4$  M<sup>-1</sup> and  $2.468 \times 10^5$  M<sup>-1</sup> corresponding to complexes **1**, **2** and **3** respectively. The observed value of binding constant ( $K_b$ ) revealed that the complex **3** is strongly bound with CT-DNA than the complexes **1** and **2** and the order of binding affinity is **2** < **1** < **3**.

#### 4.6.2. Competitive binding between EB and complexes for CT-DNA

Steady-state competitive binding experiments using complexes **1**, **2** and **3** as quenchers were undertaken to get further proof for the binding of the complexes to DNA. Ethidium bromide (EB) is a planar cationic dye which is widely used as a sensitive fluorescence probe for native DNA. EB emits intense fluorescent light in the presence of DNA due to its strong intercalation between the adjacent DNA base pairs. The displacement technique is based on the decrease of fluorescence resulting from the displacement of EB from a DNA sequence by a quencher and the quenching is due to the reduction of the number of binding sites on the DNA that is available to the EB. As the concentration of the metal—hydrazone complexes increases, the fluorescence spectra showed a significant shift (red) in wavelength with a reduction in the fluorescence intensity clearly indicating that the EB molecules are displaced from their DNA binding sites and are replaced by the metal complexes under investigation.

The fluorescence quenching spectra of DNA-bound EB by complexes **1**, **2** and **3** shown in Figs. 7, 8 and 9 illustrate that the



**Fig. 10.** Gel electrophoresis showing the chemical nuclease activity of the pUC19 DNA incubated at 37 °C for 1 h with different concentration of complex **3** in the presence of H<sub>2</sub>O<sub>2</sub> as an oxidizing agent: lane 1, C – Control (pUC19 DNA); lane 2, PC - pUC19 DNA + H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M); lane 3, pUC19 DNA + H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M) + complex **3** (25  $\mu$ M); lane 4, pUC19 DNA + H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M) + complex **3** (35  $\mu$ M); lane 5, pUC19 DNA + H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M) + complex **3** (35  $\mu$ M); lane 5, pUC19 DNA + H<sub>2</sub>O<sub>2</sub> (50  $\mu$ M) + complex **3** (55  $\mu$ M).





**Fig. 12.** Gel electrophoresis showing the chemical nuclease activity of the pUC19 DNA incubated at 37 °C for a period of 1 h, 2 h, 3 h and 4 h at a concentration of 35  $\mu$ M of complex **3** in the presence of H<sub>2</sub>O<sub>2</sub> as an oxidizing agent: lane 1, C – Control (pUC19 DNA); lane 2, PC - pUC19 DNA + H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M); lane 3, pUC19 DNA + H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M) + complex **3** (35  $\mu$ M) at 1 h incubation; lane 4, pUC19 DNA + H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M) + complex **3** (35  $\mu$ M) at 2 h incubation; lane 5, pUC19 DNA + H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M) + complex **3** (35  $\mu$ M) at 3 h incubation; lane 6, pUC19 DNA + H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M) + complex **3** (35  $\mu$ M) at 3 h incubation.

**Fig. 11.** Gel electrophoresis showing the chemical nuclease activity of the pUC19 DNA incubated at 37 °C for 2 h with different concentration of complex **3** in the presence of H<sub>2</sub>O<sub>2</sub> as an oxidizing agent: lane 1, C – Control (pUC19 DNA); lane 2, PC - pUC19 DNA + H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M); lane 3, pUC19 DNA + H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M) + complex **3** (25  $\mu$ M); lane 4, pUC19 DNA + H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M) + complex **3** (35  $\mu$ M); lane 5, pUC19 DNA + H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M) + complex **3** (45  $\mu$ M); lane 6, pUC19 DNA + H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M) + complex **3** (55  $\mu$ M).

quenching of EB bound to DNA by the test complex is in good agreement with the linear Stern-Volmer equation. The ratio of the slope to the intercept obtained by plotting  $I_0/I$  vs [Q] yielded the value of  $K_a$  corresponding to the three complexes as 7.487  $\times$  10<sup>3</sup> M<sup>-1</sup>,  $4.959 \times 10^3$  M<sup>-1</sup> and  $2.074 \times 10^4$  M<sup>-1</sup> respectively. These values suggested that the complex 3 showed higher quenching efficiency than the other complexes **1** and **2**. Further, the  $K_{app}$  values obtained for the three different complexes using the equation  $K_{\text{EB}}$  [EB] =  $K_{\text{app}}$ [complex] (where the complex concentration has the value at a 50% reduction of the fluorescence intensity of EB and  $K_{\rm EB} = 1.0 \times 10^7 \, {\rm M}^{-1}$ , ([EB] = 10  $\mu M$ )) were 6.842  $\times$   $10^5$   $M^{-1}$ , 5.832  $\times$   $10^5$   $M^{-1}$  and  $1.596 \times 10^6 \,\mathrm{M}^{-1}$ . The binding constant ( $K_b$ ) values obtained from the plot of log[(F\_0-F)/F] vs log [Q] were found to be 2.564  $\times$   $10^4$   $M^{-1}$  $1.796 \times 10^3 \ \text{M}^{-1}$  and  $1.107 \times 10^5 \ \text{M}^{-1}$  for complexes 1, 2 and 3 respectively, reflecting more binding of complex 3 with DNA to leach out more number of EB molecules originally bound to DNA than that of the complexes **1** and **2**. All these results showed clearly that the complex **3** possesses strong tendency to bind with DNA which is consistent with the absorption spectral results.

#### 4.7. DNA cleavage activity

The interaction of plasmid pUC19 DNA with complexes **1**, **2** and **3** was studied in order to determine the efficiencies with which these





**Fig. 13.** (A). Emission spectrum of BSA ( $1 \times 10^{-6}$  M;  $\lambda exi = 280$  nm;  $\lambda emi = 345$  nm) as a function of concentration of the complex **1** (1, 2, 3, 4, 5, 6, 7, 8, 9 and  $10 \times 10^{-6}$  M). Arrow indicates the effect of metal complex **1** on the fluorescence emission of BSA. (B). Plot of log  $[(F_0-F)/F]$  vs log [Q].



**Fig. 14.** (A). Emission spectrum of BSA ( $1 \times 10^{-6}$  M;  $\lambda exi = 280$  nm;  $\lambda emi = 345$  nm) as a function of concentration of the complex **3** (1, 2, 3, 4, 5, 6, 7, 8, 9 and  $10 \times 10^{-6}$  M). Arrow indicates the effect of metal complex **3** on the fluorescence emission of BSA. (B). Plot of log  $[(F_0-F)/F]$  vs log [Q].

both of which are capable of damaging DNA by Fenton type chemistry [43].

#### 4.8. Protein binding studies

#### 4.8.1. Fluorescence quenching of BSA by metal complexes (1-3)

Qualitative analysis of chemical compounds bound to BSA can be undertaken by examining the respective fluorescence spectra. Generally, the fluorescence of a protein is caused by three intrinsic characteristics of the protein, namely tryptophan, tyrosine, and phenylalanine residues. Fluorescence quenching refers to any process, which decreases the fluorescence intensity of a fluorophore due to variety of molecular interactions including excitedstate reactions, molecular rearrangements, energy transfer, ground—state complex formation, and collision quenching. Figs. 13A and 14A and Fig. S7A show the effect of increasing the concentration of metal complexes on the fluorescence emission of BSA. Addition of metal complexes to the solution of BSA resulted in the quenching of its fluorescence emission without any shift suggesting that the complex formed between the metal hydrazones and BSA is responsible for the quenching of BSA.

The fluorescence quenching is described by Stern–Volmer relation:  $I_0/I = 1 + K_{SV}[Q]$ ; where  $I_0$  and I are the fluorescence intensities of the fluorophore in the absence and presence of quencher respectively,  $K_{SV}$  is the Stern–Volmer quenching constant and [Q] is the quencher concentration. The  $K_{SV}$  value obtained from the plot of  $I_0/I$  vs [Q] was found to be  $1.550 \times 10^5 \text{ M}^{-1}$ ,  $1.226 \times 10^5 \text{ M}^{-1}$  and  $1.905 \times 10^5 \text{ M}^{-1}$  corresponding to the complexes **1**, **2** and **3**. When small molecules bind independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is represented by the Scatchard equation [44,45]: log [ $(F_0-F)/F$ ] = log [K] + nlog[Q]; where K and n are the binding constant and the number of binding sites, respectively.

Binding constants obtained from the plot of log  $[(F_0-F)/F]$  vs log [Q] (Figs. 13B and 14B, Fig. S7B) corresponding to the complexes **1**, **2** and **3** were  $6.272 \times 10^3 \text{ M}^{-1}$ ,  $2.295 \times 10^3 \text{ M}^{-1}$  and  $6.677 \times 10^3 \text{ M}^{-1}$ .

Quenching can occur by different mechanisms, which are usually classified as dynamic quenching and static quenching; dynamic quenching refers to a process in which the fluorophore and the quencher come into contact during the transient existence of the excited state. Static quenching refers to fluorophore–quencher complex formation in the ground state. A simple method to explore the type of quenching is UV–visible absorption spectroscopy. A representative absorption spectrum of pure BSA and BSA-complex **3** is shown in Fig. S8. The absorption band obtained for the BSA at 278 nm in the absence of metal complex showed an increase in the intensity of absorption after the addition of complex **3**, without any shift revealing that there exists a static



**Fig. 15.** Synchronous spectra of BSA ( $1 \times 10^{-6}$  M) as a function of concentration of the complex **1** (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and  $10 \times 10^{-6}$  M) with wavelength difference of  $\Delta = 15$  nm (A) and  $\Delta = 60$  nm (B). Arrow indicates the decrease in emission intensity accompanied by red shift (A) and blue shift (B) w.r.t various concentration of complex **1**.



**Fig. 16.** Synchronous spectra of BSA ( $1 \times 10^{-6}$  M) as a function of concentration of the complex **2** (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and  $10 \times 10^{-6}$  M) with wavelength difference of  $\Delta = 15$  nm (A) and  $\Delta = 60$  nm (B). Arrow indicates the emission intensity increase accompanied by red shift (A) and blue shift (B) w.r.t various concentration of complex **2**.

interaction between BSA and the added complex **3** due to the formation of ground state complex of the type BSA-complex **3** as reported earlier [46].

#### 4.8.2. Characteristics of synchronous fluorescence spectra

To investigate the structural changes occurred to BSA upon the addition of metal complexes, we measured synchronous fluorescence spectra of BSA before and after the addition of metal hydrazone complexes to get valuable information on the molecular microenvironment, particularly in the vicinity of the fluorophore functional groups [47]. It is a well known fact that the fluorescence of BSA is normally due to the presence of tyrosine, tryptophan and phenylalanine residues and hence spectroscopic methods are usually applied to study the conformation of serum protein. According to Miller [48], in synchronous fluorescence spectroscopy, the difference between excitation and emission wavelength  $(\Delta \lambda = \lambda_{emi} - \lambda_{exc})$  reflects the spectra of a different nature of chromophores. If the  $\Delta\lambda$  value is small (15 nm) the synchronous fluorescence of BSA is characteristic of tyrosine residue whereas a larger  $\Delta\lambda$  value of 60 nm is characteristic of tryptophan [49]. The synchronous fluorescence spectra of BSA with various concentrations of metal complexes **1–3** recorded at  $\Delta \lambda = 15$  nm and  $\Delta \lambda = 60$  nm are shown in Figs. 15 and 16 and Fig. S9. From the spectra, we understand that an increase in the concentration of metal complexes 1 and 3 resulted in a decrease in the intensity of the synchronous fluorescence spectral band corresponding to tyrosine residue with a slight red -shift. However, after the addition of complex 2 to BSA there occurred an increase in the intensity of the above said band with a slight red shift as observed for the complexes 1 and 3. In addition to this, a gradual decrease of fluorescence intensity of tryptophan residues together with slight blue -shift of emission wavelength were also observed with complexes 1ndash3. These experimental results indicate that the metal complexes do affect the microenvironment of both tyrosine and tryptophan residues during the binding process and synchronous measurements confirmed the effective binding of all the complexes with BSA. Similar behaviour behavior was observed for the interaction between the BSA and  $[Y^{III}(pdta)(H_2O)]_2^{2minus}$  [50].

#### 4.9. Cytotoxicities

Cytotoxicity is a common limitation in terms of the introduction of new compounds into the pharmaceutical industry. In general, macrocyclic complexes exhibit only slight cytotoxic effects and no dose-response effects are observed. In order to understand the in vitro cytotoxicities of the hydrazone metal complexes 1, 2 and 3, experiments were carried out using HeLa cell lines (tumor cells) and NIH 3T3 (normal cells). Complexes 1, 2 and 3 were dissolved in DMSO and blank samples containing same volume of DMSO are taken as controls to identify the activity of solvent in this cytotoxicity experiment. Cisplatin was used as a standard to assess the cytotoxicity of complexes **1**. **2** and **3** (not shown in graph) [51,52]. The capabilities of complexes 1, 2 and 3 to arrest the proliferation of tumor cells without causing any damage to normal cells were evaluated after 48 h of incubation. The results were analyzed by means of cell viability curves and expressed as IC<sub>50</sub> values. Fig. 17 displays the effect of complexes 1 and 3 on cell growth at different concentrations. The biological assays of the metal hydrazone complexes revealed that complex **3** (IC<sub>50</sub> = 173  $\mu$ M) exhibits enhanced activity against HeLa cell lines when compared with complex  $1 (IC_{50} = 201.5 \,\mu\text{M})$  [53]. On the other hand, complex 2 was found to be inactive against the above cell lines in the dosage range 31  $\mu$ M-1000  $\mu$ M. Both the complexes **1** and **3** exhibited lesser in vitro cytotoxicity against tumor cell lines than cisplatin



Fig. 17. %inhibition of NIH 3T3 and HeLa cell lines as a function of concentration of complexes 1 and 3.

 $(IC_{50} = 16.4 \ \mu\text{M})$  [51]. Increasing the concentration of complexes **1** and **3** from 31  $\mu$ M to 1000  $\mu$ M resulted in an increase in the percentage of cell inhibition. As seen from the figure, no cell death was apparent due to the treatment of complexes **1**–**3** with NIH 3T3 cells. The results of the cell viability tests imply that these complexes can be used to arrest the proliferation of tumor cells without causing perceptible damage to the normal cells.

#### 5. Conclusions

Three new divalent transition metal hydrazone complexes have been synthesised and characterised using various spectroscopic methods. Single-crystal X-ray diffraction study revealed that the hydrazone ligand used in this study forms mononuclear Co(II), Ni(II) and Cu(II) complexes of distorted octahedral geometry with 1:2 stoichiometry between metal and ligand. All the newly synthesised complexes were evaluated for DNA binding, DNA cleavage, protein binding and cytotoxicity studies. The DNA binding of metal complexes examined by absorption and fluorescence spectral techniques revealed an intercalative interaction between them and CT-DNA with binding constants ranging from 10<sup>3</sup>-10<sup>5</sup> M<sup>-1</sup>. Among the investigated complexes, the one containing copper as the central metal ion showed better binding affinity than the other two complexes containing cobalt and nickel ions as metal counterparts respectively. DNA cleavage studies revealed that all the three complexes have the ability to cleave nucleic acids and the extent of the cleavage was found to be dose dependent. Among the three complexes tested for DNA cleavage activity, complex **3** effected almost complete conversion of pUC19 DNA from the supercoiled form (Form I) to the nicked circular form (Form II). Binding of the metal complexes with BSA monitored by UV-visible spectroscopy revealed the presence of static quenching and the results of synchronous spectral studies indicated that the complexes bound with BSA in both tyrosine and tryptophan residues. The results of cytotoxicity study showed a linear relationship between the concentration of the metal complexes and the percentage inhibition of HeLa tumor cell growth without apparent damage to the normal cells. From the biological activity experiments, we observed that the hydrazone complex 3 containing copper metal ion exhibited more potential than the complexes 1 and 2 containing cobalt and nickel ions, which can be correlated to the presence of biologically essential copper ion in the molecular architecture.

#### 6. Experimental protocols

#### 6.1. Materials used for synthesis

Reagent grade chemicals were used without further purification in all the synthetic work. All solvents were purified by standard methods. The compounds CoCl<sub>2</sub>·6H<sub>2</sub>O, NiCl<sub>2</sub>·6H<sub>2</sub>O, CuCl<sub>2</sub>·2H<sub>2</sub>O, triphenylphosphine, benzhydrazide, tetrabutylammonium perchlorate (TBAP) and 2-benzoylpyridine were purchased from Sigma—Aldrich Chemie and Alfa Aesar, respectively and used as received. Calf-thymus (CT-DNA) and bouvine serum albumin (BSA) were purchased from Himedia. The plasmid supercoiled (SC) pUC19 DNA was purchased from Bangalore GeNei, Bangalore, India. The human cervical cancer cell line, HeLa and the NIH 3T3 mouse embryonic fibroblasts were obtained from National Center for Cell Science (NCCS), Pune, India. All other chemicals and reagents used for the pharmacological studies were of high quality and procured commercially from reputable suppliers.

#### 6.2. Instrumentation

Microanalyses (%C, H and N) of the ligand (HL) and metal complexes **1**, **2** and **3** were performed on a Vario EL III CHNS

analyzer. The infrared spectra of the ligand and the three complexes were recorded as KBr pellets on a Nicolet Avatar spectrometer in the range of  $400-4000 \text{ cm}^{-1}$ . The electronic absorption spectra of the complexes were recorded in methanol solution using a Jasco V-630 spectrophotometer. The emission spectra of complexes **1–3** were measured on a Jasco FP 6600 spectrofluorometer. The cyclic voltammetric studies were performed on a CH electrochemical analyzer using tetrabutylammonium perchlorate (TBAP) as the supporting electrolyte. All solutions were purged with nitrogen gas prior to making measurements at room temperature. A three-electrode assembly comprising of a glassy carbon working electrode, a platinum wire auxiliary electrode and a Ag/AgCl reference electrode was employed for the electrochemical studies.

#### 6.3. X-ray structure determination

The X-ray diffraction data for complexes **2** and **3** were collected on a Rigaku AFC-12 Saturn 724 + CCD diffractometer equipped with a graphite-monochromated Mo K $\alpha$  radiation source ( $\lambda = 0.71075$  Å) and a Rigaku XStream low-temperature device cooled to 100 K. Corrections for Lorentz and polarization effects were applied. The structure was solved by direct methods and refined by full-matrix least–squares cycles on F<sup>2</sup> using the Siemens SHELXTL PLUS 5.0 (PC) software package [54,55] and PLATON [56]. All non - hydrogen atoms were refined anisotropically and the hydrogen atoms were placed in fixed, calculated positions using a riding model.

#### 6.4. Preparations

## 6.4.1. Synthesis of the ligand N'-(phenyl(pyridine-2-yl) methylidene)benzohydrazide (HL)

The hydrazone ligand N'-(phenyl(pyridine-2-yl)methylidene) benzohydrazide (HL) was isolated from the condensation reaction of 2-benzoylpyridine and benzhydrazide in ethanol under reflux for 5 h [57]. After cooling the reaction mixture to room temperature, the solid product formed was filtered, washed with ethanol and dried in vacuo (Scheme 1). Yield: 72%. Color: pale yellow. mp: 128–133 °C. Anal. Found (%) for C<sub>19</sub>H<sub>15</sub>N<sub>3</sub>O (Mol. wt = 301.349): C, 75.24%; H, 4.81%; N, 13.52%. Calculated (%) for C, 75.73%; H, 5.01%; N, 13.94%. Selected IR bands ( $\nu_{max}/cm^{-1}$ ): 3143 (N–H); 1678 (C= O); 1581 (C=N) and 1075 (N–N).

#### 6.4.2. Preparation of complexes 1-3

The complex  $[Co(L)_2]$  (1) was prepared by refluxing equimolar quantities of  $[CoCl_2(PPh_3)_2]$  (0.300 g; 0.153 mM) and the hydrazone ligand (HL) (0.138 g; 0.153 mM) (Scheme 1) in 40 mL of methanol (Scheme 2). After 10 min, a few drops of methanolic KOH were added to the reaction mixture and refluxing was continued for an additional 5 h. The reaction mixture was then cooled to room temperature, following which the resulting precipitate was filtered off, washed with methanol and dried under vacuum. The purity of the product was checked by TLC. Slow evaporation of a MeOH/ CHCl<sub>3</sub> solution of 1 afforded crystals suitable for X-ray diffraction studies. Yield: 68%. Color: brown. mp: 265–270 °C. Anal. Found (%) for CoC<sub>38</sub>H<sub>28</sub>N<sub>6</sub>O<sub>2</sub> (Mol. wt = 659.612): C, 68.98%; H, 4.11%; N, 12.39%. Calculated (%) for C, 69.19%; H, 4.27%; N, 12.74%. Selected IR bands ( $\nu_{max}/cm^{-1}$ ): 1593 & 1488 (C=N–N=C); 1373 (C–O); 1081 (N–N).

Complex **2** was synthesised as described above for **1** by utilizing [NiCl<sub>2</sub>(PPh<sub>3</sub>)<sub>2</sub>] (0.300 g; 0.153 mM) and the hydrazone ligand (HL) (0.138 g; 0.153 mM) as in Scheme 2. Yield: 62%. Color: violet. mp: 268–272 °C. Anal. Found (%) for NiC<sub>38</sub>H<sub>28</sub>N<sub>6</sub>O<sub>2</sub> (Mol. wt = 659.372): C, 69.02%; H, 4.03%; N, 12.52%. Calculated (%) for C, 69.22%; H, 4.28%; N, 12.74%. Selected IR bands ( $\nu_{max}$ /cm<sup>-1</sup>): 1591 & 1494 (C=N–N=C); 1359 (C–O); 1086 (N–N).

Complex **3** was prepared by a procedure similar to that described for complex **1** using [CuCl<sub>2</sub>(PPh<sub>3</sub>)<sub>2</sub>] (0.300 g; 0.151 mM) and the HL ligand (0.135 g; 0.151 mM) as starting materials (Scheme 2). Examination of the reaction mixture by TLC revealed the presence of two distinct complexes, **3** and **3a**. Complex **3** was eluted from the column using a mixture of petroleum ether and ethylacetate (75:25) as the eluant. Slow evaporation of **3** from a MeOH/CHCl<sub>3</sub> mixture afforded single-crystals suitable for X-ray diffraction studies. [Cu(L)<sub>2</sub>]: Yield: 54%. Color: violet. mp: 274–278 °C. Anal. Found (%) for CuC<sub>38</sub>H<sub>28</sub>N<sub>6</sub>O<sub>2</sub> (Mol. wt = 664.222): C, 68.24%; H, 4.01%; N, 12.12%. Calculated (%) for C, 68.73%; H, 4.24%; N, 12.65%. Selected IR bands ( $\nu_{max}$ /cm<sup>-1</sup>): 1586 & 1493 (C=N–N=C); 1357 (C–O); 1078 (N–N).

Complex **3a** was isolated by using petroleum ether and ethylacetate (85:15) as the eluent and a crop of crystals suitable for single-crystal X-ray diffraction studies were obtained from a solvent system similar to that described for **3**. The structure of **3a** was confirmed by single-crystal X-ray diffraction and shown to correspond to that of the known compound  $[Cu(Cl)(PPh_3)_3]$  [32]. The reduction of Cu (+2) to Cu (+1) is believed to occur by hydrazone serving as a reducing agent.

#### 6.5. Pharmacological evaluations

#### 6.5.1. DNA binding studies

6.5.1.1. Electronic absorption spectroscopy. Electronic absorption titration experiments were performed with a fixed concentration of metal complex (25  $\mu$ M) but variable nucleotide concentration ranging from 0 to 25  $\mu$ M and after each addition of DNA to the metal complex, the readings were noted. The data were then fit to the following equation to obtain intrinsic binding constant  $K_b$  [58]: [DNA]/[ $\varepsilon_a - \varepsilon_f$ ] = [DNA]/[ $\varepsilon_b - \varepsilon_f$ ] + 1/ $K_b$ [ $\varepsilon_b - \varepsilon_f$ ]; where [DNA] is the concentration of DNA base pairs,  $\varepsilon_a$  is the extinction coefficient of the complex in free solution and  $\varepsilon_b$  is the extinction coefficient of the complex when fully bound to DNA. A plot of [DNA]/[ $\varepsilon_b - \varepsilon_f$ ] versus [DNA] gave a slope and the intercept equal to 1/[ $\varepsilon_a - \varepsilon_f$ ] and (1/ $K_b$ ][ $\varepsilon_b - \varepsilon_f$ ], respectively. The intrinsic binding constant  $K_b$  is calculated from the ratio of the slope to the intercept.

6.5.1.2. Competitive binding fluorescence measurements. The apparent binding constant  $(K_{app})$  of the complexes was determined by a fluorescence spectral technique using ethidium bromide (EB)-bound CT-DNA solution in Tris-HCl buffer (pH, 7.10). The changes in fluorescence intensities at 605 nm (545 nm excitation) of EB bound to DNA were measured with respect to concentration of the complex. EB was non-emissive in Tris-HCl buffer solution (pH 7.10) due to fluorescence quenching of the free EB by the solvent molecules. In the presence of DNA, EB showed enhanced emission intensity due to its intercalative binding to DNA. A competitive binding of the metal complexes to CT-DNA resulted in the displacement of the bound EB, thereby decreasing its emission intensity. The quenching constant  $(K_q)$  was calculated using the classical Stern–Volmer equation [59]:  $I_0/$  $I = K_q[Q] + 1$ ;  $I_0$  is the emission intensity in the absence of quencher, I is the emission intensity in the presence of quencher,  $K_q$  is the quenching constant, [Q] is the quencher concentration.  $K_q$  is the slope, obtained from the plot of  $I_0/I$  vs [Q]. The apparent binding constant ( $K_{app}$ ) has been calculated from the equation,  $K_{\text{EB}}[\text{EB}] = K_{\text{app}}$  [complex].

#### 6.5.2. DNA cleavage experiments

In each experiment, the extent of DNA cleavage was monitored by agarose gel electrophoresis. A solution containing 25  $\mu$ L of pUC19 DNA (1  $\mu$ g), HCl (50 mM, pH7.5), NaCl (50 mM), the metal complex (30  $\mu$ M), and H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M) was incubated for 1–4 h at 37 °C. Subsequently, 4  $\mu$ L of 6X DNA loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol and 60% glycerol was added to the reaction mixture and loaded onto a 1% agarose gel containing 1.0  $\mu$ g/mL of ethidium bromide. The electrophoresis was performed at 5 V/cm for 2 h in a TBE buffer. The bands were visualized under UV light and photographed. The cleavage efficiencies were measured by determination of the ability of each complex to convert the supercoiled DNA (SC) to the nicked circular form (NC). After electrophoresis, the proportion of DNA in each fraction was estimated quantitatively on the basis of the band intensities using the BIORAD Gel Documentation System. The intensity of each band relative to that of the plasmid supercoiled form was multiplied by 1.43 to take account of the reduced affinity for ethidium bromide [29].

#### 6.5.3. Protein binding studies

The binding of metal complexes with bovine serum albumin (BSA) were studied using fluorescence spectra recorded at a fixed excitation wavelength corresponding to bovine serum albumin (BSA) as 280 nm and monitoring the emission at 345 nm. The excitation and emission slit widths and scan rates were constantly maintained for all the experiments. Samples were carefully degassed using pure nitrogen gas for 15 min by using quartz cells  $(4 \times 1 \times 1 \text{ cm})$  with high vacuum Teflon stopcocks. Stock solution of BSA was prepared in 50 mM phosphate buffer (pH = 7.2) and stored in the dark at 4 °C for further use. Concentrated stock solutions of metal complexes were prepared by dissolving them in DMSO: phosphate buffer (1:100) and diluted suitably with phosphate buffer to required concentrations. 2.5 ml of BSA solution  $(10^{-6} \text{ M})$ was titrated by successive additions of a 25 µl stock solution of complexes  $(10^{-6} \text{ M})$  using a micropipette. Synchronous fluorescence spectra was also recorded using the same concentration of BSA and complexes as mentioned above with two different  $\Delta \lambda$ (difference between the excitation and emission wavelengths of BSA) values such as 15 and 60 nm.

#### 6.5.4. Cytotoxicity studies

The *in vitro* cytotoxicity assays ( $IC_{50}$ ) were performed on the human cervical cancer cell line HeLa and the NIH 3T3 mouse embryonic cell line. The HeLa tumor cell lines used in this work were grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS) and the NIH 3T3 fibroblasts were grown in Dulbeccos Modified Eagles Medium (DMEM) containing 10% FBS.

For the screening experiments, the cells were seeded into 96well plates in 100  $\mu$ l of the respective medium containing 10% FBS, at a plating density of 10 000 cells/well. The cells were incubated at 37 °C in 5% CO<sub>2</sub> and 95% air at a relative humidity of 100% for 24 h prior to the addition of the test compounds. The test compounds were solubilized in dimethylsulfoxide and diluted in the respective serum free medium. After 24 h, 100  $\mu$ l of the medium containing the test compounds with various concentrations (e.g. 0.031, 0.062, 0.125, 0.5, 1 mM) was added and incubated at 37 °C in an atmosphere of 5% CO<sub>2</sub> and 95% air with 100% relative humidity for 48 h. All measurements were made in triplicate and the medium containing no test compounds served as the control.

After 48 h, 15  $\mu$ L of MTT (5 mg/mL) in phosphate buffered saline (PBS) was added to each well and incubated at 37 °C for 4 h. The medium with MTT was then flicked off and the formazan crystals that had formed were solubilized in 100  $\mu$ L of DMSO and the absorbance at 570 nm was measured using a micro plate reader. The % cell inhibition was determined using the following formula and the graph was plotted of %cell inhibition versus concentration. The IC<sub>50</sub> values were calculated from the graph.

By means of the equation:

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#### Supplementary data

CCDC 801468 and 796258 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Center, 12 Union Road, Cambridge CB21EZ, UK) Fax: (+44) 1223-336-033; Email: deposit@ccdc.cam. ac.uk or www.http://www.ccdc.cam.ac.uk.

#### Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.05.001.

#### References

- [1] B.K. Kaymakcioglu, S. Rollas, Farmaco 57 (2002) 595-599.
- [2] S. Guniz Kucukguzel, Sevim Rollas, Ilkay Kucukguzel, Muammer Kiraz, Eur. J. Med. Chem. 34 (1999) 1093-1100.
- [3] S. Zhang, A.D. Sherry, J. Solid State Chem. 171 (2003) 38-43.
- [4] Z.Y. Yang, Synth. React. Inorg. Met.-Org. Chem. 30 (2000) 1265-1272.
- [5] B.H. Geierstanger, M. Mrksich, P.B. Dervan, D.E. Wemmer, Science 266 (1994) 646 - 650
- [6] C. Liu, J. Zhou, Q. Li, L. Wang, Z. Liao, H. Xu, J. Inorg, Biochem 75 (1996) 233-240. [7] G. Pratviel, I. Bernadou, B. Meunier, Angew, Chem., Int. Ed. Engl. 34 (1995) 746-749
- S.E. Livingstone, Coord. Chem. 20 (1980) 141-148. [8]
- A.K. Mesmaeker, J.P. Lecomte, J.M. Kelly, Top. Curr. Chem. 177 (1996) 25-76. [9]
- [10] Liang-Nian Ji, Xiao-Hua Zou, Jin-Gang Liu, Coord. Chem. Rev. 216 (2001) 513-536.
- N.A.P. Kane-Maguire, J.F. Wheeler, Coord. Chem. Rev. 211 (2001) 145-162. [11]
- [12] Y. Xiong, Liang-Nian . Ji, Coord. Chem. Rev. 185 (1999) 711-733
- [13] J.K. Barton, A.L. Raphael, J. Am. Chem. Soc. 106 (1984) 2466-2468.
- [14] M.T. Carter, M. Rodriguez, A.J. Bard, J. Am. Chem. Soc. 111 (1989) 8901-8911. [15] J.E. Patterson, D.M. Geller, Biochem. Biophys. Res. Commun. 74 (1977)
- 1220 1226
- [16] R.T.A. McGillivray, D.W. Chung, E.W. Davie, Eur. J. Biochem. 98 (1979) 477-485. K. Hirayama, S. Akashi, M. Furuya, K.I. Fukuhara, Biochem. Biophys. Res. [17] Commun. 173 (1990) 639-646.
- [18] K.H. Ulrich, J. Pharmacol. Rev. 33 (1981) 17-53.
- [19] D.S. Goodman, J. Am. Chem. Soc. 80 (1958) 3802-3808.
- [20] W.E. Klopfenstein, Biochim. Biophys. Acta 181 (1969) 323-325.
- [21] W.G. Unger, J. Pharm. Pharmacol. 24 (1972) 470-477.
- [22] A. Schepartez, B. Cuenoud, J. Am. Chem. Soc. 112 (1990) 3247-3249.
- [23] C.C. Cheng, C. Burrows, Angew. Chem. 105 (1993) 290-292.

- [24] M.C. Rodriguez-Arguelles, M.B. Ferrari, F. Bisceglie, C. Pelizzi, G. Pelosi, S. Pinelli, M. Sassi, J. Inorg. Biochem. 98 (2004) 313-321.
- [25] M.C. R-Arguelles, S. M-Vazquez, P. T-Touceda, J. S-Matalobos, M. Ana, G. -Deibe, M.B. -Ferrari, G. Pelosi, C. Pelizzi, F. Zani, J. Inorg. Biochem. 101 (2007) 138-147.
- [26] A. Shadia Galal, H. Khaled Hegab, S. Ahmed Kassab, L. Mireya Rodriguez, M. Sean Kerwin, A. Abdel-Momen El-Khamry, I. Hoda El Diwani, Eur. J. Med. Chem. 44 (2009) 1500-1508.
- I. Venanzi, J. Chem. Soc. (1958) 719-724.
- [28] G.N. Rao, Ch. Janardhana, K. Pasupathy, P. Mahesh Kumar, Ind. J. Chem. 39B (2000) 151-153. [29]
- J. Bernadou, G. Pratviel, F. Bennis, M. Girardet, B. Meunier, Biochemistry 28 . (1989) 7268–7275.
- [30] M. Blagosklonny, W.S. El-Diery, Int. J. Cancer 67 (1996) 386-392.
- [31] V. Suni, M.R.P. Kurup, M. Nethaji, Polyhedron 26 (2007) 3097–3102.
  [32] P.F. Barron, J.C. Dyason, P.C. Healy, L.M. Engelhardt, C. Packawatchai, V.A. Patrick, A.H. White, J. Chem. Soc. Dalton Trans. (1987) 1099-1106.
- [33] M. Mini Kuriakose, R. Prathapachandra Kurup, E. Suresh, Polyhedron 26 (2007) 2713 - 2718
- [34] P. Bindu, M.R.P. Kurup, T.R. Satyakeerthy, Polyhedron 18 (1999) 321-331.
- [35] R.P. John, A. Sreekanth, M.R.P. Kurup, Anwar Usman, Abdul Razak Ibrahim, H.K. Fun, Spectrochim, Acta Part A 59 (2003) 1349-1358.
- [36] S. Karabocek, N. Karabocek, A. Armutcu, Trans. Met. Chem. 31 (2006) 459-464.
- [37] Tapas Ghosh Bipul Mondal, Tarun Ghosh. Manas Sutradhar Gurunath Mukherjee, Michael G.B. Drew, Inorg. Chim. Acta 360 (2007) 1753 - 1761
- [38] Md. Abu Affan, S.W. Foo, I. Jusoh, S. Hanapi, E.R.T. Tiekink, Inorg. Chim. Acta 362 (2009) 5031-5037.
- Yingzhong Shen, Yiying Zhang, Ye Li, Xian Tao, Yan Wang, Inorg. Chim. Acta [39] 360 (2007) 1628-1632.
- [40]A.W. Wallace, W.R. Murphy Jr., J.D. Peterson, Inorg. Chim. Acta 166 (1989) 47 - 54
- [41] M.C. Hughes, D.J. Macero, Inorg. Chem. 13 (1974) 2739-2744.
- [42] Wen J. Mei, Jie Liu, C. Kang Zheng, Li J. Lin, Hui Chao, An X. Li, Feng C. Yun, Liang N. Ji, Dalton Trans. (2003) 1352-1359.
- [43] A.Y. Louie, T.J. Meade, Chem. Rev. 99 (1999) 2711-2734.
- [44] A. Kathiravan, M. Chandramohan, R. Renganathan, S. Sekar, J. Mol. Struct. 919 (2009) 210-214.
- [45] J.R. Lakowicz, Fluorescence Quenching: Theory and Applications, Principles of Fluorescence Spectroscopy. Kluwer Academic/Plenum Publishers, New York, 1999, pp 53-127.
- X.Z. Feng, Z. Yang, L.J. Wang, C. Bai, Talanta 47 (1998) 1223-1229. [46]
- G.Z. Chen, X.Z. Huang, Z.Z. Zheng, J.G. Xu, Z.B. Wang, Methods of Fluorescence [47] Analysis, second ed. Science Press, Beijing, 1990.
- [48] J.N. Miller, Proc. Anal. Div. Chem. Soc. 16 (1979) 203-208.
- J.H. Tang, F. Luan, X.G. Chen, Bioorg. Med. Chem. 149 (2006) 3210-3217. [49]
- Bin Liua, Jun Wang, Xin Wang, Bing-Mi Liu, Ling-Ling Hea, Shu-Kun Xua, [50] Spectrochim. Acta Part A 77 (2010) 1115-1121.
- [51] Yun-Jun Liu, Cheng-Hui Zeng, Fu-Hai Wu, Jun-Hua Yao, Li-Xin He, Hong-Liang Huang, J. Mol. Struct. 932 (2009) 105-111.
- [52] Qin Jiang, Jianhui Zhu, Yangmiao Zhang, Nan Xiao, Zijian Guo, Biometals 22 (2009) 297-305.
- [53] Zeng-Chen Liu, Bao-Dui Wang, Bo Li, Qin Wang, Zheng-Yin Yang, Tian-Rong Li, Yong Li, Eur. J. Med. Chem. 45 (2010) 5353-5361.
- G.M. Sheldrick, SHELXTL-PC, Version 5.03. Siemens Analytical X-ray Instru-[54] ments Inc., Madison, Wisconsin, USA, 1994.
- [55] G.M. Sheldrick, Acta Cryst. A 64 (2008) 112-122.
- [56] A.L. Spek, Acta Cryst. A46 (1990) 194-201.
- N.A. Mangalam, S. Sivakumar, S.R. Sheeja, M.R.P. Kurup, E.R.T. Tiekink, Inorg. [57] Chim. Acta 362 (2009) 4191-4197.
- A. Wolfe, G.H. Shimer Jr., T. Meehan, Biochemistry 26 (1987) 6392-6396.
- M. Lee, A.L. Rhodes, M.D. Wyatt, S. Forrow, J.A. Hartley, Biochemistry 32 (1993) 4237-4245.