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Photo-induced DNA Cleavage, Antibacterial and Anticancer activities of Mononuclear Ruthenium(II) Complexes

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ABSTRACT

Two mononuclear mixed ligand Ruthenium(II) complexes $[Ru(bpy)_2(L1)](PF_6)_2$ (**1**) and $[Ru(bpy)_2(L2)](PF_6)_2$ (**2**), where bpy=2, 2'-bipyridine, L1=4,5-bis[(*E*)-2-(4-methoxyphenyl)ethenyl]-1*H*,1'*H*-2,2'-biimidazole and L2=4,5-bis[(*E*)-2-(4-bromophenyl)ethenyl]-1*H*,1'*H*-2,2'-biimidazole, have been synthesized and characterized. Their redox and spectroscopic properties have been studied. Both the complexes have intense MLCT absorbance at approximately 475 nm in acetonitrile. And also the complexes showed an efficient photocleavage of plasmid DNA when exposed to light at 440 nm. The antibacterial activities of the ligand and their metal complexes were screened by agar diffusion method and found that the metal complexes have higher antimicrobial activity than the free ligand. They also exhibited better antitumor activity.

Keywords: Antibacterial, biimidazole, DNA, Ruthenium(II).

1. INTRODUCTION

In the search for drugs with improved clinical effectiveness, reduced toxicity and a broader spectrum of activity, other metals than platinum have been considered, such as rhodium and ruthenium. Non-platinum active compounds are likely to have different mechanisms of action, biodistribution and toxicities than platinum-based drugs and might therefore be active against human malignancies that have either an intrinsic or an acquired resistance to them. Ruthenium complexes are very promising, especially from the viewpoint of overcoming cisplatin resistance with a low general toxicity. Ruthenium has found its way into the clinic, where its properties are exploited for very miscellaneous uses. The radiophysical properties of ^{97}Ru can be applied to radiodiagnostic imaging [1-2]. Other ruthenium compounds have potential as immunosuppressants (*cis*-$Ru(III)(NH_3)_4(HIm)_2]^{3+}$), antimicrobials (e.g. organic drugs coordinated to ruthenium centres, such as $[Ru(II)Cl_2(chloroquine)_2]$ against malaria and others for the treatment of Chaga´s disease), antibiotics (ruthenium complexes of organic antibiotic compounds, e.g. the Ru(III) derivative of thiosemicarbazone against *Salmonella typhi* and *Enterobacteria faecalis*), nitrosyl delivery/scavenger tools (e.g. the Ru(III) polyaminocarboxylates known as AMD6245 and AMD1226 to treat stroke, septic shock, arthritis, epilepsy and diabetes), vasodilator/ vasoconstrictor agents and, as above mentioned, as drugs for cancer chemotherapy [3]. Ruthenium(II) and ruthenium(III) complexes have similar ligand-exchange kinetics to those of platinum(II) complexes. This property makes them the first choice in the search for compounds that display similar biological effects to platinum(II) drugs [3-4]. The range of accessible oxidation states of ruthenium under physiological conditions makes this metal unique amongst the platinum group. The ruthenium centre, predominantly octahedral, can be Ru(II), Ru(III) or Ru(IV). Ru(III) complexes tend to be more biologically inert than related Ru(II) and Ru(IV) complexes. The redox potential of a metal complex can be modified by varying the ligands. One more property of ruthenium that makes it very appreciated in medicinal chemistry is its tendency to selectively bind biomolecules, which partly accounts for the low toxicity of ruthenium drugs [3-4].

Ruthenium complexes are attracting attention as potential chemotherapeutic agents against a variety of diseases, in view of searching for synthetic antioxidants. A quite a lot of properties have been attributed to the ruthenium complexes like antitumor activity [5] and antioxidant activity [6]. In this work, we have synthesized and studied the physicochemical properties of mixed-ligand ruthenium(II) complexes of the type $[Ru(II)(bpy)_2(H_2Y\text{-}biim)]$ {Y = -OMe and -Br groups}. In addition we report photocleavage activity, antibacterial and anticancer activities of these ruthenium complexes.

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2. EXPERIMENTAL SECTION

2.1 Materials and methods

Ruthenium chloride trihydrate, imidazole-2-carboxaldehyde, 4-methoxybenzaldehyde, 4-bromobenzaldehyde, biacetyl and piperidine were purchased from Sigma-Aldrich. Acetic acid, ammonium acetate, methanol, acetonitrile and ethanol were purchased from SD Fine chemicals. All the tetrabutylammonium salt of anions were received from Sigma-Aldrich and used as received. 1,4-bis(4-methoxyphenyl)butane-2,3-dione, 1,4-bis(4-bromophenyl)butane-2,3-dione were synthesized by adopting literature procedure[7].

Absorption spectra were recorded on Shimadzu UV-160A UV-Visible spectrophotometer. Cyclic (CV) and differential pulse voltammetries (DPV) were performed by using CH instrument (USA) model CH-620 B electrochemical analyzer. A conventional three electrode system consisting of platinum disc as a working electrode, platinum wire as an auxiliary electrode and saturated calomel (SCE) as a reference electrode was used for the electrochemical measurements. 0.1 M tetrabutyl ammonium perchlorate (TBAP) was used as the supporting electrolyte for all the experiments. Positive ion electrospray ionization mass spectra of the complexes were obtained by using Thermo Finnigan LCQ 6000 advantage max ion trap mass spectrometer. Tris(hydroxymethyl)aminomethane–HCl (Tris–HCl, pH 7.2), a buffer used for DNA cleavage studies was prepared using deionised triple distilled water. All the DNA gel images were taken using UVITEC gel documentation system and fragments were analysed using UVIchem and UVI-band software.

2.2 Synthesis of Ligands

2.3

2.2.1 Synthesis of 4, 5-bis[(*E*)-2-(4-methoxy phenylethenyl]-1*H*,1'*H*-2,2'-biimidazole (L1)

1, 4-bis(4-methoxyphenyl)butane-2,3-dione (0.2 g, 0.62 mmol), imidazole-2-carboxaldehyde (0.062 g, 0.65 mmol) and ammonium acetate (2 g, 25 mmol) were dissolved in 15 mL acetic acid and heated to reflux for 3 h. After cooling, cold water (10 mL) was added to the solution, during which orange precipitate was appeared. It was filtered and purified by column chromatography on silica using ethyl acetate: hexane (1:4) as an eluent (Yield 0.12 g, 49 %). ESI MS: m/z (relative intensity): 399.3 (M^++1). Anal. Calc. for $C_{24}H_{22}N_4O_2$: C, 72.34; H, 14.71; N, 14.06. Found: C, 72.32; H, 14.63; N, 14.04; IR, cm^{-1}(KBr pellet) 3424, 2932, 1602. 1510, 1249, 771, 424.

2.2.2 Synthesis of 4, 5-bis[(*E*)-2-(4-bromophenyl)ethenyl]-1*H*,1'*H*-2,2'-biimidazole (L2)

An analogous synthetic procedure using 1,4-bis(4-bromophenyl)butane-2,3-dione instead of 1,4-bis(4-methoxyphenyl)butane-2,3-dione was used to prepare 4,5-bis[(*E*)-2-(4-bromophenyl)ethenyl]-1*H*,1'*H*-2,2'-biimidazole (Yield 0.17 g, 56 %). ESI MS: m/z (relative intensity): 495.2 (M^+-1). Anal. Calc. for $C_{22}H_{16}Br_2N_4$: C, 53.25; H, 3.25; N, 11.29. Found: C, 53.22; H, 3.21; N, 11.26; IR, cm^{-1}(KBr pellet) 3732, 2308, 1219, 772, 424.

2.3 Synthesis of Complexes

2.3.1 Synthesis of $[Ru(bpy)_2(L1)](PF_6)_2$ (1).

A mixture of [*cis*-$Ru(bpy)_2Cl_2]\cdot 2H_2O$[8] (0.651 g, 1.25 mmol) and **L1** (0.498 g, 1.25 mmol) was suspended in an ethanol/water solvent mixture (3/1, v/v). The mixture was refluxed under an inert atmosphere for 4 h while vigorous stirring was maintained. The reaction mixture was cooled to room temperature; the solvent was reduced under vacuum to one-third of its initial volume. A saturated aqueous solution of NH_4PF_6 was added to precipitate $[Ru(bpy)_2(L1)]^{2+}$ as its hexafluorophosphate salt. The product was filtered and washed with water (3 × 10 mL) and then purified by column chromatography on neutral alumina using acetonitrile/toluene (1.5/1, v/v) as an eluent. Yield: 1.27 g, 92 %. Anal. Calc. for $C_{44}H_{38}F_{12}N_8P_2O_2Ru$: C, 47.96; H, 4.03; N, 10.17. Found: C, 47.93; H, 4.01; N, 10.12. ESI-MS: m/z 811.33 $(M - 2PF_6)^+$; IR, cm^{-1}(KBr pellet) 3282, 2952, 1597, 1228, 842, 762, 556.

2.3.2 Synthesis of $[Ru(bpy)_2(L2)](PF_6)_2$ (2).

The synthesis and purification of compound **2** were similar to those of **1** using $[Ru(bpy)_2Cl_2]\cdot 2H_2O$ (0.52 g, 1.0 mmol) and **L2** (0.496 g, 1.0 mmol). Yield: 1.04 g, 87 %, Anal. Calc. for $C_{44}H_{38}F_{12}N_8P_2Br_2Ru$: C, 42.05; H, 2.69; N, 9.34. Found: C, 42.01; H, 2.66; N, 9.29; ESI-MS: m/z 909.13 $(M - 2PF_6)^+$; IR, cm^{-1}(KBr pellet) 3701, 2363, 1516, 845, 555.

2.4 DNA Cleavage Activity

Photonuclease activity of the complexes was monitored using gel electrophoresis of plasmid DNA (pUC19). The solutions were prepared for the photolysis experiment containing 3 μL of 100 μg mL^{-1} plasmid DNA in Tris buffer and varying amounts of complexes **1** and **2** (0–48 μM). Each solution was incubated for 1 hour and then irradiated at 440 nm for various time intervals varying from 10 min to 60 min. The samples were then subjected to electrophoresis in 0.8 % agarose gel (tris-boric acid-EDTA buffer, pH 8.0) at 50 V for 2 h. The gel was stained with 0.5 μg mL^{-1} of ethidium bromide. The stained gel was illuminated under UV lamp and gel documented. In a separate experiment the DNA was incubated with 48 μM of the metal complex and 10 mM of histidine and irradiated at 440 nm. The photolysed solution was subsequently subjected to electrophoresis.

2.5 Antibacterial Assay

Micro-organisms used

Five species of bacteria, two gram - positive (*Streptococcus faecalis* & *Bacillus subtilis*) and three gram negative (*Escherichia coli*, *Klebsiella pneumoniae* & *Salmonella paratyphi*) were obtained from KMCH, Coimbatore.

Preparation of Inoculum

A loopful of strain was inoculated in 30mL of nutrient broth in a conical flask and incubated on a rotary shaker at 37° C for 24 hours to activate the strain.

Bioassay

The bioassay used was the standard Agar Disc Diffusion assay. Mueller Hinton Agar was prepared for the study. Mueller Hinton agar plates were swabbed with a suspension of each bacterial species, using a sterile cotton swab. Subsequently, the sterilized filter paper discs were completely saturated with the test compound. The impregnated dried discs were placed on the surface of each inoculated plate. The plates were incubated overnight at 37º C. Methanol was used as negative control. Standard discs of Ampicillin served as positive antibacterial control. The test materials having antimicrobial activity inhibited the growth of the micro organisms and a clear, distinct zone of inhibition was visualized surrounding the disc. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition in mm.

2.6 In vitro Cytotoxicity Assay

3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

Keratenocyles cancer cell line (A431) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at 37^0 C, 5% CO_2, 95% air and 100% relative humidity. For screening experiments, the cells were seeded into 96-well plates in 100 mL of the respective medium containing 10% FBS, at a plating density of 10 000 cells/well and incubated at 37 ◦C, 5% CO2, 95% air and 100% relative humidity for 24 h prior to the addition of compounds. The compounds were dissolved in DMSO and diluted in the respective medium containing 1% FBS. After 24 h, the medium was replaced with the respective medium with 1% FBS containing the compounds at various concentrations and incubated at 37 ◦C, 5% CO2, 95% air and 100% relative humidity for 48 h. Experiments were performed in triplicate and the medium without the compounds served as control. After 48 h, 15 μL of MTT (5 mg mL-1) in phosphate buffered saline (PBS) was added to each well and incubated at 37 ◦C for 4 h. The medium with MTT was then removed and the formed formazan crystals were dissolved in 100 mL of DMSO and the absorbance measured at 570 nm using a micro plate reader. The % cell inhibition was determined using the following formula, and a graph was plotted between % of cell inhibition and concentration. From this plot, the IC_{50} value was calculated. The % cell inhibition was determined using the following formula.

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% cell Inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between % Cell inhibition and Log concentration and IC_{50} was determined using GraphPad Prism software.

3. RESULTS AND DISCUSSION

3.1 Synthesis and Characterisation

1,4-bis(4-methoxyphenyl)butane-2,3-dione and 1,4-bis(4-bromophenyl)butane-2,3-dione were condensed individually with imidazol-2-aldehyde in the presence of ammonium acetate. Ligands (**L1** and **L2)** were obtained in moderate yield after column chromatography on silica gel using 1:4 ethylacetate : hexane as an eluent. Synthesized receptors which were subjected to ESI mass spectrometry showed base peaks of protonated adduct at 399.3 and 495.2 for **L1** and **L2** respectively. The mixed ligand complexes $[Ru(bpy)_2(L)](PF_6)_2$ **(1** and **2)** have been isolated from ethanolic solution containing $[Ru(bpy)_2Cl_2].2H_2O$ as the starting material by reacting with substituted biimidazole derivatives under nitrogen atmosphere followed by precipitating the complexes as hexafluorophosphate salts. The analytical data obtained for the new complexes agree well with the proposed molecular formula. The ESI mass spectra of **1** and **2** displayed the molecular ion peaks at m/z 811.33 and 909.13 respectively. These peaks are reliable with the proposed molecular formula of the corresponding ruthenium(II) complexes. The structure of the complexes is revealed in Fig. 1.

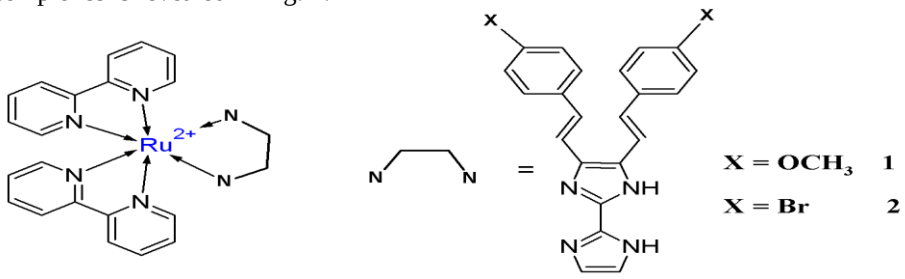

Figure 1. Structure of Ruthenium(II) complexes

The absorption spectra of all the complexes are similar, showing two strong bands in the UV region and a broad band in the visible region. The complexes display a strong absorption band at 290 nm, which is attributed to the π -π* transition of bipyridine ligand. Bands appeared at 346 nm of 1 and 343 nm of 2 were attributed to the π -π* transition of ligands L1 and L2 respectively. Broad ^{1}MLCT absorption bands have been observed at 476 and 476.2 nm for complexes **1** and **2** respectively. The redox behavior of ruthenium complexes is studied with the help of cyclic voltammetry and differential pulse voltammetry. The cyclic and differential pulse voltammograms of the Ru(II) complexes show oxidation peaks at higher potentials thereby suggesting stable Ru(II) complexes.

Table 1 Spectroscopy and electro chemical data's for the complexes **1** and **2**.

Complex	UV-Visible λ_{max}, nm	ESI-MS m/z	$E_{1/2}$ ($Ru^{(II)}/Ru^{(III)}$) vs SCE	ΔE (mV)
$[Ru(bpy)_2(L1)](PF_6)_2$ (1)	474.6, 342.0 290.4, 243.2	811.33 $(M-2PF_6)^+$	+1.097 V	138
$[Ru(bpy)_2(L2)](PF_6)_2$ (2)	478.0, 344.3 290.2, 244.4	909.13 $(M-2PF_6)^+$	+1.074 V	133

Electrochemical studies carried out for the present complexes are summarized in Table 1. The cyclic voltammograms of the complexes reveal a fairly reversible one electron redox process involving the $Ru^{(II)/}Ru^{(III)}$ couple, as judged from the peak potential separation of ~130 mV. Complex **1** gives an $E_{1/2}$ value of 1.097 V at 100 mVs^{-1}. Whereas complex **2** shows voltammetric response at 1.074 V. The $E_{1/2}$ values follow the order **2** > **1**. Complex **2** showing a greater stabilization for the $Ru^{(II)/(III)}$ species could be related to the presence of methoxy group enhancing the π –acidity of the ligand.

3.2 DNA Cleavage Activity of the Complexes

Gel electrophoresis experiment of ruthenium complexes was performed by irradiating the complexes with DNA to light for about 60 min at a wavelength of 440 nm. The present complexes did not show any apparent cleavage in the absence of light. Hence the possibility of hydrolytic cleavage of DNA involving the phosphodiester bond is eliminated. But in the presence of light radiation they are able to cleave plasmid DNA. They exhibited significant DNA cleavage at a concentration of 24 µM, when they are exposed to light at a wavelength of 440 nm. In order to understand the cleavage mechanism, we attempted to do the experiment in the presence of singlet oxygen quenchers and hydroxyl radical scavengers like histidine and DMSO respectively. Interestingly, on addition of histidine and DMSO to the reaction mixture, the DNA cleavage activity was not inhibited greatly. This conclusively shows that DNA cleavage occurs via guanine base oxidation of DNA by excited state of ruthenium complexes. DNA scission by the ruthenium complexes is shown in Fig. 2.

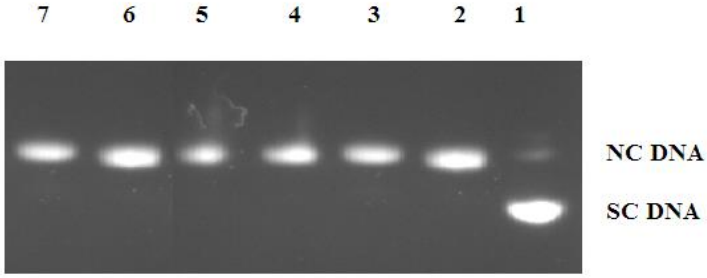

Figure 2. Cleavage of pUC19 DNA by complexes (**1** and **2**) when exposed to light at 440 nm. DNA was incubated with complex for 60 min in Tris buffer (pH 7.2) at 37˚C. Lane 1, DNA control; lane 2, DNA + 1 (24 µM); lane 3, DNA + 1 (24 µM) + DMSO (10 mM); lane 4, DNA + 1 (24 µM) + Histidine (10 mM); lane 5, DNA + 2 (24 µM); lane 6, DNA + 2 (24 µM) + DMSO (10 mM); lane 7, DNA + 2 (24 µM) + Histidine (10 mM).

3.3 Antibacterial Activity

The biological activities of many coordination compounds have been related to the ability of metal ions to form stable complexes[9-12]. The results have led to an understanding of coordination sphere and electronic properties of the metal ions. Several workers have reported that heterocyclic rings containing sulfur, nitrogen, and/or oxygen are responsible for the biological activity of ligands and their metal complexes[13].

The series of complexes **1-5** bearing heterocyclic ligands were tested for their *in vitro* antimicrobial activity against a number of standard microorganisms. Ampicillin (commercial antibiotic) was used as control. Three out of the five bacterial pathogens were negative (*Escherichia coli*, *Klebsiella pneumonia*, *Salmonella paratyphi*) and two were positive (*Bacillus subtilis*, *Streptococcus faecalis*). Disc diffusion method was used to evaluate the antibacterial activity of taken samples. After 24 hours the minimum inhibitory zone of methanolic solution of complexes and control were measured.

Table 2. Antibacterial activity of Ligands and Complexes

S. No	Bacteria	Control Ampicillin (mm)	Zone of inhibition (mm)			
			L1	1	L2	2
1	*Streptococcus faecalis*	16.3 ± 0.34	5.7 ± 0.18	8.0 ± 0.13	5.9 ± 0.14	8.8 ± 0.72

2	*Bacillus subtilis*	4.3 ± 0.36	7.7± 0.12	11.6 ± 0.04	7.3 ± 0.18	7.9 ± 0.23
3	*Klebsiella pneumonia*	15 ± 0.04	7.1 ± 0.43	7.5 ± 0.22	7.8 ± 0.42	10.2 ± 0.64
4	*Salmonella paratyphi*	14 ± 0.12	5.2 ± 0.71	8.3± 0.17	8.7 ± 0.20	11.3± 0.11
5	*Escherichia coli*	6.5± 0.82	6.8 ± 0.55	7.9 ± 0.81	5.9 ± 0.65	7.1 ± 0.54

The results of antibacterial assay of the test compounds are provided in Table 2.
The ligands are all derivatives of biimidazole. They all have the imidazole moiety as the pharmacophoric group which may confer hydrophobicity or lipophilicity to the organic molecules facilitating their permeation through the lipid membranes of the bacterial cells. Further, the organic ligands are all aromatic in character. The delocalization of π electrons over the molecular skeleton may ease the diffusion of the molecules through the cell membrane.

In complex **1**, the observed order of zone of inhibition was *B. subtilis* > *S. paratyphi* > *S. faecalis* > *E. coli* > *K. pneumonia*. On the other hand the observed zone of inhibition order of complex **2** was *S. paratyphi* > *K. pneumonia* > *S. faecalis* > *B. subtilis* > *E. coli*. In all cases, the metal complexes are more active than the ligand expectedly due to chelation, which reduced the polarity of the metal atom, mainly because of partial sharing of its positive charge with donor groups of the ligand and possible π-electron delocalisation on the aromatic rings. This increased the lipophilic character, favouring its permeation into the bacterial membrane, causing the death of the organisms [14-16].

3.4 Antitumor Activity Evaluation by MTT Assay

MTT assay was used to explore the antitumor potential of newly synthesized ruthenium(II) complexes (**1** and **2**) on cancerous keratenocyles cells A431. A431 cell line was treated with various concentrations of ruthenium(II) complexes. 50 % inhibitory concentration value (IC50) was determined for all complexes and reported in Table 3. The obtained results revealed that complex **2** was found to behave as a good antitumor agent on cancerous keratenocyles cells A431. Whereas complex **1** exhibited weaker activity against A431 cell line in comparison with **2**. The results highlight the relationship between substitutions at the para position of the phenyl ring and cytotoxic activity. Based on IC50 value the potency of cytotoxic activity of ruthenium(II) complexes is given in the order: **2** > **1**.

Table 3. *In vitro* anticancer activity of synthesized ruthenium(II) complexes

S.No.	**Complex**	**IC_{50} value (µM)**
1	$[Ru(bpy)_2(L4)](PF_6)_2$	27.08
2	$[Ru(bpy)_2(L5)](PF_6)_2$	26.94

4. CONCLUSIONS

Two new ruthenium(II) mixed ligand complexes were synthesized and fully characterized by spectroscopic methods. Complexes 1 and 2 are able to cleave plasmid DNA more effectively in the presence of light at the wavelength of 440 nm. The antibacterial activity of the complexes is found to be higher than that of the ligands. Furthermore, these complexes are found to be effective in inhibiting the growth of cancer cell.

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