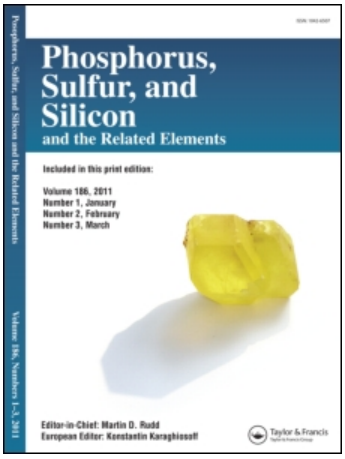

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Synthesis, Characterization of Some Transition-Metal Complexes of a New Heptadentate N_5S_2 Schiff-Base Ligand and the Effects of These Metal Complexes on U2OS Cells Cytotoxicity and DNA Cleavage Activity

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Metal complexes of a heptadentate N_5S_2 donor Schiff-base ligand were synthesized by the template reaction between 2,6-bis(2-aminothiophenoxymethyl)pyridine and 2,2′-bipyridine-6,6′-dicarboxaldehyde in the presence of Zn(II), Cd(II), Hg(II) and Pb(II) perchlorate salts. The complexes were characterized by IR, ^{1}H NMR, MS-FAB$^+$, and elemental analyses. The mitochondrial activity of U2OS cells after exposure to these complexes was determined by colorimetric assay, which detects the conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) to formazon. DNA-binding activity of all complexes was also investigated using plasmid DNA pUC18 purified from E. coli.

Keywords Mitocondrial activity; MTT; Schiff base; template synthesis

INTRODUCTION

The synthesis of Schiff-base macrocyclic metal complexes received considerable attention during the last decade because of their antitumor and cytotoxic activity.[1,2] The potential biological activity of complexes containing sulfur and nitrogen may be a reason for this increased interest. In particular complexes with N and/or S donor ligands of copper(II),[3,4] cobalt(II),[5] nickel(II),[3,5] platinum(II),[1–3,6] and iron(II/III)[6]

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are known sometimes to act as antitumor or therapeutic agents. Many efforts have been made to establish further metal-containing cytostatics however, without great success. Only little is known about the antitumor efficacy of some transition metals such as zinc(II),[7] cadmium(II), lead(II), and mercury(II). For this reason, we synthesised some new N_5S_2 Schiff-base complexes of these transition metals. Recently, we also synthesized and characterized some new similar macrocyclic and acyclic Schiff-base complexes using these metals.[8–10]

Here, we describe the complexes of a N_5S_2 donor ligand, which were obtained from the reaction of the Schiff base, resulting from the condensations of 2,6-bis(2-aminothiophenoxymethyl)pyridine and 2,2′-bipyridine-6,6′-dicarboxaldehyde with Zn(II), Cd(II), Hg(II) and Pb(II) perchlorate in methanol. The complexes were characterized by IR, ^{1}H NMR, FAB mass spectrometry, and elemental analyses.

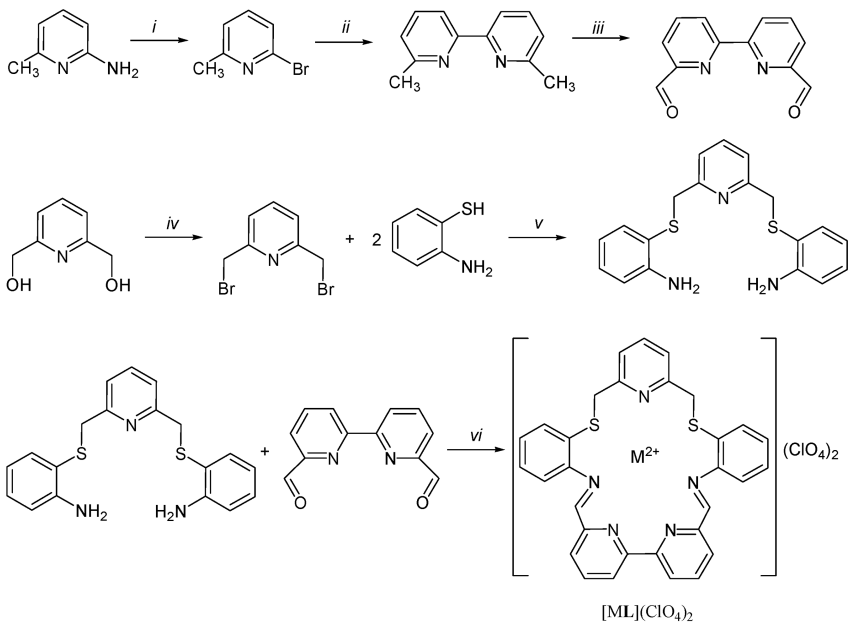
The mitocondrial activity of U2OS cells after exposure to these complexes was determined by colorimetric assay, which detects the conversion of 3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) to formazon.[11] DNA cleavage activity of all these complexes was also investigated by using plasmid DNA pUC18 purified from *E. Coli.*[12]

RESULTS AND DISCUSSION

Chemistry

All the complexes were prepared by a template synthesis, in which the Schiff base macrocyclic ligand resulted from the condensation of 2,2′-bipyridine-6,6′-dicarboxaldehyde with 2,6-bis(2- aminothiophenoxymethyl)pyridine in the presence of Zn(II), Cd(II), Hg(II) and Pb(II) ions (Scheme 1). For the synthesis of the dialdehyde compound, 2,2′-bipyridine-6,6′-dicarboxaldehyde, 6-amino-2-methylpyridine (commercially available) was brominated with concentrated aqueous hydrobromic acid and bromine.[13] Then 6,6′-dimethyl-2,2′-bipyridine was prepared using 6-bromo-2-methylpyridine by a modification of the method described by Iyoda et al.[14] and was oxidized to the dialdehyde by selenium dioxide in 1,4-dioxane. The precursor diamino compound, 2,6-bis(2-aminothiophenoxymethyl)pyridine,[15] was prepared starting from 2,6-bis(hydroxymethyl)pyridine, which was converted to 2,6-bis(bromomethyl)pyridine[16] by reaction with 47% hydrobromic acid. Then, one equivalent of 2,6-bis(bromomethyl)pyridine was reacted with two equivalents of 2-aminothiophenol in ethanol under an inert atmosphere. Physical data for all compounds are given in the Experimental Section.

The complexes were obtained with 40–80% yields. The infrared spectra of all metal complexes in the region of 400–4000 cm^{-1} show a strong



i : 47% HBr, Br_2, $NaNO_2$ then NaOH ; *ii* : $NiCl_2(PPh_3)_2$, Zn, Et_4NI, THF, under argon gas ; *iii* : SeO_2, 1,4-dioxan ; *iv* : 47% HBr, NaOH ; *v* : Na, EtOH, under argon gas ; *vi* : MeOH, $[M(ClO_4)_2]$ {M=Zn^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}}.

SCHEME 1 Synthetic route to the title complexes.

absorption band around 1616–1638 cm^{-1}, which is assigned to the C=N streching vibration, indicating the formation of the Schiff-base products. Furthermore, the absence of C=O and N–H streching vibrations in the spectra of the complexes, as compared to the aldehyde and diamine, respectively, further indicate the formation of the Schiff base. For the metal complexes absorbtions at 1091–1094 and 624 cm^{-1} were assigned to the ν_3 and ν_4 streching modes of ionic perchlorate.[17] The proton NMR spectrum of the soluble complex $[PbL](ClO_4)_2$ shows 23 peaks, which is consistent with the given structure. The composition of the complexes was also confirmed by elemental analyses and by fast atom bombardment mass spectrometry. In the mass spectrum of all complexes two fragmentation patterns were observed corresponding to the sequential loss of the counterion.

Cytotoxicity

Cytotoxic effects of metal complexes were tested using MTT assay as described in Experimental. MTT is commonly employed as an indicator

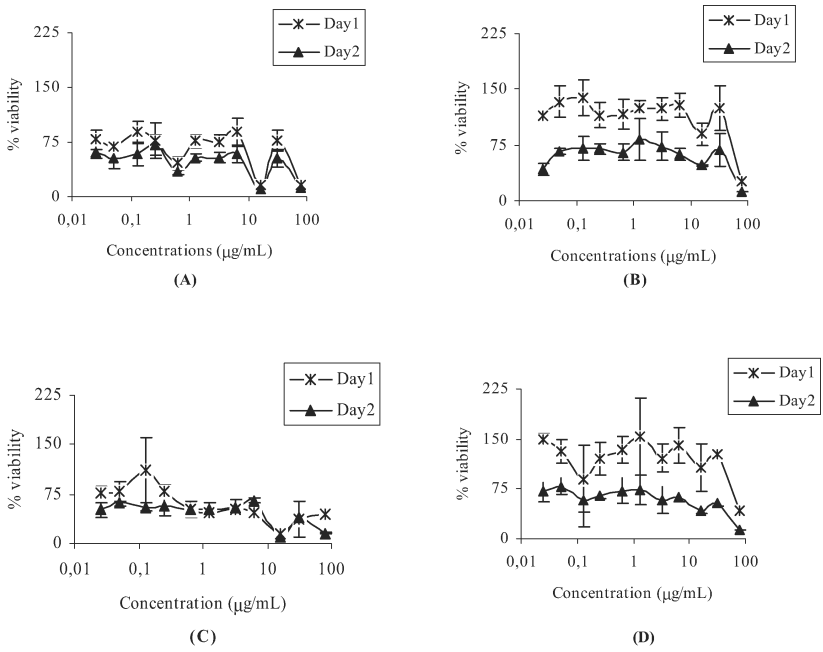


FIGURE 1 Toxicity of metal complexes for U2OS cells. Cells were incubated with various concentrations of the complexes. At each time point, MTT assay was performed. The results are the mean of triplicate wells.

of cell number and viability, since it is converted to a coloured formazan derivative via mitocondrial dehyrogenase activity only by viable cells.

The results are shown in Figure 1 and the IC_{50} value of each compounds is given in Table I. It was observed that cytotoxic activity of $[HgL](ClO_4)_2$ and $[PbL](ClO_4)_2$ increased considerably as time collapsed. The cells were exposed to 0.0256–40 μg/mL of compounds $[HgL](ClO_4)_2$ and $[PbL](ClO_4)_2$ for 24 h, and these two compounds showed no cytotoxicity for this cell line (Figures 1b and 1d). However, the cells treated with 80 μg/mL of either $[HgL](ClO_4)_2$ or $[PbL](ClO_4)_2$ showed 26 $\pm$ 5% and 41 $\pm$ 0.9% of cell viability, respectively. These

TABLE I IC_{50} Values of the Complexes with Ligand (L)

Compounds	$[ZnL](ClO_4)_2$	$[CdL](ClO_4)_2$	$[HgL](ClO_4)_2$	$[PbL](ClO_4)_2$
U2OS cell lines IC_{50} (μg/mL)	10	0,7	70	70

two compounds have IC_{50} values of 70 μg/mL as showed in Table I. However compounds $[ZnL](ClO_4)_2$ and $[CdL](ClO_4)_2$ increased the cytotoxic function, demonstrating that these two complexes give cytotoxic derivatives. The IC_{50} value was found 10 ± 0.4 for $[ZnL](ClO_4)_2$ and 0.7 ± 0.04 μg/mL for $[CdL](ClO_4)_2$. The marked increase in cytotoxicity of $[CdL](ClO_4)_2$ (IC_{50} value was 0.7 μg/mL) suggests that this complex is very important for the cytotoxic activity in this particular cell line (Figure 1a).

The ability of the metal complexes to interact with plasmid DNA pUC18 purified from E.coli in vitro was investigated by electrophoretic mobility method. The electrophoretic mobility of pUC18 DNA from the supercoiled forms to circular form was compared with plasmid DNA after incubation with the derivatives for 24 h at 37°C in the dark (Figure 2). $[ZnL](ClO_4)_2$ and $[PbL](ClO_4)_2$ allowed the change in mobility of Form I band to Form II band. The increase in concentrations

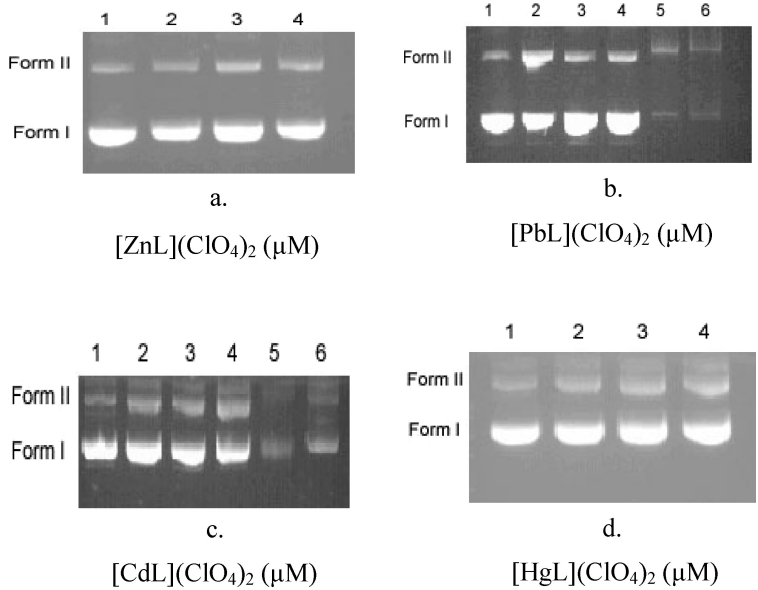


FIGURE 2 Effects of metal complexes on the electrophoretic mobility of supercoiled and open forms of the DNA plasmid pUC18. Electrophoresis was carried out after incubation of the plasmid pUC18 with (a) $[ZnL](ClO_4)_2$, (b) $[PbL](ClO_4)_2$, (c) $[CdL](ClO_4)_2$, and (d) $[HgL](ClO_4)_2$ at concentrations of the complexes ranging from 0,1–60 μM; Lane 1 applies to pUC18 plasmid DNA only, lanes 2–6 apply to plasmid interacted with increasing concentrations of the compounds.

(30 and 60 μM) is due to unwinding of the supercoiled Form I DNA to the relaxed circular Form II DNA. On the other hand, the plasmid DNA treated with high concentrations of $[CdL](ClO_4)_2$ and $[HgL](ClO_4)_2$ did not appear on the agarose gel, which might be due to complete cleavage of the plasmid DNA.

EXPERIMENTAL

Chemistry

Melting points were determined using an Gallenkamp MPD350.BM2.5 digital melting point apparatus and were uncorrected. The compounds were checked for purity by TLC on silica gel 60 F_{254} (Merck). Elemental analyses were performed on a CHNS-O Carlo Erba EA 1108 elemental analyser; IR spectra were obtained with a Shimadzu 470 IR spectrophotometer using nujol mulls or KBr disc; ^{1}H NMR spectra were recorded with a Varian (300 MHz) or Bruker NMR spectrometer (250 MHz) in $CDCl_3$ or DMF-$d_7$as solvent. ^{13}C NMR spectra were recorded with a Varian spectrometer at 75.5 MHz in $CDCl_3$ as solvent; MS-FAB$^+$ spectra were obtained with a Finnigan Mat 95 mass spectrometer.

General Synthesis Procedure

THF was distilled from sodium metal in the presence of benzophenone immediately prior to use. 1,4-Dioxane was distilled prior to use. Et_4NI was dried at 100°C under reduced pressure. All other reagents were used as purchased from commercial suppliers without further purification. Preparation of $NiCl_2(PPh_3)_2$ and purification of Zn powder both followed a literature method.[18]

6-Bromo-2-methylpyridine[13]

6-Amino-2-methylpyridine (1.08 g, 0.1 mol) in 47% hydrobromic acid (34.5 mL, 0.3 mol) was placed in an ice-salt-bath with stirring. Bromide (15.4 mL, 0.3 mol) was added dropwise to this solution with stirring while the temperature was kept at 0°C. A solution of sodium nitrite (17 g, 0.25 mol) in water (25 mL) was then introduced at such a rate that the temperature of the reaction mixture did not rise above 5°C. After completion of the reaction the dark brown solution was made alkaline with aqueous sodium hydroxide solution. The light yellow reaction mixture was extracted with diethyl ether. The extract was dried with sodium sulfate and the solvent was then removed in vacuo. Yield: 12.6 g (75.2%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.45$ (s, 3H, CH_3), 7.03 (d, $J = 7.5$ Hz, 1H), 7.20 (d, $J = 7.9$ Hz, 1H), 7.35 (t, $J = 7.7$ Hz, 1H).

^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta = 24.3$ (CH_3), 122.3, 125.2, 138.8, 141.5, 160.2.

6,6′-Dimethyl-2,2′-bipypridine[14]

$NiCl_2(PPh_3)_2$(4.9 g, 7.5 mmol), Zn powder (1.47 g, 22.5 mmol), dry Et_4NI (3.86 g, 15 mmol) and dry THF (20 mL) were stirred at room temperature under argon. 6-Bromo-2-methylpyridine (2.58 g, 15 mmol) in dry THF (5 mL) was added to the reaction mixture. The mixture was stirred at 50°C for two days. The reaction solution was poured into a mixture of 2 M aqueous ammonia (100 mL), diethyl ether (50 mL) and hexane (50 mL). The precipitate was filtered and the organic layer was separated. The aqueous layer was extracted with diethyl ether: hexane (1:1). The organic layer was washed successively with water and saturated aqueous NaCl solution, dried with anhydrous $MgSO_4$, and the solvent was evaporated in vacuo. The resulting white solid was suspended in water and heated. The suspension was acidified with concentrated HCl and filtered hot. The filtrate was cooled to ambient temperature, neutralised with saturated aqueous NaOH solution, and the solid formed was filtreted to give desired product as a cream solid (0.94 g, 68%). M.p. 88–89°C. ^{1}H NMR (300 MHz; $CDCl_3$): $\delta = 2.63$ (s, 6H, CH_3), 7.15 (d, $J = 7.4$ Hz, 2H), 7.69 (t, $J = 7.6$ Hz, 2H), 8.21 (d, $J = 7.9$ Hz, 2H). ^{13}C NMR (75.5 MHz; $CDCl_3$): $\delta = 24.8$ (CH_3), 118.5, 123.4, 137.3, 155.9, 158.1.

2,2′-Bipyridine-6,6′-dicarboxaldehyde[19]

A mixture of 6,6′-dimethyl-2,2′-bipyridine (2.0 g, 20 mmol) and selenium dioxide (10 g, 90 mmol) in 1,4-dioxane containing 4% of water (250 mL) was heated under reflux for 4 h. The reaction mixture was filtered through celite while hot. The product separated from the cold filtrate as cream crystals (1.91 g, 45%). IR (KBr): $\nu(cm^{-1}) = 1699$ (C=O), 1577 (pyridine). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.25$ (d, $J = 8.7$ Hz, 2H), 8.05 (t, $J = 6.6$ Hz, 2H), 8.82 (d, $J = 7.0$ Hz, 2H), 10.18 (s, 2H, HC=O). ^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta = 122.2$, 125.6, 138.5, 152.8, 155.8, 193.6 (C=O).

2,6-Bis(bromomethyl)pyridine[16]

2,6-Bis(hydroxymethyl)pyridine (20 g, 0.145 mol) and 47% aqueous hybrobromic acid (33.3 mL, 0.29 mol) was refluxed for 2 h. The mixture was then neutralised at 0°C by the addition of a concentrated aqueous solution of sodium hydroxide. The solid product was collected, washed with water, and dried. Recrystallization from petroleum ether (b.p. 60–80°C) gave a white crystalline solid (21.9 g, 57%). M.p. 87–88°C. IR

(KBr): ν (cm^{-1}) = 1590, 1571 (pyridine), 1206 (CH_2-Br). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 4.52 (s, 4H, CH_2), 7.36 (d, J = 7.7 Hz, 2H), 7.69 (t, J = 7.7 Hz, 1H).^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 33.6 (CH_2), 123.0, 138.3, 157.0.

2,6-Bis(2-aminothiophenoxymethyl)pyridine[15]

To a strirred solution of sodium ethoxide (sodium 2.3 g, 0.1 mol, absolute ethanol 50 mL), 2-aminothiophenol (12.5 g, 0.1 mol) in absolute ethanol (250 mL) was added dropwise over a period of 30 min at room temperature, under argon. Freshly prepared 2,6-bis(bromomethyl)pyridine (13.2 g, 0.05 mol) in absolute ethanol (100 mL) was added dropwise, and the reaction mixture was refluxed for 4 h. On cooling, the reaction mixture was poured into an equal volume of water, where upon standing cream flocculent crystals were obtained. The product was collected by filtration, washed with water, and dried over phosphorus pentoxide (14.5 g, 82%). M.p. 80–82°C. IR (KBr): ν (cm^{-1}) = 3408, 3312 (N—H); 1606, 1587 (aromatic ring). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 3.99 (s, 4H, CH_2), 4.37 (s, 4H, NH_2), 6.59 (t, J = 7.4 Hz, 2H), 6.66 (d, J = 8.0 Hz, 2H), 6.81 (d, J = 7.7 Hz, 2H), 7.09 (t, J = 7.7 Hz, 2H), 7.22 (d, J = 7.7 Hz, 2H), 7.34 (t, J = 7.7 Hz, 1H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 41.5 (CH_2), 115.1, 117.3, 118.4, 121.6, 130.3, 136.7, 136.8, 149.0, 158,0.

Metal Ion-Controlled Synthesis of L Complexes in the Presence of Zn(II), Cd(II), Hg(II), and Pb(II) Perchlorate Salts

2,2′-Bipyridine-6,6′-dicarboxaldehyde (0.18 g, 1 mmol) and the appropriate metal perchlorate salt (1 mmol) were dissolved in hot MeOH (25 mL) and 2,6-bis(2-aminothiophenoxymethyl)pyridine (0.36 g, 1 mmol) in methanol (25 mL) was added dropwise with stirring. The mixture was refluxed for 3–4 h and filtered hot. The solvent of the reaction mixture was reduced to half its original volume, and then the mixture was placed in a refrigerator to induce crystallization. The crystallized product was filtered and dried.

[ZnL](ClO$_4$)$_2$

Pale yellow solid, yield: 0.32 g (40%). IR (KBr): ν(cm^{-1}) = 1628 (C=N), 1587 (pyridine), 1094 and 624 (perchlorate anion). m/z: 594 $[ZnL(ClO_4)]^+$, 693 $[ZnL]^{2+}$. Found: C, 46.6; H, 3.0; N, 8.5; S, 8.5%. Calcd. for $C_{31}H_{23}N_5S_2O_8Cl_2Zn$: C, 46.9; H, 2.9; N, 8.8; S, 8.1%.

[CdL](ClO$_4$)$_2$

Yellow solid, yield: 0.67 g (80%). IR (KBr): ν(cm^{-1}) = 1638 (C=N), 1590 (pyridine), 1091 and 624 (perchlorate anion). m/z: 742

[CdL (ClO_4)]$^+$, 642 [CdL]$^{2+}$. Found: C, 44.2; H, 2.8; N, 8.0; S, 7.5%. Calcd. for $C_{31}H_{23}N_5S_2O_8Cl_2Cd$: C, 44.3; H, 2.7; N, 8.3; S, 7.6%.

[HgL](ClO$_4$)$_2$

Yellow solid, yield: 0.69 g (74%). IR (KBr): $\nu(cm^{-1}) = 1628$ (C=N), 1587 (pyridine), 1094 and 624 (perchlorate anion). m/z: 830 [HgL (ClO_4)]$^+$, 731 [HgL]$^{2+}$. Found: C, 39.1; H, 2.5; N, 7.1; S, 7.5%. Calcd. for $C_{31}H_{23}N_5S_2O_8Cl_2Hg \cdot H_2O$: C, 39.3; H, 2.6; N, 7.4; S, 6.8%.

[PbL](ClO$_4$)$_2$

Orange solid, yield: 0.374 g (40%). IR (KBr): $\nu(cm^{-1}) = 1616$ (C=N), 1587 (pyridine), 1091 and 624 (perchlorate anion). m/z: 837 [PbL (ClO_4)]$^+$, 736 [PbL]$^{2+}$. ^{1}H NMR (300 MHz, d_7-DMF): $\delta = 4.36$ (s, 4H, CH_2), 6.64 (t, $J = 7.0$ Hz, 2H), 6.95 (d, $J = 7.6$ Hz, 2H), 7.10–7.88 (m, 8H), 8.62 (d, $J = 7.9$ Hz, 2H), 8.80 (t, $J = 7.6$ Hz, 1H), 9.12 (d, $J = 7.0$ Hz, 2H), 9.60 (s, 2H, HC=N).

Cell Culture

U2OS cells were obtained and cultured as recommended by the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in McCoy's medium (Sigma) supplemented with 10% (v/v) of Foetal Bovine Serum (FBS) (Gibco) as adherent monolayers. Cells were incubated at 37°C under 5% CO_2 and 95% air in a humidified atmosphere.

Cytotoxicity Assays

U2OS cells in exponential growth phase were harvested and the cell number was determined using a haemocytometer. Samples were resuspended in fresh medium to give a density of 2.5×10^4/mL and then stock solutions of the complexes (2.57, 2.58, 2.60, and 2.61) were prepared as 1 mg/mL in DMSO. Final concentrations (ranging from 0.0256 to 80 μg/mL) were prepared with fresh cell-culture medium (DMSO concentration $< 0.1\%$). 200 μL of cells was placed into each of a 96-well microtitre plate and incubated for 24 and 48 h. At the end of the exposure time, 20 μL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] (5 mg/mL, Sigma) stock solution was added to each well, and the plates were incubated for a further 2 h. The medium was removed and discarded and 200 μL of DMSO were added to each well. Cells were incubated at 25°C for 10 min further, and then the absorbance was read on a Bio-Tek (ELX 808 IU) ELISA reader at a wavelength of 540 nm. The signal generated is directly proportional to

the number of viable (metabolically active) cells in the wells. The values of the blank wells were subtracted from each well of treated and control cells, and the % viability was determined according to Kumi-Diaka[20] as given below:

$$\%\ \text{viable cells} = \frac{(\text{the absorbance of the treated cells}) - (\text{the absorbance of the blank})}{(\text{the absorbance of the control}) - (\text{the absorbance of the blank})} \times 100 \quad (1)$$

Interaction with pUC18 Plasmid DNA

Interaction between the complexes and pUC18 plasmid DNA was studied by agarose gel electrophoresis. pUC18 plasmid DNA aliquots (0.5 μg/mL) were incubated in the presence of the increasing concentrations of compounds ranging from 0.1 to 60 μM. Incubation was carried out in dark at 37°C for 24 h 15 μL of drug-DNA mixture containing 0.5 μg DNA was loaded onto the 1% agarose gel and electrophoresis was carried under Tris buffer for 2 h at 5 $V \cdot cm^{-1}$. At the end of the electrophoresis, the gel was stained in the Tris buffer containing ethidium bromide (0.5 mg/mL) and visualized under UV light (Kodak).[21]

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