Research Article

Synthesis, Characterization, DNA-binding, Photocleavage, Cytotoxicity of Co(III) Mixed Polypyridyl Complexes

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ABSTRACT

Three new complexes of the type $[Co(phen)_2PIP\text{-}Br]$(**1**) $[Co(bpy)_2PIP\text{-}Br]$ (**2**) and $[Co(dmp)_2PIP\text{-}Br]$(**3**) (phen = 1,10-phenanthroline; bpy = 2,2'-bipyridine; dmb = 4,4-Dimethyl-(2,2')bipyridine), PIP-Br = 2-(4'-Bromo-phenol) imidazo[4,5-f][1,10]phenanthroline) were synthesized and characterized by using UV–VIS, IR and ^{1}H-NMR,^{13}C-NMR spectral methods. Absorption spectroscopy, emission spectroscopy, and viscosity measurements were used to investigate the binding of these Co(III) complexes with calf thymus DNA and photocleavage studies were used to investigate the binding of these complexes with plasmid DNA. The spectroscopic studies and viscosity measurements studies supported the fact that Co(III) complexes bind to CT DNA(calf thymus DNA) by an intercalation mode via PIP-Br into the base pairs of DNA. Upon irradiation, these novel Co(III) complexes cleave the plasmid pBR322 DNA from the supercoiled form I to the open circular form II. Anti-tumor activity of these Co complexes was evaluated on ovarian cancer celline, SKOV3. Cytotoxicity (MTT assay) of cobalt complexes showed growth inhibition in dose dependent manner. Cell cycle analysis by flow cytometry data showed increase in Sub G1 population. Annexin V FITC/PI staining and DNA ladder assay confirms that these complexes cause cell death by the induction of apoptosis.

Keywords: Cobalt (III) complex; Cytotoxicity; Apoptosis.

INTRODUCTION

Discovery of Cisplatin and its analogs for cancer treatment offers new insight into the field of medicinal (bio)inorganic chemistry. Although a range of their clinical use, these platinum drugs have serious limitations due to their various side effects to the normal cells, high toxicity and their inactiveness against many other cancer cell lines and occurrence of metastasis[1] led the scientists, to discover other potential metal based anticancer drug alternatives and hence complexes of various transition metals were synthesized and examined for their anticancer activities[2]. The search for new metallo-anticancer drugs, which drives much current research, currently includes a focus on ruthenium complexes. The known ruthenium complexes $[Ru(phen)_2(dppz)]^{2+}$ and $[Ru(bpy)_2(dppz)]^{2+}$ are the most extensively investigated, because they were found to be excellent DNA-molecular "light switches", which can exhibit a negligible background emission in water, but exhibit an intense luminescence in the presence of double-stranded DNA[3-5]. Hakan Niazi et al, reported crystal structure with oligonucleotide of octahedral complexes with a dipyridophenazine(dppz) ligand[6]. There have been intensive efforts to investigate factors that determine affinity and selectivity the in binding of small molecules to DNA[7], since information about these factors would be valuable for the design of sequence-specific DNA-binding molecules for applications in chemotherapy and in the development of tools for biotechnology[8]. In our group, much effort has been devoted to studying the DNA interactions and cytotoxicities of novel polypyridyl complexes containing different intercalative ligands[9-13].

In this article we report three Co(III) mixed ligand polypyridyl complexes, $[Co(phen)_2PIP\text{-}Br](ClO_4)_3$ (1), $[Co(bpy)_2\ PIP\text{-}Br](ClO_4)_3$ (2), and, $[Co(dmb)_2\ PIP\text{-}Br](ClO_4)_3$ (3), and their DNA binding behavior their abilities to induce cleavage of pBR322 DNA. Cell viability experiments indicated that the Co(III) complexes showed significant dose dependent cytotoxicities against human ovarian cancer cell line, namely SKOV3. The complexes were also tested for antimicrobial activity, and docked into DNA base-pairs using a docking program[14, 15].

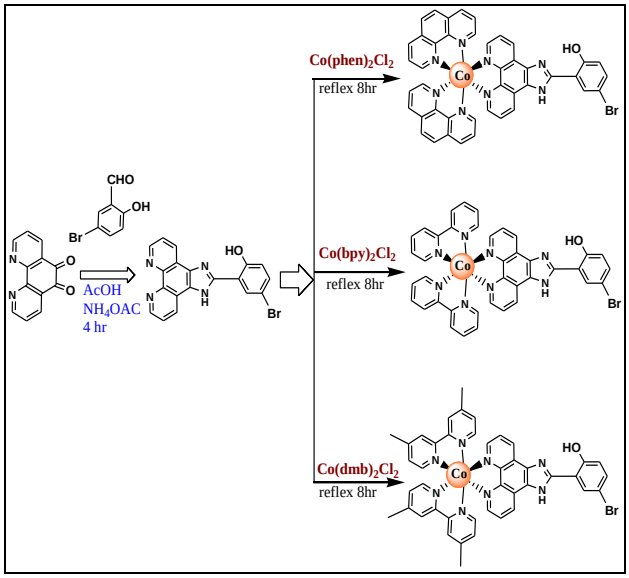
MATERIALS AND METHODS

$CoCl_2.6H_2O$. 1,10-phenanthroline monohydrate, 2,2'-bipyridine and 4,4'-dimethyl-2,2'-bipyridine were purchased from Merck. CT-DNA and super coiled (CsCl purified) pBR322 DNA (Bangalore Genei, India) was used as received. All other common chemicals and solvents were procured from locally available sources; solvents were purified before use by standard procedures[16]. Deionized, double distilled water was used for preparing various buffers. Solutions of DNA in 5mMTris-HCl buffer (pH = 7.2), 50 mM NaCl gave a ratio of UV absorbance at 260 and 280 nm of about 1.8 - 1.9:1, indicating the DNA was sufficiently free of protein[17]. The concentration of CT-DNA was determined spectrophotometrically using the molar absorption coefficient 6600 M^{-1} cm^{-1} (260 nm)[18]. Human tumor cell lines were obtained from NCCS, Pune, and maintained in RPMI 1640 medium (Sigma Aldrich)

medium supplemented with 10% fetal bovine serum, 1% penicillin, and streptomycin in a humidified 5% CO_2 atmosphere.

Synthesis and characterization

1,10-Phenanthroline-5,6-dione[19], *cis*-$[Co(phen)_2Br_2]Br.2H_2O$, *cis*-$[Co(bpy)_2Br_2]Br.2H_2O$ and *cis*-$[Co(bpy)_2Br_2]Br.2H_2O$ [20,21] were prepared according to the methods used in our previous study and the literature respectively[22,23]. The syntheses of the free ligands and their Co(III) complexes are shown in scheme 1.



Scheme 1: Synthetic route for the preparation of Co(III) (1-3) complexes.

Synthesis of PIP-Br

A mixture of phen-dione (0.53 g, 2.50 mmol), 5-bromo-2-hydroxybenzaldehyde (0.69 g, 3.50 mM), ammonium acetate (3.88 g, 50.0 mmol) and glacial acetic acid (15 mL) was refluxed for 4 h. The above solution was cooled to the room temperature and diluted with water. Drop wise addition of concentrated aqueous ammonia gave a yellow precipitate which was collected, washed with H_2O and dried. The crude product thus obtained was purified by recrystallization with $CHCl_3$/MeOH (4:1 v/v) and dried. Yield = 0.73 g (68%). Analytical data: $C_{19}H_{11}BrN_4O$) Cacld (%): C, 58.33; H, 2.83; N, 14.32; Found (%): C, 58.80; H, 2.90; N, 14.48; ESI-MS m/z: Cacld: 390, Found: 391 $[M+H]^+$. IR (KBr, cm^{-1}) 3420-2953 (broad) (N-H, O-H), 1626 (C=N), 1585 (C=C). ^{1}H NMR (DMSO-d_6, 400MHz, δ-ppm):8.30 (d, 2H), 8.09 (d, 2H), 7.68-7.51 (m, 2H), 7.26-7.22 (m, 2H),7.20 (s, 1H).

Synthesis of Complex-1

A mixture of *cis*-$[Co(phen)_2Br_2]Br.2H_2O$ (0.57 g, 1.0 mmol), Pip-Br (0.56 g, 1.5 mmol) in EtOH (50 cm^3) was refluxed for 4 h to give a yellow solution. After filtration, the complex was precipitated by addition of a saturated ethanolic solution of $NaClO_4$. The complex was filtered off and dried under vacuum before recrystallization (Me_2CO–Et_2O). Yield: (79%). $C_{43}H_{27}Cl_3CoN_8O_{13}$; Calcd (%); Analytical data: $RuC_{43}H_{27}N_8BrCl_2O_9$ Calcd (%): C:49.11; H:2.59; N:10.66; Found(%): C: 4.52; H:2.70; N:10.68; ESI-MS (m/z): Calcd:1049, Found:1050 $[M+H]^+$. IR: 3484-3408 (N-H. O-H), 1638 (C=N), 1616 (C=C), 730 (Ru–N (PIP-Br), 620 cm^{-1} (Ru–N (phen). ^{1}H-NMR (DMSO-d_6, 400 MH_Z δ ppm): 9.06 (d, 6H), 8.79 (d, 1H), 8.40 (d, 6H), 8.27 (d, 4H), 8.15 (d, 2H), 8.02 (d, 2H),7.88-7.75 (m, 2H), 7.34-7.31 (d, 2H). ^{13}C-NMR (100 MHz, DMSO-d_6, 298 K, δ ppm): 155.8, 152.3, 150.9, 147.8, 145.1, 138.2, 132.60,, 125.3, 126.9, 127.0, 124.1, 119.7.

Synthesis of Complex-2

This complex was obtained by a procedure similar to that described above, except that $[Co(bpy)_2Br_2]Br.2H_2O$ (0.53 g, 1.0 mmol) was used in place of *cis*-$[Co(phen)_2Br_2]Br.2H_2O$. (Yield: 72%). $C_{39}H_{26}Cl_3CoN_8O_{13}$; Analytical data: $RuC_{39}H_{27}BrCl_2N_8O_9$ Calcd (%): C:46.68; H:2.71; N:11.17; Found(%): C:46.90; H:2.81; N:11.20;. ESI-MS (m/z): Cacld:1001. Found:1002 $[M+H]^+$. IR: 3450 (N-H), 1610 (C=N), 1465 (C=C), 718 (Ru–N (L)), 615 cm^{-1} (Ru–N(PIP-Br)). ^{1}H-NMR (DMSO d_6, 400 MH_Z δ ppm): 8.93 (d, 2H), 8.86 (d, 4H), 8.53 (d, 4H), 8.24 (t, 2H), 8.12 (d, 4H), 7.54-7.46 (m, 6H), 7.21 (d,4H).^{13}C-NMR (100 MHz, DMSO-d_6, 298K): 158.2, 157.0, 152.1, 146.8, 139.2, 132.1 , 127.2, 126.5, 125.1, 123.7, 119.1, 116.2.

Synthesis of complex-3

This complex was obtained by a procedure similar to that described above, but using *cis*-$[Co(dmb)_2Br_2]Br.2H_2O$ (0.587 g, 1.0 mmol) in place of *cis*-$[Co(phen)_2Br_2]Br.2H_2O$. Yield: (65%). $C_{43}H_{35}Cl_3CoN_8O_{13}$; Analytical data: $RuC_{43}H_{35}N_8BrCl_2O_9$: Calcd (%) C:48.74; H:3.33; N:10.57; Found(%): C:49.14; H:3.73; N:10.75. ESI-MS (m/z): Calcd: 1058, Found 1059 $[M+H]^+$. IR: 1475 (C=C), 1617 (C=N), 717 (Ru–N (L)), 609 cm^{-1} (Ru–N(PIP-Br). ^{1}H-NMR (DMSO-d_6, 400 MH_Z δ ppm): 9.12 (d, 6H), 8.94 (d, 4H), 8.80 (s, 1H), 8.15 (d, 6H), 8.09 (t, 6H), 7.92 (d, 1H), 7.71 (d, 1H), 2.58 (s, 12H). ^{13}C-NMR (100 MHz, DMSO-d_6, 298K): 157.5, 154.1, 152.1, 150.1, 149.7, 146.7, 130.1, 129.1, 127.6 126.4, 125.1, 124.7, 119.2, 115.0, 21.1.

Physical measurements

^{1}H and ^{13}C [^{1}H]NMR spectra were measured on a Bruker Z-Gradient single axis fitted with a high resolution probe and 400 MHz standard spectrometer using DMSO-d_6 as the solvent and TMS as an internal standard. IR spectra were recorded in KBr discs on a Perkin-Elmer FT-IR-1605 spectrometer. Microanalysis obtained on a Perkin-Elmer 240 elemental analyzer. UV-Visible spectra were recorded with an Elico Biospectrophotometer, *model BL198*. Fluorescence spectra were recorded with an Elico spectrofluorimeter model SL 174.

The DNA binding experiments were performed in Tris-HCl buffer at room temperature. The absorption titrations were performed at a fixed complex concentration, to which the DNA stock solution was gradually added up to the point of saturation. The mixture was allowed to equilibrate for 5 min before the spectra were recorded.

The emission intensities were recorded in the range of 530-690 nm. In these emission studies fixed complex concentrations (10 µM) was taken and to this varying concentration (0-100 µM) of DNA were added. The excitation wavelength was fixed and the emission range was adjusted before measurements. The fraction of the ligand bound was calculated from the relation,

$$C_b = C_t [(F-F_0)/F_{max}-F_0)]$$

where C_t is the total complex concentration, F is the observed fluorescence emission intensity at a given DNA concentration, F_0 is the intensity in the absence of DNA and F_{max} is when the complex is fully bound to DNA. The binding constant (K_b) was obtained from a modified Scatcherd equation[24], From a Scatcherd plot of r/C_f *versus* r, where r is C_b/[DNA] and C_f is the concentration of free complex.

Viscosity experiments were carried out with an Ostwald viscometer. DNA samples approximately 200 base pairs in average length were prepared by sonication in order to minimize complexities arising from DNA flexibility[25]. Data were analyzed as $(\eta/\eta º)^{1/3}$ versus [Co]/[DNA], where η is viscosity of DNA in the presence of complex and ηº is the viscosity of DNA alone. Viscosity values were calculated from the observed flow time of DNA-containing solutions (t >100 s) corrected for the flow time of buffer alone (t^0), $\eta = t-t_0$[26].

For the gel electrophoresis experiments, super coiled pBR322 DNA (0.1 µg/µL) was treated with 20 to 80 µM of Cobalt(III) and the solution were irradiated under the UV light (360 nm) for half an hour. The samples were analyzed by electrophoresis for 2.5 h at 50 V on a 0.8% agarose gel in 1x TAE buffer, pH 8.2. The gel was stained with 1 µg/mL ethidium bromide and then photographed under UV light. The gels were viewed in a Gel doc system and photographed using a CCD camera (Gel Documentation System Merck- Genei Model: 120544GB).

Antimicrobial tests were performed by the standard disc diffusion method[27]. The antibacterial activities of the complexes were studied against *Staphylococcus aureus* (MTCC 96) and *Escherichia coli* (MTCC 443). Each complex was dissolved in DMSO at 1mg/mL^{-1}. Paper discs of Whatman filter paper no. 1 were cut and sterilized in an autoclave. The paper discs were saturated with 10 mL of the cobalt complex dissolved in DMSO, so DMSO as negative control and placed aseptically in Petri dishes containing M-test agar media inoculated with *S.aureus or E. coli*. The Petri dishes were incubated at 37º C and the inhibition zones were recorded after 24 h. The experiments were repeated and the average of the two runs was taken. The results were also compared with the standard antibacterial drug streptomycin at the same concentration.

In Vitro studies

SKOV3, an human ovarian carcinoma cell line was cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 mg/ml streptomycin (Invitrogen Corporation, Grand Island, NY) 2mM l-glutamine, 10mM HEPES, and 1.0mM sodium pyruvate in 5% CO_2 incubator at 37 °C.

MTT cytotoxic test

The cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, USA) and performed as per standard protocol.SKOV3 cells were seeded 7,000/well in 96 well plate and treated with Co complexes 1,2 and 3 in varying concentrations from 100µM to 1.56µM for 48hrs where as untreated cells were taken as controls. After treatment, MTT reagent was added (5mg/ml) and incubated in dark for 4hrs at 37 °C. After incubation, media was aspirated and DMSO (200µl) was added to each well to dissolve the purple colored precipitate of formazan crystals. The optical density was measured at 570 nm using a microtitre plate reader. Then IC_{50} of Co complexes were calculated.

Morphologic observation by phase contrast microscope

SKOV3 cells at a density 5×10^4 cells/well were seeded in 24-well plates and then cells were treated with Co complexes 1, 2 and 3 at 25µM concentration for 48hrs.Cell morphology was examined and photographed using a phase contrast microscope.

Cell cycle analysis

To analyse the cell cycle dynamics, briefly 3×10^5 SKOV3 cells were seeded and then treated with Co complexes 1, 2 and 3 of 25µM for 48hrs.After stipulated time of treatment, cells were collected by centrifugation and wash with PBS and fixed in 70% ethanol for 30-60min at room temperature and then cells were centrifuged, alcohol was discarded and pellet was washed with PBS. Then cells were again resuspended in 500µl of PBS which consists of 50µg/ml PI stain and 100µg/ml of RNase and incubate for 20min in dark at 37°C before analysis. Cells with only medium was used as control. Cell cycle distribution was determined using a FACScan flow cytometer and Cell Quest software (FACS Calibur; Becton-Dickinson, San Jose, CA).A total of 10000 cells were gated and analyzed. All experiments were performed in triplicates.

Apoptotic analysis with Annexin V FITC PI Dual Staining:

For Apoptotic analysis, One million cells were seeded in T25 flasks and then treated with Co complexes 1, 2 and 3 of 25µM concentration for 48hrs and cells without treatment were kept as controls. Campothecin was used as early apoptotic inducer. After the treatment with Co complexes, cells were pelleted down, washed with PBS and remaining experiment was performed according to the sigma Annexin V FITC PI kit protocol.

Detection of DNA fragmentation (ladder assay)

For DNA isolation, 5 million cells were treated with complexes 1, 2 and 3 of Cobalt of 25 µM concentrations

for 72hrs.Untreated cells were used as controls. After incubation, cells were pelleted, washed with PBS and then lysed with 700µl of lysis buffer (10 mM EDTA, 0.5% SDS, 10 mM Trizma Base and 0.5 µg/mL Proteinase K; pH 7.5) and incubated for 2hrs at 37 °C. After this, cells were treated with 0.5 µg/mL RNase A for 2 h at 55 °C. Then DNA was extracted by phenol-chloroform isoamyl alcohol method and DNA thus obtained was dissolved in 50ul of TE buffer. Concentration of DNA was calculated by Nano drop. A total of 2.5µg DNA was loaded on 2% agarose gel and ran at 20V and then stained with Ethidium bromide and finally DNA was visualized by UV illumination.

RESULTS AND DISCUSSION

Characterization

All the three synthesized complexes were confirmed using different spectral techniques. The proton spectrum of ligand (PIP-Br) gave a singlet peak at 9.1 corresponding to OH proton, and a broad singlet peak at 5.0 corresponding to –NH proton, but in the complexes 1, 2 and 3 it is shifted to downfield indicates complex confirmation. The $^{13}C[^{1}H]$-NMR spectrum, C-OH carbon of ligand(PIP-Br) gave a singlet peak at 146 ppm, whereas in complexes it is at 153,153 and 150ppm respectively, carbon next to nitrogen in ligand at 145 ppm, but in complexes 149, 149 and 152 ppm respectively. The FT-IR spectrum of this complex-1 showed bands at 1634 (C=N) and 1432 (C=C), complex-2 is 1607 (C=N) and 1434 (C=C) and complex-3 is 1607 (C=N) and 1434 (C=C), all are shifted to a lower frequency when compared to free ligand consistent with complexation. New bands at 620, 610, 612 are Co–N (PIP-Br) and 456, 575, 570 cm^{-1} assigned to Co–N(phen, bpy, dmb) of complexes 1-3 respectively were support complex formation.

Electronic absorption

The absorption spectra of three complexes were studied to identify the interaction between metal complexes and DNA.

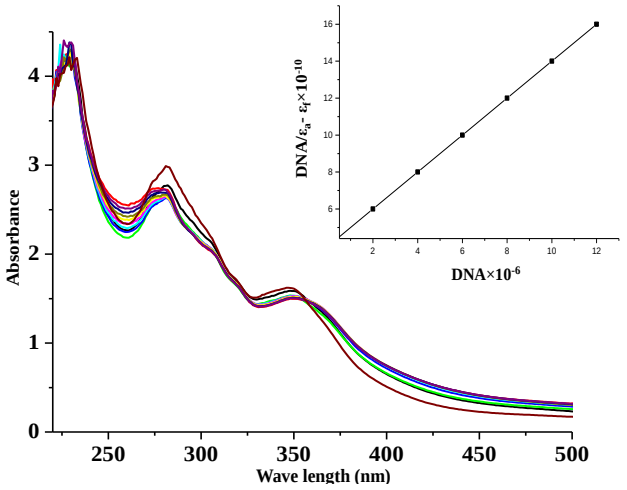


Figure 1: Absorption changes of $[Co(bpy)_2(pip\text{-}Br)]^{2+}$ upon increasing DNA concentration (5 mM Tris, pH 7.5). Inset shows the fit of the absorption changes at 350 nm

The spectra of these complexes in the absence and presence of DNA are shown in Figure 1, bands at λ_{max} 375 nm (**1**), 350 nm (**2**), and 350 nm (**3**) are assigned to MLCT[28]. These spectrums were absorbed with increasing the concentration of CT-DNA, results in decreasing intensities at MLCT region. As the DNA concentration is increased, the MLCT bands of complexes **1**- **3** exhibited hypochromism of 18%, 13%, and 9% respectively, and bathochromism of about 3-6 nm. Intrinsic DNA binding constants K_b were determined by monitoring the change of absorbance of the MLCT bands of the complexes with increasing concentration of DNA[29], according to following equation[24]

$$[DNA]/(\varepsilon_a-\varepsilon_f) = [DNA]/(\varepsilon_b-\varepsilon_f) + 1/(K(\varepsilon_b-\varepsilon_f))$$

where [DNA] is the concentration in the base pairs, the apparent absorption coefficients ε_a, ε_f, and ε_b correspond to $A_{obsd}/[Co]$, the extinction coefficients for the free cobalt complex, the complex in the presence of DNA, and the cobalt complex in the fully bound form, respectively. In plots of $[DNA]/(\varepsilon_a-\varepsilon_f)$ versus [DNA], K_b is given by the ratio of slope to intercept. The K_b values of are 5.12×0^5, 4.15×10^5 and 3.10×10^5 M^{-1}, for complex **1**, **2** and **3** respectively. The difference in binding strength of complexes **1** and **2** could be attributed to the different ancillary ligands; phenanthroline is more planar than bipyridyl hence the binding constant of complex **1** is higher than complex **2**. Similarly, the two additional methyl groups of dimethyl bipyridyl in complex **3** exert steric hindrance, hence complex **2** binds strongly than complex **3**. The ligand PIP-Br contains a free hydroxy group, which may form intramolecular hydrogen bonds with the nitrogens of DNA.

Fluorescence studies

The emission studies were performed to characterize the binding mode of the complexes with CT-DNA. It gives additional supportive information of complexes binding to CT- DNA. Luminescence titration experiments were performed in Tris buffer (pH = 7.2) at a fixed metal complex concentration (10 µM) at room temperature. The change of emission intensity is related to the extent to which the complex enters into the hydrophobic environment within the DNA. Figure 2 shows the fluorescence excitation and emission spectra for the free and bound complexes in the presence of different amounts of CT-DNA. Excitation wavelengths of ≈ 350- 375 nm and emission wavelengths of ≈ 420, 430 and 425 nm for complexes **1**, **2** and **3** respectively. An increasing the DNA concentration of the resultant solution to each complex increase in fluorescence intensity. The intrinsic binding constant was obtained from the fluorescence data using a modified form of one scatcherd equation[24] with a plot of r/c_f *versus* r, where r is the binding ratio $C_b/[DNA]$ and C_f is the free ligand concentration. These plots gave binding constants (K_b) of $(5.14 \pm 0.1) \times10^5$ M^{-1}, $(3.96 \pm 0.1) \times10^5$ M^{-1}, and $(3.27 \pm 0.1) \times 10^5$ M^{-1} for complexes **1**, **2** and **3** respectively. The order of Kb values agrees with the results of the absorption studies.

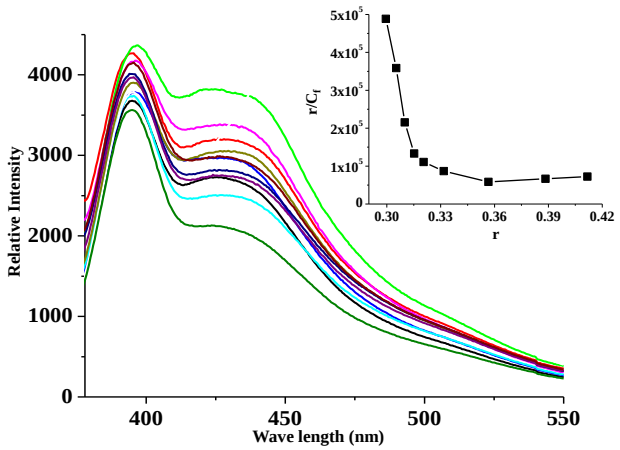


Figure 2: Emission spectra of $[Co(bpy)_2(pip\text{-}Br)]^{2+}$ in Tris–HCl buffer upon addition of CT-DNA. Inset: Scatchard plot of above complex, which gives binding constant (K_b).

Viscosity studies

Viscosity measurement is sensitive to DNA length change and it is least ambiguous, most critical test of a binding model. In classical intercalation, ligand bound in between the base pairs of DNA, so the DNA helix lengthens and an increase in the viscosity of the DNA solution[30, 31]. In case of partial or nonclassical intercalation, ligand may bind sides of DNA base pairs, resulting in a decreases DNA length and its viscosity. As the concentration of cobalt(III) complexes increases, the relative viscosity of DNA also increases Figure 3, it is similar to $[Ru(phen)_2dppz]^{2+}$, proven DNA intercalator[32]. Although the intercalating ligand is the same in all three complexes and follows order is 1 > 2 > 3, the small differences in the viscosity is due to the difference in ancillary ligands.

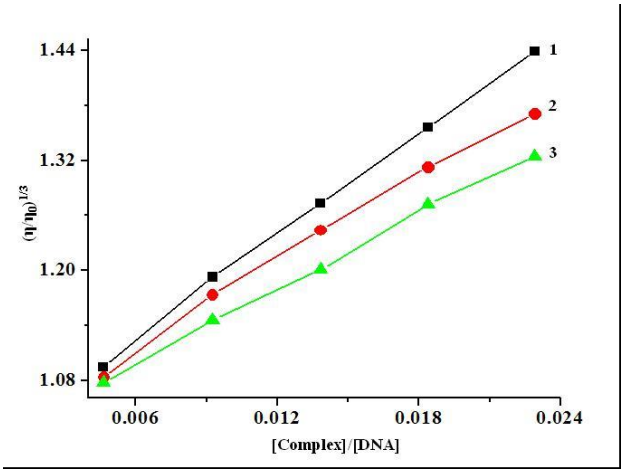


Figure 3: Effect of increasing amounts of complexes 1-3 on the relative viscosity of calf thymus DNA at room temperature.

Photo activated cleavage of pBR322 DNA

Many cobalt(III) complexes can cleave DNA under irradiation by UV or Visible light. When circular plasmid DNA is subject to electrophoresis, relatively fast migration will be observed in the intact super coiled form (Form I); if scission occurs on one strand (nicked), the super coiled will relax to generate a slower-moving open circular form (Form II)[24]. Plasmid pBR322 DNA was subjected to gel electrophoresis after incubation with the cobalt(III) complexes and irradiation at 365 nm for 60 min. In control or the DNA-complex mixtures were incubated in the dark no cleavage was observed, but the complexes were irradiation under UV light the scission was occurred, with increasing concentrations of complexes from 5-60µM resulted in a decrease in the amount of supercoiled DNA, whereas the nicked (form II) increased which is slower moving[33] as shown in Figure 4. These results indicate that scission occurs on one strand (nicked). Here, all three complexes are effective for photo sensitized cleavage of DNA.

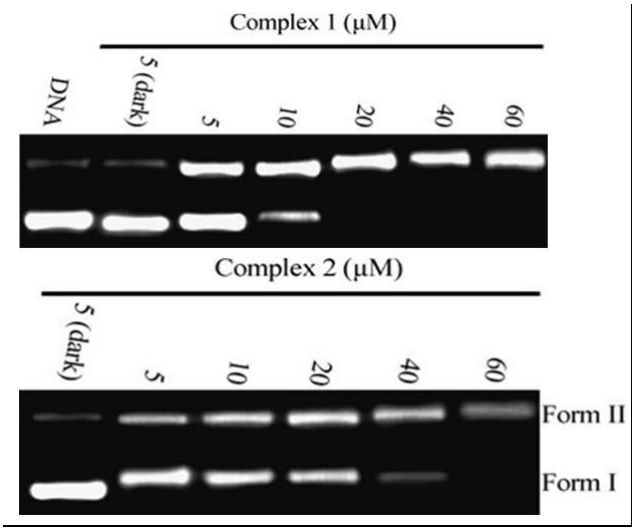


Figure 4: Photocleavage studies of pBR322 DNA, in the absence and presence of complexes 1 and 2. Control plasmid DNA (absence of complexes) (DNA) and in presence of complexes with 5µM (dark), 5µM, 10µM, 20 µM, 40 µM and 60µM respectively.

Antimicrobial activities

The antibacterial activity results indicate that the three cobalt(III) complexes showed good activity against *E. coli, S. aureus*. The results were expressed as zone inhibition diameter (inmm) versus the control (DMSO). DMSO control showed a negligible activity. Complex **1** shows the highest activity (19 mm) against *S. aureus* and 18 mm inhibition against *E.coli* at 1 mg/mL concentration. Complexes **2** and **3** showed less activity against these bacteria than complex-1, but these three complexes were shown less effective than the standard drug ampicillin. Earlier studies have given results which were also similar to this study[34, 5].

In vitro studies

MTT Assay

Cytotoxicity of cobalt complexes 1, 2 and 3 were showed significant cell growth inhibition in dose dependent manner on SKOV3 cells on incubation for 48hrs with Co complexes in comparision to untreated cells. IC_{50} value calculated from MTT assay for complex 1 was 15µM

whereas for complex 2 and 3 it was 12 and 13.3 µM as shown in figure 5.

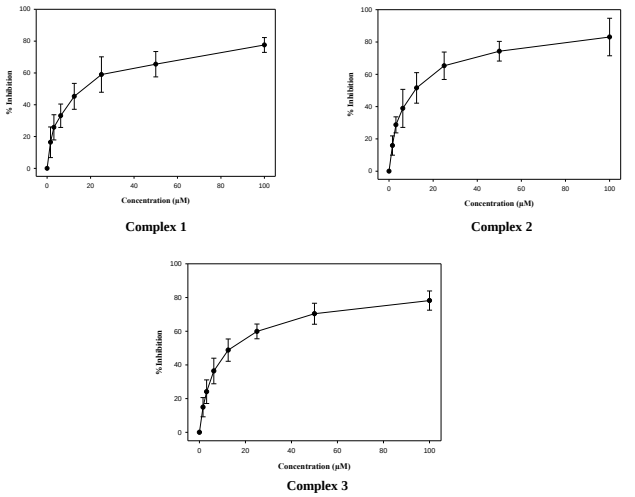


Figure 5: Assessment of cytotoxicity of complexes 1, 2 and 3 on SKOV3 cells by MTT assay. Cells were treated with complexes 1, 2 and 3 in varying concentrations from 100µM to 1.56µM for 48hours.Untreated cells were used as controls. IC_{50} was calculated. Data illustrated here are from three replicates (n=3) of experiments and represented as mean ± S.D.

Morphological observation

The effect of three cobalt complexes on morphology of SKOV3 was observed by phase contrast microscope. When cells treated with cobalt complexes 1, 2 and 3 at 25µM concentration for 48hrs, these complexes inhibited cell growth in significant manner compared to untreated cells. As shown in the figure 6, Apart from drastic decrease in cell number, cell morphology also changed. Notably cells become round and shrunken.

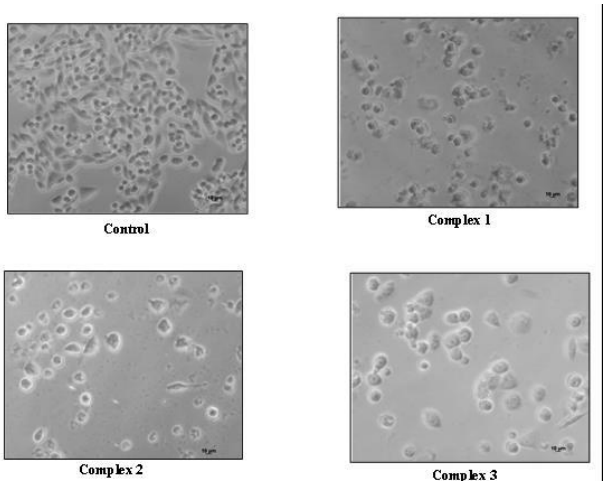


Figure 6: Morphological changes occurred in SKOV3 cells. Cells were treated with complexes 1, 2 and 3 with 25µM for 48 hours and then observed under phase contrast microscope.

Cell cycle analysis

Since Cobalt complexes 1, 2 and 3 treatment induces cytotoxicity in SKOV3 cells, so we were interested to know whether these are disturbing the cell cycle dynamics. For this, Cell cycle studies were performed using flow cytometry. Results had shown that a notable change was observed that complex 1 treatment causes increase in S phase population of some cells which means it was arresting S phase of cell cycle but some remaining cells were in sub G1 phase. Sub G1 represents the hypodiploid nuclei, DNA fragmentation and cell death. Due to complex 2 and complex 3 treatments, 47.8% cells and 28% cells went to Sub G1 phase. All these complexes had shown increase in SubG1 population as compared to control as shown in figure 7. These results suggest that all three complexes were effective in causing cell death.

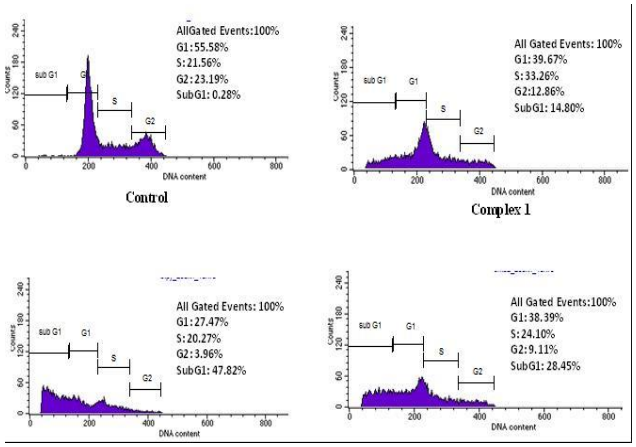


Figure 7: Effect of Co complexes on cell cycle progression.SKOV3 cells were treated with complexes 1, 2 and 3 with 25µM. After 48hours of treatment, cells were fixed and stained with PI and distribution of cell cycle phases were quantified by flow cytometry.

Dual staining by Annexin V FITC

Since Co complexes was causing cell death,we were interested to investigate the mechanism of cell death. For this, Annexin V FITC/ Propidium Iodide (PI) dual staining followed by flow cytometric analysis was performed in SKOV3 cells that were treated with Co complexes for 48hours. Results had shown that four populations.

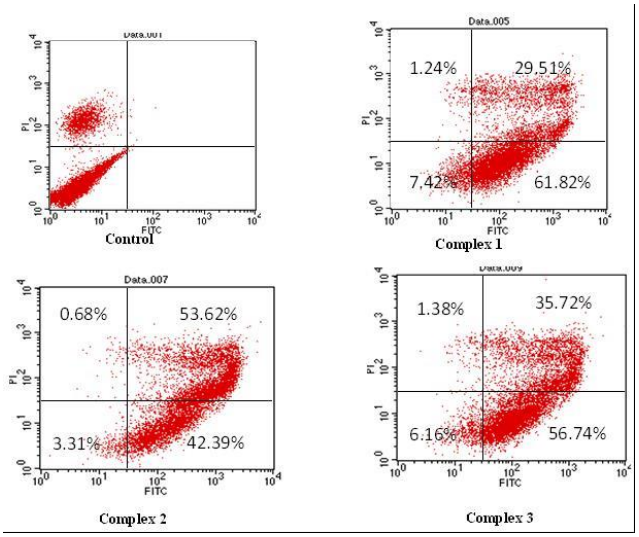


Figure 8: Evaluation of Apoptosis by flow cytometry. SKOV3 cells were treated with 25 µM complexes 1, 2 and

3 for 48hours.After treatment, cells were stained with Annexin V FITC/PI and analyzed by flow cytometry.

As shown in the figure 8, cells in the lower left quadrant were negative to both Annexin V and PI which indicates viable cells. lower right quadrant was positive to Annexin V FITC, which indicated cells were in early stages of apoptosis; upper left quadrant is positive for PI which represents dead cells whereas upper right quadrant is positive for both Annexin V FITC and PI which indicates late stage of apoptosis. Results showed that due to Co complex 1 treatment, 61.8% cells underwent early apoptosis and 29.5% cells to late apoptosis. Complex 2 treatment causes 42.39% cells to early apoptotic phase and 53.6% cells to late apoptosis whereas complex 3 treatment causes 56.7% cells to early apoptosis and 35.7% cells to late apoptosis. These results demonstrated that these complexes causes cell death by the induction of apoptosis.

DNA fragmentation Assay

To further delineate the mechanism of cell death induced by Co complexes, DNA fragmentation assay was performed which is characteristic for apoptosis. Genomic DNA fragmentation is a hallmark of apoptosis where DNA is cleaved into oligonucleosomal fragments by the activation of endonucleases. (Zhang et al, 2000) Figure 9 shows typical ladder pattern of internucleosomal fragmentation when SKOV3 cells treated with Co complexes 1, 2 and 3 at 25 µM concentration for 72 hrs whereas untreated cells showed intact DNA. This assay further confirms that these three complexes induce cell death by means of apoptosis.

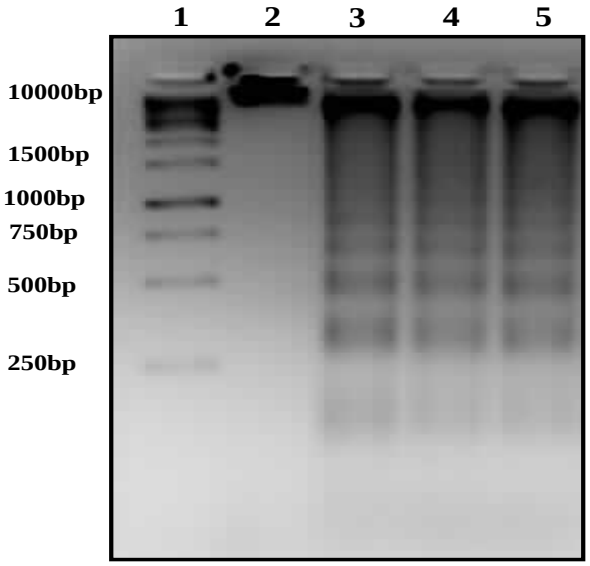


Figure 9: Detection of DNA Fragmentation in SKOV3 cells. Cells were treated with complexes 1, 2 and 3 with 25µM for 72 hours and DNA was isolated by phenol Chloroform method and 2.5µg DNA /well was loaded onto the well of 2% gel, ran at 20V and finally stained with EtBr.1) 1Kb ladder; 2) Control; 3) complex1; 4) Complex2; 5) Complex 3.

CONCLUSION

In this report, we synthesized three Co(III) polypyridyl complexes and characterized. From spectroscopic titration, complexes binds to CT DNA effectively in the order 1>2>3 through intercalative mode which was revealed by viscosity experiments. Upon irradiation, these complexes can effectively cleave plasmid pBR322 DNA. These complexes showed the ability to inhibit the bacterial growth at given conditions. These complexes are cytotoxic to ovarian cancer cells, SKOV3. These complexes were perturbing the cell cycle and finally causing cell death by means of apoptosis. The most lipophilicity and planarity of complex 1, with the ancillary ligand phen, strongly bind between DNA base pairs through π-π stacking. So complex 1, shown higher cytotoxicity and DNA binding constant (K_b) than complexes containing bpy (2) and dmb (3)

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